

Effect of Formoterol on Eosinophil Trans-Basement Membrane Migration Induced by Interleukin-8-Stimulated Neutrophils

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Key Words

Asthma · Eosinophil · Trans-basement membrane migration · Neutrophil · Interleukin-8 · Formoterol

Abstract

Background: Neutrophils are often increased in the airways of either chronic severe asthma or acute exacerbations. Neutrophils that have migrated in response to interleukin-8 (IL-8) may lead eosinophils to accumulate in the airways of patients with asthma and possibly aggravate the disease. In this study, we investigated whether formoterol modified the trans-basement membrane migration (TBM) of eosinophils stimulated with neutrophils and IL-8. **Methods:** Neutrophils and eosinophils were isolated from peripheral blood obtained from healthy donors. Eosinophil TBM was examined using a modified Boyden's chamber technique. Neutrophils were preincubated with or without formoterol (0.1 μ M) at 37°C for 30 min. Eosinophils were added to the upper compartment of a chamber with a Matrigel-coated transwell insert. Medium containing preincubated neutrophils and IL-8 was added to the lower compartment of the chamber. After a 90-minute incubation, the eosinophils that had migrated into the lower chamber were calculated using eosinophil

peroxidase assays. **Results:** A combination of neutrophils and IL-8 significantly induced the eosinophil TBM; formoterol alone had no effect. However, formoterol modestly but significantly attenuated the TBM of eosinophils stimulated with neutrophils and IL-8. **Conclusion:** These results suggest that formoterol may act as a therapeutic agent on enhanced eosinophilic inflammation in acute exacerbation or persistent, severe asthma. The effect of formoterol likely involves the inhibition of neutrophil activation.

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Introduction

Inhaled corticosteroids (ICSs) have drastically improved asthma control [1, 2]. However, there is also evidence that increasing ICSs is not effective when asthma is exacerbated [3]. Infiltration of neutrophils into the airways, which is often observed with asthma exacerbation, may be associated with this phenomenon. In general, the infiltration of eosinophils into airways with asthma is well known, and the sputum eosinophil count is often used as a marker of asthma exacerbation [4, 5]. On the other hand, the presence of neutrophilic inflammation is also

observed in persistent asthma [6, 7]. Green et al. [8] reported that asthma patients with predominantly neutrophils in their sputum do not respond as well to treatment with ICSs.

Although sputum neutrophilia is often clinically observed in severe asthma or asthma exacerbation, the mechanisms by which it occurs are still under discussion. According to the ENFUMOSA (European Network Study for Understanding the Mechanisms of Severe Asthma), both neutrophilic and eosinophilic inflammation persist in the airways of patients with severe asthma [9]. We observed a similar phenomenon in severe, persistent asthma in Japanese patients [10]. We recently reported that neutrophils stimulated with interleukin-8 (IL-8) play a role in the regulation of eosinophilic inflammation in asthma [11]. That is to say, in the presence of IL-8, neutrophils induce eosinophil trans-basement membrane migration (TBM) *in vitro*, which is one of the key processes in which eosinophils accumulate in the airways of asthma patients [11].

The combination of ICSs and long-acting β_2 agonists (LABA) is now widely known to improve asthma control and to reduce exacerbations as demonstrated in a number of studies, including the OPTIMA (Optimal Protocol for Treatment Initiation with Methotrexate and Adalimumab Combination Therapy in Patients with Early Rheumatoid Arthritis) study and the FACET (Formoterol and Corticosteroids Establishing Therapy) study [12, 13]. O'Byrne [14], in a double-blind, randomized, parallel-group study, demonstrated that a combination of budesonide (an ICS) and formoterol (a LABA) could replace short-acting β_2 agonist (SABA) reliever medications in asthma exacerbations. Another study showed that formoterol reduced neutrophilic airway inflammation in asthma patients [15]. Formoterol has been known to have anti-inflammatory effects on neutrophils, inhibiting the release of oxidants [16] and the adhesion to postcapillary venules in rat airways [17]. Another study reported that allergen-induced sputum eosinophilia was significantly reduced by combination treatment with budesonide/formoterol, but not by budesonide alone [18].

Taken together, we hypothesized that formoterol would provide an inhibitory effect on the neutrophil-eosinophil interaction (which may be important in the exacerbation of asthma) via an inhibitory effect on neutrophil activation. In this study, we report that the TBM of eosinophils, which is induced by IL-8-stimulated neutrophils, is inhibited by formoterol.

