



Requirement of JNK1 for endothelial cell injury in atherogenesis

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ABSTRACT

Objective: The c-Jun N-terminal kinase (JNK) family regulates fundamental physiological processes including apoptosis and metabolism. Although JNK2 is known to promote foam cell formation during atherosclerosis, the potential role of JNK1 is uncertain. We examined the potential influence of JNK1 and its negative regulator, MAP kinase phosphatase-1 (MKP-1), on endothelial cell (EC) injury and early lesion formation using hypercholesterolemic LDLR^{-/-} mice.

Methods and results: To assess the function of JNK1 in early atherogenesis, we measured EC apoptosis and lesion formation in LDLR^{-/-} or LDLR^{-/-}/JNK1^{-/-} mice exposed to a high fat diet for 6 weeks. En face staining using antibodies that recognise active, cleaved caspase-3 (apoptosis) or using Sudan IV (lipid deposition) revealed that genetic deletion of JNK1 reduced EC apoptosis and lesion formation in hypercholesterolemic mice. By contrast, although EC apoptosis was enhanced in LDLR^{-/-}/MKP-1^{-/-} mice compared to LDLR^{-/-} mice, lesion formation was unaltered.

Conclusion: We conclude that JNK1 is required for EC apoptosis and lipid deposition during early atherogenesis. Thus pharmacological inhibitors of JNK may reduce atherosclerosis by preventing EC injury as well as by influencing foam cell formation.

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1. Introduction

The JNK family comprises three genes. JNK1 and JNK2 are both ubiquitously expressed, whereas JNK3 expression is limited to the brain [1]. Despite overlapping expression patterns, JNK1 and JNK2 target distinct intracellular proteins for phosphorylation and have specific functions [2]. JNK family members play important roles in cardiovascular injury and disease. Animal studies have revealed that JNK can be activated in arteries in response to injury [3–5] and during the development of aneurysms [6] or lesions [7,8]. Moreover, gene transfer of a dominant negative form of JNK reduced neointimal formation in injured arteries [3]. A previous study demonstrated that JNK2 promotes atherosclerosis by enhancing lipid uptake by macrophages and subsequent foam cell formation [9].

We recently demonstrated that JNK1 induces the expression of pro-apoptotic genes at a disease-prone region of the arterial tree [10]. By contrast, EC at atheroprotected sites expressed MAP kinase phosphatase-1 (MKP-1), a nuclear-localised dual specificity

phosphatase that inactivates JNK (and its sister molecule p38) by dephosphorylating threonine and tyrosine residues in the activation loop [10–12]. Despite these insights, the potential role of JNK1 in atherosclerosis remains uncertain. Here we tested the novel hypothesis that a balance between MKP-1 and JNK1 activities regulates EC apoptosis and lesion formation in early atherogenesis.

2. Materials and methods

2.1. Crossing and maintenance of animals

LDLR^{-/-} mice on a C57BL/6 genetic background were obtained commercially from Jackson laboratories and then bred in-house [13]. JNK1^{-/-} mice were generated by Professor Flavell [14] and obtained from Professor Roger Davis from the University of Massachusetts, USA. JNK1-deficient mice were crossed with LDLR^{-/-} mice to produce the LDLR^{-/-}/JNK1^{-/-} strain (Supplemental Fig. 1). MKP-1^{-/-} mice (C57BL/6 background) were obtained from Bristol-Myers Squibb (via Professor Andy Clark, University of Birmingham, UK) and crossed in-house with LDLR^{-/-} to produce the LDLR^{-/-}/MKP-1^{-/-} strain (Supplemental Fig. 2). The JNK1^{-/-}/LDLR^{-/-} and

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MKP-1^{-/-}/LDLR^{-/-} mice appeared phenotypically normal although litter sizes were reduced in the former compared to LDLR^{-/-} mice (data not shown). All mice used in this study were female. Mice were housed under specific-pathogen free conditions and studied according to UK Home Office regulations.

2.2. Composition of Western-type diet

Where indicated, normal chow diet was replaced at 10 weeks of age with Diet W; a cholate-free high fat Western-type diet (Arie-blok Diet W, 4021.06; Woerden, Netherlands) for 2–12 weeks. The Diet W (High fat diet) consisted of (w/w) cocoa butter (15%), cholesterol (0.25%), sucrose (40.5%), cornstarch (10%), corn oil (1%), cellulose (5.95%), casein (20%), 50% choline chloride (2%), methionine (0.2%) and mineral mixture (5.1%), total fat content (16%).

