Chemokines, leukocytes and human ovarian cancer

R. P. M. Negus

A thesis submitted for the Degree of
Doctor of Philosophy at the University of London.

Imperial College of Science, Technology and Medicine,
St. Mary's Hospital,
Paddington,
Declaration

I declare that to the best of my knowledge, the results of this thesis are original, and that where work has been performed by other individuals, they are appropriately credited.
Abstract

Many solid tumours of epithelial origin contain a mononuclear cell infiltrate. The relationship of these cells to members of the chemokine family was examined in human ovarian cancer. Using immunohistochemical staining, the predominate infiltrating cell types identified were CD68+ macrophages and CD3+, CD8+, CD45RO+ T cells. Both cell types occurred throughout the tumours, but were predominantly found in stroma. However, macrophages, unlike T cells, clustered at highest density in regions of necrosis. The expression of four CC chemokines, MCP-1, MIP-1α, MIP-1β and RANTES, was studied by RT-PCR and in situ hybridisation. Although all four were expressed, the number of cells expressing MCP-1 was greater than any other chemokine. MCP-1 expression was associated with tumour cells and some stromal cells, but did not occur adjacent to necrotic areas. MCP-1 protein was detected by ELISA in ascites from patients with ovarian carcinomas and supernatants from freshly isolated tumour cells. In tissue sections, the number of MCP-1 expressing cells correlated with the number of infiltrating CD68+ and CD8+ cells. TNF-α was a potent stimulus to MCP-1 production by ovarian carcinoma cell lines. Since the oxygen tension within areas of necrosis is likely to be extremely low, the effect of hypoxia on TNF-α induced MCP-1 mRNA expression was investigated. Anoxia downregulated TNF-α induced MCP-1 expression in three ovarian carcinoma cell lines. The effect of anoxia on MCP-1 gene expression was consistent with a specific oxygen sensing system and may explain why MCP-1 is not expressed near necrotic regions. Anoxia also decreased the migration of monocytes in response to MCP-1. The regulation of MCP-1 gene expression and monocyte migration by anoxia may in part explain the observed distribution of macrophages within human ovarian carcinomas.
Acknowledgements

Many people have helped me with various aspects of this thesis. I would particularly like to thank Professor Alberto Mantovani and his group, who provided support throughout the project; Professor Gordon Stamp, who helped with morphometry and histopathology; Miss Shanti Raju, Mr Peter Mason and Dr Hilary Russel for providing clinical samples; Mr George Elia and his team for their help with immunohistochemistry and section cutting; Dr Stuart Naylor, for introducing me to the techniques required to start the project; Dr Frances Burke for collaboration with both the PCR and hypoxia studies; Dr Thomas Leber for collaboration particularly on northern analysis; Dr Sergio Dias for immunological advice; Dr Lynn Turner for performing the migration assays; Dr Patrick Maxwell for advice on hypoxically regulated genes; Dr Dai Chaplin for access to equipment at the Gray Laboratories; Mr Robert Moore, for providing technical support; and Dr Graeme Wistow for help with MIF. Finally, I would like to thank Dr Frances Balkwill, for welcoming me into the laboratory, for enabling me to pursue my ideas and for providing encouragement and support at every stage. Whilst working at the Imperial Cancer Research Fund (ICRF) I was supported by a MRC Clinical Training Fellowship and the ICRF.
## Contents

### Chapter 1. Introduction ........................................................................ 16
  1.1. Cytokines ........................................................................... 16
  1.2. Chemokines ........................................................................ 19
    1.2.1. Introduction ................................................................ 19
    1.2.2. Chemokine families .................................................... 19
    1.2.3. Chemokine structure ................................................... 20
    1.2.4. Chemokines and their actions on target cells .................. 24
      1.2.4.i. CXC chemokines ............................................ 24
      1.2.4.ii. CC chemokines ............................................. 25
      1.2.4.iii. C chemokines .............................................. 27
      1.2.4.iv. CXXXC chemokines ...................................... 27
    1.2.5. Chemokine receptors .................................................. 28
    1.2.6. Mechanism of action of chemokines on target cells ............ 31
  1.3. Cytokines and ovarian cancer .................................................... 32
    1.3.1. Growth factors ......................................................... 33
    1.3.2. Colony stimulating factors ............................................ 33
    1.3.3. Angiogenic factors ..................................................... 34
  1.4. Clinical and pathological aspects of ovarian cancer ............................ 35
    1.4.1. Epidemiology and risk factors ........................................ 35
    1.4.2. Clinical features and diagnosis of ovarian cancer ............... 35
    1.4.3. Pathological classification of ovarian tumours .................. 36
    1.4.4. Molecular markers in ovarian cancer ................................. 37
    1.4.5. Management of ovarian cancers ...................................... 40
  1.5. The physical environment of solid tumours .................................... 42
    1.5.1. Introduction ............................................................ 42
    1.5.2. Angiogenesis, oxygen delivery and tumour growth ............... 42
    1.5.3. Tumour vascularisation and prognosis .............................. 44
  1.6. Aims of the thesis .................................................................. 45

### Chapter 2. The leukocyte infiltrate in human ovarian cancer ............... 46
  2.1. Introduction ........................................................................ 46
  2.2. Aims of the chapter ................................................................ 48
  2.3. Methods ............................................................................ 49
    2.3.1. Patient selection and sample collection ......................... 49
    2.3.2. Immunohistochemistry ............................................. 49
Chapter 2. The morphometrical and statistical analysis of ovarian tumour samples

2.3.2. i. Antibodies .................................................... 49
2.3.2. ii. Streptavidin-peroxidase immunostaining method .................................................... 50
2.3.3. Morphometric analysis of tissue sections .................................................... 52
2.3.4. Statistical methods ..................................................... 53

2.4. Results .............................................................................. 54
2.4.1. Estimation of mean tangent nuclear diameters .................................................... 54
2.4.2. Estimation of tumour compartment volumes .................................................... 54
2.4.3. Estimation of the number and distribution of infiltrating leukocytes ... 56
2.4.3. i. Comparison of phenotypic markers .................................................... 56
2.4.3. ii. Comparison of T cell markers .................................................... 66
2.4.4. T-cell functional markers .............................................. 67
2.4.5. HLA-DR expression and macrophage distribution ................................................... 70

2.5. Summary and discussion ......................................................... 71

2.6. Conclusions ........................................................................ 74

Chapter 3. The expression of MCP-1 and other CC chemokines by ovarian tumours and their relationship to tumour infiltrating leukocytes .................................................... 75

3.1. Introduction ........................................................................ 75
3.2. Aims of the chapter ................................................................ 79
3.3. Materials and methods ............................................................ 80
3.3.1. Ovarian samples ................................................................ 80
3.3.1. i. Tissue collection ..................................................... 80
3.3.1. ii. Characteristics of tumour samples used to compare CC chemokine expression by RT-PCR .................................................... 80
3.3.1. iii. Characteristics of tumour samples used to compare CC chemokine expression and the cellular infiltrate .................................................... 80
3.3.1. iii. Characteristics of patients used in analysis of ascites for MCP-1 expression .................................................... 81
3.3.2. Cell lines and culture ...................................................... 81
3.3.2. i. Ovarian carcinoma cell lines .............................................. 81
3.3.2. ii. Mononuclear cell lines .................................................... 81
3.3.2. iii. Preparation of cytospins .................................................. 81
3.3.3. Isolation of TAM and ovarian carcinoma cells .................................................... 82
3.3.4. RT-PCR ........................................................................ 82
3.3.4.i. Preparation of RNA from tissue samples and cell lines ......................................................... 82
3.3.4.ii. PCR ................................................................................................................................. 82
3.3.5. Probes ............................................................................................................................... 83
3.3.6. Southern blotting .................................................................................................................. 84
3.3.7. In situ hybridisation ............................................................................................................. 85
3.3.7.i. Prehybridisation ............................................................................................................... 85
3.3.7.ii. Probe labelling ............................................................................................................... 86
3.3.7.iii. Hybridisation ............................................................................................................... 87
3.3.7.iv. Post-hybridisation washes ............................................................................................ 87
3.3.7.v. Autoradiography ............................................................................................................ 88
3.3.7.vi. Development, counterstaining and mounting ................................................................ 88
3.3.7.vii. Assessing chemokine expression by in situ hybridisation ............................................. 88
3.3.8. ELISA for MCP-1 in ascites, TAM and tumour cell preparations .......................................... 88
3.3.9. Immunohistochemistry and cell counts in frozen sections ................................................... 88
3.3.10. Statistical methods ........................................................................................................... 89
3.4. Results ................................................................................................................................. 90
3.4.1. Expression of CC chemokines by human ovarian carcinomas and tumour cell lines .............. 90
3.4.1.i. RT-PCR for chemokines in cell lines ............................................................................... 90
3.4.1.ii. RT-PCR for chemokines in tumour samples .................................................................... 91
3.4.1.iii. In situ hybridisation for CC chemokines and quantification of chemokine expression ......... 91
3.4.1.iv. Cell types expressing MIP-1α ...................................................................................... 98
3.4.1.v. Cell types expressing MIP-1β and RANTES ............................................................... 99
3.4.2. Production of MCP-1 protein in ovarian cancer .................................................................... 102
3.4.2.i. Estimation of MCP-1 production in ascites .................................................................... 102
3.4.2.ii. MCP-1 production by freshly isolated TAM and tumour cells ........................................ 103
3.4.3. CD8+ and CD68+ cell counts in frozen sections ................................................................... 103
3.4.3.i. Correlation of cell counts in frozen and paraffin embedded specimens ............................. 103
3.4.3.ii. Leukocyte-chemokine correlations ............................................................................... 103
3.5. Summary and discussion ...................................................................................................... 105
3.6. Conclusions .......................................................................................................................... 107
Chapter 4. The effect of cytokines and other inflammatory mediators on MCP-1 expression in human ovarian cancer cell lines

4.1. Introduction ................................................................. 108
4.2. Aims of chapter ............................................................. 111
4.3. Materials and methods .................................................... 112
   4.3.1. Reagents ................................................................. 112
   4.3.2. Cell lines and culture .................................................. 112
   4.3.3. Probes ................................................................... 112
   4.3.4. Northern analysis ...................................................... 112
   4.3.5. RT-PCR ................................................................. 113
   4.3.6. ELISA ................................................................... 113
4.4. Results ........................................................................... 114
   4.4.1. Constitutive and inducible production of MCP-1 .......... 114
      4.4.1.i. Constitutive production of MCP-1 ......................... 114
      4.4.1.ii. The effect of growth factors on MCP-1 production ................................................. 114
      4.4.1.iii. The effect of inflammatory cytokines on MCP-1 production ................................................. 114
      4.4.1.iv. The effect of thrombin on MCP-1 expression ................................................. 116
   4.4.2. TNF-α induced MCP-1 expression in ovarian cancer cell lines ................................................. 116
      4.4.2.i. Time-course of TNF-α induced MCP-1 expression ................................................. 116
      4.4.2.ii. Dose-response of MCP-1 expression to TNF-α ................................................. 116
      4.4.2.iii. TNF-α receptor expression ................................................. 116
      4.4.2.iv. Effect of TGF-β1 on TNF-α induced MCP-1 expression ................................................. 116
      4.4.2.v. Effect of TNF-α on MCP-1 expression in other cell lines ................................................. 121
4.5. Summary and discussion .................................................. 122
4.6. Conclusions ..................................................................... 123
Chapter 5. The action of hypoxia on TNF-α induced MCP-1 expression and on macrophage migration ................................................................. 124

5.1. Introduction ........................................................................ 124

5.2. Aims of the chapter ............................................................ 127

5.3. Materials and methods ...................................................... 128

5.3.1. Reagents ...................................................................... 128

5.3.2. Cell lines and culture .................................................... 128

5.3.3. Hypoxic culture .......................................................... 128

5.3.4. Probes ........................................................................ 129

5.3.5. Northern analysis ........................................................ 129

5.3.6. ELISA ...................................................................... 129

5.3.7. Western analysis ........................................................... 129

5.3.8. Electrophoretic-mobility shift assays (EMSA) ............... 130

5.3.9. Preparation of PBMC .................................................. 130

5.3.10. Migration assays ........................................................ 130

5.3.11. Image analysis ........................................................ 131

5.4. Results .............................................................................. 132

5.4.1. Response of TNF-α stimulated cells to hypoxia ............ 132

5.4.1.i. Effect of hypoxia on constitutive and TNF-α induced MCP-1 expression ................................................................. 132

5.4.1.ii. Time-course of action of hypoxia .................................. 139

5.4.1.iii. Effect of degree of hypoxia on MCP-1 expression ........ 140

5.4.1.iv. Off-response to hypoxia .......................................... 141

5.4.2. Mechanism of hypoxic response .................................... 142

5.4.2.i. Effect of cobalt chloride and desferrioxamine on TNF-α induced MCP-1 expression ................................................................. 142

5.4.2.ii. Effect of potassium cyanide on TNF-α induced MCP-1 expression ................................................................. 143

5.4.2.iii. Effect of antioxidants on TNF-α induced MCP-1 expression ................................................................. 144

5.4.2.iv. Effect of hydrogen peroxide on MCP-1 expression .... 146

5.4.3. Effect of hypoxia on TNF-α signalling ......................... 146

5.4.3.i. Effect of hypoxia on TNF receptor expression ............. 146

5.4.3.ii. Effect of hypoxia on NF-κB mobilisation ................... 148

5.4.3.iii. Effect of antioxidants on NF-κB mobilisation ............ 148
5.4.3.iv. Effect of hydrogen peroxide on NF-κB mobilisation ............................ 148

5.4.4. Effect of hypoxia on monocyte migration in response to MCP-1 ......................... 151
  5.4.4.i. Effect of hypoxia on THP-1 migration .................................. 151
  5.4.4.ii. Effect of hypoxia on PBMC migration ............................... 151

5.4.5. The expression and production of migration inhibitory factor .................. 154
  5.4.5.i. Effect of hypoxia and TNF-α on MIF expression and production by monocytes 154
  5.4.5.ii. Effect of hypoxia and TNF-α on MIF expression by monocytes isolated from whole blood 154
  5.4.5.iii. Effect of hypoxia and TNF-α on MIF expression by PEO14 cells ............ 154

5.5. Summary and discussion ........................................................................ 157

5.6. Conclusions .......................................................................................... 160

Chapter 6. Summary, hypothesis and future plans ............................................ 161
  6.1. Summary .............................................................................. 161
  6.2. Hypothesis .......................................................................... 162
  6.3. Future plans ........................................................................ 163

Publications .................................................................................................. 166
  Published work .................................................................................. 166
  In press ............................................................................................ 166

References .................................................................................................... 167
Figures

Figure 1.1. The monomeric structures of CC chemokines .................................. 21
Figure 1.2. The dimeric structure of chemokines ............................................ 22
Figure 2.1. The histological appearance of an epithelial ovarian cancer .......... 55
Figure 2.2. Scatter plots of the calculated numbers of infiltrating leukocytes .... 58
Figure 2.3. The distribution of T cells in human ovarian carcinomas ............... 56
Figure 2.4. The distribution of T cells in ovarian carcinomas ......................... 57
Figure 2.5. Natural killer cells and B cells in ovarian carcinomas .................... 61
Figure 2.6. Mast cells and eosinophils in ovarian carcinomas......................... 62
Figure 2.7. Macrophage and T cell densities in areas of necrosis .................... 63
Figure 2.8. The distribution of macrophages within ovarian carcinomas ......... 64
Figure 2.9. The typical appearance of macrophages within necrotic areas ....... 65
Figure 2.10. Correlations between T cell phenotypic markers ....................... 66
Figure 2.11. Immunohistochemistry for granzyme B .................................... 67
Figure 2.12. Immunohistochemistry for TIA-1 ........................................... 68
Figure 2.13. Controls for T cell activation markers ..................................... 69
Figure 2.14. MHC class II expression in ovarian tumours ............................. 70
Figure 3.1. Expression of CC chemokines in ovarian cancer cell lines ............. 90
Figure 3.2. Positive controls for in situ hybridisation .................................. 92
Figure 3.3. CC chemokine expression in ovarian carcinomas ....................... 93
Figure 3.4. In situ hybridisation for MCP-1 .............................................. 94
Figure 3.5. In situ hybridisation for MCP-1 .............................................. 95
Figure 3.6. In situ hybridisation for MCP-1 .............................................. 96
Figure 3.7. The absence of MCP-1 expression adjacent to necrotic regions .... 97
Figure 3.8. MIP-1α mRNA detected by in situ hybridisation ......................... 98
Figure 3.9. Expression of MIP-1β detected by isotopic in situ hybridisation ..... 99
Figure 3.10. Expression of MIP-1β detected by isotopic in situ hybridisation .. 100
Figure 3.11. RANTES expression in ovarian carcinomas ............................. 101
Figure 3.12. MCP-1 protein levels in serum and ascites ................................ 102
Figure 3.13. Chemokine-leukocyte correlations in ovarian carcinomas .......... 104
Figure 4.1. The effect of growth factors and inflammatory cytokines on MCP- 
1 production .......................................................... 115
Figure 4.2. Time-course of response of PEO14 cells to stimulation with TNF- 
α .......................................................... 117
Figure 4.3. Dose-response of PEO14 cells to stimulation with TNF-α ............ 118
Figure 4.4. Expression of p55 and p75 in PEO1 and PEO14 cells as 
determined by RT-PCR .................................................. 119
Figure 4.5. The effect of TGF-β1 on MCP-1 expression ................................... 120
Figure 5.1. The change in oxygen content in a modular incubation chamber. 132
Figure 5.2. The effect of anoxia on TNF-α induced MCP-1 expression in PEO14 cells. .......................................................... 134
Figure 5.3. The effect of anoxia on TNF-α induced MCP-1 expression in PEO1 cells ......................................................................................... 135
Figure 5.4. The effect of anoxia on TNF-α induced MCP-1 expression in OVCAR-3 cells .................................................................................... 136
Figure 5.5. The effect of anoxia on MCP-1 protein production by ovarian cell lines........................................................................................ 137
Figure 5.6. The effect of anoxia on MCP-1 expression by mono-mac-6 cells. 138
Figure 5.7. Time-course of the response of PEO14 cells to anoxia 139
Figure 5.8. The effect of different oxygen concentrations on TNF-α induced MCP-1 expression .......................................................... 140
Figure 5.9. The off-response to hypoxia ...................................................... 141
Figure 5.10. Evidence for the existence of a specific oxygen-sensing mechanism in PEO14 cells .......................................................... 142
Figure 5.11. The effect of KCN on MCP-1 and GAPDH gene expression 143
Figure 5.12. The effect of PDTC on TNF-α expression in the cell line mono-mac-6 144
Figure 5.13. The effect of PDTC on TNF-α induced expression of MCP-1 in PEO14 cells .................................................................................... 145
Figure 5.14. The effect of anoxia and TNF-α on mRNA levels of TNFR p55 in PEO1 and PEO14 cells .................................................................................... 146
Figure 5.15. The effect of CoCl2 on expression of TNFR p55 in PEO14 cells .................................................................................... 147
Figure 5.16. The effect of hypoxia on the mobilisation of the transcription factor NF-κB in PEO14 cells .......................................................... 149
Figure 5.17. NF-κB mobilisation in response to oxidants and anti-oxidants in PEO14 cells. .................................................................................... 150
Figure 5.18. The effect of anoxia on the migration of THP-1 cells to MCP-1 152
Figure 5.19. Migration of monocytes in PBMC preparations in response to MCP-1 .................................................................................... 153
Figure 5.20. The effect of hypoxia and TNF-α on MIF mRNA and protein levels .................................................................................... 155
Figure 5.21. MIF expression in peripheral blood monocytes 156
Tables

Table 1.1. Human chemokines cloned and sequenced ........................................... 23
Table 1.2. Murine homologues of some human chemokines ................................. 24
Table 1.3. Chemokine receptors and their ligands ............................................. 29
Table 1.4. A simplified version of the WHO classification for ovarian neoplasms ................................................................. 38
Table 1.5. FIGO staging for primary carcinoma of the ovary .............................. 41
Table 2.1. Antibodies used to define the leukocyte phenotypes in human ovarian carcinomas ................................................................. 50
Table 2.2. The mean tangent diameter of different leukocyte phenotypes ................ 54
Table 2.3. The proportion of tumour volume occupied by its various components ................................................................. 55
Table 2.4. The estimated number of infiltrating cells/mm³ of whole tumour ............ 56
Table 3.1. Primer sequences used in RT-PCR reactions .................................... 83
Table 4.1. MCP-1 expression by tumour cell lines .......................................... 121
Abbreviations

Below are listed some of the more commonly used abbreviations used in this thesis.

ATP  adenosine triphosphate
BSA  bovine serum albumin
bp   base pair
CHX  cycloheximide
CD   cluster differentiation
cDNA complementary DNA
CO₂  carbon dioxide
CoCl₂ cobalt chloride
CTP  cytidine triphosphate
DAB  diaminobenzidine
DEPC diethylpyrocarbonate
DFO  desferrioxamine
DNA  deoxyribonucleic acid
DTT  dithiothreitol
EDTA disodium ethylene diamine tetraacetate.2H₂O
EGF  epidermal growth factor
EGFR epidermal growth factor receptor
Epo  erythropoietin
EST  expressed sequence tag
FCS  foetal calf serum
GAPDH glyceraldehyde-3-phosphate dehydrogenase
GTP  guanosine triphosphate
HCL  hydrochloric acid
HIF-1 hypoxia inducible factor 1
HLA  human leukocyte antigen
hpf  high power field
H₂O₂ hydrogen peroxide
IFN  interferon
I-κB inhibitor of kappa B
IL   interleukin
KCN  potassium cyanide
kDa  kilo Dalton
LPS  lipopolysaccharide
MCP  monocyte chemoattractant protein 1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MIF</td>
<td>migration inhibitory factor</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>N₂</td>
<td>nitrogen</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>³²P</td>
<td>phosphorus-32</td>
</tr>
<tr>
<td>p55</td>
<td>p55 component of the TNFR</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PDTC</td>
<td>pyrrolidine dithiocarbamate</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated on activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RNasin</td>
<td>RNase inhibitor</td>
</tr>
<tr>
<td>RPMI</td>
<td>Rockwell Park Memorial Institute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>³⁵S-UTP</td>
<td>Sulphur-35</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium chloride, sodium citrate buffer</td>
</tr>
<tr>
<td>TAM</td>
<td>tumour associated macrophage</td>
</tr>
<tr>
<td>TDCF</td>
<td>tumour derived chemotactic factor</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TIL</td>
<td>tumour associated lymphocyte</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>tumour necrosis factor receptor</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris[hydroxymethyl]aminomethane</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

1.1. Cytokines

This thesis is primarily concerned with an examination of chemokine expression by human ovarian cancers. It focuses on one in particular, monocyte chemoattractant protein 1 (MCP-1), and an attempt has been made to determine some of the factors present within tumours that may regulate the expression of this chemokine. Chemokines do not act in isolation, but are part of a network that incorporates other cytokines and links the cells producing and responding to them. In this chapter a definition of the term cytokine is given and the relationship of chemokines to this family of biological mediators is described. An overview of those cytokines known to be expressed by ovarian cancers is given; those directly relevant to this thesis are expanded upon in the appropriate chapter sections.

Cytokines tend to be small proteins or glycoproteins which act through specific cell surface receptors coupled to second messenger or signal transduction systems (3, 4). The term cytokine includes the interleukins, interferons, tumour necrosis factors, growth factors, angiogenic factors, colony stimulating factors and chemokines. Cytokines are produced by virtually every nucleated cell type, although most are derived from, and act within, the haematopoietic system. Many cytokines have overlapping functions; while the reason for this apparent redundancy is not known, it may help to confer stability in biological systems (5). Most cytokines are growth or differentiation factors but they also regulate functions such as the release of other cytokines, the activation of leukocytes and leukocyte migration. They usually act in an autocrine or paracrine fashion, although exceptions are easy to come by. For instance migration inhibitory factor (MIF) is detectable in the circulation and can be secreted by cells of the anterior pituitary and erythropoietin is produced by the kidney and acts on the bone marrow.

Cytokines can also be classified on the basis of structural differences. The haematopoietins, which include IL-2 to IL-7, IL-9 and IL-10, the colony stimulating factors, the interferons and erythropoietin (Epo), consist of 4 α-helical bundles; epidermal growth factor (EGF) and transforming growth factor α (TGF-α), which can both bind to the EGF receptor (EGFR) have a β-sheet structure; IL-1α and β and the IL-1R antagonist, like the fibroblast growth factors (FGFs), consist of a β-trefoil; the
TNFs have a jelly-roll configuration; various growth factors including TGF-β, platelet derived growth factor (PDGF), nerve growth factor (NGF) and vascular endothelial growth factor (VEGF) are cysteine knots; and the chemokines consist of a triple-stranded anti-parallel β-sheet in a Greek key motif linked to an α-helix (3).

Among the first activities subsequently classed as cytokines were interferon (IFN) and MIF. Interferon was identified in 1957 as a factor released from chick chorio-allantoic membranes incubated with heat-inactivated influenza virus, that interfered with viral infection of fresh chorio-allantoic membranes (6). In the 1960s, MIF was characterised as a T cell derived activity that inhibited the random migration of macrophages (7). Kasakura and Lowenstein (8) subsequently recognised that supernatants from mixed leukocyte cultures were blastogenic for lymphocytes. The collective term ‘lymphokine’ was suggested by Dumonde in 1969 to describe biochemically undefined lymphocyte-derived activities (9). By the early 1970s it was clear that non-haematopoietic cells were capable of secreting lymphokine-like activities and the term ‘cytokine’ was coined (10). As the number of leukocyte-derived activities increased further an attempt was made to make the naming of monocyte- and lymphocyte-derived activities more systematic by the introduction of the name interleukin (IL) at the Second International Lymphokine Workshop. Even this system is not perfect, for instance IL-8 is a chemokine rather than possessing structural or functional similarities with other interleukins.

Cytokine receptors (R) form a superfamily based on their structural and functional homologies. Most are glycoproteins in which the amino-terminus is external to the plasma membrane and the carboxy-terminals are characterised by a lack of motifs for kinase activity (11). A shared common structure in the extracellular region is the cytokine module, which consists of a repeated fibronectin-like barrel of 7 β strands (12). The simplest cytokine receptors are homodimers in which two receptor molecules bind to one molecule of ligand. The EpoR, growth hormone receptor, granulocyte-CSF receptor and prolactin receptor are all examples. More complicated cytokine receptors have different subunits, one of which may serve to bind ligand and the other to transduce signals. Granulocyte-macrophage CSF, IL-3 and IL-5 all consist of a ligand-specific α subunit and a β subunit common to all three receptors. Some cytokine receptors contain three subunits; for instance the IL-2R consists of an α chain (or tac), and a β and γ chain (13). The TNF-receptor family is different from other members of the cytokine receptor family. TNFRs bind ligands including TNF-α, TNF-β, nerve growth factor, Fas and CD40. The extracellular domain of these receptors is characterised by a recurring cysteine-rich amino-acid motif. Some TNFRs
have a cytoplasmic motif known as the 'death domain', which is essential for the
transduction of apoptotic signals (14). Chemokine receptors form another distinct
group of cytokine receptors. As described below, they are all heptahelical rhodopsin-
like receptors that frequently bind more than one ligand (15).

The study of cytokines has led to the recognition of novel signalling molecules (see
(16) for review). Although some cytokines, particularly those that are predominantly
growth factors, such as PDGF and EGF, signal through receptors that belong to the
tyrosine kinase family, many other cytokine receptors (e.g. those for the interleukins,
colony stimulating factors and the TNFR superfamily) do not possess intrinsic
tyrosine or serine/threonine kinase activity. Instead, ligand-receptor binding is linked
to signal transduction through two novel families, the Janus kinases (JAKs) and
signal transducers and activators of transcription (STATs). Other novel molecules
involved in cytokine signal transduction, include the 'death domain' containing
proteins through which TNFRs signal (17), and the SMAD proteins, which were
originally identified on the basis of sequence homologies with Drosophila Mad and
sma genes in Xenopus, mouse and man (see (18) for a review) and which mediate
downstream signalling events for members of the TGF-β family.
1.2. Chemokines

1.2.1. Introduction
Leukocyte chemoattractant factors have been known for some time, but factors such as C5a, leukotriene B4, platelet-activating factor and the bacterial N-formyl peptides do not have sufficient specificity to explain the distinct nature of the inflammatory infiltrate seen in different diseases. Over the past decade, considerable progress has been made in characterising a subfamily of chemotactic cytokines, the chemokines. A variety of different functions have been ascribed to members of the chemokine family, but one activity shared by many is their ability to stimulate the chemotactic migration of distinct sets of leukocytes. As a group, they share structural and functional similarities (see (1) for review). They are all small proteins (8-10kDa), apart from fractalkine, and are frequently glycosylated. Their amino acid sequences show 20-80% homology (more than other cytokine families), and their tertiary structures have considerable similarity. The first members of the chemokine family were identified on the basis of their biological activities in culture supernatants and were purified biochemically before being cloned. Platelet factor 4 (PF4) was sequenced in 1977 (19, 20) and β thromboglobulin (β-TG) followed in 1978 (21). However, it was not until the late 1980s that chemokines were considered to be a distinct family. One of the first chemokines recognised specifically as a leukocyte chemoattractant was interleukin 8 (IL-8). IL-8 was identified both as a monocyte-derived neutrophil chemoattractant (22) and as an 'anionic' neutrophil activating peptide (ANAP (23). It was cloned in 1988 by Matsushima (24) and Lindley (25). More recently techniques such as subtractive hybridisation, screening cDNA libraries and database searching for expressed sequence tags (ESTs) have been used to find homologous cytokines. At present more than 30 chemokines have been cloned and more than 40 are recognised on the basis of their ESTs. In this section a brief description of the types and activities of human chemokines is given.

1.2.2. Chemokine families
Chemokines can be divided into two main families based on their primary structure (26). Both families contain two pairs of cysteine half residues found at the amino and carboxy terminals of the molecule. In the CXC, or α, family, the amino terminal cysteine residues are separated by a single amino acid, whereas in the CC, or β family they are adjacent. Well known members of the CC family include the monocyte chemoattractant proteins (MCPs), macrophage inflammatory proteins (MIPs), eotaxin and regulated upon activation, normal T cell expressed and secreted (RANTES; for a more complete definition, see (27)). CXC chemokines include IL-8, neutrophil
activating peptide 2 (NAP-2), platelet basic protein (PBP and its derivatives, PF4 and β-TG), and stromal cell-derived factor 1 (SDF-1). Recently other structural families have been identified. Lymphotactin is a chemokine which acts on lymphocytes but has only a single pair of cysteines (28), whereas fractalkine has two pairs of cysteine residues, but the N-terminal cysteines are separated by 3 amino acid residues, CXXXC. Fractalkine can exist as a membrane bound or secreted form (29).

In addition to the differences in chemokine primary structure there are differences in their genomic organisation. Many genes for CC chemokines are located on chromosome 17 in man (30), while those for CXC chemokines are on chromosome 4 (31). The open reading frame for several CC chemokines (e.g. MCP-1, MIP-1α and MIP-1β) is organised into 3 exons and 2 introns, while that for CXC chemokines such as IL-8, GROα and IP-10 consists of 4 exons and 3 introns. The gene for human lymphotactin is located on chromosome 1q (28). Table 1.1 lists the different members of the chemokine families cloned and characterised so far, and table 1.2 gives some of the known murine homologues of human chemokines.

1.2.3. Chemokine structure
Chemokines display similarities in their higher order structure. Using NMR and crystallographic approaches, the structures of PF4 (32), IL-8 (33), MIP-1β (34), MCP-1 (35), MCP-3 (36) and RANTES (37) have been established. They consist of a relatively disordered amino terminus followed by 3 antiparallel β-pleated sheets forming a Greek key configuration and a carboxy-terminal α-helix that extends over the top of the β sheets. The monomeric structures of chemokines are virtually superimposable (figure 1.1). However, higher order structures have been more difficult to define. Under the conditions required for structural analysis, MCP-1, MIP-1β, RANTES and IL-8 form dimers (figure 1.2) and PF-4 can form tetramers (32).

One reason for the formation of higher order structures is the charge distribution along the chemokine molecule which allows electrostatic interactions between different molecules to occur. Multimer formation may be in part responsible for the bell-shaped dose-response curve that is a feature of migration assays. However, whether chemokines act physiologically as dimers or as monomers has not been resolved. IL-8 mutants which are incapable of dimerising due to the replacement of Arg-26 with a N-methyl group, are nonetheless still active (38). Clore (39) makes the point that the circulating concentrations of chemokines (in the nM range) are well below the
equilibrium constant for dimerisation (approx. 0.1µM). Using a combination of size exclusion high performance liquid chromatography, sedimentation equilibrium ultracentrifugation and chemical cross-linking, IL-8, MCP-1 and I309 have all been found to be predominantly dimeric at concentrations above 100µM, but to exist as monomers at concentrations at which they display chemotactic activity. On the other hand Rollins et al found that dominant negative JE mutants form dimers with wild type protein but these dimers do not bind efficiently to MCP-1 receptors (40). Several lines of evidence suggest that chemokines bind to components of the cell surface, such as glycosaminoglycans (41). It may be that by binding to the cell surface, chemokines achieve high enough local concentrations to be presented to target cells as dimers.

Figure 1.1. The monomeric structures of CC chemokines. The structures of monomeric MIP-1β (yellow), RANTES (red) and MCP-1 (blue) have been superimposed, showing regions of similarity and difference, particularly at the amino terminal (from (42)).
Figure 1.2. The dimeric structure of chemokines.
Schematic ribbon drawings of the IL-8 dimer (A) and the MIP-1β dimer (B). The subunits are in red and blue in each drawing (from (43)).
<table>
<thead>
<tr>
<th>Family</th>
<th>Name</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXC</td>
<td>IL-8</td>
<td>(24)</td>
</tr>
<tr>
<td></td>
<td>MGSA/GROα</td>
<td>(44)</td>
</tr>
<tr>
<td></td>
<td>Groβ</td>
<td>(45)</td>
</tr>
<tr>
<td></td>
<td>Groγ</td>
<td>(45)</td>
</tr>
<tr>
<td></td>
<td>PBP</td>
<td>(46)</td>
</tr>
<tr>
<td></td>
<td>CTAP III</td>
<td>(47)</td>
</tr>
<tr>
<td></td>
<td>β-TG</td>
<td>(48)</td>
</tr>
<tr>
<td></td>
<td>NAP-2</td>
<td>(49)</td>
</tr>
<tr>
<td></td>
<td>ENA-78</td>
<td>(50)</td>
</tr>
<tr>
<td></td>
<td>GCP-2</td>
<td>(51)</td>
</tr>
<tr>
<td></td>
<td>PF4</td>
<td>(52)</td>
</tr>
<tr>
<td></td>
<td>IP-10</td>
<td>(53)</td>
</tr>
<tr>
<td></td>
<td>MIG</td>
<td>(54)</td>
</tr>
<tr>
<td></td>
<td>SDF-1</td>
<td>(55)</td>
</tr>
<tr>
<td>CC</td>
<td>MCP-1</td>
<td>(56)</td>
</tr>
<tr>
<td></td>
<td>MCP-2</td>
<td>(57)</td>
</tr>
<tr>
<td></td>
<td>MCP-3</td>
<td>(58)</td>
</tr>
<tr>
<td></td>
<td>MCP-4</td>
<td>(59)</td>
</tr>
<tr>
<td></td>
<td>MIP-1α</td>
<td>(60)</td>
</tr>
<tr>
<td></td>
<td>MIP-1β</td>
<td>(61)</td>
</tr>
<tr>
<td></td>
<td>HCC-1</td>
<td>(62)</td>
</tr>
<tr>
<td></td>
<td>I-309</td>
<td>(63)</td>
</tr>
<tr>
<td></td>
<td>RANTES</td>
<td>(64)</td>
</tr>
<tr>
<td></td>
<td>eotaxin</td>
<td>(65)</td>
</tr>
<tr>
<td></td>
<td>LARC</td>
<td>(66)</td>
</tr>
<tr>
<td></td>
<td>CK β8</td>
<td>(67)</td>
</tr>
<tr>
<td></td>
<td>ELC</td>
<td>(68)</td>
</tr>
<tr>
<td></td>
<td>TARC</td>
<td>(69)</td>
</tr>
<tr>
<td></td>
<td>MDC</td>
<td>(70)</td>
</tr>
<tr>
<td>C</td>
<td>lymphotactin</td>
<td>(28)</td>
</tr>
<tr>
<td>CXXXC</td>
<td>fractalkine</td>
<td>(29)</td>
</tr>
</tbody>
</table>

Table 1.1. Human chemokines cloned and sequenced.
1.2.4. Chemokines and their actions on target cells

In addition to CC and CXC chemokines being separable on grounds of their primary structure and genomic organisation, they also have different activities on leukocyte subtypes. CXC chemokines act principally on neutrophils, while CC chemokines act mainly on mononuclear cells. Other chemokine functions have been identified in addition to those relevant to leukocyte physiology. In this section the major actions identified for members of the various chemokine families are reviewed.

1.2.4.i. CXC chemokines

The ability of CXC chemokines to act on neutrophils may depend on the presence of a glutamate-leucine-arginine (ELR) motif close to the amino-terminal. The ELR motif appears to be important for signal transduction involved in neutrophil chemotaxis and is absent in CC chemokines. It may also be important in determining the angiogenic properties of some CXC chemokines (71).

One of the best known CXC chemokines is interleukin 8 (IL-8). This was first identified as a selective in vitro chemoattractant for neutrophils but not monocytes (23, 72). In addition to acting as a chemoattractant, IL-8 can stimulate degranulation in neutrophils (73), increase adhesion of neutrophils to endothelial cells, fibrinogen and matrix proteins (74, 75), increase expression of complement receptor 1 (76) and enhance the cytostatic effects of neutrophils on Candida albicans (77). IL-8 can also act as a chemoattractant for T cells (78). In response to the inflammatory cytokines TNF-α and IL-1, IL-8 is expressed by vascular endothelium (79), monocytes (24), dermal fibroblasts and keratinocytes (80).

GRO-α, another neutrophil chemoattractant (81), is identical to melanocyte growth stimulatory activity (MGSA) (44). This CXC chemokine was initially characterised as 1309.

### Table 1.2. Murine homologues of some human chemokines.

<table>
<thead>
<tr>
<th>Murine chemokine</th>
<th>Human homologue</th>
</tr>
</thead>
<tbody>
<tr>
<td>JE</td>
<td>MCP-1</td>
</tr>
<tr>
<td>KC</td>
<td>GRO-α</td>
</tr>
<tr>
<td>IP-10</td>
<td>IP-10</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>Eotaxin</td>
</tr>
<tr>
<td>TCA3</td>
<td>I309</td>
</tr>
</tbody>
</table>

Table 1.2. Murine homologues of some human chemokines.
from transformed hamster cells with abnormal growth control (82). Two other related proteins with similar activities were discovered later and designated GRO-β and GRO-γ (45). ENA78 (50) and GCP-2 (83), both contain ELR motifs and are good neutrophil chemoattractants, but neither is as potent as IL-8. Platelet basic protein can be cleaved proteolytically to yield connective tissue activating peptide III (CTAP-III), β-TG and NAP-2 (46). Although these three chemokines contain an ELR motif, they are weaker neutrophil chemoattractants than the others described so far. CTAP-III is a weak mitogen for fibroblasts (84) and β-TG has chemoattractant activity for fibroblasts (85).

The non-ELR containing CXC chemokines include PF4, IP-10 and MIG. PF4 was recognised as a major component of platelet α granules in 1966 and IP-10 as the product of the monocytic cell line U937 stimulated with IFN-γ (86). Although chemokines structurally, these proteins have only weak neutrophil chemoattractant activity (87, 88). PF4 may have a role in inhibiting the formation of new blood vessels. Recombinant human PF4 can inhibit blood vessel proliferation in chick chorio-allantoic membranes and proliferation of human umbilical vein endothelial cells (HUVECs) (89). MIG and SDF-1 are both T cell chemoattractants (54, 90).

1.2.4. ii. CC chemokines

Within the CC family, the MCPs are the most potent group of monocyte chemoattractants and of these, MCP-1 is the best characterised. Reflecting its expression by many different cell types, MCP-1 was characterised by three groups independently, from baboon aortic smooth muscle (91), the myelomonocytic cell line HL-60 (92) and the malignant glioma cell line UMG105 (56). Tumour derived chemotactic factor, a factor that had been partially characterised from a number of different human and murine tumours and tumour cell lines, was also shown to be homologous to MCP-1 (93). Furthermore, the amino acid sequence of MCP-1 was identical to that predicted for a murine early response gene known as JE (94), cloned from PDGF stimulated murine 3T3 cells (95).

MCP-1 is produced by a wide variety of cells, particularly in response to stimulation with inflammatory cytokines. MCP-1 expression can be stimulated in human monocytes and mesangial cells by IFN-γ, TNF-α and IL-1 (96, 97) and in alveolar macrophages by both IL-1 and TNF-α (98). Fibroblasts produce MCP-1 in response to TNF-α, IL-1 (99) and PDGF (100). Synoviocytes (101) and human renal proximal tubular epithelial cells stimulated with inflammatory cytokines also produce this chemokine (102). Endothelial cells express MCP-1 in response to stimulation with IL-
1 and TNF-α (79) and to mechanical strain (103). Other stimuli which can result in MCP-1 expression include LPS (79), thrombin (104), Fcγ receptor cross-linking in peripheral blood mononuclear cells (105) and possibly hypoxia (106). The main activity of MCP-1 is to stimulate the chemotactic migration of monocytes. It may also play a role in monocyte activation, such as the production of superoxide anions (107). MCP-1 causes migratory responses in both CD4+ and CD8+ T cells (108) and can stimulate the degranulation of basophils (109).

Closely homologous to MCP-1 are MCP-2 and MCP-3 (110). These chemokines are less potent monocyte chemoattractants than MCP-1, but they can activate basophils (111) and chemoattract T cells. In addition, MCP-3 is active on eosinophils (112) and dendritic cells (113). MCP-4, like MCP-3, is active on eosinophils as well as monocytes and T cells (59). In the mouse a fifth MCP, MCP-5 has been identified (114).

