Ketotifen inhibits PAF-induced actin polymerization in a human eosinophilic leukaemia cell line, EoL-1

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Ketotifen inhibits PAF-induced actin polymerization in a human eosinophilic leukaemia cell line, EoL-1, was examined by flow cytometry with the use of reagents specific for the filamentous form of actin (F-actin). Actin polymerization has been considered to be essential for locomotion of cells, chemotaxis and chemokinesis, and thus it reflects the chemotactic reaction of EoL-1 cells stimulated by PAF. Unstimulated EoL-1 cells showed little PAF-induced actin polymerization, whereas EoL-1 cells cultured for 9 days with the supernatant of a human ATL cell line, HL-3 (HL-3 sup), showed marked actin polymerization when stimulated with PAF. The actin polymerization in EoL-1 cells induced by PAF was seen in a dose-dependent manner at concentrations of 10^-4M to 10^-6M of PAF, and the maximum effect was seen at 10^-5M of PAF. CV-3988, a specific antagonist of PAF, inhibited 80% of the actin polymerization in EoL-1 cells induced by PAF at a concentration of 10^-6M. Ketotifen inhibited up to 40% of the PAF-induced actin polymerization of EoL-1 cells in a dose-dependent manner at concentrations of 10^-6M to 10^-4M. These results suggest that ketotifen may play an important role in the prevention of eosinophil-induced inflammation in allergic disorders by inhibiting PAF-induced chemotaxis of eosinophils.

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An anti-allergic agent, ketotifen, has been shown to be clinically useful in allergic disorders, such as bronchial asthma, allergic rhinitis and atopic dermatitis [1-6]. Ketotifen has been considered to suppress or alleviate such allergic disorders by a combination of several effects: antihistamine effect [7], suppression of the release of slow-reactive substance of anaphylaxis [8], and an increase of the number of β-adrenergic receptors [9, 10]. The bronchoalveolar lavage fluids (BALF) of patients with bronchial asthma seem to contain fewer eosinophils after ketotifen treatment [11]. It is well known that eosinophils accumulate towards the locus of an allergic reaction in response to platelet activating factor (PAF), eosinophil chemotactic factor of anaphylaxis and other chemokinetic factors of eosinophils. Eosinophils in allergic reactions have been considered to prolong and worsen allergic inflammation by secreting cytotoxic substances, such as major basic protein, eosinophil cationic protein, eosinophil peroxidase and eosinophil-derived neurotoxin [12]. Therefore, the inhibitory effect of ketotifen on the accumulation of eosinophils at the locus of an allergic reaction has been considered to be part of its anti-allergic activity [11].

Experiments using normal eosinophils, however, are rather difficult because eosinophils are a minor population of white blood cells and because the purification of them is difficult. Recently, a human eosinophilic cell line, EoL-1 was established from a patient with eosinophilic leukaemia [13]. EoL-1 cells have a myeloblastic feature and few eosinophilic granules under normal culture conditions, but they differentiate into eosinophilic granule-containing cells and not into other cell lineages when cultured in an alkaline medium, or after the addition of dimethyl sulfoxide [13], or tumour necrosis factor [14]. Thus, EoL-1 cells seem to be "committed" eosinophil precursor cells. We showed that EoL-1 cells differentiate to contain eosinophilic granules and to express CD23 and an eosinophil differentiation antigen, EO-1 [15] on their surfaces when stimulated with the supernatant of a human ATL cell line, HL-3 (Morita et al., manuscript submitted for publication). EoL-1 thus seems to be a useful in vitro model for examinations of the functions and differentiation of eosinophils. Using this in vitro system, we investigated the anti-PAF effect of ketotifen and found that ketotifen inhibits PAF-induced actin polymerization in EoL-1 cells.
Materials and methods

