

Eosinophil peroxidase stimulates the release of granulocyte-macrophage colony-stimulating factor from bronchial epithelial cells

Shinji Motojima, MD,^a Tetsuya Adachi, MD,^a Ken-ichi Manaka, PhD,^b
Masafumi Arima, MD,^a Takeshi Fukuda, MD,^a and Sohei Makino, MD^a
Mibu, Japan

Background: Asthma is characterized by an accumulation of activated eosinophils in the airway. Eosinophil viability-enhancing activity is present in the sputum of patients with asthma, largely because of granulocyte-macrophage colony-stimulating factor (GM-CSF). Bronchial epithelial cells have been shown to release cytokines including GM-CSF when stimulated with IL-1 β or tumor necrosis factor- α .

Objective: The study was designed to determine whether eosinophil peroxidase (EPO) stimulates the release of GM-CSF from bronchial epithelial cells.

Methods: Epithelial cells (BEAS-2B) were cultured in serum free HD-F12 medium in a 24-well tissue culture plate until they became confluent. The cells were then exposed to EPO (5.9×10^{-8} to 5.9×10^{-7} mol/L) for 15 minutes, washed twice, and cultured in 1 ml of HD-F12. The supernatants were harvested at 3, 6, or 24 hours, and GM-CSF concentration was measured by ELISA. BEAS-2B cells were also treated with a system comprising EPO (1.9×10^{-9} to 5.9×10^{-8} mol/L) + 10^{-5} mol/L H_2O_2 + 10^{-4} mol/L Br for 24 hours.

Results: The GM-CSF concentration in the supernatant pretreated with EPO increased in a time- and concentration-dependent manner compared with control. The release of GM-CSF was not inhibited by catalase but was inhibited by cyclohexamide and by mixing of EPO with heparin, suggesting that the action is due to the cationic property of EPO. When EPO was combined with H_2O_2 and Br, 5.9×10^{-9} mol/L EPO + 10^{-5} mol/L H_2O_2 released two times more GM-CSF into the supernatants compared with control.

Conclusion: These results suggest that EPO stimulates epithelial cells to release GM-CSF and forms a self-stimulatory cycle. (*J Allergy Clin Immunol* 1996;98:S216-23.)

Key words: Asthma, BEAS-2B cell, bronchial epithelium, eosinophil peroxidase, granulocyte-macrophage colony-stimulating factor

Eosinophils are one of the most dominant infiltrating cells in the airways of patients with asthma,^{1,2} in whom they play an important role in the pathogenesis of asthma by damaging respiratory epithelium and by releasing leukotriene C4 and platelet-activating factor.³⁻⁵ When eosinophil activation in the airways was evaluated by measuring the concentration of eosinophil cationic protein in

Abbreviations used

CHX:	Cyclohexamide
EPO:	Eosinophil peroxidase
EVEA:	Eosinophil viability-enhancing activity
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
TNF- α :	Tumor necrosis factor- α

From ^athe Department of Medicine and Clinical Immunology and ^bthe Laboratory of Tissue Culture, Dokkyo University School of Medicine.

Reprint requests: Shinji Motojima, MD, Department of Medicine and Clinical Immunology, Dokkyo University School of Medicine, Mibu, Tochigi, 321-02, Japan.

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0091-6749/96 \$5.00 + 0 1/0/76962

sputum, it was very high in acute asthma and decreased gradually with intensive treatments including intravenous corticosteroids.⁶

Among many factors that can activate eosinophils, cytokines such as IL-3, IL-5, and granulo-

cyte-macrophage colony-stimulating factor (GM-CSF) are thought to be important.^{3,7} We have shown that there is eosinophil viability-enhancing activity (EVEA) in the sputum of persons with acute asthmatic, revealed to be attributable mainly to GM-CSF and partially to IL-5 by inhibition assay. In addition, the EVEA significantly correlated with the concentration of eosinophil cationic protein in the sputum, indicating that these cytokines are at least in part responsible for the eosinophil activation in the airways of patients with asthma.⁸