The macrophage inflammatory proteins, MIP-1α and MIP-1β were originally characterised from LPS-treated monocytic cell lines (115, 116). Their cellular sources appear to be restricted to monocytes and T cells. They are chemoattractants for monocytes, although not as potent as MCP-1, and are active on distinct T cell subsets. In microchemotaxis assays, MIP-1α attracts predominantly CD8+ cells while MIP-1β is active on CD4+ cells (117, 118). In transendothelial assays, however, both MIPs attract CD4+ cells better than CD8+ cells (119). A novel action of MIP-1α is its ability to inhibit haematopoietic stem cell proliferation (120) and it is identical to stem-cell inhibitory factor (121). This activity has potential clinical application in aiding bone marrow rescue following therapy for haematological malignancies.

RANTES was discovered using a subtractive hybridisation approach in T cells (64). The mature protein is 8kDa and may be O-glycosylated at two serine residues in the amino-terminal (122). Like the MIPS, the cellular sources of RANTES are more restricted than MCP-1, although it is expressed by platelets (454) some tumour cell lines (123) and synoviocytes from rheumatoid joints (124). In migration assays, RANTES preferentially attracts CD4+, CD45RO+ T cells (125), but in the presence of endothelium, both CD4+ and CD8+ cells respond to RANTES (119).

Eotaxin is chemoattractant for eosinophils. It was first recognised in bronchoalveolar lavage fluid in a guinea pig model of allergic airways disease. Challenge of sensitised animals was associated with a massive accumulation of eosinophils within the lungs (65). Eotaxin appears to work in concert with IL-5. In an isolated perfused hind-limb
model, IL-5 promoted the release of eosinophils from the bone marrow into the circulation (126). Human eotaxin has also been cloned (127).

Other CC chemokines have been less well characterised. I-309 is expressed by γδ T cells, from which it was originally identified by subtractive hybridisation (63). I-309 is active on monocytes alone (128). Another monocyte specific CC chemokine is CK β8. This chemokine is expressed by monocytes induced with IL-1β and is also expressed in normal human liver and gut (67). HCC-1, isolated from the plasma of patients with chronic renal failure, is expressed by a variety of different tissues. It can produce a calcium flux in responding cells, but does not have chemotactic activity (62). TARC (thymus and activation-regulated chemokine) is a T cell chemoattractant expressed constitutively by the thymus (69). ELC was identified as a result of searching the EST data base. The complete cDNA encodes a novel, highly basic polypeptide. ELC is most strongly expressed in the thymus and lymph nodes. Searching for ESTs has also identified liver and activation regulated chemokine (LARC). As well as being constitutively expressed by the liver, LARC can be strongly induced in several human cell lines. LARC is weakly chemotactic for granulocytes but is not active on the monocyte cell line, THP-1, or blood monocytes (66). The cDNA for macrophage-derived chemokine (MDC) was isolated by random sequencing of cDNA clones from human monocyte-derived macrophages. It appears to be synthesised specifically by cells of the macrophage lineage. MDC causes the migration of monocytes and IL-2-activated natural killer cells (70).

1.2.4.iii. C chemokines
The only member of this family known so far is lymphotactin, a T cell chemoattractant identified in a cDNA library generated from activated mouse progenitor T cells (28). Lymphotactin has an amino acid sequence similar to MIP-1β and GRO-α, but contains only a single pair of cysteines. Northern blotting of lymphocyte subsets revealed high levels of expression by activated thymic and splenic CD8+, CD3+ T cells. Lymphotactin was particularly active at stimulating the chemotactic migration of CD8+ cells and in at least some T cells a Ca2+ flux is generated by lymphotactin.

1.2.4.iv. CXXXC chemokines
Until the discovery of fractalkine, only small soluble chemokines were known. However, fractalkine can exist in two forms, either membrane-anchored or as a shed 95K glycoprotein. The polypeptide chain of this CXXXC chemokine is predicted to be part of a 373-amino-acid protein that carries the chemokine domain on top of an extended mucin-like stalk (29). The soluble CXXXC chemokine has potent
chemoattractant activity for T cells and monocytes, and the cell-surface-bound protein, which is induced on activated primary endothelial cells, promotes strong adhesion of these leukocytes.

1.2.5. Chemokine receptors

Chemokine receptors are members of the heptahelical, rhodopsin-like, G-protein-coupled family. They share structural homology with receptors for other leukocyte chemoattractants such as fMLP (129), the complement component C5a, receptors for other signalling molecules, such as adrenaline, retinoic acid and angiotensin II and herpesvirus homologues (130). However, they also possess features that appear to be unique, such as the amino acid sequence DRYLAIV in the second intracellular domain (131). Chemokine receptors can be divided into three groups on the basis of their ligand specificities, CXC receptors (CXC), CC receptors (CCR) and the promiscuous chemokine receptor, DARC (Duffy antigen receptor for chemokines), that is identical to the Duffy blood group antigen (summarised in table 1.3). Chemokine receptors are often encoded by a single open reading frame of about 1000bp, yielding a protein of approximately 400 amino acids with molecular masses of ≈40kDa. Chemokine receptors all appear to be coupled to Gαi proteins and are therefore susceptible to inhibition with pertussis toxin [see (15) for review]. Ligand binding is usually associated with a Ca²⁺ flux (15). However, other signal transduction pathways are also probably involved.

The first chemokine receptors to be cloned were those for IL-8. Studies on IL-8 binding revealed about 20000 high affinity (Kₐ<8 x10⁻¹⁰M) receptors per cell (132) on neutrophils, whereas T cells carried less than 300 such receptors per cell (133). Ligand displacement studies (134) and SDS-PAGE analysis of chemically cross-linked ¹²⁵I-IL-8 indicated (133) that there might be two IL-8 receptors, one which bound IL-8 alone and a second which could also bind GRO proteins, ENA-78 and NAP-2. The existence of two IL-8 receptors was confirmed by the finding of unique cDNAs which encoded highly homologous IL-8 receptors (135, 136). The IL-8 receptors, originally designated IL-8RA and IL-8RB, are now more usually known as CXCR1 and CXCR2. Two other CXC receptors have been identified, that for IP10 and MIG, CXCR3 (137), and that for SDF-1, CXCR4 (138). The cDNA for CXCR4 was isolated from a human monocyte cDNA library and was initially called LESTR (leukocyte-derived seven-transmembrane domain receptor). LESTR was then found to be identical to the fusin protein, the co-receptor to CD4 for laboratory-adapted syncytiom-inducing strains of HIV-1 (139).
Table 1.3. Chemokine receptors and their ligands (adapted from Rollins, and Premack and Schall (1, 2)).

<table>
<thead>
<tr>
<th>Family</th>
<th>Receptor</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR</td>
<td>CXCR-1</td>
<td>IL-8</td>
</tr>
<tr>
<td></td>
<td>CXCR-2</td>
<td>IL-8, GRO α, β, γ, ENA-78, NAP-2</td>
</tr>
<tr>
<td></td>
<td>CXCR-3</td>
<td>IP-10, Mig</td>
</tr>
<tr>
<td></td>
<td>CXCR-4</td>
<td>SDF-1α</td>
</tr>
<tr>
<td>CCR</td>
<td>CCR1</td>
<td>MIP-1α, MCP-3, RANTES</td>
</tr>
<tr>
<td></td>
<td>CCR2</td>
<td>MCP-1, MCP-3</td>
</tr>
<tr>
<td></td>
<td>CCR3</td>
<td>Eotaxin, RANTES, MCP-2, MCP-3</td>
</tr>
<tr>
<td></td>
<td>CCR4</td>
<td>MIP-1α, RANTES, MCP-1, TARC</td>
</tr>
<tr>
<td></td>
<td>CCR5</td>
<td>MIP-1α, MIP-1β, RANTES</td>
</tr>
<tr>
<td></td>
<td>CCR6</td>
<td>MIP-3α/LARC</td>
</tr>
<tr>
<td></td>
<td>CCR7</td>
<td>MIP-3β/ELC</td>
</tr>
<tr>
<td>Duffy antigen</td>
<td>DARC</td>
<td>RANTES, MCP-1, IL-8, GRO, NAP-2</td>
</tr>
<tr>
<td>Viral receptors</td>
<td>CMV US28</td>
<td>human CC chemokines</td>
</tr>
<tr>
<td></td>
<td>HSV ECRF3</td>
<td>IL-8, GRO</td>
</tr>
</tbody>
</table>

More CC than CXC chemokine receptors have been identified so far and they display greater overlap in their ligand specificities. CCR1 binds MIP-1α, RANTES and MCP-3 with high affinity (140, 141). The MCP-1 receptor comes in two forms, CCR2A and CCR2B, the result of alternative splicing at the carboxy-terminal (142). CCR2 binds MCP-1 with high affinity, but also binds MCP-3 (143). Peripheral blood monocytes express CCR2 at 1700/cell with a $K_d$ of 1.9 $\times$ 10^{-9}M (144). Both CCR3 and CCR4 were cloned from granulocytes. CCR3 was cloned following the recognition that eotaxin binds to a specific receptor on eosinophils (127). It also binds RANTES, MCP-2 and MCP-3 (145). CCR4 was cloned from basophils and binds the major basophil activating chemokines, MIP-1α, MCP-1 and RANTES (146). CCR5 was identified by homology cloning (147) and binds MIP-1α, MIP-1β and RANTES with high affinity.

A chemokine receptor also exists which binds multiple chemokines, both CC and CXC (148), with high affinity, but without any apparent functional consequences. This promiscuous receptor was identified on the surface of erythrocytes (149) and was subsequently found to be identical to the Duffy blood group antigen (150). It has therefore been termed the Duffy antigen receptor for chemokines (DARC). It is also
expressed by the endothelium of endothelial venules (151) even in Duffy negative individuals. Furthermore, DARC is the receptor by which the malarial parasite *Plasmodium vivax* gains entry into red cells (152). At present the consensus view is that DARC functions as a chemokine 'sink', removing chemokines from the circulation in order that they do not bind and attract leukocytes elsewhere in the circulation.

Receptors homologous to chemokine receptors are expressed by several members of the Herpes virus family. The gene ECRF3 of *Herpesvirus saimiri* encodes a membrane protein with structural similarities to CXCR1 and CXCR2 (153). Recombinant protein expressed in frog oocytes will bind to IL-8, GRO-α and NAP-2. The US28 protein of cytomegalovirus binds multiple CC chemokines with high affinity (MCP-1 $K_d=6 \times 10^{-10}$M; RANTES $K_d=2.7 \times 10^{-10}$M) (154).

Of particular importance recently has been the realisation that chemokine receptors are involved in the entry of the HIV virus into human cells. HIV is the causative agent of the acquired immunodeficiency syndrome (AIDS) in humans (155), which occurs as a result of depletion of the CD4+ T cell population (156). The CD4 glycoprotein was found to serve as a receptor for the HIV virus (157), but some human and animal cells were resistant to HIV-1 infection even when CD4 was expressed on the cell surface (158). Studies with somatic hybrids raised the possibility that other factors were involved in complementing the block to fusion in resistant cells (159). In 1995 Cocchi *et al* (160) showed that RANTES, MIP-1α and MIP-1β were HIV suppressive factors expressed by CD8+ cells. These chemokines all bind to CCR5. When expressed with CD4, CCR5 allowed entry of HIV isolates into cell lines. The following year, a G protein-coupled receptor was cloned and termed 'fusin' since it served as a cofactor for HIV-1 fusion and entry (139). Fusin is now known to be identical to the chemokine receptor CXCR4 (90). The picture that is emerging is that macrophage-tropic (M-tropic) primary HIV isolates gain entry to cells using CD4 and CCR5 as a co-receptor, whereas T cell-tropic (T-tropic) HIV isolates use CD4 in conjunction with CXCR4. This difference in tropism and receptor usage is probably important in disease progression, since M-tropic isolates tend to occur early in disease, whereas T-tropic syncytium inducing isolates are associated with disease progression. Tropism appears to depend on the amino acid sequence of the third variable region (V3) of the HIV envelope protein (Env), gp120 (161).
1.2.6. Mechanism of action of chemokines on target cells

Chemokines are best known for their ability to cause the directed migration of leukocytes. As described earlier, over 40 human chemokines have been identified. However, studies on how they act have lagged behind. In this section a brief account will be given of how chemokines may be presented to target cells and of events following ligand-receptor binding. Other aspects of chemokine induced leukocyte activation, some of which have already been mentioned, will also be discussed.

Chemokines are basic proteins, raising the possibility that they are bound to the extracellular matrix by non-covalent interactions with acidic groups. One current view is that chemokines, released from cells at sites of inflammation, bind locally to proteoglycans in the extracellular matrix and are presented to circulating leukocytes. Receptor-ligand interactions are followed by integrin activation, strong adhesion and transmigration into tissue spaces. Within tissues, chemokine gradients exist to direct leukocyte migration. Any chemokine molecules escaping into the circulation are mopped up by DARC on erythrocytes, to prevent binding and leukocyte extravasation at irrelevant sites. Directed cell migration by a gradient of chemoattractant bound to a substrate is termed haptotaxis. Most work on the presentation of chemokines at extracellular sites has involved IL-8. Radiolabelled exogenous IL-8 was detected bound to endothelial cells (162), and in the presence of heparan sulphate, neutrophil chemotaxis in vitro was enhanced (163).

Binding of chemoattractants to serpentine receptors is frequently followed by a rise in intracellular Ca\(^{2+}\), mobilised from intracellular stores. This is associated with activation of phospholipase C (PLC) which then cleaves phosphatidylinositol (4,5)-bisphosphate to 1,2-diacylglycerol and inositol triphosphate. The latter is in turn involved in the mobilisation of Ca\(^{2+}\) from intracellular stores (164). Chemotactic responses to RANTES in T cells can be inhibited by wortmannin, implicating PI3 kinase in the signalling pathway (165). Neutrophil migration in response to IL-8 may similarly depend on PI3 kinase (166). Other signal transduction pathways are involved in chemokine signalling, since activation of G protein coupled receptors may lead to reorganisation of the actin cytoskeleton independently of activated PLC (167) and activation of mitogen activated protein kinases (MAPKs) has been detected in MCP-1 stimulated human monocytes (168). The regulation of adhesion through integrins and the formation of lamellipodia and fillopodia related to the activation of Rho, Rac and Cdc42 respectively (169).
The actions of chemokines on cellular adhesion molecules have largely been studied in monocytes and granulocytes. MCP-1, MIP-1α and RANTES induced an increase in the expression of the β2 integrin α subunits, CD11b and CD11c, in human monocytes isolated from peripheral blood. This was associated with mobilisation of Ca²⁺ from intracellular stores and resulted in enhanced binding of monocytes to endothelial cells (170). CD18 is implicated in the adhesion of neutrophils to fibrinogen stimulated by C5a, fMLP, IL-8 and GROα (171). In eosinophils, eotaxin upregulated the expression of CD11b and mobilise Ca²⁺ from intracellular stores, as well as activating the respiratory burst and increasing actin polymerisation (164).

The action of chemokines on T cells has been less explored. RANTES can increase the expression of a variety of adhesion molecules in T cells and T cell clones that might be important not only in binding to endothelium, but also in homotypic adhesion, which may be important in recruiting cells to inflammatory sites (172). Furthermore, RANTES stimulation of T lymphocytes isolated from peripheral blood led to an increase in the number of polarised cells (165). There is also evidence that CC chemokines can cause degranulation and proliferation in cytotoxic T cells (173). In T cells, chemokines induced changes in the distribution of the receptors CCR2 and CCR5. Following treatment with chemokines these receptors concentrated at the leading edge of the cell and the effect could be blocked by pertussis toxin (174).

The net effect of all these changes is to enhance leukocyte adhesion to vascular endothelium at sites of chemokine production and to orientate cells so that they can migrate haptotactically towards the source of a chemokine gradient. Further activation of leukocytes, such as the respiratory burst and degranulation, occurs particularly in granulocytes, where non-specific killing mechanisms are appropriate.

1.3. Cytokines and ovarian cancer
The cytokines that have been detected in ovarian cancers can be divided into different categories, including inflammatory cytokines, such as TNF-α, IL-6 and IL-1; growth factors, such as TGF-α, TGF-β1, PDGF, insulin like growth factor 1 (IGF-1) and EGF; those that regulate monocytes/macrophages, such as IL-10, macrophage colony stimulating factor (M-CSF) and MIF; and a variety of angiogenic factors, including platelet derived endothelial cell growth factor (PD-ECGF) and VEGF. In an RT-PCR based examination of mRNA derived from malignant and non-malignant ovarian tissue, Burke et al (175) found IGF-1, PDGF, TGF-α, TGF-β and M-CSF expression in more than 80% of tumours studied. The inflammatory cytokines IL-1α, IL-6,
TNF-α and lymphotoxin (LT) were also present in a similar proportion of tumours, but the results of studies examining the production of IL-1 have been conflicting. Of almost equal interest to those cytokines that have been detected, are those that have not. For instance, cytokines associated with T helper cell differentiation, such as IL-2, and IFN-γ were not detected in any tumours and IL-4 was found in less than 40% of cases. The role of all these factors is still under assessment, but there are likely to be interactions between different cell types within the tumour that contribute to the growth of the whole tumour. EGF and TGF-α, the colony stimulating factor M-CSF and angiogenic factors are discussed briefly here. TNF-α, IL-1, TGF-β1 and PDGF are described in more detail in chapter 4 and MIF in chapter 5.

1.3.1. Growth factors
Several growth factors and their receptors are known to be expressed by ovarian tumours. They include TGF-α, TGF-β1, EGF and PDGF. The primary structure of EGF was determined in 1972 (176). Since then other members of this family have been characterised, including TGF-α, the pox virus growth factors and amphiregulin. All of these EGF-like molecules bind to the EGFR with high affinity, produce mitogenic responses in EGF-sensitive cells and have similar primary structures (177). In ovarian cancer, the presence of TGF-α and its interaction with the EGFR may be more important than EGF. In a study of samples from 133 patients, EGF was detected in 27.6% but TGF-α was found in 88.5% (178). The presence of immunoreactive TGF-α in the ascites of patients with ovarian cancer is predictive of poor survival (179). Stromberg et al detected the EGFR in 17/17 ovarian cancer cell lines and 16 of these also expressed TGF-α (180). Since antibodies to TGF-α, but not to EGF can inhibit growth in ovarian cell lines (181), the presence of TGF-α and EGFR may represent an autocrine loop involved in the proliferation of these cells.

1.3.2. Colony stimulating factors
As its name suggests, M-CSF is a lineage specific haematopoietin that stimulates proliferation and survival in mononuclear phagocytes (182). It is a 45-90kDa homodimeric protein that is principally secreted by fibroblasts, but it can also be produced by endothelial cells and monocytes (183). Both M-CSF and its receptor c-fms have been found in ovarian tumours. Kacinski et al (184) found that c-fms mRNA and protein localised to tumour epithelium. In the same study M-CSF was detected in 6/14 cases by immunohistochemistry and again localised to the epithelial areas. However in breast cancer, both macrophages and tumour cells were positive.
for M-CSF (183). Elevated circulating levels of M-CSF have also been detected in ovarian, endometrial and breast cancers (185).

1.3.3. Angiogenic factors
The best known angiogenic factor is VEGF (186). Isolated from both human and animal tumour cell lines, VEGF is particularly potent at inducing new blood vessel formation due to its mitogenic activity on endothelial cells (see (187) for review). A number of human tumours express VEGF, including renal and bladder carcinomas (188), ovarian cancers (189) and central nervous system tumours (190). VEGF is a powerful promoter of microvascular permeability (191), thereby allowing the extravasation of fibrinogen and the development of tumour stroma by the formation of cross-linked fibrin monomers (192). Increased vascular permeability mediated by VEGF may account for the occurrence of pleural effusions and ascites which can be significant clinical problems (193). In human ovarian cancer patients, both VEGF protein and the KDR receptor have been detected in tumour cells (194). VEGF detected immunohistochemically correlated significantly with poor survival (195).

Other cytokines implicated in angiogenesis include fibroblast growth factors (FGFs), which are heparin binding and may be sequestered by the extracellular matrix (196). These factors are mitogenic for endothelial cells and have been detected in many different human tumours (197). PD-ECGF is angiogenic in vivo and is chemotactic for endothelial cells in vitro. Levels of PD-ECGF are elevated in patients with advanced cancers (198) and Reynolds et al (199) have shown that although PD-ECGF is expressed by both benign and malignant tumours of the ovary mRNA levels were significantly higher in malignant disease.
1.4. Clinical and pathological aspects of ovarian cancer

1.4.1. Epidemiology and risk factors

The incidence of ovarian cancer is approximately 5000 cases per annum in the UK and 26,600 per annum in the USA (200). Of these over 60% will be expected to die of their disease within 5 years. The highest incidence of cancers of the ovary occurs in the white population in Western and Northern Europe and North America (201, 202). Compared with other malignancies, ovarian cancers account for a disproportionate number of deaths. This is largely due to the fact that these tumours tend to spread silently throughout the peritoneal cavity often presenting as stage III or IV disease. However, matched stage for stage, the five year survival rate from all ovarian tumours is comparable to that for breast, colorectal and cervical carcinomas. At present Stage I ovarian cancers are only diagnosed in about 20% of cases as compared with more than 50% of breast cancers (203). It is this skew towards late presentation, rather than marked differences in mortality at any given stage, that results in the high death rate associated with ovarian cancer.

Ovulation appears to be a major risk factor for ovarian cancer, since pregnancy (204), the contraceptive pill (205), and breast feeding (206) are all associated with a decreased relative risk of developing the disease, while there is evidence that ovarian hyperstimulation may increase the risk (207). Rarely, ovarian cancer is a familial disease and these cases have been associated with the genes BRCA1 and BRCA2 (208). Familial cases probably account for less than 5% of all ovarian cancers (200). Other factors which have been implicated include socio-economic status (209), childhood infections, particularly mumps (210), obesity (211), diet (212) and the use of talc (213). The relationship between benign epithelial tumours and ovarian malignancies has not been resolved and there is still dispute as to whether borderline tumours necessarily progress to invasive disease. There is some evidence that endometriosis plays a role in the development of endometrioid tumours (214).

1.4.2. Clinical features and diagnosis of ovarian cancer

Ovarian cancers often spread asymptptomatically throughout the peritoneal cavity. The first symptoms may simply be those of general malaise. Irregular menses can occur in pre-menopausal women. Other symptoms include abdominal distension; abnormal vaginal bleeding; symptoms referable to involvement of the gastrointestinal tract, such as constipation and pain; and symptoms referable to involvement of the urinary tract such as dysuria, haematuria and symptoms of renal failure. Weight loss is another
common presenting symptom. The most important physical sign is the presence of a fixed, irregular pelvic mass. Patients are frequently cachectic and there may be ascites. The presenting symptoms and signs reflect the pattern of spread of ovarian tumours. Direct extension of tumour into neighbouring structures, exfoliation of malignant cells into the peritoneal cavity and spread to the retroperitoneal lymph nodes (215) are the most frequent means of tumour dissemination. Blood borne metastases are rare (216).

The most sensitive and specific combination of tests to determine whether a pelvic mass is malignant, is the serum CA125 level together with transvaginal ultrasonography (217), although the value of this combination for routine screening is still disputed (218). By themselves these tests are not so useful. The marker CA125 is raised in only 50% of Stage I cancers (219) and may be elevated in a variety of other diseases. Transvaginal ultrasonography is specific for ovarian tumours of all kinds, but is not particularly sensitive (220). Other serum markers, such OVX-1, M-CSF (221) and HER-2/neu (222) have also been evaluated. The role of newer imaging modalities, such as colour Doppler scanning, computerised tomographic scanning, magnetic resonance imaging and positron emission tomography, remains to be explored.

### 1.4.3. Pathological classification of ovarian tumours

A simplified version of the WHO Histological Classification of Ovarian Tumours is given in table 2 (223). Ovarian carcinomas account for more than 90% of ovarian malignancies. Six types are recognised in the classification of epithelial ovarian tumours; serous, mucinous, endometrioid, clear cell, Brenner and unclassifiable. In common with other peritoneal tumours, ovarian carcinomas arise mainly from the coelomic epithelium which covers the ovary (224) either directly or from inclusion cysts (225). A number of studies indicate a clonal origin for malignant epithelial cells (226).

Serous tumours consist of tall columnar epithelial cells, lining cysts filled with serous fluid. Microscopic papillae are another characteristic features. Malignant tumours are recognised by epithelial cells piling up into more than one layer. Serous cystadenocarcinomas account for approximately 40% of all cancers of the ovary. Mucinous tumours have a similar histological appearance, but they tend to produce larger multiloculated cystic structures filled with viscid fluid rich in glycoproteins. They are not as common as serous tumours and make up about 10% of ovarian malignancies. Endometrioid carcinomas account for 20% of ovarian carcinomas.
Their distinguishing feature is the presence of tubular gland-like structures which resemble those seen in endometrial carcinomas. Clear cell tumours and Brenner tumours are both much less common. Clear cell carcinomas may be solid or cystic whereas Brenner tumours consist of nests of cells within a dense stroma (223).

1.4.4. Molecular markers in ovarian cancer
Changes in a large number of proteins involved in the normal regulation of cell growth and proliferation have been identified in ovarian cancer. In this section, some of these will be described, with particular emphasis on growth factors. This list cannot be exhaustive, but will serve to illustrate some of the major findings to date. Changes have also been reported in oestrogen and progesterone receptors, androgen receptors, gonadotropin releasing hormone receptors and somatostatin receptors in ovarian tumours (see (227) for review). A variety of other tumour markers, whose function is not yet known, have also been described, such as CA125, a surface glycoprotein, cancer associated serum antigen and inhibin and activin, which are regulators of gonadal cell growth.
<table>
<thead>
<tr>
<th>Tumours of surface epithelium</th>
<th>Serous tumours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>serous cystadenoma</em></td>
</tr>
<tr>
<td></td>
<td><em>borderline serous tumour</em></td>
</tr>
<tr>
<td></td>
<td><em>serous cystadenocarcinoma</em></td>
</tr>
<tr>
<td></td>
<td><em>adenofibroma and cystadenofibroma</em></td>
</tr>
<tr>
<td>Mucinous tumours</td>
<td><em>mucinous cystadenoma</em></td>
</tr>
<tr>
<td></td>
<td><em>borderline mucinous tumour</em></td>
</tr>
<tr>
<td></td>
<td><em>mucinous cystadenocarcinoma</em></td>
</tr>
<tr>
<td>Endometrioid carcinoma</td>
<td></td>
</tr>
<tr>
<td>Clear cell adenocarcinoma</td>
<td></td>
</tr>
<tr>
<td>Brenner tumour</td>
<td></td>
</tr>
<tr>
<td>Undifferentiated carcinoma</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Germ cell tumours</th>
<th>Teratoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Benign</em></td>
</tr>
<tr>
<td></td>
<td><em>cystic teratoma</em></td>
</tr>
<tr>
<td></td>
<td><em>solid teratoma</em></td>
</tr>
<tr>
<td></td>
<td><em>malignant</em></td>
</tr>
<tr>
<td></td>
<td><em>monodermal or specialised</em></td>
</tr>
</tbody>
</table>

| Sex cord-stromal tumours     | Granulosa-theca cell tumours |
|------------------------------| *granulosa cell tumour* |
|                              | *thecoma* |
|                              | *fibroma* |
| Sertoli-Leydig cell tumour   |                            |
| Gonadoblastoma               |                            |

| Unclassified tumours         |                            |
| Metastatic tumours           |                            |

Table 1.4. A simplified version of the WHO classification for ovarian neoplasms.
Growth factor receptors may also be proto-oncogenes. For instance c-erbB1 encodes the epidermal growth factor receptor (EGFR), to which both TGF-α and EGF can bind. The reported expression of EGFR in ovarian carcinoma is variable. In one immunohistochemical study, 77% of 87 ovarian carcinomas were positive, with staining localised predominantly to malignant epithelial cells (228). On the other hand Stromberg et al found expression of EGFR in all 17 ovarian cancer cell lines examined (180). EGFR status has been correlated with prognosis (229). A mutant EGFR exists, which is constitutively active even though it fails to bind EGF (230). However, DNA isolated from ovarian tumours has not revealed any apparent amplifications or rearrangements in the EGFR gene (231).

Another proto-oncogene that has tyrosine kinase activity and structural similarity to the EGFR is c-erbB2 (also known as HER-2/neu). This protein is expressed by normal ovary (232) and is over expressed in ovarian cancers (233). Over expression of this gene and survival of patients with ovarian tumours correlated significantly (234). Two other similar proteins that may be important in disease, c-erbB3 and c-erbB4, have been identified (235). Alterations in ras gene expression are known in ovarian cancer. Ras is involved in signal transduction between G proteins and downstream elements. It is both mutated (236) and over expressed (237) in ovarian tumours. C-myc amplification also occurs, particularly in serous adenocarcinomas (238).

Changes in the genes described above produce dominant effects. Alterations in tumour suppressor genes, which act in a recessive fashion have also been found. One of the best know such genes is p53. This is involved in the regulation of the cell cycle and controls the entry of cells into phase by controlling the activity of Rb. P53 is also involved in pathways leading to apoptosis. Mutations in p53 have been demonstrated in 50% of ovarian cancers (239), but not in borderline or benign disease (240). Mutations in p53 also lend support to the clonal nature of ovarian cancer (226). Mutations in the gene BRCA1 occur in familial breast and ovarian cancer. BRCA1 codes for a novel protein with an amino-terminal RING finger motif. It is involved in transcriptional regulation, DNA repair and site-specific recombination. Mutations in BRCA1 have been identified in up to half the members of breast-ovarian families (241). Like p53, the BRCA1 gene is located on the short arm of chromosome 17 in humans. This seems to be particularly susceptible to allele loss in ovarian cancer. For instance, of 120 ovarian cancers, 53% had allele loss and of these 87% had losses at multiple loci (242).
1.4.5. Management of ovarian cancers

Cytoreductive surgery remains the mainstay of treatment for ovarian cancer. At surgery, the extent of the disease is staged in order to assess the prognosis and to guide subsequent therapy. The International Federation of Gynaecology and Obstetrics (FIGO) staging system is given in table 1.5.

It is possible to treat some stage I or II tumours with surgery alone (243). However the majority of cases require chemotherapy or radiotherapy as well. The principle aim of surgery is to debulk the tumour mass as much as possible. Many studies have shown that in advanced ovarian cancer, the amount of residual tumour after the first laparotomy is the most important prognostic factor (244, 245). Surgery is not only performed to reduce the tumour mass, but may be performed palliatively, for instance to relieve bowel obstruction.

Adjuvant therapies include chemotherapy and radiotherapy. Because of the high morbidity associated with radiotherapy, its role is limited (246). A variety of chemotherapeutic agents are used in the treatment of ovarian cancers. They include cyclophosphamide, doxorubicin and cisplatin-based compounds. To date, two-drug combination regimens that include platinum-based compounds would appear to be as effective as multi-drug regimens, but with less toxicity (247). Of the platinum-based compounds available, cisplatin in combination with cyclophosphamide appears to give a better progression-free survival than carboplatin and cyclophosphamide (248). Newer agents include paclitaxel (derived from the bark of the Pacific yew, Taxus brevifolia) and synthetic analogues such as taxotere. Paclitaxel has now been licensed in the UK for treatment of patients with advanced ovarian cancer in combination with cisplatin (249). Initial trials with these drugs are promising. The benefits of chemotherapy are most apparent in high grade, advanced stage disease (250).
<table>
<thead>
<tr>
<th>Stage</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Growth limited to the ovaries</td>
</tr>
<tr>
<td></td>
<td>Ia</td>
</tr>
<tr>
<td></td>
<td>Ib</td>
</tr>
<tr>
<td></td>
<td>Ic</td>
</tr>
<tr>
<td>II</td>
<td>Growth involving one or both ovaries with pelvic extension</td>
</tr>
<tr>
<td></td>
<td>IIa</td>
</tr>
<tr>
<td></td>
<td>Ib</td>
</tr>
<tr>
<td></td>
<td>IIc</td>
</tr>
<tr>
<td>III</td>
<td>Tumour involving one or both ovaries with peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes; superficial liver metastases equals stage III; tumour is limited to the true pelvis but with histologically proven malignant extension to small bowel or omentum</td>
</tr>
<tr>
<td></td>
<td>IIIa</td>
</tr>
<tr>
<td></td>
<td>IIIb</td>
</tr>
<tr>
<td></td>
<td>IIIc</td>
</tr>
<tr>
<td>IV</td>
<td>Growth involving one or both ovaries with distant metastases; pleural effusion with positive cytology; parenchymal liver metastases.</td>
</tr>
</tbody>
</table>

Table 1.5. FIGO staging for primary carcinoma of the ovary.
1.5. The physical environment of solid tumours

1.5.1. Introduction
In conventional histopathology texts, most emphasis is placed on descriptions of the tumour cells themselves. But supporting these cells and often accounting for a large proportion of the tumour volume is the stroma. This consists of connective tissue, fibroblasts, blood vessels and infiltrating cells of the host immune system. The stroma provides a scaffold on which cells can grow and also serves to carry the blood supply to the tumour. The microvasculature is critical to tumour growth and has been the subject of several studies attempting to relate neovascularisation to clinical outcome.

Infiltrating leukocytes were first recognised as a feature of solid tumours by Virchow in the 1860s (251). He attributed their presence to the fact that tumours frequently arise in sites of chronic inflammation. A description of these cells in human ovarian cancers forms the subject of chapter 2. Another common feature of tumours are the microscopic regions of cell death which contain necrotic cells, apoptotic bodies, red cell fragments and eosinophilic hyaline. These regions may develop as a result of inadequacies in their blood supply. As will be contended within this thesis, the presence of necrotic regions may in part account for the distribution of infiltrating cells. Oxygen is one factor delivered to tumour cells, which as well as being necessary for efficient cellular metabolism, regulates the expression of a wide variety of genes. In this section, the relationship between tumour vasculature, tumour growth and tumour oxygenation will be described, with particular reference to ovarian carcinomas.

1.5.2. Angiogenesis, oxygen delivery and tumour growth
The process of angiogenesis is central not only to the growth of primary tumours but also for the development of metastases. Experimental tumours separated from their blood supply stop growing at a size of 1-2mm³, but growth can be reinstated if vascularisation is permitted (252). The idea that tumour growth is dependent on nutrient delivery was strengthened by an early finding, in a mouse model, that tumour cells surround capillaries in a cylindrical configuration to a radius of 150-200µm, which was the estimated diffusion distance for oxygen (253). In fact, despite neovascularisation, direct measurements of tumour oxygenation show that the median oxygen tension within tumours is significantly lower than that in surrounding normal tissues. Using the polarographic (Eppendorf) needle electrode, in which polarisation is proportional to oxygen tension, Nordsmark et al (254) determined that the median oxygen tension in the majority of head and neck nodes from 14 patients with head and
neck cancers was significantly lower (≤10mmHg in 40% of nodes) than in the surrounding subcutaneous tissues (30-60mmHg). Part of the reason for the low observed oxygen tensions may be the presence of tumour necrosis. In C3H mouse mammary carcinomas implanted into the feet of female mice the percentage of oxygen tensions ≤5mmHg increased with tumour size, but this increase was lost by taking into account the amount of the total tumour volume that was necrotic, implying that such regions make a significant contribution to low oxygen values (255). Other studies indicate that oxygen tension adjacent to necrotic regions is very low. The gene for VEGF is upregulated by hypoxia (256) and in situ analysis of glioblastomas has shown that it is expressed immediately adjacent to necrotic foci (257). Another technique used to measure oxygen tension directly is phosphorescence quenching microscopy. In a human colonic carcinoma xenograft model in SCID mice (258) oxygen tension declined away from blood vessels, such that values at ≥150µm were nearly anoxic (0-0.5mmHg). The distances in animal models are consistent with those in human tumours. In a histological analysis of carcinoma of the oesophagus, the mean distance between tumour capillaries and the onset of necrosis was 92±34µm (259). The effect of hypoxia on the proliferation of ovarian cell lines has also been studied. OW-1, SAU, and SKA cells cultured at ≤10mmHg for 24 hours remained viable as determined by trypan blue exclusion, but the decrease in the incorporation of [3H] thymidine indicated that there was an 80-90% inhibition of DNA synthesis (260).

As well as some regions existing permanently at low oxygen tensions, tumour perfusion may fluctuate rapidly. Using a laser Doppler system, Hill et al demonstrated that erythrocyte flux in over a quarter of human tumours changed 2-fold over a study period of 60 minutes and that more than half of these changes occurred within 20 minutes (261). The reason for these fluctuations in perfusion may include the architecture of the tumour vasculature. Ovarian carcinoma microvasculature has been reconstructed from factor VIII stained serial sections and compared to that of normal ovary (262). Tumour vasculature has a bizarre, chaotic pattern, quite distinct from the regular array of vessels in normal ovary. Other reasons for fluctuations in blood supply may include transient occlusion by circulating cells or by cells derived from the tumour. This can lead to a complete cessation of blood flow, or to the dissociation of plasma flow from the cellular elements in blood (D. Chaplin, personal communication). Thus both the distance from the microvasculature and fluctuations in the local delivery of oxygen and nutrients can contribute to the low oxygen tensions within tumours.
1.5.3. Tumour vascularisation and prognosis

Several studies of human solid tumours have demonstrated the importance of tumour vasculature in disease progression. Weidner et al assessed neovascularisation in histological sections from 49 patients with carcinoma of the breast, by immunohistochemical staining for factor VIII. They found that the number of microvessels in the most intensively vascularised high power fields was associated with a significant increase in the risk of local or distant metastases (263). Similar findings have been reported for cutaneous melanoma (264). Vascular 'hot spots' have been linked with malignancy in ovarian tumours. In advanced stage ovarian cancers (stage III or IV), higher vessel counts were associated with a worse prognosis (265). Microvessel counts determined by staining endothelial cells for factor VIII were significantly higher in carcinomas than benign cystadenomas (266). More recently, Schoell et al related the mean endothelial area to outcome in histological sections of tumours from 28 patients with stage IIIc ovarian carcinomas (267). There was a highly significant difference in the endothelial area between those who survived for a prolonged period (9.8±3.2 years) and those who died (1.9±1.2 years).
1.6. Aims of the thesis

The initial aim of this thesis was to assess and quantify the types and distribution of infiltrating leukocytes in human ovarian tumours, both to assess the contribution that different cell types might make to the infiltrate and to compare the infiltrate in these tumours with those described in other tumours.

The second aim was to assess the expression of CC chemokines likely to be involved in promoting the cellular infiltrate. The production of MCP-1 was analysed in particular detail, since evidence from other groups indicated that it might be expressed by human ovarian cancers.

The final aim of the thesis was to determine those factors that might be responsible for regulating chemokine expression within ovarian tumours, with particular reference to the role of TNF-α and oxygen tension.
Chapter 2

The leukocyte infiltrate in human ovarian cancer

2.1. Introduction
Since Virchow (251) many studies have examined the leukocyte infiltrate of human tumours using histochemical or immunohistochemical staining of tumour sections or by analysing the cell populations in disaggregated tumours. Attempts were made to correlate the infiltrate with prognosis (268) and the expression of MHC antigens (269). Furthermore, since tumours tend to grow despite the presence of an infiltrate, factors have been sought that inhibit leukocyte activities (270, 271).

Even before the advent of immunohistochemistry, numerous studies recognised the presence of lymphocytes and round cells in human tumours, including carcinomas of the breast, stomach, colon, bladder, kidney and skin (reviewed in (268)). Two studies in the late 1970s concluded that lymphocytes and macrophages were the main infiltrating cell types. Tötterman et al examined the cellular infiltrate in carcinomas of the thyroid and breast and in testicular seminomas. They found a predominance of monocytes, tissue macrophages and lymphocytes in the carcinomas and T cells and plasma cells in the seminomas (272). Mechanically-prepared tumour cell suspensions from carcinomas of the breast, colon, stomach, lung and rectum were found to contain both lymphocytes (0.5-5%, mean 2%) and macrophages (1-28%, mean 7.4%) (273). Macrophages were identified in four tumours by staining for α-napthyl esterase. Although they were mainly found within and around the tumour parenchyma, they also localised to necrotic areas. In a subsequent study (274) lymphocytes were counted in haematoxylin and eosin stained tissue sections and were found to be generally more numerous in the stroma. These infiltrating cells were considered to be part of a cellular immune response. Other cell types, particularly granulocytes have not been found so consistently. For instance, Wood and Gollahon found that the number of Fc-receptor positive cells in suspensions prepared form 39 tumours varied from 2-56%, but they were able to identify very few granulocytes morphologically (275).

More recent studies have used immunohistochemistry with specific monoclonal antibodies to phenotype infiltrating cells. CD3+, CD45 RO+ and CD68+ populations were all found in a series of 75 formalin-fixed, paraffin embedded, thyroid carcinomas...
CD3+ T cells and macrophages occurred in comparable numbers, but there were generally more CD3+ T cells than CD45RO+ cells. However, in malignant melanoma Strohal et al (277) found more CD3+ T cells than macrophages. They looked at frozen sections and concluded that CD4+ and CD45RA+ cells were more numerous than CD8+ and CD45RO+ cells respectively. However, the proportion of CD8+ cells increased in more advanced tumours. Frozen sections of colon carcinomas have been similarly examined (278). The majority of the infiltrate consisted of macrophages and CD4+ α:β T cells. However, CD8+ T cells were also abundant, particularly in close association with tumour cells. In carcinoma of the breast high numbers of CD45RO+ T cells, CD68+ macrophages and B cells have been found (279) in paraffin-embedded material. Lwin et al (280) found similar results. Within the tumour epithelium, the infiltrating population consisted largely of suppressor/cytotoxic T lymphocytes with a smaller number of macrophages. T helper cells were seen in only 1 out of 10 subjects and were present in very low numbers. In the stroma, there were roughly equal numbers of suppressor/cytotoxic cells, macrophages and plasma cells. CD8+ cells have also been reported to predominate in hepatocellular carcinoma (281).