Cell line

A human eosinophilic leukaemia cell line, EoL-1 was kindly donated by Dr H. Saito, Yamagata Central Prefectural Hospital, Japan, and maintained in RPMI 1640 medium (Nissui Pharmaceutical Co. Ltd, Tokyo) supplemented with 13% fotal calf serum (FCS), 2 mM L-glutamine, 100 U·ml⁻¹ penicillin and 100 µg·ml⁻¹ streptomycin; this is referred to as "the culture medium" in this paper. A human T-cell line, HIL-3 was established from peripheral blood mononuclear cells of an adult T-cell leukaemia patient. HIL-3 cells expressed the mRNA of interleukin-3, granulocyte/macrophage colony-stimulating factor and macrophage colony-stimulating factor as determined by Northern blot hybridization. The expression of interleukin-3 mRNA was marginal, and mRNA of interleukin-2 and interleukin-4 was not expressed (MoRI, unpublished data). HIL-3 cells were maintained in the same culture medium with or without 10% FCS, stored at 4°C, 25°C or 37°C for a certain number of seconds. Cells were fixed with 50 µl of 37% phosphate-buffered formalin and then permeabilized and stained in a single step with an argon ion laser at 300 mW. For each sample, 10,000 light-scatter-gated cells were analysed. Fluorescence data were collected with linear amplification, and specific cell immunofluorescence was expressed as channel numbers on a linear scale.

Assays for chemotaxis

The chemotactic response of EoL-1 cells was determined by the Boyden Chamber method [16] and actin polymerization [17]. Briefly, EoL-1 cells cultured with or without 10% HIL-3 sup for 9–12 days were placed in chemotactic chambers consisting of upper and lower compartments separated by a Millipore filter of 8 µm pore size (SMWP01300, Millipore Corporation). The culture medium with or without 10⁻⁷M PAF was placed in the lower compartment, and the cell suspension (4x10⁶ cells in 0.5 ml of the culture medium) was placed in the upper compartment. The chambers were incubated at 37°C with 5% CO₂ and 95% air in a humidified atmosphere for 3 h. The filters were then removed, fixed in methanol, stained with Giemsa, dried and cleared in xylene. The total number of cells reaching the lowest surface of the filter per 5 high power fields (chemotactic activity) was counted. For the analysis of actin polymerization, cells were resuspended in HBSS at a concentration of 1x10⁶ cells·450 µl⁻¹ and incubated with or without various concentrations of PAF at 4°C, 25°C or 37°C for a certain number of seconds. Cells were fixed with 50 µl of 37% phosphate-buffered formalin and then permeabilized and stained in a single step with an argon ion laser at 300 mW. For each sample, 10,000 light-scatter-gated cells were analysed. Fluorescence data were collected with linear amplification, and specific cell immunofluorescence was expressed as channel numbers on a linear scale.

Inhibitory effect of CV-3988 and ketotifen against actin polymerization of EoL-1 cells stimulated with PAF

EoL-1 cells cultured with 10% HIL-3 sup for 9 days were resuspended in HBSS at a concentration of 1x10⁶ cells·450 µl⁻¹ and pre-incubated with or without various concentrations of CV-3988 or ketotifen for 60 min at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Then 10⁻⁷M PAF was added, and incubation continued for 45 s at 25°C. The cells were fixed, permeabilized, stained and analysed by FACS as described above. The percentage inhibition of CV-3988 or ketotifen was calculated as follows:

\[
\frac{(1 - \text{MFI of the cells pre-incubated with CV-3988 or ketotifen}) \times 100}{\text{MFI of the cells pre-incubated without CV-3988 or ketotifen}}
\]

MFI: mean fluorescent intensity.
Cytotoxic effects of CV-3988 and ketotifen

The 10% HIL-3 sup-induced EoL-1 cells were incubated with CV-3988 or ketotifen at a concentration of 10^-5 M for 60 min. After cell viability and viable cell counts had been determined by 0.3% trypan blue exclusion, the cells were washed twice with PBS, resuspended in the culture medium at a concentration of 5x10^5 cells·ml^-1, and incubated for another 24 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Then viable cell counts and cell viability were again determined.

Statistical analysis

Student’s t-tests were used in the statistical analysis.

