

Endothelial protein C receptor–dependent inhibition of human eosinophil chemotaxis by protein C

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Background: Eosinophil infiltration is a characteristic feature of allergic inflammation. Allergic responses are associated with local activation of the coagulation pathway and accumulation of fibrin.

Objective: We tested whether protein C and activated protein C (APC), which are endogenous anti-inflammatory coagulation inhibitors, affect eosinophil function.

Methods: Eosinophils were from venous blood of healthy donors. Cell migration and apoptosis were studied by using micropore filter assays and fluorometry, respectively. Receptor expression was investigated by means of RT-PCR and SDS-PAGE of immunoprecipitated protein.

Results: Protein C and APC had no significant chemotactic effects on eosinophils. Eosinophils pretreated with protein C or APC showed significantly reduced migration toward chemoattractants. No effect of either protein C preparation was seen in eosinophil apoptosis assays. The inhibiting effect on migration was reversed by an antibody against the endothelial protein C receptor (EPCR). Synthesis of EPCR by eosinophils is suggested by demonstration of receptor mRNA expression and detection of metabolically labeled receptor protein.

Conclusions: Data suggest that an EPCR is expressed by eosinophils whose activation with protein C or APC arrests directed migration. Protein C–affected eosinophil chemotaxis is a novel thrombin-independent component of the protein C pathway. (*J Allergy Clin Immunol* 2003;112:375–81.)

Key words: Eosinophils, coagulation, allergy, asthma, protein C

Eosinophilic granulocytes are implicated in a great variety of diseases, including allergic disorders, vasculitic granulomatous diseases, immunologic disorders, intestinal and pulmonary diseases, and, not least, parasitic infections.^{1,2} Eosinophils are potent inflammatory cells that secrete a number of lipid mediators and proteins relevant to the pathophysiology of inflammation and allergic diseases.^{3,4}

Allergic cutaneous reactions in human subjects lead to an activation of the coagulation pathway and accumulation of fibrin formations.^{5,6} Moreover, increased throm-

Abbreviations used

APC: Activated protein C
EPCR: Endothelial protein C receptor
FACS: Fluorescence-activated cell sorting
MACS: Magnetic-activated cell sorting
PAR: Protease-activated receptor
PC: Protein C

bin generation occurs in the airways of patients with asthma bronchiale.⁷ A reduced activation of the protein C pathway and decreased expression of thrombomodulin and protein C and its receptor were demonstrated in the airways of asthmatic patients.⁸

The protein C pathway is one of the main modulators of coagulation activation.⁹ Studies have revealed that components of this pathway might also inhibit inflammatory responses, such as inhibition of leukocyte adhesion to vascular endothelial cells and accumulation in rat lungs.¹⁰ Protein C also inhibits proinflammatory cytokine release in monocytes⁹ that were shown to express endothelial protein C receptor (EPCR).¹¹

In this study we examine the effects of protein C and activated protein C (APC) on eosinophils and show that eosinophils express an EPCR that might be involved in the inhibitory effects of protein C and APC on cell migration.

METHODS

Reagents

All stock solutions were stored at –20°C before use. RPMI 1640 with phenol red was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). BSA was from Dade Behring (Marburg, Germany). C5a, gelatin, dextran, thrombin receptor activator peptide SFLLRNPNDKYEPF, hirudin, staurosporine, penicillin G, and streptomycin were from Sigma Chemical (St Louis, Mo). Eotaxin was from R&D Systems (Minneapolis, Minn). Protein C (Ceprotin) was from Baxter (Deerfield, Ill), and APC recombinant human protein C activated by thrombin (Xigris) was from Lilly (Indianapolis, Ind). GM-CSF (Leucomax) was from Novartis (Vienna, Austria). Lymphoprep was from Nycomed Pharma AS (Oslo, Norway). Dulbecco's PBS and L-glutamine were from PAA Laboratories (Linz, Austria), HBSS without phenol red was from Invitrogen (Carlsbad, Calif), RPMI without L-methionine and L-glutamine was from Biochrom (Berlin, Germany), EasyTag EXPRE^{35S} Protein Labeling Mix [³⁵S] was from PerkinElmer (Wellesley, Mass), and the Protein A Sepharose was from Amersham Biosciences (Buckinghamshire, England). Anti-EPCR antibodies RCR2, RCR92, and RCR252 were a gift of Kenji Fukudome (Saga Medical School,

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Saga, Japan). The biotinylated mouse anti-rat antibody and the IgG isotype control were from eBioscience (San Diego, Calif), streptavidin-phycoerythrin was from Becton-Dickinson (San Jose, Calif). Magnetic-activated cell sorting (MACS) separation columns and microbeads were from Miltenyi Biotec (Auburn, Calif). The microchemotaxis chambers were from Neuroprobe (Bethesda, Md), and cellulose nitrate filters were from Sartorius (Goettingen, Germany). RNA-Bee was from Tel-Test Inc (Friendswood, Tex), reverse transcriptase was from Gibco BRL (Life Technologies, Vienna, Austria), hot Star Taq polymerase was purchased from Quiagen Inc (Valencia, Calif), and primers were from MWG Biotech (Ebersdorf, Germany). Certified PCR Agarose was from Bio-Rad (Hercules, Calif). Protein S was from Enzyme Research Laboratories Inc (South Bend, Ind). The thrombin receptor antibody ATAP2 and protease-activated receptor (PAR) 2 antibody (c-17) was from Santa Cruz Biotechnology (Santa Cruz, Calif), and the PAR-2 agonist was from Neosystems (Strasbourg, France). The Gln antibody was from Amerca Diagnostica Inc (Grennwich, Conn).

