

Airway epithelial cells release eosinophil survival-promoting factors (GM-CSF) after stimulation of proteinase-activated receptor 2

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Background: Epithelium is considered an active participant in allergic inflammation. Proteinase-activated receptor (PAR) 2 is expressed in a variety of cell types, including epithelial cells, and has been implicated in inflammation.

Objective: PAR-2-mediated activation of airway epithelial cells induces the release of mediators that could promote eosinophil survival and mediate eosinophil recruitment.

Methods: PAR-2-activating peptides were used to activate the human airway epithelial cell line A549, as well as primary cultures of small airway epithelial cells (SAECs). Human peripheral blood eosinophils were cultured in the presence or absence of epithelial cell supernatants. Survival was assessed by using an Annexin V apoptosis detection kit. GM-CSF and eotaxin were measured by using ELISA.

Results: Eosinophils undergo apoptosis in the absence of growth factors. Supernatants from PAR-2-activated A549 epithelial cells increased eosinophil survival. Supernatants from resting SAECs also increased eosinophil survival, but supernatants from PAR-2-activated SAECs showed a greater effect. The effect of PAR-2-activated epithelial cell supernatants on eosinophil survival was completely inhibited by a neutralizing anti-GM-CSF antibody but not an anti-IL-5 antibody. Resting A549 cells did not release any detectable GM-CSF, whereas PAR-2-activated cells released 35 pg/10⁶ cells. Resting SAECs released 754.3 pg/10⁶ cells of GM-CSF, which was further increased to 1360.5 pg/10⁶ cells after PAR-2-mediated activation. Budesonide inhibited this PAR-2 effect. PAR-2-activated epithelial cells also released eotaxin.

Conclusion: PAR-2-mediated activation of airway epithelial cells induced release of GM-CSF, which promoted eosinophil survival and activation. It also induced release of eotaxin, which could mediate eosinophil recruitment to the airways. (*J Allergy Clin Immunol* 2001;107:679-85.)

Key words: Inflammation, lung, inflammatory mediators, cell surface molecules, protease-activated receptor 2, GM-CSF, eotaxin, corticosteroids

Respiratory epithelium is considered an active participant in allergic inflammation.¹ Airway epithelial cells are the first mucosa cells to be exposed to environmental stimuli associated with airway inflammation.²⁻⁵ These noxious substances, together with mediators released by inflammatory cells, activate epithelial cells to produce a plethora of proinflammatory mediators, such as eotaxin,⁶ GM-CSF,⁷ RANTES,^{8,9} IL-6,¹⁰ IL-8,¹¹ and matrix metalloproteinases.^{12,13} These mediators induce the accumulation of inflammatory cells and prolong their survival.^{7,10} Furthermore, GM-CSF expression in nasal epithelial cells increases in patients with allergic rhinitis, supporting a role for epithelial cells in eosinophil accumulation in allergic inflammation.¹⁰ The particular stimuli that initiate the upregulation of these inflammatory mediators during allergic inflammation are not clearly defined.

Many of the stimuli that might activate airway epithelial cells, including allergens and mast cell and neutrophil products, are proteases. Proteases mediate some of their effects through protease-activated receptors (PARs). Four PAR receptors (PAR-1 to PAR-4) have been cloned. PAR-1 and PAR-3 are activated primarily by thrombin, and PAR-2 and PAR-4 are activated primarily by trypsin.^{14,15}

PAR-2 receptors are expressed in many structural cells¹⁵⁻¹⁷ and in inflammatory cells, such as neutrophils,¹⁸ but their exact physiologic roles are not clear.¹⁹ Inflammatory cytokines, such as TNF and IL-1 α , upregulate PAR-2 mRNA expression,²⁰ and PAR-2 is involved in inflammation in the rat.²¹ PAR-2 activation induces vasodilation^{22,23} and hypotension²² in vivo. Short-term PAR-2 activation has been shown to inhibit acetylcholine-induced bronchoconstriction in the airways,²⁴ but the long-term effects on the production of inflammatory mediators have not been studied. Mast cell tryptase,^{25,26} various trypsin-like proteases that localize to the airway epithelium,^{24,27} allergens,²⁸ and other proteases may activate PAR-2 receptors in the airways and mediate proinflammatory effects.