2.3. Lipoprotein, cholesterol and triglyceride analysis

Analysis for lipoprotein profiles and serum total cholesterol and triglycerides was performed by fast protein liquid chromatography using a size-exclusion column (Superose 6, PC 3.2/30, GE Healthcare, UK) and Infinity™ Cholesterol (TR13421) or Triglycerides (TR22421) Liquid Stable Reagents (Thermo Electron Corporation, USA) as described [13].

2.4. Lesion analysis

En face lipid staining was carried out using Sudan IV as described [15]. Briefly, after termination by CO₂ overdose, perfusion fixation was carried out using 2% formalin (neutral buffer pH 7.0) at 37 °C at a pressure of 110 cm water for 5 min, via a cannula (Venofix 23G; Medisave, UK) inserted in the left ventricle. Each aorta was then stained using 5 ml Sudan IV (S4261-25; Sigma Aldrich, UK) which was introduced into the left ventricle over a 5 min period. The aorta was isolated and adventitial fat was removed. After cutting along the outer curvature, the aorta was mounted and photographed (Olympus SZX16). En face plaque quantification was performed using ImagePro Plus software (version 5.1, Media Cybernetics, Silver Spring, USA) by a single operator blinded to the group allocation, and expressed as percentage of total aortic area (lesion area fraction).

Cryosections of the aortic root were stained with Oil Red O and Mayer's haematoxylin and lesions were analysed as we described [13]. Briefly, sectioning of the heart at the level of the aortic root was carried out following the method of Paigen and colleagues [16]. Five cryosections at 100 µm intervals were taken for each individual heart, rinsed in water and then immersed in isopropanol for 30 s before staining with Oil Red O (00625-25G; Sigma–Aldrich) for 15 min followed by counterstaining using Harris' haematoxylin (351945S; Sigma–Aldrich). The sections were mounted and visualized using an Olympus BX50 light microscope in identical light conditions. The lesion area was quantified using ImagePro Plus software by an experienced researcher who was blinded to the experimental design. Mean lesion area values were obtained by averaging data from 5 aortic root sections. To correct for variation in the alignment of sections, mean lesion area values were divided by the mean area of the aortic wall and expressed as a percentage.

2.5. Immunostaining of aortic root cross sections

Cryosections of the aortic root were stained with antibodies that recognise JNK1 (Cell Signalling Technology), CD68 (AbD Serotec; macrophage marker), smooth muscle actin (Dako). After stringent washing, sections were incubated with appropriate secondary antibodies conjugated to biotin (Vector Laboratories) and with Avidin

and Biotinylated horseradish peroxidase macromolecular Complex prior to the application of VECTASTAIN substrate (Vector Laboratories). Alternatively, sections were stained using Gomori's trichrome (Sigma) following the manufacturer's recommendations. Sections were mounted and visualized by bright field microscopy (Nikon).

2.5.1. En face staining

Activation of caspase-3 in EC was measured by en face staining of the murine aortic arch following [17]. Antibodies that recognise active, cleaved caspase-3 were used (9661S; Cell Signalling Technology) [10]. Briefly, aortae were perfusion fixed and isolated prior to permeabilisation using 0.5% Triton X-100 (Sigma–Aldrich), and blocking with 20% goat serum overnight at room temperature. After washing with PBS, the tissue was incubated with anti-active caspase-3 rabbit IgG antibodies overnight at 4 °C. The tissue was then washed with PBS and incubated with goat anti-rabbit IgG antibodies conjugated to Alexa fluor-568 (A11036; Invitrogen) for 2–3 h at room temperature followed by incubation with anti-CD31 antibodies (directly conjugated to Alexa fluor 488) for 72 h at 4 °C. After washing with PBS, the tissue was incubated with TOPRO-3 to counterstain nuclei. To control for specific binding, tissues were incubated with irrelevant rabbit IgG antibodies and appropriate fluorescent secondary antibodies, or were incubated with secondary antibodies alone. The ascending aorta and arch were mounted and images of the EC monolayer were obtained using an inverted laser-scanning confocal microscopy (LSM 510 Meta inverted; Zeiss, Oberkochen, Germany). Protected (outer curvature) and susceptible (inner curvature) regions of the aortic arch were located using anatomical landmarks described by Iiyama and colleagues [17]. The proportion of positive cells at each site was quantified by analysis of multiple fields of view from atherosusceptible or atheroprotected sites, and expressed as percentage positivity.