Preparation of human eosinophils

Granulocytes were obtained from the peripheral blood of healthy donors with no history of atopic or hypereosinophilic conditions by means of dextran sedimentation and centrifugation through Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). This step was repeated once to remove mononuclear cells and was followed by hypotonic lysis of contaminating erythrocytes with sodium chloride solution. After washing, cells were resuspended in $50 \mu\text{L}/5 \times 10^7$ cells ice-cold MACS buffer (PBS with 5 mmol/L EDTA and 0.5% BSA); an equal volume of MACS colloidal superparamagnetic microbeads conjugated with anti-human CD16 mAb was added and incubated (30 minutes at 6°C). Recommended volumes of ice-cold MACS buffer were added to the cell-microbead mixture, and the cell suspension was loaded onto the separation column. The eluate containing CD16⁺ eosinophils was collected, washed, and resuspended in RPMI 1640/0.5% BSA, and the separation procedure was repeated to increase purity. The purity of sorted eosinophils was greater than 98%, as determined by means of morphology and fluorescence-activated cell sorting (FACS) analysis. Contaminating cells comprised less than 1% lymphocytes, less than 1% neutrophils and basophils, and negligible numbers of monocytes-macrophages.

Preparation of monocytes

PBMCs were isolated from EDTA-prepared blood of healthy volunteers. After Lymphoprep density gradient centrifugation, PBMCs were collected and washed 3 times with normal saline solution. Positive selection of monocytes was performed by adding MACS colloidal superparamagnetic microbeads conjugated with anti-human CD14 mAbs to cooled freshly prepared PBMC preparations in MACS buffer (PBS with 5 mmol/L EDTA and 0.5% BSA) according to the manufacturer's instructions. Cells and microbeads were incubated for 15 minutes at 4°C to 6°C. In the meantime, the separation column was positioned in the MACS magnetic field and washed with MACS buffer at room temperature. The cells were washed with MACS buffer, resuspended, and loaded onto the top of the separation column. The eluent containing the CD14⁺ cells was withdrawn, and after removal of the column from the magnet, trapped monocytes (CD14⁺) were eluted with a 6-fold amount of cold MACS buffer, centrifuged, and resuspended in medium containing 0.5% BSA. Preparations yielded a purity of approximately 98%.

Eosinophil migration assay

Migration assays were performed by using a modified 48-well Boyden microchemotaxis chamber (Neuroprobe, Bethesda, Md) in which a 5- μm -pore-size cellulose nitrate filter separated the upper

and lower chambers. In various experiments eosinophil migration was tested toward protein C or APC (0.1 pg/mL to 10 $\mu\text{g/mL}$), eotaxin, (1 nmol/L), RANTES, or C5a (both 10 nmol/L). For deactivation of migration, eosinophils were preincubated for 20 minutes at various concentrations of protein C or APC (0.1 pg/mL to 10 $\mu\text{g/mL}$). After washing twice in HBSS, eosinophil migration was assessed toward eotaxin, RANTES, or C5a in the lower wells of the chamber for 30 minutes at 37°C in a humidified atmosphere (5% CO_2). In some experiments cells were copreincubated with either function-blocking (RCR-252) or nonblocking (RCR-92) antibodies (20 $\mu\text{g/mL}$) against the EPCR and protein C preparations, followed by migration toward chemoattractants, as described above.

After the migration period, the nitrocellulose filters were dehydrated, fixed, and stained with hematoxylin. Migration depth of the cells into the filters was quantified by means of microscopy, measuring the distance (micrometers) from the surface of the filter to the leading front of 3 cells. Data are expressed as a chemotaxis index, which is the ratio between the distance of directed and random migration without attractants of eosinophils into the nitrocellulose filters.

Apoptosis of eosinophils

The effect of protein C or APC on early apoptosis (5 hours) of eosinophils was tested by using the Annexin-V/FITC kit (Bender MedSystems, Vienna, Austria). Freshly prepared eosinophils were incubated in medium, protein C, or APC (0.01 $\mu\text{g/mL}$) for 5 hours at 37°C in a humidified atmosphere. Staurosporine (1 $\mu\text{g/mL}$; proapoptotic) and GM-CSF (40 ng/mL; antiapoptotic) served as controls and were used with or without protein C or APC. For quantification, eosinophils were washed twice in PBS and resuspended in binding buffer (1×10^6 cells/mL); 195 μL was incubated with 5 μL of Annexin V-FITC for 10 minutes at room temperature, washed, and analyzed on a FACScan (Becton-Dickinson FACScan with Cellquest software, San Jose, Calif).