Materials and Methods

Reagents

Anti-CD16 antibody-coated magnetic beads were purchased from Miltenyi Biotec (Auburn, Calif., USA). HBSS was purchased from Gibco BRL (Grand Island, N.Y., USA). PBS was obtained from Wako (Osaka, Japan). Recombinant human IL-8 was purchased from R&D Systems (Minneapolis, Minn., USA). Fetal calf serum was purchased from MP Biomedicals (Aurora, Ohio, USA). BSA was purchased from Sigma-Aldrich (St. Louis, Mo., USA). Formoterol and budesonide were supplied by AstraZeneca (Lund, Sweden).

Preparation of Neutrophils and Eosinophils

Neutrophils and eosinophils were isolated from peripheral blood collected from nonatopic healthy donors whose eosinophil content was <5% of their peripheral leukocytes. The numbers of males and females were comparable among donors, with similar age distributions ranging from 26 to 34 years. Informed consent was obtained before collection of each blood sample. Briefly, 10 ml of dextran was added to 40 ml of heparinized blood and erythrocytes were removed as sediment. The system-HISTOPAQUE (Sigma-Aldrich) was performed for isolation according to the manufacturer's instructions. Separated lymphocytes were removed, 2 ml of sterile distilled water and 2 ml of 1.8% salt solution were added, followed by 20 ml of the sterilized PBS and the neutrophils (>95% purity and >99% viability) were separated by centrifuge. Neutrophils (0.5×10^5 cells/ml) with 0.2% BSA/HBSS were prepared, and IL-8, formoterol and budesonide were added to the neutrophil suspension and preincubated for 30 min in 5% CO₂ at 37°C. The remaining cells were washed with HBSS at 4°C supplemented with 5% FCS in PBS, then incubated with anti-CD16 antibody-coated magnetic beads for 30 min at 4°C, and finally filtered with a column containing steel wool placed in a magnetic field (Miltenyi Biotec). Eosinophils (>98% purity and >99% viability) which passed through the column were collected and washed, and the number of cells was adjusted to 2.5×10^5 cells/ml with 0.2% BSA/HBSS buffer. Eosinophils (2.5×10^5 cells/ml) were added to the upper compartment of a Matrigel-coated transwell (3 μ m; Becton Dickinson Labware, Franklin Lakes, N.J., USA). Neutrophils and IL-8 or formoterol, budesonide and buffer were added to the lower compartment of the chamber and incubated for 90 min in 5% CO₂ at 37°C. The study was approved by the IRB and all subjects gave written informed consent.

Trans-Basement Membrane Migration

The peroxidase activity of migrated eosinophils in the lower compartment of the chamber was determined and the number of migrated eosinophils was calculated from the activity of the standard media which contained known numbers of eosinophils [11]. To determine the peroxidase activity of eosinophils, the medium of the lower compartment was incubated with a substrate (1 mM OPD, 1 mM H₂O₂ and 0.1% Triton X-100 in Tris-HCl, pH 8.0) for 30 min at room temperature. The reaction was stopped by adding 100 μ l of 4 N H₂SO₄ and absorbance at 490 nm was determined. The effect of neutrophils on the outer density value in this assay was negligible: 0.046 ± 0.002 for 0% control and 0.064 ± 0.006 for 100% control of neutrophils, respectively (n = 4) [11]. Similarly, the addition of neutrophils to 100% control of eosinophils did not modify the outer density value [1.566 ± 0.023 addition of 0% con-

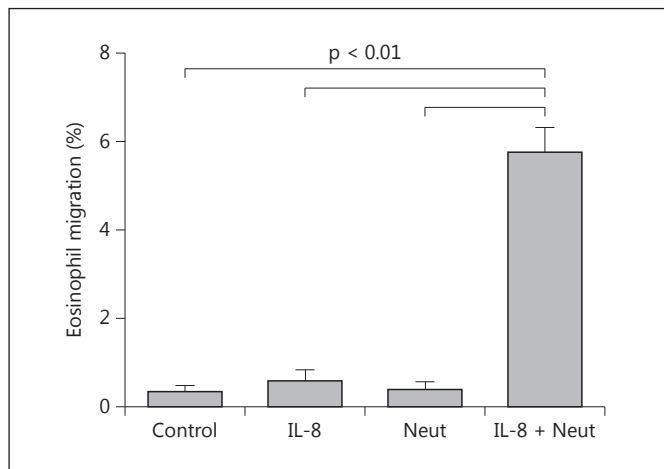


Fig. 1. A combination of IL-8 and neutrophils induced eosinophil TBM. The graph represents the percentage of migrated eosinophils. Buffer (Control), IL-8 in buffer (IL-8), neutrophils (Neut) or the combination of IL-8 and neutrophils (IL-8 + Neut) are in the bottom compartment of the transwell, and eosinophils are added to the upper compartment. Values are mean $\pm$ SEM. The combination of IL-8 and neutrophils is compared with control, IL-8 and neutrophils only ($n = 8$).