Several studies have specifically addressed the nature of the leukocyte infiltrate in ovarian cancer. Haskill et al (282) used sedimentation-velocity to separate the components of the inflammatory infiltrate in 38 ovarian carcinomas. They concluded that T cells and macrophages were the dominant components and that very few B cells and NK cells were present. However, Kabawat et al (269) in an immunohistochemical analysis of cryostat sections from 70 tumours concluded that the majority of the infiltrate consisted of CD4+ T-cells with very few macrophages. Again they found few B cells and NK cells. In another study on frozen sections, CD4+ cells again made up the majority of the T cell infiltrate (283). However, other authors consider that CD4+ T cells are difficult to assess due to the expression of CD4 by other mononuclear cells (284). In the only study so far of ovarian tumours using paraffin embedded sections, Dietl et al concluded that in dysgerminoma of the ovary the tumour infiltrating lymphocytes were predominantly CD8+ and that most of the intra-tumoural T-cells expressed the α:β T cell receptor (285). Other studies have employed FACS analysis of either disaggregated whole tumours or cells from ascites (286, 287). All of these studies conclude that T cells form the majority of the infiltrate and that B cells and NK cells account for less than 5% of the infiltrating cell population.
Although antibodies to most of the well known leukocyte markers are available for use in frozen sections, previous experience in our laboratory with frozen specimens of ovarian tumours indicated that tissue architecture is frequently poorly preserved. The main reason for this is that these tumours are very friable, leading to damage particularly during processing for immunohistochemistry. Ice crystal formation due to slow freezing or partial freeze-thawing, for instance while cutting sections, is another problem. Water frozen rapidly does not crystallise, but forms a glass with little increase in its volume and hence minimal tissue damage. However, water can crystallise at temperatures just below 0°C. Therefore, a tissue block does not need to thaw in order for damage to occur. Another feature of frozen sections is that areas of necrosis appear to be ripped out when sections are cut, again leading to a loss of important morphological features.

For these reasons paraffin embedded material was used for this part of the study. In these sections, tissue architecture is generally well preserved and it is possible to distinguish all the main components with haematoxylin and eosin staining. For immunohistochemistry with some antibodies, pre-processing with trypsin or by microwaving was required to enhance antigen exposure. This can produce some damage to the section, particularly microwaving, but this was not found to be a significant problem. The main disadvantage of using paraffin sections was that antibodies to CD4 were not available.

Conventional morphometric techniques, as described in Aherne and Dunhill (288), were used to assess the relative number of infiltrating cells. Differences in cell size can produce apparent differences in cell number when assessed in a 2-dimensional tissue section. Counts were therefore corrected using the equivalent nuclear diameter for each cell type.

2.2. Aims of the chapter
The aims of this chapter were to define the phenotypes of leukocytes infiltrating human ovarian carcinomas and to assess their distribution quantitatively. A limited study was performed using markers of activation for both T cells and macrophages to assess functional aspects of the infiltrating cells.
2.3. Methods

2.3.1. Patient selection and sample collection
The infiltrating leukocyte population was assessed in twenty human epithelial ovarian tumours. Nine were collected at the time of operation from St. Thomas's Hospital, Lambeth and The Samaritan Women's Hospital, Paddington and were fixed in formol saline for 24 hours. Eleven tumours were obtained from the Hammersmith Hospital (London, UK) archives. Fifteen tumours were classified as papillary serous adenocarcinomas (five grade 1, five grade 2 and five grade 3), two were borderline serous tumours, two were papillary endometrioid tumours (grade 2) and there was one solid endometrioid tumour (grade 3). Of the 17 patients whose ages were known, the mean age was 66.88 years (SD 9.51). All tissue samples were paraffin embedded prior to cutting 4μm sections onto silane coated slides. The histological type and grade of all specimens was reassessed at the time of the study by Prof. Gordon Stamp, using previously defined criteria (289).

2.3.2. Immunohistochemistry
2.3.2.i. Antibodies
In order to phenotype the infiltrating cell population, a panel of mainly mouse monoclonal anti-human antibodies was used. Most of these antibodies recognise epitopes on cluster differentiation (CD) antigens, the exceptions being mast cell tryptase and neutrophil elastase. In addition sections were stained for 2 markers of lymphocyte cytotoxic activity and for the presence of the HLA class II molecule HLA-DR. Table 2.1 gives the specifications of the individual antibodies used. The presence of eosinophils was determined in haematoxylin and eosin stained sections by their characteristic staining and morphology.
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Antigen</th>
<th>Antibody</th>
<th>Type</th>
<th>Isotype or subclass</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>CD68</td>
<td>PG-M1</td>
<td>mo*</td>
<td>IgG3, κ</td>
<td>DAKO</td>
</tr>
<tr>
<td>T cells</td>
<td>CD3</td>
<td>anti-T cell</td>
<td>rab#</td>
<td>IgG1, κ</td>
<td>DAKO</td>
</tr>
<tr>
<td>Cytotoxic T cells</td>
<td>CD8</td>
<td>C8/144B</td>
<td>mo</td>
<td>IgG2a</td>
<td>DAKO</td>
</tr>
<tr>
<td>Memory T cell</td>
<td>CD45RO+</td>
<td>UCHL1</td>
<td>mo</td>
<td>IgG2a, κ</td>
<td>DAKO</td>
</tr>
<tr>
<td>B cells</td>
<td>CD20</td>
<td>L26</td>
<td>mo</td>
<td>IgG2a, κ</td>
<td>DAKO</td>
</tr>
<tr>
<td>Natural killer cells</td>
<td>CD57</td>
<td>NK1</td>
<td>mo</td>
<td>IgM, κ</td>
<td>DAKO</td>
</tr>
<tr>
<td>Mast cells</td>
<td>mast cell tryptase</td>
<td>AA1</td>
<td>mo</td>
<td>IgG1, κ</td>
<td>DAKO</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>neutrophil elastase</td>
<td></td>
<td>mo</td>
<td>IgG1, κ</td>
<td>DAKO</td>
</tr>
<tr>
<td>Cytotoxic lymphocytes</td>
<td>membrane associated protein</td>
<td>TIA-1</td>
<td>mo</td>
<td>IgG1, κ</td>
<td>Coulter Clone</td>
</tr>
<tr>
<td>Cytotoxic lymphocytes</td>
<td>granzyme B</td>
<td>GrB7</td>
<td>mo</td>
<td>IgG2a</td>
<td>Monosan</td>
</tr>
<tr>
<td>HLA Class II</td>
<td>HLA-DR</td>
<td>TAL-1B5</td>
<td>mo</td>
<td>IgG2b</td>
<td>DAKO</td>
</tr>
</tbody>
</table>

*mouse monoclonal; #rabbit polyclonal

Table 2.1. Antibodies used to define the leukocyte phenotypes in human ovarian carcinomas.

2.3.2.ii Streptavidin-peroxidase immunostaining method

For mouse monoclonal antibodies
1. Deparaffinise sections and take down through graded alcohols (50%, 70%, 95%, 100%) to water.
2. Block endogenous peroxidase (A), 10 minutes.
3. Wash in running tap water. Trypsinise (B) or microwave as necessary (C). Rinse in PBS.
4. Incubate for 35 min. in primary antibody at optimal concentration.
5. Wash in 2 changes of PBS, 5 min. each.
6. Incubate in biotinylated rabbit anti-mouse (DAKO), diluted 1:300.
7. Wash in 2 changes of PBS, 5 min. each.
8. Incubate for 30 min. in streptavidin-peroxidase (DAKO), dilute 1:500.
9. Wash in 2 changes PBS, 5 min. each.
10. Prepare peroxidase substrate 3,5-diaminobenzidine (DAB); 5mg DAB, 10ml PBS, 20µl 30% H₂O₂.
11. Incubate sections in peroxidase substrate for 2 minutes.
12. Wash in water and counterstain in haematoxylin for 30s-2 min.
13. Dehydrate through graded alcohols
15. Mount in DPX-type mountant (BDH Laboratory Supplies, Poole, Dorset UK).

For rabbit polyclonals
Steps 1-3 as above
4. Incubate sections in normal swine serum (DAKO) diluted 1:25 fro 15 min. Drain.
5. Incubate for 35 min. in primary antibody at optimal concentration
6. Wash in 2 changes of PBS, 5 min. each
7. Incubate for 35 min. in biotinylated swine anti-rabbit (DAKO), diluted 1:500.
Then proceed from step 7 above.

A. Blocking of endogenous peroxidase.
2.4ml of 30% H₂O₂, in 400ml methanol.

B. Trypsinisation
1. 400mg bovine trypsin, 400mg CaCl₂, 400 distilled H₂O.
2. Mix and incubate at 37°C. Adjust pH to 7.8 with 0.1M NaOH.
3. Incubate sections for 15 min.

C. Microwaving
1. make up 0.01M sodium citrate and adjust pH to 6
2. microwave sections in boiling solution for 10 min.

Steps 1, 2, 12 and 13 were performed using a Bayer DRS 601 automatic slide stainer.
Slides were mounted using a Tissue-Tek automatic mounter.
2.3.3. Morphometric analysis of tissue sections

All cell counts were performed using a Nikon Labophot II microscope (Nikon, Kingston, Surrey, UK) at a magnification of x200 (x20 objective, x10 eyepiece). In order to achieve consistency in counting, only cells with cytoplasmic staining and a nucleus were included, even though this may have led to an underestimation of some cell types by excluding effete cells. This was particularly applicable to macrophages in areas of necrosis. The area counted in each section was chosen randomly from a representative field of tumour, but areas that were predominantly stroma were excluded. Cell counts were performed with the aid of a 10x10 index grid (Graticules Ltd, Tonbridge, Kent, UK). For each section, 9 areas were assessed with the grid arranged in a 3x3 pattern, to give a total area of 2.3mm².

Area counts were carried out using a modified Chalkley array (Graticules Ltd) over the same area as the cell counts. The modified Chalkley array consists of 25 randomly arranged points; the proportion of points coincident with any tissue component is proportional to its area and volume fractions (290). In addition to total cell counts, four different components within the tumours were assessed: tumour cell islands, stroma, areas of necrosis and areas of space (figure 1). Areas of necrosis were defined as having cell debris, apoptotic bodies, or red cell fragments. Areas of space included both real spaces, for instances between tumour papillae and those created by fixation and processing.

The method of DeHoff and Rhines (291) as described by Aherne and Dunill (288) was used to estimate the number of cells per mm³. Equivalent nuclear diameters for each cell type were determined by tracing a minimum of 100 cell circumferences using an image analysis programme (Lucia, Nikon, UK) and a x100 objective oil immersion lens. A frequency histogram of equivalent diameters was constructed for each cell type. The left hand tail of each histogram was estimated to correct for 'optically lost caps'. The mean tangent diameter (D) was then calculated from the mean of the set of equivalent diameters (d), according to the formula:

\[ D = \frac{4d}{\pi} \]

Estimates of equivalent diameters derived from cell circumference tracing were validated by assessing the mean diameter of 40 erythrocytes contained within the sections (mean=6.61µm, standard deviation=0.66µm).
Cell counts were combined with area counts to produce an estimate of the number of cells within each tumour compartment/mm. This was then converted to an estimate of cells/mm³ using the formula:

$$N_v = \frac{N_A}{(D+t)}$$

where $N_v =$ number of cells/mm³, $N_A =$ number of cells/mm², $D =$ mean tangent diameter and $t =$ section thickness, taken to be 4µm.

### 2.3.4. Statistical methods

Since the data were not normally distributed, medians were used to summarise the data and non-parametric methods of analysis were used to calculate P values. Wilcoxon's matched pairs signed rank test was used for all comparisons between matched pairs of data. Spearman's rank correlation was used to determine the relationship between the CD3+, CD8+ and CD45RO+ T-cell populations and to test whether a correlation existed between the number of chemokine expressing cells and the number of CD68+ or CD8+ cells. The Mann-Whitney U test was used to test whether there was a significant difference between the number of CD68+ and CD8+ cells in frozen and paraffin sections and also to assess the relationships between the leukocyte populations, chemokines and tumour grade (292). Within each analysis up to 10 comparisons were made. Using the Bonferroni method to correct for multiple comparisons (293), only P values <0.005 were considered to be statistically significant. However, since this method is highly conservative P values <0.05 are also shown.
2.4. Results

2.4.1. Estimation of mean tangent nuclear diameters
The nature of the cellular infiltrate was assessed in 20 epithelial carcinomas. For each cell type, 9 high power fields were counted at a magnification of x200 (x20 objective, x10 eyepiece). The mean tangent nuclear diameters were calculated from the measured equivalent nuclear diameters (Table 2.2) for each phenotype and were used to estimate the number of cells found per unit volume from the actual number counted per unit area.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Mean tangent nuclear diameters (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+</td>
<td>5.3</td>
</tr>
<tr>
<td>CD8+</td>
<td>5.4</td>
</tr>
<tr>
<td>CD45RO+</td>
<td>5.1</td>
</tr>
<tr>
<td>CD68+</td>
<td>6.5</td>
</tr>
<tr>
<td>B cell</td>
<td>4.7</td>
</tr>
<tr>
<td>Natural killer cell</td>
<td>4.3</td>
</tr>
<tr>
<td>Mast cell</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Table 2.2. The mean tangent diameter of different leukocyte phenotypes. Mean tangent diameters were estimated from a population of equivalent nuclear diameters for each phenotype.

2.4.2. Estimation of tumour compartment volumes
The Chalkley array was used to determine the volume of tumour occupied by each compartment. The compartments were defined as tumour parenchyma, stroma, necrotic areas (defined by the presence of cell debris, apoptotic bodies, or red cell fragments) and areas of space between these components (figure 2.1). The proportion of whole tumour occupied by each of these compartments is given in Table 2.3.

The volume of whole tumour occupied by tumour parenchyma and stroma were similar (43% and 37% respectively). Necrosis accounted for approximately 4% of the volume of the tumour and that occupied by real and artefactual space, 16%. There were no significant differences between the volumes occupied by each compartment.
with tumour grade. Typically, necrotic regions were seen to consist of apoptotic bodies, eosinophilic hyaline, red cell fragments and mononuclear cells.

<table>
<thead>
<tr>
<th>Parenchyma</th>
<th>Stroma</th>
<th>Necrosis</th>
<th>Space</th>
</tr>
</thead>
<tbody>
<tr>
<td>43%</td>
<td>37%</td>
<td>4%</td>
<td>16%</td>
</tr>
<tr>
<td>(14-90%)</td>
<td>(8-82%)</td>
<td>(0-37%)</td>
<td>(0-54%)</td>
</tr>
</tbody>
</table>

Table 2.3. The proportion of tumour volume occupied by its various components. The proportion of total tumour volume occupied by parenchyma, stroma, necrosis and space was estimated for 20 tumours using Chalkley point counting. The mean and range are given for each tumour component.

![Figure 2.1. The histological appearance of an epithelial ovarian cancer. An example of a grade 2 serous papillary adenocarcinoma stained with haematoxylin and eosin and viewed at low power (x20 objective). Cell counts were assessed in the tumour parenchyma (T), stroma (S), necrotic regions (N) and areas of space (Sp).](image-url)
2.4.3. Estimation of the number and distribution of infiltrating leukocytes

2.4.3.i. Comparison of phenotypic markers

Data from the estimation of cell counts and tumour compartment volumes was combined to produce estimates of the cell densities (cells/mm$^3$) both for individual compartments and the tumour as a whole. These estimates are based on the assumption that the microscopic structure of the tumour is relatively homogenous. Two patterns of infiltrate were distinguishable. Cells present in large numbers were CD68+ macrophages, CD3+, CD8+ and CD45RO+ T cells, while those found in much lower numbers were B cells, natural killer (NK) cells and mast cells. Table 2.4 gives the median and range for each of 7 different cell types per mm$^3$ of total tumour.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Median (cells/mm$^3$)</th>
<th>% total infiltrate</th>
<th>Range (cells/mm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+</td>
<td>2,200</td>
<td>18.2</td>
<td>50-40,300</td>
</tr>
<tr>
<td>CD8+</td>
<td>2,800</td>
<td>23.5</td>
<td>0-18,200</td>
</tr>
<tr>
<td>CD45RO+</td>
<td>2,900</td>
<td>24.5</td>
<td>200-43,200</td>
</tr>
<tr>
<td>CD68+</td>
<td>3,700</td>
<td>31.5</td>
<td>600-15,200</td>
</tr>
<tr>
<td>NK cells</td>
<td>100</td>
<td>1.1</td>
<td>0-1,000</td>
</tr>
<tr>
<td>B cells</td>
<td>0</td>
<td>0</td>
<td>0-2,900</td>
</tr>
<tr>
<td>Mast cells</td>
<td>200</td>
<td>1.5</td>
<td>0-1,600</td>
</tr>
</tbody>
</table>

Table 2.4. The estimated number of infiltrating cells/mm$^3$ of whole tumour. Median values, percentage of the total infiltrate counted and ranges for 7 tumour infiltrating leukocyte phenotypes/mm$^3$ of total tumour are given.

All the tumours studied contained cells which were positive for the macrophage marker CD68 (see Weiss (294) for review) and for the markers CD3, CD8 and CD45RO, within both the stromal and epithelial areas. There was a significant difference between those cells present in high (CD3+, CD8+ and CD45RO+ T cells and CD68+ macrophages) and low (CD20+ B cells, CD57+ NK cells and mast cells) numbers ($P<0.0005$), but not between the cell types within each group (figure 2.2A). In general the infiltrating cell density was significantly higher in the stroma compared with tumour parenchyma (figures 2.2B and C). For instance the median value for the number of CD3+ T cells found within the stroma was 3,800 cells/mm$^3$ compared with
1,600 cells/mm³ in the tumour parenchyma (P<0.005). Similar values were obtained for CD45RO+ T cells. Median values for CD68+ macrophages were 6,700 cells/mm³ and 2,100 cells/mm³ in stroma and parenchyma respectively (P<0.05). In a previous study (data not shown), the proportion of macrophages, assessed by the anti-CD68 antibody EBM11 was estimated in frozen sections of serous and mucinous adenocarcinomas as between 1 and 28% cells/hpf (mean 7.8%) (295). Unlike the CD3+ population, there was no significant difference between the number of CD8+ cells found in the stroma and the tumour parenchyma (median values 4,300 cells/mm³ and 2,500 cells/mm³ respectively, P=0.083).

In the stroma, T cells occurred singly or in clusters but when associated with tumour cell islands they tended to occur alone (figures 2.3 and 2.4). There was no correlation between the number of infiltrating CD68+, CD3+, CD8+ or CD45RO+ cells and tumour grade. Of the cell types identified to be present in low numbers, there were no significant differences between the number of natural killer cells (figure 2.5A) and B cells (figure 2.5B) found in the stroma or tumour parenchyma (P=0.01 and P=0.08 respectively), but mast cells were virtually confined to the stroma (P<0.0005, figure 2.6A). These cells types were rarely seen in necrotic regions. Although eosinophils were seen in occasional sections within the stroma and associated with tumour cells, they were rare (figure 2.6B). Neutrophils were assessed using morphological criteria and a monoclonal antibody to neutrophil elastase. In contrast to the other cell types they appeared to be largely confined to blood vessels.

The most striking differences in cell density were seen in regions of necrosis (figure 2.7). Here a significantly greater number of macrophages were observed than any other cell type (P<0.0005, for all comparisons; median 12,700 cells/mm³, range 0-128,000 cells/mm³). Macrophages in the parenchyma and stroma appeared to be smaller than those found in regions of necrosis (figure 2.8). Macrophages in necrotic areas frequently had an activated morphology with abundant, foamy cytoplasm (figure 2.9). Since cells that appeared effete were excluded from the counts, the total number of macrophages may have been underestimated. In small areas of necrosis macrophages were present throughout, but in larger areas they tended to be restricted to the periphery of the region. Macrophages occasionally occurred alone within gland spaces.
Figure 2.2. Scatter plots of the calculated numbers of infiltrating leukocytes. Graphs of results in whole tumour (A), tumour parenchyma (B) and stroma (C). Cell numbers are expressed as (cells/mm³) for each of the 7 phenotypic markers. Horizontal bars represent median values. For each tumour compartment there was a significant difference (P<0.0005) between the number of cells positive for CD3, CD8, CD45RO and CD68 and those positive for CD57, CD20 and mast cell tryptase.
Figure 2.3. The distribution of T cells in human ovarian carcinomas. Low power view of CD3+ cells (A) and CD8+ cells (B) in a grade 2 papillary serous adenocarcinoma. The similarity in distribution of these two phenotypes is apparent in these serial sections. T cells were often clustered in the stroma, but were more dispersed in the tumour parenchyma.
Figure 2.4. The distribution of T cells in ovarian carcinomas. CD45RO+ cells in the same tumour region as figures 2.3, demonstrating the similarity in distribution (A). At high power CD8+ cells within the tumour parenchyma were closely apposed to the tumour cells, but without evidence of apoptosis or necrosis (B).
Figure 2.5. Natural killer cells and B cells in ovarian carcinomas.
In paraffin embedded sections of ovarian carcinomas, natural killer cells (A) and B cells (B) were found in much lower numbers than T cells and macrophages.
Figure 2.6. Mast cells and eosinophils in ovarian carcinomas. Mast cells (A) were found within the tumours. Eosinophils were rarely seen and were most obvious in haematoxylin and eosin stained frozen sections (B).
Figure 2.7. Macrophage and T cell densities in areas of necrosis. Scatter plot of the number of cells positive for the T cell phenotype markers versus the number positive for CD68 in regions of necrosis. Macrophages in these areas occurred at significantly higher density than any other cell type. The horizontal bars represent median values.
Figure 2.8. The distribution of macrophages within ovarian carcinomas. CD68+ macrophages within two grade 3 ovarian carcinomas. In both parenchyma (A) and stroma (B), macrophages tended to occur alone or in loose clusters.
Figure 2.9. The typical appearance of macrophages within necrotic areas. Macrophages within a necrotic region (A) of a grade 3 solid endometrioid tumour. Here these cells had abundant foamy cytoplasm and occurred at higher density than either the tumour parenchyma or stroma.
2.4.3.ii. Comparison of T cell markers

Spearman's rank correlation was used to examine the relationship between the three T cell markers, both for total cell counts and for cells found within tumour cell areas and stroma. In all cases there was a significant correlation between the three markers ($r_s > 0.7$ and $P < 0.005$). Since there was no significant difference between the total number of CD3+, CD8+ or CD45RO+ T cells, this suggests that the majority of infiltrating T-cells have a cytotoxic memory phenotype (figure 2.10).

![Figure 2.10. Correlations between T cell phenotypic markers. Scatter plot demonstrating the correlation between CD3+ T cells and CD8+ T cells. Using Spearman's rank correlation, $r_s = 0.85$ and $P < 0.005$.]

There was no correlation between these T-cell markers and the CD68+ macrophage population. Staining for CD4+ T cells was undertaken in frozen sections using the antibody MT310 (DAKO) in order to determine their contribution to the total T cell infiltrate. However, due to concerns that there might be cross-reactivity with other mononuclear cells (296) and since it was not always possible to discriminate accurately between the different cell types in these sections, CD4+ cells were not included in the final assessment.
2.4.4 T-cell functional markers

The assessment of the sections for the T cell phenotypic markers had indicated that they were rarely associated with regions of necrosis. Furthermore, inspection of T cells within the tumour parenchyma did not reveal evidence of local apoptosis. To assess the cytotoxic potential of these cells \textit{in vivo}, sections were stained for two different markers of T cell activation, the presence of granzyme B and the activation marker TIA-1. No staining for granzyme B (figure 2.11) was seen in any section and only weak occasional staining for TIA-1 was present (figure 2.12). Inflamed tonsil was used as a positive control for both of these markers. In stained cells a typical granular cytoplasmic staining pattern was found (figure 2.13).

![Figure 2.11. Immunohistochemistry for granzyme B.](image)

Serial sections of a grade 2 papillary endometrioid carcinoma stained for the markers CD8 (A) and granzyme B (B). Despite the presence of CD8+ cells, there was no staining for granzyme B in any of the sections examined.
Figure 2.12. Immunohistochemistry for TIA-1.
Staining for the T cell activation marker TIA-1 in a grade 1 papillary serous adenocarcinoma. Many CD8+ cells were present (A), but in a serial section there was very little staining for TIA-1 (B).
Figure 2.13. Controls for T cell activation markers.
Sections of inflamed tonsil were used as positive controls for staining with antibodies to granzyme B (A) and TIA-1 (B). In both cases numerous cells were present with a granular cytoplasmic pattern of staining.
2.4.5 HLA-DR expression and macrophage distribution
Since macrophages were found at highest density in necrotic regions and appeared to have an activated morphology, sections were stained with the antibody TAL-1B5 in order to assess the expression of class II HLA. Positive cells were seen throughout the stroma and parenchyma and in some areas tumour cells were also positive for 1B5. However, macrophages in necrotic regions were universally negative for this marker (figure 2.14).

![Figure 2.14. MHC class II expression in ovarian tumours.](image)

Figure 2.14. MHC class II expression in ovarian tumours.
Cells positive for HLA-DR, identified with the antibody TAL-1B5 were found both within the parenchyma and stroma, but within necrotic regions, CD68+ macrophages were not also positive for TAL-IB5.
2.5. Summary and discussion

The results indicate that the host cell infiltrate in human ovarian carcinomas consists largely of macrophages and T cells. Macrophages were the most numerous cell type present, although there were no statistically significant differences in the number of macrophages and CD3+ cells in either the tumour parenchyma or stroma. The high degree of correlation between cells positive for the markers CD3+, CD8+ and CD45RO+, implies that the majority of infiltrating T-cells have a cytotoxic memory phenotype but there was no evidence that these cells were actively cytotoxic judging by staining for TIA-1 and granzyme B. Other leukocytes such as B cells, NK cells and mast cells were present in low numbers, while eosinophils were seen only occasionally and neutrophils, with one exception, appeared to be largely confined to blood vessels.

Most CD3+ and CD45RO+ T cells were found in the stroma, but there was no significant difference between the number of stromal CD8+ cells and the number found in the tumour parenchyma. Although infiltrating cells in the stroma often occurred in aggregates (described by some pathologists as follicles), they were usually found alone when associated with tumour cells. In many tumours, it is the CD4+ phenotype that predominates, but in the ovarian tumours used in this study, the number of CD3+ and CD8+ cells were similar and correlated significantly. The reason for the difference may simply reflect variations between the infiltrate in different tumour types. However, the means of assessing the infiltrate may also contribute to the differences. In this study the infiltrate was assessed specifically in and around tumour parenchyma. In carcinoma of the colon the T cell populations were reported to vary according to their location, with a greater proportion of CD8+ cells associated with the tumour parenchyma (278). Anti-CD4 has also been reported to cross-react with monocytes and which may lead to an overestimation of the number of CD4+ T cells (275). For this reason it was not considered possible to assess the number of CD4+ cells in frozen sections accurately and they were not included in this analysis.

The existence of a lymphocyte infiltrate has attracted attention because of the potential of these cells to destroy tumour cells. T cells can be activated in vitro and will kill autologous tumour cells, particularly when stimulated with cytokines such as IL-2 and IFN-γ which are not normally expressed in these tumours (175). This approach has been used in clinical trials of therapy for advanced ovarian cancers with modest success. For instance, therapy with autologous T cells activated with IL-2 and retargeted using F(ab')2 fragments of the bispecific monoclonal antibody OC/TR, which is directed both to CD3 molecules on T lymphocytes and to folate receptors on
ovarian carcinoma cells, resulted in complete surgical and histological responses in 3/19 patients with stage III and IV disease, which lasted up to 26 months (297). Cytotoxic T cells from ovarian tumours can recognise the mucin (Muc-1) core peptide of polymorphic epithelial mucins that is expressed by a variety of epithelial cancers (298). Furthermore, in a study examining T cell clonotypes, Yamamoto et al (299) found accumulations of clonally expanded T cells in lymph nodes of patients with metastatic cancer in a pattern that would support the idea that the T cell infiltrate is the result of an antigen-driven immune response. On the other hand defective expression of the TCR ζ-chain has been found in T cells and NK cells from patients with ovarian cancer (300). This may in part explain the observation that isolated T cells do not display tumouricidal activity when unstimulated (272, 301). Alternatively, the relationship between the host and the tumour is being viewed in the wrong way. If the tumour is regarded as non-self, then a specific immune response would be expected. However, if it is not, then the results with freshly isolated T cells are hardly surprising (302).

CD68+ cells were assumed to represent tumour associated macrophages. They were found predominantly in the stroma, but also occurred in close proximity to tumour cells. Within tumour areas, the proportion of macrophages was up to 28% with a mean of 7.8% (295). The biggest difference in distribution of macrophages and the other cell types was their very high density in regions of necrosis. This phenomenon has been observed in other tumours. Furthermore, experiments with tumour cell spheroids, employing a variety of different tumour cell lines, have shown that monocytes accumulate preferentially at the margin of the central region of necrosis, while lymphocytes do not (C. Lewis, personnel communication). The intra-tumoural macrophages in necrotic regions were larger than those associated with the stroma or parenchyma and frequently had a foamy cytoplasm. These features are indicative of cells that are actively phagocytosing. In order to examine the possible functional status of macrophages associated with necrotic regions, the expression of HLA-DR was examined in situ with the antibody TAL-1B5 (303). Within both the tumour stroma and parenchyma, cells staining positive with this antibody were detected. Some tumour cells also expressed MHC class II. However, immunoreactivity to TAL-1B5 was not detected among CD68+ cells within necrotic regions. The explanation for this apparent lack of class II expression is not clear, but may contribute to the lack of tumour-specific immune responses.

Like T cells, macrophages freshly isolated from ovarian tumours are poor at killing tumour cell targets (304, 305). Similarly, this activity can be stimulated in vitro with
cytokines such as IL-1 and TNF-α, or with LPS (306). The function of tumour associated macrophages (TAM) remains disputed. Mantovani (307) tested the effect on tumour cell proliferation of TAM isolated from a chemically-induced murine sarcoma. He found that at low macrophage to tumour cell ratios (≤2:1), proliferation of tumour cells was consistently observed, although at high ratios (≥20:1), non-specific cytotoxicity occurred. Neither of these two ratios is inconsistent with the local concentrations of macrophages that were observed within the different tumour compartments. Macrophages are a rich source of cytokines and other growth factors. They are associated with TNF-α production in ovarian tumours (308) and macrophages isolated from ovarian tumours can secrete IL-1 and IL-6 (309). Furthermore, TNF-α can act as a growth factor for ovarian cancer cells (310) and may promote angiogenesis (311), although this is likely to be an indirect effect (312).

In this study, no evidence was found for cytotoxic T cells in vivo. Several reasons for this may exist. If the majority of tumour infiltrating cells are indeed CD8+, then there may be a lack of T cell help. There is little evidence that cytokines associated with Th1/Th2 differentiation, particularly IFN-γ and IL-2, are expressed in ovarian tumours (175). Combined with a relative paucity of CD4+ cells this could contribute to the lack of markers of T cell cytotoxicity found in situ. Both class I MHC antigens and class II are expressed by ovarian tumours (269). However, this study found a lack of class II expression in necrotic regions, regions that might be expected to be rich in potential antigens derived from tumour cells. Other reasons for the observed lack of cytotoxic T cells have also been proposed. In breast carcinomas, there is evidence that expression of the mucin antigen DF3/MUC1 can induce apoptosis in activated T cells (313) and in hepatocellular carcinoma, not only was there a significant loss of the CD95 receptor (Fas) from tumour cells, but in co-culture experiments, HepG2 hepatoblastoma cells expressing CD95L (FasL) mRNA were capable of killing CD95+ Jurkat lymphocytes (314). Another factor that may be important is the lack of co-stimulatory molecules that characterises some tumours (315). Finally, members of the chemokine family may inhibit the activation of T cells (316).

Little is known of the function of other leukocytes in solid tumours, although almost all studies agree that there are relatively few of them compared with T cells and macrophages. B cells usually comprise 5% or less of the infiltrating cell population. Due to their ability to kill tumour cells in vitro, attention has focused on the potential of NK cell as anti-tumour agents. However, the effector to tumour cell ratios used in vitro are completely at variance with the ratios seen in vivo and it is difficult to imagine how NK cells can do much damage to the malignant cell population at the numbers
observed, particularly since most are confined to the stroma. In a study on 17 ascites and 7 solid ovarian tumours, Introna et al found lower natural killer activity than in peripheral blood lymphocytes (317). The role of mast cells is not clear (see (318) for review), although, partly due to their ability to release vasoactive agents, few are required for them to be significant (Dr H Gould, personal communication). The role of neutrophils and eosinophils is also not known.

On the basis of the leukocyte phenotypes determined by immunohistochemistry, predictions were made as to those chemokines that might be expressed by human ovarian carcinomas. An assessment of the expression of four CC chemokines, MCP-1, MIP-1α, MIP-1β and RANTES forms the subject of chapter 3.

2.6. Conclusions
- CD68+ macrophages and CD3+, CD8+, CD45RO+ T cells accounted for most of the leukocyte infiltrate in human ovarian carcinomas.
- The high degree of correlation between the different T cell markers indicated that CD8+, CD45RO+ cells form the majority of the infiltrate.
- Macrophages were found throughout tumour stroma and parenchyma, but occurred at highest density in regions of necrosis.
- Macrophages in necrotic regions had a foamy appearance and did not express HLA-DR.
- T cells, particularly CD8+, were more evenly distributed and were not associated with apoptotic cells or regions of necrosis.
- Markers of T cell activation were virtually absent.
- B cells, natural killer cells and mast cells were present in low numbers. Their functional significance is not known.
- Neutrophils were virtually only seen within the lumena of blood vessels.
- Eosinophils were rarely seen.
Chapter 3

The expression of MCP-1 and other CC chemokines by ovarian tumours and their relationship to tumour infiltrating leukocytes

3.1. Introduction

The presence of a predominantly mononuclear infiltrate in human ovarian cancers, described in chapter 2, together with the relative absence of granulocytes, suggested that CC chemokines may play an important role in determining the nature of the leukocyte infiltrate. Furthermore, the presence of large numbers of macrophages and earlier work by Mantovani’s group establishing the presence of a tumour derived chemotactic factor (TDCF) for monocytes in ovarian cancers, provided some evidence that MCP-1 was produced by these tumours. In the early 1980s, Bottazzi et al used chemotaxis assays to determine the chemotactic activity for monocytes of culture supernatants from both murine sarcomas (319) and freshly disaggregated human ovarian carcinomas (320). A significant correlation was found between the activity of the supernatants from cultured murine tumour cells and the percentage of TAMs within the tumours. In both human and murine tumours, the activity appeared to be a protein, since the ability to chemoattract macrophages was lost after treatment with heat or proteases. Fractionation of the chemotactic activity suggested a molecular weight of approximately 12kDa. Other tumour cells were also able to produce monocyte chemotactic activity. Graves et al (321) demonstrated that antiserum to baboon smooth muscle cell-derived chemotactic factor (SMC-CF) blocked both SMC-CF induced monocyte migration and the activity released from cells derived from a melanoma, osteosarcoma, glioblastoma, fibrosarcoma and rhabdomyosarcoma.

MCP-1 was purified from the glioma cell line U-105MG (322), which constitutively expresses high levels, and cloned from both this cell line (56) and stimulated myelomonocytic cell lines (323) in 1989. Shortly afterwards, TDCF derived from experimental sarcomas was found to be the same as MCP-1 (93). Although it was highly likely that the chemotactic activity associated with ovarian tumours was MCP-1, this had not been shown conclusively.
Monocyte chemoattractant protein 1 (MCP-1) can be expressed by a wide variety of cell types in response to many different stimuli (see (324) for review). Despite the discovery of other chemokines that act on monocytes, MCP-1 remains the most potent chemoattractant for these cells. There has been much interest in whether MCP-1 is expressed in pathological processes where macrophages accumulate, ranging from atherosclerosis to infectious diseases and including cancer. In addition, the in vivo actions of MCP-1 have been assessed in animal models using both transfected cells and transgenic animals.

MCP-1 has been found in atherosclerotic lesions. In frozen sections of human carotid endarterectomy specimens examined by isotopic in situ hybridisation, MCP-1 was expressed by vascular smooth muscle cells, intimal cells and macrophages, particularly those bordering the necrotic lipid core (325). Smooth muscle cells cultured from specimens of human atheroma expressed MCP-1 in response to stimulation with IFN-γ and TNF-α. Endothelial cells and macrophages in human atheroma have also been found to be positive for MCP-1 by immunohistochemistry using an anti-MCP-1 monoclonal antibody (326), although less staining was observed for smooth muscle cells. Other mesenchymal cells express MCP-1 in response to inflammatory cytokines. Synoviocytes from patients with osteoarthritis and rheumatoid arthritis expressed MCP-1 in response to stimuli including IL-1, TNF-α, LPS, PDGF and TGF-β1 (327). Synovial fibroblasts from patients with rheumatoid arthritis have been found to express not only MCP-1, but MIP-1α, MIP-1β, IL-8 and GRO in response to IL-1α (328).

Several studies have looked at MCP-1 expression in renal diseases which are frequently associated with a mononuclear infiltrate. Prodjosudjadi et al studied 50 biopsies representing a number of different conditions and described an association between the intensity of immunohistochemical staining for MCP-1 and the infiltration of macrophages (329). The sources of MCP-1 included mesangial cells and tubular-epithelial cells. In renal transplants, both RANTES (330) and MCP-1 (331) are expressed by tubular cells and infiltrating macrophages. Other inflammatory conditions in which MCP-1 has been detected include idiopathic pulmonary fibrosis, in which pulmonary epithelial cells, macrophages, smooth muscle cells and vascular endothelial cells, all expressed MCP-1 (332), inflammatory bowel disease (333) and viral hepatitis (334).

Human tumours can express MCP-1. Using in situ hybridisation and immunohistochemistry Yoshimura et al detected MCP-1 in 8/8 surgical specimens of
human malignant glioma (322). MCP-1 localised to glioma cells with large pleiomorphic nuclei (335). In Kaposi's sarcoma (336) and malignant fibrous histiocytoma (337) it was again the tumour cells which appeared to be the major source of MCP-1. However, in prostatic carcinomas, MCP-1 transcripts occurred in the fibromuscular stroma not in carcinomatous cells (338). These studies demonstrate that although human tumour cells can produce MCP-1, it is not always the case.

Further evidence that tumour cells, in addition to other components of the tumour, can produce MCP-1 has been provided by the study of tumour cell lines. MCP-1 can be expressed constitutively by glioma cell lines, although there is variability between cell lines (335). Monocytic cell lines (323), smooth muscle cell lines (339), fibroblasts (340) and vascular endothelium (79) can all be induced to produce MCP-1. Human proximal tubular epithelial cell lines expressed MCP-1 in response to TNF-α and IL-1α (102). TDCF was also produced by other murine and human tumour cell lines, including cell lines derived from human sarcomas, melanomas, a lymphoma, leukaemias and breast, stomach and bladder carcinomas (319). Similarly, TDCF was detected in the ovarian cell line OVCAR-3 (320).

Analysis of the functional role of MCP-1 in disease processes has been extended by animal studies. In a mouse model of dermal wounding, murine MCP-1 (JE) was expressed by monocyte and macrophage-like cells as early as 12 hours after wounding and the number of infiltrating macrophages correlated with JE expression assessed by northern blotting (341). In a rat model of cutaneous hypersensitivity, MCP-1 expression correlated with both the monocyte and T cell infiltrate (342). The infiltration of these cells could be almost completely abolished if antibodies to MCP-1 were given intravenously at the same time as the subcutaneous antigen challenge. Recently, Gong et al have developed a truncated analogue of MCP-1 which acts as an antagonist. It has been used in a mouse model of joint disease (343). The MRL-lpr mouse normally develops a chronic inflammatory arthritis with many features similar to rheumatoid arthritis. Daily injection of the MCP-1 antagonist resulted in a delayed onset of arthritis, measured by joint swelling and histopathological examination of the joints. Even when the antagonist was given after the disease had developed, there was still a decrease in signs of the disease. MCP-1 expression has been found in several other animal models which involve tissue injury, including experimental autoimmune encephalomyelitis (EAE) in the rat (344) and mouse (345), a rat lung organ culture model of lung injury (346) and a mouse model of hydronephrosis (347).
Mice transgenic and knockout for MCP-1 now exist and have been used to assess the in vivo role of MCP-1 in monocyte recruitment. In transgenic mice in which the expression of MCP-1 is controlled by the mouse mammary virus long terminal repeat (348), high circulating levels of MCP-1 were found. But while protein extracts from organs displayed monocyte chemoattractant activity in vitro, monocytes did not infiltrate organs in vivo. Furthermore, transgenic lines expressing the highest levels of MCP-1 were the most susceptible to infection with the intracellular pathogen, Listeria monocytogenes. On the other hand, when transgenic expression was engineered to be organ specific, recruitment of monocytes and macrophages did not occur (349). The phenotype of MCP-1 knockout mice has not yet been described, but macrophage recruitment to the peritoneum of these animals following intra-peritoneal thyoglycolate is reduced (Barrett Rollins, personal communication). Knockout mice for the MCP-1 receptor, CCR2, have also been made (350). Leukocytes from these animals failed to migrate to MCP-1 in chemotaxis assays. Furthermore, the development of granulomata in response to a purified mycobacterial protein, together with the production of Th1-type cytokines, was impaired.