RT-PCR

Total RNA was isolated from 10^7 cells by using an acid guanidinium thiocyanate-phenol-chloroform mixture. A reverse transcriptase reaction was performed on 1 μg of RNA by using random hexamers reverse transcriptase. One microgram of the resulting cDNA was then subjected to 33 cycles of PCR in a 50- μL reaction mixture containing 1 pmol of sense and antisense primer pairs in a Biometra thermocycler at 95°C for 60 seconds (denaturation), 54°C for 60 seconds (annealing), and 72°C for 60 seconds (extension). Primers were designed to amplify a 409-bp coding sequence of human EPCR. The sense primer sequence was GGC AGT TTC ATC ATT GCT GG. The antisense primer sequence was TTG AAC GCC TCA GGT GAT TC. The PCR products were subjected to agarose gel analysis.

FACS analysis of EPCR expression on eosinophils

A total of 5×10^5 cells were washed twice in Dulbecco's PBS containing 0.5% BSA and incubated with 150 $\mu\text{g/mL}$ human IgG for 20 minutes at 4°C. After pelleting, cells were incubated with 10 $\mu\text{g/mL}$ anti-EPCR antibody RCR-252 or the respective isotype-matched control IgG (eBioscience) for 30 minutes at 4°C. After washing, 10 $\mu\text{g/mL}$ biotinylated mouse anti-rat IgG (eBioscience) was incubated for another 30 minutes. Cells were washed twice, and the eosinophils were subsequently incubated with a 1:25 dilution of streptavidin-phycoerythrin, washed twice, and then immediately analyzed on a FACScan with Cellquest software (Becton-Dickinson).

SDS-PAGE analysis of EPCR in leukocytes

Cells (5×10^6) were washed and incubated for 10 minutes in 2 mL of met-/cys⁻ RPMI in a humidified atmosphere at 37°C. Easy-

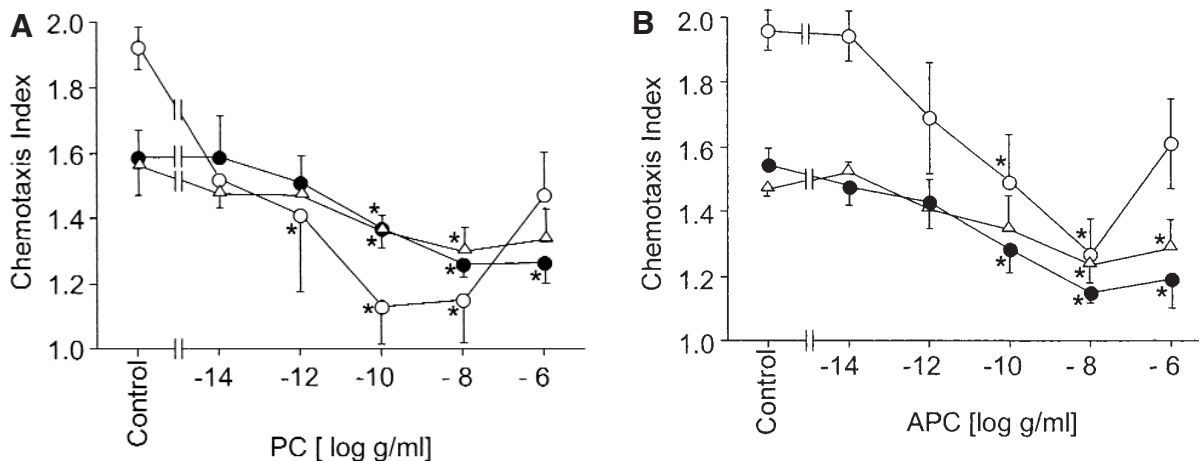


FIG 1. Inhibition of chemokine-induced migration of eosinophils by protein C (PC; **A**) and recombinant human APC (**B**). Cells were preincubated with varying protein C-APC concentrations for 30 minutes. Then chemotaxis toward eotaxin (10^{-8} mol/L, open circles), RANTES (10^{-8} mol/L, filled circles), and C5a (10^{-8} mol/L, open triangles) was tested. Results are given as the mean $\pm$ SEM of the migration index, which is the ratio of the distance of migration (in micrometers) toward attractant and the distance toward medium. Mean distance of random migration was 83.7 ± 6.1 μ m. * $P < .05$, Mann-Whitney U test versus medium incubation after multiple group comparison by using the Kruskal-Wallis test ($n = 3$).

Tag EXPRESS Protein Labeling Mix [^{35} S] was added to the pelleted cells for an incubation period of 40 minutes at 37°C . Then cells were washed in cold PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$, and 500 μL of Nonidet P-40 lysis buffer was added. After 10 minutes of incubation on ice, samples were centrifuged at 4°C . One microgram of the anti-EPCR antibody RCR-2 was added to the 500 μL of supernatant for an overnight incubation on a turnover table at 4°C . Then 1 μg of a secondary anti-rat IgG antibody was added for an additional 4 hours at 4°C . Immune complexes were captured with protein A sepharose beads for 1 hour at 4°C . Beads were then washed in PBS containing decreasing amounts of Triton X (1%, 0.1%, and 0.05%). The eluted proteins were applied to 7.5% SDS-PAGE and analyzed by means of autoradiography after treatment with enhancer solution (DuPont, Wilmington, Del).