trol vs. 1.569 ± 0.012 addition of 100% control of neutrophils ($n = 4$, $p = \text{n.s.}$) [11]. The numbers of migrated eosinophils were determined by measuring fluorescence in the medium using the Fluoromark (Bio-Rad Laboratories, Hercules, Calif., USA) microplate fluorometer. The viability of both eosinophils and neutrophils after migration exceeded 98% by trypan blue exclusion.

Statistical Analysis

Values are expressed as means $\pm$ SEM. Parametric data were compared by using the Student t test for mean values analysis of variance (ANOVA) with correction for multiple comparisons (Scheffé's constants) when more than two variables were analyzed using GraphPad software.

Results

A Combination of IL-8 and Neutrophils Induced Eosinophil TBM

We have reported that a combination of IL-8 and neutrophils induced eosinophil TBM when both cell types were placed simultaneously in the upper room of a Boyden chamber [11]; this is thought to be a model of asthma exacerbation like that which follows a viral infection. In this study, to test the hypothesis that formoterol may modify eosinophil TBM via the inhibition of neutrophil activation, we first explored whether the induction of

eosinophil TBM also occurred when neutrophils were placed in the bottom chamber. Neutrophils (0.5×10^5 cells/ml) were placed in the bottom chamber of the transwell, and eosinophils (2.5×10^5 cells/ml) were added to the upper chamber. The experiments were run in the presence or absence of 10 nM of IL-8 in the bottom chamber. After a 90-minute incubation at 37°C , we counted the migration of eosinophils with eosinophil peroxidase assay as described in Methods. As illustrated in figure 1, only the combination of IL-8 and neutrophils significantly induced eosinophil migration. Neutrophils or IL-8 alone had no effect on eosinophil migration. Therefore, although the experimental condition was different (neutrophils in the lower vs. the upper chamber), we confirmed the results from our previous study [11]; neutrophils stimulated by IL-8 can induce eosinophil TBM with our current system.

Budesonide Did Not Attenuate TBM of Eosinophils by IL-8-Stimulated Neutrophils

It is well known that corticosteroids provide anti-inflammatory effects on airway inflammation such as bronchial asthma [1] and that ICS therapy has a pivotal anti-inflammatory role in the treatment of mild asthma [14], although recent reports have demonstrated that simply increasing ICSs does not improve asthma exacerbations [3, 12, 13]. To investigate the effects of corticosteroids on eosinophil TBM, we added $0.1 \mu\text{M}$ of budesonide to the bottom compartment of the transwell with the IL-8 (10 nM) and the neutrophils (0.5×10^5 cells/ml). They were preincubated together for 30 min at 37°C . After the incubation, we put eosinophils (2.5×10^5 cells/ml) into the upper compartment and incubated for 90 min at 37°C . IL-8-stimulated neutrophils induced eosinophil TBM (fig. 2). However, budesonide did not attenuate the TBM of eosinophils stimulated by IL-8 and neutrophils (fig. 2). In selected experiments, we also used higher concentrations of budesonide (up to $1 \mu\text{M}$) to observe the effect on eosinophil TBM, but no inhibitory effect of budesonide was seen (data not shown).

Formoterol Attenuates the TBM of Eosinophils Stimulated by IL-8 and Neutrophils

The effect of combination treatment with budesonide/formoterol for both the maintenance and relief of severe asthma exacerbation is unbeaten [12, 13]. Following the results shown in figure 2, we investigated the effect of formoterol on eosinophil TBM by adding $0.1 \mu\text{M}$ of formoterol, rather than budesonide, to the bottom chamber. As illustrated in figure 3, formoterol modestly (18.2% in-