T lymphocytes, eosinophils, and mast cells are regarded as the source of GM-CSF in the airways,^{5,7} but bronchial epithelial cells are also regarded as its source. Cultured bronchial epithelial cells express the messenger RNA (mRNA) and release the molecule of GM-CSF in vitro, a process enhanced by tumor necrosis factor- α (TNF- α), IL-1 β ,⁹ NO₂,¹⁰ and respiratory syncytial virus.¹¹ In patients with asthma, the mRNA and the molecule of GM-CSF are expressed on the bronchial epithelium and are reduced by the use of inhaled corticosteroids.¹²⁻¹⁵

Eosinophil peroxidase (EPO), one of the four eosinophil granule basic proteins,³ is present in the sputum of persons with asthma at a high concentration¹⁶ and can induce damage to the respiratory epithelium with or without H₂O₂ and halide in vitro.¹⁷ In this study we show enhancement of GM-CSF release from bronchial epithelial cells by EPO at concentrations that do not induce epithelial damage.

METHODS

Culture of bronchial epithelial cells

Human bronchial epithelial cell line transformed by SV-40 (BEAS-2B)^{11,18} was used. BEAS-2B cells were provided by Dr. Marie Jackson (National Cancer Institute, Bethesda, Md.). BEAS-2B cells were cultured in serum free Ham's F-12 medium (HD-F12) supplemented with hormones. The added hormones were hydrocortisone (Sigma, St. Louis, Mo.; 10^{-7} mol/L), epidermal growth factor (Sigma; 25 ng/ml), insulin (Gibco, Grand Island, N.Y.; 5 μ g/ml), transferrin (Gibco; 5 μ g/ml), triiodothyronine (Sigma; 2×10^{-10} mol/L), and endothelial cell growth supplement (Sigma; 15 μ g/ml), as suggested.¹⁹ BEAS-2B cells were cultured in a 24-well flat-bottom tissue culture plate (Falcon 3047, Becton Dickinson, Franklin Lakes, N.J.) with 1 ml of HD-F12 in each well. The medium was changed twice a week until the cells became confluent, and then the cells were used for experiments. At this point of culture, each well contained approximately 3×10^5 cells.

Stimulation of BEAS-2B cells with EPO alone

BEAS-2B cells cultured until they became confluent were washed once with phosphate-buffered saline. Human EPO, provided by Dr. Gerald J. Gleich (Mayo Clinic, Rochester, Minn.), was diluted to 5.9×10^{-8} , 1.9×10^{-7} , and 5.9×10^{-7} mol/L with 0.5 ml HD-F12 and was applied to BEAS-2B cells in the wells for 15 minutes at 37° C. HD-F12 alone was applied to control wells. After the cells were washed twice with HD-F12, 1 ml of HD-F12 was added to each well and the culture plate was kept in a CO₂ incubator in an atmosphere of 5% CO₂ in air at 37° C. Culture supernatants were harvested at 3, 6, or 24 hours of incubation; spun down at 400g for 5 minutes; and kept at -20° C until assay. BEAS-2B cells attached to the bottom of the plate were exfoliated with 50 U/ml of trypsin (Mochida Pharmaceuticals, Japan), and the viability was checked with 0.1% erythrocin-B. The concentration of GM-CSF in the supernatants were measured with the GM-CSF ELISA System (Amersham, UK) according to the manufacturer's instructions.

