The antiallergic drug oxatomide promotes human eosinophil apoptosis and suppresses IL-5–induced eosinophil survival

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Background: Eosinophils accumulated in sites of allergic inflammation are thought to play a crucial role in the pathogenesis of allergic disorders including asthma, allergic rhinitis, and atopic dermatitis, and tissue eosinophilia is attributable to increased eosinophil survival or decreased eosinophil apoptosis.
Objective: Effects of the antiallergic, histamine H_1 blocker oxatomide on viability and apoptosis of eosinophils isolated from the peripheral blood of atopic subjects were studied.
Methods: Eosinophil viability and apoptosis were evaluated by using a colorimetric assay and annexin V–labeling, caspase-3 activity, and DNA fragmentation assay.
Results: The viability of eosinophils increased in the presence of IL-5 (10 ng/mL), confirming that IL-5 prolongs eosinophil survival in vitro. Application of oxatomide at concentrations over 20 μmol/L for 24 hours decreased the IL-5–induced enhancement of eosinophil viability. Double staining of the cells with annexin V and propidium iodide showed that deprivation of IL-5 promoted spontaneous eosinophil apoptosis and that oxatomide facilitated apoptosis and suppressed the prolongation of eosinophil survival stimulated by IL-5. In the absence of IL-5, approximately 71% and 96% of eosinophils after 24 and 48 hours, respectively, underwent spontaneous apoptosis. IL-5 decreased the rate of eosinophil apoptosis to 38% and 52% after 24 and 48 hours, respectively. Oxatomide increased eosinophil apoptosis in a concentration-dependent manner in the presence of IL-5. Furthermore, oxatomide increased caspase-3 activity and DNA fragmentation.
Conclusion: We demonstrated that oxatomide possesses a novel therapeutic effect of apoptosis promotion on eosinophils and prevents the antiapoptotic effects of IL-5, suggesting that oxatomide may contribute to resolution of tissue eosinophilia in allergic inflammation. (J Allergy Clin Immunol 2003;111:567-72.)

Key words: *Eosinophil, apoptosis, oxatomide, allergic inflammation*

Abbreviations used
LAR: Late asthmatic response
PI: Propidium iodide
PS: Phosphatidylserine
WST-1: 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate
z-VAD: Benzyloxycarbonyl-Val-Ala-Asp-(Ome) fluoromethyl ketone

For the past decade, pharmacologic management of atopic diseases including bronchial asthma, allergic rhinitis, and atopic dermatitis has substantially progressed. This success is thought to be derived from advances in understanding pathophysiology of these diseases and development of anti-inflammatory drugs and improvement of their use, based on newly discovered mechanisms of the diseases.[1-6] Most of the anti-inflammatory drugs inhibit infiltration and activation of eosinophils at the sites of diseases including the bronchial and nasal mucosa and the skin. The cytokines such as IL-3, IL-5, and GM-CSF are crucial mediators for eosinophil accumulation and survival in the allergic inflammatory sites, and depletion of these cytokines promotes spontaneous eosinophil apoptosis in vivo and in vitro.[7,8] Caspases, a family of cysteine proteases, are activated in the interior of cells during the process to apoptosis and cleave specific death substrates, thus modulating the morphologic and biochemical changes characterizing the apoptotic process in many cells. The activation of initiator caspase-8, caspase-9, or the death stimuli results in the activation of effector caspases, caspase-3, -6 and -7, in a direct and indirect manner.[9] Suppression of prolonged eosinophil survival induced by IL-5 is a potential therapeutic strategy for resolution of allergic inflammation in asthma and dermatitis.

Glucocorticoids are the most potent drugs to treat eosinophilic inflammation and allergic diseases.[10] Although precise mechanisms of glucocorticoid resolution of allergic inflammation are not fully clarified, glucocorticoids exert their therapeutic actions on diverse levels to inhibit eosinophilic inflammatory processes. Glucocorticoids suppress generation of IL-3, IL-5, and GM-CSF by T lymphocytes, leading to deprivation of the eosinophil survival cytokines,[11,12] and directly inhibit prolongation of eosinophil survival by these cytokines.[13,14] Theophylline is also known to promote eosinophil apoptosis.[15,16] These drugs are actually used for treatment for allergic inflammatory diseases such as bronchial asthma; however, their clinical use is dimin-

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Supported by The Japan Health Science Foundation, Tokyo, Japan (KH71067) and by The Kyowa Hakko Research grant.
Received for publication January 23, 2002; revised November 8, 2002; accepted for publication November 18, 2002.
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0091-6749/2003 $30.00 + 0
doi:10.1067/mai.2003.136