Studies on the effect of MCP-1 expression on tumour growth and development have largely used transfected cell lines. Rollins et al (351) found that stable transfection of Chinese hamster ovary cells with MCP-1 did not alter their phenotype in vitro. However, while wild-type cells were able to form large tumours in nude mice, no tumours were found in mice injected with transfected cells. Tumours developing from transfected cells elicited an abundant cellular infiltrate consisting primarily of monocytes and eosinophils. Similarly injection of a murine melanoma cell line transfected with MCP-1 into C57Bl6 mice was accompanied by a two-fold increase in the percentage of tumour associated macrophages. These MCP-1 producing clones exhibited slower rates of growth in vivo, but their growth rate in vitro was unaffected. However, in another study, MCP-1 producing melanoma cells were found to produce more tumours despite their slower growth (352). Tumour cells do not have to be the source of MCP-1, since growth of the renal adenocarcinoma cell line, RENCA, can be reduced by mixing it with syngeneic fibroblasts engineered to produce MCP-1 (353). Recently, Zhang et al (354) have examined the growth and macrophage content of four human tumour cell lines that express variable amounts of MCP-1, in a nude mouse model. They found a close correlation between MCP-1 expression and macrophage content. The macrophage content of two MCP-1 expressing tumours could be significantly reduced by treatment with a monoclonal antibody to MCP-1 and macrophage content correlated inversely with tumour size. While this seems to demonstrate that any level of MCP-1 expression can lead to tumour regression, similar
experiments have not been done in syngeneic systems in animals with intact immune systems. The results of the experiments with transfected murine melanoma cells (352) would support the idea that MCP-1 and macrophage infiltration can contribute to tumour development.

Prior to this study, there was no direct evidence that ovarian cancers express CC chemokines, although the presence of TDCF supported the idea that they produce MCP-1. The phenotypes of the infiltrating leukocytes that were found immunohistochemically suggested that other chemoattractants might be present and in view of the large number of CD8+ cells, MIP-1α was considered a likely candidate. MIP-1α can be produced by both macrophages (116) and CD8+ cells (355) and is chemoattractant for CD8+ cells in vitro (117). On the other hand, those chemokines known to act on CD4+ cells, such as MIP-1β and RANTES, were not expected to be present. The main source of MIP-1β is macrophages (116) and MIP-1β is reported to be chemoattractant for CD4+ cells. RANTES is expressed by T cells (64) and is preferentially chemoattractant for CD4+, CD45RO+ T cells (125). RANTES has been shown to be present where CD4+ cells accumulate, for instance in inflammatory states such as rejection of renal transplants (330) and liver disease (334), but none of these chemokines has so far been identified in a human tumour.

3.2. Aims of the chapter
The main aim of this chapter was to establish whether MCP-1 is present within human ovarian carcinomas. In addition it was hoped to identify the cell types that produce this chemokine and to describe their distribution. A further objective was to examine the distribution of expression of MIP-1α, MIP-1β and RANTES, to compare the presence of these chemokines with that of MCP-1 and to determine the likely cellular sources of these chemokines. Finally, the relationship between the number of chemokine expressing cells and the number of infiltrating CD8+ and CD68+ cells was examined.
3.3. Materials and methods

3.3.1. Ovarian samples

3.3.1.1. Tissue collection
Frozen sections were used for all the in situ hybridisation studies. Tissue samples, cut to approximately 5mm in thickness, were frozen in isopropanol which had been cooled to near freezing on liquid nitrogen. Frozen specimens were stored at -70°C. Since isopropanol penetrates tissues better than liquid nitrogen, freezing in isopropanol on liquid nitrogen improves tissue preservation by enhancing the speed at which the cooling occurs. If water cools slowly, ice crystal formation can occur with consequent tissue damage. However, rapidly frozen water forms a glass and this leads to better preservation of tissue morphology. To keep tissue samples as cold as possible and to avoid ice crystal formation once they had been collected, frozen specimens were always handled on a bed of dry ice.

3.3.1.2. Characteristics of tumour samples used to compare CC chemokine expression by RT-PCR
Between 10 and 12 human tumours derived taken from those already banked in the Biological Therapies Laboratory were used to screen for chemokine expression by RT-PCR. They included seven serous adenocarcinomas, one mucinous carcinoma, one endometrioid carcinoma, one granulosa cell tumour, one clear cell carcinoma and one Brenner tumour. These tumours had been collected from a variety of sources and had been used in previous studies in the laboratory.

3.3.1.3. Characteristics of tumour samples used to compare CC chemokine expression and the cellular infiltrate
Twenty frozen epithelial tumours were assessed for CC chemokine expression by in situ hybridisation. Ten of these tumours were from the same patients as the paraffin embedded material described in chapter 2 and 10 samples were obtained from the Queen’s Hospital, Belfast. There were 15 serous tumours (five grade 1, two grade 2 and eight grade 3), two mucinous tumours (grade 2) and three endometrioid tumours (grade 2). For both in situ hybridisation and immunohistochemical studies, 6µm cryostat sections were cut onto baked glass 4-well slides coated with 3-aminopropyl-triethoxy-silane (TESPA, Sigma Chemicals Ltd, Poole, Dorset UK), air dried and stored at -70°C. Sections were fixed in 4% paraformaldehyde for 20 minutes, or formol saline for five minutes for in situ hybridisation and immunohistochemistry respectively.
3.3.1.iii. Characteristics of patients used in analysis of ascites for MCP-1 expression

This work was performed by Professor Alberto Mantovani's laboratory at the 'Mario Negri' Institute, Milan, Italy. Ascites was collected by paracentesis from a total of 46 patients with ovarian cancer. Thirty two patients were subsequently shown histologically to have serous adenocarcinomas, three mucinous adenocarcinomas, five clear cell carcinomas, four endometrioid carcinomas, three undifferentiated carcinomas and one an anaplastic mucous secreting tumour. Twenty patients had received some treatment before their ascites was analysed and 26 were untreated.

3.3.2. Cell lines and culture

3.3.2.i. Ovarian carcinoma cell lines

PEO1, PEO4, PEO14 and PEA2 (obtained from S. Langdon, ICRF Oncology Unit, Western General Hospital, Edinburgh, UK) (356), SKOV-3 (American Type Culture Collection, ATCC, Rockville, Maryland, USA) (357) and OVCAR-3 ovarian carcinoma cells (obtained from T. Hamilton, NCI, Bethesda, USA) were used to obtain RNA for RT-PCR and culture supernatants to assay for MCP-1 expression. All these cell lines grow as adherent monolayers. PEO1, PEO4, PEO14 and PEA2 cells were grown in RPMI 1640 (Gibco, Paisley, UK), supplemented with 10% FCS (Gibco) and 2.5μg/ml bovine insulin (Sigma). OVCAR-3 cells were grown in RPMI 1640 in the presence of 5μg/ml insulin. E4 was substituted for RPMI 1640 when culturing SKOV-3 cells. 2mM L-glutamine, 100U/ml penicillin and 100μg/ml of streptomycin were included in all tissue culture media. All cell lines were grown under pyrogen free conditions, and in an humidified atmosphere of 5% CO₂ in air. They were routinely split 1:2 or 1:3 when confluent (up to 3 times per week).

3.3.2.ii. Mononuclear cell lines

The human promyelocytic cell line HL60 (358) was used as a positive control for in situ hybridisation and RT-PCR for MCP-1. This cell line was originally obtained from the American Type Tissue Collection (ATCC) and is held at ICRF. HL60 cells were routinely maintained in RPMI 1640 supplemented with 5% FCS and split to a concentration of 5 x10⁵/ml up to 3 times per week. They were stimulated with 10ng/ml human recombinant TNF-α for 6 hours prior to preparing cytospins or total RNA.

3.3.2.iii. Preparation of cytospins

Cytospins for in situ hybridisation were prepared using a Shandon Cytospin 3. 5 x10⁵ cells/ml were loaded per chamber and spun onto TESPA coated baked slides at
500rpm for five minutes. Cytospin preparations were allowed to air dry before fixation in 4% paraformaldehyde and dehydration through graded alcohols. Slides were stored at -70°C with desiccant. Prior to in situ hybridisation, slides were brought to room temperature, rehydrated though graded alcohols and refixed in 4% PFA for 15 minutes. For cytospins, the first 11 steps of the pre-hybridisation protocol (section 3.3.7.i.) were omitted.

3.3.3. Isolation of TAM and ovarian carcinoma cells
This work was undertaken in Professor Mantovani’s laboratory. TAM and ovarian carcinoma cells were separated from ascites using discontinuous Ficoll Hypaque and Percoll gradients, essentially as described (320). Highly enriched TAM preparations (>95% by morphology), were obtained using CD14-coated magnetic beads (Unipath, Italy). Tumour cell preparations were depleted of CD14+ cells. TAM and tumour cells were cultured at 3 x10^6/ml in RPMI 1640 with 10% FCS. After 24 hours supernatants were collected for MCP-1 measurement.

3.3.4. RT-PCR
3.3.4.i. Preparation of RNA from tissue samples and cell lines
Two methods of preparing total RNA and its subsequent reverse transcription for PCR were employed during the course of the thesis. For ovarian cell lines, total RNA was obtained from cells grown in 175cm² tissue culture flasks (Nalge Nunc International, Denmark) by the method of Chomczynski (359). RNA was quantified by absorbence at 260nm and the samples were treated with DNase before being used for cDNA synthesis. cDNA was prepared using the methods and reagents of the Boehringer Mannheim cDNA strand synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany), for the first strand synthesis only and starting with 2µg total RNA.

This method was superseded by the introduction of Tri-Reagent™. For the assessment of CC chemokine expression in a panel of ovarian tumours, 100mg of ovarian samples were lysed into Tri-Reagent™ (Molecular Research Centre Inc., Cincinnati, OH, USA) according to the manufacturers protocol. cDNA was reverse transcribed from 5µg of total RNA using the Ready To Go, T-primed First-Strand Kit (Pharmacia Biotech, Inc., USA).

3.3.4.ii. PCR
MCP-1 primers were designed from the sequences submitted to Genbank using Primer programme version 0.5 May 1991. The MCP-1 sequences were chosen to span the protein coding region within the MCP-1 cDNA PstI restriction sites. Those for MIP-
1α, MIP-1β were taken from the work of Hosaka et al (328) and those for RANTES from Mattei et al (360). Primer sequences are given in table 3.1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used in all PCR reactions to control for reverse transcription of total RNA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>CAAACTGAAGCTCGCACTCTCGCC ATTCTTGAGTTGTGGAGTGAGTGTTCA</td>
<td>361</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>CCTTGCTGTCTCCCTCTTGCA CACTCAGCTCTAGGTCGCTG</td>
<td>254</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>TGTCTCTCCTCATGCTAGTA GTACTCTGGACCCAGGAT</td>
<td>233</td>
</tr>
<tr>
<td>RANTES</td>
<td>CCTCTCCACAGGTACCATT CAAGGTATTCAGGACTC</td>
<td>314</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGAAGGTCGGAGTCAACGGGATTGGA ACGAGTACTCAGCGCCAGCACTCGC</td>
<td>277</td>
</tr>
</tbody>
</table>

Table 3.1. Primer sequences used in RT-PCR reactions.
The primer sequences for each chemokine and their expected reaction product sizes are given.

PCR reactions were performed using the Perkin-Elmer Gene Amp PCR Kit (Perkin-Elmer Corporation, Norwalk, CT, USA). 2µl (~200ng) of cDNA was used for each amplification. A master mix consisting of all reagents except the cDNA was made up for each primer pair. A final volume of 25µl per reaction was overlaid with mineral oil and the following protocol used in a Techne PCR machine (Cambridge UK.): 1 cycle of 94°C (5 min), 60°C (1 min), 72°C (0.5 min); 35 cycles of 94°C (45 sec), 60°C (45 sec), 72°C (2 min), 1 cycle of 72°C (7 min). 10µl of reaction mixture was electrophoresed on a 1.2% agarose gel and visualised by ethidium bromide staining. 123bp markers (Gibco BRL) were used to estimate band sizes. A positive control for the PCR reaction was provided in the kit. For negative controls distilled water was used instead of cDNA.

3.3.5. Probes
cDNAs for MCP-1, MIP-1α, MIP-1β and RANTES were the kind gift of Professor Alberto Mantovani ('Mario Negri' Institute, Milan, Italy). β-actin cDNA was obtained from Dr L Kedes (Stanford University, Stanford, Ca., USA). For in situ
hybridisation experiments cDNAs for MCP-1, MIP-1α, MIP-1β and RANTES were subcloned, where necessary, into suitable transcription vectors (pGEM series, Promega, UK) prior to generating 35S labelled sense and anti-sense riboprobes. All cDNAs were assessed by restriction digests. Antisense β-actin was used as a positive control for all in situ hybridisation experiments and the appropriate sense riboprobes were used as negative controls.

3.3.6. Southern blotting

Southern blotting with randomly primed chemokine cDNA probes was undertaken to validate PCR results. 1.2% agarose gels were depurinated in 250ml 0.25M HCl until the bromophenol blue turned green/yellow (or for not longer than 15 minutes), neutralised in 0.4m NaOH for five minutes and alkaline blotted onto Hybond N+. Southern blotting was by capillary transfer with the membrane wetted before setting up the blot. Blotting was carried out overnight. After transfer, the membrane was rinsed twice in 250ml SSC for five minutes at room temperature and hybridised to 32P labelled probes under the conditions outlined by Church and Gilbert (361). For this part of the study, probes were labelled with 32P-dCTP by the random priming method of Feinberg and Vogelstein (362). The following mixture was used to pre-hybridise and hybridise the membranes:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume/amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M sodium phosphate buffer</td>
<td>10ml</td>
</tr>
<tr>
<td>0.1M EDTA pH 8</td>
<td>0.5ml</td>
</tr>
<tr>
<td>BSA</td>
<td>0.5g</td>
</tr>
<tr>
<td>SDS</td>
<td>3.5g</td>
</tr>
<tr>
<td>formamide (ultra pure)</td>
<td>7.5ml</td>
</tr>
<tr>
<td>distilled water</td>
<td>32ml</td>
</tr>
</tbody>
</table>

Hybridisation was performed at 42°C overnight. Post-hybridisation the membrane was washed three times for 20 minutes at 65°C in a buffer consisting of 40mM sodium phosphate buffer, pH 7.2, 1mM EDTA, pH 8, and 1% SDS. Membranes were then exposed to Kodak XAR5 film at room temperature for 10-30 minutes.
3.3.7. **In situ hybridisation**

3.3.7.i. **Prehybridisation**

The method described by Naylor *et al* (363) was followed. All incubations were carried out at room temperature in RNase free buffers (DEPC treated) and baked dishes. Frozen sections for *in situ* hybridisation were brought to room temperature and air dried for one hour prior to further processing. The following steps were then carried out:

1. 4% PFA  
2. PBS  
3. distilled water  
4. 0.2M HCl  
5. PBS  
6. 4% PFA  
7. PBS  
8. 5µg/ml proteinase K in PK buffer (50mM Tris, 5mM EDTA, pH 7.5)  
9. PBS  
10. 4% PFA  
11. distilled water  
12. 0.1M triethanolamine + acetic anhydride (1ml/400ml)  
13. PBS  
14. Dehydrate through graded alcohols (50%, 70%, 95%, 100%)  
15. Air dry in hood

The purpose of the pre-hybridisation steps was to optimise tissue penetration by the probes, whilst preserving tissue morphology.
3.3.7.ii. Probe labelling

The following reagents were incubated together at 37°C for one hour:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>i. 5x transcription buffer</td>
<td>4µl</td>
</tr>
<tr>
<td>ii. 100mM DTT</td>
<td>2µl</td>
</tr>
<tr>
<td>iii. RNasin</td>
<td>0.8µl</td>
</tr>
<tr>
<td>iv. ATP, CTP, GTP (2.5mM of each)</td>
<td>4µl</td>
</tr>
<tr>
<td>v. Linearised template</td>
<td>1µl (=1µg)</td>
</tr>
<tr>
<td>vi. 35S-UTP</td>
<td>10µl</td>
</tr>
<tr>
<td>vii. Relevant polymerase</td>
<td>1µl</td>
</tr>
</tbody>
</table>

Two units (=2µl) of RQ1 DNase were added to the mix, which was incubated at 37°C for 30 minutes. The reaction volume was made up to 100µl with distilled water and labelled probes were phenol extracted (100µl) and precipitated with 20µl 10M ammonium acetate, 300µl absolute alcohol and 2µl 10mg/ml tRNA at -70°C for 15 minutes. Pellets were air dried and taken up in 100µl alkaline digestion buffer (40mM NaHCO₃, 60mM Na₂CO₃, pH 10.2, 10mM DTT) and incubated at 60°C. The incubation time was determined according to the formula:

\[ t = \frac{(L₀ - L_f)}{0.11 \cdot L₀ \cdot L_f} \]

where \( t \) is time in minutes, \( L₀ \) is the initial probe length and \( L_f \) is the desired probe length in order to achieve a probe of approximately 200bp.

The reaction was stopped with 10µl 1M sodium acetate pH 6 and 10µl 5% acetic acid. 2µl 10mg/ml tRNA was added and the probe phenol extracted twice, precipitated as above, dried and resuspended in 40µl 10mM DTT. Labelled probes were counted in a Beckman LS 6000IC scintillation counter and used if counts were greater than 2 x10⁶/µl.
3.3.7.iii. Hybridisation

The following reagents were used to make approximately 1ml of hybridisation mix:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount (µl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>i. 1M DTT (-20°C)</td>
<td>10</td>
<td>10mM</td>
</tr>
<tr>
<td>ii. Deionised formamide</td>
<td>600</td>
<td>60%</td>
</tr>
<tr>
<td>iii. 100x Denhardt’s</td>
<td>10</td>
<td>1x</td>
</tr>
<tr>
<td>iv. 1M Tris pH 8</td>
<td>10</td>
<td>10mM</td>
</tr>
<tr>
<td>v. 5M NaCl</td>
<td>60</td>
<td>0.3M</td>
</tr>
<tr>
<td>vi. 0.5M EDTA</td>
<td>10</td>
<td>1mM</td>
</tr>
<tr>
<td>vii. 10mg/ml poly A</td>
<td>30</td>
<td>300µg/ml</td>
</tr>
<tr>
<td>viii. 10mg/ml carrier RNA</td>
<td>30</td>
<td>300µg/ml</td>
</tr>
<tr>
<td>ix.. 20mM cold S-UTP</td>
<td>22</td>
<td>500µM</td>
</tr>
<tr>
<td>x. 50% dextran sulphate</td>
<td>200</td>
<td>10%</td>
</tr>
</tbody>
</table>

Probe was added to the hybridisation mixture to a final concentration of 5 x10⁴cpm/µl and the whole mixture heated to 80°C for two minutes. 20µl of hybridisation mixture was applied to each section, which were then covered with a siliconised coverslip and incubated overnight at 50°C in sealed slide box humidified with tissue soaked in 50% formamide, 5x SSC.

3.3.7.iv. Post-hybridisation washes

Sections were washed to high stringency in the following washes:

<table>
<thead>
<tr>
<th>Wash buffer</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 5x SSC, 0.1% β-ME</td>
<td>50°C</td>
<td>3x 20 min</td>
</tr>
<tr>
<td>2. 50% formamide, 2x SSC, 0.1% β-mercaptoethanol</td>
<td>65°C</td>
<td>30 min</td>
</tr>
<tr>
<td>3. RNase buffer (0.5M NaCl, 10mM Tris, 5mM EDTA, pH 8)</td>
<td>37°C</td>
<td>2x 10 min</td>
</tr>
<tr>
<td>4. RNase A, 20µg/ml in the above</td>
<td>37°C</td>
<td>30 min</td>
</tr>
<tr>
<td>5. RNase buffer</td>
<td>37°C</td>
<td>15 min</td>
</tr>
<tr>
<td>6. 50% formamide, 2x SSC, 0.1% β-ME</td>
<td>65°C</td>
<td>30 min</td>
</tr>
<tr>
<td>7. 2x SSC</td>
<td>RT</td>
<td>15 min</td>
</tr>
<tr>
<td>8. 0.1x SSC</td>
<td>RT</td>
<td>15 min</td>
</tr>
</tbody>
</table>

After the final wash, slides were dehydrated through graded alcohols and air dried.
3.3.7.5. Autoradiography
Slides were dipped in 0.1% gelatine, air dried and coated in Ilford K5 emulsion. After air drying in a darkroom, they were placed in racks in a box containing desiccant, wrapped in tin foil and stored at 4°C for 10 days.

3.3.7.6. Development, counterstaining and mounting
Slides were developed in D19 (Kodak, Paris, France) for two minutes, 30 seconds, stopped in 1% acetic acid for 30 seconds and fixed in 30% sodium thiosulphate (fresh) for five minutes. They were then rinsed in distilled water for one hour, counterstained in 1% toluidine blue for seven minutes and dehydrated through graded alcohols. Residual water was removed with acetone, before the slides were cleared in xylene and mounted in DePeX (BDH).

3.3.7.7. Assessing chemokine expression by in situ hybridisation
To compare the expression of different chemokine mRNAs, the total number of positive cells were counted in 10 adjacent high power fields (x40 objective, x10 eyepiece), giving a total area of 0.73mm². Fields were chosen to include at least one area of high expression, but to avoid the edge of the section and areas of artefact. The definition of a positive cell was essentially subjective as there was no better method to distinguish between signal, background and artefact. The proportion of tumour and stroma was assessed over the same area using the modified Chalkley array as described in chapter 2. Cell counts were expressed as cells/mm².

3.3.8. ELISA for MCP-1 in ascites, TAM and tumour cell preparations
MCP-1 was measured using a sandwich ELISA assay based on a rabbit antiserum and a mAb (5D3-F7), as described (364). This assay had a sensitivity of approximately 80pg/ml in peritoneal fluids and 30pg/ml in culture supernatants. It was specific for human MCP-1 and did not detect the mouse equivalent, JE, or the closely related human chemokines MCP-2 and MCP-3. This work was performed in Prof. Mantovani’s laboratory.

3.3.9. Immunohistochemistry and cell counts in frozen sections
Frozen sections were air dried for 1 hour at room temperature prior to fixation for five minutes. Paraformaldehyde or formalin fixed frozen sections were stained for CD68+ macrophages and CD8+ T-cells with the antibodies EBM11 (DAKO) and DK125 (DAKO), respectively. CD4+ T-cells were detected in paraformaldehyde fixed frozen sections using the antibody MT310 (DAKO). The staining procedure was otherwise
identical to that for paraffin embedded sections. Counterstaining was performed with 1% toluidine blue. Slides were prepared for microscopy as described in section 3.2.6.vi. Cells counts in 10 randomly chosen high power fields (x40 objective, x10 eyepiece, total area 0.73mm²) were expressed as cells/mm².

3.3.10. Statistical methods
As for the cell counts in chapter 2, infiltrating cells and cells expressing chemokines in the frozen sections were not normally distributed. Wilcoxon’s matched pairs signed rank test was used for comparisons between matched pairs of data. Spearman’s rank correlation was used to test whether a correlation existed between the number of chemokine expressing cells and the number of CD68+ or CD8+ cells. The Mann-Whitney U test was used to test whether there was a significant difference between the number of CD68+ and CD8+ cells in frozen and paraffin sections. P values were again only considered statistically significant if P<0.005, but values P<0.05 are also shown. Student’s unpaired t-test was used to compare MCP-1 expression in plasma and ascites between the different groups.
3.4. Results

3.4.1. Expression of CC chemokines by human ovarian carcinomas and tumour cell lines

3.4.1.i. RT-PCR for chemokines in cell lines

RT-PCR was performed for chemokine expression in 6 ovarian carcinoma cell lines. MCP-1 was detected in 6/6, although at extremely low levels in SKOV-3 and PEA2 cells; MIP-1α in 2/6; MIP-1β in 1/6; and RANTES in 1/6 (figure 3.1A). The result for MCP-1 was confirmed by Southern blotting (figure 3.1B).

Figure 3.1. Expression of CC chemokines in ovarian cancer cell lines. CC chemokine expression was assessed by RT-PCR (A) in 6 ovarian cancer cell lines; 123, 123bp size markers; +, positive control for PCR; -, negative control for PCR; 1, SKOV-3 cells; 2, OVCAR-3 cells; 3, PEO1 cells; 4, PEO4 cells; 5, PEO14 cells; 6, PEA2 cells; con, positive control for chemokine expression. GAPDH was used to control for reverse transcription of RNA. The expression of MCP-1 by cell lines was also confirmed by Southern blotting (B).
3.4.1.ii. RT-PCR for chemokines in tumour samples

RT-PCR for each of the 4 CC chemokines was undertaken on 12 ovarian carcinoma samples. Expression of MCP-1 was detected in 11/12 samples, MIP-1α in 11/11, MIP-1β in 9/10 and RANTES in 9/10 (data not shown).

3.4.1.iii. In situ hybridisation for CC chemokines and quantification of chemokine expression

The number of cells expressing each of the CC chemokines MCP-1, MIP-1α, MIP-1β and RANTES was counted in 10 high power fields per cryostat section (x40 objective, x10 eyepiece), in a panel of 20 ovarian carcinomas. Final cell counts were expressed per mm². Because the in situ hybridisation studies were performed on cryostat sections counterstained with toluidine blue, areas of necrosis were not always well preserved. Therefore, cell counts were only assessed for tumour or stromal areas and the total area. For none of the chemokines was any positive labelling seen with sense probes. In situ hybridisation for β-actin was positive in all the samples (figure 3.2A). HL60 cells stimulated with TNF-α were used as positive controls for MCP-1 expression (figure 3.2B). Approximately 20% of HL60 cells were positive for MCP-1 mRNA in cytopsins probed with antisense riboprobes.

The total number of cells expressing MCP-1 or MIP-1α was significantly greater than the number expressing MIP-1β or RANTES (P<0.005, figure 3.3A). MCP-1 was expressed by more cells within tumour islands than any other chemokine (P<0.0005, median value 72.5 cells/mm², figure 3.3B), but there was no significant difference between the number of MCP-1 expressing cells in tumour and stroma (P<0.03). No difference was found in the number of MCP-1 expressing cells with grade. Within the stroma (figure 3.3C) the number of cells expressing MCP-1 and MIP-1α were similar (median values 21 cells/mm² and 26 cells/mm² respectively). MIP-1α was also expressed by more stromal cells than MIP-1β or RANTES (P<0.0005). MCP-1 expressing cells within the tumour islands frequently occurred in clusters (figure 3.4 and fig 3.5A), although in one borderline tumour, the expression of MCP-1 by discrete tumour cells was observed (figure 3.5B). MCP-1 expressing cells in stroma tended to occur alone (figure 3.6A). In a previous study, the mean percentage of tumour cells expressing MCP-1 within a cluster was 8.2% (±5.8%) (295). In the stroma the median number of cells expressing MIP-1β and RANTES was 2/mm² and 7/mm² respectively. While silver grains indicating MCP-1 expression were frequently associated with typical tumour cell nuclei, MIP-1α, MIP-1β and RANTES were associated with smaller and more intensely stained nuclei, suggesting that they might
be derived from the infiltrating leukocyte population. Cells positive for MCP-1 signal were also occasionally seen within gland spaces. In one case these cells had nuclear morphology typical of macrophages (figure 3.6B). In 9/20 frozen sections processed for in situ hybridisation for MCP-1 it was possible to distinguish regions of necrosis. However, none of these was associated with expression of MCP-1 (figure 3.7).

Figure 3.2. Positive controls for in situ hybridisation.
Tumour sections, such as this serous adenocarcinoma, were probed for expression of the 'housekeeping gene' β-actin (A). As a positive control for MCP-1 the monocytic cells HL60 were stimulated with 10ng/ml TNF-α for 6 hours and cytospins prepared. Approximately 10% of cells expressed MCP-1 mRNA as determined by in situ hybridisation (B).
Figure 3.3. CC chemokine expression in ovarian carcinomas.
Scatter plots of the calculated numbers of chemokine expressing cells/mm³ in whole tumour (A), tumour parenchyma (B) and stroma (C). The horizontal bars represent median values.
Figure 3.4. *In situ* hybridisation for MCP-1.

Expression of MCP-1 mRNA was detected by *in situ* hybridisation using an anti-sense $^{35}$S labelled riboprobe generated from the 5' region of the MCP-1 coding sequence. Signal for MCP-1 in a grade 2 papillary endometrioid carcinoma viewed under brightfield (A) and darkfield (B). Arrows indicate several MCP-1 expressing cells.
Figure 3.5. *In situ* hybridisation for MCP-1.
MCP-1 expression by tumour cells (A) in a grade 3 serous adenocarcinoma seen at high power and in a borderline tumour (B). The focal nature of MCP-1 expression is clearly visible in the borderline tumour.
Figure 3.6. *In situ* hybridisation for MCP-1.
Expression of MCP-1 by stromal cells in a grade 2 mucinous adenocarcinoma (A) and by cells with typically macrophage shaped nuclei lying within a gland space (B) in the same tumour.
Figure 3.7. The absence of MCP-1 expression adjacent to necrotic regions. In some frozen sections necrotic regions were discernible. However, MCP-1 expression was not found within or adjacent to these areas in any of these cases. In this case of a grade 3 papillary serous adenocarcinoma, the nearest cells expressing MCP-1 (arrowed) in the stroma are >200µm from the necrotic region (N).
3.4.1.iv. Cell types expressing MIP-1α

MIP-1α was the second most highly expressed chemokine, but MIP-1α expressing cells were confined to the stroma. There was no significant difference between the number of stromal cells expressing MIP-1α and those expressing MCP-1. Like MCP-1, MIP-1α expressing cells tended to occur singly (figure 3.8A). In some tumours it was possible to identify MIP-1α expression and CD8+ staining in the same regions (figure 3.8B).

![Figure 3.8A](image1.png)

![Figure 3.8B](image2.png)

Figure 3.8. MIP-1α mRNA detected by in situ hybridisation. Individual cells expressing MIP-1α are clearly visible within the stroma of a serous adenocarcinoma (A). CD8+ cells (B) localised to the same region as expression of MIP-1α. In this particular region there was no staining for CD68.
3.4.1.v. Cell types expressing MIP-1β and RANTES

In some tumours, ice crystal formation produced a ring-shaped artefact in the nucleus of tumour cells, but not mononuclear cells, allowing these cell types to be distinguished. Neither MIP-1β nor RANTES expression appeared to be associated with tumour cells, even though these chemokines were detected in tumour areas (figures 3.9-11). Occasional cells within gland spaces were positive for MIP-1β signal, implying that macrophages are a source of this chemokine in ovarian tumours. Expression of RANTES was weak in every sample studied and it was difficult to define its cellular sources.

Figure 3.9. Expression of MIP-1β detected by isotopic in situ hybridisation. Individual cells within a grade 3 carcinoma area can be seen associated with in situ signal under both brightfield (A) and darkfield (B).
Figure 3.10. Expression of MIP-1β detected by isotopic in situ hybridisation. High power of a single cell associated with in situ signal for MIP-1β seen in the centre of figure 3.9A.
Figure 3.11. RANTES expression in ovarian carcinomas. RANTES expression in a papillary serous adenocarcinoma tumour viewed under brightfield (A) and darkfield (B). In nearly every case, the number of silver grains appeared lower in RANTES expressing cells than for the other CC chemokines.
3.4.2. Production of MCP-1 protein in ovarian cancer

3.4.2.i. Estimation of MCP-1 production in ascites

MCP-1 protein was detected by ELISA in ascites from patients with ovarian cancer (figure 3.12). The mean level (4.28ng/ml) was significantly higher than that in ascites from patients with cirrhosis (mean 0.76ng/ml, P<0.00001). Low but detectable levels were found in plasma from normal laboratory donors (mean 0.23ng/ml), patients with benign gynaecological disease (mean 0.39ng/ml) and patients with ovarian carcinoma (0.49ng/ml), but in ovarian cancer patients, the ascites levels were significantly higher than plasma levels in any of these groups (P<0.00001).

Figure 3.12. MCP-1 protein levels in serum and ascites.

Scatter plots of the MCP-1 protein levels found in the ascites of patients with ovarian tumours, ascites from patients with hepatic cirrhosis, the serum of patients with ovarian tumours and the serum of normal laboratory volunteers. The horizontal bars represent the median for each group. There was a significant difference between the amount of MCP-1 found in the ascites of patients with ovarian tumours and all the other groups (P<0.00001).
3.4.2. ii. MCP-1 production by freshly isolated TAM and tumour cells
In three patients highly enriched TAM and ovarian tumour cells were fractionated from ascites and their capacity to release MCP-1 assessed over a 24 hour period. MCP-1 production was essentially confined to the ovarian carcinoma-enriched population. The tumour cell enriched population produced 2.3, 40.9 and 47.2 ng/ml compared with 0.3, 0.2 and 0.1 ng/ml respectively produced by TAM.

3.4.3. CD8+ and CD68+ cell counts in frozen sections

3.4.3. i. Correlation of cell counts in frozen and paraffin embedded specimens
The total number of CD68+ macrophages and CD8+ T cells were assessed in cryostat sections in order to explore the relationship between them and the number of chemokine expressing cells. The antibody used to detect macrophages in frozen sections (EBM 11) was not as specific as that for paraffin embedded material (PG-M1), therefore only cells with positive cytoplasmic staining and characteristic nuclear morphology were included in the cell counts. No significant difference was found between the total number of macrophages or CD8+ cells per mm² in the frozen and paraffin sections (data not shown). All the frozen sections assessed contained cells which were positive for CD68, within both the tumour parenchyma and stroma. The distribution of CD68 positive cells was similar to that found in paraffin embedded sections.

3.4.3. ii. Leukocyte-chemokine correlations
A possible correlation was found between the number of cells expressing MCP-1 and the number of CD68+ cells ($r_s=0.50$, $P=0.026$). A significant correlation was found between the CD8+ population and MCP-1 expression ($r_s=0.63$, $P<0.005$) and the CD8+ population and RANTES expression ($r_s=0.6$, $P<0.005$). There were no significant correlations between the number of CD68+ or CD8+ cells and expression of the other chemokines (figure 3.13).
Figure 3.13. Chemokine-leukocyte correlations in ovarian carcinomas. The relationship between MCP-1 expression and the number of CD8+ (A) and CD68+ (B) cells identified in frozen sections of 20 ovarian tumours was assessed. The correlation between CD8+ and MCP-1 ($r_s=0.63$, $P<0.005$) was stronger than that for CD68+ and MCP-1 ($r_s=0.50$, $P=0.026$).
3.5. Summary and discussion

MCP-1 mRNA expression was detected in several ovarian carcinoma cell lines and in frozen sections of ovarian tumours. MCP-1 protein production was detected in ascites from patients with ovarian carcinomas and the supernatants of ovarian cell lines and tumour cells freshly isolated from ascites. In the latter it was present at much higher levels than culture supernatants of macrophages from the same patients. The expression of MIP-1α, MIP-1β and RANTES was detected by RT-PCR in tumour samples, but in fewer cell lines. MCP-1 expression in cryostat sections localised to tumour cells as well as stromal elements and was expressed by significantly more cells than any of the other chemokines examined. MIP-1α expression was confined to tumour stroma and there was no significant difference here between the number of cells expressing MIP-1α and MCP-1. The other two chemokines, MIP-1β and RANTES were expressed by far fewer cells, and cells expressing RANTES were only weakly positive, consistent with the RT-PCR results. There was a weak correlation between the total number of cells expressing MCP-1 per section and the total number of CD68+ cells. However, the correlation between MCP-1 expression and CD8+ cells was stronger. A correlation was also found between RANTES expression and the number of CD8+ cells, although because RANTES expression was so weak, the functional significance of this is not clear. CD68+ cells and MCP-1 expression were found in the stroma of normal ovaries.

As in the original study by Bottazzi (320), production of MCP-1 from ovarian cell lines was variable. Expression of mRNA by SKOV-3 and PEA2 cells was barely detectable and is unlikely to be functionally significant. Other studies have also concluded that SKOV-3 does not produce MCP-1 (354) and although MCP-1 was detected by RT-PCR in OVCAR-3 cells they are known to produce only low levels of chemotactic activity (320). The production of MCP-1 by SKOV-3, OVCAR-3, PEO1 and PEO14 cells is examined in more detail in chapter 4. Both the in situ hybridisation studies and the ascites data indicate that there is also a wide variation in the expression and production of MCP-1 from whole tumours. In situ hybridisation indicated that MCP-1 expression occurs within discrete clusters of cells in the tumour parenchyma. The cell line data had already indicated that tumour cells themselves could be a source and this was confirmed by determining the expression of MCP-1 in isolated tumour cells. The existence of a chemokine gradient between tumour and blood was indicated by the presence of MCP-1 in ascites but not in plasma of patients with ovarian tumours. A potential gradient of chemotactic activity has also been described in carcinoma of the lung (365).
On the basis of the leukocyte phenotyping data, several predictions had been made regarding the likely presence of MIP-1α, MIP-1β and RANTES. These were borne out by the *in situ* analysis. The functional significance of the expression of these chemokines is not clear, although given the level of expression of MIP-1β and RANTES it seems unlikely that they play a significant role. MIP-1α, however, was expressed by a large number of cells within the stroma and in several instances it was possible to relate these directly to the CD8+ population. It is likely that both the CD8+ population and macrophages contribute to the expression of MIP-1α which may recruit more CD8+ cells. However, MCP-1 is also capable of attracting CD8+ lymphocytes (108) and a statistically significant relationship was found between the number of cells expressing this chemokine and the number of CD8+ cells. MIP-1α may serve to amplify the recruitment of CD8+ cells, from a population initially attracted by MCP-1. How the expression of MIP-1α is regulated within these tumours is not known.

In this study, only a weak correlation existed between MCP-1 expression and the number of TAM. Previous studies have found significant correlations between chemotactic activity and the number of TAM. For instance, in the study on the identification of chemotactic activity from ovarian tumours, there was a significant correlation between TAM and chemotactic activity \( r=0.62, P=0.00097 \) (320). In the present study, several factors may have led to a decrease in the significance of the correlation between the number of MCP-1 expressing cells and the number of TAM. The number of TAMs were correlated with MCP-1 mRNA rather than protein and the level of mRNA expression was considered in terms of whole cells. The method of assessing TAM was deliberately stringent. Only cells with nuclear and cytoplasmic staining were counted and this may have led to an underestimation of the number of cells present. Tissue preservation may also have played a role. In several sections it appeared that areas of necrosis with their associated TAM may have been ripped out when preparing cryostat sections, again leading to the number of these cells being underestimated. Furthermore, when the results of the distribution of CD68+ cells in paraffin embedded sections was compared with the results of the distribution of MCP-1 expressing cells in frozen sections, a discrepancy in the spatial relationship between MCP-1 expression and macrophage accumulation became apparent. Although the highest density of macrophages was observed in necrotic regions, MCP-1 expression was not found in or adjacent to these areas. It was reasoned that either another chemoattractant was involved or that some other variable(s) must interact with MCP-1 to create the observed patterns of macrophage distribution and chemokine expression.
In order to try and resolve this apparent paradox, the regulation of the expression of MCP-1 was investigated. In the first instance, inflammatory cytokines and growth factors were tested for their ability to stimulate MCP-1 production. This work is described in chapter 4.

3.6. Conclusions

• MCP-1 is a major CC chemokine in human ovarian carcinomas.
• Tumour cells are capable of producing significant quantities of MCP-1.
• Other sources of MCP-1 within the tumour are likely to exist, particularly macrophages.
• A significant correlation exists between the number of CD8+ T cells and the number of cells expressing MCP-1.
• The relationship between MCP-1 mRNA expression and the number of TAM was weak.
• The major sites of MCP-1 expression were spatially separate from the sites where TAM accumulate at highest density.
• MIP-1α is expressed by an equal number of cells in the stroma to MCP-1.
• MIP-1α is likely to be expressed by CD8+ T cells and TAM.
• MIP-1β and RANTES were expressed by a small number of cells. Their main sources were considered to be mononuclear cells.
Chapter 4

The effect of cytokines and other inflammatory mediators on MCP-1 expression in human ovarian cancer cell lines

4.1. Introduction

The results of chapter 3 demonstrated that MCP-1 expression occurs in discrete clusters of cells within human ovarian carcinomas. Constitutive expression of this chemokine between different ovarian tumour cell lines was also variable. MCP-1 production can be stimulated by a large number of cytokines and other factors in a variety of different cell lines. Since inflammatory cytokines and growth factors are found in ovarian cancers, the effect of several of these on MCP-1 expression and production was tested. A brief description of the cytokines tested in this chapter is given first, together with evidence for their presence in ovarian tumours. In chapter 2, the presence of necrotic regions within the tumours examined was described. Since many of these areas contain red cell fragments, it was assumed that components of the coagulation system might also be present. One clotting factor, thrombin, can stimulate MCP-1 production and the effect of thrombin on MCP-1 expression by an ovarian tumour cell line was also tested.

One cytokine that consistently stimulates MCP-1 production and that is present in ovarian cancers is TNF-α. mRNA and protein have both been detected in human ovarian tumours and the amount of TNF-α produced correlated with tumour grade (308). However, while TNF-α mRNA was associated with tumour parenchyma, TNF-α protein localised mainly to TAMs, particularly those occurring at the stromal epithelial border. Both receptors for TNF-α were found in this study. TNFR p55 was associated with the tumour parenchyma and had a homogenous distribution. The expression of p75 was restricted to single cells and had the same distribution as TAMs. TNF-α expression has been found in other tumours, such as malignant melanoma (360), some brain tumours (366) and carcinoma of the breast (367). In contrast to the destructive effect of high doses of exogenous TNF-α on methylcholanthrene induced sarcomas in mice (368), more recent evidence suggests a role for low doses of endogenous TNF-α in the growth of solid tumours. TNF-α stimulated the proliferation of several ovarian cancer cell lines (310) and in a human
tumour xenograft model of ovarian cancer, exogenous TNF-α caused malignant cells in ascites to adhere to the peritoneal surface and form solid tumours (369). TNF-α has been implicated in angiogenesis (370), although this effect may be indirect and it can induce chemokine expression, including MCP-1 and IL-8. How production of this cytokine is regulated within tumours is not known. TNF-α can induce its own production and there is evidence that TNF-α production in PMA stimulated U937 cells can be augmented by low oxygen tensions (371).