Stimulation with an EPO + H₂O₂ + halide system

BEAS-2B cells cultured until they became confluent were washed once with phosphate-buffered saline. Human EPO was diluted to 1.9×10^{-9} , 5.9×10^{-9} , 1.9×10^{-8} , and 5.9×10^{-8} mol/L with 0.5 ml HD-F12 and was applied to BEAS-2B cells in the wells for 15 minutes at 37° C. HD-F12 alone was applied to control wells. After the cells were washed twice with HD-F12, 1 ml of HD-F12 was added to each well. H₂O₂ and NaBr (Wako Pure Chemical, Japan) at final concentrations of 10^{-5} and 10^{-4} mol/L, respectively, were added, and the culture plate was kept in a CO₂ incubator in an atmosphere of 5% CO₂ in air at 37° C. Culture supernatants were harvested at 24 hours of incubation, spun down at 400g for 5 minutes, and kept at -20° C until assay. Measurements of cell viability and the concentration of GM-CSF in the supernatants were done as described in the preceding section.

Northern blot hybridization

BEAS-2B cells were cultured in a tissue culture flask (3108, Becton Dickinson) until they became confluent. EPO diluted to 5.9×10^{-7} mol/L with 5 ml of HD-F12 was applied to the cells for 15 minutes at 37° C. After the cells were washed with HD-F12 twice, 15 ml of HD-F12 was added to the flask and kept in the CO₂ incubator. Negative control cells were treated with HD-F12 alone instead of EPO in HD-F12. Positive control cells were incubated with 20 ng/ml of human recombinant IL-1 β (Genzyme, Cambridge, Mass.). After 3 hours of incubation, cells were harvested with a scraper.

mRNA was extracted from 10^7 cells with the Quick Prep mRNA Purification Kit (Pharmacia, Sweden) with the use of extraction buffer containing guanidium thio-

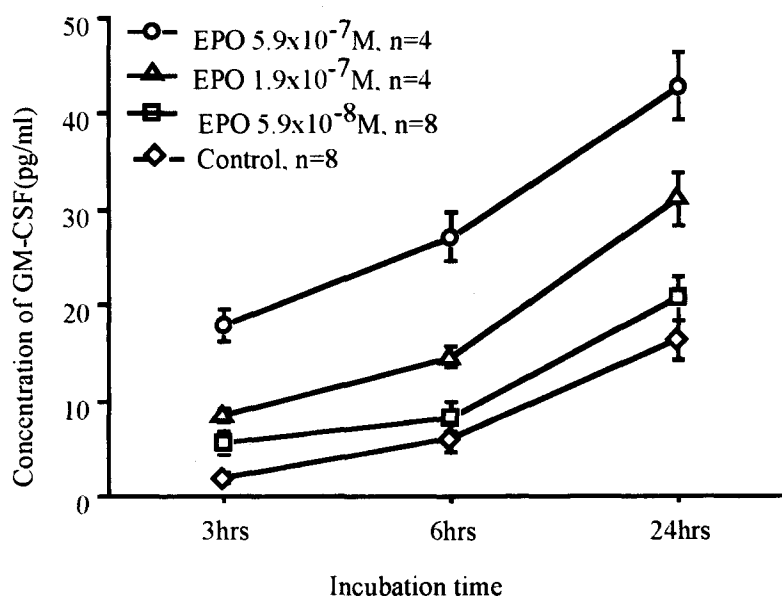


FIG. 1. Effect of EPO on release of GM-CSF from BEAS-2B cells. BEAS-2B cells were first washed with phosphate-buffered saline. EPO was then applied to BEAS-2B cells and incubated for 15 minutes at 37° C. Cells were washed twice with HD-F12 medium and were again incubated with 1 ml of HD-F12 at 37° C. Supernatants were harvested at 3, 6, or 24 hours, and concentration of GM-CSF was measured.