Statistical methods

Data are expressed as mean $\pm$ SEM. Means were compared by using the Mann-Whitney U test, the paired t test, and Kruskal-Wallis ANOVA. A difference with a P value of less than .05 was considered to be significant. Statistical analyses were performed by using the StatView software package (Abacus Concepts, Berkeley, Calif).

RESULTS

Effects of protein C and APC on eosinophil migration

Arrest of inflammatory cells at sites of coagulation is an important component of the response to injury. The effects of protein C and APC on eosinophil motility were therefore tested. Eosinophils were pretreated at 37°C for 20 minutes with various concentrations of protein C or APC to investigate the effects on migration toward different chemoattractants. Both protein C and APC preparations inhibited migration of eosinophils toward eotaxin (10 nmol/L), C5a (10 nmol/L), and RANTES (10 nmol/L) in a dose-dependent manner; the plasma zymogen and the activated protein were similarly active (Fig 1).

Freshly prepared eosinophils were allowed to migrate toward different concentrations of protein C or APC (1 $\mu\text{g}/\text{mL}$ to 1 pg/mL) to explore for chemotactic properties of protein C and APC in the absence of chemoattractants; eotaxin (10^{-8} mol/L) was used as a positive control. Neither protein C nor APC induced a significant migratory response of eosinophils (data not shown).

Receptor mechanisms in the deactivation of eosinophil chemotaxis by protein C and APC

The inhibitory effects of protein C and APC on migration might be mediated through EPCR on eosinophils. Therefore the effects of a function-blocking anti-EPCR antibody on eosinophil chemotaxis were compared with those of a nonblocking anti-EPCR antibody. In the presence of the function-blocking anti-EPCR antibody RCR-252 (20 $\mu\text{g}/\text{mL}$), protein C and APC failed to inhibit chemotaxis toward various chemoattractants. Coincubation of either protein C or APC with the anti-EPCR antibody RCR-92 (20 $\mu\text{g}/\text{mL}$), which does not block its protein C activator function, had no significant effect on the inhibition of chemotaxis (Fig 2).

It was previously described that PAR signaling might play a role in mediating effects of protein C in endothelial cells.¹² Therefore it was tested whether PAR agonists or antagonists are able to influence protein C- or APC-mediated effects in eosinophils.

Treatment of eosinophils with protein C or APC in the presence of a PAR-1 agonist (SFLLRNPNDKYEPF, 1 $\mu\text{mol}/\text{L}$) and a PAR-2 agonist (SLIGKV, 100 $\mu\text{mol}/\text{L}$) did not significantly affect the inhibitory actions of protein C or APC on directed migration toward eotaxin (1 nmol/L). Also, no effect was observed after coincubation with antibodies against PAR-1 (ATAP2, 10 $\mu\text{g}/\text{mL}$) and PAR-2 (c-17, 1 $\mu\text{g}/\text{mL}$). To exclude the effects of thrombin, which might be present in contaminating doses and