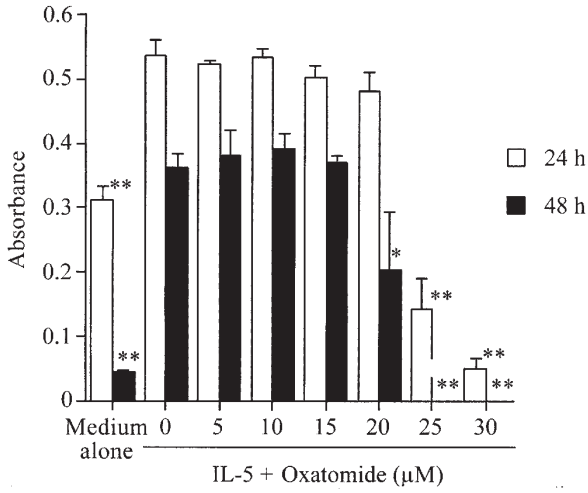

FIG 1. Oxatomide inhibits viability of eosinophils stimulated by IL-5. The viability is expressed as values of absorbance. Data are expressed as mean ± SE (n = 3) and representative of 3 independent experiments. *$P < .05$, **$P < .01$; significantly different from the IL-5–treated and 0 μmol/L oxatomide–treated group.

ished by adverse effects.[17-19] Furthermore, it has been shown that some patients with bronchial asthma are resistant to glucocorticoid treatment.[20,21] Therefore, development of a new drug capable of promoting eosinophil apoptosis without severe side effects has been expected.

Therapeutic effects of oxatomide, an antiallergic drug effective against allergic rhinitis and atopic dermatitis, are mediated by blockade of histamine H_1 receptor and inhibition of release of both preformed mediators such as histamine and de novo synthesized mediators such as leukotrienes and prostaglandins from mast cells and basophils.[22] In addition, it has also been reported that oxatomide has inhibitory effects on oxygen radical production and peptide-leukotriene release from guinea pig eosinophils,[23] implying that this drug may affect diverse functions in inflammatory cells including eosinophils.

In this report, the effects of oxatomide on apoptosis of peripheral eosinophils isolated from atopic subjects were examined. We demonstrate that oxatomide is a potent inducer of eosinophil apoptosis and suppresses prolongation of eosinophil survival induced by IL-5.

METHODS

Materials

RPMI 1640 and FCS were purchased from GIBCO-RBL (Rockville, Md). IL-5 was obtained from Genzyme (Cambridge, Mass). Annexin-V-FLUOS Staining Kit and Cell Proliferation Reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Benzyloxycarbonyl-Val-Ala-Asp-(Ome) fluoromethyl ketone (z-VAD) was obtained from Peptide Institute (Minoh, Japan). Other chemicals were obtained from Sigma (St Louis, Mo). Oxatomide was provided by Kyowa Hakko Kogyo (Tokyo, Japan) and was dissolved in 0.1N HCl at the concentration of 2.0×10^{-3} mol/L. The vehicle HCl did not interfere with experiments at concentrations up to 1.5%.

Isolation of eosinophils

Peripheral venous blood from atopic donors was anticoagulated with heparin (100 U/mL), and eosinophils were isolated by using an immunomagnetic procedure[24] as previously described,[25] except that contaminating red blood cells were removed by hypotonic lysis to avoid the known effects of ammonium chloride on eosinophil responses.[26] Eosinophil viability, assessed by exclusion of trypan blue, and purity, assessed by Randolf staining, were greater than 99.5%.

Measurement of eosinophil viability

Cell viability was examined by using a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 to formazan by mitochondorial dehydrogenases in viable cells, according to the manufacturer's instruction. Briefly, cells were cultured with oxatomide or vehicle at the density of 2×10^5 cells/well in 100 μL RPMI 1640 containing 2% heat-inactivated FCS in the presence or absence of IL-5 (10 ng/mL) for indicated time periods and were thereafter cultured with WST-1 for 4 hours. The absorbance of formazan product was measured at 440-nm wavelength. The assay was performed in triplicate, and the cell viability was expressed as a value of absorbance.