cyanate and *n*-lauroyl sarcosine, and an oligo (dT)-cellulose spun column. Five micrograms of mRNA was electrophoresed on a 1.5% agarose gel in a buffer containing 0.2 mol/L 3-[*N*-Morpholino]propanesulfonic acid and 0.8 mol/L formaldehyde, transferred to a nylon membrane, and cross-linked with ultraviolet light. A membrane was prehybridized in 50% formamide, 1% sodium dodecyl sulfate, 1 mol/L NaCl, 10% dextran sulfate, and sheared salmon sperm DNA (100 μ g/ml) at 42° C for 1 hour. The membrane was then hybridized overnight at 42° C with denatured ^{32} P-labeled complementary DNA probe (3.2 kb, Oncogene Science). The probe was radiolabeled with [α - ^{32} P]dCTP (New England Nuclear) by using a DNA-labeling kit (Pharmacia). The membrane was washed once in 2 $\times$ standard saline citrate containing 0.1% sodium dodecyl sulfate at room temperature for 5 minutes, twice in 2 $\times$ standard saline citrate containing 0.1% sodium dodecyl sulfate at 50° C for 30 minutes, and once in 0.1 $\times$ standard saline citrate containing 0.1% sodium dodecyl sulfate at room temperature for 30 minutes. Finally, the membranes were subjected to autoradiography.

Analysis

Values were expressed in mean $\pm$ SEM. Differences between groups were analyzed by one-way or two-way analysis of variance. Fisher's protected least significant difference (PLSD) was used as a post hoc test, and the difference was regarded as significant when p was <0.05 .

RESULTS

Effect of EPO alone on the release of GM-CSF

The release of GM-CSF into the supernatants of BEAS-2B cells is shown in Fig. 1. The release of GM-CSF into the supernatants increased depending on the incubation time ($n = 4$, $p < 0.0001$) and the concentration of EPO used in the pretreatment as well ($p < 0.0001$). Cell viability at 24 hours was $>98\%$ irrespective of the treatment.

Effect of various reagents on the release of GM-CSF

The concentration of GM-CSF in the supernatants of BEAS-2B cells treated with various reagents is shown in Fig. 2. The GM-CSF concentration in the control supernatant was 10.9 ± 0.6 pg/ml ($n = 4$). The GM-CSF concentration was 20.7 ± 0.3 pg/ml ($n = 4$) in supernatant pretreated with 5.9×10^{-7} mol/L EPO and was significantly higher than that in the control supernatant ($p < 0.005$). When BEAS-2B cells were incubated with 100 U/ml of catalase (from *Aspergillus niger*, Sigma) after pretreatment with 5.9×10^{-7} mol/L EPO, GM-CSF concentration in the supernatant was 22.4 ± 1.6 pg/ml ($n = 4$), and the GM-CSF release was not inhibited.

Treatment with cyclohexamide (CHX), a protein synthesis inhibitor, was done as follows.