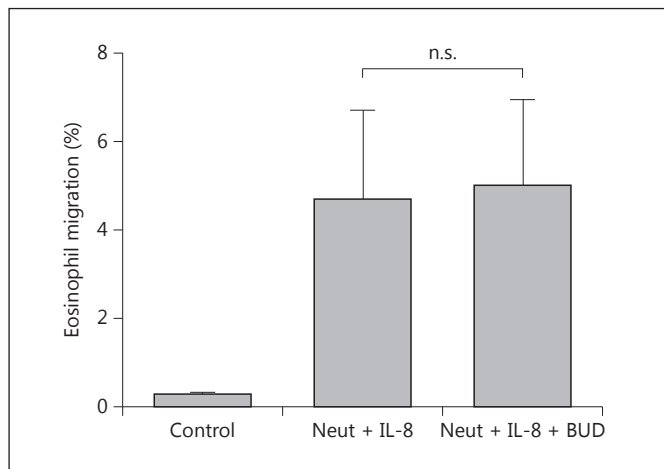


Fig. 2. Budesonide does not attenuate the TBM of eosinophils stimulated by IL-8 and neutrophils. The graph shows the percentage of migrated eosinophils. Buffer (Control), neutrophils and IL-8 (Neut + IL-8) or budesonide with IL-8 and neutrophils (Neut + IL-8 + BUD) are in the bottom compartment of the transwell, and eosinophils are added to the upper compartment. Values are mean $\pm$ SEM; n.s. = not significant for the comparison between Neut + IL-8 and Neut + IL-8 + BUD ($n = 3$).

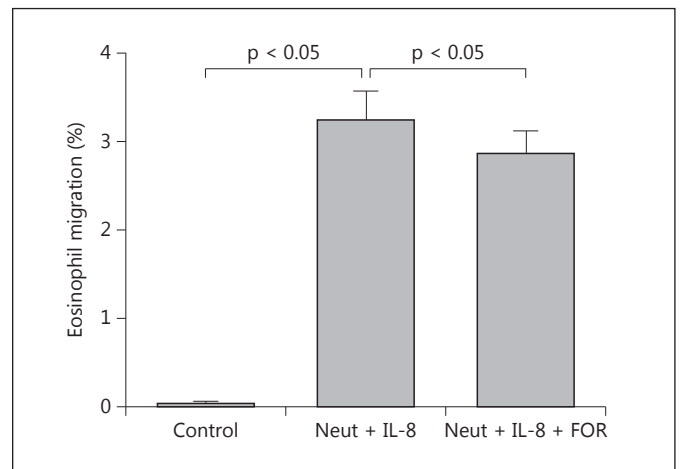


Fig. 3. Formoterol significantly attenuates the TBM of eosinophils stimulated by IL-8 and neutrophils. The graph indicates the percentage of migrated eosinophils. Buffer (Control), neutrophils and IL-8 (Neut + IL-8) or formoterol with IL-8 and neutrophils (Neut + IL-8 + FOR) are in the bottom compartment of the transwell, and eosinophils are added to the upper compartment. Values are mean $\pm$ SEM; $p < 0.05$ for Neut + IL-8 versus Neut + IL-8 + FOR or for Control versus Neut + IL-8 ($n = 5$).

hibition) but significantly attenuated the TBM of eosinophils stimulated by 10 nM of IL-8 and neutrophils. Formoterol inhibited TBM of eosinophils in a dose-dependent manner from 10^{-8} to 10^{-6} M (data not shown). This result suggests that formoterol provides an inhibitory effect via the inactivation of neutrophils.

Discussion

We provide evidence that formoterol inhibits the TBM of eosinophils induced by IL-8-activated neutrophils. Our data suggest that one of the mechanisms by which formoterol inhibits airway inflammation in asthma may be associated with neutrophilic inflammation. Moreover, this may explain why the combination of budesonide/formoterol relieves asthma exacerbation and persistent severe asthma.

The coexistence of eosinophils and neutrophils in the airways of severe asthma and exacerbation following viral infection is well known [9, 10, 19]. Our previous report suggests that neutrophils can regulate the accumulation of eosinophils in asthma airways [10]. As mentioned previously, TBM is the pivotal process by which circulating eosinophils accumulate in the airways of asthma patients. In this study, eosinophil TBM was induced by a combi-

nation of IL-8 and neutrophils, but not by either alone (fig. 1). The potential mechanism of this phenomenon is thought to be that neutrophils stimulated with a chemoattractant (such as IL-8) augment the TBM of eosinophils. Further, matrix metalloproteinase (MMP)-9, leukotriene B₄ (LTB₄), platelet-activating factor (PAF) and tumor necrosis factor (TNF)- α are thought to be involved as mediators in the augmentation of eosinophil TBM by IL-8-stimulated neutrophils, because the inhibitors or the antagonists of these factors inhibit this augmentation [11].

