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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. M. Morita, S. Tsuruta, K.J. Mori, M. Mayumi, H. Mikawa.

ABSTRACT: The inhibitory effect of ketotifen on platelet activating factor (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, HIL-3 (HIL-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^{-10}M to 10^{-6}M of PAF, and the maximum effect was seen at 10^{-7}M 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^{-5}M. Ketotifen inhibited up to 40% of the PAF-induced actin polymerization of EoL-1 cells in a dose-dependent manner at concentrations of 10^{-9}M to 10^{-5}M. 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. *Eur Respir J., 1990, 3, 1173–1178.*

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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 histamine release [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 chemoattractants 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 sulphoxide [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, HIL-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% foetal 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-5, 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 supplemented with 5 U·ml^{-1} of recombinant human interleukin-2, a gift from Takeda Chemical Industries Ltd, Osaka.

Reagents

Conditioned medium of HIL-3 cells (HIL-3 sup) was obtained from the supernatant of a 3 day culture of HIL-3 cells at an initial concentration of 1×10^6 cells·ml^{-1}, collected in large amounts and stored at -40°C until use. Platelet activating factor (PAF), 1-0-hexadecy1-2-0-acetyl-sn-glycero-3-phosphorylcholine purchased from Bachem Feinchemikalien AG, was dissolved in Dulbecco's phosphate buffered saline (PBS) with 0.1% FCS at a concentration of 10^{-4}M and diluted with Hanks balanced salt solution (HBSS) (Nissui Pharmaceutical Co. Ltd) at the time of use. Ketotifen, a gift from Sandoz Pharmaceutical Co. Ltd, Tokyo, was dissolved in the same PBS and diluted with HBSS at the time of use. CV-3988, rac-3-(N-n-octadecylcarbamoyloxy)-2-methoxypropyl 2-thiazolioethyl phosphate, a gift from Takeda Chemical Industries Ltd, Osaka, was dissolved in ethanol, stored at -40°C and diluted with HBSS at the time of use.

Cell culture

EoL-1 cells were suspended in the culture medium at a concentration of 5×10^5 cells·ml^{-1} with 10% HIL-3 sup and cultured for 9 days at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium containing HIL-3 sup was renewed, and cell counts were readjusted to a concentration of 5×10^5 cells·ml^{-1} on days 3 and 6.

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^{-7}M PAF was placed in the lower compartment, and the cell suspension (4×10^6 cells in 0.8 ml of the culture medium) was placed in the upper compartment. The chambers were incubated at 37°C with 5% CO_2 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 1×10^6 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 HBSS containing 1.65×10^{-6}M nitrobenzoxadiazol (NBD)-phallacidin (Molecular Probes, Junction City, OR) and 100 μg·ml^{-1} of lysophosphatidyl-choline and analysed by FACS (FACS 440, Becton Dickinson, Mountain View, CA) with the 488 nm line from 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 1×10^6 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% CO_2 and 95% air. Then 10^{-7}M 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:

$$\left(1 - \frac{\text{MFI of the cells pre-incubated with CV-3988 or ketotifen}}{\text{MFI of the cells pre-incubated without CV-3988 or ketotifen}}\right) \times 100\ (\%)$$

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 5×10^5 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

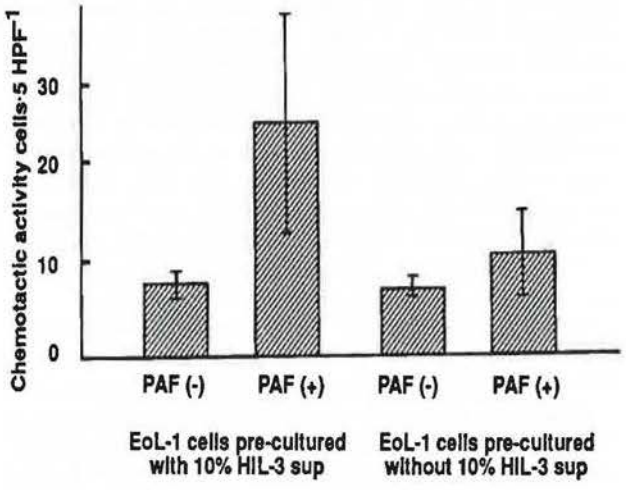

Fig. 1. – PAF-induced chemotaxis of EoL-1 cells. Eol-1 cells precultured with or without 10% HIL-3 sup for 9–12 days were stimulated with 10^{-7}M PAF, and chemotactic activity was determined by the Boyden chamber method. Representative data of three experiments are shown. PAF: platelet activating factor; HIL-3 sup: conditioned medium of human T-cell line: EoL-1: human eosinophilic leukaemia cell line.

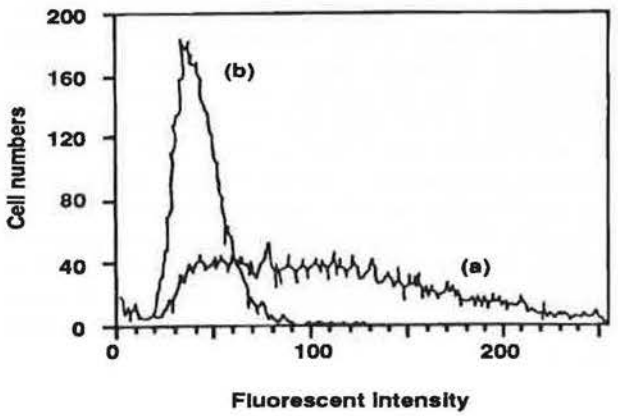

Fig. 2. – PAF-induced actin polymerization in EoL-1 cells. Data shown are FACS-generated histograms of 10,000 EoL-1 cells pre-cultured with 10% HIL-3 sup for 9 days. Fluorescent intensity was determined (a) with and (b) without the addition of 10^{-7}M PAF at 25°C for 45 s. Fluorescence data were collected with linear amplification, and the specific cell immunofluorescence was expressed as channel numbers on a linear scale. The pattern was confirmed by repeated experiments. For abbreviations see legend to figure 1.