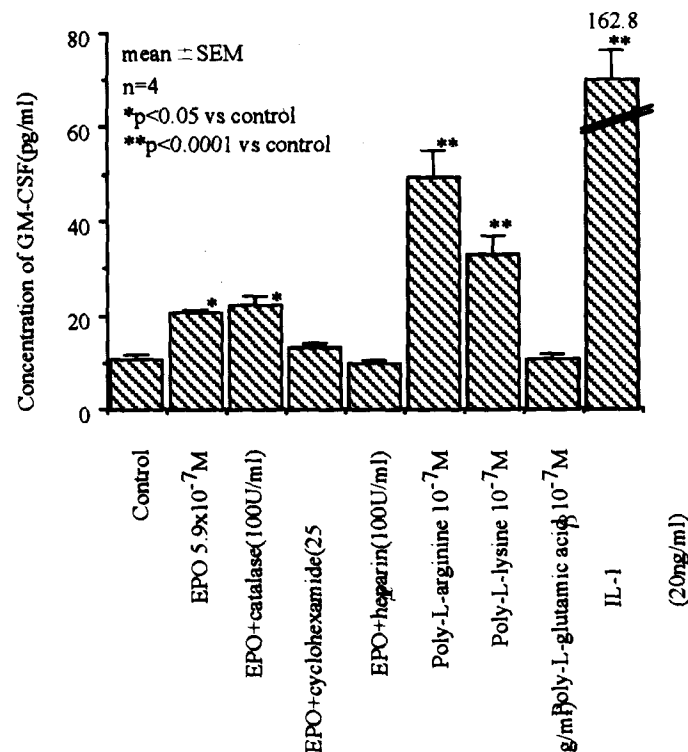


FIG. 2. Effect of various substances on release of GM-CSF from BEAS-2B cells in 24-hour culture supernatants. BEAS-2B cells were exposed to EPO, poly-L-arginine, poly-L-lysine, and poly-L-glutamic acid for 15 minutes and washed before incubation with 1 ml of HD-F12. Human recombinant IL-1 β and catalase were cocultured with cells for entire 24-hour incubation period. Cells were exposed to CHX for 30 minutes before exposure to EPO and then were cultured in its presence for 24 hours. Heparin was mixed with EPO before addition to wells with BEAS-2B cells.

BEAS-2B cells were first incubated with 25 μ g/ml of CHX in HD-F12 for 30 minutes. The cells were next exposed to 5.9×10^{-7} mol/L EPO for 15 minutes, washed with HD-F12, and finally incubated with 1 ml of HD-F12 with 25 μ g/ml of CHX for 24 hours. The GM-CSF concentration in the supernatants thus obtained was 13.5 ± 0.6 pg/ml ($n = 4$). Although the difference did not reach a significant level ($0.05 < p < 0.1$), about three fourths of the EPO-stimulated GM-CSF release was inhibited by using CHX.

Treatment with heparin was done as follows. EPO was diluted to 5.9×10^{-7} mol/L with HD-F12 containing 100 U/ml heparin (from porcine intestine, Sigma) and then applied to BEAS-2B cells for 45 minutes. The cells were washed and incubated with 1 ml HD-F12 for 24 hours. The GM-CSF concentration in the supernatants thus obtained was 9.7 ± 0.7 pg/ml ($n = 4$). The increase in GM-CSF release by EPO was completely abolished by using heparin.

Pretreatment with 10^{-7} mol/L poly-L-arginine (45,500 d, Sigma) poly-L-lysine (53,900 d, Sigma,

USA), or poly-L-glutamic acid (17,500 d, Sigma) was done as with EPO. The GM-CSF concentrations in the supernatants pretreated with 10^{-7} mol/L poly-L-arginine, poly-L-lysine, and poly-L-glutamic acid were 49.5 ± 5.5 pg/ml ($n = 4$), 33.1 ± 3.7 pg/ml ($n = 4$), and 10.9 ± 0.6 pg/ml ($n = 4$), respectively. Pretreatment with poly-L-arginine and poly-L-lysine significantly increased the release of GM-CSF ($p < 0.0001$).

Human recombinant IL-1 β (20 μ g/ml) was cocultured with BEAS-2B cells for the entire 24-hour incubation period. The GM-CSF concentration in the supernatant cultured with human recombinant IL-1 β was 162.8 ± 6.3 pg/ml ($n = 4$) and was significantly higher than those with any other treatments ($p < 0.0001$).

Effect of the EPO + H₂O₂ + halide system on the release of GM-CSF

The effect of the EPO + H₂O₂ + halide system (EPO system) on the release of GM-CSF is shown in Fig. 3. EPO at a concentration of 1.9×10^{-9} mol/L combined with 10^{-5} mol/L