Flow cytometry of eosinophils for detection of apoptosis

The exposure of phosphatidylserine (PS) to the outer side of plasma membrane has been shown to be a sensitive marker of apoptosis in eosinophils and other cell types.[27,28] PS was detected with annexin V, and the integrity of cell membrane was examined by using propidium iodide (PI). Briefly, cells were cultured as described above in 200 μL culture medium and were washed with PBS and stained with FITC-conjugated annexin V and PI for 10 minutes according to the manufacturer's instructions. The cells were then analyzed by a fluorescence-activated cell sorter Calibur analyzer (Becton Dickinson, Mountain View, Calif), with excitation at 488 nm and detection with a 515-nm bandpass filter for FITC and a 580-nm longpass filter for PI. Flow cytometry histograms of 10,000 events were obtained.

Determination of caspase-3 activity

The caspase-3 activity in cell lysate was colorimetrically measured by using CaspACE Assay System (Promega, Madison, Wis).

DNA fragmentation assay

Cell lysis, DNA isolation, and agarose gel electrophoresis were carried out by using Apoptosis Ladder Detection Kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's procedure.

Statistics

Results are expressed as mean ± SE. Comparisons between groups were performed with a 1-way analysis of variance followed by Dunnett test (between a control and all means) or by Bonferroni multicomparison test (between 2 groups).

RESULTS

In the absence of IL-5, the viability of eosinophils spontaneously decreased. The viability after 24- and 48-hour culture periods was 60% and 15%, respectively, of the IL-5–treated cells. In the presence of 10 ng/mL IL-5, eosinophil viability markedly increased. Application of oxatomide concentration-dependently inhibited the IL-5–promoted eosinophil viability at concentrations ranging from 20 to 30 μmol/L after 24-hour treatment. The