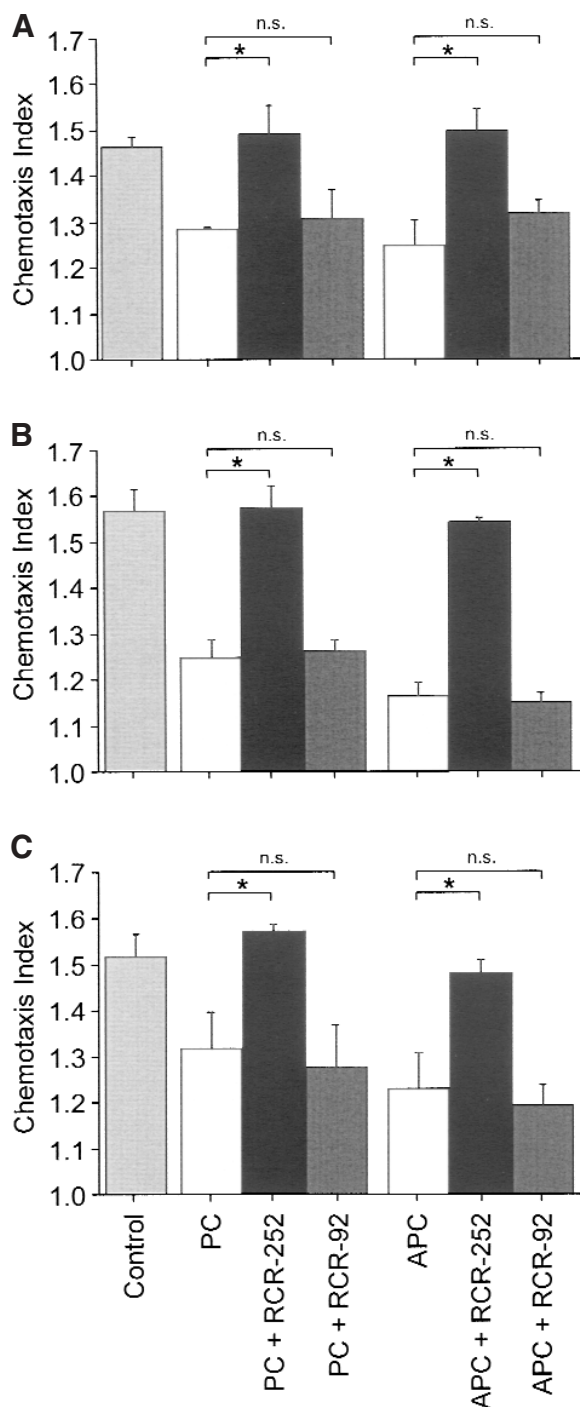


FIG 2. Inhibition of the effects of protein C (PC) and recombinant human APC on chemoattractant-induced migration of eosinophils through anti-EPCR mAbs. Cells were concomitantly incubated with either protein C or APC and the function-blocking anti-EPCR antibody RCR252 or the nonfunction-blocking anti-EPCR antibody RCR92 for 30 minutes. Then chemotaxis toward eotaxin (10^{-8} mol/L, **A**), RANTES (10^{-8} mol/L, **B**), and C5a (10^{-8} mol/L, **C**) was tested. Results are given as the mean $\pm$ SEM of the migration index, which is the ratio of the distance of migration (in micrometers) toward attractant and the distance toward medium. The mean distance of random migration was $94.1 \pm 2.0 \mu\text{m}$. * $P < .05$, Mann-Whitney U test versus medium incubation after multiple group comparison by using the Kruskal-Wallis test ($n = 3$).

complex with protein C, thrombomodulin, and EPCR, coincubation of protein C or APC and hirudin (1 U/mL) was also performed. Again, no effect on the inhibitory properties of protein C or APC on eosinophil migration of interfering with a thrombin-dependent pathway was seen. Coincubation of protein C-APC and protein S (10 $\mu\text{g/mL}$) also had no significant influence on the migration-inhibitory effects of protein C-APC (Fig 3).

Because the Gla domain of protein C has been shown to be important in the protein C-EPCR interaction,¹³ we tested whether blocking the Gla domain can influence protein C-APC effects. Coincubation with a Gla antibody (1 $\mu\text{g/mL}$) was able to diminish the migration-inhibitory effects of protein C or APC (Fig 3).

Expression of EPCR in eosinophils

EPCR has been proposed to mediate the anti-inflammatory effects of APC,¹⁴ and its expression in monocytes has been described.¹¹ To determine whether the *EPCR* gene is expressed in eosinophils, RT-PCR was performed. Data confirm that *EPCR* mRNA is expressed in human eosinophils, as well as in monocytes that were used as the control cell type (Fig 4, A).

Experiments were performed to further confirm de novo synthesis of EPCR protein analysis after radiolabeling and immunoprecipitation by SDS-PAGE. Eosinophils and purified CD14⁺ PBMCs (monocytes) were metabolically radiolabeled with EXPRESS [³⁵S], and EPCR was recovered from cell lysates by means of immunoprecipitation with the anti-EPCR antibody RCR-2. Newly synthesized EPCR was clearly discernible on SDS-PAGE autoradiographs as a 45-kd band for both eosinophils and monocytes (Fig 4, C). The presence of EPCR-like immunoreactivity on the surface of eosinophils was tested by means of FACS analysis. A slight shift of fluorescence was observed by the anti-EPCR antibody RCR-252, which is indicative of the presence of low-grade cell-surface EPCR (Fig 4, B).

Apoptosis of eosinophils

The effects of protein C or APC on spontaneous apoptosis of isolated eosinophils were studied by using the Annexin-V/FITC labeling kit. The number of apoptotic cells was quantified by using FACS analysis after 5 hours of incubation with serum-free medium, protein C, or APC (0.01 $\mu\text{g/mL}$). Staurosporine (1 $\mu\text{g/mL}$; proapoptotic) and GM-CSF (40 ng/mL; antiapoptotic) were used as controls. After 5 hours, $54.3\% \pm 4.6\%$ ($n = 3$) of eosinophils underwent apoptosis. Incubation with GM-CSF reduced this number to $44.0\% \pm 5.2\%$ ($P < .05$ vs medium), and with staurosporine, $90.6\% \pm 3.3\%$ ($P < .05$ vs medium) of the cells were apoptotic. Neither protein C ($52.5\% \pm 6.6\%$) nor APC ($49.2\% \pm 8.7\%$) altered the rate of eosinophil apoptosis ($P > .1$ vs medium).

