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Glucocorticoid Receptor Nuclear Translocation in CD4 T Cells from Severe and Moderate Asthmatic Patients Treated with Fluticasone/Vilanterol

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ABSTRACT

Different phenotypes of asthma from mild to severe are categorized based on diverse clinical features. A guideline for the recognition and treatment of asthma has been provided by Global Initiative for Asthma (GINA). To control symptoms and prevent asthma exacerbation in most patients combinational therapy with inhaled corticosteroids (ICS) and a long acting B2-adrenreceptor agonist (LABA) are recommended. Understanding asthma phenotypes would be helpful to improve asthma diagnosis and treatment. The aim of this study was to verify glucocorticoid receptor glcococorticoid receptor (GR) nuclear translocation in CD4 T cells treated with fluticasone furoate (FF), vilanterol (V) and FF/V combination in severe asthmatic patients compare to patients with moderate asthma and healthy controls using Immunocytochemistry (ICC).

After taking blood and separating PBMCs from each subject, CD4 T cells were isolated from PBMCs using CD4+ T cell isolation kit. Isolated CD4 T cells were cultured in presence of FF, V and FF/V combination for 1 hour and after cytocentrifugation, cells were incubated with anti GR-antibody and subsequently stained with FITC bound secondary antibody and GR nuclear translocation was observed under microscope.

The results showed significant increasing in GR nuclear translocation in treated CD4 T cells from patients with moderate asthma and controls compare to those severe asthmatic patients, along with treating cells with FF/V combination no significant GR nuclear translocation was observed compare to that of using mono treatment of cells with FF and V.

Based on our findings, it can be concluded different mechanisms are responsible for severe asthma and moderate asthma.

Keywords: Corticosteroid; Glucocorticoid receptor; Long-acting b2 agonist; Severe asthma

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INTRODUCTION

Asthma syndrome comprises distinct variable disorders and airway inflammation including wheezing, coughing, chest tightness, dyspnea and airway hyper responsiveness (AHR).[1] Different phenotypes of asthma from mild to severe are categorized based on diverse clinical features. Guideline for the recognition and treatment of asthma has been provided by Global Initiative for Asthma (GINA).[1] To control symptoms and prevent asthma exacerbation in most patients combinational therapy with inhaled corticosteroids (ICS) and a long acting β2-adrenreceptor agonist (LABA) are indicated.[2] In 2015, FDA approved inhaled fluticasone furoate/vilanterol (FF/V) once daily as a treatment strategy for 18 years and older asthmatic patients.[1] Fluticasone furoate is a novel synthetic glucocorticoid with high affinity binding to glucocorticoid receptors (GR).[3] Upon binding of the ligand, GRs translocate from cytoplasm to the nucleus and binds to the glucocorticoid response elements (GRE) in the promoter region of steroid sensitive genes. This results in switching on β2-adrenergic receptors (2-ARs) and anti inflammatory genes and suppression of cytokines, chemokines, enzymes and receptors which are associated with inflammatory responses.[4] β2-adrenegic receptors are expressed on many cells including lymphocytes, mast cells, macrophages, epithelial and endothelial cells.[5]Vilanterol is one of the potent, selectiveβ2-agonist with a rapid and prolonged bronchodilation (over 24 hours) in patients with asthma and COPD (5). The benefit of combinational therapy of FF/V therapy was assessed in patients with moderate to severe asthma.[6-9] The symptoms of most asthmatic patients controlled by ICS and maximum dose of ICS/LABA is required to control symptoms of about 10% of asthmatic patients and there is a small proportion of patients who are completely corticosteroid resistance and no clinical improvement was defined even after high dose of oral corticosteroid.[4] The airway inflammation of these steroid insensitive patients is comparable to those with COPD.[4] This may indicate that there are some common mechanisms underlying in these diseases.[4] Understanding asthma phenotypes and its diverse clinical features, molecular, cellular, morphological and functional characteristics of each individual patient would be instructive to improve asthma diagnosis and treatment.[10] Thus, the aim of this study was to verify GR translocation in CD4 T cells treated with FF, V and in FF/V combination in severe asthmatic patients compare to patients with moderate asthma and healthy controls using Immunocytochemistry (ICC).

MATERIALS AND METHODS

Reagents

Fluticasone furoate (FF) and vilanterol (V) were provided by Glaxo SmithKline (Stevenage, UK). FF was dissolved in DMSO at 10 mM concentration and V dissolved in PBS at 10 mM concentration, proper dilutions were used in the assays ensuring that the DMSO concentration was less than 0.5% in the assays.