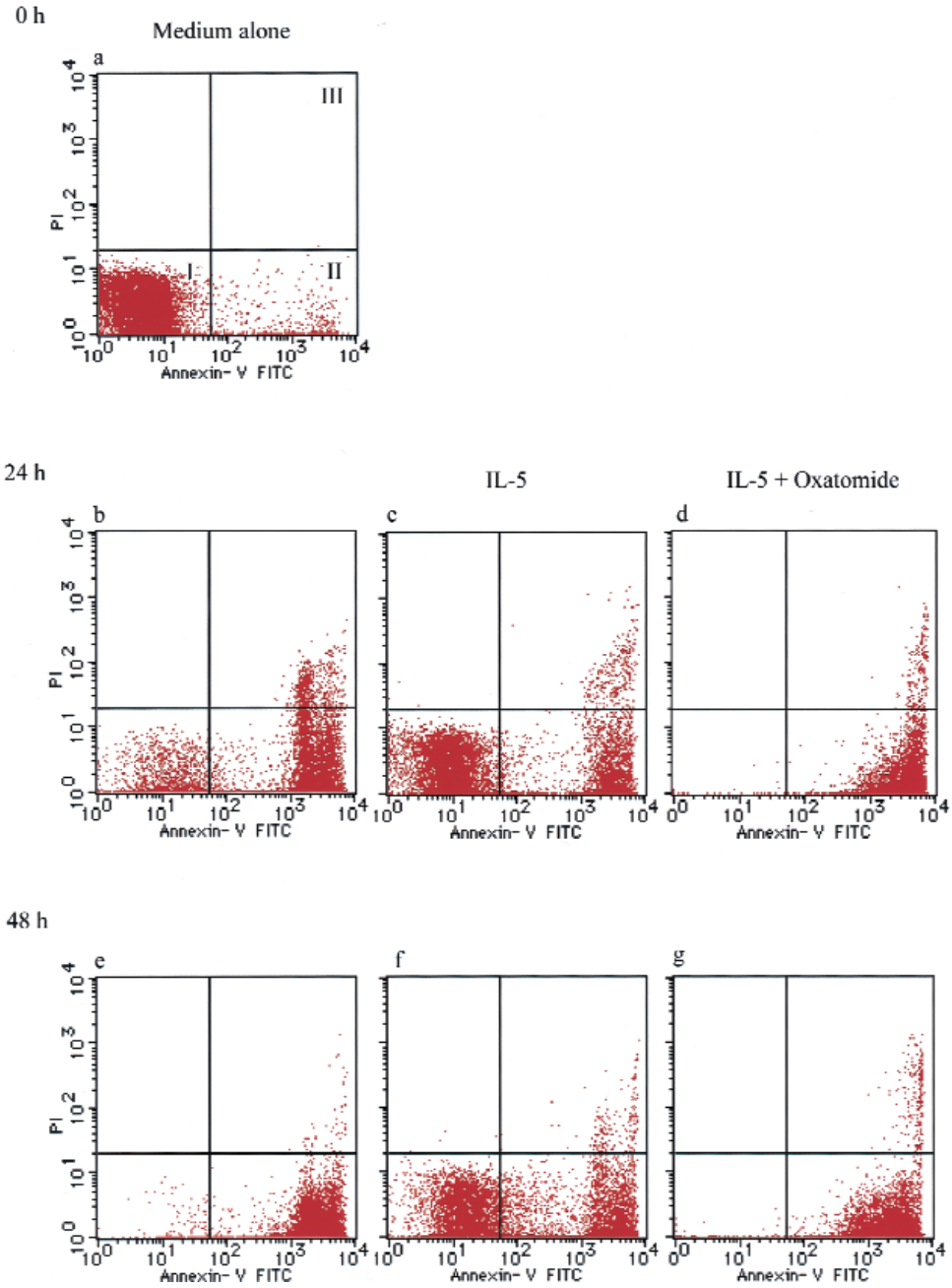

FIG 2. Flow cytometric analysis of eosinophil apoptosis. Cells were cultured with vehicle, 10 ng/mL IL-5, and 30 μmol/L oxatomide as indicated. Annexin V– and PI-negative cells are regarded as living (*I*), annexin V–positive and PI-negative cells as apoptotic (*II*), and annexin V– and PI-positive cells as late apoptotic (*III*). Data are representative of 4 separate experiments.

oxatomide-induced reduction was seen at a lower concentration of 20 μmol/L after 48 hours (Fig 1).

To determine whether the inhibitory effect of oxatomide on eosinophil viability is caused by apoptosis, the translocation of PS from the inner side of the plasma membrane to the outer layer was assessed by using the specific binding of annexin V to PS (Fig 2, Table I). More than 97% of the freshly isolated cells were in a normal survival condition negative to annexin V and PI. In the absence of IL-5, approximately 71% and 96% of the cells after 24 and 48 hours, respectively, were annexin V–positive and PI-negative, indicating that deprivation of IL-5 in culture medium promoted spontaneous eosinophil apoptosis. Application of 10 ng/mL IL-5 suppressed the induction of eosinophil apoptosis in that annexin V–positive cells were 38% and 52% after 24 and 48 hours, respectively, confirming the previous reports that IL-5 strongly prevented apoptosis and promoted eosinophil

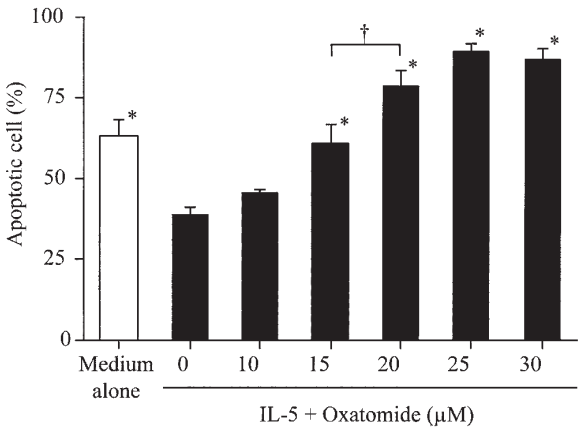

FIG 3. Oxatomide concentration-dependently promotes eosinophil apoptosis. Cells were incubated with various concentrations of oxatomide in the presence of 10 ng/mL IL-5 for 24 h. Data are expressed as mean ± SE (n = 6). **P* < .05, ***P* < .01; significantly different from the IL-5–treated and 0 μmol/L oxatomide–treated group. †*P* < .05.

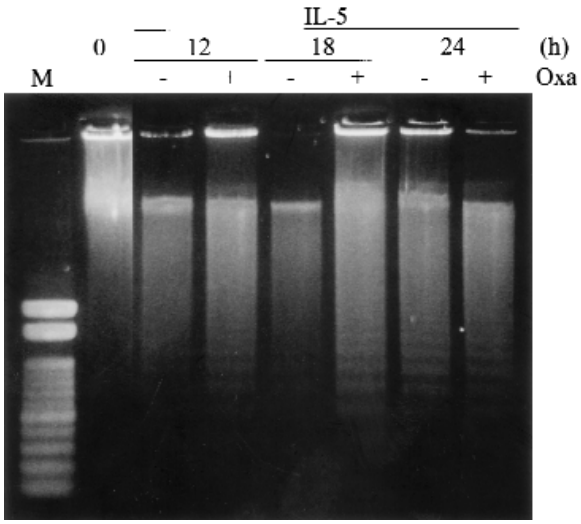

FIG 5. Oxatomide (*OXA*)-induced DNA fragmentation in eosinophils. Cells were treated with 30 μmol/L oxatomide in the presence of 10 ng/mL IL-5. *M*, 100 bp DNA marker.