DISCUSSION

Chemotactic migration of eosinophils is a prerequisite for the cells to reach their site of biologic action. Our

working hypothesis is that protein C and APC might modulate eosinophil chemotaxis directly and independently of other components of the coagulation cascade. The present study implicates EPCR of eosinophils in the effects of protein C on cell migration. Protein C and APC blocked the chemotaxis of eosinophils toward chemokines and other attractants. This effect could be reversed by an anti-EPCR antibody directed against a protein C–APC binding site on EPCR. Expression of EPCR in eosinophils is confirmed by means of identification of expression of mRNA and newly synthesized protein.

Chronic asthma might lead to subepithelial fibrosis, extracellular matrix deposition, smooth muscle hypertrophy, and goblet cell hyperplasia in the airways.¹⁵ Inflammatory cells, such as eosinophils, contribute to airway structural changes by releasing proinflammatory cytokines and growth factors.¹⁶ Eosinophil-derived elastase¹⁷ and metalloproteases¹⁸ are involved in the process of tissue remodeling and fibrosis. Additionally, eosinophils act as direct modulatory cells in fibroblast proliferation and collagen synthesis through transforming growth factor β .¹⁹

Beside inflammation, activation of the coagulation and clotting system plays an important role in the pathogenesis of allergic diseases.^{20,21} Studies have suggested that excessive procoagulant activity and abnormal fibrin turnover play relevant roles in the pathogenesis of atopic dermatitis and asthma bronchiale. Interstitial and intra-alveolar fibrin, its degradation products, and local thrombin generation might potentiate the inflammatory response by their effects on chemotaxis, vascular permeability, and immunomodulation.²² Activation of protein C, the most important pathway in the anticoagulant system within the vasculature that also occurs in the alveolar space, is decreased in patients with asthma bronchiale and might cause increased procoagulant activity and inhibited fibrinolysis, thus promoting intra-alveolar fibrosis in these disorders.^{8,23} Results of the present study suggest that the effects of the protein C pathway extend beyond that within the coagulation system.

In the present study it was observed that protein C and APC inhibit directed migration of eosinophils in an EPCR-dependent fashion because the inhibitory effect could be reversed with an anti-EPCR antibody (Fig 2). Migration of eosinophils was tested toward various chemoattractants that elicit the migratory response through different signaling pathways. Protein C and APC were able to inhibit migration toward each of these substances in a dose-dependent manner (Fig 1). Nearly complete abrogation of stimulated migration was seen at concentrations in the range of 1 $\mu\text{g/mL}$ to 10 ng/mL, which is the amount of protein C circulating in animals and human subjects with normal functioning endothelium.²⁴ Protein C and APC inhibited migration of eosinophils toward attractants of different classes that differ in their receptor signaling cascades. This observation might indicate that the protein C pathway of eosinophils affects mechanisms central to the process of cell migration rather than chemoattractant or postreceptor signaling.

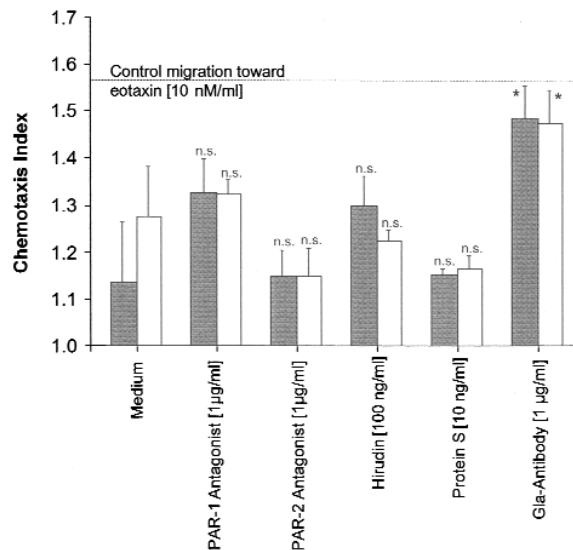


FIG 3. Abrogation of the effects of protein C (PC) and recombinant human APC on chemokine-induced migration by Gla antibodies. Cells were coincubated with protein C (gray bars) or APC (white bars; both 10 ng/mL) and various test substances for 20 minutes. Then chemotaxis toward eotaxin (10 nmol/L per milliliter) was monitored. Results are given as the mean $\pm$ SEM of the chemotaxis index, which is the ratio of the distance of unstimulated cells toward medium. Chemotaxis of untreated eosinophils toward eotaxin (10 nmol/L per milliliter) served as a positive control (chemotaxis index = 1.563 ± 0.049). * $P < .05$, Mann-Whitney U test versus medium after multiple group comparison by using the Kruskal-Wallis test.

Diffuse exposure of eosinophils to circulating protein C in the intravascular compartment might prevent eosinophils from premature activation.