Subjects

Five healthy nonsmoking controls, 4 moderate and 4 severe asthmatic patients (these severe asthmatic patients were uncontrolled cases) who defined by GINA guidelines (http://www.ginasthma.com) were entered the study also the asthma severity of the patients were based on ERS/ATS criteria.[10] After taking written informed consent, peripheral blood mononuclear cells (PBMCs) of the subjects were isolated using ficoll-paque gradients (Ceaderlane, Canada). Subsequently, CD4 T cells were separated from PBMCs by magnetic cell separation using CD4+ T cell isolation kit (Miltenyi Biotec, Germany) according to the manufacturer, the purity of the cells was confirmed by flowcytometry.

Cell Culture

CD4+ T cells were cultured in plate-bound Anti-CD3 antibody (eBioscience, USA). 96 well plates containing RPMI-1640 media (Gibco, USA) and 0.1% FBS (Gibco, USA) and Anti-CD28 antibody (eBioscience, USA) at a density of 200,000 cells/well were used. After 18 hours stimulation, cells were treated with FF (10^{-7} M), V (10^{-7} M) and FF/V (10^{-7} M/10^{-7} M) for 1 hour and subsequently prepared for ICC. The *MTT* assay was performed before conducting the experiment. The used concentration of FF and V was based on the previous studies.[11,12] The rational for

choosing high concentrations of these drugs was based on using high dose treatment of ICS/LABA for severe asthmatic patients.

Immuocytochemistry

Single CD4 T cell suspensions were adhered onto microscopic slides using cytocentrifuge to get a monolayer cells on the slides. Consequently, cells were fixed with formaldehyde solution for 20 minutes at room temperature, fixed cells were permeablized with Triton X-100 3% and after adding blocking reagent, cells were individually incubated with GR (M-20, Santa Cruz Biotechnology) at 4˚C over night. Slides were incubated with secondary antibody sheep anti-rabbit IgG (H&L) FITC conjugated (Agrisera, Sweden) for 40 minutes. After washing cells 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI), a fluorescent blue nuclear indole chromatin stain was used for nuclear staining. Subsequently slides were visualized with fluorescent microscope. The images were analyzed in the image J program.

Statistics Analysis

The results of the experiments are presented as mean±SD. One way ANOVA test was used to compare treatment groups. Graph Pad Prism 5 software (Graph Pad Prism, San Diego, CA, USA) was used to analyze data, results were considered significant if p Value was less than 0.05.

This study was approved by ethics committee and research committee of Immunology, Asthma and Allergy Research Institute (IAARI), Tehran University of Medical Sciences (Numbers 92-01-40-21242 and 412/p/277 respectively).

RESULTS

Subjects

Four patients with moderate asthma (2 females, 2 males) and 4 patients with severe asthma (3 females, 1 male) and five healthy controls (3 females, 2 males) were entered the study. The mean age of healthy controls, patients with moderate asthma and patients with severe asthma were 31.2±3.4, 37.2±14.5 and 47±10.2, respectively. All patients were under medication for their asthma management (Table 1). Except one patient with moderate asthma, all other patients used both ICS/LABA for controlling their asthma.

GR Nuclear Translocation in CD4+ T Cells from Healthy Controls Compare to the Patients with Moderate Asthma (MA) and Severe Asthma (SA)

CD4 T cells and stimulated CD4 T cells with CD3/CD28 antibodies from healthy controls and patients with moderate and severe asthma were subjected to ICC to evaluate the expression of GR in these cells before treatments. All cells in these three groups expressed GR diffusely in the cytoplasm around the nuclei membrane under microscopy while, GR nuclear expression in CD4 T cells of control subjects and patients with moderate asthma before CD3/CD28 stimulation were 6% and 3%, respectively. After stimulating CD4 T cells with CD3/CD28 nuclear translocation of GR was increased to 18% in cells of control patients and 14% in those of MA patients. Very rare nuclear translocation was detected in the cells of severe asthmatic patients before and after stimulation (Figure 1).