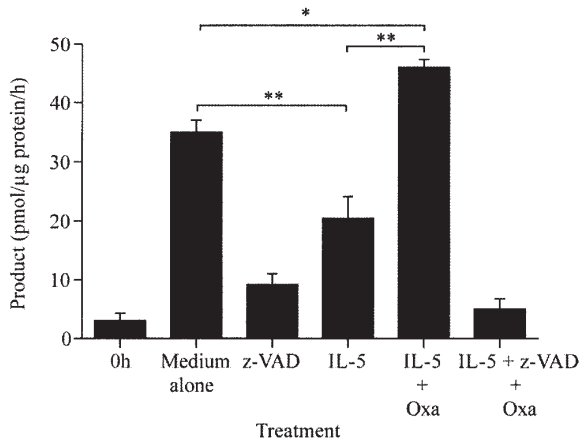

FIG 4. Effects of oxatomide (*OXA*) on caspase-3 activity in eosinophils. Cells were treated with drugs indicated for 18 h. Concentrations are as follows: 10 ng/mL IL-5, 50 μmol/L z-VAD-fmk, 30 μmol/L oxatomide. Data are expressed as means ± SE (n = 3) and representative of 3 independent experiments. **P* < .05, ***P* < .01.

survival. Oxatomide (30 μmol/L) induced eosinophil apoptosis in the presence or absence of IL-5 in that annexin V–positive apoptotic cells were greater than 95% and greater than 97% after 24- and 48-hour treatment, respectively. Late apoptotic eosinophils that were simultaneously positive to both annexin V and PI were less than 9% under all experimental conditions. Annexin V–negative and PI-positive cells were less than 0.1% in these conditions. In the presence of IL-5, treatment with oxatomide for 24 hours increased the rate of apoptotic cells in a concentration-dependent manner (Fig 3).

Executioner caspases including caspase-3 are thought to be responsible for the actual destruction of the cell.[8] The activity of caspase-3 in the cell lysates obtained from eosinophils just after isolation (0 hour) and treatment with various drugs for 18 hours were determined (Fig 4). Caspase-3 activities increased in the absence of IL-5 (medium alone), and application of the broad-spectrum caspase inhibitor z-VAD (50 μmol/L) inhibited the enzymatic activities. Although treatment with IL-5 suppressed an increase in spontaneous activities, oxatomide overcame the effect of IL-5, suggesting that oxatomide induces activation of caspase-3.

Next, effects of oxatomide on cellular DNA fragmentation were examined in the presence of IL-5. Whereas spontaneous DNA fragmentation was observed even in the presence of IL-5 (10 ng/mL) after 24 hours, 30 μmol/L oxatomide facilitated the progress of cellular DNA fragmentation in that oxatomide-induced DNA ladder was observed after 12 hours, indicating that oxatomide accelerates eosinophil apoptosis (Fig 5).

DISCUSSION

Eosinophils have been considered as major inflammatory cells that play a critical role in allergic inflammation including the late asthmatic response (LAR) subsequently evoked after immediate asthmatic response by allergen challenge.[29-33] A scenario has been proposed that suggests that eosinophil migration into the airway on the LAR is initiated by mast cell activation in the local microenvironment. One of the rapidly developing areas in the fields of allergy and immunology is the study of programmed cell death, apoptosis. Apoptosis is characterized by condensation of nuclear chromatin, shrinkage of the cell, destruction of both the nucleus and cytoplasm into multiple apoptotic bodies, and elimination of the cell by phagocytic cells. In vitro, cultured eosinophils are known to die within several days as a result of apoptosis, and their survival can be prolonged when incubated with eosinophilopoietic cytokines such as IL-3, GM-CSF, and IL-5.[34-36] IL-5 inhibits eosinophil apoptosis with induction of new RNA and protein synthesis, but the details are unclear.[37]

TABLE I. Oxatomide promotes eosinophil apoptosis and suppresses IL-5–induced eosinophil survival