The ability of APC to inhibit thrombin generation has the potential to reduce the proinflammatory activities of thrombin, which are mediated by PAR-1, PAR-3, and PAR-4.²⁵ PARs mediate thrombin-induced inflammation by generating signals that provide the intracellular and functional link between inflammation at sites of vascular injury, modulating platelet and endothelial cell activation.^{26–28} Recently, Riewald et al¹² demonstrated that PAR-1, the prototypical thrombin receptor, is a target for EPCR-dependent APC signaling in endothelial cells and that PAR-1 signaling could account for all APC-induced protective genes against sepsis. Because thrombin is a potential contamination of protein C preparations, control experiments in which thrombin was directly inactivated by hirudin were also performed. Protein C and APC effects appeared independent of thrombin. Human eosinophils constitutively transcribe mRNA for PAR-2 and PAR-3 but not those for PAR-1 and PAR-4, and further investigations demonstrated the expression of a functional PAR-2 receptor on human eosinophils.²⁹ We investigated the effect of selective PAR-1 or PAR-2 agonists and an antibody against PAR-1 and PAR-2 on eosinophil chemotaxis. Neither of the agonists nor the antibodies against PAR-1 and PAR-2 were able to affect protein C- or APC-induced inhibition of eosinophil

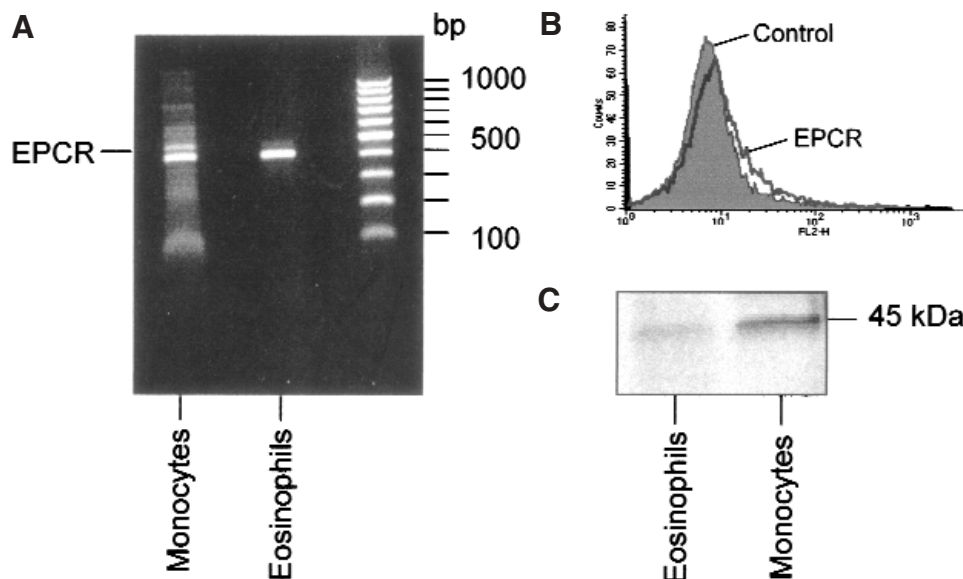


FIG 4. RT-PCR and immunoprecipitation analysis of EPCR in eosinophils. **A**, *EPCR* mRNA in eosinophils and monocytes. One microgram of total RNA from each sample was reverse transcribed into cDNA and amplified for the *EPCR* gene by using PCR. EPCR is represented by the 409-bp product. **B**, FACS analysis of anti-EPCR mAb binding to eosinophils. Fluorescence analysis used a FACScan Flow cytometer, and a histogram of phycoerythrin fluorescence is shown. Cells were either incubated with isotype-matched control IgG (gray curve) or anti-EPCR mAb (black line) and stained with phycoerythrin-conjugated streptavidin. **C**, Synthesis of EPCR in eosinophils and monocytes. Cells were radiolabeled for 40 minutes, followed by lysis and immunoprecipitation with the anti-EPCR antibody RCR-2, SDS-PAGE, and autoradiography.

chemotaxis. According to these data, involvement of PAR-1 or PAR-2 in the effects of protein C or APC on eosinophils is unlikely.

Previous studies have demonstrated that EPCR expression is not restricted to the endothelium. It has also been detected in monocytes, as well as in a number of cancer cell lines, including those derived from monoblastic leukemia (U937), glioblastoma (U87,T98G), osteosarcoma (HOS), and erythroleukemia (HEL, K562).¹¹ Thus EPCR might also be involved in the eosinophil's response to protein C and APC. A blocking antibody against EPCR was able to diminish the effects of protein C and APC on eosinophil migration. A control antibody that binds to EPCR but does not affect EPCR in the activation of protein C³⁰ failed to affect protein C- or APC-dependent inhibition of migration. This might indicate that ligation of the high-affinity binding site of EPCR³¹ for protein C and APC is required for the functional response to occur. Data show that eosinophils express the *EPCR* gene (Fig 4). By using immunoprecipitation, we identified a metabolically labeled, newly synthesized EPCR in the lysate of eosinophils. A slight expression of EPCR on the cell surface of eosinophils was also seen by means of FACS analysis, thus confirming functional and molecular data.

For detecting biologic activity of EPCR in a second cell function system, we investigated the effects of protein C and APC on eosinophil apoptosis. It was previously described in endothelial cells that the number of cells undergoing apoptosis is significantly reduced by

APC.³² In our experiments protein C and APC failed to significantly affect eosinophil apoptosis. Stimulation of apoptosis has important implications for the resolution of inflammatory disorders. Vignola et al,³³ for example, demonstrated that the absolute number of apoptotic eosinophils was inversely correlated with clinical severity of asthma. Inhibition of apoptosis with protein C or APC, such as in endothelial cells,³² was not observed in eosinophils.