A striking feature of asthma is the abundance of eosinophils in the airway mucosa. These bone marrow-derived cells are recruited to sites of inflammation in response to locally produced soluble mediators.²⁹ Eosinophil numbers, particularly those with activated

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Abbreviations used

DMEM: Dulbecco's modified Eagle's medium
 MAP kinase: Mitogen-activated protein kinase
 PAR: Proteinase-activated receptor
 SAEC: Small airway epithelial cell

phenotype, increase in BAL fluid of symptomatic asthmatic patients.³⁰⁻³² Similarly, eosinophil granule proteins are elevated in BAL fluid, and their levels correlate with bronchial hyperresponsiveness.^{33,34} Eosinophils are thought to induce respiratory damage and dysfunction through the release of arachidonic acid metabolites, granular cationic proteins, and reactive oxygen species. Disruption of the airway epithelium by these mediators increases bronchial hyperresponsiveness. Because of the importance of eosinophils in the physiologic changes seen in asthma, stimuli that further enhance their accumulation, activation, and survival in the airways may be very important in asthma pathophysiology.

This article reports that PAR-2-mediated activation of airway epithelial cells induces the production and release of the eosinophil survival promoter GM-CSF. PAR-2-mediated activation also induces the release of eotaxin, which could mediate eosinophil recruitment to the airways.

METHODS**Materials**

Recombinant human TNF and goat anti-mouse IgG-phycoerythrin-conjugated antibody (Cedarlane Laboratories Ltd, Ontario, Canada); recombinant human GM-CSF and IL-5 (Genzyme, Cambridge, Mass); anti-GM-CSF and anti-IL-5 neutralizing antibodies, GM-CSF, and Eotaxin ELISA kits (R&D Systems, Minneapolis, Minn); Annexin V apoptosis detection kit and anti-CD69 antibody (BD Biosciences, Mississauga, Ontario, Canada); and Dulbecco's modified Eagle's medium (DMEM), RPMI-1640, FBS, penicillin and streptomycin, and L-glutamine (Biowhittaker, Walkersville, Md) were purchased as shown. Budesonide was generously provided by Astra Pharma Inc (Mississauga, Ontario, Canada). PAR-1 (Aparafluoro-FRCyclohexylACitY-NH₂ and the inactive control peptide FSLRLY-NH₂), PAR-2 (transcinnamoyl-LIGRLO-NH₂ and SLIGRL-NH₂ and the inactive control peptides LRGILS-NH₂ and tc-OLRGIL-NH₂), and PAR-4 (GYPGKF-NH₂) activating peptides were synthesized by the Peptide Synthesis Facility of the Faculty of Medicine, University of Calgary. These peptides were greater than 95% pure, as determined by means of HPLC and mass spectrometry criteria.

Airway epithelial cell culture and activation

The human pulmonary type II-like epithelial cell line A549 (American Type Culture Collection, Rockville, Md) was cultured in DMEM supplemented with 10% FBS, 4 mmol/L L-glutamine, and 100 µg/mL penicillin/streptomycin (complete medium). A549 cells were grown in 12-well plates in complete medium until confluent. They were then maintained for 24 hours in DMEM without supplements and subsequently activated for 24 hours in DMEM with the indicated concentrations of PAR-2-activating peptides. The supernatants were collected and kept at -70°C until analyzed by means of ELISA or used for eosinophil culture.

Small airway epithelial cells (SAECs) isolated from human airways (Clonetics, Walkersville, Md) were cultured in serum-free medium (Clonetics), according to the manufacturer's instructions. SAECs were cultured in 12-well plates in serum-free medium sup-

plemented with growth factors (bovine pituitary extract, 30 µg/mL; hydrocortisone, 0.5 µg/mL; human recombinant epidermal growth factor, 0.5 ng/mL; epinephrine, 0.5 µg/mL; transferrin, 10 µg/mL; insulin, 5 µg/mL; retinoic acid, 0.1 ng/mL; triiodothyronine, 6 ng/mL; gentamicin, 50 µg/mL; amphotericin B, 50 ng/mL; and human epithelial growth factor, 0.5 ng/mL) until they were 80% to 90% confluent and then maintained in DMEM without supplements for a further 24 hours. Cells were then activated in DMEM for 24 hours. Supernatants were collected and analyzed by means of ELISA for GM-CSF and eotaxin content or used for eosinophil culture.

Eosinophil isolation

Eosinophils were isolated from peripheral blood of atopic individuals, as previously described.³⁵ Granulocytes were isolated from peripheral blood by means of gradient centrifugation with Ficoll, and eosinophils were purified from granulocytes after elimination of neutrophils with anti-CD16-coated magnetic beads. Eosinophil preparations obtained with this method had greater than 97% purity (in most cases >99%).

