Inhibition of Eosinophil Survival by a Selective Inhibitor of Phosphodiesterase 4 via the Induction of Apoptosis

Way Wang, Kazuko Masu, Gen Tamura, Ko Suzuki, Keiko Ohwada, Kaori Okuyama, Kunio Shirato, Motoaki Takayanagi, and Isao Ohno*

a Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine; and b Department of Respiratory and Infectious Diseases, Tohoku University Graduate School of Medicine; 1–1 Seiryo-machi, Aoba-ku, Sendai 980–8574, Japan; c Miyagi Red Cross Blood Center; 2–37 Syoun-wa-machi, Aoba-ku, Sendai 981–0913, Japan; and d Department of Pathophysiology, Tohoku Pharmaceutical University; 4–4–1 Komatsushima, Aoba-ku, Sendai 981–8558, Japan. Received September 1, 2004; accepted December 4, 2004

Selective inhibitors of phosphodiesterases (PDEs) have been suggested to have anti-inflammatory effects on bronchial asthma through the inhibition of chemotaxis, adhesion, degranulation, the respiratory burst, and survival prolongation of eosinophils. However, the mechanisms by which these agents inhibit eosinophil survival remain unclear. We therefore investigated the possible mechanisms of inhibitory effects of selective inhibitors of PDE 3 (cilostazol) and PDE 4 (rolipram) on granulocyte-macrophage colony-stimulating factor (GM-CSF)-mediated eosinophil survival. Purified blood eosinophils were cultured with medium alone or GM-CSF (0.01 ng/ml) in the presence or absence of the agents for up to 6 d. DNA was extracted from freshly isolated eosinophils and eosinophils cultured for 2 d with medium alone, GM-CSF, or GM-CSF in the presence of the agents, and analyzed using agarose gel electrophoresis. The presence of rolipram (10⁻⁴, 10⁻⁵, 10⁻⁶M), but not cilostazol, significantly inhibited eosinophil survival at days 2, 4, and 6. A laddering pattern was observed in the DNA of eosinophils cultured with medium alone and with GM-CSF in the presence of rolipram. The results reveal that selective PDE 4 inhibitors inhibit GM-CSF-mediated eosinophil survival through the induction of apoptosis.

Key words eosinophil; phosphodiesterase inhibitor; apoptosis; survival; granulocyte-macrophage colony-stimulating factor (GM-CSF)

Bronchial asthma is a disease characterized by chronic airway inflammation associated with the accumulation and activation of inflammatory cells, such as eosinophils, in the bronchial wall and airway lumen. Eosinophils at the site of inflammation release preformed and newly synthesized mediators, including eosinophil cationic protein, major basic protein, leukotriene C₄, and reactive oxygen. These mediators contribute to the formation of the pathological features of asthmatic airways such as bronchoconstriction, mucus hypersecretion, microvascular leakage, submucosal edema, and epithelial shedding, which lead to the clinical features of asthma, hyperresponsiveness, and airway narrowing.1–5

Eosinophils cultured in the absence of hematopoietic cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-5 undergo apoptosis, which is physiologic cell death characterized by morphologic changes such as membrane blebbing and nuclear condensation, and by the degradation of DNA into oligonucleosome-sized fragments showing a “ladder” pattern on agarose gel.6–8 Apoptotic eosinophils have been shown to be removed from the tissue without releasing inflammatory mediators after ingestion by macrophages,8 which is believed to be one of the important mechanisms in the resolution of inflammation.9 GM-CSF and IL-5, which have been detected in sputum, bronchoalveolar lavage fluid, and bronchial tissues from asthmatics,9 prolong the survival of tissue eosinophils through the prevention of apoptosis,10,11 leading to the development and maintenance of airway inflammation. Therefore the induction of apoptosis in eosinophils would be beneficial in the treatment of asthma. The induction of apoptosis in airway eosinophils by the administration of anti-Fas antibody or inhaled steroid is reported to be associated with the reduction or termination of asthmatic airway inflammation in vivo.12,13 cAMP is well known as a second messenger mediating intracellular signal transduction evoked by the binding of agonists to their cell-surface receptors. The concentration of intracellular cAMP is dependent on the catalysis rate of ATP to cAMP by adenylate cyclase and of cAMP to 5′-adenosine monophosphate by cyclic nucleotide phosphodiesterases (PDEs).14 PDEs comprise at least 11 families containing, in total, more than 50 different PDE enzyme variants, the differentiation of which is based on the primary protein and cDNA sequences, substrate specificity, regulation of enzymatic activity, and calcium/calmodulin dependence.15 The increased concentration of intracellular cAMP is known to suppress the functions of inflammatory cells.16,17 With regard to eosinophils of which the PDE isoenzyme is exclusively type 4,17 selective inhibitors of PDE 4 and theophylline, a nonselective PDE inhibitor, have been shown to inhibit chemotaxis, adhesion, degranulation, and the release of active oxygen by an increase in the cAMP content.14,16,18 Furthermore, PDE 4 inhibitors and theophylline, as well dibutyryl cAMP (d-cAMP), a cAMP analogue, have been also demonstrated to reduce eosinophil survival prolonged by GM-CSF or IL-5.19–24 Together with the pathogenic roles of eosinophils in asthma, there has been increased interest in the therapeutic benefit of these agents in asthma.

