Original Article

Inhibitory effects of pemirolast potassium and FK506 on degranulation and IL-8 production of eosinophils

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ABSTRACT

Eosinophils are the key effector cells for allergic inflammation. In order to clarify drugs which can regulate eosinophil function, we investigated the direct effect of pemirolast potassium (anti-allergic drug) and FK506 (immunosupressant) on eosinophil degranulation and cytokine production. Eosinophil degranulation induced by granulocyte-macrophage colony stimulating factor and/or platelet activating factor was inhibited from 10^{-4} to 10^{-6} mol/L pemirolast (inhibitory effect 66 and 33%, respectively) and 10^{-8} mol/L FK506 (inhibitory effect 45%). Pemirolast and FK506 also inhibit interleukin (IL)-8 production by eosinophil. Our data suggest that both pemirolast potassium and FK506 possess direct regulatory effects on human eosinophils and a potential to suppress allergic inflammation.

Key words: anti-allergic drug, cytokine, eosinophil, degranulation.

INTRODUCTION

Recently, the importance of allergic inflammation in the pathogenesis of asthma has become widely accepted.[1] Eosinophils are the key effector cells for allergic inflammation. Factors such as major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil derived neurotoxin (EDN, eosinophil protein × (EPX)), which are released from eosinophil granules, damage bronchial epithelial cells, resulting in bronchial hyperreactivity.[1–4] In addition, recent reports have revealed that eosinophils produce cytokines and play a pivotal role in allergic inflammation.[5–8] When we are able to interrupt this cascade of reaction, the allergic inflammation may be controlled. Therefore, recent treatments for asthma target not only the immediate spastic reaction of the bronchus but also the inflammatory reaction.

Granulocyte-macrophage colony stimulating factor (GM-CSF) plays an important role not only in the differentiation but also in the activation of eosinophils.[1,9,10] GM-CSF is abundant at the site of allergic inflammation[11] Granulocyte-macrophage colony stimulating factor gene transfer in the lungs of normal mice causes accumulation of inflammatory cells and irreversible bronchial remodeling.[12] Platelet activating factor (PAF) is another important chemical mediator which activates eosinophils as well as neutrophils.[1] Thus, GM-CSF and PAF constitute the inflammatory process of asthma. Pemirolast is an anti-allergic drug which was originally found to inhibit immunogobulin E (IgE)-mediated release of a chemical mediator from mast cells.[13,14] It has been shown that pemirolast inhibited eosinophil chemotaxis.[15] Although pemirolast inhibits LTD4 release from eosinophils induced by calcium ionophore,[16] the effect of pemirolast on eosinophil degranulation by physiological stimuli such as GM-CSF has not been clarified. Moreover, the effect of pemirolast on cytokine production is unknown.

In order to compare the effect of pemirolast, we examined the effect of FK506, an immunosuppressant which also inhibits allergic reaction.[17] FK506 prominently suppresses GM-CSF and IL-3 release from eosinophils[18] and inhibits mast cell degranulation.[19] However, the inhibition of eosinophil degranulation by FK506 has not been fully

explored. Thus, in this study we investigated the effect of pemirolast and FK506 on eosinophil degranulation in conjunction with IL-8 production, which plays a critical role of accumulation for leukocytes.[20–22]

METHODS

Eosinophil purification

Whole blood was obtained from patients with moderate eosinophilia (6–20%), collected in tubes containing EDTA. Peripheral blood mononuclear cells were separated by Ficoll-Hypaque centrifugation. The remaining cells were separated into erythrocytes and granulocytes by 6% dexran sedimentation. In order to isolate eosinophils, the granulocyte population was incubated with mouse anti-human CD16 monoclonal antibodies (Immunotech SA, Marseille, France). CD16-positive neutrophils were then depleted using beads coated with a goat antimouse antibody (Dynabeads, Dynal AS, Oslo, Norway).[8] The purity of the negatively selected eosinophils was checked by staining with Diff-Quick (Baxter, Dudigen, Switzerland) and was greater than 95%.