Eosinophil apoptosis detection

Purified eosinophils were incubated for up to 48 hours with or without growth factors or with conditioned supernatants from resting or PAR-2-activated lung epithelial cells (A549, SAECs, or both) in 250 µL of medium in 24-well plates. For coculture experiments, A549 cells were grown in 12-well plates for 3 days. The medium was changed to fresh medium, and then eosinophils were added at 10⁶ per milliliter in 1 mL of complete medium and cocultured with the epithelial cells for up to 72 hours.

Eosinophil survival was studied by using an Annexin V apoptosis detection kit, according to the manufacturer's instructions. Cells were washed twice with PBS, incubated for 15 minutes at room temperature with FITC-Annexin V and propidium iodide, and analyzed with a FACScan flow cytometer (Becton Dickinson). Neutralizing antibodies against human GM-CSF, IL-5, or both were added to determine the importance of these cytokines for supporting eosinophil survival under the experimental conditions studied.

Flow cytometry for CD69 expression

Resting or activated eosinophils were incubated for 30 minutes at 4°C with 10 µg/mL anti-CD69 antibody in flow buffer (PBS with 0.1% BSA and 0.05% sodium azide), washed twice, and then incubated for 30 minutes at 4°C with goat anti-mouse IgG-phycoerythrin-conjugated antibody. The cells were analyzed with a FAC-Scan flow cytometer (Becton Dickinson).

Statistical analysis

Data are presented as means ± SEM of viable eosinophils or as means ± SEM of GM-CSF and eotaxin content. Statistical differences among the means of multiple groups were determined by using one-way ANOVA. The paired Student *t* test was used to compare the means of 2 groups. A *P* value of less than .05 was considered statistically significant.

RESULTS

Freshly isolated human peripheral blood eosinophils were found to be greater than 92% viable by means of Annexin V and propidium iodide staining, and they underwent apoptosis after culture in the absence of growth factors; 23.4% ± 8.5% of the cells remained viable after 48 hours of culture. Culture of eosinophils in the presence of supernatants from resting A549 cells induced a small but significance increase in eosinophil survival (34.9% ±

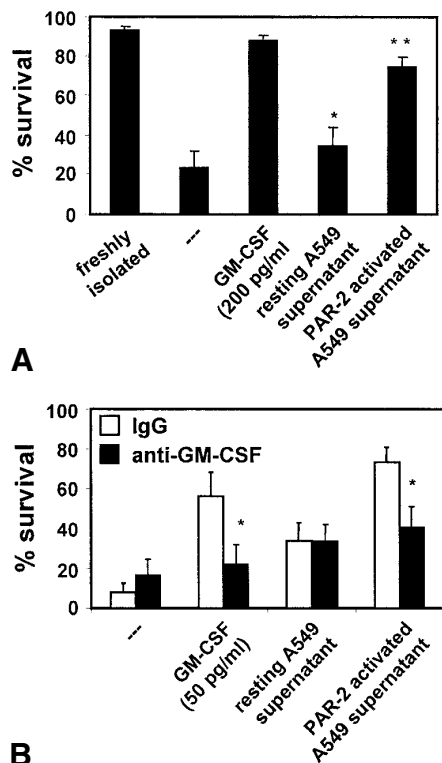


FIG 1. A, Survival of freshly isolated eosinophils or eosinophils after 48 hours of culture in the absence of growth factors, in the presence of GM-CSF (200 pg/mL), or in the presence of resting or PAR-2-activated A549 epithelial cell supernatants (1:1 with RPMI-1640 supplemented with 10% FBS; $n = 5$). * $P < .02$ compared with no supplements. ** $P < .01$ compared with no supplements and resting A549 supernatants. **B**, Effect of neutralizing anti-GM-CSF antibody (5 µg/mL) on eosinophil survival in the presence of GM-CSF or A549 epithelial cell supernatants ($n = 4$). * $P < .01$ compared with the same condition without anti-GM-CSF.