Treatment	Living (%)	Apoptotic (%)	Late apoptotic (%)
24 h			
Medium alone	20.2 ± 1.1*	71.4 ± 1.5*	8.6 ± 1.9
Oxatomide	3.8 ± 3.5*	95.4 ± 3.4*	0.8 ± 0.2
IL-5	57.6 ± 0.6	37.4 ± 0.2	4.5 ± 0.7
IL-5 + oxatomide	0.3 ± 0.02*	97.7 ± 0.3*	2.0 ± 0.3
48 h			
Medium alone	2.6 ± 0.4*	95.6 ± 1.1*	1.8 ± 0.7
Oxatomide	0.4 ± 0.1*	98.8 ± 0.2*	0.8 ± 0.2
IL-5	46.1 ± 2.0	52.0 ± 2.1	1.9 ± 0.7
IL-5 + oxatomide	0.8 ± 0.3*	97.2 ± 0.3*	1.9 ± 0.5

Cells were cultured with 30 μmol/L oxatomide or vehicle in the presence or absence of 10 ng/mL IL-5 for 24 or 48 h. Living cells (annexin V– and PI-negative), apoptotic cells (annexin V–positive and PI-negative), and late apoptotic cells (annexin V– and PI-positive). Data are expressed as mean ± SE (n = 3).
*$P < .01$, significantly different from the respective IL-5–treated groups.

We demonstrated that the antiallergic, histamine H_1 receptor blocker oxatomide is a potent promoter of eosinophil apoptosis. Oxatomide promotion of PS externalization, caspase-3 activation, and cellular DNA fragmentation indicates definitive evidence that oxatomide induces apoptosis of eosinophils via a caspase-dependent mechanism. IL-3, IL-5, and GM-CSF derived from other inflammatory cells such as T lymphocytes and mast cells induce eosinophil survival, and accumulation of eosinophils in the airway of asthmatic patients, ie, tissue eosinophilia, propagates allergic inflammation and correlates with severity of the disease.[38-40] IL-5 is a most important cytokine for eosinophil survival in sites of allergic inflammation. The balance between migration of eosinophils from the peripheral blood and clearance of the cells from the tissue is a critical mechanism for determination of eosinophil numbers in allergic inflammatory tissues. Elimination of eosinophils via apoptosis without histotoxic mediator release may be one of the therapeutic strategies for the resolution of allergic inflammation. Although the endogenous cytokines such as IL-12 are known to promote eosinophil apoptosis, IL-5 overcomes the IL-12–induced apoptosis.[41] The antiasthmatic drugs such as glucocorticoids and theophylline are also known to promote eosinophil apoptosis.[14-16] However, application of these drugs for long-term therapy is restricted because of many adverse effects.[17-19] In contrast, oxatomide so far has not been reported to have severe side effects.

Pharmacologic evidence indicates the expression of histamine H_1 receptors on human eosinophils.[42] Cetirizine resembles oxatomide in pharmacologic profiles, ie, an antiallergic, histamine H_1 receptor blocker.[43] Because cetirizine did not decrease eosinophil viability at concentrations up to 100 μmol/L (unpublished observation), it seems that oxatomide-induced apoptosis is not mediated by histamine H_1 receptor blockade on eosinophils. In addition to previously reported actions of oxatomide such as the histamine H_1 receptor blockade and the inhibition of chemical mediator release,[44] the promotion of eosinophil apoptosis is one of the beneficial therapeutic effects as an antiallergic drug.

Oxatomide promoted eosinophil apoptosis at concentrations greater than 15 μmol/L. In a similar concentration range, it has been shown that the drug exerts several actions on inflammatory cells such as mast cells, neutrophils, and eosinophils. In the mucosal mast cell line rat basophilic leukemia cells, oxatomide inhibits influx of extracellular calcium and degranulation.[45] Oxatomide reduces superoxide generation stimulated by fMet-Leu-Phe in neutrophils and oxygen radical generation and peptide-leukotriene release in eosinophils.[23,46] The inhibitory effect of oxatomide on calcium influx is attributable to the inhibition of degranulation from mast cells and of arachidonic acid metabolism in inflammatory cells.[44] Although it has been shown that inhibitors of calcium influx such as ketotifen and econazole are capable of promoting the mast cell to apoptosis,[47] precise mechanisms underlying promotion of eosinophil apoptosis by oxatomide remain unclear.

It is demonstrated that the antiallergic, histamine H_1 receptor blocker oxatomide promotes eosinophil apoptosis. Oxatomide seems to have multiple therapeutic effects on allergic inflammation via the induction of eosinophil apoptosis in addition to the inhibition of histamine H_1 receptors and chemical mediator release.

We would like to thank Ms Hiroko Kawazu and Ms Yasuko Nonaka for their expert technical assistance.

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