Human studies committee approval was obtained and an individual consent form was completed and signed prior to the period in which we conducted these studies.

Eosinophil stimulation

Polystyrene tissue culture plates with 96 or 48 wells were coated with 2% human serum albumin (HAS; Sigma Chemical Co., St Louis, MO, USA), as described by Horie and Kita.[23] For the degranulation assay, eosinophils (2×10^5 cells/well) in Pipes A buffer were simultaneously stimulated using recombinant GM-CSF (10 ng/mL; a donation from Sherring-Plough Brinny Co., NJ, USA) and PAF (10^{-6} mol/L; Bachem Fine Chem. AG, Bubendorf, Switzerland) for 2 h at 37°C in a 5% CO_2 incubator. Following this, the supernatants were harvested. The levels of ECP and eosinophil protein X (EPX) were assayed using radioimmunoassay kits (Pharmacia Fine Chemicals, Division of Pharmacia Biotechnology International AB, Uppsala, Sweden). For IL-8 production, eosinophils (1×10^6 cells/well) in 5% fetal calf serum (FCS) in RPMI 1640 were treated with 5 μg/mL of cytochalasin B (Sigma Chemical Co.) and were simultaneously stimulated by recombinant 10 ng/mL GM-CSF and 5000 units/mL tumor necrosis factor (TNF)-α (R&D System Inc., Minneapolis, MN, USA) for 16 h at 37°C in a 5% CO_2 incubator.
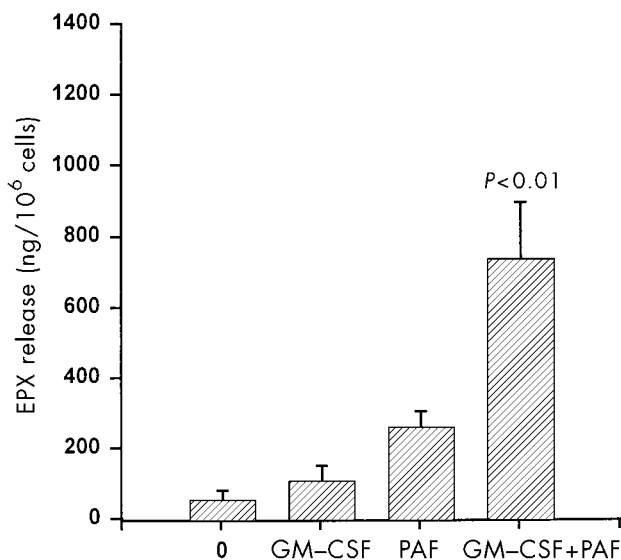

Fig. 1 Eosinophil degranulation induced by granulocyte-macrophage colony stimulating factor (GM-CSF) and platelet activating factor (PAF). Highly purified eosinophils were incubated with GM-CSF (10 ng/mL) and PAF (1 μmol) for 2 h. The data were expressed as mean ± SEM of four different experiments from four different donors.
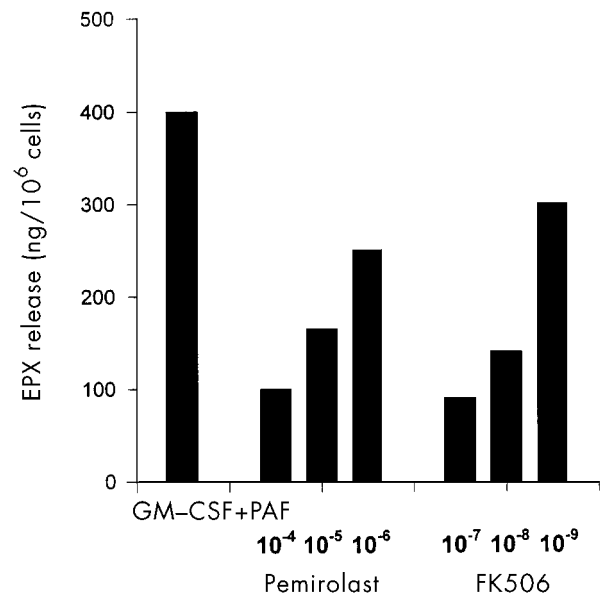

Fig. 2 The inhibitory effects of pemirolast and FK506 on eosinophil degranulation induced by platelet activating factor (PAF) and granulocyte-macrophage colony stimulating factor (GM-CSF). Eosinophils were preincubated with the indicated concentration of the drugs for 30 min and then degranulation was induced as described in the legend to Fig. 1. The amount of eosinophil protein X (EPX) released was measured using an RIA kit. The assay was performed in duplicate. This is representative data; pooled data is shown in Fig. 3.