Another inflammatory cytokine identified in ovarian cancers is IL-1. This consists of two forms, IL-1α and IL-1β (see (372) for review). Like TNF-α, IL-1 can specifically induce the transcription of a wide variety of genes, including interleukins, tumour necrosis factors, colony stimulating factors, complement components, growth factors, collagenases and chemokines. However, the role of this cytokine in ovarian tumours is not clear and reports on the production of IL-1α and IL-1β are conflicting. Naylor et al found low levels of IL-1β mRNA by in situ hybridisation in a similar distribution to the macrophage population (373). However, in another study, TAM isolated from ascites were not found to release appreciable levels of IL-1 spontaneously or following LPS stimulation and were poor producers of this cytokine compared with blood monocytes (309). IL-1α expression was detected in 9/11 ovarian tumours by RT-PCR (175). Transcripts for both IL-1α and IL-1β have been found in ovarian tumour cell lines and in ovarian tumour cells isolated from ascites (374), but IL-1α and IL-1β protein have not been found (375, 376).

IL-6 can stimulate MCP-1 production. It is a 21-28kDa glycoprotein involved in the regulation of immune responses, acute-phase reactions and haematopoiesis (see (377) for review). Both lymphoid and non-lymphoid cells can produce IL-6, including T cells (378), monocytes (379) and fibroblasts (380). It has been detected in ovarian tumours by in situ hybridisation, but was expressed by fewer cells than either TNF-α or IL-1β (373). IL-6 has also been found in ascites from patients with ovarian cancer (375, 376, 381). Both macrophages (309) and tumour cells are potential sources of IL-6. In one study IL-6 production was detected in ovarian cancer cell lines and primary tumour cell cultures (382).

Of the growth factors that can stimulate the production of MCP-1, the best known is PDGF. The murine homologue of MCP-1, JE, was originally identified from a cDNA library prepared from mouse 3T3 cells stimulated with PDGF (94). The JE gene was subsequently shown to encode a chemokine (95) that is highly homologous (68%) to human MCP-1 (56). PDGF itself was identified as a mitogen released from platelets.
(383). It is a cationic protein of approximately 30 kDa (384), consisting of two distinct chains, A and B (385). Naturally occurring PDGF can exist as either a homodimer or heterodimer of the two chains.

As well as its mitogenic activity, PDGF can stimulate eicosanoid production (386), collagen synthesis (387) and collagenase activity (388). It has chemotactic activity for fibroblasts (389) and smooth muscle cells (390) and mitogenic activity for connective tissue cells (see (391) for review). Ovarian epithelial tumour cell lines expressed both PDGF A and B, but not PDGF receptors (392). There was no evidence of PDGF gene rearrangement or amplification in these cell lines. In serous ovarian carcinomas, immunoreactive PDGF has been detected in tumour cells and the PDGFRα but not PDGFRβ was detected in some tumours. PDGFRα can bind all three PDGF isoforms, while PDGFRβ is specific for PDGF-BB (393). In colorectal carcinoma on the other hand, PDGFRβ staining was confined to tumour stroma, and PDGF AB and PDGF BB isoforms were expressed by macrophages (394). PDGF has been linked to tumour progression by both autocrine stimulation of tumour cell growth and development of tumour stroma.

The reported effects of TGF-β1 on MCP-1 expression are conflicting, although all refer to members of the monocyte/macrophage lineage. TGF-β1 upregulated MCP-1 expression from both osteoblasts (395) and astrocytes (396). Recently, Kitamura reported the isolation of a factor from the conditioned medium of glomerular mesangial cells that repressed the induction of MCP-1 by LPS in J774.2 macrophages (397). This activity was neutralised by anti-TGF-β1 antibodies and could be reproduced by exogenous TGF-β1. TGF-β1 is involved in cell growth, differentiation, extracellular matrix production and apoptosis (see (398) for review). The relationship between TGF-β1 and the growth of ovarian tumours is not clear. Malignant ovarian tumours have been associated with a loss of autocrine growth inhibition by TGF-β1 (399). Furthermore, in some ovarian cell lines, TGF-β1 can induce apoptosis (400). Isolated carcinoma cells in suspension produce TGF-β1 (401) and TGF-β receptors have been detected in ovarian cancer cell lines (402). But this cytokine can also induce cytotoxicity in T cells (401).

Another factor that has been shown to stimulate MCP-1 production, and that may be present in ovarian tumours is thrombin. This serine protease is an important component of the coagulation cascade and has multiple cell activating functions. For instance, it is mitogenic for fibroblasts and vascular smooth muscle cells (403, 404) and can stimulate the production of PDGF in endothelial cells (405). Since thrombin
is released at sites of vascular injury, a potent cause of necrotic tissue damage, it might be expected to be present in the necrotic regions of ovarian carcinomas. Thrombin can stimulate MCP-1 production by mesangial cells (104) and increased mRNA levels have been detected in human peripheral blood monocytes, endothelial cells (406) and liver fat storing cells (407).

4.2. Aims of chapter
The main aim of this chapter was to examine the effect of the inflammatory cytokines TNF-α, IL-1β and IL-6, and the growth factors PDGF and TGF-β1, on MCP-1 production by ovarian cancer cell lines. In addition, the effect of thrombin on MCP-1 expression was studied. Further studies on TNF-α induced MCP-1 expression were performed and the effect of TGF-β1 on MCP-1 expression was examined.
4.3. Materials and methods

4.3.1. Reagents
Recombinant human TNF-α was supplied by BASF/Knoll (Maidenhead, Berkshire, UK). Recombinant human IL-6, IL-1α, human TGF-β1 and human PDGF were purchased from R&D Systems (Abingdon, Oxon, UK). Thrombin was purchased from Sigma (Poole, Dorset, UK).

4.3.2. Cell lines and culture
The ovarian cancer cell lines PEO1, PEO14, OVCAR-3 and SKOV-3 have been described (chapter 3, section 3.3.2.1.). The hepatoblastoma cell line HepG2 (408) and the breast carcinoma cell lines MCF-7 (409) and MDA-MB-231 (410) are held at ICRF, having originally been obtained from the ATCC. All cell lines were grown in a humidified atmosphere, containing 5% CO₂ at 37°C under pyrogen free conditions. HepG2, MCF-7 and MDA-MB-231 grow as adherent monolayers and were routinely cultured in E4 supplemented with 10% FCS. For stimulation experiments, cells were seeded at 8 x10⁴/cm² into 6- or 24-well plates in serum-free medium and allowed to adhere overnight. They were washed twice with fresh medium prior to being stimulated.

4.3.3. Probes
The cDNA for MCP-1 used to make probes for northern analysis was an approximately 400bp PstI restriction fragment which spanned the entire protein coding region. The sources of MCP-1 and β-actin cDNA have been given in chapter 3, section 3.3.5.

4.3.4. Northern analysis
Total RNA was prepared from approximately 2 x10⁶ cells using Tri-Reagent™ (Molecular Research Centre Inc., Cincinnati, OH, USA) according to the manufacturers protocol. 15µg of total RNA was subjected to electrophoresis through a 1% agarose-formaldehyde gel and blotted by capillary transfer onto nylon membranes (Hybond N+, Amersham, UK). After transfer, membranes were UV crosslinked (1200J, Stratalinker, Stratagene, La Jolla, CA, USA). Blots were prehybridised for 2-4 hours at 42°C in formamide (45%), phosphate buffer (0.2M), BSA (1%), SDS (7%) and EDTA (1mM). Probes were labelled by random priming using a commercially available kit (Prime-It, Stratagene). Hybridisation was performed overnight at 42°C. Following hybridisation, membranes were washed twice in 2x SSC, 0.1% SDS for 5 minutes at room temperature, twice in 0.1% SSC, 0.1% SDS
for 15 minutes at 70°C and finally 2x SSC for 10 minutes at room temperature. Blots were wrapped in Saran wrap (Dow Chemical Company) and exposed to film (X-OMAT or Biomax MS, Eastman Kodak Co., Rochester, NY, USA) with an intensifying screen at -70°C.

4.3.5. RT-PCR
Primers for TNFR p55 and p75 were obtained from Clontech (Palo Alto, USA). Primers for MCP-1 and GAPDH have been described (chapter 3, section 3.3.4.ii). Total RNA was prepared as before (section 4.2.4). Reverse transcription and PCR reactions were performed as described previously (chapter 3, section 3.3.4).

4.3.6. ELISA
Supernatants were assayed for MCP-1 with a commercially available ELISA (Quantikine, R&D), according to the manufacturer's protocol. This assay has a sensitivity of 5pg/ml. After addition of the chromogen, plates were read in a Dynatech MR 5000 plate reader at 450nm. Data was analysed using the computer software Biolinx™ (Dynatech Laboratories, Inc., Chantilly, VA, USA).
4.4. Results

4.4.1. Constitutive and inducible production of MCP-1

4.4.1.i. Constitutive production of MCP-1
MCP-1 production was detected by ELISA in unstimulated PEO14 cells grown for 24 hours in the absence of serum, but not in PEO1 cells (figure 4.1A and B). Pooling the results of the control groups used for the stimulation experiments gave a mean production of 376pg/ml over 24 hours (maximum 975.92pg/ml, minimum 23.6pg/ml). Constitutive production of MCP-1 was not found in any other cell line tested.

4.4.1.ii. The effect of growth factors on MCP-1 production
The effect of stimulating PEO1 and PEO14 cells with 10ng/ml PDGF and TGF-β1 on MCP-1 expression was determined by ELISA. Neither of these factors increased MCP-1 production over basal levels (figure 4.1A and B).

4.4.1.iii. The effect of inflammatory cytokines on MCP-1 production
The effect of the inflammatory cytokines TNF-α, IL-1α and IL-6 on MCP-1 production by PEO1 and PEO14 cells was determined by ELISA. Both IL-1α and TNF-α were strong inducers of MCP-1 expression whereas IL-6 had no effect (figure 4.1A and B). MCP-1 protein was also induced in OVCAR-3 cells in response to TNF-α. Since there is evidence for significant production of TNF-α by ovarian tumours, the effect of this cytokine on the expression of MCP-1 by ovarian cancer cell lines was studied in more detail.
Figure 4.1. The effect of growth factors and inflammatory cytokines on MCP-1 production. PEO1 (A) and PEO14 (B) cells were stimulated with TNF-α, IL-1α, IL-6, PDGF and TGF-β1. MCP-1 production was determined by ELISA. Data are shown for stimulation with 10 ng/ml of each cytokine. PEO1 cells did not show constitutive expression of MCP-1, but production was stimulated in response to both TNF-α and IL-1α. PEO14 cells showed variable constitutive expression of MCP-1. Like the PEO1 cells, production was only significantly increased by TNF-α and IL-1α (P<0.0005 for all comparisons, unpaired t-test). The results are the means (±SD) of three separate cultures.
4.4.1. iv. The effect of thrombin on MCP-1 expression
The effect of 2U/ml thrombin was tested on PEO1 cells seeded serum-free into 24-well plates. Cells were cultured for up to 24 hours, but expression of MCP-1, determined by RT-PCR, could not be detected (data not shown).

4.4.2. TNF-α induced MCP-1 expression in ovarian cancer cell lines

4.4.2.1. Time-course of TNF-α induced MCP-1 expression
Since TNF-α was a potent inducer of MCP-1 production, its action was investigated further. PEO14 cells were cultured for up to 48 hours in the continued presence of TNF-α. Stimulation of MCP-1 expression was seen as early as 30 minutes after addition of TNF-α and was maximal between two and four hours. In the continued presence of TNF-α, MCP-1 expression was still elevated at 48 hours (figure 4.2), but cells washed twice in medium alone at three hours did not continue to express MCP-1 at high levels at 24 hours (CJ Scotton, personal communication).

4.4.2. ii. Dose-response of MCP-1 expression to TNF-α
PEO14 cells were exposed to doses of TNF-α ranging from 0.01ng-100ng for three hours. Induction of MCP-1 expression could be seen with doses as low as 1ng/ml (figure 4.3).

4.4.2. iii. TNF-α receptor expression
Expression of TNFR p55 and p75 by the cell lines PEO1 and PEO14 was assessed by RT-PCR. Only expression of p55 could be detected in either cell line (figure 4.4). There was no obvious effect on receptor expression following stimulation of cells with TNF-α for three hours.

4.4.2. iv. Effect of TGF-β1 on TNF-α induced MCP-1 expression
Since TGF-β1 has been reported to down-regulate MCP-1 expression, the effect of various doses of this cytokine on TNF-α induced MCP-1 expression was also tried. However, there was no apparent effect on MCP-1 expression (figure 4.5).
Figure 4.2. Time-course of response of PEO14 cells to stimulation with TNF-α. In a time-course experiment PEO14 cells were cultured for up to 48 hours in the presence of 10ng/ml TNF-α and MCP-1 expression was assessed by northern analysis (A). β-actin was used to control for loading. The relative expression of MCP-1, corrected for loading against β-actin (B) emphasises that MCP-1 expression was maximal between two and four hours and was sustained throughout the whole 48 hour period of the experiment.
Figure 4.3. Dose-response of PEO14 cells to stimulation with TNF-α. In dose-response experiments MCP-1 mRNA could be detected after three hours of stimulation with 1ng/ml TNF-α. Northern analysis (A) showing MCP-1 expression by PEO14 cells in response to doses of TNF-a from $1 \times 10^{-2}$ to 100ng/ml for three hours. Integrated densities indicating the relative change in MCP-1 levels corrected for β-actin are also shown (B). These results are representative of two separate experiments.
Figure 4.4. Expression of p55 and p75 in PEO1 and PEO14 cells as determined by RT-PCR.

123, 123bp markers; +, positive control for PCR; -, negative control for PCR; con, positive controls for receptor expression; lane 1, PEO1 cells alone; lane 2, PEO1 cells stimulated with 10ng/ml TNF-α for three hours; lane 3, PEO14 cells alone; lane 4, PEO14 cells stimulated with 10ng/ml TNF-α for three hours. Only expression of the p55 component of the receptor was detected, even in stimulated cells.
Figure 4.5. The effect of TGF-β1 on MCP-1 expression.

PEO14 cells stimulated with 10ng/ml TNF-α were cultured in the presence of increasing concentrations of TGF-β1. MCP-1 expression was by detected by northern analysis. Lane 1, cells alone; lane 2, TNF-α alone; lane 3, 10ng/ml TGF-β1 alone; lane 4, TNF-α + 0.1ng/ml TGF-β1; lane 5, TNF-α + 1ng/ml TGF-β1; lane 6, TNF-α + 10ng/ml TGF-β1.
4.4.2. **Effect of TNF-α on MCP-1 expression in other cell lines.**

The response of several other cell lines to stimulation with TNF-α was assessed. TNF-α induced MCP-1 expression could not be detected in HepG2 cells by RT-PCR. Similarly in the breast cancer cell lines MCF-7 and MDA-MB-231, mRNA for MCP-1 could not be detected by northern analysis when these cells were stimulated with TNF-α and in the ovarian cancer cell line SKOV-3, MCP-1 expression and production could not be detected by northern or ELISA respectively. The results of MCP-1 expression by epithelial tumour cell lines, alone or in response to TNF-α are summarised in table 4.1.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Constitutive MCP-1 expression</th>
<th>TNF-α inducible MCP-1 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEO1</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>PEO14</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>OVCAR-3</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>MCF-7</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>HepG2</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 4.1. MCP-1 expression by tumour cell lines.
Summary of constitutive and inducible expression of MCP-1 in ovarian cancer cell lines and three other epithelial tumour cell lines.
4.5. Summary and discussion

In this chapter an attempt was made to establish some of those factors that might affect MCP-1 expression by tumour cells. Of the inflammatory cytokines and growth factors, TNF-α and IL-1α were potent stimulants to the production of MCP-1, whilst the others were unable to stimulate production of this chemokine from either PEO1 or PEO14 cells. In addition, TNF-α stimulated the expression of MCP-1 from OVCAR-3, but not SKOV-3 ovarian cancer cell lines. Thrombin was tested since several other cell types have been shown to respond to this factor (406), and it might reasonably be expected to occur within the necrotic regions of tumours, along with other members of the coagulation cascade (411). TGF-β1 had no effect on MCP-1 expression alone or in combination with TNF-α.

As demonstrated by northern analysis, TNF-α regulates the expression of MCP-1 at the level of mRNA. Low doses of TNF-α could stimulate MCP-1 expression and in the continued presence of TNF-α, MCP-1 mRNA levels were sustained in PEO14 cells. The results of RT-PCR for the different components of the TNFR support the idea, based on the results of in situ studies, that tumour cells express only p55 (308). Therefore, TNF-α induced MCP-1 expression probably occurs via this receptor.

Both constitutive and inducible MCP-1 expression varied between the ovarian cancer cell lines PEO1 and PEO14. No expression was found in the other epithelial cell lines tested. In the previous chapter, constitutive expression had been detected in all the cell lines tested, but this was using RT-PCR and the PEO14 cells gave the strongest band. These results are consistent with earlier observations that different cell types produce different amounts of TDCF and notably, MCF-7 cells (319) were poor producers of this activity. In a separate study, MCP-1 production by SKOV-3 cells (412) could not be detected.

The variation in the expression of MCP-1 by different cell lines and the stimulation of expression of MCP-1 by TNF-α goes some way to explaining the patchy pattern of MCP-1 expression that is seen in tumours. If some cells express MCP-1 more highly than others, then even in the absence of other stimuli, variations in the in situ signal might be expected. In the presence of TNF-α, these signals might be further modified. However, as described in chapter 2, the highest density of macrophages appeared to be in and around necrotic regions within these tumours, whereas in none of the sections examined was MCP-1 expression located in or adjacent to these areas. This spatial separation between some macrophages and the sources of MCP-1 might explain the lower correlation that was found between the number of macrophages and
the number of MCP-1 expressing cells. As discussed in the introduction, these regions are likely to be very hypoxic. Furthermore, hypoxia can enhance TNF-α production in mitogen stimulated U937 cells and in frozen sections, immunoreactivity to TNF-α localised to clusters of macrophages, which would be consistent with those in necrotic regions in paraffin embedded sections. Since MCP-1 expression is stimulated by TNF-α, it was surprising that in situ signal was absent adjacent to necrotic regions. In order to investigate this potential paradox, the direct effect of low oxygen tension on TNF-α induced MCP-1 expression was examined. This forms the subject of the final results chapter.

4.6. Conclusions

- Of the inflammatory cytokines and growth factors that might potentially induce MCP-1 in ovarian carcinomas, TNF-α and IL-1α are the most potent in the cell lines PEO1 and PEO14.
- Thrombin does not induce MCP-1 expression in these cell lines.
- TGF-β1 does not affect MCP-1 expression in the ovarian line PEO14.
- There is considerable heterogeneity in MCP-1 expression by different cell lines.
- TNF-α induced MCP-1 expression can be sustained in the continued presence of TNF-α.
5.1. Introduction

The results presented in chapters 2 and 3 indicated that a discrepancy exists between the distribution of TAMs associated with necrotic regions of tumour, and the distribution of MCP-1 expression. In chapter 4, TNF-α was found to be a potent stimulus to MCP-1 production in 2 ovarian carcinoma cell lines. Other factors that might explain variability in MCP-1 expression, such as differences in constitutive expression between cell lines and the effect of TGF-β1 were also investigated, but neither of these appeared to have a significant effect on MCP-1 gene expression. In particular, they did not explain the lack of MCP-1 expression adjacent to regions of necrosis. Since the oxygen tension in necrotic regions is very low, the effect of anoxia on TNF-α induced MCP-1 expression, monocyte migration in response to MCP-1 and MIF expression were tested.

Necrotic regions are likely to be anoxic. There is increasing evidence that in mammals a specific oxygen sensing system mediates many of the responses to low oxygen tensions. This system regulates the expression of genes, including enzymes involved in glucose metabolism, erythropoietin (Epo) and VEGF. It also plays a part in physiological responses to hypoxia, such as changes in the respiratory rate. Many of these effects enhance oxygen utilisation or delivery. One of the first genes to be examined in the context of its response to hypoxia was Epo. This cytokine stimulates the production of erythrocytes from the bone marrow (413). In mammals the main sources of Epo are the foetal liver (414) and the adult kidney (415). In 1988 Goldberg et al used the human hepatoma cell line Hep3B to study the oxygen sensing system responsible for mediating the increased production of Epo in response to hypoxia (416). Since cobalt chloride was known to increase the red cell mass in man (417) and cobalt (418) and nickel (419) were known to stimulate Epo production in perfused kidneys, the effects of these and other metal ions were tested on Hep3B cells. Manganese, in addition to cobalt and nickel also induced Epo production, but iron, zinc, cadmium and tin did not. Incubation of cells in the presence of cycloheximide (CHX) decreased the level of Epo expression in response to cobalt and hypoxia, indicating that protein synthesis is a necessary step prior to Epo production. Since manganese,
cobalt and nickel can all bind to haemoglobin and lock the molecule in the deoxy conformation, a heme-like oxygen sensor was proposed as an intermediate between the hypoxic stimulus and the production of Epo. On the basis of these results, it was also predicted that carbon monoxide, which mimics oxygen but binds to haemoglobin with very high affinity, should inhibit the effect of hypoxia. Hep3B cells cultured in the presence of 1% oxygen and 10% carbon monoxide were found to express only 1/3-1/4 the amount of Epo compared with those cultured in 1% oxygen alone. These results strongly suggested that the response to hypoxia in these cells was via a specific oxygen sensor rather than through non-specific effects on oxidative phosphorylation.

An Epo enhancer sequence at the 3' end of the gene and cis-acting sequences responsible for the regulation of Epo production were subsequently defined (419). The existence of a specific oxygen sensing system was then demonstrated in a wide variety of cells including human hepatoma, human foetal fibroblast, human skin fibroblast, pig renal epithelium, monkey renal fibroblast, rat aortic endothelium human myelomonocytes, Chinese hamster lung fibroblast, Chinese hamster ovary and mouse renal adenocarcinoma (420). When these cell lines were transfected with plasmids containing the Epo 3' enhancer linked to the α1-globin gene, hypoxia induced activity was detected by an increase in reporter transcripts. Thus although tissue expression of Epo is tightly restricted, the oxygen-sensing mechanism appears to be widespread.

Further analysis of the Epo 3' enhancer region showed that an approximately 50-nucleotide sequence confers enhancer activity and that a transcription factor, termed hypoxia inducible factor 1 (HIF-1) acted through this sequence. HIF-1 bound the target sequence 5'-TACGTGCT-3' in the Epo enhancer. This site-specific binding enabled HIF-1 to be purified and cloned (421). HIF-1 is a heterodimer of α and β subunits each of which contains a basic helix-loop-helix motif and a PAS protein-protein interaction domain that had been found in a variety of known or suspected transcription factors (422). Comparison of the β subunit of HIF 1 revealed it to be identical to the aryl nuclear receptor translocator that had been implicated in xenobiotic responses (421). Whilst the HIF-1β subunit is constitutively expressed, HIF-1α expression is induced by hypoxia, thereby accounting for the effects of CHX described by Goldberg et al (416).

A wide variety of other genes, particularly those related to glucose metabolism are now known to be regulated by hypoxia. Both phosphoglycerate kinase 1 (PGK1) and lactate dehydrogenase A genes have control elements similar to the Epo 3' enhancer (423). The expression of these genes could be induced in a variety of cells in response
to 50µM cobalt chloride, but expression was not affected by 100µM cyanide. Furthermore, electrophoretic-mobility shift assays showed that oligonucleotides from the PGK 1 and Epo enhancer cross-compete for DNA-binding with hypoxia-inducible factors. The hypoxia response elements in the Ldha (lactate dehydrogenase A) and ENO1 (enolase 1) gene promoters have also been found to contain functionally essential HIF-1 sites (424). Other genes which show responses to hypoxia, cobalt or electron transport inhibitors consistent with a system involving a specific oxygen-sensor and HIF-1, include glycolytic enzymes, glucose transporters and mitochondrial genes (425). Not all these genes are upregulated by hypoxia. For instance, the expression of the glucose transporter, GLUT-2, was repressed by hypoxia or cobalt.

Angiogenic factors and growth factors are under the control of similar systems. Hypoxia can upregulate the expression of VEGF (256) and FGFs (426). These effects are mimicked by desferrioxamine (DFO) and cobalt respectively. Growth factors under hypoxic regulation include PDGF-A and PDGF-B and TGF-β1 (256). Recently Maxwell et al have shown that tumours derived from Hepa-1 cells functionally deficient in HIF-1 are less vascular, express less VEGF and generally grow more slowly than wild type tumours (427). Chemokine expression can be directly upregulated by hypoxia. In human endothelial cells incubated at oxygen tensions of less than 5% (14-18mmHg) Karakurum et al found an increase in IL-8 mRNA expression and protein production (106). IL-8 expression in response to hypoxia was associated with enhanced binding of nuclear factor κB (NF-κB) to the IL-8 promoter in electrophoretic-mobility shift assays.

Other studies have suggested that MCP-1 can be regulated by oxygen tension. For instance, MCP-1 has been found to be expressed in models of brain ischaemia (428). In human endothelial cells in culture, Karakurum et al claimed that hypoxia alone induced MCP-1 expression (106). However, they used GAPDH as an internal control, which is itself hypoxically regulated (429).

As well as examining the effect of hypoxia on MCP-1 expression, two potential factors that might affect macrophage migration in regions of necrosis were investigated. These were the direct effects of hypoxia on monocyte migration and the effect of hypoxia on expression and production of migration inhibitory factor (MIF). MIF was identified over 30 years ago as a T cell derived factor, capable of inhibiting the random migration of macrophages (7). It was subsequently found to be present in the circulation and to be expressed by the pituitary (430). Recently macrophages were also recognised as a source of this cytokine (431). MIF has been identified in
macrophages that accumulate in the alveoli of patients with the adult respiratory distress syndrome (432), in which hypoxia is a characteristic feature. Production of MIF can be upregulated by TNF-α, but the effect of hypoxia on its expression is not known. MIF can be expressed by normal ovary (433), and has been identified in ascites from patients with ovarian tumours (434). Although macrophages are recruited to inflammatory sites where blood flow might be expected to be altered, there do not appear to be any previous studies directly addressing the effect of low oxygen tension on their response to chemoattractants.

5.2. Aims of the chapter
The principle aim of this chapter was to investigate the effects of low oxygen tension on MCP-1 expression by ovarian cancer cells stimulated with TNF-α, and to determine whether this response was consistent with a specific oxygen-sensing system. Since TNF-α acts through the transcription factor NF-κB, which in turn can be regulated by the availability of oxygen free-radicals, an attempt was made to determine whether the effect of hypoxia was equivalent to removing free-radicals. The migration of monocytes to MCP-1 under normoxic and hypoxic conditions was examined in order to try and explain the high density of these cells in necrotic regions of tumour.
5.3. Materials and methods

5.3.1. Reagents
Cobalt chloride (CoCl₂), desferrioxamine (DFO), potassium cyanide (KCN) and pyrroldine dithiocarbamate (PDTC) were obtained from Sigma. Hydrogen peroxide (H₂O₂) was from BDH Laboratory Supplies (Poole, UK). Recombinant human MCP-1 was purchased from PeproTech EC Ltd. (London, UK). Antibodies to MIF (polyclonal goat anti-human IgG) were purchased from R&D Systems (R&D). Secondary antibodies (rabbit anti-goat) were from DAKO. Gas cylinders were purchased from BOC (Manchester, UK).

5.3.2. Cell lines and culture
Cell culture was essentially as described in chapters 3 and 4. The ovarian cancer cell line PEO14 was used for the majority of the experiments described in this chapter. Additional experiments employed PEO1 and OVCAR-3 cells. For all experiments, adherent cell lines were seeded at a density of 8 x 10⁴/cm², in 6-well plates (Nalge Nunc International, Denmark) THP-1 cells (435), used in the migration assays, were purchased from the American Type Culture Collection (Rockville, Maryland, USA). Mono-mac-6 cells, used to investigate MIF expression, were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). These myelomonocytic cell lines were chosen because both are known to express the MCP-1 receptor CCR2 (142). These cell lines were cultured in RPMI 1640 with 10% FCS and were split up to 3 times per week to a density of 5 x 10⁵/ml. Mono-mac-6 cells were supplemented with 9µg/ml bovine insulin (Sigma) and non-essential amino acids (Gibco BRL). THP-1 cells were supplemented with 50µM β-mercaptoethanol (Sigma).

5.3.3. Hypoxic culture
Cells were gassed with the desired mixture for 30 minutes in a modular incubation chamber (Flow Laboratories, Del Mar, Ca., USA) prior to incubation at 37°C. The length of time required to equilibrate the chamber was determined by gassing with 95% N₂, 5% CO₂ and measuring the oxygen tension of the effluent gas with a Thermox 1 analyser (Thermolab Instruments Inc., Pennsylvania, USA). Oxygen levels of 0.01% were achieved after 10 minutes and the chamber equilibrated after 20 minutes.
5.3.4. Probes
The cDNAs for MCP-1 and β-actin have been described. cDNA for GAPDH was kindly provided by Dr Sylvie Kossodo (Massachusetts General Hospital, MA, USA), TNF-α by TNF receptor p55 (TNFR p55) by Prof. M Feldman (Charing Cross Sunley Research Centre, London, UK) and MIF by Dr Graeme Wistow (National Institutes of Health, Bethesda, Maryland 20892, USA).

5.3.5. Northern analysis
Northern analysis was performed as described in chapter 4, section 4.3.4.

5.3.6. ELISA
The Quantikine ELISA kit (R&D) was again used for the detection of MCP-1 in culture supernatants (chapter 4, section 4.3.6.)

5.3.7. Western analysis
MIF production by mono-mac-6 cells was assessed by western analysis of cell lysates under non-reducing conditions. 1 x 10^6 cells were harvested by centrifugation at 4000rpm for 15 minutes. Cells were lysed in 1.5% Triton X1114 in PBS for 15 minutes at 4°C. The lysate was then spun, incubated for two minutes at 37°C, spun again and the supernatant transferred to a fresh tube on ice. Loading buffer (5x stock solution: 5% SDS; 0.5M Tris-HCl, pH 6.5; 50% glycerol; bromophenol blue crystals) was added to the lysate and the whole incubated at 65-70°C for 10 minutes prior to loading. For storage (-20°C) 20µl 0.5M EDTA and 2.5µl of 2.5mg/ml aprotinin were added to the samples. Size markers were run on each gel.

Samples were run on 15% SDS-PAGE gels at 150-180V in running buffer consisting of 0.025M Tris, 0.192M glycine and 0.1% SDS. Proteins were then transferred onto nitrocellulose membranes (Hybond™ ECL™, Amersham International plc, Bucks, UK) at 40V for 1-2 hours, in transfer buffer consisting of 7.2g glycine, 1.46 TrisBase and 200ml methanol made up to one litre with water.

Before probing membranes, markers were visualised with Ponceau S solution (Sigma) and marked with a scalpel on the membrane. Membranes were blocked for a minimum of one hour with 0.1% Tween-20 (National Diagnostics, Atlanta, Georgia, USA), 10% fat-free dried skimmed milk (Marvel, Premier Beverages, Adbaston, Stafford, UK) in PBS. After blocking, and between incubations with each antibody, membranes were rinsed and washed twice with PBS + 0.1% Tween-20 (PBS-T) for 15 minutes and twice with PBS-T for 5 minutes. Both primary and secondary
antibodies were diluted in 3% marvel, PBS-T and incubated at room temperature for one hour. Detection was performed using the ECL™ system (Amersham). Membranes were exposed to film (Fujifilm RX, Fuji Photo Film Co., Ltd., Tokyo, Japan) for between 30 seconds and 10 minutes.

5.3.8. Electrophoretic-mobility shift assays (EMSA)
EMSAs were performed by Dr Frances Burke in the Biological Therapies Laboratory, ICRF. DNA-binding protein extracts were prepared as described previously (436) with minor modifications. Briefly, 1-2 x10⁶ cells were washed in ice-cold PBS, pelletted and resuspended in lysis buffer (10mM Hepes, pH 7.9, 10mM KCl, 1mM DTT, 1mM EDTA, 1mM EGTA, 0.2% NP40, 0.5mM PMSF, 1mM sodium vanadate, 1mM β-glycerol phosphate, 20mM sodium fluoride and 1µg/ml each of aprotinin, leupeptin and pepstatin). The extracts were resuspended in buffer containing 25% glycerol and stored at -70°C prior to use.

For EMSA, double stranded oligonucleotide for NF-κB (Promega, UK) was end labelled with γ-32P ATP according to standard procedures. Binding reactions were performed in a total volume of 20µl in 40mM Hepes, pH 7.9, 2mM MgCl₂, 8% Ficoll, 2mM DTT, 100mM NaCl, and poly(deoxyinosine-deoxycytidine) 50µg/ml (Pharmacia Biotech, Herts, UK). Extracts were incubated with labelled oligonucleotides and incubated at room temperature for 20 minutes. Duplicate samples were also set up containing 50x excess of cold oligonucleotide to act as a competitor. Complexes were separated on 4% acrylamide gels in 0.25x tris-borate EDTA at 4°C, dried down and detected by autoradiography.

5.3.9. Preparation of PBMC
Peripheral blood mononuclear cells (PBMC) were prepared as described previously (165). In brief, heparinised blood samples were centrifuged on Lymphoprep™ (density gradient of 1.077), and the mononuclear cell layer was removed, washed twice and resuspended in RPMI 1640/1% BSA to a final total cell concentration of 1.5 x10⁶ cells/ml. Following migration assays, monocytes were identified by their characteristic morphology.

5.3.10. Migration assays
Migration assays were performed by Dr. Lynn Turner in the Biological Therapies Laboratory, ICRF. THP-1 cell and monocyte chemotaxis were examined using a 48-well microchemotaxis chamber (Neuro Probe, Cabin John, MD, USA). The lower wells were filled with 27µl chemoattractant in RPMI/1% BSA and covered with a 5µm
pore size polycarbonate membrane. 52μl of cell suspension was added to each upper well. THP-1 cells were used at a concentration of 1 x 10⁷ cells/ml. Migration assays under anoxic conditions were performed by placing the chemotaxis chamber in a modular incubation chamber and gassing with 95% N₂, 5% CO₂ for the entire duration of the assay. After incubation at 37°C for 90 minutes the membrane was removed and the non-cell side washed with PBS. The membrane was then fixed in methanol for two minutes before staining with Diff-Quik (Baxter, UK) according to the manufacturer’s protocol. THP-1 and monocyte migration was assessed by counting the number of cells attached to the lower surface of the membrane, in five high power fields (100x objective lens, 10x eyepiece) per well.

5.3.11. Image analysis
Autoradiographs were scanned with a UMAX scanner and manipulated with Adobe Photoshop 4.0. The determination of integrated density was performed using NIH Image 1.58. Scanners were calibrated with a Kodak photographic step tablet no.2. The films used for autoradiography were assumed to be linear over the range of exposures used. The relative level of MCP-1 expression was corrected by dividing by the corresponding density for β-actin.
5.4. Results

5.4.1. Response of TNF-α stimulated cells to hypoxia

5.4.1.i. Effect of hypoxia on constitutive and TNF-α induced MCP-1 expression

The effect of anoxia on TNF-α induced MCP-1 expression was tested in three ovarian cancer cell lines, PEO1, PEO14 and OVCAR-3. Cells were all plated at 8 x 10^4/cm^2 and allowed to adhere for 24 hours prior to stimulating with TNF-α and anoxia, alone and in combination, for 3 or 24 hours. To achieve anoxic conditions, the chambers were gassed for 30 minutes with 5% CO₂ and 95% N₂ (figure 5.1).

Figure 5.1. The change in oxygen content in a modular incubation chamber. Continuous gassing with 95% N₂ and 5% CO₂ at a flow rate of 5 litres/min, resulted in the oxygen content of the chamber being equilibrated with that of the gas supply after approximately 20 minutes. The volume of the chamber was approximately 7 litres.
In all three cell lines tested, the induction of MCP-1 expression by TNF-α was downregulated by hypoxia (figs 5.2-4). This effect was apparent at three hours in PEO14 cells and in the other cell lines at 24 hours. Fold-changes in gene expression were determined by performing integrated densitometry on autoradiographs, and correcting the results for the corresponding levels of β-actin expression. At 24 hours, there was over an 100-fold decrease in MCP-1 expression in PEO14 cells exposed to anoxia and an approximately 2-fold decrease in PEO1 and OVCAR-3 cells respectively. In none of the cell lines did hypoxia alone stimulate MCP-1 expression. GAPDH expression has been shown to be upregulated by hypoxia (429). This was found in all three ovarian cancer cell lines in response to hypoxia. In PEO14 cells there was a 10-fold increase in GAPDH in response to hypoxia (figureS2A). GAPDH was also upregulated in the other cell lines, but the changes were not so great (2-fold for PEO1 cells and OVCAR-3 cells).

There was also a statistically significant reduction in TNF-α inducible MCP-1 protein production in response to culture under anoxic conditions for all three cell lines (figure 5.5). A decrease in the constitutive production of MCP-1 protein by PEO14 cells was observed, although this did not achieve statistical significance. The effect of hypoxia on TNF-α induced and constitutive MCP-1 expression was not confined to the ovarian tumour cell lines, since a 6-fold decrease in MCP-1 mRNA levels was also found in mono-mac-6 cells (figure 5.6).
Figure 5.2. The effect of anoxia on TNF-α induced MCP-1 expression in PEO14 cells.

The effect of anoxia (95% N₂, 5% CO₂), on MCP-1 mRNA levels in PEO14 cells stimulated with 10ng/ml TNF-α for three and 24 hours was assessed by northern analysis (A). Over an 100-fold reduction in MCP-1 mRNA levels occurred in response to anoxia at 24 hours. There was a reciprocal up-regulation in the expression of GAPDH. β-actin was used as a loading control. Integrated densitometry corrected for β-actin emphasises the downregulation of MCP-1 expression particularly at the 24 hour time-point (B). These results are representative of at least three independent experiments.
Figure 5.3. The effect of anoxia on TNF-α induced MCP-1 expression in PEO1 cells. Cells were stimulated with 10ng/ml TNF-α for three and 24 hours in the presence or absence of oxygen and MCP-1 mRNA levels were assessed by northern analysis. The changes in expression of both MCP-1 and GAPDH were less marked than those seen with PEO14 cells, but were still apparent, particularly at 24 hours (A). Integrated densitometry corrected for β-actin is also shown (B).
Figure 5.4. The effect of anoxia on TNF-α induced MCP-1 expression in OVCAR-3 cells.

The effect of anoxia (95% N₂, 5% CO₂), on MCP-1 mRNA levels in OVCAR-3 cells stimulated with 10ng/ml TNF-α for three and 24 hours assessed by northern analysis (A). The changes in expression of MCP-1 and GAPDH were similar to those seen in PEO1 cells. Integrated densitometry was again performed and corrected for β-actin (B).
Figure 5.5. The effect of anoxia on MCP-1 protein production by ovarian cell lines. PEO1, PEO14 and OVCAR-3 were stimulated with 10ng/ml TNF-α in the presence and absence of oxygen. In each case there was a significant decrease in the amount of TNF-α inducible MCP-1 protein when cells were cultured under anoxic conditions, as opposed to normoxia (P<0.005 for PEO1 and PEO14 cells, P<0.05 for OVCAR-3, two sample t test). There was also a decrease in constitutive production by PEO14 cells, but this was not statistically significant.
Figure 5.6. The effect of anoxia on MCP-1 expression by mono-mac-6 cells. Mono-mac-6 cells were cultured for 24 hours under the conditions indicated. Northern analysis (A) and integrated densitometry (B) revealed constitutive expression of MCP-1 mRNA by these cells and a 6-fold downregulation of TNF-α inducible MCP-1 by anoxia.
5.4.1.ii. Time-course of action of hypoxia

PEO14 cells were cultured between one and 24 hours under anoxic conditions in the presence of TNF-α in order to try and establish when the response to hypoxia was first detectable, since in PEO14 cells an effect had already been seen at three hours. The earliest detectable response in this cell line occurred after two hours of anoxic culture (figure 5.7).

Figure 5.7. Time-course of the response of PEO14 cells to anoxia.

PEO14 cells were stimulated for three hours with 10ng/ml TNF-α, then cultured under anoxic conditions for up to 24 hours. MCP-1 expression was determined by northern analysis at the time points shown (A). +, cells cultured for three hours with 10ng/ml TNF-α under normoxic conditions; -, cells alone cultured under normoxic conditions. Integrated densitometry (B) demonstrates the effect of anoxic culture on MCP-1 expression at two hours.
5.4.1.iii. Effect of degree of hypoxia on MCP-1 expression

The effect of various concentrations of oxygen were tested on PEO14 cells. Cells were cultured for 24 hours in the continued presence of 10ng/ml TNF-α. Cultures were gassed with 0%, 1% or 5% O₂ (balanced with 5% CO₂ and N₂), or were cultured in normoxia with 5% CO₂. There was little difference in the level of MCP-1 expression in cells cultured in 5% O₂ compared with those cultured in air. However, there was a significant reduction in MCP-1 expression in 1% O₂ which was similar to that under anoxic conditions (figure 5.8).

![Figure 5.8. The effect of different oxygen concentrations on TNF-α induced MCP-1 expression.](image)

The effect of different concentrations of O₂ on TNF-α induced MCP-1 expression assessed by northern analysis (A). At 5% O₂ MCP-1 expression was unchanged, but at 1% O₂ it was reduced. Integrated densities corrected for loading by comparison with β-actin are also shown (B).
### 5.4.1.iv. Off-response to hypoxia

PEO14 cells were cultured under hypoxic conditions for 24 hours. They were then removed from the hypoxic chamber and 10ng/ml TNF-α was added to the culture medium. The cells were regassed with 5% CO₂, 95% N₂. Cells were estimated to have been exposed to more than 5% O₂ for a maximum of 20 minutes, including the time taken to regas the chambers. After a further three hours of incubation, cells were lysed. Northern analysis of RNA preparations revealed that the level of MCP-1 expression was greater than that seen in cells cultured for 24 hours with TNF-α under anoxic conditions, indicating that the hypoxic response had been switched off during the exposure of the cells to oxygen concentrations greater than 5% (figure 5.9).