Table 1. Medications used by asthmatic patient who entered in immunocytochemistry study of CD4 T cells

Medications	MA1	MA2	MA3	MA4	SA1	SA2	SA3	SA4
inhaled corticosteroid (fluticasone/beclomethasone)	+	+	+	+	+	+	+	+
ICS/LABA	-	+	+	+	+	+	+	+
Inhaled Salbutamol	+	+	+	+	+	+	+	+
Leukotriene modifier (Montelukast)	-	+	+	-	-	-	-	+
Xanthine (Theophylline, Aminophylline)	-	-	+	-	+	+	+	-

ICS: inhaled corticosteroid,
LABA: long acting B2-adrenreceptor agonist
MA: moderate asthma, SA: severe asthma
+: medication was used, -: medication was not used

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Induction of GR Nclear Translocation in CD4+ T Cells Treated with FF, V and FF/V from Healthy Controls Compare to the Patients with MA and SA

Stimulated CD4 T cells from healthy controls and patients with MA and SA were treated with FF, V and FF/V for 1 hour. The results of ICC showed increased translocation of GR in the cells of controls and MA patients after treatments while no GR translocation was detected in case of severe asthma. More GR translocation was observed in cells from patients with MA which treated with FF, V and FF/Vcombination compare to those of controls (Figure 2). Also, these results showed more translocation of GR in treating cells with FF and V(the difference is not significant) compare to treating them with FF/V combination in patients with MA, while no difference of GR nuclear

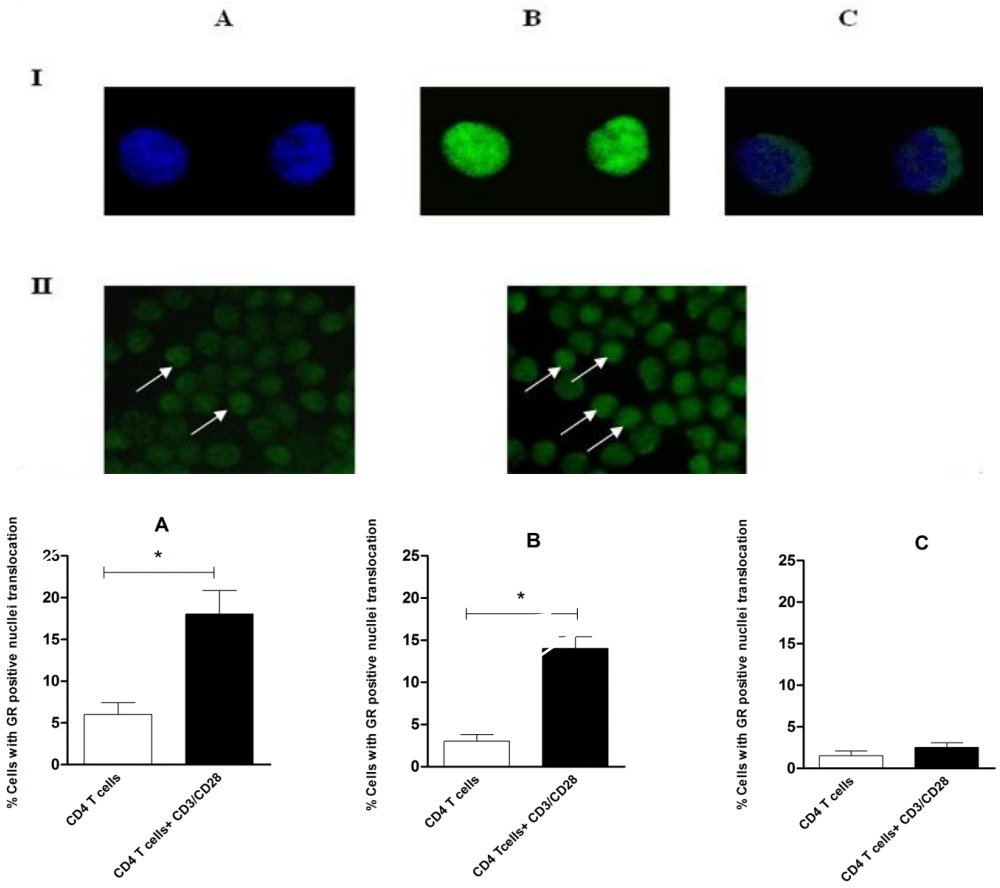
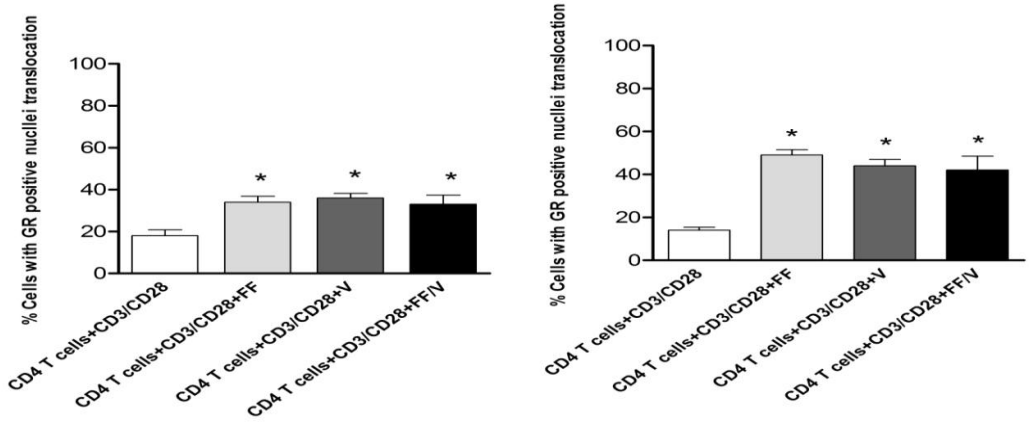