However, the mechanisms by which PDE 4 inhibitors suppress eosinophil survival remain to be elucidated,23,24 while theophylline and d-cAMP were shown to decrease the survival rate via the induction of apoptosis.19–22 Therefore, to evaluate whether PDE 4 inhibitors have the ability to cause eosinophils to undergo apoptosis, we examined the effects of selective PDE isoenzyme inhibitors on eosinophil survival prolonged by GM-CSF.

* To whom correspondence should be addressed. e-mail: iohno@tohoku-pharm.ac.jp © 2005 Pharmaceutical Society of Japan
MATERIALS AND METHODS

Eosinophil Purification Eosinophils were isolated from the peripheral blood of healthy donors after removal of mononuclear cells by Percoll density-gradient centrifugation and depletion of neutrophils by immunomagnetic negative selection as previously described. Peripheral blood anticoagulated with sodium citrate 85 mm, citrate 42 mm, and 2.2% (w/v) glucose was obtained from Miyagi Red Cross Blood Center (Sendai, Japan). The purity of eosinophils as determined by light microscopic examination of cytospin preparations stained with Diff-Quik (International Chemical Ltd., Osaka, Japan) was higher than 99%. The viability assessed by trypan blue dye exclusion was higher than 99%.

Eosinophil Culture Freshly purified eosinophils were resuspended at a concentration of 1×10⁶/ml in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Cansers International Inc., Toronto, Canada), penicillin G (Meiji Seika Co. Ltd., Tokyo, Japan) 100 U/ml, and streptomycin (Meiji) 100 μg/ml. Two hundred microliters of the cell suspension were cultured at 37°C in 5% CO₂ in the presence or absence of GM-CSF (Genzyme, Cambridge, MA, U.S.A.) (0.01, 0.1, or 1 ng/ml) with or without theophylline (Wako Pure Chemical Industries Ltd., Osaka, Japan) (10⁻³ M), rolipram (Biomol. Research Laboratories, Inc., Plymouth Meeting, PA, U.S.A.), a selective inhibitor of PDE 4, (10⁻⁶, 10⁻⁵, or 10⁻⁴ M) or cilostazol (provided by Ohtsuka Pharmaceutical Co. Ltd., Tokyo, Japan), a selective inhibitor of PDE 3, (10⁻⁶, 10⁻⁵ or 3×10⁻⁵ M) in 96-well flat-bottomed tissue culture plates (Corning Glass Works, Corning, NY, U.S.A.). Every 2 d eosinophils were counted in a hemocytometer and their viability assessed using trypan blue dye exclusion. The survival rate was calculated with the formula: survival, %=(the number of live cells/2×10⁵ which was the number of live cells at the beginning of culture)×100. The culture was performed in duplicate for each culture condition and all results represent the mean of four experiments.