![Figure 5.9. The off-response to hypoxia.](image)

Lane 1, PEO14 cells were stimulated with 10ng/ml TNF-α and cultured in oxygen for three hours; lane 2, cells similarly TNF-α stimulated, but cultured anoxically for three hours; lane 3, cells cultured under anoxic conditions for 24 hours before the addition of TNF-α and then cultured for a further three hours in oxygen; lane 4, as for lane 3, but cells were returned to anoxic conditions after the addition of TNF-α; lane 5, cells cultured in the presence of TNF-α under anoxic conditions for 24 hours. The ethidium bromide stained gel indicates loading.
5.4.2. Mechanism of hypoxic response

5.4.2.i. Effect of cobalt chloride and desferrioxamine on TNF-α induced MCP-1 expression

The effect of CoCl₂ and DFO on TNF-α induced MCP-1 expression was tested in PEO14 cells. Neither reagent alone had any effect on MCP-1 expression and little effect was seen on TNF-α induced expression at 10µM. However, 100µM of CoCl₂ and DFO resulted in a downregulation of MCP-1 expression and GAPDH was reciprocally upregulated (figure 5.10). Similar results to DFO were found for TNF-α induced MCP-1 expression in PEO1 cells (data not shown).

![Figure 5.10](image)

Figure 5.10. Evidence for the existence of a specific oxygen-sensing mechanism in PEO14 cells. CoCl₂ (A) and DFO (B) were used to mimic the effect of hypoxia in PEO14 cells. Little effect was apparent at 10µM (C10 and D10), but at a dose of 100µM of both reagents, (C100 and D100), a reduction in MCP-1 mRNA expression in response to TNF-α was seen. GAPDH expression was stimulated by DFO and CoCl₂ alone. β-actin is used as a loading control. Unstimulated (con) and TNF-α stimulated (T) cells were used as negative and positive controls respectively.
5.4.2.ii. Effect of potassium cyanide on TNF-α induced MCP-1 expression

In order to exclude the possibility that general effects of hypoxia on cellular metabolism are involved in the downregulation of MCP-1, PEO14 cells were cultured in the presence of TNF-α and 10μM and 100μM potassium cyanide (KCN) for 24 hours. Neither dose of KCN had an effect on MCP-1 or GAPDH expression either alone or in the presence of TNF-α (figure 5.11).

Figure 5.11. The effect of KCN on MCP-1 and GAPDH gene expression. In contrast to the effects of DFO and CoCl₂, neither 10μM (K10) nor 100μM potassium cyanide (K100) had any effect on MCP-1 or GAPDH expression by PEO14 cells. Unstimulated (con) and TNF-α stimulated (T) PEO14 cells were used as negative and positive controls respectively.
5.4.2.iii. Effect of antioxidants on TNF-α induced MCP-1 expression

The anti-oxidant PDTC was used to determine whether inhibiting NF-κB mobilisation would affect MCP-1 expression. The effect of PDTC on TNF-α induced MCP-1 expression was compared to LPS induced TNF-α expression in mono-mac-6 cells (figure 5.12). PDTC has been shown to almost completely inhibit TNF-α expression by LPS in mono-mac-6 cells and is associated with a lack of mobilisation of NF-κB (437). Pre-treatment of PEO14 cells with 300mM PDTC for one, two or three hours resulted in a decrease in MCP-1 expression of up to 10-fold (figure 5.13), compared with over 100-fold following anoxic culture. Furthermore, the effect was not as great as that of PDTC on LPS induced TNF-α expression by mono-mac-6 cells.

Figure 5.12. The effect of PDTC on TNF-α expression in the cell line mono-mac-6. Cells were stimulated with 30ng/ml Salmonella minnesota LPS. Changes in TNF-α expression were assessed by northern analysis (A) and integrated densitometry (B). Lane 1, unstimulated cells; lane 2, cells treated with 300mM PDTC alone for three hours; lane 3, cells stimulated with LPS alone for three hours; lane 4, cells pre-treated with PDTC for one hour, then stimulated with LPS, in the presence of PDTC for three hours. In this experiment, PDTC resulted in an approximately 80-fold decrease in TNF-α expression.
Figure 5.13. The effect of PDTC on TNF-α induced expression of MCP-1 in PEO14 cells.

The results of northern analysis (A) and the corresponding integrated densities (B) are shown. Lane 1, the expression of MCP-1 in response to TNF-α alone for three hours; lane 2, cells pre-incubated with PDTC for three hours; lane 3, cells pre-incubated with PDTC for two hours; lane 4 cells pre-incubated with PDTC for one hour prior to stimulation with TNF-α. Pre-incubation with PDTC resulted in a 10-fold decrease in MCP-1 expression. Unstimulated cells provided negative controls (con).
5.4.2. iv. Effect of hydrogen peroxide on MCP-1 expression

Since PDTC was capable of inhibiting MCP-1 expression, the effect of H$_2$O$_2$ was also tested to determine whether the presence of exogenous oxygen free radicals was capable of switching on MCP-1 production. Induction of MCP-1 expression by H$_2$O$_2$ could not be demonstrated (data not shown).

5.4.3. Effect of hypoxia on TNF-α signalling

5.4.3. i. Effect of hypoxia on TNF receptor expression

The effect of TNF-α and hypoxia, alone and in combination, was tested on the expression of the p55 component of the TNFR in the cell lines PEO1 and PEO14. There did not appear to be any change in receptor expression to either stimulus alone or in combination in the two cell lines tested (figure 5.14). In PEO14 cells cultured in the presence of 100μM CoCl$_2$, the expression of TNFR p55 also remained unaltered (figure 5.15).

![Figure 5.14. The effect of anoxia and TNF-α on mRNA levels of TNFR p55 in PEO1 and PEO14 cells. PEO1 or PEO14 cells were cultured with 10ng/ml TNF-α and anoxia alone and in combination for 24 hours. Neither stimulus appeared to have any appreciable effect on constitutive expression of TNFR p55 mRNA determined by northern analysis in either cell line.](image-url)
Figure 5.15. The effect of CoCl₂ on expression of TNFR p55 in PEO14 cells. Like anoxia, there was no obvious change in expression of p55 in response to stimulation with up to 100µM CoCl₂ for 24 hours; con, unstimulated cells; T, 10ng/ml TNF-α; C₁₀, 10µM CoCl₂; C₁₀₀, 100µM CoCl₂.
5.4.3.ii. Effect of hypoxia on NF-κB mobilisation

The effect of hypoxia on the mobilisation of NF-κB was assessed by EMSA. PEO14 cells were cultured under normoxic or hypoxic conditions, with and without 10 ng/ml TNF-α for a total of one hour, including the time for gassing the chamber. Hypoxia alone had no effect on NF-κB activity, whereas activation was seen in the presence of TNF-α. Under hypoxic conditions, the formation of DNA-protein complexes in the presence of double-stranded NF-κB oligonucleotides was not diminished (figure 5.16).

5.4.3.iii. Effect of antioxidants on NF-κB mobilisation

Anti-oxidants can downregulate the mobilisation of NF-κB (438). PEO14 cells were stimulated with TNF-α for one hour after pre-incubation with 300 mM PDTC for two hours. Mobilisation of the transcription factor was almost completely inhibited (figure 5.17).

5.4.3.iv. Effect of hydrogen peroxide on NF-κB mobilisation

To determine whether the addition of an exogenous source of oxygen radicals could enhance NF-κB mobilisation, PEO14 cells were incubated with 10 μM and 500 μM H₂O₂. Activation of NF-κB was not apparent at either concentration of H₂O₂ (figure 5.17).
Figure 5.16. The effect of hypoxia on the mobilisation of the transcription factor NF-κB in PEO14 cells.

Cells were stimulated with combinations of TNF-α and anoxia for one hour and the formation of DNA-protein complexes assessed by EMSA. Anoxia alone had no effect on NF-κB, whereas DNA-protein complex formation was detected in the presence of TNF-α. Unlike treatment with the anti-oxidant PDTC, TNF-α induced mobilisation of NF-κB was sustained under anoxic conditions. These results are representative of two experiments.
Figure 5.17. NF-κB mobilisation in response to oxidants and anti-oxidants in PEO14 cells.

Lane 1, unstimulated cells cultured normoxically; lane 2, unstimulated cells cultured anoxically; lane 3, TNF-α stimulated cells cultured normoxically; lane 4, TNF-α stimulated cells cultured anoxically; lane 5, cells treated with 300mM PDTC alone; lane 6, cells pre-treated with 300mM PDTC for two hours prior to stimulation with TNF-α for one hour. Pre-treatment with PDTC resulted in a complete inhibition of DNA-NF-κB complex formation in response to TNF-α.
5.4.4. Effect of hypoxia on monocyte migration in response to MCP-1

5.4.4.i. Effect of hypoxia on THP-1 migration
In addition to the effect of low oxygen tension on MCP-1 expression, the effect of anoxia was tested on monocyte migration in microchemotaxis assays. The migration of monocytic THP-1 cells was tested in response to MCP-1. Under normoxic conditions, the migratory response of THP-1 to increasing concentrations of MCP-1, was bell-shaped, with optimal migration at 100ng/ml. However, under anoxic conditions, the mean number of cells migrating was diminished at every dose of MCP-1. The difference between the migration of THP-1 cells under normoxia and hypoxia was significantly different at each dose (P<0.005) except the highest (figure 5.18).

5.4.4.ii. Effect of hypoxia on PBMC migration
The migration experiments performed with THP-1 cells were repeated on preparations of mononuclear cells from whole blood. Migration assays were performed for 90 minutes under normoxia or hypoxia and monocyte migration was assessed in stained filters by counting cells with characteristic monocyte morphology. These cells were typically larger than lymphocytes with a greater cytoplasmic:nuclear ratio and a kidney-bean shaped nucleus. Optimal migration of monocytes was found at 10ng/ml MCP-1 under normoxic conditions. The mean number of migrating cells was again reduced under hypoxic conditions, at all except the highest dose of MCP-1 used. The differences were statistically significant (P<0.05) when 0.1 and 1ng/ml MCP-1 were used in the assays (figure 5.19).
Figure 5.18. The effect of anoxia on the migration of THP-1 cells to MCP-1. THP-1 cells migrated in response to MCP-1 under normoxic conditions giving a bell-shaped dose response curve (A). However, under anoxic conditions (B) the mean number of migrating cells were significantly reduced (P<0.005) at all except the highest dose of MCP-1 used. The results represent the mean (±SD) of three independent experiments.
Figure 5.19. Migration of monocytes in PBMC preparations in response to MCP-1. Under normoxia monocytes migrated optimally to 10ng/ml MCP-1 (A). Under anoxic conditions (B) there was a significant reduction in migration at 0.1 and 1ng/ml (P<0.05). There were no differences at the higher doses used. These results represent the mean (±SD) of two experiments.
5.4.5. The expression and production of migration inhibitory factor

5.4.5.i. Effect of hypoxia and TNF-α on MIF expression and production by monocytes

Preliminary experiments were performed to establish whether TNF-α and anoxia could affect MIF gene expression. Mono-mac-6 cells, seeded at a density of 5 x 10⁵/ml in 1% serum (under the conditions used, these cells aggregated in the absence of serum), were stimulated with combinations of TNF-α and anoxia for 24 hours to determine the effect on MIF mRNA and protein levels. Neither hypoxia nor TNF-α, alone or in combination, appeared to affect MIF levels in these cells (figure 5.20).

5.4.5.ii. Effect of hypoxia and TNF-α on MIF expression by monocytes isolated from whole blood

The effects of anoxia and TNF-α were also tested on freshly isolated monocytes. Cells were seeded at a density of 8 x 10⁴ in 6-well plates. Unlike mono-mac-6 cells, both TNF-α and anoxic culture alone led to an increase in MIF expression detected by northern blot, after 16 hours of culture. Furthermore, these two stimuli appeared to be additive in combination (figure 5.21).

5.4.5.iii. Effect of hypoxia and TNF-α on MIF expression by PEO14 cells

MIF expression by the ovarian cancer cell line PEO14 was assessed by northern analysis. Unstimulated PEO14 cells expressed high levels of MIF, which were unchanged following stimulation with 10ng/ml TNF-α for three hours (data not shown).
Figure 5.20. The effect of hypoxia and TNF-α on MIF mRNA and protein levels in monocytes.

MIF mRNA was determined by northern analysis (A) and protein by western analysis (B) in the monocytic cell line mono-mac-6. The integrated densities of the northern analyses are also shown (C). There was no change in either MIF mRNA or protein levels under any of the experimental conditions used.
Figure 5.21. MIF expression in peripheral blood monocytes. Northern analysis (A) and integrated densities (B), showing the effect of TNF-α and anoxic culture on levels of MIF expression in freshly isolated peripheral blood monocytes. For northern blotting, 2 µg of total RNA were loaded per lane. Both TNF-α and anoxic culture alone enhanced MIF expression. The effect of these stimuli together appeared to be additive.
5.5. Summary and discussion
This chapter describes the effect of anoxia on MCP-1 expression in response to stimulation with TNF-α by three ovarian cancer cell lines. In all three cell lines, anoxic culture resulted in a decrease in the expression of MCP-1. The mechanism of action of hypoxia was studied further in the cell line PEO14. The effects of CoCl₂ and DFO in mimicking the effect of anoxia and the lack of an effect with KCN are consistent with a specific oxygen-sensing system acting through the transcription factor HIF. Further evidence for this comes from the fact that the earliest response occurs after approximately two hours of anoxic culture, but on exposure to atmospheric oxygen the effects of anoxia are rapidly reversed. The effect of anoxia could be inhibited by CHX (CI Scotton, personal communication), implying that de novo protein synthesis is required. There was little effect of anoxia on either the expression of the TNFR p55 or the mobilisation of NK-κB, indicating that anoxia acts downstream of the TNFR and through a functionally separate system from that mediated by reactive oxygen intermediates (ROIs). The effects of anoxia were not limited to the ovarian cell lines, but were apparent in the monocytic cell line, monomac-6. Collectively these results suggest that low oxygen tensions, particularly those that might occur in and around regions of necrosis, might switch off MCP-1 production, even in the presence of a potent stimulus, such as TNF-α. Although other genes have been identified which are downregulated in response to hypoxia (425), this appears to be the first description of a chemokine gene that is hypoxically downregulated.

The signalling pathways of TNF-α downstream of its receptors are complex. Members of the TNFR superfamily do not possess intrinsic tyrosine kinase or serine/threonine kinase domains, but various kinases have been reported to become activated by TNF-α including protein kinase A (439), protein kinase C (440) and mitogen-activated kinases (441, 442). Most work has been concerned with the p55 receptor, through which both gene activation and apoptotic cell death can be stimulated. p55 is associated with TRADD, a death domain containing protein that not only mediates the induction of apoptosis, but also NF-κB activation (443). Unlike the TNFRs themselves, downstream components of the signalling pathway do possess intrinsic kinase activity. A variety of other intermediates have been implicated in subsequent signalling, including Traf2, a protein originally identified in association with signalling through p75 and a novel protein kinase called NIK (444). NIK binds Traf2 and NF-κB is not inducible by TNF-α in cells expressing kinase-deficient NIK mutants. Stress-activated protein kinase, SAPK, which belongs to the c-jun kinase
family (JNK), is also activated through a non-cytotoxic TRAF2 dependent pathway (445).

At least part of the TNF-α induced MCP-1 expression is mediated through NF-κB, since EMSA analysis showed mobilisation of this transcription factor in response to TNF-α, and the effect of TNF-α could be partially inhibited by PDTC an anti-oxidant and iron-chelator that inhibits the release of NF-κB from I-κB. NF-κB is a member of the Rel family of eukaryotic transcription factors (446). Rel family members interact with and are modulated by members of the IκB family, which includes IκBa, IκBβ, IκB-γ and Bcl-3 (reviewed in (447)). In some cell lines, reactive oxygen intermediates (ROIs) can stimulate the release of NF-κB directly (448) and ROI scavengers, such as PDTC, have been shown to inhibit activation of NF-κB (438). In the experiments described here, hypoxia did not seem to affect NF-κB mobilisation.

The involvement of ROIs is an unusual element in the p55 signalling cascade. ROIs are generated by mitochondria at the level of ubiquinone in the electron transfer cascade. Major ROI species are H2O2, the superoxide radical, O2•-, and hydroxyl radicals, OH-. The dissociation of NF-κB from I-κB in response to TNF-α requires the participation of ROIs, but it is not clear at exactly which point in the pathway ROIs work. ROIs were originally implicated in cell death induced by TNF-α, but it now appears that they may also be involved in the regulation of gene expression, particularly with respect to NF-κB (449). TNF-α signal transduction may not rely on ROIs universally. Brennan and O’Neill (450) found that while H2O2 activated NF-κB in Jurkat cells, it did not do so in EL4.NOβ-1 T cells and KB epidermal cells. Similarly, N-acetylcysteine didn’t inhibit TNF-α activated NF-κB in EL4.NOβ or KB cells, while doing so in Jurkat cells, although the metal ion chelator and anti-oxidant PDTC was inhibitory in all three cell lines. Sphingomyelinase has been implicated in downstream signalling of TNF-α through its p55 receptor (451).

The effect of anoxia on the migration of monocytes in microchemotaxis assays was assessed in order to try and explain why these cells accumulate at high density around necrotic regions. In response to MCP-1 alone, THP-1 cells responded maximally at 100ng/ml. However, when the assay was performed under anoxic conditions, the normally bell-shaped dose-response curve was flattened. A similar result was obtained with monocytes in a peripheral blood mononuclear cell preparation. The change in shape of the dose-response curve suggests a change in efficacy of MCP-1, rather than in potency. At present it is not clear how this may come about. The time-course of the effect (the assays were performed over 90 minutes), is too fast to be
consistent with an HIF mediated response. TNF-α and LPS have both been shown to
downregulate expression of the MCP-1 receptor, CCR2 (452), although this might be
expected to change the potency of MCP-1. Whether anoxia has direct effects on
chemokine signal transduction, or the cytoskeleton are not known.

The expression and production of MIF was examined as another potential reason for
the accumulation of macrophages in necrotic regions. In preliminary experiments, on
mono-mac-6 cells, anoxia did not appear to affect expression of MIF, or MIF protein
levels in cell lysates, following 24 hours of exposure to TNF-α. However, in
monocytes isolated from peripheral blood, both anoxia and TNF-α upregulated the
expression of MIF. MIF is stored preformed within macrophages (431). It will
therefore be relevant to determine whether anoxia can cause an early release of MIF,
which might contribute to the inhibition of monocyte migration in the microchemotaxis
assays, or whether there is only a later upregulation of MIF expression, which may
nevertheless contribute to macrophage accumulation in vivo.

The results presented in this chapter may explain why MCP-1 expression was not
found adjacent to necrotic regions in in situ hybridisation studies. In the next chapter,
an attempt is made to synthesise the results of this and the preceding results chapters
into an hypothesis as to how chemokines may interact with other cytokines and the
physical environment of the tumour to produce the observed pattern of distribution of
macrophages and lymphocytes.
5.6. Conclusions

- Anoxia diminishes the expression of MCP-1 in response to TNF-α in three ovarian cancer cell lines and the monocytic line mono-mac-6.
- The effect of anoxia does not appear to be due to changes in the level of expression of the p55 TNF-α receptor.
- The action of CHX, CoCl₂, DFO and the time-courses of the responses to anoxia are all consistent with a specific-oxygen sensing mechanism mediated by HIF.
- Both NF-κB and ROIs are probably involved in the normal induction of MCP-1 expression by TNF-α, but not in its downregulation by anoxia.
- The response of these cell lines to anoxia may explain why MCP-1 expression was not seen in association with regions of necrosis.
- Monocyte migration response to MCP-1 was also inhibited by anoxia.
- In freshly isolated monocytes, but not the cell line mono-mac-6, anoxia and TNF-α, alone or in combination, can increase expression of MIF.
Chapter 6
Summary, hypothesis and future plans

6.1. Summary
In human ovarian carcinomas, macrophages and CD3+, CD8+, CD45RO+ T cells formed the bulk of the cellular infiltrate. These cells occurred both in the tumour stroma and the tumour parenchyma. Despite the close proximity of T cells to tumour cells, markers of T cell activation were not detected. Necrotic regions made up approximately 5% of the tumour areas studied. However, in these regions, macrophages occurred at high density whereas other infiltrating cells were rarely found. Macrophages in these regions expressed little MHC class II.

The CC chemokine and potent macrophage/monocyte chemoattractant MCP-1 was expressed by tumour cells and some stromal cells. MCP-1 protein was detected in the ascites of patients with ovarian carcinomas. Freshly isolated tumour cells expressed MCP-1 protein at higher levels than macrophages. However, MCP-1 expression was restricted to small patches of tumour cells and was not noted in or adjacent to necrotic regions, raising the question as to why macrophages cluster in these areas. The number of cells expressing MCP-1 correlated with the number of CD8+ and CD68+ cells within tumour sections. Other chemokines were also detected within these tumours. MIP-1α mRNA was expressed by an equivalent number of cells to MCP-1 in the stroma, but was not present in the tumour parenchyma. CD8+ cells may represent at least one source of MIP-1α. MIP-1β and RANTES were only detected at low levels.

A wide variety of inflammatory cytokines and growth factors are produced by ovarian cancers. Some of these were tested in an in vitro system. TNF-α was a potent stimulus to MCP-1 production by ovarian carcinoma cell lines. Since this cytokine is known to be expressed by ovarian carcinomas, it was studied further. In one cell line, TNF-α could stimulate sustained expression of MCP-1 and could induce MCP-1 expression at low doses.

The effect of low oxygen tension on TNF-α induced MCP-1 expression was investigated to address the question as to why MCP-1 was not detected adjacent to necrotic regions. Anoxia produced a profound decrease in MCP-1 expression and a significant decrease in protein production in three ovarian cell lines tested. A
monocytic cell line showed a similar response to anoxia. The effects of anoxia on MCP-1 expression were due to specific effects on gene expression, since the expression of GAPDH was upregulated and KCN did not reproduce the effect. Other experiments indicated that anoxia was acting through a specific oxygen sensing system, consistent with that involved in the upregulation of a number of genes, including Epo and enzymes involved in glucose metabolism. The effects of anoxia were distinct from those of oxygen free radicals, and were not mediated by changes in TNFR p55 expression.

The effect of anoxia was also tested on the migratory response of monocytes to MCP-1 in microchemotaxis assays. Anoxia consistently reduced the response of a monocytic cell line, THP-1, to MCP-1 and freshly isolated human peripheral blood monocytes behaved similarly. The effect on monocyte migration was more rapid than the effect of anoxia on MCP-1 gene expression. In freshly isolated human blood monocytes, preliminary data suggested that both TNF-α and anoxia could stimulate the expression of MIF, although this was not found in a monocytic cell line.

In the next section, an hypothesis is proposed to account for the in vivo and experimental observations.

6.2. Hypothesis

Tumours cells produce MCP-1, the level of production being dependent on at least two variables, local concentrations of molecular oxygen and TNF-α. Oxygen tension depends in turn on erythrocyte flux, which can vary rapidly (over minutes) and randomly, throughout the tumour. Unlike MCP-1, TNF-α concentrations may be enhanced by low oxygen tensions.

Blood vessels represent the source of infiltrating cells. For the bulk of the tumour (both stroma and parenchyma), variations in oxygen delivery lead to variations in MCP-1 expression. Because cells enter the tumour via the stroma, and MCP-1 levels vary randomly, infiltrating TAMs and lymphocytes tend to become evenly distributed throughout the tumour stroma and parenchyma, with time.

Necrotic regions occur when tumour cell growth results in cells becoming too far from a blood vessel to receive an adequate supply of oxygen and other nutrients, and to remove metabolic waste products. In addition, blood vessels may become occluded, leading to local areas of ischaemia and necrosis. The oxygen tension in necrotic
regions is extremely low. Due to the effect of hypoxia on the MCP-1 gene, this chemokine is not expressed adjacent to such areas. When macrophages enter a necrotic region, two mechanisms may result in their chances of leaving being reduced. In the short term, migration in response to any available MCP-1 gradients is inhibited. In the longer term, MIF expression is increased and MCP-1 receptor expression decreased.

However, in the absence of MCP-1 expression, why do macrophages enter necrotic regions? Two explanations are proposed here. The simplest solution is that chemokine gradients can traverse areas of necrosis. Therefore, if a region of necrosis lies between a macrophage and the chemokine source to which it is responding, the cell will migrate towards the source until the effects of low oxygen tension inhibit its further progress. A more complex solution is that gradients of other chemoattractants, not necessarily chemokines, may be set up around necrotic regions. For instance, VEGF is a hypoxically upregulated gene(256). The expression of VEGF adjacent to necrotic regions has been demonstrated by in situ hybridisation in human malignant glioma (257), and VEGF can act as a chemoattractant for monocytes. Thus in the absence of MCP-1, the predominant chemoattractant adjacent to necrotic regions could be VEGF. A similar two step paradigm for leukocyte migration has recently been proposed for IL-8 and fMLP in which neutrophils responded to a second chemokine when the gradient of the first became saturated (453).

6.3. Future plans
A wide variety of questions emerged as a result of the work of this thesis. This section will focus on those areas that I hope to investigate, or that will be taken further in the Biological Therapies Laboratory.

The bulk of the important questions arise from chapter 5. Future studies can be divided into several sections. The first will be to determine whether the effect of oxygen tension on MCP-1 expression is restricted to ovarian cancer cells or is a more general phenomenon, either of transformed cell lines, or of cells in general. It will be particularly interesting to know whether epithelial cells behave differently to endothelial cells in this respect. The action of hypoxia on the expression of other chemokines needs be investigated. MCP-1 appears to be expressed by a much wider variety of cells than any other chemokine, with the exception of IL-8. It will be interesting to know if IL-8 behaves similarly to MCP-1. These chemokines may represent a first line of response to tissue damage.
Experiments with DFO and CoCl₂ indicated that a specific oxygen sensing system, similar to that described for Epo, is responsible for regulating the expression of the MCP-1 gene. Deletional mutagenesis studies on the MCP-1 promoter will provide direct evidence as to the nature of the cis-and trans-acting factors. It is likely that HIF is involved in the regulation of the MCP-1 gene. The position of the HIF binding sites may determine whether this transcription factor up- or downregulates gene expression.

It is proposed that two mechanisms could account for the accumulation of macrophages in regions of low oxygen tension. The first is a rapid effect which occurs immediately on exposure to low oxygen tensions; the second is a longer term effect, mediated by MIF, which serves to hold macrophages in these regions. The action of anoxia on monocyte migration was probably too rapid to be consistent with it being mediated through a specific oxygen sensor. While it may be due to direct effects on cell metabolism, there may be other effects on factors involved in cell motility, for instance the cytoskeleton and the activation of adhesion molecules. Alternatively, hypoxia may act directly on the MCP-1 signal transduction pathway. Preliminary evidence suggests that hypoxia downregulates CCR2 expression, but with a time-course suggestive of an oxygen sensor. The short term effects of oxygen tension on cell surface receptors needs to be assessed. It is important to know whether this effect is restricted to a particular cell line or the CCR2 receptor, or whether it is a more general phenomenon.

A potential candidate to mediate some of the longer term effects is MIF. In a preliminary experiment hypoxia upregulated the expression of the MIF gene. Not only does this work need to be repeated, but the effect of hypoxia on MIF release needs to be examined. MIF can be stored preformed in macrophages and although MIF expression was not affected in mono-mac-6 cells by TNF-α or hypoxia, the effect of these stimuli on MIF release is not known. This appears to be the first time that the effect of hypoxia on monocyte migration effect has been described. It is a potentially important mechanism for regulating local events within tumours and other sites of inflammation. Both the short and longer term effects of hypoxia on monocytes should be reversible when oxygen tension is restored to normal.

Other questions that merit further investigation include the observed lack of class II MHC expression in necrotic regions suggesting that hypoxia also affects the expression of these molecules. The effect of oxygen tension on expression of co-stimulatory molecules and the TCR would also be interesting. Finally, it must be
remembered that oxygen is only one factor delivered by the vasculature and many relatively simple studies could be derived from looking at the effects of glucose deprivation, or the effect of waste products, such as hydrogen ions, in similar systems to those described above.

It is likely that many of the effects observed in human ovarian cancer are involved more generally in inflammatory processes. The study of this disease should therefore not only give insights into the biology of solid tumours, but also to a wide range of other inflammatory and immunological processes.
Published work


In press


References

15. Murphy, P. M. Chemokine receptors: structure, function and role in microbial pathogenesis. [Review] [174 refs], Cytokine and Growth Factor Reviews. 7: 47-64, 1996.


42. Handel, T. M. and Domaille, P. J. Heteronuclear (1H, 13C, 15N) NMR assignments and solution structure of the monocyte chemoattractant protein-1 (MCP-1) dimer, Biochemistry. 35: 6569-84, 1996.


174
175


123. Schall, T. J. Biology of the RANTES/SIS cytokine family. [Review] [93 refs], Cytokine. 3: 165-83 94305., 1991.


130. Ahuja, S. K., Gao, J. L., and Murphy, P. M. Chemokine receptors and molecular mimicry. [Review] [42 refs], Immunology Today. 15: 281-7, 1994.


301. Lotzova, E. Role of human circulating and tumor-infiltrating lymphocytes in cancer defense and treatment. [Review] [37 refs], Natural Immunity & Cell Growth Regulation. 9: 253-64, 1990.

302. Fuchs, E. J. and Matzinger, P. Is cancer dangerous to the immune system? [Review] [46 refs], Seminars in Immunology. 8: 271-80, 1996.


194


326. Takeya, M., Yoshimura, T., Leonard, E. J., and Takahashi, K. Detection of monocyte chemoattractant protein-1 in human atherosclerotic lesions by an anti-


368. Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N., and Williamson, B. An endotoxin-induced serum factor that causes necrosis of tumors,


372. Dinarello, C. A. Biologic basis for interleukin-1 in disease. [Review] [586 refs], Blood. 87: 2095-147, 1996.


205


BEST COPY

AVAILABLE

Poor text in the original thesis.
Some text bound close to the spine.
Some images distorted
Hypoxia down-regulates MCP-1 expression: implications for macrophage distribution in tumors

Rupert P. M. Negus, Lynn Turner, Frances Burke, and Frances R. Balkwill

Biological Therapies Laboratory, Imperial Cancer Research Fund, London, United Kingdom

Abstract: Monocyte chemotactic protein 1 (MCP-1) is likely to contribute to the macrophage infiltrate in human ovarian carcinomas. Although MCP-1 is predominantly expressed by the tumor parenchyma, macrophages accumulate at highest density in necrotic regions, which are associated with low oxygen tensions. Tumor necrosis factor (TNF-α) can stimulate MCP-1 production and is also present within ovarian carcinomas. We have investigated the effect of hypoxia on both MCP-1 expression in ovarian cancer cell lines and monocyte migration. Hypoxia down-regulated TNF-α-induced MCP-1 mRNA and protein production by ovarian cancer cells. The effect was mimicked by cobalt chloride and desferrioxamine, consistent with a specific oxygen-sensing mechanism. Unlike antioxidants, hypoxia did not inhibit nuclear factor κB mobilization. Monocyte migration in response to MCP-1 was also diminished by hypoxic conditions. Down-regulation of MCP-1 expression and the inhibition of monocyte migration are independent effects of hypoxia that may contribute to the distribution of macrophages within ovarian tumors. J. Leukoc. Biol. 63: 758-765; 1998.

Key Words: chemokines • chemotaxis • ovarian cancer

INTRODUCTION

Monocyte chemoattractant protein-1 (MCP-1) is a member of the CC family of chemokines [1]. It is a potent chemoattractant for macrophages [2] but is also active on CD4+ and CD8+ T cells [3]. MCP-1 protein has been detected in the ascites of patients with ovarian cancer and in situ hybridization studies have localized MCP-1 mRNA predominantly to tumor cells [4]. MCP-1 expression is not associated with necrotic regions in this disease [our unpublished data]. In common with other solid human tumors of epithelial origin, ovarian carcinomas contain a leukocyte infiltrate made up largely of macrophages and CD8+ T cells. Both of these cell types are found in tumor parenchyma and stroma, but the highest density of macrophages is in microscopic areas of necrosis that occur even within macroscopically normal tissue. Furthermore, MCP-1 expression in these areas correlates with the extent of the CD8+ infiltrate but is only weakly correlated with the CD68+ macrophage infiltrate [5]. The expression of MCP-1 can be induced by a variety of factors, including inflammatory cytokines such as tumor necrosis factor (TNF-α) [6] and interleukin-6 (IL-6) [7] and growth factors such as platelet-derived growth factor (PDGF) [8,9] and transforming growth factor β1 (TGF-β1) [7]. These cytokines have also been reported to be expressed by ovarian tumors and ovarian cancer cell lines [10-12]. In particular, mRNA for TNF-α has been localized to both ovarian tumor parenchyma and infiltrating macrophages but TNF-α immunoreactivity was mainly associated with macrophages within and adjacent to tumor areas [13].

Studies using polarographic needle electrodes have demonstrated that the oxygen tension within tumors is significantly lower than adjacent normal tissue [14]. This is likely to be due to the chaotic nature of the tumor vasculature [15] and to dynamic changes in microregional perfusion that can occur over relatively short periods of time [16]. Therefore, while regions of necrosis in solid tumors are likely to be very hypoxic (<1% O2) [17], even histologically normal tumor tissue may be subjected to transiently low oxygen tensions. Although many genes are up-regulated by hypoxia, including cytokines such as erythropoietin [18], vascular endothelial growth factor [19], and IL-8 [20], some, such as GLUT-2 have been found to be down-regulated [21]. There is a widespread oxygen-sensing system in mammalian cells that is probably based on a heme-like cytoplasmic protein [22].

Within ovarian carcinomas, macrophages are found in necrotic regions, but MCP-1 expression is largely confined to tumor parenchyma. However, even here MCP-1 expression is patchy. This suggested that hypoxia may regulate MCP-1 expression and perhaps macrophage migration within tumors. In this study we examined the production of MCP-1 by tumor cells in response to a variety of cytokines that are found in ovarian tumors. We report that TNF-α stimulated tumor cell lines to produce MCP-1 and that this was diminished by low oxygen tension. Furthermore, we have found that hypoxia reduced the migration of monocytes in response to MCP-1. This...

Abbreviations: MCP-1, monocyte chemotactic protein-1; TNF-α, tumor necrosis factor α; IL-6, interleukin-6; PDGF, platelet-derived growth factor; TGF-β1, transforming growth factor β1; GAPDH, glyceraldehyde phosphate dehydrogenase; DFO, desferrioxamine; FCS, fetal calf serum; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor κB; FBMC, peripheral blood mononuclear cells; RT-PCR, reverse transcriptase-polymerase chain reaction.

Correspondence: Dr. F. R. Balkwill, Biological Therapies Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London, WCA 3PX, UK.

Received December 1, 1997; revised February 2, 1998; accepted February 4, 1998.
combination of these effects of hypoxia might help to explain the observed distribution of MCP-1 mRNA and macrophages within human ovarian cancers.

METHODS
Reagents
Recombinant human TNF-α was supplied by BASF/Knoll (Maidenhead, Berkshire, UK). Recombinant human IL-6, human TGF-β1, and human PTDG were purchased from R & D Systems (Abingdon, Oxon, UK). Gases were purchased from BOC (Manchester, UK). cDNA for MCP-1 was kindly provided by Prof. Alberto Mantovani (Marino Negri Institute, Milan, Italy). β-actin was from Dr. L. Kedes (Stanford University, Stanford, CA), glyceraldehyde phosphate dehydrogenase (GAPDH) from Dr. S. De Kossodo (Massachusetts General Hospital, Boston, MA), and TNF receptor p55 (TNFR p55) from Prof. M. Feldman (Kennedy Institute of Rheumatology, London, UK). Primers for TNFR p55 and p75 were purchased from Clontech (Palo Alto, CA). Cobalt chloride (CoCl2), desferrioxamine (DFO), potassium cyanide (KCN), and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma (Poole, Dorset, UK). Recombinant human MCP-1 was purchased from PeproTech EC Ltd. (London, UK).

Cell lines and culture
The ovarian cancer cell lines PEO1 and PEO14 were provided by Dr. S. Langdon (ICRF, Edinburgh, UK) [23]. OVCAR-3 cells and the monocytic cell line THP-1 were purchased from the American Type Culture Collection (Rockville, MD). All cell lines were grown in a humidified atmosphere at 37°C (5% CO2) under pyrogen-free conditions. Cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS). With the exception of THP-1, all cell lines were also grown in the presence of insulin (2.5 μg/mL). Two millimolar L-glutamine, 100 U/mL penicillin, and 100 μg/mL of streptomycin were included in all tissue culture media. THP-1 cells were supplemented with 50 μM β-mercaptoethanol (Sigma). For cell stimulation experiments, 2 mL of samples were centrifuged on Lymphoprep° (density gradient of 1.077). The mononuclear cell layer was then removed, washed twice, and resuspended in RPMI 1640/1% BSA to a final total cell concentration of 1.5 X 106 cells/mL. After migration assays, monocytes were identified by their characteristic morphology.

Conditions for hypoxic culture
Cells were gassed with the desired mixture for 30 min in a modular incubation chamber (Flow Laboratories, Del Mar, CA) before incubation at 37°C. The length of time required to equilibrate the chamber was determined by gassing with 95% N2, 5% CO2 and measuring the oxygen tension of the effluent gas with a Thermox 1 analyzer (Thermolab Instruments, Inc.). After 10 min, 0.01% oxygen was achieved and 0.005% after 30 min. GAPDH expression, which has been shown to be up-regulated by hypoxia [24], was used to indicate hypoxic responses in Northern analyses.

Northern analysis
Total RNA was prepared from 2 X 106 cells using Tri-Reagent (Molecular Research Centre Inc., Cincinnati, OH) according to the manufacturer’s protocol. Fifteen micrograms of total RNA was subjected to electrophoresis through a 1% agarose-formaldehyde gel and blotted by capillary transfer onto nylon membranes (Hybond N+, Amersham, UK). After transfer, membranes were UV cross-linked. Blots were prehybridized for 2-4 h at 42°C in formamide (45%), phosphate buffer (0.2 M), bovine serum albumin (BSA; 1%), sodium dodecyl sulfate (SDS; 7%), and ethylenediaminetetraacetic acid EDTA; 1 mM). Probes were labeled by random priming using a commercially available kit (Prime-It, Stratagene, La Jolla, CA). Hybridization was performed overnight at 42°C. After hybridization, membranes were washed twice in 2X saline sodium citrate (SSC), 0.1% SDS for 5 min at room temperature, twice in 0.1X SSC, 0.1% SDS for 15 min at 70°C, and finally in 2X SSC for 10 min at room temperature. Blots were exposed to film (Biomax MS, Eastman Kodak Co., Rochester, NY) with an intensifying screen at -70°C.

Enzyme-linked immunosorbent assay (ELISA)
Supernatants were assayed for MCP-1 with a commercially available ELISA (Quantikine, R & D) according to the manufacturer’s protocol. This assay has a sensitivity of 5 pg/mL. After the addition of chromosome, plates were read in a Dynatech MR 5000 plate reader at 450 nm. Data were analyzed using the computer software Biolinx® (Dynatech Laboratories, Inc., Chantilly, VA).

Electrophoretic mobility shift assays (EMSA)
DNA-binding protein extracts were prepared as described previously with minor modifications [25]. Briefly, 1 to 2 X 106 cells were washed in ice-cold phosphate-buffered saline (PBS), pelleted, and resuspended in lysate buffer [10 mM NaCl, 25% glycerol, 0.5% Triton X-100, 100 mM NaCl, 50 μg/mL poly(deoxyinosine-deoxycytidine) (Pharmacia Biotech, Herts, UK). Nuclear extracts were mixed with labeled oligonucleotides and incubated at room temperature for 20 min. Duplicate samples were also set up containing a 50X excess of cold oligonucleotide to act as competitor. Complexes were separated on 4% acrylamide gels in 0.25X Tris-borate EDTA at 4°C, dried, and detected by autoradiography (Fuji Film, Fuji Photo Film Co., Ltd., Tokyo, Japan) at -70°C.

Isolation of peripheral blood mononuclear cells (PBMC)
PBMC were prepared as described previously [26]. In brief, heparinized blood samples were centrifuged on Lymphoprep® (density gradient of 1.077). The mononuclear cell layer was then removed, washed twice, and resuspended in RPMI 1640/1% BSA to a final total cell concentration of 1.5 X 106 cells/mL. After migration assays, monocytes were identified by their characteristic morphology.

THP-1 and monocyte migration assay
THP-1 and monocyte chemotaxis were examined using a 48-well microchemotaxis chamber (Neuro Probe, Cabin John, MD). The lower wells were filled with 27 μL chemotactic agent in RPMI/1% BSA and covered with a 5-μm pore size polycarbonate membrane. Fifty-two microliters of cell suspension was added to each upper well. THP-1 cells were used at a concentration of 1 X 107 cells/mL. Migration assays under anoxic conditions were performed by placing the chemotaxis chamber in a modular incubation chamber and gassing with 95% N2, 5% CO2 for the entire duration of the assay. After incubation at 37°C for 90 min the membrane was removed and the non-cell side washed with PBS. The membrane was then fixed in methanol for 2 min before staining with Diff-Quik (Baxter, Newbury, UK). THP-1 and monocyte migration was assessed by counting the number of cells attached to the lower surface of the membrane in five high power fields (X100 objective lens, X10 eyepiece) per well.

Image analysis
Autoradiographs were scanned with a UMAX Mirage D-16L scanner and manipulated with Adobe Photoshop 4.0. The determination of integrated density was performed using NIH Image 1.58. The scanner was calibrated with a Kodak photographic step tablet no. 2. Films used for autoradiography were assumed to be linear over the range of exposures used. The relative level of MCP-1 expression was corrected by dividing the corresponding density for β-actin.