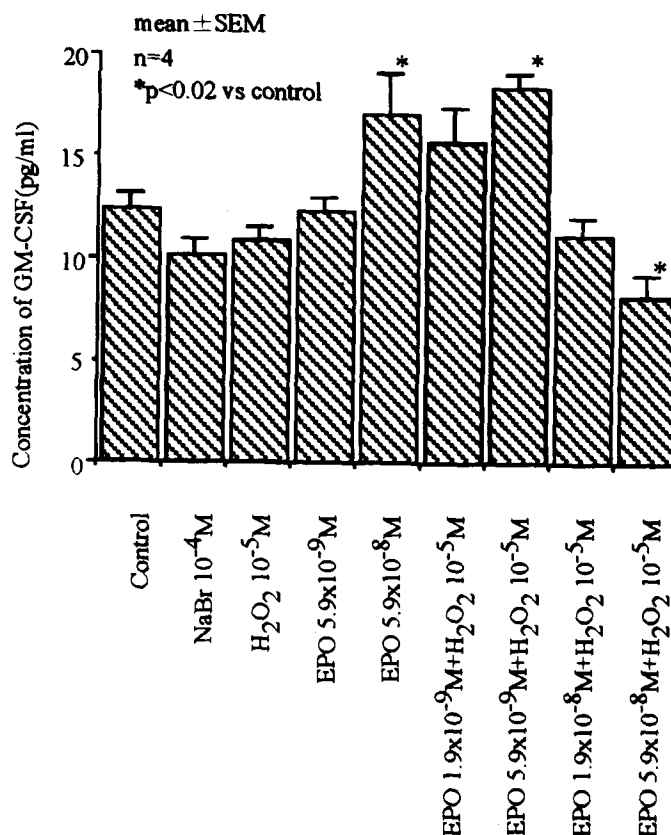


FIG. 3. Effect of EPO + H₂O₂ + halide system on release of GM-CSF from BEAS-2B cells in 24-hour culture supernatants. BEAS-2B cells were first washed with phosphate-buffered saline. EPO was then applied to BEAS-2B cells and incubated for 15 minutes at 37° C. Cells were washed twice with HD-F12 medium and were again incubated with 1 ml of HD-F12 at 37° C in presence of 10^{-5} mol/L H₂O₂ and 10^{-4} mol/L NaBr. To compare with EPO system, individual components included in EPO system were added separately.

H₂O₂ and 10^{-4} mol/L Br did not affect the release of GM-CSF. The system comprising EPO (5.9×10^{-9} mol/L) + 10^{-5} mol/L H₂O₂ + 10^{-4} mol/L Br significantly enhanced the release of GM-CSF (EPO system vs control: 18.4 ± 0.7 pg/ml vs 12.4 ± 0.8 pg/ml, $n = 4$, $p < 0.002$). EPO alone at a concentration of 5.9×10^{-8} mol/L again enhanced the release of GM-CSF compared with control (EPO vs control: 17.0 ± 2.0 pg/ml vs 12.4 ± 0.8 pg/ml, $n = 4$, $p < 0.02$). However, 1.9×10^{-8} or 5.9×10^{-8} mol/L EPO + 10^{-5} mol/L H₂O₂ + 10^{-4} mol/L Br did not increase the release of GM-CSF. In these experimental conditions, cell viability evaluated by using 0.1% erythrocin-B was >98% and was not different from the control. Treatment of BEAS-2B cells with 5.9×10^{-7} mol/L EPO + 10^{-5} mol/L H₂O₂ + 10^{-4} mol/L Br destroyed most of the cells, and the GM-CSF concentration in the supernatant was nearly zero (data not shown).

Northern blot analysis

An example of the expression of mRNA for GM-CSF is shown in Fig. 4. mRNA for β -actin was used for the control. The ratios of the integrated optical density of the mRNA signal for GM-CSF to that for β -actin were 3.63 and 5.75 in the control and the EPO-pretreated cells, respectively. Pretreatment with EPO increased the expression of mRNA for GM-CSF by approximately 60%.