The supernatants were harvested and the cell pellets were lysed by freezing and thawing three times and by the addition of 1% triton X. The IL-8 levels were measured using an ELISA kit (R&D System Inc.). The detection limit of this assay was 10 pg/mL. Eosinophil viability did not significantly change with the addition of pemirolast potassium and FK506 at the dose we used as assessed by trypan blue exclusion technique and propium iodine (PI) staining (data not shown)

RESULTS

Degranulation induced by GM-CSF and PAF

Eosinophils released EPX in response to activation by GM-CSF (Fig. 1). Platelet activating factor also induced the release of EPX. When eosinophils were stimulated by GM-CSF and PAF simultaneously, larger amounts of EPX were released ($P < 0.01$, Fig. 1), indicating that a combination of GM-CSF and PAF has a synergistic effect on PAF-induced degranulation.
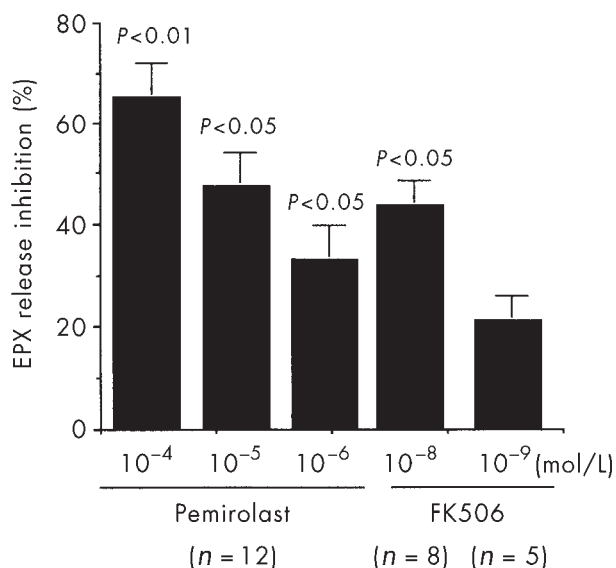
Effects of pemirolast and FK506 on eosinophil degranulation

We next evaluated the inhibitory effect of pemirolast and FK506 on eosinophil degranulation induced by the combination of GM-CSF and PAF. Representative data is shown in Fig. 2. Pemirolast and FK506 inhibited eosinophil degranulation dose-dependently (Fig. 2). A summary of the inhibitory effects of pemirolast on GM-CSF + PAF-induced EPX release from eosinophils of 12 different donors is shown in Fig. 3. The mean inhibitory effect of pemirolast was 66% at 10^{-4} mol/L ($P < 0.01$, multiple comparison using corrected P by Bonferroni), 47% at 10^{-5} mol/L ($P < 0.05$), and 33% at 10^{-6} mol/L ($P < 0.05$). FK506 (10^{-8} mol/L) also significantly inhibited eosinophil degranulation at $45 \pm 4.3\%$ ($P < 0.05$, $n = 8$). These data suggest that pemirolast and FK506 effectively suppress eosinophil degranulation.
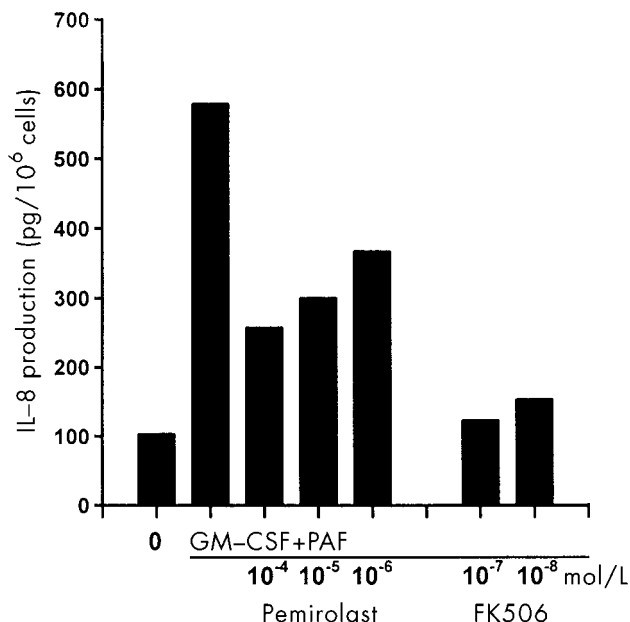