Negus et al. Hypoxia, MCP-1 expression, and macrophage...
RESULTS

TNF-α induction of MCP-1 in ovarian tumor cell lines

PEO1 and PEO14 cells were stimulated with four cytokines known to be present in ovarian carcinomas, TNF-α, IL-6, PDGF, and TGF-β1. Cells were cultured in the presence of 10 ng/mL of each cytokine for 24 h and the amount of MCP-1 released assayed by ELISA. Of the cytokines tested, only TNF-α induced MCP-1 production. In these experiments the mean (± sd) amount of MCP-1 produced by PEO1 cells stimulated with 10 ng/mL TNF-α was 4,680 ± 790 pg/mL and by PEO14 cells 16,400 ± 870 pg/mL. The response of a third cell line, OVCAR-3, to TNF-α stimulation was also tested and found to be similar to PEO1 cells (5,120 ± 690 pg/mL). There was no significant production of MCP-1 by unstimulated PEO1 cells, but variable constitutive production of MCP-1 (up to 600 pg/mL) was detected in PEO14 cells.

TNF-α-induced MCP-1 expression was regulated at the level of mRNA. Dose-response experiments on PEO14 cells revealed expression of MCP-1 mRNA to be stimulated with 0.1 ng/mL TNF-α at 3 h and to be greatly increased by 10 ng/mL (Fig. 1). In a time-course experiment, MCP-1 mRNA was detectable by Northern analysis 30 min after stimulation with TNF-α at 10 ng/mL and was maximal 2–4 h later. TNF-α was used at a concentration of 10 ng/mL in subsequent experiments and cells were stimulated with TNF-α for 3 h, unless otherwise stated.

Hypoxia down-regulates MCP-1 induction in response to TNF-α

The effect of extremely low oxygen tension (anoxia) on ovarian cell lines was determined by culturing PEO1, PEO14, and OVCAR-3 cells in 95% N₂, 5% CO₂ for 24 h. Commercially available gas cylinders containing this mixture have an oxygen content of approximately 5 ppm. Cells were stimulated with TNF-α at the time of gassing. Under conditions of anoxia alone, there was no stimulation of MCP-1 expression, but in all cell lines, anoxic culture resulted in a decrease in TNF-α-induced MCP-1 mRNA levels assessed by Northern blot analysis (Fig. 2). In the PEO14 cells, this reduction, determined by densitometry, was as much as 150-fold compared to cells stimulated with TNF-α and cultured under normoxic conditions for 24 h. The expression of GAPDH, which is known to be up-regulated by hypoxia, was also increased at 24 h and was particularly striking in PEO14 cells (Fig. 2). In the cell line PEO14, preincubated with TNF-α for 3 h before gassing, the effect of anoxia was apparent after 2 h (data not shown). The decrease in MCP-1 mRNA expression was reflected by a significant decrease in MCP-1 protein production (P < 0.005 for PEO1 and PEO14 cells, P < 0.05 for OVCAR-3, Fig. 3). The low levels of constitutive expression of MCP-1 detected in PEO14 cells (0.6 ± 0.09 ng/mL) were also reduced by anoxic culture.

Fig. 1. Dose-response of MCP-1 expression in PEO14 cells stimulated with TNF-α determined by Northern blot analysis. PEO14 cells were stimulated with TNF-α for 3 h under serum-free conditions at doses ranging from 0.01 to 100 ng/mL. MCP-1 expression was visible in response to 0.1 ng/mL TNF-α. Expression of β-actin was used to control for RNA loading and transfer. The results are representative of two experiments.

Fig. 2. Northern analysis demonstrating the reduction in MCP-1 mRNA levels when the cell lines PEO1, PEO14, and OVCAR-3 were stimulated with TNF-α and cultured under anoxic conditions for 24 h. The response of GAPDH mRNA levels to hypoxia is also shown. The increase in expression of GAPDH was particularly marked in PEO14 cells, in which the down-regulation of MCP-1 mRNA was greatest. The results for PEO1 and PEO14 cells are representative of at least three experiments.

Fig. 3. MCP-1 protein production under normoxic and anoxic conditions in response to stimulation with TNF-α was determined in PEO1, PEO14, and OVCAR-3 cells by ELISA. Results were compared using the two sample t test. Anoxia significantly reduced TNF-α-induced MCP-1 production at 24 h in all three cell lines compared with MCP-1 production in cells cultured under normoxic conditions (P < 0.005 for PEO1 and PEO14 cells, P < 0.05 for OVCAR-3). The histogram shows the mean protein level (± sd) of three separate cultures under each set of conditions.
Evidence for a heme-like oxygen-sensing system in ovarian cancer cell lines

Transition metal ions, such as Co(II), and chelating agents, such as DFO, mimic the effects of hypoxia where a specific oxygen-sensing system exists [18]. To investigate the existence of this system in the ovarian cell lines, PEO14 cells were stimulated with TNF-α in the presence of increasing doses of either cobalt chloride (CoCl₂) or DFO. Both CoCl₂ and DFO inhibited induction of MCP-1 by TNF-α at an optimal dose of 100 μM. Neither inhibitor alone had any effect on MCP-1 expression (Fig. 5). The effect of the metabolic inhibitor potassium cyanide (KCN) was tested to see whether the effects of anoxia were mediated via a generalized effect on cellular metabolism. At a dose of 100 μM, KCN did not have any effect on TNF-α-induced expression of MCP-1 (Fig. 5).

The effect of hypoxia on TNFR p55 and NF-κB

The effects of TNF-α may be mediated by either the p55 or the p75 component of the receptor and frequently involve mobilization of the transcription factor NF-κB. Expression of p55 and p75 in the cell line PEO14 was studied by reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern analysis. Only the p55 component of the TNF-α receptor could be detected by RT-PCR (data not shown) and Northern analysis. Culture under anoxic conditions did not affect the expression of the mRNA for p55 (data not shown).

NF-κB is implicated in downstream signaling events resulting in the stimulation of MCP-1 expression by TNF-α [27]. Mobilization of NF-κB in response to stimulation with TNF-α for 1 h was confirmed by EMSA of PEO14 cells. Anoxia alone did not appear to alter TNF-α-induced NF-κB (Fig. 6). PEO14 cells pretreated with the antioxidant PDTC for 2 h before stimulation with TNF-α for 1 h also expressed lower levels of MCP-1 than cells stimulated with TNF-α alone. However, unlike anoxia, PDTC pretreatment inhibited the mobilization of NF-κB (data not shown).

Fig. 5. Evidence for the existence of a specific oxygen-sensing mechanism. Cobalt chloride (A) and desferrioxamine (B) were used to mimic the effect of hypoxia in PEO14 cells. At a dose of 100 μM desferrioxamine (D100) or cobalt chloride (C100), a reduction in MCP-1 mRNA levels in response to 10 ng/mL TNF-α (T) was seen. GAPDH expression was stimulated by both desferrioxamine and cobalt chloride. In contrast, culture of cells in the presence of 100 μM potassium cyanide (K100) had no effect on MCP-1 or GAPDH expression (C).
Inhibition of monocyte migration to MCP-1 by hypoxia in microchemotaxis assays

To explore the possibility that low oxygen tension has direct effects on cell mobility, preliminary experiments were performed to compare the migration of the monocyte cell line THP-1 and monocytes in a PBMC preparation, to MCP-1 under normoxic and anoxic conditions. Under normoxic conditions, THP-1 cells migrated maximally to 100 ng/mL MCP-1 (Fig. 7A). Under anoxic conditions, the mean number of THP-1 cells migrating in response to MCP-1 was reduced at all doses used and these differences were statistically significant for all except the highest dose of MCP-1 \((P < 0.005\), Fig. 7B). The greatest response in monocyte migration in preparations of PBMC was seen with 10 ng/mL MCP-1 (Fig. 7C). Anoxia significantly reduced the response to MCP-1 when concentrations of 0.1 and 1 ng/mL were used \((P < 0.05)\). The response to 10 ng/mL was also lower under anoxic conditions (Fig. 7D), but did not achieve statistical significance \((P = 0.56)\).

Discussion

We have demonstrated that hypoxia can down-regulate the expression of MCP-1 in response to TNF-α and that a specific oxygen-sensing mechanism is likely to account for this effect. This may explain the lack of MCP-1 expression adjacent to necrotic regions in human ovarian cancers. Unlike the antioxidant PDTC, hypoxia did not affect the mobilization of NF-κB in response to TNF-α, which indicates that at least some of the effects of molecular oxygen are distinct from those of oxygen free radicals. In order to understand why macrophages accumulate preferentially in necrotic regions, preliminary experiments were performed which demonstrated that both the monocyte cell line THP-1 and in monocytes derived from whole blood, the chemotactic response to MCP-1 under anoxic conditions was diminished.

Of the cytokines tested, only TNF-α was able to induce MCP-1 production in ovarian tumor cell lines. The amount of MCP-1 protein detected by ELISA in stimulated cell lines (means 5.1-20.9 ng/mL) was comparable to that determined previously in culture supernatants of tumor cells isolated from the ascites of patients with ovarian cancer (2.3-47.2 ng/mL) [4]. The effect of TNF-α on MCP-1 expression by ovarian tumor cells was inhibited by 1% oxygen and anoxia. Down-regulation of TNF-α-induced MCP-1 mRNA by anoxia was apparent at 24 h in three cell lines tested and occurred after 2 h of anoxic culture in PEO14 cells. Although there was a significant reduction in the amount of MCP-1 protein detected by ELISA in all three cell lines, the magnitude of this effect was not as great as the reduction in MCP-1 mRNA levels. At least part of the reason for this discrepancy may be the time courses of the different effects. The induction of MCP-1 expression was maximal at 2-4 h, whereas the earliest response to hypoxia occurred at 2 h. It is likely that MCP-1 protein accumulated before the down-regulation of AICNI gene expression was complete. The time-course of the response to anoxia and the action of CoCl₂ and DFO in mimicking the effect of low oxygen tensions, compared with the lack of effect of KCN, are all consistent with the involvement of a specific oxygen-sensing system [18].

There is evidence that the oxygen tension within tumors is much lower than that of the surrounding normal tissues. Nordmark et al. [14] found that, although the median oxygen tension in the subcutaneous tissues of 14 patients with head and neck cancers was 30-60 mmHg, in the majority of head and neck nodes it was less than 30 mmHg and in over 40% it was less than 10 mmHg (1.3%). Tumor perfusion may also fluctuate quite rapidly. Using a laser Doppler system, Hill et al. found that the erythrocyte flux in 26% of human tumors changed twofold over a study period of 60 min and that more than 50% of these changes occurred within 20 min [16]. Although tumor tissue oxygen tension depends on the proximity of local blood vessels [28] and the degree of perfusion, the necrotic fraction of a tumor has been found to correlate with the percentage of oxygen values less than 1% [17], implying that these regions are persistently hypoxic.

To date, the best described oxygen-sensing system is proposed to consist of a heme-like cytoplasmic oxygen sensor.
acting through a heterodimeric transcription factor, hypoxia-inducible factor 1 (HIF-1) [29]. Evidence for activation of HIF-1 has recently been demonstrated adjacent to necrotic areas in a murine tumor model [30]. Database searching of the known MCP-1 promoter sequence [31] has identified an HIF-1 consensus sequence approximately 2170 bp upstream of the transcription initiation site. Although HIF is usually associated with the up-regulation of gene expression, there are a number of genes known to be down-regulated by hypoxia which have responses to iron chelators and transition metal ions consistent with an erythropoietin-like oxygen-sensing system [21]. Other transcriptional regulators that are responsive to low oxygen tensions probably exist, for instance elevated levels of IL-6 mRNA and protein appeared to result from stimulation of the NF-IL-6 site in the IL-6 promoter [32].

The response to hypoxia of two components of the TNF-α signaling pathway was assessed. TNF-α signals through two receptors, termed p55 and p75 [33] and gene expression in response to TNF-α frequently involves the transcription factor NF-κB [34]. We were only able to detect the p55 component of the TNF-α receptor in ovarian cancer cell lines and there was no change in the level of expression of this receptor under anoxic conditions. In an in situ hybridization study in frozen sections of ovarian cancers, Naylor et al. [13] found that mRNA for p55 was associated with tumor cells but expression of p75 was restricted to infiltrating mononuclear cells.

NF-κB is a transcription factor involved in the expression of many genes concerned with inflammatory and immune response [35]. It has also been implicated in the regulation of transcription of MCP-1 in response to TNF-α [31]. In both endothelial cells [20] and NIH3T3 [36] cells hypoxia alone can mobilize NF-κB. In the case of endothelial cells hypoxia also stimulated the production of IL-8 and MCP-1. We were unable to demonstrate activation of NF-κB or stimulation of expression of MCP-1 by hypoxia alone in ovarian tumor cells. Neither did hypoxia interfere with NF-κB mobilization in response to TNF-α. The antioxidant PDTC also inhibited expression of MCP-1 in response to TNF-α, but in the presence of PDTC mobilization of NF-κB was not detected. This is consistent with other studies in which PDTC has been shown to interfere with the dissociation of NF-κB from IκB [37]. These results indicate that hypoxia is likely to act downstream of p55 and that the redox regulation of NF-κB is not directly involved in the down-regulation of MCP-1 by low oxygen tensions. However, the exact point of action of hypoxia on the pathway from TNF-α to MCP-1 remains to be determined.

It is generally assumed that migrating leukocytes respond to a gradient of the appropriate chemokine and that their final
position within an inflammatory site is determined by the source of the chemokine [38]. In the case of MCP-1 this idea is supported by data from microchemotaxis assays [39] and by in vivo studies. For instance, organ-specific expression of MCP-1 in transgenic mice results in a corresponding monocyte infiltrate [40]. This is consistent with our observation that patients with ovarian tumors have significant levels of MCP-1 protein in ascites but not in plasma [4]. However, this alone does not explain the spatial separation we have observed between the sites of MCP-1 expression and the sites of highest density of macrophages within these tumors. The apparent paradox might be explained by the effects of hypoxia on MCP-1 expression and monocyte migration.

If MCP-1 expression is modulated by oxygen tension, as our results suggest, then the pattern of expression of MCP-1 could vary dynamically with the degree of local oxygenation, resulting in shifting gradients of MCP-1 within the tumor. This in turn would lead to a random walk of macrophages through the tumor as they responded to changing gradients of MCP-1 and might account for the observed distribution of macrophages in the tumor parenchyma and stroma [5]. Under anoxic conditions in microchemotaxis assays monocytes responded less well to MCP-1. If this mechanism also operates in vivo, then an encounter region of necrosis macrophage migration would be inhibited, resulting in a net accumulation of these cells. The results of microchemotaxis assays, with both THP-1 cells and peripheral blood monocytes, suggest that this could happen even in the presence of a chemokine gradient. But if hypoxia down-regulates MCP-1 expression, how are macrophages drawn to necrotic regions? One possibility is that hypoxia up-regulates the expression of another factor, adjacent to necrotic regions, which can also act as a macrophage chemotactant. Vascular endothelial growth factor is one cytokine that fits this description [19, 41]. Alternatively, MCP-1 may be able to traverse necrotic regions. Thus, macrophages sensing a chemokine gradient generated by cells on the far side of an area of necrosis would migrate toward the stimulus until inhibited by low oxygen tension.

The action of hypoxia on monocyte migration may occur at some point on the MCP-1 signaling pathway. Sica et al. [42] have shown that both TNF-α and lipopolysaccharide can down-regulate the expression of the MCP-1 receptor, CCR2. Because the expression of TNF-α from the monocyte cell line U937 stimulated with PHA is increased by hypoxia [43], it is possible that a paracrine loop exists in very hypoxic regions such as areas of necrosis. This may also be consistent with in vivo observations that the greatest immunoreactivity to TNF-α is found associated with macrophages [13]. On the other hand, the major effect on the migration dose-response curves appeared to be a change in the efficacy of MCP-1, suggesting that receptor numbers remain unchanged under hypoxic conditions.

Our results support the idea that in ovarian tumors at least two mechanisms determine the final distribution of infiltrating macrophages; the expression of MCP-1 by carcinoma cells, which varies according to changes in the local oxygen tension, and the inhibition of macrophage migration in regions of very low oxygen tension, even in the presence of a chemokine gradient. The down-regulation of MCP-1 expression and the inhibition of macrophage migration by hypoxia are novel mechanisms that are likely to play a role in the distribution of macrophages in both malignancy and chronic inflammatory conditions characterized by a mononuclear infiltrate.

ACKNOWLEDGMENTS

R. P. M. Negus is supported by a MRC Clinical Training Fellowship.

REFERENCES


We defined a cytokine mRNA profile of 12 ovarian cancer biopsies, 10 normal/benign biopsies, six ovarian cancer cell lines and three ovarian cancer xenografts, using RT-PCR. The profile, based on screening for 25 cytokines and 12 receptor mRNAs, was rich in growth factors, pro-inflammatory cytokines and chemokines, but weak in lymphocyte-associated cytokines. The pattern was unique to ovarian tissue, but similar in normal, benign and malignant biopsies, with >80% samples expressing 16 cytokines in common. Fourteen of these were also expressed by >65% cell lines, but fewer were detected in xenografts. Potential autocrine loops existed for IL-1, IGF-1, M-CSF, GM-CSF and TNF-α. IL-4 and IFN-γ receptors were expressed in absence of ligand. Chemokines RANTES, MIP-1α and MIP-1β were expressed in biopsies, but were rarely detected in cell lines and absent from xenografts. IGF-1 and its receptor was expressed in every sample, as was IFN-γ receptor. Another 10 cytokine mRNAs and six receptors were expressed in >80% samples. These may contribute to key survival/growth loops. Similarities between normal and malignant biopsies suggest that analogous processes of remodelling and repair occur. RT-PCR proved a rapid, reproducible screen, but further assays are required to detect quantitative differences between normal and malignant tissues and tumour models.

As regulators of malignant cell behaviour and communication with stroma, cytokines have proved useful in understanding cancer biology and developing novel therapies. However, cytokines have yet to make a major impact in cancer treatment, particularly in the common epithelial tumours. The reasons for this include inappropriate interpretation of animal model data, problems with systemic delivery of locally acting agents, and, perhaps most importantly, incomplete understanding of the cytokine network which already exists within the tumour microenvironment. This cytokine network may be involved in many aspects of tumour growth and spread, such as proliferation, motility and survival of malignant cells, cell:cell or cell: matrix adhesion, neovascularization, extracellular matrix remodelling, host cell infiltration, local immune response and nutritional balance.

Ovarian cancer is one epithelial malignancy in which there is evidence for a complex network. Published information on individual cytokines has suggested that a number of autocrine and paracrine cytokine loops influence the biology of this tumour, but there have to date been few systematic studies of the cytokine network at the level of mRNA or protein.

We have used RT-PCR as a rapid and reproducible screen for cytokine and cytokine receptor mRNA expression in biopsies of malignant ovary, and compared the results with biopsies of normal ovary, ovarian cancer cell lines, and xenografts established directly from patient samples. Our aims in doing this were threefold: to identify 'key' cytokines that, being found in all groups, may be central to growth, differentiation and cell survival of ovarian tissue; to see if the cytokine mRNA profile of in vitro and animal models of ovarian cancer resembled that of the malignant biopsies, and to examine differences in cytokine expression between normal and malignant biopsies.

RESULTS

RT-PCR for cytokines and their receptors

We screened for expression of mRNA for 25 cytokines that could be divided into the following groups (see Fig. 2): pro-inflammatory cytokines IL-1α, IL1β, IL-6, TNF-α, and LT(β) growth factors IGF-1, PDGF-A, PDGF-B, EGF, TGF-α, TGF-β1, M-CSF(α) chemokines IL-8, MCP-1, RANTES, MIP-1α and MIP-1β(α) lymphocyte-associated cytokines IL-2, IL-4, IL-7, IL-10, IFN-γ and GM-CSF(α), and the angiogenic factors ECGF and PDECGF(α). Samples were also screened for expression of receptors for IL-1, IL-4, IL-6, TNF-α and LT (p55 and p75), IGF-1 (types I and II), M-CSF, GM-CSF, IL-2 (α and β), IL-7 and IFN-γ. A
positive control for cytokine mRNA expression was included in every experiment. A positive signal was also validated by either Southern blotting or restriction enzyme digestion for some of the cytokines screened. An example of the RT-PCR for the p55 TNF receptor and its validation is shown in Figure 1.

Expression of cytokine and cytokine receptor mRNA in biopsies from epithelial ovarian cancer

We analysed 10-12 biopsies from malignant ovary for each cytokine and receptor. As shown in Figure 2, a consistent pattern of mRNA expression was found within this group of samples. Sixteen cytokines, IL-1α, IL-6, TNF-α, LT, IGF-1, PDGF-A, PDGF-B, TGF-α, TGF-β, M-CSF, IL-8, MCP-1, RANTES, MIP-1α, MIP-1β and PDECGF, were expressed by a majority (>80%) of biopsies. IL-10 and GM-CSF mRNA was detected in 58% biopsies, EGF in 42%, IL-7 in 25% and IL-2 in 17%. mRNA for IL-1β was only detected in one sample. mRNA for IL-4, IFN-γ and ECGF was not detected. As shown in Figure 3, potential autocrine/paracrine loops were suggested with the expression of receptors for IL-1, TNF-α and LT, M-CSF, GM-CSF and IGF-1. IL-4 and IFN-γ receptors were expressed in all biopsies in the absence of mRNA for their ligand. 83% of tumours expressed the IL-2α and 66% the IL-2β.

Expression of cytokine and cytokine receptor mRNA in biopsies from normal ovaries and those with benign disease

We analysed mRNA expression in three biopsies from ovaries with benign disease and seven biopsies from normal ovary. As there were no obvious differences in results from normal and benign disease, these were considered as one group. Eight to 10 samples from this group were assessed for each cytokine or receptor mRNA. As shown in Figure 2, a consistent pattern of mRNA expression was found which was similar to that found in the malignant biopsies. Thus the same sixteen cytokines, IL-1α, IL-6, TNF-α, LT, IGF-1, PDGF-A, PDGF-B, TGF-α, TGF-β, M-CSF, IL-8, MCP-1,
TABLE 1. Cytokine mRNA expression in ovarian samples. Percentage positive samples (all groups combined)

<table>
<thead>
<tr>
<th>Cytokine mRNA</th>
<th>100%</th>
<th>90-99%+</th>
<th>80-89%</th>
<th>50-79%</th>
<th>25-49%</th>
<th>&lt;10%</th>
<th>0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1</td>
<td>IL-6</td>
<td>PDGF-A</td>
<td>IL-1α</td>
<td>EGF</td>
<td>IL-1β</td>
<td>IFN-γ</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>PDGF-B</td>
<td>TNF-αp55</td>
<td>GM-CSF</td>
<td>IL-2</td>
<td>IL-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-α</td>
<td>TGF-β</td>
<td>MCP-1</td>
<td>MIP-1α</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-CSF</td>
<td></td>
<td>MCP-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td></td>
<td>MCP-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDEC1GF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

mRNA from 10-12 ovarian tumours, 8-10 normal biopsies, six cell lines and three xenografts was screened by RT-PCR for the cytokines detailed above. The sum of the positive samples in each group is expressed as a percentage of the total number of samples screened for each cytokine.

TABLE 2. Cytokine receptor mRNA expression in all ovarian samples. Percentage positive samples (all groups combined)

<table>
<thead>
<tr>
<th>Cytokine mRNA</th>
<th>100%</th>
<th>90-99%+</th>
<th>80-89%</th>
<th>50-79%</th>
<th>25-49%</th>
<th>&lt;10%</th>
<th>0%</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-tylI</td>
<td>IL-1tyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>IL-4</td>
<td>TNF-αp75</td>
<td>GM-CSF</td>
<td>IL-2β</td>
<td>IL-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-CSF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

mRNA from 10-12 ovarian tumours, 8-10 normal biopsies, six cell lines and three xenografts was screened by RT-PCR for the cytokine receptors detailed above. The sum of the positive samples in each group was expressed as a percentage of the total number of samples screened for each cytokine receptor.

**Defining the 'core' cytokines and receptors**

Because of the consistency of expression between the groups, we analysed the data for the cytokine and receptor mRNAs that were most commonly found in all the samples from every group. Table 1 summarizes the data for cytokines and Table 2 for cytokine receptors. IGF-1 was the only cytokine whose mRNA was expressed in all samples. A further six cytokines (IL-6, TNF-α, TGF-α, M-CSF, IL-8 and PDEC1GF) were expressed in over 90% of all samples and a further four cytokines (PDGF-A, PDGF-B TGF-β and MCP-1) expressed in over 80% of all samples. As described above, the expression of the chemokines RANTES, MIP-1α and MIP-1β was high in the biopsies but low or absent in the xenografts and cell lines, but the other cytokines were infrequently expressed in all groups. mRNA for IFN-γ was not detected in any of the samples. The potential importance of IGF-1 was stressed by the presence of mRNA for its type I receptor in all of the samples. The IFN-γ-receptor was also universally present even though mRNA for its ligand was never expressed. Over 80% of all samples expressed the type II IGF receptor as well as mRNA for the IL-1, IL-4, TNF-α (p55 and p75), and M-CSF receptors. In spite of the presence of its ligand and a signal in the positive control, gp80IL-6 receptor expression was not detected in any of the samples.

**DISCUSSION**

We have found RT-PCR to be a rapid and useful screen for determining the overall cytokine context of tissues and cell lines. This study confirmed the presence of several cytokines reported using other techniques, and found additional cytokines and receptors that may be of importance to ovarian cancer biology. We have also identified cell lines and xenografts that most closely resemble malignant tissues in terms of their cytokine context. The cytokine mRNA profile of the normal and malignant ovary is rich in pro-inflammatory cytokines, growth factors and chemokines but lymphocyte-associated cytokines are less commonly found.

Similarities between normal and malignant biopsies may reflect the fact that analogous processes of repair and remodelling occur in these tissues even when the epithelial cells are transformed. However, there could still be differences in mRNA levels, mRNA stability and expression of the protein product. RT-PCR is a highly sensitive technique and the method we used would not necessarily be expected to detect quantitative differences in mRNA. Ribonuclease protection assays, for instance, detected differences in mRNA levels between normal and malignant ovarian tissues. Comparison of the cell line and biopsy data, taken together with previously published information on the site of MCP-1 and TNF-α expression suggests that
4, may be of therapeutic benefit, as both have been known to inhibit tumour cell growth\textsuperscript{22,23} and their receptors are expressed in a majority of samples in the absence of their ligand.

**MATERIALS AND METHODS**

**Patient material**

Biopsy samples were obtained at the time of surgery, cut into 5 mm cubes and snap-frozen in isopentane which had been cooled almost to its freezing point in liquid nitrogen. Samples were stored at -80°C until required. Twelve samples were obtained from patients with ovarian carcinomas (seven serous adenocarcinomas, one mucinous carcinoma, one endometrioid carcinoma, one granulosa cell tumour, one clear cell carcinoma, one Brenner tumour), three from patients with benign ovarian tumours (cellular fibroma, benign cyst and benign serous cystadenoma) and seven from patients with histologically normal ovaries.

**Cell lines**

PEO1 and PEO4 were derived from the ascites of a patient with a poorly differentiated adenocarcinoma. PEA2 was obtained after chemotherapy from a patient with a poorly differentiated adenocarcinoma. PEO14 was obtained prior to therapy from a patient with a well differentiated serous adenocarcinoma. (All the above were obtained from and described by Langdon et al.) OVCAR-3 was obtained from a patient with malignant ascites associated with a poorly differentiated papillary adenocarcinoma (obtained from and described by Hamilton et al.\textsuperscript{10}) SKOV-3 originated from ascites in a patient with an ovarian adenocarcinoma and was obtained from the American Tissue Culture Collection (ATCC) (Rockville, MD). Cell lines were all grown under endotoxin and pyrogen free conditions in RPMI 1640 or D-MEM (SKOV 3 only) supplemented with 10% fetal calf serum. With the exception of SKOV-3, insulin (10 µg/ml) was added to the culture medium. Cell lines were grown until they just reached confluency before total RNA was extracted for cDNA synthesis.

**Xenografts**

HU, LA and OS xenografts\textsuperscript{11} were obtained directly from human ovarian cancer specimens, and were grown as intraperitoneal ascites, passaged from mouse to mouse. Tumour material was obtained from the mice by peritoneal lavage and snap frozen in liquid nitrogen. Samples were stored at -80°C.

**cDNA synthesis**

Unless otherwise stated, all reagents were obtained from Sigma (Poole, Dorset, UK). Total RNA was extracted following the method of Chomczynski and Sacchi\textsuperscript{12} with modification by Puissant and Houdebine.\textsuperscript{13} Solid tissues were homogenized directly into solution D 4 M guanidium thiocyanate, 25 mM sodium citrate pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol), using an ultraturrax T25 (Janke and Kunkel, Staufen, Germany) with 3 × 10-s bursts on ice using maximum speed. The RNA was subsequently precipitated and the pellet resuspended in 50 µl of DNase buffer containing 2 units of RNase-free DNase 1 (Promega, Southampton) and 2 units RNase inhibitor (Promega). This was incubated for 2 h at 37°C to remove any contaminating genomic DNA. Following phenol/chloroform extraction, the RNA was redissolved in DEPC treated water. First strand cDNA was synthesized from total RNA using a cDNA synthesis kit (Boehringer Mannheim AG Switzerland) according to the manufacturers instructions and starting with 2 µg total RNA.

**PCR**

Primers were either obtained from Clontech (Palo Alto, CA) complete with suitable positive control, or designed from the sequences submitted to genbank using Primer programme version 0.5 May 1991. Those designed from genbank are listed below.

- **MCP-1**
  - 5' primer CAA ACT GAA GCT CGC ACT TTC GCC
  - 3' primer ATT CTT GGG TTG TGG AGT GAG TGT TCA
- **β-ACTIN**
  - 5' primer GTG GGG CGC CCC AGG CAC CA
  - 3' primer CTC CTT AAT GTC ACG CAC GAT TTC
- **PDEGF**
  - 5' primer TGT CAT CCA GAG CCC AGA G
  - 3' primer AAC CAG CGT CTT TGC CAG
- **M-CSF and RANTES sequences** were taken from Mattei et al.\textsuperscript{14} MIP-1α and β sequences were taken from Hosaka et al.\textsuperscript{15}

Two µl (200 ng) of cDNA was used for each amplification. A master mix consisting of all reagents except the cDNA was made up for each primer pair. A final volume of 25 µl per reaction was overlaid with mineral oil and the following protocol was used in a Techne PCR machine (Cambridge, UK): 1 cycle 94°C (5 min); 60°C (1 min); 72°C (0.5 min); 35 cycles 94°C (45 s); 60°C (45 s); 72°C (2 min); 72°C (7 min). Ten µl of reaction mixture was subsequently electrophoresed on a 1.2% agarose gel and visualized by ethidium bromide staining. 123 bp markers (Gibco BRL) were used to estimate band sizes. Positive control cDNAs were provided with the amplimer kit. The promyelomonocytic cell line HL-60 (obtained from ATCC) stimulated with TNF-α (10 ng/ml) for 3 h was used as a positive control for MCP-1 and MIP-1β. HL-60 cells stimulated with PMA (500 µg/ml) and retinoic acid (1 µg/ml) for 2 days provided a positive control for MIP-1α. Mono-Mac-6 cells (obtained from the German Collection of microorganisms and cell cultures, Department Human and Animal Cell Cultures, Braunschweig, Germany) were a positive control for RANTES. For the negative controls water was used in place of the cDNA.

**Southern blotting techniques**

In order to validate the PCR results, in four cases, agarose gels were depurinated in 0.25 M HCl for 15 min, neutralized in 0.4 M NaOH for 5 min and alkaline-blotted onto Hybond


The Detection and Localization of Monocyte Chemoattractant Protein-1 (MCP-1) in Human Ovarian Cancer

Rupert P. M. Negus, Gordon W. H. Stamp, Michele G. Reif, Frances Burke, Saleem T. A. Malik, Sergio Bernasconi, Paola Allavena, Silvano Sozzani, Alberto Mantovani, and Frances R. Balkwill

*Biological Therapies Laboratory, Imperial Cancer Research Fund, London, WC2A 3PX, United Kingdom; †Department of Histopathology, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 0HS, United Kingdom; ‡Thunder Bay Regional Cancer Centre, Thunder Bay, Ontario P7A 7T1, Canada; §Istituto di Ricerche Farmacologiche “Mario Negri,” 20157 Milano, Italy; and ††Section of Pathology, Department of Biotechnology, University of Brescia, 25123 Brescia, Italy

Abstract

Chemokines may control the macrophage infiltrate found in many solid tumors. In human ovarian cancer, in situ hybridization detected mRNA for the macrophage chemo- kine monocyte chemoattractant protein-1 (MCP-1) in 16/17 serous carcinomas, 4/4 mucinous carcinomas, 2/2 endometrioid carcinomas, and 1/3 borderline tumors. In serous tumors, mRNA expression mainly localized to the epithelial areas, as did immunoreactive MCP-1 protein. In the other tumors, both stromal and epithelial expression were seen. All tumors contained variable numbers of cells positive for the macrophage marker CD68. MCP-1 mRNA was also detected in the stroma of 5/5 normal ovaries. RT-PCR demonstrated mRNA for MCP-1 in 7/7 serous carcinomas and 6/6 ovarian cancer cell lines. MCP-1 protein was detected by ELISA in ascites from patients with ovarian cancer (mean 4.28 ng/ml) and was produced primarily by the cancer cells. Human MCP-1 protein was also detected in culture supernatants from cell lines and in ascites from human ovarian tumor xenografts which induce a peritoneal monocytosis. We conclude that the macrophage chemoattractant factors (13, 14) and there is a correlation between MCP-1 levels and the number of TAMs present. These findings were related to the in vitro production of MCP-1 by ovarian cancer cell lines and the production of MCP-1 by two human ovarian cancer xenografts that induced a monocytosis in the nude mouse hosts.

Introduction

Leukocytes infiltrate many solid malignant tumors (1). In human epithelial ovarian cancer, the infiltrating cells are predominantly macrophages and T lymphocytes (2). Tumor-associated macrophages (TAM) are found both within the stromal (3) and epithelial tumor areas (4).

Although TAM may be cytotoxic to tumor cells when activated in vivo (5) or in vitro (6), the evidence that they normally have a tumoricidal role is scant. Indeed TAM may promote tumorigenesis by the production of cytokines such as EGF, PDGF, TGF-β, TNF-α, IL-1, and IL-6 (7). TNF-α can alter the behavior of ovarian cancer xenografts, causing tumor cells in ascites to form solid peritoneal tumors with well developed stroma (8). In situ hybridization studies show that TNF-α is expressed both by infiltrating macrophages and tumor epithelial cells, although TNF-α protein is largely restricted to TAM (4). TNF-α derived from TAM may also stimulate angiogenesis (9, 10) and TAM may contribute to tumor spread by producing matrix metalloproteases (11, 12).

A variety of human and murine tumor cells produce monocyte chemoattractant factors (13, 14) and there is a correlation between the amount of activity in culture supernatants and the number of TAMs when these cells produce tumors in vivo (15). Certain tumors, such as freshly disaggregated ovarian carcinomas, primary ovarian carcinoma cultures, and established ovarian carcinoma cell lines, produce a protein with an apparent molecular mass of 12 kD (16) which has chemotactic activity for peripheral blood monocytes. A protein, designated monocyte chemoattractant protein-1 (MCP-1), which has very similar properties, has been purified to homogeneity and the cDNA cloned from the glioma cell line U-105MG (17), the myelomonocytic lines U937 and THP-1 (18, 19), and a variety of normal and transformed cells (20).

The aim of this study was to discover whether MCP-1 is expressed in human ovarian cancer, to establish its cellular sources in vivo and to determine whether there is a correlation between MCP-1 levels and the number of TAMs present. These findings were related to the in vitro production of MCP-1 by ovarian cancer cell lines and the production of MCP-1 by two human ovarian cancer xenografts that induced a monocytosis in their nude mouse hosts.

Methods

Tissue samples. Biopsy specimens of ovarian tumors from untreated patients were obtained at operation and snap frozen into liquid nitrogen.

1. Abbreviations used in this paper: MCP, monocyte chemoattractant protein; RT, reverse transcription; TAM, tumor-associated macrophage.
 DEVELOPMENT OF ENZYMHO-IMMUNOASSAYS (EIA) FOR MACROPHAGE COLONY-STIMULATING FACTOR (M-CSF) AND LEUKAEMIA INHIBITORY FACTOR (LIF) BY USING THE SAME CAPTURE AND SIGNAL GENERATING POLyclonal ANTIBODY

Philippe Fixe, Valérie Lorgeot, Yannick Le Meur, Lionel Coupey, Dominique Heymann, Anne Godard, Vincent Praloran

Specific high titre polyclonal antibodies rapidly obtained by intralymphnode immunization of rabbits with recombinant M-CSF and LIF (<60 μg/animal) have been used to develop specific, accurate and sensitive EIAs. The same batch of purified anti-M-CSF or anti-LIF Igs has been used for the coating of 96-well plates (capture antibody) and for the quantitative detection of the bound cytokine molecules (soluble biotinylated Igs). The sensitivity (M-CSF: 10 IU/ml, LIF: 20 pg/ml), accuracy (intra-assay-CV: 8.2 to 12.8% for M-CSF; 0 to 19.9% for LIF) and reproducibility (inter-assay-CV: 7.9 to 13.6% for M-CSF; 4.9 to 17.5% for LIF) are equivalent to those for previously published RIAs or EIAs. These assays are highly specific since 11 other cytokines (Epo: 3 IU/ml; G-CSF: 100 IU/ml; CNTF, OSM, SCF, IL-1β, IL-2, IL-3, IL-6, IL-11, IL-13: 5 ng/ml) tested in both EIAs were not detectable. Finally, the M-CSF and LIF concentrations measured in various biological fluids were found to be similar to those measured by us and others with different assays. In conclusion, the methodology used for M-CSF and LIF EIAs presented in this work represents a valuable approach for most cytokines, particularly when they are still available in reduced amounts.

Over the last decade, studies have emphasized the role of cytokines in inflammatory reactions as well as in haematological and immunological diseases. Cytokines play an important role in cell-to-cell communications through specific cell surface receptors. They regulate cell functions such as proliferation, differentiation and regulation of gene expression (including cytokine synthesis). Several cytokines have been reported to be interesting markers and/or prognosis factors in various pathological situations such as: Interleukin-6 (IL-6) in multiple myeloma and neonatal bacterial infection, macrophage colony-stimulating-factor (M-CSF) in epithelial ovarian or breast cancer, and leukaemia inhibitory factor (LIF) in giant cell arteritis. Most enzymo-immunoassays (EIA) for cytokines use monoclonal antibodies. However, their development is often rather long and costly and their monospecificity may be sometimes a disadvantage for antigens having variable peptide sequences and/or glycosylation sites, or being bound to soluble receptors.

M-CSF and LIF are two pleiotropic cytokines playing a role in haematopoiesis, immune response, pregnancy and in various inflammatory and malignant pathologies. We have developed a reproducible and sensitive sandwich EIA for each of them. These EIAs are characterized by the fact that we used the same batch of purified anti-M-CSF or anti-LIF rabbit polyclonal immunoglobulins (Igs) for the solid phase capture of M-CSF or LIF and for their revelation by soluble biotinylated secondary antibody. They have then been tested for their sensitivity and specificity as well as for their reliability. Furthermore, these EIAs have been used to assess M-CSF and LIF concentrations in serum, plasma, joint or pleural effusions and culture supernatants of various origins. Our results were compared with results obtained by other techniques on the same samples or with those published by us or others.

© 1996 Academic Press Limited
They were then stored at −70°C before cutting 5-7-µm sections. Sections for in situ hybridization were mounted on baked glass slides coated with 3-aminopropyl-triethoxy-silane (Sigma Chemicals Ltd., Poole, Dorset, United Kingdom), air dried, and stored at −70°C until use. Sections for immunohistochemistry were cut onto poly-L-lysine-coated slides and stored at −70°C.

Cell lines. The following cell lines were used to prepare cDNA for reverse transcription (RT)-PCR: PEO 1, PEO 4, PEO 14, and PEA 2 (obtained from S. Langdon, Imperial Cancer Research Fund Ovocoitology Unit, Western General Hospital, Edinburgh, United Kingdom), SKOV-3 (American Type Culture Collection, Rockville, MD), and OVCAR-3 (obtained from T. Hamilton, National Cancer Institute, Bethesda, MD). These lines grow as adherent monolayers. PEO 1, PEO 4, PEO 14, and PEA 2 were grown in RPMI 1640 medium supplemented with 10% FCS. OVCAR-3 was also grown in RPMI 1640, but in the presence of 10 µg/ml insulin.

Mice and xenografts. 6–12-wk-old specific pathogen-free female nu/nu (nude) mice of mixed genetic background were maintained as described previously (21). The ovarian cancer xenografts LA and HU were established from primary human tumors as described previously (22). The ascites were confined to the peritoneum and were passaged in vivo. For protein estimation, the mice were injected intraperitoneally with 4 ml of RPMI 1640. Once removed, the ascites were spun and the supernatant was stored at −70°C.

Riboprobes. A template consisting of an ~360-bp fragment of MCP-1 cDNA in the expression vector pGEM 3Z (Sigma Chemicals Ltd.) was linearized with BamHI (Promega Corp., Southampton, United Kingdom) and transcribed with SP6 RNA polymerase (Promega Corp.) to generate antisense riboprobes. Sense probes were made using HindIII and T7 polymerase (Promega Corp.). Antisense β-actin (obtained from Dr. L. Kedes, Stanford University, Stanford, CA) was used as a positive control for all in situ hybridization experiments. Probes were generated from HindIII-linearized Bluescript containing human β-actin cDNA using T7 RNA polymerase. Both sense and antisense MCP-1 riboprobes were used to probe the TNF-α-stimulated HL60 cytopsin.

In situ hybridization. The method described by Naylor et al. was followed. In brief, cytospin preparations were incubated at 37°C for 4 h with 10 mg/ml human recombinant TNF-α for 6 h before preparing cytopsins or RNA.

Immunohistochemistry. The avidin-biotin-peroxidase complex method was used to stain both for macrophages and MCP-1 protein (24). Sections were air dried overnight and fixed in 4% paraformaldehyde in PBS for 5 min before staining. They were preincubated with normal rabbit serum (DAKO, High Wycombe, Bucks, United Kingdom) and mounted in DPX (BDH Laboratory Supplies, Merck Ltd., Lutterworth, United Kingdom).