DNA Fragmentation Analysis DNA was extracted, as previously described, with some modifications, from 3 to 4 million freshly purified eosinophils and those cultured as described above for 2 d with medium alone, GM-CSF 0.01 ng/ml, or GM-CSF 0.01 ng/ml in the presence of rolipram 10⁻³ M or d-cAMP 10⁻³ M (Sigma Chemical Co., St. Louis, MO, U.S.A.). Two micrograms of each DNA sample and a molecular size marker (Roche Diagnostics GmbH, Mannheim, Germany) were electrophoresed through 2.8% agarose gel (Nippon Gene Co. Ltd., Toyama, Japan) containing ethidium bromide (Sigma) (10 μg/ml) and viewed under UV light.

Data Analysis All data are expressed as mean±S.D. The significance of differences in survival was determined using the Mann-Whitney U-test. A p value of less than 0.05 was considered significant.

RESULTS

Effects of Theophylline on the Survival of Eosinophils Cultured with Various Concentrations of GM-CSF Only 4.8±3.3% (mean±S.D.) of eosinophils cultured without GM-CSF survived. In contrast, the addition of GM-CSF to eosinophil cultures resulted in a dose-dependent increase in survival on day 4 (Fig. 1). The survival rate when cultured with GM-CSF at concentrations of 0.01, 0.1, and 1 ng/ml was 42.2±13.3%, 86.5±10.3%, and 78.3±17.1%, respectively (n=4), as previously reported. Theophylline (10⁻³ M) potently inhibited the prolongation of survival induced by GM-CSF in eosinophil cultures at concentrations of 0.01 and 0.1 ng/ml, but not at 1 ng/ml, on day 4 (Fig. 1). Based on these results, GM-CSF was used at the concentration of 0.01 ng/ml to evaluate the effects of agents on eosinophil survival in the subsequent experiments.

Effects of Rolipram on Prolongation of Eosinophil Survival by GM-CSF The presence of rolipram at a concentration of 10⁻⁴ M in eosinophil cultures significantly decreased GM-CSF-mediated eosinophil survival from 71.1±3.2% to 49.5±7.0%, from 32.5±2.4% to 18.0±5.6%, and from 10.0±4.7% to 2.1±2.0% at 2, 4, and 6 d, respectively (n=4) (Fig. 2A). Dose dependency in the inhibition of eosinophil survival by rolipram was demonstrated on day 4 when survival was inhibited to 68.5±5.2%, 57.0±9.5% and 50.0±4.0% of that in eosinophils cultured with GM-CSF in the absence of rolipram at rolipram 10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M, respectively (n=4) (Fig. 2B).
Effects of Cilostazol on Prolongation of Eosinophil Survival by GM-CSF The addition of cilostazol had no significant effects on the prolongation of eosinophil survival by GM-CSF at any time point (Fig. 3A) or at any concentration (Fig. 3B) (n = 4).

Effects of Theophylline and Rolipram on Spontaneous Survival of Eosinophils The addition of either theophylline (10^{-3} M) or rolipram (10^{-4} M) alone to the culture medium did not significantly decrease eosinophil survival on day 4. The survival rate of eosinophils cultured with theophylline or rolipram alone was 97.5 ± 4.3% or 95.3 ± 7.1% of that of eosinophils cultured with medium alone.

Agarose Gel Electrophoresis of Eosinophil DNA To explore whether the mechanism of the inhibitory effect of rolipram on eosinophil survival was through the induction of apoptosis, we analyzed DNA from eosinophils using agarose gel electrophoresis (Fig. 4). Although the DNA in freshly isolated eosinophils was intact, that from eosinophils cultured with medium alone showed a ladder pattern of 180 oligomers which is characteristic of apoptosis. DNA fragmentation was prevented in eosinophils in the presence of GM-CSF (0.01 ng/ml), whereas DNA in eosinophils cultured with rolipram (10^{-4} M) even in the presence of GM-CSF (0.01 ng/ml) showed a fragmentation pattern.

To determine the involvement of cAMP in the apoptosis induced by rolipram, DNA in eosinophils cultured with GM-CSF (0.01 ng/ml) in the presence of d-cAMP (10^{-4} M) was electrophoresed. As shown in Fig. 5, the fragmentation pattern was also observed in this DNA.