DISCUSSION

EPO is one of four eosinophil granule basic proteins and is characterized by both a cationic property and peroxidase activity.³ It has been reported that EPO is toxic to many kinds of tissues. Specifically, the EPO + H₂O₂ + halide system is toxic to schistosomula of *Schistosoma mansoni*,²⁰ *Brugia pahangi*, and *B. malayi*. EPO alone also is toxic, although a higher concentration is needed.²¹ EPO is toxic to guinea pig tracheal epithelium at a

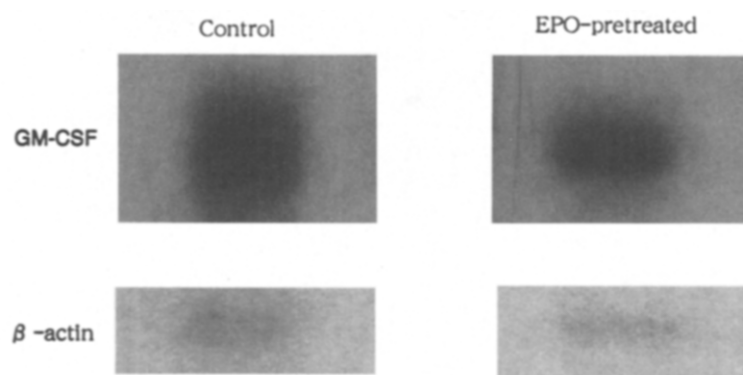


FIG. 4. Expression of mRNA for GM-CSF and β -actin detected by Northern blotting. RNA extracted from BEAS-2B cells pretreated with 5.9×10^{-7} mol/L or those incubated with medium alone (control) were subjected to Northern blotting.

concentration of 10^{-8} mol/L when combined with H_2O_2 and halide. EPO alone at a concentration of 10^{-6} mol/L is also toxic to the respiratory epithelium, probably because of its cationic property.¹⁷

On the other hand, a low concentration of EPO has been reported to be stimulatory. For example, the EPO + H_2O_2 + halide system at a concentration that is not toxic stimulates histamine release from rat mast cells.²² EPO alone stimulates platelets to release serotonin without release of lactate dehydrogenase.²³ Thus it is suggested that EPO at a high concentration is toxic and that at a low concentration it is stimulatory.

We have found EVEA in the sputum of patients with acute asthma and attributed it mainly to GM-CSF.⁸ Bronchial epithelium is one of the candidates for the source of GM-CSF in the airways. IL-1 β , TNF- α ,⁹ NO₂¹⁰ and a respiratory syncytial virus¹¹ have been reported to stimulate release of GM-CSF from bronchial epithelium. We conducted this experiment hypothesizing that eosinophil granule basic proteins, abundant in the sputum of persons with asthma,^{6, 16} may be one of the stimuli for GM-CSF release.

As shown by the current results, EPO alone stimulated the release of GM-CSF from BEAS-2B cells in a time- and concentration-dependent manner. The release is probably not attributable to cell destruction, because of the following findings: (1) CHX, a protein synthesis inhibitor, abolished most of the enhancement of GM-CSF; (2) >98% of the cells were viable at the end of the experiments; and (3) GM-CSF release was not recognized when BEAS-2B cells were destroyed by a high concentration of the EPO + H_2O_2 + halide system. In addition, mRNA for GM-CSF was up-regulated by using EPO, although not to a high degree.

Peroxidase activity probably does not contribute to the GM-CSF release with EPO alone, because catalase did not inhibit the increase in GM-CSF release. Therefore, the cationic property of EPO seems to be related to the release of GM-CSF, a possibility supported by the finding that highly cationic peptides such as poly-L-arginine and poly-L-lysine also stimulated the release of GM-CSF. On the other hand, an acidic peptide, poly-L-glutamic acid, did not show such an action. Application of EPO after mixing with heparin abolished the increase in GM-CSF release with EPO. Heparin binds to eosinophil granule basic proteins with its high acidic property and has been reported to abolish their toxicity.^{17, 24}