Results

PAF-induced chemotaxis of EoL-1 cells

EoL-1 cells cultured for 9-12 days with 10% HIL-3 sup contained about 20% eosinophilic granule-positive cells and showed significant chemotaxis when stimulated by 10^-7 M PAF, as determined by the Boyden chamber method, whereas EoL-1 cells under normal culture conditions contained less than 3% eosinophilic granule-positive cells and showed little chemotaxis when stimulated by PAF (fig. 1). The chemotactic response was weak and sometimes inadequate for further quantitative analysis (data not shown). On the other hand, when the actin polymerization was examined, PAF at a concentration of 10^-7 M induced actin polymerization with a marked increase of F-actin contents in EoL-1 cells pre-cultured with 10% HIL-3 sup for 9 days (fig. 2). EoL-1 cells without pre-incubation with 10% HIL-3 sup showed little actin polymerization when stimulated with PAF (data not shown). Actin polymerization occurred in a short time. Our preliminary experiments showed that the F-actin content increased rapidly and reached a plateau 30-45 s after the addition of PAF at 25°C and then decreased. At 37°C, the maximum F-actin content
appeared within 30 s and then decreased rapidly, making detection of the maximum level or plateau technically difficult (data not shown). Therefore, although the F-actin content was approximately 1.5 times larger at 37°C than at 25°C, we examined actin polymerization induced by PAF at 25°C in the following experiments. The maximum F-actin content was much lower at 4°C (approximately 8% of that induced at 37°C). Actin polymerization was induced by stimulation with PAF at concentrations of $10^{-10}$M to $10^{-4}$M, and the maximum increase of F-actin content was seen at a concentration of $10^{-7}$M PAF (fig. 3).

**Inhibition by CV-3988 and ketotifen of actin polymerization in EoL-1 cells stimulated by PAF**

As shown in figure 4, CV-3988 inhibited PAF-induced actin polymerization in EoL-1 cells in a dose-dependent manner at concentrations of $10^{-11}$M to $10^{-3}$M, and the maximum inhibition reached 80% at a concentration of $10^{-3}$M. Ketotifen also showed a dose-dependent inhibition of actin polymerization at concentrations of $10^{-9}$M to $10^{-4}$M. The maximum inhibition reached 40% at a concentration of $10^{-9}$M of ketotifen.

**Cytotoxic effect of CV-3988 and ketotifen**

It is possible that the inhibitory effects of CV-3988 and ketotifen on PAF-induced actin polymerization in EoL-1 cells was due to the non-specific cytotoxic effect of these substances. In order to rule out this possibility, the cytotoxic effects of CV-3988 and ketotifen were examined. There was no decrease in the cell viability and the viable cell number after treatment for 60 min with CV-3988 or ketotifen (data not shown). Moreover, as shown in table 1, EoL-1 cells pre-incubated with either CV-3988 or ketotifen for 60 min proliferated as well as those without such pre-incubation.

**Table 1. - Viable cell counts and cell viability of EoL-1 cells treated with CV-3988 or ketotifen**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CV-3988</th>
<th>Ketotifen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell counts (x10^6)</td>
<td>10.0±0.8</td>
<td>8.9±0.7</td>
<td>10.7±1.3</td>
</tr>
<tr>
<td>Viability %</td>
<td>83.6±1.4</td>
<td>82.8±4.4</td>
<td>83.1±3.0</td>
</tr>
<tr>
<td>(p&gt;0.05)</td>
<td></td>
<td>p&gt;0.05</td>
<td></td>
</tr>
</tbody>
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EoL-1 cells cultured with 10% HIL-3 sup for 9 days were incubated with or without CV-3988 or ketotifen and viable cell counts and cell viability were determined (see Materials and methods). The data represent the mean±so (n=4).

**Discussion**

EoL-1 cells cultured for 9 days with 10% HIL-3 sup showed a chemotactic response when stimulated with PAF, as detected by both the Boyden chamber method and the actin polymerization method. The chemotaxis of the EoL-1 cells detected by the Boyden chamber method, however, was weak and quantitative analysis using this method was difficult. Determination of the F-actin content, on the other hand, showed that EoL-1 cells cultured with 10% HIL-3 sup for 9 days had marked actin polymerization when stimulated with PAF. This discrepancy seemed to be due to the difference in sensitivity of the two methods and to the insufficient maturation of EoL-1 cells examined by the Boyden chamber method. Actin polymerization is caused by a change of actin assembly from a globular form to a filamentous form (F-actin), the production of which is essential for and precedes changes in the motile behaviour of cells, such as chemotaxis [17, 18]. Therefore, we examined the effect of ketotifen on PAF-induced chemotaxis in EoL-1 cells as reflected by the change of actin polymerization, although actin polymerization does not represent the whole process of chemotaxis.