En face TUNEL staining was carried out using an In situ Cell Death Detection Kit (11684817910; Roche, Germany) as described [10]. Aortae were fixed and permeabilised before incubation with TUNEL reaction mixture at 37 °C for 1 h. After washing with PBS, tissues were incubated with anti-FITC-biotin antibodies for 3 h at room temperature and then with Streptavidin Alexa Fluor 488 for 2 h at room temperature. EC were co-stained using Griffonia lectin conjugated to Rhodamine (Vector labs, USA) and nuclei were counter-stained using TOPRO-3. Staining was assessed by laser-scanning confocal microscopy (as above). EC were considered apoptotic when TUNEL staining co-localized with pyknotic or fragmented nuclei. To control for specific binding, tissues were incubated with TUNEL reaction mixture that lacked labelled nucleotides. The technique was validated by demonstrating that DNase treatment of portions of the murine aorta generated enhanced TUNEL signals in EC (Supplemental Fig. 3).

2.5.2. Statistics

Differences between samples were analysed using an unpaired or paired Student's *t*-test or one-way ANOVA with Bonferroni's adjustment.

3. Results

En face staining revealed that exposure of LDLR^{-/-} mice to high fat feeding for 2 or 6 weeks enhanced EC apoptosis at an atherosusceptible site but not at a protected site, indicating that EC apoptosis co-localises with early atherogenesis, suggesting a contributing role (Supplemental Fig. 4). We examined the potential role of JNK1 in EC injury and early lesion formation by studying LDLR^{-/-} and LDLR^{-/-}/JNK1^{-/-} mice exposed to a high fat diet for 6

weeks. Genetic deletion of JNK1 protected EC at the susceptible site from caspase-3 activation and DNA fragmentation (Fig. 1A and B, compare 1 and 5) and reduced lesion area in the aorta (Fig. 2A) and aortic root (Fig. 2B), indicating that JNK1 contributes to EC apoptosis and atherosclerosis. Analysis of macrophage, smooth muscle cell and collagen content of aortic root lesions suggested that deletion of JNK1 had little or no effect on plaque composition (Supplementary Figs. 5, 6 and 7). It is unlikely that JNK1 promotes atherogenesis by altering lipid metabolism since deletion of JNK1 did not influence cholesterol levels and had a modest effect on plasma triglyceride levels (Fig. 2C and Supplemental Fig. 8).

We tested the hypothesis that MKP-1 reduces apoptosis and lesion formation at atheroprotected sites by comparing EC injury and lesion formation in $LDLR^{-/-}/MKP-1^{-/-}$ compared to $LDLR^{-/-}$ mice. Deletion of MKP-1 enhanced EC apoptosis at an atheroprotected site (Fig. 1A and B, compare 2 and 4), but this intervention did not modify lesion formation (Fig. 2A and B), composition (Supplementary Figs. 5, 6 and 7), or plasma lipid profiles (Fig. 2C and Supplemental Fig. 3) in mice exposed to a high fat diet for 6 weeks. We also determined the potential influence of MKP-1 deletion on a later stage of atherosclerosis but observed that lesion sizes in the aorta were similar in $LDLR^{-/-}/MKP-1^{-/-}$ and $LDLR^{-/-}$ mice exposed to a