DISCUSSION

Our study showed for the first time that a selective inhibitor of PDE 4 (rolipram), but not a selective inhibitor of PDE 3 (cilostazol), inhibited the prolongation of eosinophil survival by GM-CSF through inducing apoptosis as assessed by DNA fragmentation. Although PDE 4 inhibitors including rolipram have been reported to reduce eosinophil survival prolonged by culture with IL-5 or GM-CSF, whether the reduction is due to the induction of apoptosis had not been investigated. The increased apoptosis in cells of eosinophil lineage due to theophylline and specific PDE 4 inhibitors was reported only in circulating progenitor cells from asthmatics. Whether the inhibitory effects on eosinophil survival in either the previous or our study, because the PDE isoenzyme of human eosinophils is exclusively type 4.

There has been evidence showing that selective PDE 4 inhibitors increase the cAMP content, leading to the inhibition of degranulation, respiratory burst, mediator synthesis, chemotaxis, and adhesion in guinea pig and human eosinophils. The increase in intracellular cAMP by d-cAMP, cholera toxin, or theophylline also facilitates apoptosis in such type of cells as thymocytes, myeloid cell line IPC-81, and eosinophils. In the present study, we observed the induction of apoptosis, in terms of DNA fragmentation, by d-cAMP in eosinophils cultured with GM-CSF. Rolipram has been reported to cause an increase in the intracellular cAMP content of human eosinophils at the concentration of $\approx 10^{-7} \text{M}$. The cAMP content of eosinophils in-

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**Fig. 3. Effects of Cilostazol on GM-CSF-Mediated Eosinophil Survival**

(A) Eosinophils were cultured with medium alone (closed squares) or GM-CSF (0.01 ng/ml) in the presence (closed triangles) or absence (closed circles) of cilostazol ($10^{-4}$ M). Survival was assessed on days 2, 4, and 6 and expressed as mean ± S.D. (n = 4). Data were compared at each time point between the culture with GM-CSF in the presence and absence of cilostazol. (B) Eosinophils were cultured with GM-CSF (0.01 ng/ml) in the presence (closed columns) or absence (a hatched column) of cilostazol. Survival was assessed on day 4. Data are expressed as the percentage of survival in the culture with GM-CSF alone (mean ± S.D., n = 4).

**Fig. 4. Agarose Gel Electrophoresis of DNA Extracted from Eosinophils**

DNA 2 µg extracted from freshly isolated eosinophils (lane 1) and from eosinophils cultured for 2 d with medium alone (lane 2), GM-CSF (0.01 ng/ml) (lane 3), or GM-CSF (0.01 ng/ml) in the presence of rolipram ($10^{-4}$ M) (lane 4) was electrophoresed on 2.8% agarose gel. This figure represents experiments with eosinophils from three different donors. M, size marker.

**Fig. 5. Agarose Gel Electrophoresis of DNA from Eosinophils Cultured with d-cAMP**

DNA 2 µg extracted from eosinophils cultured for 2 d with GM-CSF (0.01 ng/ml) in the presence of d-cAMP ($10^{-4}$ M) (lane 1) was electrophoresed as in Fig. 4. M, size marker.
creases to approximately 400 fmol/10^6 cells in the presence of rolipram 10^{-6} M.\textsuperscript{30} Since the content of intracellular cAMP at which apoptosis in eosinophils can be observed was shown to be more than 250 fmol/10^6 cells,\textsuperscript{23} it is likely that even rolipram 10^{-8} M exhibits apoptotic activity through a cAMP-dependent mechanism in our study. Therefore it is conceivable that, although the content of cAMP in eosinophils was not measured in the current study, the PDE 4 inhibitor caused eosinophils to undergo apoptosis by the elevation of the cAMP content in eosinophils. Hallsworth \textit{et al.} reported that cholera toxin and d-cAMP inhibited the survival of eosinophils cultured with GM-CSF through the increase in intracellular cAMP. However, in contrast to our results, rolipram failed to reduce the survival rate.\textsuperscript{21} As discussed by the authors, it is possible that the level of cAMP elicited by rolipram was lower than that required to overcome the effects of a relatively high concentration (1000 pg/ml) of GM-CSF. This possibility was also reported in the case of IL-5, in which the inhibitory effect of aminophylline on eosinophil survival was significantly greater at low concentrations of IL-5 than at high concentrations.\textsuperscript{19} The concentration of GM-CSF used in our study, which was the same as in a previous report showing the inhibitory effect of rolipram on the survival rate of eosinophils cultured with GM-CSF,\textsuperscript{24} was within the range of 10 to 200 pg/ml observed in sputum and in bronchoalveolar lavage fluid from asymptomatic and symptomatic asthmatics after allergen challenge.\textsuperscript{5} Accordingly, PDE 4 inhibitors would be expected to exert their inhibitory effect on eosinophil survival in vivo.