9.1%), whereas supernatants from PAR-2-activated A549 cells induced a much higher increase ($74.3\% \pm 5.6\%$; Fig 1, A). The experiments presented in Fig 1, A, were performed by using the transcinamoyl-LIGRLO-NH₂ PAR-2-activating peptide. However, the second PAR-2-activating peptide (SLIGRL-NH₂) had similar effects. Supernatants from epithelial cells cultured in the presence of the PAR-2-inactive peptides had no effect on eosinophil survival (data not shown). Furthermore, supernatants from PAR-1- and PAR-3-activated epithelial cells also had no effect on eosinophil survival (data not shown). None of the peptides used had a direct effect on eosinophil survival, whether added in the presence or absence of GM-CSF (data not shown). Budesonide (10^{-6} to 10^{-10} mol/L) inhibited the effect of PAR-2-activated A549 epithelial cell supernatants on eosinophil survival and also inhibited the effect of GM-CSF on eosinophil survival.

Epithelial cells are known to produce factors that prolong eosinophil survival, with GM-CSF being the most prominent.⁷ To identify the factors produced by PAR-2-activated A549 epithelial cells that prolong eosinophil survival, we used neutralizing antibodies. A neutralizing anti-GM-CSF antibody (5 µg/mL) inhibited the effect of

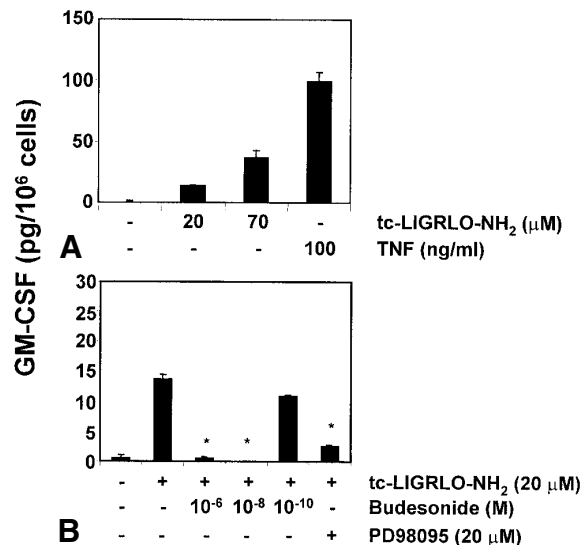


FIG 2. A, Release of GM-CSF by A549 epithelial cells after 24 hours of activation with PAR-2-activating peptides or TNF ($n = 3$). **B**, Effect of budesonide and the MAP kinase inhibitor PD98095 on GM-CSF release from A549 epithelial cells after PAR-2-mediated activation ($n = 3$). * $P < .01$ compared with no inhibitors.

GM-CSF on eosinophil survival (Fig 1, B). It also inhibited the effect of PAR-2-activated A549 supernatants on eosinophil survival (from $73.3\% \pm 7.7\%$ viable cells in the presence of PAR-2-activated A549 cell supernatants to $40.9\% \pm 11.1\%$ in the presence of the same supernatant with anti-GM-CSF neutralizing antibody). However, anti-GM-CSF antibodies had no effect on the small but significant eosinophil survival advantage in the presence of supernatants from resting A549 epithelial cells, indicating that resting A549 cells may also release other factors that are important for eosinophil survival. Anti-IL-5 neutralizing antibodies inhibited the IL-5 effect on eosinophil survival but did not alter the effect of GM-CSF or PAR-2-activated A549 supernatants on eosinophil survival (data not shown).

Supernatants from resting A549 cells contained very little if any (below the detection limit of the ELISA used) GM-CSF. After PAR-2-mediated activation, A549 cells released up to 37.8 ± 4 pg GM-CSF/10⁶ cells after 24 hours of stimulation with 70 µmol/L tc-LIGRLO-NH₂ (Fig 2, A). TNF has been shown to induce GM-CSF release from A549 cells³⁶ and bronchial epithelial cells³⁷ and was used as a positive control. Preincubation of A549 epithelial cells with the corticosteroid budesonide (as low as 10^{-8} mol/L) completely abolished GM-CSF release (Fig 2, B). Also, preincubation of epithelial cells with the mitogen-activated protein kinase (MAP kinase) inhibitor PD98095 (10 µmol/L) almost completely inhibited PAR-2-mediated GM-CSF release (Fig 2, B). In all the above experiments, A549 cells were incubated with PAR-2-activating peptides in the absence of FBS. In the presence of 10% FBS, the amount of GM-CSF released after PAR-2 activation was 2 to 3 times higher, and again budesonide completely abolished the PAR-2 effect (data not shown).