ICSs have considerably contributed to our ability to improve asthma control; however, not all patients achieve control of asthma symptoms and airway inflammation with ICS therapy. In particular, increasing ICSs is not effective in treating the exacerbation of asthma [3]. Both sputum eosinophilia and neutrophilia are seen clinically in severe asthma and in asthma exacerbation. Neutrophil accumulation is generally understood to be resistant to glucocorticoid therapy [20]. Our data, in which budesonide does not attenuate the TBM of eosinophils stimulated by IL-8 and neutrophils, (fig. 2) support these clinical findings. In contrast, the question that the effect of budesonide on eosinophil TBM might be underestimated still remains, because budesonide works via binding with glucocorticoid receptor protein. The incubation

time of budesonide might be too short for the inhibition of eosinophil TBM to show enough suppression. The longer incubation time of budesonide was not used in this study; that is another assignment.

The combination therapy of budesonide/formoterol has been efficacious as a reliever medication in cases of asthma exacerbation. The mechanism of this efficacy might partially depend upon an anti-inflammatory effect of formoterol. Kelly et al. [18] demonstrated that allergen-induced sputum eosinophilia was significantly reduced by combination treatment with budesonide/formoterol but not by budesonide monotherapy. Formoterol itself has various kinds of anti-inflammatory effects. Formoterol is known to suppress the production of superoxide-anion and the release of elastase by formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated neutrophils in vitro [21]. Formoterol also attenuates neutrophil numbers and IL-8 levels in the sputum of asthmatic patients [15]. In this study, formoterol significantly reduced the TBM of eosinophils stimulated by IL-8 and neutrophils (fig. 3). Another kind of β_2 agonist is known to have a similar effect, as Procaterol inhibits eosinophil migration and the release of eosinophil chemotactic activity from epithelial cells [22]. Our data suggest that formoterol has an anti-inflammatory effect via the suppression of neutrophil activation.

Following these results, our interest moved to the mechanisms by which formoterol, but not budesonide, attenuates eosinophil TBM by IL-8-stimulated neutrophils. As mentioned previously, there is evidence that MMP-9, LTB₄ or PAF are involved in the augmentation of eosinophil TBM stimulated by activated neutrophils, because the inhibitors for MMP-9, LTB₄ and PAF blocked the eosinophil migration [11]. Therefore, formoterol may affect these chemoattractants and reduce the TBM of eosinophils. A recent study indicates that formoterol attenuates production of superoxide and LTB₄ on activated neutrophils by fMLP via association with increases of cyclic adenosine monophosphate [21]. MMP-9 may be a target as another potential mechanism. Tacon et al. [23] recently demonstrated that formoterol reduces transcription factor, Fos-related Ag-1 (Fra-1, part of the AP-1 family) expression and DNA binding activity and MMP-9 on lung epithelial cells induced by rhinovirus infection, via inhibition of the MAPK pathway. Rhinovirus causes the majority of common colds and is known to cause asthma exacerbation. According to their study, the combination of formoterol/dexamethasone therapy suppressed MMP-9 expression more than either monotherapy did [23]. In this study, we also examined the effect of formoterol/

budesonide combination therapy on eosinophil TBM by IL-8-stimulated neutrophils; however, we could not find any additional effects (data not shown).

These discussions remain speculations, however, as the molecular mechanisms by which formoterol attenuates eosinophil TBM by activated neutrophils are still uncertain. Nonetheless, our results support the clinical efficacy of formoterol on sputum eosinophilia in asthma exacerbation, which is not altered by ICS monotherapy. Although we did not investigate MMP-9, LTB₄ and PAF, they should be part of the targets for the mechanism of inhibition of eosinophil TBM by activated neutrophils in further study.

In summary, we have presented the novel finding that formoterol reduced the TBM of eosinophils by IL-8-stimulated neutrophils. In our study, formoterol may have contributed to inhibit airway eosinophilia induced by activated neutrophils (which assumes exacerbation of asthma). Our finding suggests that formoterol may be useful as a therapeutic agent for enhanced eosinophilic inflammation that occurs in persistent, severe asthma. This formoterol effect likely involves inhibiting neutrophil activation.

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Disclosure Statement

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this paper.

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