Fig. 3 Percent inhibitory effect of pemirolast and FK506 on eosinophil degranulation induced by granulocyte-macrophage colony stimulating factor (GM-CSF) + platelet activating factor (PAF). Eosinophils were preincubated with the indicated concentration of the drugs for 30 min and then degranulation was induced as described in the legend to Fig. 2. The percentage inhibitory effect was calculated as EPX released without pemirolast or FK506–EPX released with pemirolast or FK506–EPX released without pemirolast or FK506. The bar indicates the mean ± SEM of 12 patients for pemirolast and examinations and 8 patients for FK506.

Fig. 4 Inhibitory effects of pemirolast and FK506 on IL-8 production induced by granulocyte-macrophage colony stimulating factor (GM-CSF) + TNF-α. Eosinophils were preincubated with various concentrations of pemirolast and FK506 for 30 min, and were then stimulated with GM-CSF and TNF-α simultaneously. After a 16 h stimulation period, the supernatant and the cells were harvested. The amount of IL-8 content was measured in the supernatant of 0.5×10^6 lysed eosinophils in 200 μL of medium by ELISA. The bar indicates the mean of duplicate determinations. Similar results were obtained from seven different experiments, with summarized data being shown in Fig. 5.
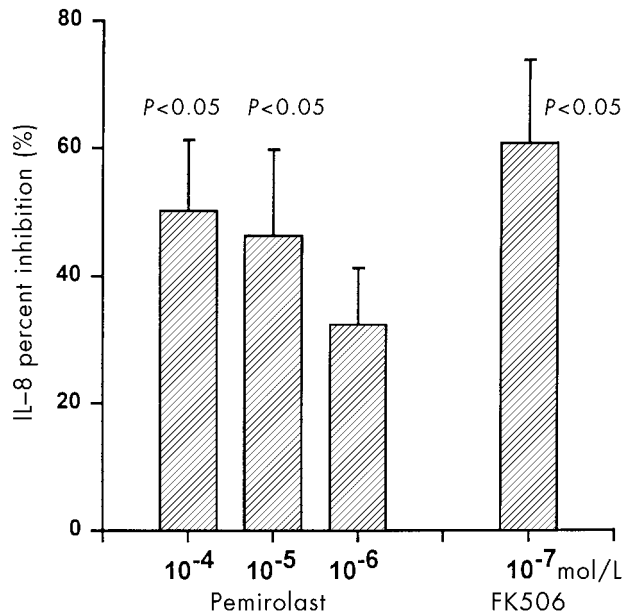
Fig. 5 Percent inhibitory effects of pemirolast and FK506 on IL-8 production. Inhibitory effects of pemirolast and FK506 IL-8 production by eosinophils were examined as described in the legend to Fig. 4. Percent inhibition was calculated as IL-8 production without pemirolast or FK506–IL-8 production with pemirolast or FK506–IL-8 production without pemirolast or FK506. The bar represents mean ± SEM of results from seven different donors.