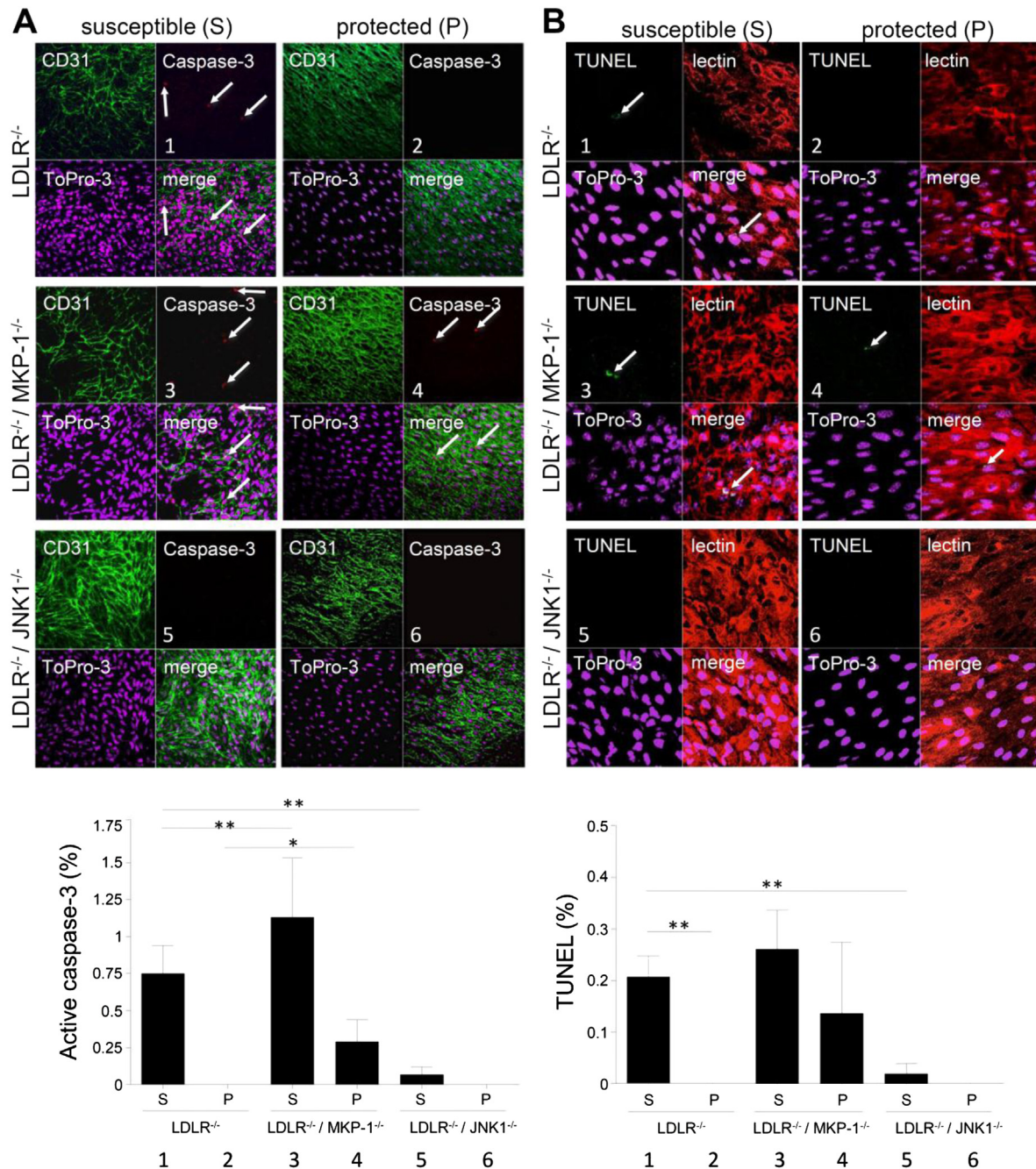


Fig. 1. JNK1 regulates endothelial injury in hypercholesterolemia. $LDLR^{-/-}$, $LDLR^{-/-}/JNK1^{-/-}$ or $LDLR^{-/-}/MKP-1^{-/-}$ mice were exposed to a high fat diet for 6 weeks. (A) Caspase-3 activation was measured in EC by en face staining of susceptible (S) or protected (P) regions of the aorta (red; $n = 6$ per group). Endothelial marker is CD31 (green) and nuclei were stained using ToPro-3 (purple). (B) En face TUNEL staining was carried out at susceptible (S) or protected (P) regions of the aorta (green; $n = 6$ per group). Endothelial marker is lectin (red). Representative images (positive cells indicated by arrows) and quantitation of active caspase-3 and TUNEL-positive cells (mean $\pm$ SD) are shown. * $p < 0.05$, ** $p < 0.01$.