Both ketotifen and CV-3988, a specific antagonist of PAF, inhibited PAF-induced actin polymerization in 10% HIL-3 sup-induced EoL-1 cells. The serum concentration of ketotifen 3.3 h after oral administration is $10^{-4}$M to $10^{-3}$M [19] and ketotifen's inhibition of PAF-induced actin polymerization was demonstrated at these physiological concentrations.

The inhibitory effects of CV-3988 and ketotifen against actin polymerization were not due to their non-specific cytotoxic effects because pre-incubation with CV-3988 or ketotifen had no effect on cell growth even after 24 h. CV-3988 is considered to
specifically inhibit PAF-induced actin polymerization at receptor sites [20]. CV-3988 does not inhibit fMLP (formyl-methionyl-leucyl-phenylalanine)-induced actin polymerization in human peripheral blood neutrophils (Tsutada et al., unpublished data). Our preliminary experiments showed that EoL-1 cells cultured with 10% HIL-3 sup seemed to express PAF receptors; specific binding of PAF has been detected in binding experiments using 3H-PAF (Morita et al., unpublished data).

Ketotifen, on the other hand, does not appear to act as a PAF receptor antagonist, since similar levels of inhibition by ketotifen were seen against actin polymerization induced by PAF and fMLP (33% and 40%, respectively, at 10M of ketotifen) in human peripheral blood neutrophils (Tsutada et al., unpublished data). It is likely that ketotifen inhibits the signal transduction process after PAF receptors and fMLP receptors have been stimulated by their agonists.

The inhibitory effect of ketotifen on the locomotion and accumulation of eosinophils at the locus of allergic reactions may be one of the therapeutic mechanisms of the anti-allergic function of this drug. In in vitro experiments and in clinical observations, the accumulation of eosinophils at the locus of allergic reactions, such as BALF in bronchial asthma, nasal mucosa in allergic rhinitis and skin in atopic dermatitis, has been shown to be inhibited by the systemic or local treatment of bronchial asthma.

Our experiments have demonstrated the inhibitory effect of ketotifen on the PAF-induced chemotactic reaction of EoL-1 cells and suggest that ketotifen works at the stage of or preceding actin polymerization in the cells. Further investigation is needed to clarify the mechanisms of the inhibitory effect of ketotifen against PAF. Although EoL-1 cells may not have all the characteristics of normal eosinophils, they appear to be a good model for further investigations.

References


RÉSUMÉ: Les effets inhibiteurs du ketotifène sur la polymérisation de l’actine induite par le PAF dans une lignée cellulaire leucémique éosinophile de l’homme (EoL-1), a été examinée par cytométrie de flux en utilisant des réactifs spécifiques pour la forme filamenteuse d’actine (F-actine). La
polymérisation de l'actine a été considérée comme essentielle à la motilité des cellules, à la chimiotaxie, et à la chimiokinase, et dès lors reflète la réaction chemoactique des cellules EoL-1 stimulées par le PAF. Les cellules EoL-1 non stimulées ont montré peu de polymérisation de l'actine induite par le PAF, alors que les cellules EoL-1 cultivées pendant 9 jours avec le surnageant d'une lignée cellulaire humaine ATL (HIL-3) (HIL-3 sup), ont montré une polymérisation marquée de l'actine lorsqu'elles étaient stimulées par le PAF. La polymérisation de l'actine dans les cellules EoL-1 induites par le PAF est dose-dépendante à des concentrations de 10^{-10} M jusqu'à 10^{-4} M de PAF. L'effet maximum est observé à 10^{-7} M de PAF. CV-3988, un antagoniste spécifique de PAF, a inhibé 80% de la polymérisation de l'actine dans les cellules EoL-1 induites par le PAF à une concentration de 10^{-4} M. Le ketotifène a inhibé jusqu'à 40% de la polymérisation de l'actine induite par le PAF dans les cellules EoL-1, de manière dose-dépendante, à des concentrations de 10^{-6} M à 10^{-2} M. Ces résultats suggèrent que le ketotifène pourrait jouer un rôle important dans la prévention de l'inflammation induite par les éosinophiles dans les maladies allergiques, en inhibant la chimiotaxie des éosinophiles induite par le PAF.

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