IL-8 production by eosinophils

The effects of the drugs on total IL-8 production from extracellular and intracellular IL-8 was examined. In Fig. 4, a representative result was shown. Pemirolast and FK506 inhibited IL-8 production and secretion by eosinophils dose-dependently. The summary of inhibitory effects is shown in Fig. 5. The mean inhibitory effect from seven donors was 50.2% at 10^{-4} mol/L of pemirolast ($P < 0.05$, multiple comparison using corrected P by Bonferroni), 46.4% at 10^{-5} mol/L of pemirolast ($P < 0.05$), and 60.9% at 10^{-7} mol/L of FK506 ($P < 0.05$) (Fig. 4). Thus, pemirolast and FK506 inhibit IL-8 production from eosinophils.

Discussion

The need for anti-allergic drugs that regulate eosinophil function has been clear since asthma was first considered to be an inflammatory response of the airway.[1] In this report, we have shown that pemirolast and FK506 can directly act on eosinophils to suppress degranulation and

cytokine production, which may result in the disruption of the inflammatory cascade of asthma *in vivo*.

It has been reported that GM-CSF has a priming effect for the expression of adhesion molecules on eosinophils induced by N-formyl-methionyl-leucyl-phenylalanine or PAF.[10] Interleukin-5 has a priming effect for cytokine production induced by RANTES or IL-8.[24] We showed that GM-CSF had a synergistic effect on eosinophil degranulation induced by PAF. Such effects of chemical mediators and cytokines will lead to the activation of cytokine cascade in asthma. We examined the inhibitory effects of drugs on degranulation of eosinophils induced by GM-CSF + PAF. Pemirolast inhibited degranulation of eosinophils at concentrations as low as 10^{-6} mol/L. In humans, the maximum serum concentration at regular doses of pemirolast has been reported to be 3×10^{-6} mol/L.[25] Thus, the inhibitory effect observed in our study would occur *in vivo* at clinical dosages.

It has been reported that pemirolast inhibits chemotaxis of eosinophils,[15] and that pemirolast inhibits LTC4 release from eosinophils.[16] However, there are no studies regarding the effects of pemirolast on cytokine production. In this study, we showed that pemirolast suppressed degranulation and cytokine production. The suppressive effect on cytokine production was comparable to that of FK506. These data suggested an *in vivo* effect of pemirolast in allergic inflammatory regulation.

FK506 also directly affected eosinophil degranulation and IL-8 production. Because the suppressive effect of cytokine production on T cell is prominent,[26] we removed lymphocytes by Ficoll-Hypaque gradient separation and by using anti-CD3 monoclonal antibodies in order to eliminate the effects of FK506 through lymphocytes. We achieved contamination by neutrophils in less than 5% of purified eosinophils, and contamination by lymphocytes below 1% of purified eosinophils. FK506 also inhibited eosinophil degranulation similar to pemirolast.

It has been reported that FK506 inhibits mast cell degranulation as well as cytokine production.[19] In regard to eosinophils, it has been reported that IL-3 and GM-CSF production induced by Ca ionophore was suppressed by FK506.[18] Eosinophils produce a significant amount of IL-8 by various physiological stimuli.[8,27,28] FK506 (10^{-7} mol/L) exhibit a stronger inhibitory effect than pemirolast (10^{-4} mol/L). IL-8 is a pro-inflammatory cytokine which contributes to inflammation in asthma sufferers.[20,21] Regulating IL-8 production may be a way of controlling allergic inflammation.

Recently, the signal transduction pathways of eosinophils have been clarified.[29–34] L-3, IL-5 and GM-CSF, which use a common receptor β subunit, induce tyrosine phosphorylation of lpr/yes-related novel gene (lyn) and spleen tyrosine kinase (syk) in eosinophils.[30] Tyrosine phosphorylation of lyn and syk plays an essential role in apoptosis of eosinophils, which has been demonstrated using antisense oligonucleotides of lyn and syk.[31] We found that tyrosine phosphorylation is required for eosinophil degranulation induced by PAF and IL-8 production induced by GM-CSF + TNF-α (data not shown). It has been reported that pemirolast inhibits MAP-kinase activity on human basophilic leukemia cells.[35] Further investigation is needed in order to establish whether pemirolast inhibits degranulation via suppressive effects on MAP-kinase.

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