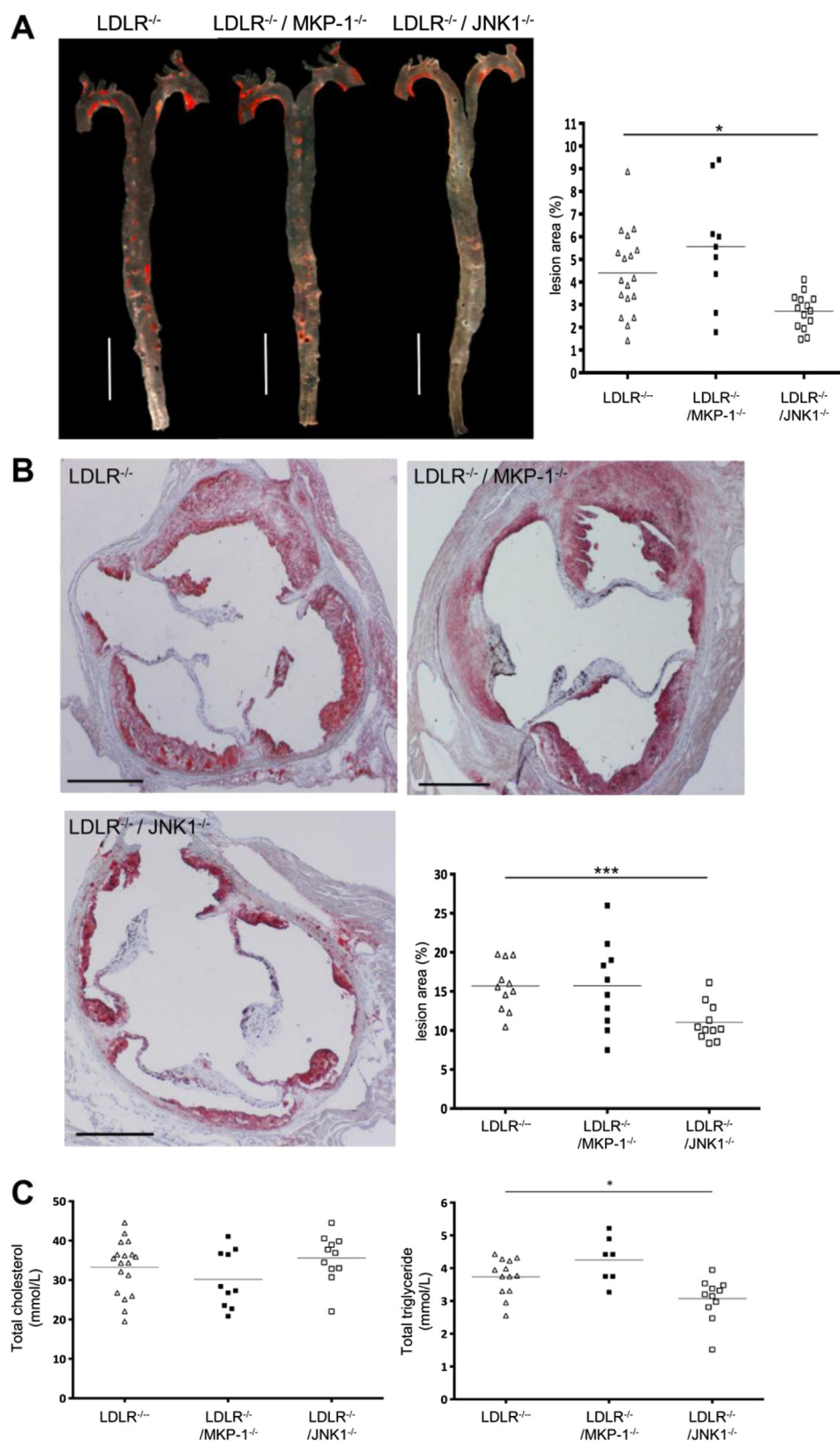


Fig. 2. JNK1 promotes lesion formation. LDLR^{-/-}, LDLR^{-/-}/JNK1^{-/-} or LDLR^{-/-}/MKP-1^{-/-} mice were exposed to a high fat diet for 6 weeks. Aortic en face lesions (A) and aortic root lesions (B) were quantified and expressed as a proportion of the entire aortic/aortic root area. (C) Total plasma cholesterol (left) or triglyceride (right) levels were measured and mean values $\pm$ SD are shown. * $p < 0.05$, *** $p < 0.001$. It was observed that lesion area in the aorta (A) or aortic root (B) was significantly higher in LDLR^{-/-} compared to LDLR^{-/-}/JNK1^{-/-} mice.

high fat diet for 12 weeks (data not shown). Thus although MKP-1 reduced EC apoptosis during atherogenesis it did not protect against early lesion formation.