Preparation of RNA from tissue samples and cell lines. Total RNA was prepared from human ovarian tissue by the method of Chomczynski (26). RNA was quantitated by absorbance at 260 nm and the samples were treated with DNase before being used for cDNA synthesis.

Oligonucleotide primers. The MCP-1 primers used were as follows: 5'-CAACAGCTGCTGGAGGAGTGTCAGTCACCTC-3' (−20 to 36) and 5'-TTTCCACGCTGCTGGAGGAGTGTCAGTCACCTC-3' (−299 to −324) giving a PCR product of 354 bp. For β-actin, used as a control, the primers were: 5'-GGGCGGCCCCGACGACACAC-3' and 5'-CTCTCTATACGTACGACCATTT-3' yielding a product of 548 bp. The MCP-1 sequences were chosen to span the protein coding region within the MCP-1 cDNA 5' untranslated regions.

RT-PCR. cDNA was prepared using the methods and reagents of the Boehringer Mannheim cDNA strand synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany) for the first strand synthesis only. PCR was carried out using the Perkin-Elmer Gene Amp PCR Kit (Perkin-Elmer Corp., Norwalk, CT), using MCP-1 or β-actin specific primers. A reaction volume of 25 µl per sample was made up. PCR was carried out on 2-µl aliquots of cDNA using 35 amplification cycles. Amplification products were electrophoresed through a 1.2% agarose gel and visualized by ethidium bromide staining.

Characteristics of patients used in analysis of ascites. Ascites were collected by paracentesis from a total of 46 patients with ovarian cancer. 30 patients were subsequently shown histologically to have serous adenocarcinoma, 3 mucinous adenocarcinoma, 5 clear cell carcinoma, 4 endometroid carcinoma, 3 undifferentiated carcinoma, and 1 anaplastic mucous-secreting tumor. 20 patients had received some treatment before their ascites were analyzed and 26 were untreated.

Isolation of TAM and ovarian carcinoma cells. TAM and ovarian carcinoma cells were separated from ascites using discontinuous Ficoll-Hypaque and Percoll gradients, essentially as described (16). Highly enriched TAM preparations (>95% by morphology) were obtained using CD14-coated magnetic beads (Unipath, Milan, Italy). Tumor cell preparations were depleted of CD14+ cells. TAM and tumor cells were cultured at 3 × 10⁵/ml in RPMI 1640 with 10% FCS. After 24 h supernatants were collected for MCP-1 measurement.

ELISA for MCP-1 in ascites, TAM and tumor cell preparations, tumor cell lines and xenografts. MCP-1 was measured using a sandwich ELISA assay based on a rabbit antisera and an mAb (5D3-F7), as described recently (25). The assay has a sensitivity of 40 pg/ml in supernatants and 80 pg/ml in peritoneal fluids. It is specific for human MCP-1 and does not detect the mouse equivalent, JE, or the closely related human chemokines MCP-2 and MCP-3.

Results

MCP-1 mRNA and protein in human ovarian cancer biopsies. In situ hybridization demonstrated mRNA for MCP-1 in a total of 23/26 ovarian tumors (Table 1). In the group of serous adenocarcinomas, mRNA expression was detected in 16/17 samples. In 13 of these cases it was possible to distinguish between stromal and epithelial expression; while some label was found at the stromal-epithelial border in all 13 cases, it was predominantly confined to the epithelium in 11/13 (Fig. 1, A and B) and to the stroma in 2/13 (Fig. 1 C). Within the epithelium, expression was confined to single cells or small groups of cells. The maximum expression was 12.4% epithelial cells/high power field and the mean was 1.4%, 4/4 mucinous tumors expressed mRNA; 1 expressed predominantly epithelial mRNA while in the other 3 it was confined to the stroma or stromal-epithelial interface. In 1/2 endometroid carcinomas, expression of MCP-1 mRNA was equal between the stroma and the epithelium.
Table 1. Frequency and Extent of MCP-1 mRNA Expression in Ovarian Cancer Biopsies Determined by In Situ Hybridization

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Number of cases</th>
<th>Total number positive ±</th>
<th>+</th>
<th>++</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serous</td>
<td>17</td>
<td>16</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Mucinous</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Borderline</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Nonmalignant ovarian tissue</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

±, < 0.2 cells/high power field; +, 0.2–2 cells/high power field; ++, 2–20 cells/high power field. The mean number of labeled cells in 10 high power fields was calculated for each sample.

Figure 1. The distribution of MCP-1 mRNA and protein as determined by in situ hybridization with a 35S-labeled antisense MCP-1 riboprobe. (A) Individual cells labeled within an epithelial area of a serous adenocarcinoma, × 200 and (B) × 650. (C) Stromal expression of MCP-1 mRNA in a serous adenocarcinoma, × 650. (D) Focal epithelial labeling in a serous borderline tumor, × 200.
TAMs in human ovarian cancer. All the tumors studied contained cells which were positive for the macrophage marker CD68 (for review see Weiss et al. [27]), within both the stromal and epithelial areas. CD68-positive cells were mainly found in groups adjacent to, or just within, the epithelial areas, but occasionally, positive cells aggregated in small areas of necrosis or were observed in glandular lumina. In serous and mucinous adenocarcinomas the proportion of CD68 positive cells varied between 1 and 28% cells/high power field with a mean of 7.8%. However, there was no statistically significant correlation between the number of cells expressing MCP-1 mRNA in a section and the number of CD68-positive cells detected by immunohistochemistry (Mann-Whitney two-sample test and Spearman’s rank correlation coefficient).

MCP-1 and macrophages in nonmalignant ovarian tissue. MCP-1 mRNA was detected in the stroma of 5/5 ovaries which had been removed for a variety of nonmalignant conditions. Stromal macrophages could be identified by CD68 staining in all cases, but it was not possible to determine whether these cells were expressing MCP-1 mRNA. Mesothelium was only preserved in one case and in this no epithelial mRNA was expressed.

MCP-1 levels in human serum and ascites. MCP-1 protein was detected by ELISA in ascites from patients with ovarian cancer (Fig. 2). The mean level (4.28 ng/ml) was significantly higher than that in ascites from patients with cirrhosis (mean 0.76 ng/ml; P < 0.00001 by Student’s t test). Low but detectable levels were found in plasma from normal laboratory donors (mean 0.23 ng/ml), patients with benign gynecological disease (mean 0.39 ng/ml), and patients with ovarian carcinoma (0.49 ng/ml). In ovarian cancer patients, the ascites levels were significantly higher than plasma levels (P < 0.00001 by Student’s t test).

MCP-1 production by freshly isolated TAM and tumor cells. In three patients highly enriched TAM and ovarian tumor cells were fractionated from ascites and their capacity to release MCP-1 was assessed over a 24-h period. MCP-1 production was essentially confined to the ovarian carcinoma–enriched population. The tumor cell–enriched population produced 2.3, 40.9, and 47.2 ng/ml compared with 0.3, 0.2, and 0.1 ng/ml, respectively, produced by TAM.

MCP-1 in cell lines. MCP-1 expression was detected by RT-PCR in the ovarian cancer cell lines SKOV-3, OVCAR-3, PEO 1, PEO 4, PEO 14, and PEA 2. This was confirmed by Southern blotting (Fig. 3). Ovarian cancer cell lines were heterogeneous in terms of MCP-1 protein production. PEO 14 produced the highest level (mean 6.77±0.01 ng/ml); low but appreciable levels were found in PEO 1 (mean 0.11±0.01 ng/ml), PEO 4 (mean 0.27±0.01 ng/ml), and OVCAR-3 (mean 0.09±0.01 ng/ml), and little or no MCP-1 was detected in supernatants from PEA 2 (mean 0.04±0.01 ng/ml) and SKOV-3 (0.06±0.01 ng/ml).

Ovarian cancer xenografts. Human MCP-1 protein was detected in peritoneal washouts from both HU (mean 0.07±0.02 ng/ml) and LA (mean 0.38±0.01 ng/ml) ovarian cancer xenografts. 7 d after intraperitoneal injection of the HU and LA xenografts, the proportion of cells positive for the mouse pan-macrophage marker F4/80 in peritoneal washouts was 65±5 and 54±4%, respectively. These levels were significantly higher (P < 0.005 for HA and P < 0.025 for LA) than those in control mice (40±1.1%).

Discussion
Using in situ hybridization we have demonstrated that MCP-1 is expressed in human epithelial ovarian cancer. Furthermore,
MCP-1 protein is detectable in significant levels in ascites from these patients. Within the serous tumor group, MCP-1 mRNA is predominantly associated with the epithelial areas, although stromal expression was occasionally detected. Where immunoreactivity for MCP-1 was found, it was also within the epithelium, and when TAMs were separated from tumor cells in ascites, the tumor cells were the major source of MCP-1. In mucinous tumors, mRNA expression was predominantly associated with the stroma, while in endometrioid tumors it was distributed throughout both the epithelium and stroma. The single borderline tumor that was positive for MCP-1 mRNA showed stromal and very focal epithelial expression. In all the tumor types, TAMs were most abundant at the tumor-stroma border, usually occurring singly or in small groups. The normal ovaries were all positive for stromal MCP-1 mRNA and all contained stromal macrophages. Human MCP-1 protein was detected by specific ELISA in peritoneal washouts of two different human ovarian cancer xenografts in nude mice. These tumors are associated with a significant increase in the macrophage population 7 days after injection. Although it is likely that the tumor epithelium is a significant source of MCP-1 in vivo, stromal elements also contribute. A shift in balance from stromal to epithelial MCP-1 production may be associated with a more malignant phenotype.

Although MCP-1 has been detected in other pathologies associated with a macrophage infiltrate, this is the first demonstration that it is produced by an epithelial tumor and that the tumor cells themselves are the likely major source. In human malignant glioma, MCP-1 mRNA and protein localized to tumor cells but not infiltrating macrophages (28). A similar pattern of MCP-1 expression has been demonstrated in Kaposi's sarcoma (29) and the fibroblast-like tumor cells derived from fibrous histiocytoma in culture (30). MCP-1 production by epithelial cells has been shown in other conditions associated with a macrophage infiltrate; human MCP-1 derived from vascular endothelium has been demonstrated both in animal models and human pathological specimens of atherosclerosis. The murine equivalent of MCP-1, JE, has been detected in renal tubular epithelium in a mouse model of hydronephrosis (31). The source of the stromal MCP-1 in the tumors and nonmalignant ovarian stroma that we studied is uncertain but a wide variety of other cell types are known to be able to produce MCP-1, including macrophages (32, 33), monocytes (34), and fibroblasts (35).

The mechanism by which MCP-1 acts as a chemoattractant is not clear. Martinet (36) suggests that there is an in vivo concentration gradient between the tumor and neighboring blood vessels on the basis of the presence of a monocyte chemoattractant activity gradient between pleural fluid and plasma in patients with pulmonary adenocarcinoma. We also found a significant difference in MCP-1 concentration between ascitic fluid and plasma. Although there was no statistically significant correlation between the amount of MCP-1 mRNA expression and the number of infiltrating macrophages, there was a topographical association, with both the majority of MCP-1 mRNA and TAMs being found at the epithelial-stromal interface. The relationship between MCP-1 expression and TAMs is unlikely to be simple. Studies with transgenic mice show that even if MCP-1 is over expressed in the gonads, there is not an accompanying macrophage infiltrate (37). Chemokines may trigger the expression of leukocyte integrins, thereby enabling strong adhesion to vascular endothelium (38). They may also stimulate the release of matrix metalloproteases and facilitate the movement of cells through the tumor stroma (39); the matrix metalloprotease MMP-9, which degrades type IV collagen found in basement membranes, has been detected in human ovarian cancer by in situ hybridization and its distribution corresponds to that of TAMs (12).

In ovarian cancer, monocyte chemoattraction may be a function of more than one chemokine; for instance, macrophage-colony stimulating factor protein has been detected by immunohistochemistry (40). Other c-c chemokines, such as RANTES, also have monocyte chemoattractant activity; furthermore, some may play a part in the recruitment of T cells, which form a prominent part of the mononuclear cell infiltrate in human ovarian cancer (2). On the other hand, MCP-1 is chemoattractant for CD56*/CD16* natural killer cells (41) but these are rarely seen in human ovarian cancer (3). The presence of MCP-1 is therefore likely to be necessary, but not by itself sufficient, for macrophage recruitment.

We conclude that MCP-1 is expressed by tumor cells in human epithelial ovarian cancer. Furthermore, human MCP-1 is produced by ovarian cancer xenografts in nude mice and is constitutively expressed by several cell lines in vitro. In vivo, other factors, such as expression of the appropriate adhesion molecules and the correct substrate for optimal chemotaxis, are likely to be required for monocyte infiltration.

**Acknowledgments**

We would like to thank Mr. Peter Mason and his staff at the Samaritans Hospital, London for their help in obtaining tumor samples, Mr. George Elia and the staff of the Imperial Cancer Research Fund Histopathology Unit for their expert technical assistance, and Mrs. Maureen Cobbing for her invaluable secretarial support.

Alberto Mantovani is funded by the Special Project Cytokines from the National Research Council and the Italian Association for Cancer Research.

**References**


Quantitative Assessment of the Leukocyte Infiltrate in Ovarian Cancer and Its Relationship to the Expression of C-C Chemokines

Rupert P. M. Negus,* Gordon W. H. Stamp,† Joanna Hadley,‡ and Frances R. Balkwill*
From the Biological Therapies Laboratory,* Imperial Cancer Research Fund, and the Department of Histopathology,† Royal Postgraduate Medical School, Hammersmith Hospital, London, and the Medical Statistics Group,‡ Imperial Cancer Research Fund, Institute of Health Sciences, Headington, Oxford, United Kingdom

We have defined the host leukocyte infiltrate in epithelial ovarian tumors and related this to the expression of C-C chemokines. Immunohistochemical analysis of 20 paraffin-embedded biopsies showed that the infiltrate was primarily composed of CD68* macrophages and CD8+/CD45RO+ T cells (median values, 3700 cells/mm³ and 2200 cells/mm³, respectively). Natural killer cells, B cells, and mast cells occurred in lower numbers (median values, 0 to 200 cells/mm³). Eosinophils were rarely seen and neutrophils were mainly confined to blood vessels. More infiltrating cells were found in stromal than in tumor areas. Only macrophages occurred in significant numbers in areas of necrosis (P < 0.0005). Using in situ hybridization to mRNA, we examined expression of the chemokines MCP-1, MIP-1α, MIP-1β, and RANTES. MCP-1 and MIP-1α were expressed by significantly more cells than MIP-1β and RANTES (P < 0.005). In tumor epithelial areas, the predominant chemokine was MCP-1. MCP-1 and MIP-1α were the predominant stromal chemokines. A significant correlation was found between the total number of CD8+ T cells and the number of cells expressing MCP-1 (r = 0.63 and P < 0.003, respectively) and between the CD8+ population and RANTES-expressing cells (r = 0.6 and P < 0.003). A correlation was also found between CD68+ macrophages and the number of cells expressing MCP-1 (r = 0.50 and P = 0.026). We suggest that MCP-1 may be responsible for the leukocyte infiltrate in ovarian carcinomas, but the expression of other chemokines may determine its exact nature. (Am J Pathol 1997, 150:1723–1734)
also lead to tumor cell destruction. This can be induced in experimental animal models and could account for the effects seen with adjuvant tumor therapies, such as Calmette-Guérin bacillus. Increasing the macrophage to tumor cell ratio may also lead to tumor cell destruction in vitro. Furthermore Chinese hamster ovary cells transfected with monocyte chemoattractant-1 (MCP-1) fail to grow in syngeneic hosts and contain a mononuclear infiltrate. These effects may be mediated by products of macrophage activation. The idea of the macrophage balance may extend to other tumor-infiltrating leukocytes, particularly T lymphocytes.

Although the role of infiltrating cells in epithelial tumors is controversial, a likely stimulus for their presence is the local production of chemokines. These are small (8 to 10 kd) cytokines that are chemoattractant for a variety of leukocytes and other cells. They are generally classified on the basis of two conserved amino-terminal cysteine residues that may either be adjacent, C-C, or separated by another amino acid, C-X-C. The C-C chemokines are active on T cells and monocyte/macrophages, but C-X-C chemokines are more specific for neutrophils. The leukocyte content of a tumor may depend on the chemokines that are expressed. In a previous study, we described the production of MCP-1 by human epithelial ovarian tumor cells and demonstrated the presence of a chemokine gradient between ascites and plasma. MCP-1 has been found in other tumors, such as malignant glioma, where macrophages are plentiful. Although the presence of this chemokine may explain the macrophage infiltrate in ovarian tumors, there are no data that correlate the level of MCP-1 expression and the extent of the infiltrate in individual tumors. Moreover, the contribution of other chemokines has not been assessed. To investigate the relationship between host immune cells, chemokines, and tumor in epithelial ovarian cancer, we have defined quantitatively the nature and distribution of the leukocyte infiltrate using a panel of antibodies in paraffin-embedded material. We also analyzed cryostat sections of epithelial tumors for expression of the C-C chemokines macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and RANTES (regulated upon activation, normal T cell expressed and stimulated) and compared these with MCP-1 expression in the same samples. The number of chemokine-expressing cells was then correlated with the infiltrating cell population.

**Materials and Methods**

**Tissue Samples**

Twenty human epithelial ovarian tumors were used to assess the infiltrating leukocyte population. Nine were collected at the time of operation and were fixed in formal saline for 24 hours. Eleven were obtained from the Hammersmith Hospital (London, UK) archives. There were 2 borderline tumors, 15 papillary serous cystadenocarcinomas (5 grade 1, 5 grade 2, and 5 grade 3), 2 papillary endometroid tumors (grade 2), and 1 solid endometroid/undifferentiated tumor (grade 3). All tissue samples were paraffin embedded before cutting 4-µm sections onto silane-coated slides. The histological type and grade of all specimens was reassessed at the time of the study, using criteria previously outlined by us.

An additional 20 frozen epithelial tumors were assessed for C-C chemokine expression by in situ hybridization. Ten of these tumors were from the same patients as the paraffin-embedded material and ten samples were obtained from the Queen’s Hospital, Belfast. One was classified as a borderline/papillary serous cystadenocarcinoma (grade 1); eleven were papillary serous cystadenocarcinomas (three grade 1, four grade 2, and four grade 3); two were mucinous cystadenocarcinomas (grade 2); three were papillary endometroid carcinomas (grade 2); one was a papillary serous adenocarcinoma (grade 3), and two were serous solid/cystic adenocarcinomas (grade 3). The 6-µm cryostat sections were cut onto 3-aminopropyl-triethoxy-silane-coated four-well slides. These sections were also used to assess the CD68$^+$ and CD8$^+$ population. Sections were air dried for 1 hour and fixed in 4% paraformaldehyde for 20 minutes or formal saline for 5 minutes, respectively, before immunostaining.

**Antibodies**

A panel of 11 antibodies was used to phenotype the infiltrating leukocyte population: anti-CD3 (DAKO, High Wycombe, UK) for all T lymphocytes, anti-CD8 (C8/144B, DAKO) for cytotoxic T lymphocytes in paraffin-embedded material, anti-CD8 (DK125, DAKO) for cytotoxic T lymphocytes in frozen material, anti-CD45RO (UCHL1, DAKO) for memory T lymphocytes, anti-CD4 (MT310, DAKO) for helper T lymphocytes in frozen material, anti-CD68 (PG-M1, DAKO) for macrophages in paraffin-embedded material, anti-CD68 (EBM 111, DAKO) for macrophages in frozen material, anti-CD20 (L26, DAKO) for B lymphocytes, anti-CD57 (NK1, DAKO) for natural killer cells, anti-mast-cell-tryptase (AA1, DAKO) for mast cells, and MAC 387 (DAKO) for granulocytes. The presence of eosinophils was determined in hematoxylin-and-eosin-stained sections according to their characteristic staining and morphology.
Immunohistochemistry

A three-step indirect streptavidin-peroxidase system was used throughout. Immunostaining was revealed by the chromogen 3,5-diaminobenzidine (DAB). All tissue sections were treated with H2O2 to block endogenous peroxidase activity before staining. In addition, sections stained with anti-CD3 were blocked with normal swine serum (DAKO) before the primary antibody was applied. Sections were incubated sequentially with primary antibody, biotinylated rabbit anti-mouse IgG (DAKO), and streptavidin-peroxidase conjugate (DAKO) for 35 minutes each. Between each step, sections were washed twice in fresh phosphate-buffered saline, pH 7.2, for 5 minutes. The final incubation with DAB was for a maximum of 2 minutes, followed by a 2-minute wash in water. Sections were counterstained with Harris' hematoxylin for 30 seconds. Positive controls were obtained by staining sections of tonsil, spleen, or stomach as appropriate. Omission of the primary antibody was used to provide negative controls.

Assessment of Cell Numbers and Distribution

All cell counts were performed using a Nikon Labophot II microscope (Nikon, Kingston, UK) at a magnification of x200 (20x objective and 10x eyepiece). Only cells with cytoplasmic staining, nuclear counterstain, and appropriate morphology were included. The area counted in each section was chosen randomly from a representative field of tumor. Cell counts were performed with the aid of a 10 x 10 index grid (Graticules, Tonbridge, UK). For each section, nine areas were assessed with the grid arranged in a 3 x 3 pattern, to give a total area of 2.3 mm².

Area counts were carried out using a modified Chalkley array (Graticules) over the same area as the cell counts. The modified Chalkley array consists of 25 randomly arranged points; the proportion of points coincident with any tissue component is proportional to its area and volume fractions. In addition to total cell counts, four different components within the tumors were assessed: tumor cell islands, stroma, areas of necrosis, and areas of space. Areas of necrosis were defined as having cell debris, apoptotic bodies, or red cell fragments. Areas of space included both real spaces, for instance, between tumor papillae, and those created by fixation and processing.

The method of DeHoff and Rhines as described by Ahern and Dunhill was used to estimate the number of cells per mm². Equivalent nuclear diameters for each cell type were determined by tracing a minimum of 100 cell circumferences using an image analysis program (Lucia, Nikon, UK) and a 100x objective oil immersion lens. A frequency histogram of equivalent diameters was constructed for each cell type. The left-hand tail of each histogram was estimated to correct for optically lost caps. The mean tangent diameter (D) was then calculated from the mean of the set of equivalent diameters (d), according to the formula $D = \frac{4d}{\pi}$. Estimates of equivalent diameters derived from cell circumference tracing were validated by assessing the mean diameter of 40 erythrocytes contained within the sections (mean = 6.61 µm, standard deviation = 0.66 µm).

Cell counts were combined with area counts to produce an estimate of the number of cells within each tumor compartment/mm². This was then converted to an estimate of cells/mm³ using the formula $N_0 = \frac{N_A}{(D + t)}$, where $N_0$ = number of cells/mm³, $N_A$ = number of cells/mm², $D$ = mean tangent diameter, and $t$ = section thickness, taken to be 4 µm.

In Situ Hybridization and Assessment of Chemokine Message Expression

All the cDNAs used in the these studies were the kind gift of Professor Alberto Mantovani (Mario Negri Institute, Milan, Italy), other than β-actin, which was obtained from Dr. L. Kedes (Stanford University, Stanford, CA). cDNAs for MCP-1, MIP-1α, MIP-1β, and RANTES were subcloned into suitable transcription vectors before generating 35S-labeled sense and antisense riboprobes as previously described. In situ hybridization was performed according to our standard protocol and incubation was at 4°C for 10 days. Message expression was seen in all antisense-probed sections but in none of the sense control sections. All sections were positive for the expression of β-actin.

To compare the expression of different chemokine mRNAs, the total number of positive cells were counted in 10 adjacent high-power fields (40x objective and 10x eyepiece), giving a total area of 0.73 mm². Fields were chosen to include at least one area of high expression but to avoid the edge of the section and areas of artifact. The proportion of tumor and stroma was assessed over the same area using the modified Chalkley array. Due to the difficulty in determining the equivalent of the mean nuclear diameter, cell counts were expressed as cells/mm².
Immunohistochemistry and Cell Counts in Frozen Sections

Paraformaldehyde- or formalin-fixed frozen sections were stained for CD68+ macrophages and CD8+ T cells with the antibodies EBM111 and DK125, respectively. CD4+ T cells were detected in paraformaldehyde-fixed frozen sections using the antibody MT310. The staining procedure was otherwise identical to that for paraffin-embedded sections. Counting was performed with 1% toluidine blue. Cells were counted in 10 randomly chosen high-power fields (40× objective and 10× eyepiece; total area, 0.73 mm²) and were expressed as cells/mm².

Statistical Analysis

As the data were not normally distributed, medians were used to summarize the data, and nonparametric methods of analysis were used to calculate P values. Wilcoxon's matched pairs signed rank test was used for all comparisons between matched pairs of data. Spearman's rank correlation was used to determine the relationship between the CD3+, CD8+, and CD45RO+ T cell populations and to test whether a correlation existed between the number of chemokine-expressing cells and the number of CD68+ or CD8+ cells. The Mann-Whitney U test was used to test whether there was a significant difference between the number of CD68+ and CD8+ cells in frozen and paraffin sections and also to assess the relationships between the leukocyte populations, chemokines, and tumor grade.11 Within each analysis, up to 10 comparisons were made. Using the Bonferroni method to correct for multiple comparisons,22 only P values <0.005 were considered to be statistically significant. However, as this method is highly conservative, P values <0.05 are also shown.

Results

Composition and Localization of the Cellular Infiltrate in Epithelial Ovarian Cancer

The nature of the cellular infiltrate was assessed in 20 epithelial carcinomas. For each cell type, 9 high-power fields were counted at a magnification of ×200 (20× objective and 10× eyepiece). After correcting for the mean tangent diameter, cell numbers were expressed as cell densities (cells/mm³), both for the total area assessed and for each of the four different compartments within the tumor. These were tumor cell islands, stroma, necrotic areas (defined by the presence of cell debris, apoptotic bodies, or red cell fragments) and areas of space between these components. The proportion of whole tumor occupied by each of these compartments is given in Table 1. The volume of whole tumor occupied by tumor cells and stroma were similar (43 and 37%, respectively). Necrosis accounted for 4% of the volume of the tumor, and that occupied by real and artificial space was 16%. There were no significant differences between the volumes occupied by each compartment with tumor grade. Two patterns of infiltrate were distinguishable. Cells present in large numbers were CD68+ macrophages, CD3+, CD8+, and CD45RO+ T cells, whereas those found in much lower numbers were B cells, natural killer (NK) cells, and mast cells (Figure 1a). Table 2 gives the median and range for each of the different cell types per mm³ of total tumor. There was a significant difference between those cells present in high (CD3+, CD6+, and CD45RO+ T cells and CD68+ macrophages) and low (CD20+ B cells, CD57+ NK cells, and mast cells) numbers (P < 0.0005) but not between the cell types within each group. In general, the infiltrating cell density was significantly higher in the stroma compared with the tumor compartment (Figure 1, b and c). For instance, the median value for the number of CD3+ T cells found within the stroma was 3800 cells/mm³ compared with 1600 cells/mm³ in the tumor cell areas (P < 0.005). Similar values were obtained for CD45RO+ T cells. Median values for CD68+ macrophages were 6700 cells/mm³ and 2100 cells/mm³ in stroma and tumor areas, respectively (P < 0.05). The exception to this was the CD8+ population in which there was no significant difference between the numbers found within the stroma and the tumor cell islands (median values, 4300 cells/mm³ and 2500 cells/mm³, respectively; P = 0.083). In the stroma, T cells occurred singly or in clusters (Figure 2a), but when associated with tumor cell islands, they were only found alone (Figure 2b). The most striking difference in cell density was seen in regions of necrosis, where a significantly greater number of macrophages were observed than any other cell type (P < 0.0005, for all comparisons; median, 12,700 cells/mm³; range, 0 to 128,000 cells/mm³; Figure 1d). Macrophages in these areas had an activated morphology with abundant, foamy cytoplasm (Figure 2c). As cells that

| Table 1. Proportion of Tumor Volume Occupied by Various Components (Means and Ranges) |
|-----------------|------|------|------|-----|
| Tumor           | Stroma | Necrosis | Space |
| 43%             | 37%   | 4%     | 16%  |
| (14-90%)        | (8-82%)| (0-37%)| (0-54%) |

Figure 1. Scatter plots demonstrating the absolute numbers of infiltrating leukocytes (cells/mm³) in whole tumor (a), tumor cell islands (b), and stromal (c) and necrotic (d) areas for each of the seven leukocyte markers examined. Only the T-cell subsets and CD68⁺ macrophages are shown for clarity. The horizontal bars represent median values.

Fellows appeared effete were excluded from the counts, the total number of macrophages may have been underestimated. There was no correlation between the number of infiltrating CD68⁺, CD3⁺, CD8⁺, or CD45RO⁺ cells and tumor grade. Neutrophils were assessed using morphological criteria and the monoclonal antibody Mac 387. In contrast to the other cell types, they appeared to be largely confined to blood vessels. Although eosinophils were seen in occasional sections within the stroma and associated with tumor cells, they were rare. Neither neutrophils nor eosinophils were included in the cell counts.

Spearman's rank correlation was used to examine the relationship between the three T-cell markers, both for total cell counts and for cells found within tumor cell areas and stroma. In all cases, there was a significant correlation between the three markers (r > 0.7 and P < 0.005). As there was no significant difference between the total number of CD3⁺, CD8⁺, or CD45RO⁺ T cells, this suggests that the majority of infiltrating T cells have a cytotoxic memory pheno-
Table 2. Median Values and Ranges for Seven Tumor-Infiltrating Leukocyte Populations/mm² of Total Tumor Cell Type

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3⁺</td>
<td>2200</td>
<td>50-40,300</td>
</tr>
<tr>
<td>CD8⁺</td>
<td>2800</td>
<td>0-18,200</td>
</tr>
<tr>
<td>CD45RO⁺</td>
<td>2900</td>
<td>200-43,200</td>
</tr>
<tr>
<td>CD68⁺</td>
<td>3700</td>
<td>600-15,200</td>
</tr>
<tr>
<td>NK cells</td>
<td>100</td>
<td>0-1,000</td>
</tr>
<tr>
<td>B cells</td>
<td>0</td>
<td>0-2,900</td>
</tr>
<tr>
<td>Mast cells</td>
<td>200</td>
<td>0-1,600</td>
</tr>
</tbody>
</table>

notype. There was no correlation between these T cell markers and the CD68⁺ macrophage population. Staining for CD4⁺ T cells was undertaken in frozen sections to determine their contribution to the total T cell infiltrate. However, there was cross-reactivity with other mononuclear cells, and as it was not always possible to discriminate accurately between the different cell types in these sections, CD4⁺ cells were not included in the final assessment.

Chemokine Expression in Epithelial Cancer

The number of cells expressing each of the C-C chemokines MCP-1, MIP-1α, MIP-1β, and RANTES was determined in 10 high-power fields per cryostat section (40× objective and 10× eyepiece). Final cell counts were expressed per mm². Because the in situ hybridization studies were performed on cryostat sections counterstained with toluidine blue, areas of necrosis could not be reliably distinguished from artifact. Therefore, cell counts were assessed only for tumor or stromal areas. The total number of cells expressing MCP-1 or MIP-1α was significantly greater than the number expressing MIP-1β or RANTES (P < 0.005; Figure 3a). MCP-1 was expressed by more cells within tumor islands than any other chemokine (P < 0.0005; median value, 72.5 cells/mm²; Figure 3b), but there was no significant difference between the number of MCP-1-expressing cells in tumor and stroma (P < 0.03). No difference was found in the number of MCP-1-expressing cells with grade. Within the stroma, the number of cells expressing MCP-1 and MIP-1α were similar (median values, 21 cells/mm² and 26 cells/mm², respectively). MIP-1α was expressed by more stromal cells than MIP-1β or RANTES (P < 0.0005; Figure 3c). MCP-1-expressing cells within the tumor islands tended to occur in discrete clusters (Figure 4a), whereas MCP-1- and MIP-1α-expressing cells in stroma occurred alone (Figure 4, b and c). The mean percentage of tumor cells expressing MCP-1 within a cluster was 8.2% (SD = 5.6%). In the stroma, the median number of cells expressing MIP-1β (Figure 4d) and RANTES were 2 cells/mm² and 7 cells/mm², respectively (Figure 3c). Although silver grains indicating MCP-1 expression were frequently associated with typical tumor cell nuclei, MIP-1α, MIP-1β, and RANTES were generally associated with smaller and more intensely stained nuclei, suggesting that they might be derived from the infiltrating leukocyte population.
Relationship between Chemokine Expression and Infiltrating Cells

CD68+ macrophage and CD8+ T cell counts were also assessed in cryostat sections to explore possible relationships between them and the number of chemokine-expressing cells. The antibody used to detect macrophages in frozen sections (EBM 111) was not as specific as that for paraffin-embedded material (PG-M1); therefore, only cells with positive cytoplasmic staining and characteristic nuclear morphology were included in the cell counts. No significant difference was found between the total number of macrophages or CD8+ cells per mm² in the frozen and paraffin sections (data not shown). A possible correlation was found between the number of cells expressing MCP-1 and the number of CD68+ cells ($r_s = 0.50; P = 0.026$). A significant correlation was found between the CD8+ population and MCP-1 expression ($r_s = 0.63; P < 0.005$; Figure 5a) and the CD8+ population and RANTES expression ($r_s = 0.6; P < 0.005$; Figure 5b). There were no significant correlations between the number of CD68+ or CD8+ cells and expression of the other chemokines.

Discussion

This is the first time that the nature and distribution of the leukocyte population in human epithelial ovarian cancer have been assessed quantitatively and com-
pared with the expression of C-C chemokines. Furthermore, it is the first demonstration that the chemokines MIP-1α, MIP-1β, and RANTES are expressed in these tumors. We have shown that the host infiltrate consists largely of macrophages and T cells. The high degree of correlation between cells positive for the markers CD3, CD8, and CD45RO implies that the majority of infiltrating T cells have a cytotoxic memory phenotype. Other leukocytes such as B cells, NK cells, and mast cells were present in low numbers, whereas eosinophils were seen only occasionally and neutrophils appeared to be largely confined to blood vessels. More cells were found in the stroma, but both T cells, particularly CD8⁺ cells, and macrophages were also found in close proximity to tumor cells. Whereas infiltrating cells in the stroma often occurred in aggregates, they were found alone much more frequently when associated with tumor cells. Only CD68⁺ macrophages were found in high numbers in areas of necrosis, and here they appeared to have an activated morphology.

Three previous studies have specifically addressed the nature of the leukocyte infiltrate in ovarian cancer, but none of these has looked quantitatively at cell distribution. Haskell et al. used sedimentation-velocity to separate out the components of the inflammatory infiltrate in 38 epithelial ovarian tumors. They concluded that T cells and macrophages were the dominant components with a lesser contribution from B cells and NK cells. Kabawat et al. analyzed cryostat sections from a series of 70 tumors with a panel of monoclonal antibodies and concluded that very few macrophages were present but that the majority of the infiltrate consisted of CD4⁺ T cells. Again, they found few B cells and NK cells. In the most recent study, again on cryostat sections, van Ravenswaay Claasen et al. concluded that the majority of infiltrating cells were macrophages and T cells expressing the αβ T-cell receptor but that the CD4⁺/CD8⁺ ratio was difficult to determine because of the reactivity of anti-CD4 with macrophages. We were also unable to assess the number of CD4⁺ cells in frozen sections due to cross-reactivity of the anti-CD4 antibody with other mononuclear cells.
Our observations on epithelial ovarian carcinoma are supported by work in other carcinomas. Studies on both paraffin-embedded and frozen specimens of breast cancers support the contention that macrophages and T cells are the dominant infiltrating cells and that most of the T cells are CD8+. An assessment of the infiltrating cell population in seven cases of dysgerminoma of the ovary again found that tumor-infiltrating lymphocytes were predominantly CD8+ and most of the intratumor T cells expressed the αβ T-cell receptor. Even before the introduction of monoclonal antibodies, studies by Svennevig et al. showed that T cells and macrophages predominate in a variety of carcinomas. They also found macrophages in large numbers in areas of intratumoral necrosis. Work done on carcinoma of the thyroid revealed the presence of macrophages and CD45RO+ T cells and in carcinoma of the colon the presence of T cells again carrying the αβ receptor.

The role of the leukocyte infiltrate in carcinomas remains unclear. When activated, both tumor-associated macrophages and tumor-infiltrating lymphocytes are capable of destroying tumor cells. However, using a reverse transcriptase polymerase chain reaction screen, we have shown that macrophage- and lymphocyte-activating cytokines such as interferon-γ and interleukin-2 are generally not expressed by ovarian tumors. The apparent lack of CD4+ cells would also imply that the infiltrate is immunologically inert. It is still not known whether macrophages associated with areas of microscopic necrosis are part of a host response to the tumor, causing local destruction, or whether they are simply there to remove necrotic debris from some other cause, such as the death of a blood vessel. The ratio between macrophages and tumor cells appears to be important; tumor-associated macrophages isolated from chemically induced tumors in mice promote tumor cell proliferation in vitro at ratios of <2:1 but exhibited nonspecific cytolytic activity at ratios of >20:1 without any specific activation.

Although the role of the infiltrate is not understood, chemokines may account for its presence. In this paper, we have demonstrated a direct association between the number of chemokine-expressing cells and the leukocyte infiltrate. MCP-1 is a potent monocyte chemoattractant and has greater in vitro activity on these cells than other MCPs, MIP-1α, MIP-1β, or RANTES. It is also active on T cells. Some work suggests that MIP-1α is specific for CD8+ T cells and MIP-1β for CD4+ cells, although other reports indicate that this relationship may not be absolute. CD45RO+ cells seem to possess greater migratory activity than CD45RA+ cells in response to chemokines and RANTES may be most active on CD4+, CD45RO+ T cells in vitro. Using in situ hybridization, we have shown that of the four C-C chemokines studied, the most frequently expressed were MCP-1 and MIP-1α. MCP-1 was more highly expressed by tumor cell islands than other chemokines. Although there was no significant statistical difference between the number of MCP-1-expressing cells in tumor and stroma after the Bonferroni correction was applied, MCP-1 expression by tumor cells is supported by our previous in situ observations and the fact that tumor cells isolated from ascites produced significantly more MCP-1 protein than macrophages. In the stroma, MIP-1α-expressing cells were present in equivalent numbers to MCP-1-expressing cells.
those expressing MCP-1 and were more abundant than either MIP-1α or RANTES. The source of MIP-1α, MIP-1β, and RANTES was not clear, but these chemokines can all be derived from lymphocytes, and in particular, MIP-1α can be strongly expressed by CD8+ T cells.30 This is supported by the observation that the in situ signal for these chemokines was usually associated with nuclei that were smaller and more intensely staining than typical tumor cell nuclei within the tumors. Furthermore, whereas reverse transcriptase polymerase chain reaction revealed the presence of mRNA for all of these chemokines in tumor specimens, they were expressed by few ovarian tumor cell lines.29

To assess the relationship between the number of infiltrating cells and the number of chemokine-expressing cells, the total number of CD68+ macrophages and CD8+ T cells counted in frozen sections was correlated with the number of cells expressing each of the four chemokines. No correlation was found between MIP-1α and either of these two cell types, but the total number CD8+ T cells correlated significantly with the number of cells expressing MCP-1 and RANTES. In vitro, MCP-1 has been shown to be chemotactrant for T cells and RANTES for CD45RO+ T cells. Although these chemokines are also active on CD4+ cells in vitro, the context in which they act in vivo may be very different. Furthermore, RANTES was expressed by a relatively low number of cells. Although the correlation between MCP-1 and the CD68+ population was not statistically significant (r = 0.5; P = 0.026), the Bonferroni method used in this study is highly conservative and a weak correlation may exist. Other factors, particularly M-CSF, which is both a proliferative and chemotactrant factor for monocytes, may contribute to the macrophage population. Resident tissue macrophages can proliferate in response to M-CSF,28 and double staining in our sections for CD68 and the proliferation marker MIB 1 revealed some cells positive for both (data not shown). In both adenocarcinoma of the breast39 and ovary,40 M-CSF is expressed by epithelial tumor cells, and its receptor, c-fms, is found on the surface of infiltrating macrophages. A positive correlation was also found between the number of CD68+ macrophages and the number of M-CSF+ tumor cells in adenocarcinoma of the breast.39

We have found that macrophages and CD8+, CD45RO+ T cells form the bulk of the infiltrating cell population, within both the tumor and stromal compartments. There are significantly more cells within the stroma than the tumor compartment, but only macrophages are found in high numbers within areas of necrosis. Of the four chemokines analyzed, MCP-1 and MIP-1α predominate. MCP-1 is expressed by significantly more tumor cells than any other chemokine, whereas MIP-1α is expressed by more stromal than tumor cells. MCP-1 may promote both the lymphocyte and macrophage infiltrate and MIP-1α may be expressed by CD8+ T cells. We shall go on to examine the factors that may be responsible for promoting chemokine expression within the ovarian tumors, particularly the relationship between tumor cells and individual leukocyte subtypes. As tumor necrosis factor-α is known to be expressed by both tumor cells and tumor-associated macrophages in this disease49 and can stimulate MCP-1 expression in a variety of cell types, this cytokine provides one possible link between the infiltrating cell population and the pattern of expression of chemokines observed within these tumors.

Acknowledgments

We thank Mr. P. Mason, Miss S. Raju, the Queen's Hospital, Belfast, and the Hammersmith Hospital for access to fresh and archival tumor specimens, Professor Alberto Mantovani (Mario Negri Institute, Milan, Italy) for the chemokine cDNAs, and George Elia and the ICRF Histopathology Unit for the preparation of both paraffin-embedded and cryostat sections.

References

infiltrates from tumour and ascites material. Br J Cancer 1982, 45:728–736
20. DeHoff RT, Rhines FN: Determination of the number of particles per unit volume from measurements made on random plane sections: the general cylinder and ellipsoid. Trans Am Inst Mining Metallurg Eng 1961, 221:975
1α, interleukin-8, and RANTES. Eur J Immunol 1995, 25:751-756