The potency of IL-1 β to release GM-CSF from BEAS-2B cells was approximately eight times higher than that of 5.9×10^{-7} mol/L EPO. The potency of 20 ng/ml TNF- α was comparable to that of 20 ng/ml IL-1 β in another experiment.²⁵ This large difference in the potency between these cytokines and EPO suggests that release of GM-CSF is largely dependent on these cytokines, and stimulation by EPO, if any, is complementary. In fact, we have found that sputum extracts from persons with asthma stimulate the release of GM-CSF from BEAS-2B cells. The activity was largely abolished by the combination of anti-IL-1 β and anti-TNF- α antibodies.²⁵

We also found that another eosinophil granule basic protein, major basic protein, stimulates the release of GM-CSF from BEAS-2B cells (unpublished observations). Thus, stimulation for GM-CSF release is a common feature of cationic proteins such as EPO, major basic protein, and poly-L-arginine, but the mechanism remains unknown. Cationic proteins are thought to adhere to negatively charged sites on the cell membrane.

Specifically, major basic protein reportedly adheres to negatively charged phospholipids and thereby contributes to cell damage.²⁶ However, the targets on the cell surface that contribute to enhancement of GM-CSF release and the signal transduction related to this phenomenon have not been elucidated. Although it seems possible that EPO adheres to receptors for IL-1 or TNF non-specifically and uses the same signal pathways, further study is needed to elucidate the mechanism of the enhancement of GM-CSF release.

When EPO + H₂O₂ (10⁻⁵ mol/L) + halide (10⁻⁴ mol/L Br) is applied to BEAS-2B cells, a very low concentration of EPO, down to 5.9 × 10⁻⁹ mol/L, enhanced GM-CSF release. However, EPO at a higher concentration inhibited GM-CSF release, and 5.9 × 10⁻⁷ mol/L EPO combined with 10⁻⁴ mol/L H₂O₂ resulted in cell death. Our experiment revealed the critical concentration of EPO at which the effect of EPO changes from stimulation to inhibition or toxicity. In the experiment in which EPO alone was applied, EPO > 5.9 × 10⁻⁷ mol/L was not used because of the limited amount of EPO available. However, because EPO at 10⁻⁶ mol/L is toxic to parasites²¹ and respiratory epithelium,¹⁷ it is likely that EPO at > 5.9 × 10⁻⁷ mol/L would have inhibited GM-CSF release.

An EPO + H₂O₂ + halide system has been shown to stimulate the release of histamine from mast cells.²² However, the mechanism is not known. It is thought that hypohalous acid generated by the EPO + H₂O₂ + halide system causes oxidation or halogenation of membrane proteins and lipids, producing cell damage as does myeloperoxidase.²⁷ It was recently reported that a reactive oxygen species, H₂O₂ at 10⁻⁵ to 10⁻⁴ mol/L, stimulates mitogen-activated protein kinase in tracheal myocytes by protein kinase-C-dependent and protein kinase C-independent pathways.²⁸ This finding may explain one of the mechanisms of GM-CSF release by the EPO system. However, further study is needed to clarify whether the same mechanism is applicable to the increase in the GM-CSF release.

Although the concentration of H₂O₂ in the airways of persons with asthma is not known, there various kinds of cells can generate superoxide anions such as macrophages, neutrophils, and eosinophils.^{5, 27} Because superoxide anions are converted rapidly to H₂O₂, and because 10⁻⁸ mol/L EPO is present in the sputum of persons with asthma,¹⁶ there is a high possibility that the EPO + H₂O₂ + halide system is working *in vivo*. In this system, the difference in EPO concentration, which is stimulatory and toxic, is only tenfold. Accord-

ingly, it seems possible that EPO acts as a toxic substance at the sites where EPO deposition is concentrated and acts as a stimulating substance at the site where EPO deposition is sparse.

The results of our study suggest the existence of a cycle in asthma whereby eosinophils in the airway stimulate epithelial cells to release GM-CSF, resulting in an accumulation of eosinophils and damage to the airway.

We thank Dr. Gerald J. Gleich, the Mayo Clinic, for providing us with EPO and Dr. Takayuki Ohtoshi, Tokyo University, for providing us with the method of serum free cell culture.

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