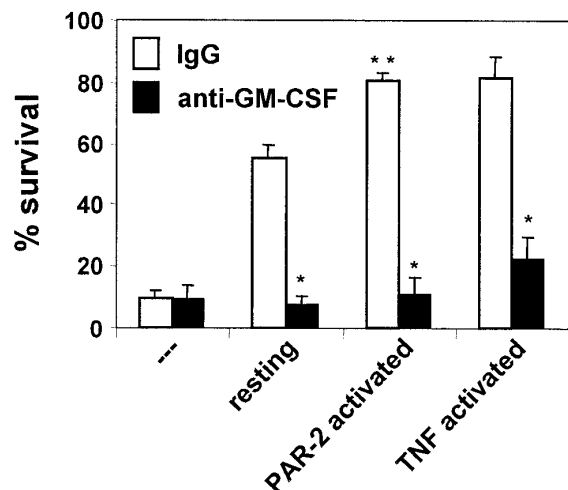


FIG 3. Survival of eosinophils after 48 hours of culture in the absence of growth factors or in the presence of SAEC supernatants after stimulation with PAR-2-activating peptides or TNF (1:10 with RPMI-1640 supplemented with 10% FBS) in the presence or absence of anti-GM-CSF neutralizing antibodies (5 μ g/mL; $n = 4$). * $P < .01$ compared with the same condition without anti-GM-CSF. ** $P < .02$ compared with supernatant of resting SAECs.

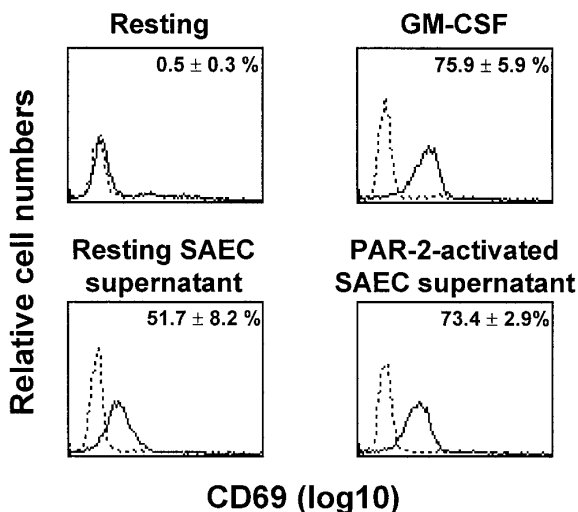


FIG 4. CD69 expression after 24 hours of culture in the absence of growth factors (upper left), in the presence of GM-CSF (2 ng/mL; upper right), or in the presence of resting (lower left) or PAR-2-activated (lower right) SAEC supernatants. Numbers indicate means $\pm$ SEM of CD69-positive cells from 3 independent experiments.

To verify the biologic significance of PAR-2-mediated GM-CSF release from airway epithelial cells, we performed similar experiments by using supernatants from SAECs. SAEC supernatants had higher eosinophil survival-promoting activity compared with A549 supernatants, and PAR-2 activation augmented this effect (Fig 3). In the experiments shown here, SAEC supernatants were used diluted 1:10 with RPMI, whereas in the A549 experiments the supernatants were diluted only 1:2. Anti-GM-CSF neutralizing antibody completely abolished the

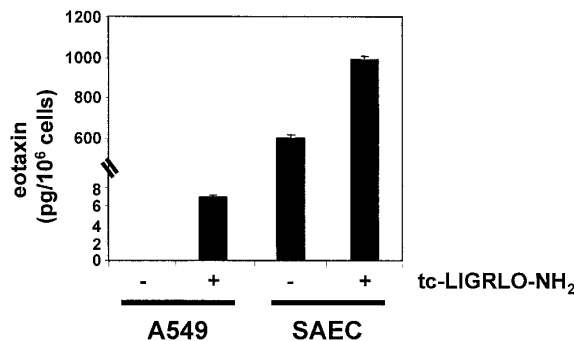


FIG 5. Eotaxin release from resting and PAR-2-activated SAECs and A549 human airway epithelial cells ($n = 3$).

effect of both resting and activated SAEC supernatants on eosinophil survival (Fig 3). By means of ELISA, we verified that SAECs released higher amounts of GM-CSF compared with A549 epithelial cells. Resting SAECs released 603.4 pg GM-CSF/10⁶ cells, and PAR-2-activated SAECs released 1088.4 pg/10⁶ cells.