Figure1. I. Immunocytochemistry of glucocorticoid receptor (GR) translocation in CD4 T cells. Nuclear stained CD4 T cells with 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (A), Cells stained with GR-Ab (M-20) and FITC-secondaryAb (B), overlay of A and B shows nuclear translocation of GR in CD4 T cells.

II. GR translocation was increased in CD4 T cells after stimulating with CD3/CD28 antibodies for one hour. In all slides bright cells with high density were considered and counted as positive GR translocation and cells with fade color were not taken into the account. Left picture is unstimulated CD4 T cells and right picture is stimulated CD4 T cells both from a control subject.

III. GR translocation was increased in CD4 T cells after stimulating with CD3/CD28 antibodies for one hour. Quantification of positive GR nuclear translocation in controls (panel A), patients with moderate asthma (panel B), and patients with severe asthma (panel C). The numbers of counted cells are from three independent experiments. 400 cells were counted from four parts of the slides, data are presented as mean + SD. (n=3, *p<0.05).**

I



II

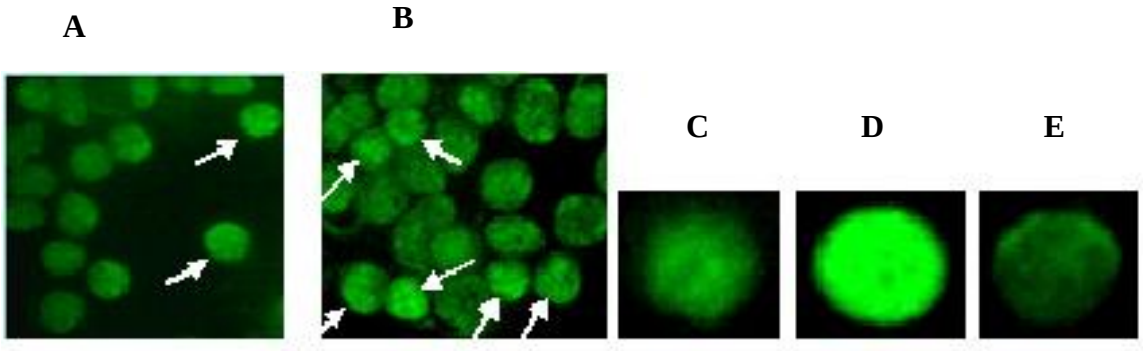


Figure 2. I. Glucocorticoid Receptor (GR) translocation was increased in CD4 T cells after treatment with fluticasone furoate (FF), vilanterol (V) and Fluticasone furoate /vilanterol (FF/V) for one hour in control subjects (left panel) and patients with moderate asthma (right panel). The numbers of counted cells are from three independent experiments. Four hundred cells were counted from four parts of the slides, data are presented as mean (n=3, *p<0.05)**
II. GR nuclear translocation was increased in CD4 T cells after treatment with fluticasone furoate (FF), vilanterol (V) and fluticasone furoate /vilanterol (FF/V) for one hour in patients with moderate asthma; before treatment (A) after treatment (B). The density of GR nuclear translocation was increased remarkably after treatment in control subjects (C), patients with moderate asthma (D) but in patients with severe asthma no nuclear GR translocation was visible (E).

translocation was detected in the cells from controls treated with FF, V and FF/V under microscopy (Figure 2). It should be noted that after treatment, CD4 T cells with positive GR translocation from MA patients were Very bright. In all treated groups compare to the stimulated cells the density of fluorescent was increased remarkably. In all slides bright cells with high density were considered and counted as positive GR translocation and cells with fade color were not taken into the account.