Protein kinase A (PKA) was shown to be involved in the induction of apoptosis in GM-CSF-stimulated eosinophils by agents elevating intracellular cAMP content.\textsuperscript{21} Since GM-CSF does not decrease the cAMP content in those cells,\textsuperscript{21} the antiapoptotic effects of the agents would not be simply due to the increase in cAMP. In eosinophils, GM-CSF activates the Ras-Raf-1-MEK-mitogen-activated protein kinase pathway, Janus kinase (JAK)-signal transducer and activator of transcription pathway, and phosphoryllysinolosit (PI)-3 kinase intracellular signaling component through the common β subunit of GM-CSF and IL-5 receptors.\textsuperscript{31} Of these kinases, Lyn, Syk, and JAK2 were demonstrated to be involved in antiapoptotic signals of GM-CSF.\textsuperscript{32,33} cAMP and PKA have been reported to inhibit the activation of these kinases in neutrophils,\textsuperscript{34} platelets,\textsuperscript{35} macrophage cell line THP-1,\textsuperscript{36} and dendritic cells.\textsuperscript{37} However, the interaction of cAMP and/or PKA with these kinases in eosinophils remains to be investigated. On the other hand, unless eosinophils are stimulated with survival-enhancing cytokines, agents increasing intracellular cAMP content such as d-cAMP, cholera toxin, prostaglandin-E2, and rolipram are reported to prolong the spontaneous survival of the cells.\textsuperscript{21,22,38} Phosphoryllysinolosit, in contrast, accelerates apoptosis.\textsuperscript{21} In our study, these effects of rolipram and theophylline were not observed. The reason might be the difference in methods (apoptosis-positive cells vs. cell survival) and duration of culture (9 h vs. 96 h) for the evaluation of apoptosis between the previous and current study. The antiapoptotic effects of cholera toxin and d-cAMP were not reduced by a PKA inhibitor, in contrast to the involvement of PKA in proapoptotic effects on GM-CSF-stimulated eosinophils.\textsuperscript{21} The proapoptotic effects of theophylline were, at least in part, due to the decrease in Bcl-2 protein expression, while d-cAMP had no effects on the expression.\textsuperscript{22} As far as Bcl-2 in eosinophils, the literature is currently unclear regarding the constitutive expression.\textsuperscript{20} GM-CSF is reported neither to induce nor to enhance the expression in the cells.\textsuperscript{22,40} These findings suggest that the effects of agents increasing intracellular cAMP content on eosinophil survival could be dependent on the condition of eosinophils. The mechanisms by which cAMP modulates eosinophil survival need to be investigated further, focusing on the interaction with intracellular signal transduction and regulatory genes for apoptosis.

The profile of PDE isoenzymes has been determined in each cell type including eosinophils, lymphocytes, and macrophages,\textsuperscript{23} which play important roles in airway hyperresponsiveness and inflammation characteristic of asthma.\textsuperscript{5} Selective PDE inhibitors have been demonstrated to regulate functions by controlling the content of cAMP and/or cGMP \textit{via} the modulation of PDE activity.\textsuperscript{34,16,27} In animal models of asthma, these agents have been shown to inhibit the induction of airway hyperresponsiveness and the accumulation of inflammatory cells including eosinophils elicited by allergen challenge.\textsuperscript{14,41,42} It is assumed from our findings that the anti-inflammatory effects of theophylline and selective PDE 4 inhibitors in allergic airway inflammation were due, at least in part, to the removal of eosinophils from the inflammatory sites by the induction of apoptosis. It would therefore be of interest to assess further these agents as antiasthma drugs.

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