We then studied the effect of PAR-2-mediated epithelial cell activation on eosinophil survival during coculture of eosinophils with epithelial cells (Table I). These experiments were performed in the presence of 10% FBS. Coculture of eosinophils with A549 epithelial cells induced a more prolonged effect on eosinophil survival compared with culture of eosinophils in the presence of A549 cell supernatants. Therefore we studied the effect of coculture on eosinophil survival for up to 72 hours. After 72 hours of coculture with A549 cells, 50.1% $\pm$ 5.4% of eosinophils were viable. PAR-2-mediated activation further increased eosinophil survival. When a permeable membrane separated the 2 cell types to inhibit adhesive interactions, this effect was lower, but there was still a significant effect of PAR-2-mediated epithelial cell activation on eosinophil survival. The effect of coculture and PAR-2 activation on eosinophil survival was inhibited by over 80% by anti-GM-CSF neutralizing antibodies but was not affected by the prostaglandin synthase inhibitor indomethacin, indicating that GM-CSF was the main eosinophil survival factor released.

Next, we investigated whether proinflammatory cytokines produced from PAR-2-activated epithelial cells have other biologic effects on eosinophils. For this, we studied CD69 expression on eosinophils after incubation in the presence or absence of SAEC supernatants. SAEC supernatants increased CD69 expression on eosinophils (Fig 4). This increase was more pronounced with supernatants of PAR-2-activated epithelial cells. A549 cell supernatants had a similar but smaller effect.

PAR-2-activated epithelial cells release GM-CSF, which can affect eosinophil survival and induce eosinophil activation. We also examined whether PAR-2-activated cells release factors that could induce eosinophil recruitment. Resting A549 did not release detectable levels of eotaxin, whereas SAECs released 598.6 $\pm$ 19.2 pg/10⁶ cells. Eotaxin release increased in both epithelial cell types after PAR-2-mediated activation (Fig 5).

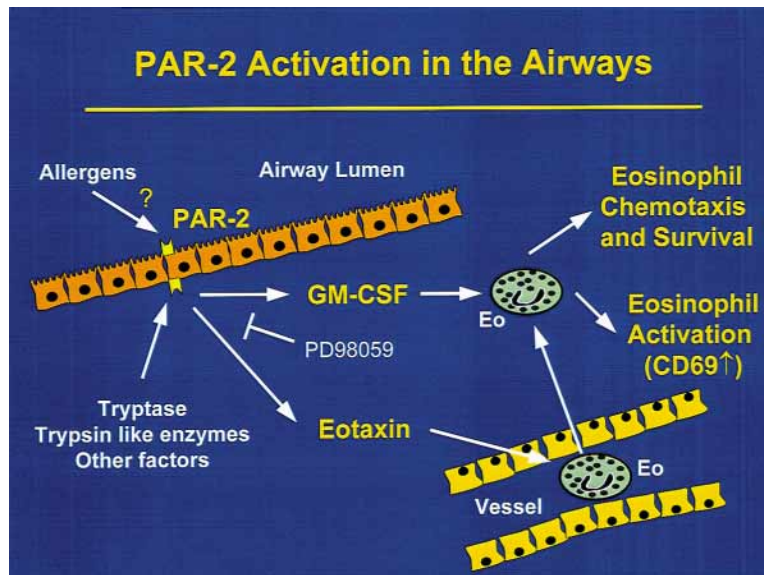


FIG 6. Eosinophil recruitment to the airways in asthma: role of PAR-2. Eo, Eosinophil.

TABLE I. Eosinophil survival after 72 hours of coculture with A549 epithelial cells: Effect of adhesion and GM-CSF

Coculture	—	Filter*	Anti-GM-CSF†
—	1.5% ± 0.2%		
A549	50.1% ± 5.4%	21.4% ± 7.1%	13.1% ± 2.1%
A549 and tc-LIGRLO (20 μmol/L)	64.4% ± 4.7%‡	36.6% ± 10.5%§	14.8% ± 4.7%
A549 and LRGILS (20 μmol/L)	56.6% ± 1.1%	19.0% ± 7.5%	

Eosinophils were cocultured with A549 epithelial cells for 72 hours. For the conditions listed under *Filter*, a permeable filter separated eosinophils from epithelial cells for the duration of the coculture. Anti-GM-CSF was added at 5 μg/mL for the duration of coculture (n = 4 for all experiments).

*P < .01 compared with no filter for every condition.