DISCUSSION

This is the first study which was intended to find out GR translocation based on ICC in CD4+ T cells from healthy controls and patients with MA and SA treated with FF, V, and FF/V combination. The results of this study showed that the GR nuclear translocation in CD4+ T cells from healthy controls and patients with MA at the base line was the same without significant difference while almost no GR translocation was detected in CD4+

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T cells of patients with uncontrolled SA. Increasing GR translocation was detected in cells of controls and MA subjects with FF, V, FF/V treatments, while no translocation was observed in cells of patients with SA with the different treatments. Previous studies have shown significant nuclear translocation of GR in PBMCs treated with FF from healthy controls and patients with MA.[3] In asthmatic smooth muscle treated with FF different GR translocation was observed in severe asthmatic patients compare to patients with moderate asthma and healthy controls, although nuclear GR baseline levels were similar in these three groups.[12] In human airway epithelial cells high-efficacy of FF as a GR agonist was reported in driving GRE-dependent transcription.[13] It is suggested that any GR agonist induces transcription in genes dependent manner and FF promote most glucocorticoid inducible anti-inflammatory genes. Epigenetic and other regulatory elements which interferes with 3 D- conformational GR could control gene expression in the presence of different gluccocorticoids.[13] Genetic studies have been indicated that GR polymorphisms and mutations are not associated with GR dysfunction in steroid resistance asthma and COPD and in a genome-wide study and some other studies in asthmatic patients a functional polymorphism was identified in the glucocorticoid induced transcript 1 (GLCCI1) which is suggested to decline pulmonary function of receiving inhaled ICS in asthmatic patients.[4,14-16] Other studies implicated the role of proinflammatory cytokines, transcription factors and epigenetic factors such as histone acetylation in expression and translocation of GR.[4]

The benefit of combinational therapy of FF/V therapy was assessed in patients with moderate to severe asthma in several clinical trials.[6,17-20] Several clinical trials suggested that one daily use of FF/V is comparable with twice daily use of other ICS/LABAs therapy.[6,20-23] Based on the results of two recent systematic reviews, although the combination of FF/V could increase FEV1 versus to FF monotherapy and twice daily use of ICS/LABA but, no significant clinical difference was observed comparing once daily use of FF/V combination to twice daily use of ICS/LABA and FF monotherapy.[24,25] Molecular studies along with research on the outcomes of the combination FF/V therapy would be beneficial for controlling asthmatic patients.

In a study on cells from induced sputum of mild asthmatic patients, increase GR translocation and expression was observed after combinational therapy.[26] The same result was reported in sputum macrophages from COPD patients using combination of fluticasone propionate and salmeterol.[27] LABA has been observed to increase the effectiveness of ICS in inflammatory diseases possibly through up regulation of GRE-dependent anti inflammatory genes.[4] The other probable mechanism is the improvement of GR translocation to the nucleus.[28] It is suggested that formoterol as a LABA could enhance glucocotticoid function by preventing GR phophorylation through protein phosphatase 2A.[29] Although, according to the ICC results of this study combination of FF/V has no significant effect on GR translocation in CD4 T cells from controls and MA patients compare to singly treatment of FF and V. To date there is no reported molecular study to imply the effect of vilanterol on asthmatic patients.

Considering GR transcription level, downstream effects of GR transcription and translocation are the limitation of our study. However, these molecular studies would be informative regarding different responsible mechanisms in MA and SA patients.

Based on the ICC result of this study, in spite of increasing GR translocation in CD4 T cells after treatment with FF, V and FF/V, no significant differences of GR translocation was observed in combination FF/V treatment compare to FF and V treatment in control subjects and patients with moderate asthma and almost no increasing GR translocation was observed in treated cells of uncontrolled severe asthmatic patients. It seems the mechanism responsible for uncontrolled asthma is different from controlled asthma. Further molecular cluster analysis, gene expression and epigenetic studies are needed to find the possible effects of these drugs and also novel therapeutic strategies should be considered for the benefit of uncontrolled SA patients.

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