4. Discussion

The JNK family of MAP kinases has been implicated in numerous and diverse cellular activities and is known to be an important regulator of vascular physiology [3–10]. However, the roles of specific JNK isoforms have remained uncertain. Broad-spectrum pharmacological inhibitors that target all three JNK isoform have been shown to reduce atherosclerosis in mice [9]. On the other hand, these inhibitors are likely to have deleterious side-effects given the critical role of JNKs in physiological homeostasis. In this study, we demonstrate that genetic deletion the JNK1 gene can reduce EC apoptosis and atherosclerosis in a murine model. Our findings complement a previous study that demonstrated a role for JNK2 in foam cell formation in hypercholesterolemic mice [9]. Taken together, these observations suggest that targeting of specific JNK isoforms, JNK1 or JNK2, may have clinical utility for the prevention of atherosclerosis whilst avoiding the potential adverse effects of inhibiting all three JNKs.

The mechanism linking JNK1 activation with lesion formation is likely to involve EC apoptosis which was reduced at atheroprone sites by deletion of JNK1. The induction of EC apoptosis in LDLR^{-/-} animals required exposure to a high fat diet and occurred predominantly at a site of disturbed blood flow, suggesting an interaction between hypercholesterolaemia, flow and JNK1-dependent EC injury. Although the levels of EC apoptosis detected were relatively low (0.2–1%), it is plausible that they influence atherosclerosis because low rates of apoptotic EC have previously been shown to promote vascular disease [18,19] and have a major effect on the permeability of the endothelial monolayer [20].

We demonstrated previously that JNK1 is activated in EC in response to disturbed flow in the murine aorta [12]. Moreover, microarray analysis of cultured EC treated with a pharmacological JNK inhibitor and subsequent siRNA-based analyses demonstrated that JNK-c-Jun signalling primes EC apoptosis by inducing proapoptotic transcripts [10]. Thus we propose that disturbed flow primes EC for apoptosis in response to hypercholesterolaemia by promoting JNK1-dependent induction of apoptotic molecules. It is important to note that other mechanisms may contribute to the proatherogenic effects of JNK1 including effects on macrophages and smooth muscle cell physiology [21–23]. Thus future studies should be carried out using conditional knockouts to dissect out the functions of JNK1 in EC and other cell types.

Our demonstration that MKP-1 deletion enhanced EC apoptosis may be related to the ability of this molecule to suppress JNK1 activity at sites exposed to uniform flow [12]. However, the increased rates of apoptosis in response to MKP-1 deletion were not sufficient to increase lesion formation which was similar in MKP-1^{-/-}/LDLR^{-/-} and LDLR^{-/-} mice. These observations contrast with previous reports where genetic deletion or pharmacological inhibition of MKP-1 suppressed macrophage accumulation in arteries and reduced lesion formation in ApoE^{-/-} mice [24–26]. These apparent discrepancies may reflect differences in the murine models used i.e. LDLR^{-/-} (current study) and ApoE^{-/-} mice [24,25]. Compared to the ApoE^{-/-} model, LDLR^{-/-} mice develop relatively mild hypercholesterolaemia and early lesions contain fewer macrophages [15]. Thus the effects of MKP-1 on macrophage infiltration may dominate in ApoE^{-/-} mice but not in the LDLR^{-/-} model. In addition, the divergence of EC apoptosis v plaque size in LDLR^{-/-}/MKP-1^{-/-} and LDLR^{-/-}/JNK1^{-/-} mice may indicate that other shear regulated dual specificity phosphatases are important in JNK1 regulation, for example dual specificity phosphatases 5, 8, and

16 [27]. Regardless of the explanation, these findings suggest that the influence of MKP-1 on atherogenesis may vary according to plasma cholesterol levels and inflammatory load. Overall, our study suggests that targeting of JNK may have clinical utility for the prevention or treatment of early atherogenesis.

Conflicts of interest

The authors report no relationships that could be construed as a conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.atherosclerosis.2014.05.950>.

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