†P < .01 compared with non-anti-GM-CSF-treated cells for each condition.

‡P < .02 compared with unstimulated and LRGILS-stimulated A549 cells.

§P < .02 compared with unstimulated and LRGILS-stimulated A549 cells in the presence of a filter.

DISCUSSION

We have shown that PAR-2-mediated activation of airway epithelial cells induces the production and release of factors that prolong eosinophil survival. Using neutralizing antibodies, we showed that the factor produced by epithelial cells under these conditions is GM-CSF. Interestingly, resting A549 epithelial cells also release factors, other than GM-CSF or IL-5, that promote eosinophil survival. This effect is not seen with SAECs. The nature of these factors is not clear. SAECs produce higher amounts of GM-CSF compared with A549 epithelial cells, and their supernatants offer greater survival advantage to eosinophils. We also showed that PAR-2-mediated GM-CSF release is inhibited by corticosteroids and requires MAP kinase activation. PAR-2-mediated activation of epithelial cells can also induce eosinophil activation, as seen by the upregulation of CD69 expression. We finally showed that under the same conditions, epithelial cells also release eotaxin, which could affect eosinophil chemotaxis to areas of release of PAR-2-activating enzymes.

PAR-2 receptors have been implicated in inflammation in the gut and the lung. Their ability to induce the

release of factors that would promote eosinophil recruitment and survival might be one of the mechanisms involved in PAR-2-mediated inflammation, especially in the lung. The proteases that activate PAR-2 receptors in the lung are not known. Apart from mast cell tryptase^{25,26} released in the respiratory tract after mast cell activation, other proteases may also activate PAR-2 in the airways. Trypsin immunoreactivity has been identified in airway epithelium,^{24,27} and a trypsin-like protease has been purified from human airways.³⁸ Many allergens are known to contain proteases, and at least one of these allergens, Der p 9 from house dust mite, can activate trypsin-activated receptors on epithelial cells.²⁸ The ability of many enzymes found in the airways during airway inflammation to activate PAR-2 receptors makes these receptors attractive candidates to mediate inflammatory responses.

Apart from eosinophil-specific growth and chemotactic factors, PAR-2 activation might also lead to the production of other inflammatory mediators. We have shown recently that PAR-2 activation leads to the release of matrix metalloproteinase 9 from epithelial cells.³⁹ Furthermore, fungal allergens induce protease-dependent release of IL-6 and IL-8 from airway epithelial cells,⁴⁰ an

effect that we speculate could be mediated through PAR-2 activation.

Mast cells are known to be involved in asthma⁴¹ and mediate the accumulation of eosinophils in the lung. They release tryptase, which can activate PAR-2 receptors.²⁵ Mast cell tryptase has been implicated in the inflammatory responses seen in asthma.^{42,43} Local release of tryptase in the lung, apart from its direct effects on smooth muscle, could also, through PAR-2 activation, induce the production of eosinophil survival-promoting and chemotaxis-promoting factors and in this way prolong the duration of inflammation. Interference with the effects of tryptase on epithelial cells could therefore diminish the recruitment and survival of eosinophils in the lung.

Corticosteroids are the main pharmacologic agents used to modulate inflammation in asthma⁴⁴ and have the potential to inhibit the development of inflammatory changes in the lung.⁴⁵ Corticosteroids inhibit growth factor-dependent eosinophil survival⁴⁶ and also inhibit the release of cytokines from different cell types.^{9,47} Our results showed that corticosteroids also inhibit the production of GM-CSF from PAR-2-activated epithelial cells, indicating that corticosteroids are potentially good anti-inflammatory tools in PAR-2-mediated inflammation.

The signal transduction pathways that lead to inflammatory mediator release in the airways are not clearly defined. Our results with the MAP kinase inhibitor PD98059 suggest that MAP kinase activation is important for PAR-2-mediated GM-CSF release from airway epithelial cells. Knowledge of these pathways could offer one more target to interfere with inflammatory mediator release during allergic airway inflammation.

In conclusion, this study points toward new receptors responsible for the release of eosinophil chemotaxis and survival-promoting factors in the airways. PAR-2 receptors have attracted a lot of interest recently because they mediate inflammatory responses in many systems. Our observations implicate epithelial cell PAR-2 receptors in proinflammatory responses in the airways. The functions of PAR-2 receptors in the airways, as proposed from the results presented in this article, are summarized in Fig 6.

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