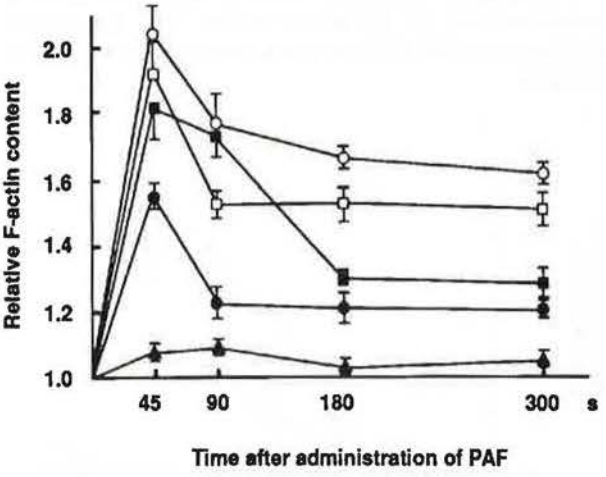

Fig. 3. – F-actin content of 10% HIL-3 sup-induced EoL-1 cells stimulated with PAF. EoL-1 cells precultured with 10% HIL-3 sup for 9 days were stimulated with 10^{-10}M (▲), 10^{-9}M (●), 10^{-8}M (■), 10^{-7}M (○) or 10^{-6}M (□) PAF at 25°C. Mean fluorescent intensity (MFI) was determined at the indicated intervals after the administration of PAF. The relative F-actin contents were expressed as the ratio of the MFI of EoL-1 cells stimulated with PAF to the MFI of EoL-1 cells not stimulated with PAF. The data represent the mean±SD (n=4). For abbreviations see legend to figure 1.

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^{-6}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^{-5}M, and the maximum inhibition reached 80% at a concentration of 10^{-5}M. Ketotifen also showed a dose-dependent inhibition of actin polymerization at concentrations of 10^{-9}M to 10^{-5}M. The maximum inhibition reached 40% at a concentration of 10^{-5}M of ketotifen.

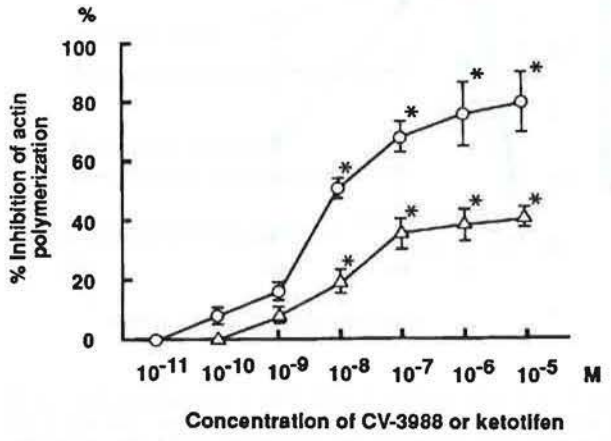

Fig. 4. – Inhibitory effect of CV-3988 and ketotifen on PAF-induced actin polymerization. HIL-3 sup-induced EoL-1 cells were pre-incubated with CV-3988 (○) or ketotifen (△). F-actin content was determined as mean fluorescent intensity (MFI) after stimulation with 10^{-7}M PAF at 25°C for 45 s. The inhibitory effects of CV-3988 and ketotifen are expressed as explained in Materials and methods. The data represent the mean±SD (n=5). *: $p<0.01$. For abbreviations see legend to figure 1.

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

	Control	CV-3988	Ketotifen
Cell counts $\times 10^5 \cdot ml^{-1}$	10.0±0.8	8.9±0.7	10.7±1.3
Viability %	83.6±1.4	82.8±4.4	83.1±3.0
		p>0.05	
			p>0.05

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±SD (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^{-8}M to 10^{-7}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 (Tsuruta *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 ^{3}H-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 10^{-7}M of ketotifen) in human peripheral blood neutrophils (Tsuruta *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 administration of ketotifen [4, 11]. 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.

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Le ketotifene inhibe la polymérisation de l'actine induite par le PAF dans une lignée cellulaire leucémique éosinophile chez l'homme. M. Morita, S. Tsuruta, K.J. Mori, M. Mayumi, H. Mikawa.

RÉSUMÉ: Les effets inhibiteurs du ketotifene sur la polymérisation de l'actine induite par le PAF dans une lignée cellulaire leucémique éosinophilique de l'homme (EoL-1), a été examinée par cytométrie de flux en utilisant des réactifs spécifiques pour la forme filamenteuse d'actine (F-actin). La

polymérisation de l'actine a été considérée comme essentielle à la motilité des cellules, à la chimiotaxie, et à la chimiokinèse, et dès lors reflète la réaction chemotactique 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^{-6} 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^{-5} M. Le ketotifene a inhibé jusqu'à 40% de la polymérisation de l'actine induite par le PAF dans less cellules EoL-1, de manière dose-dépendante, à des concentrations de 10^{-9} M à 10^{-5} M. Ces résultants suggèrent que le ketotifene pourrait jouer un rôle important dans la prévention de l'inflammation induite par les éosinophiles dans les maladies allergiques, en inhibitant la chimiotaxie des éosinophiles induite par le PAF.

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