Taken together, these results show that protein C and APC inhibit the migratory response of eosinophils to different potent chemoattractants through mechanisms that involve an EPCR of eosinophils. This furthermore indicates that modulation of eosinophil function might be among the anti-inflammatory effects of the anticoagulatory pathway of protein C.

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REFERENCES

1. Grewe M, Czech W, Morita A, Werfel T, Klammer M, Kapp A, et al. Human eosinophils produce biologically active IL-12: implications for control of T cell responses. *J Immunol* 1998;161:415-20.
2. Lim KG, Wan HC, Bozza PT, Resnick MB, Wong DT, Cruikshank WW, et al. Human eosinophils elaborate the lymphocyte chemoattractants, IL-16 (lymphocyte chemoattractant factor) and RANTES. *J Immunol* 1996;156:2566-70.
3. Giembycz MA, Lindsay MA. Pharmacology of the eosinophil. *Pharmacol Rev* 1999;51:213-340.
4. Wardlaw AJ, Moqbel R, Kay AB. Eosinophils: biology and role in disease. *Adv Immunol* 1995;60:151-266.

5. Atkins PC, Kaplan AP, von Allmen C, Moskovitz A, Zweiman B. Activation of the coagulation pathway during ongoing allergic cutaneous reactions in humans. *J Allergy Clin Immunol* 1992;89:552-9.
6. Atkins PC, von Allmen C, Moskovitz A, Valenzano M, Zweiman B. Fibrin formation during ongoing cutaneous allergic reactions: comparison of responses to antigen and codeine. *J Allergy Clin Immunol* 1993;91:956-60.
7. Gabazza EC, Taguchi O, Tamaki S, Takeya H, Kobayashi H, Yasui H, et al. Thrombin in the airways of asthmatic patients. *Lung* 1999;177:253-62.
8. Hataji O, Taguchi O, Gabazza EC, Yuda H, Fujimoto H, Suzuki K, et al. Activation of protein C pathway in the airways. *Lung* 2002;180:47-59.
9. Grey ST, Hancock WW. A physiologic anti-inflammatory pathway based on thrombomodulin expression and generation of activated protein C by human mononuclear phagocytes. *J Immunol* 1996;156:2256-63.
10. Murakami K, Okajima K, Uchiba M, John M, Nakagaki T, Okabe H, et al. Activated protein C prevents LPS-induced pulmonary vascular injury by inhibiting cytokine production. *Am J Physiol* 1997;272:L197-202.
11. Galligan L, Livingstone W, Volkov Y, Hokamp K, Murphy C, Lawler M, et al. Characterization of protein C receptor expression in monocytes. *Br J Haematol* 2001;115:408-14.
12. Riewald M, Petrovan RJ, Donner A, Mueller BM, Ruf W. Activation of endothelial cell protease activated receptor 1 by the protein C pathway. *Science* 2002;296:1880-2.
13. Regan LM, Mollica JS, Rezaie AR, Esmon CT. The interaction between the endothelial cell protein C receptor and protein C is dictated by the gamma-carboxyglutamic acid domain of protein C. *J Biol Chem* 1997;272:26279-84.
14. White B, Livingstone W, Murphy C, Hodgson A, Rafferty M, Smith OP. An open-label study of the role of adjuvant hemostatic support with protein C replacement therapy in purpura fulminans-associated meningococemia. *Blood* 2000;96:3719-24.
15. Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 2000;161:1720-45.
16. Barnes PJ, Chung KF, Page CP. Inflammatory mediators of asthma: an update. *Pharmacol Rev* 1998;50:515-96.
17. Lungarella G, Menegazzi R, Gardi C, Spessotto P, de Santi MM, Bertoncin P, et al. Identification of elastase in human eosinophils: immunolocalization, isolation, and partial characterization. *Arch Biochem Biophys* 1992;292:128-35.
18. Ohno I, Ohtani H, Nitta Y, Suzuki J, Hoshi H, Honma M, et al. Eosinophils as a source of matrix metalloproteinase-9 in asthmatic airway inflammation. *Am J Respir Cell Mol Biol* 1997;16:212-9.
19. Levi-Schaffer F, Garbuzenko E, Rubin A, Reich R, Pickholz D, Gillerly P, et al. Human eosinophils regulate human. *Proc Natl Acad Sci U S A* 1999;96:9660-5.
20. Fujisawa T, Fujisawa R, Kato Y, Nakayama T, Morita A, Katsumata H, et al. Presence of high contents of thymus and activation-regulated chemokine in platelets and elevated plasma levels of thymus and activation-regulated chemokine and macrophage-derived chemokine in patients with atopic dermatitis. *J Allergy Clin Immunol* 2002;110:139-46.
21. Moritani C, Ishioka S, Haruta Y, Kambe M, Yamakido M. Activation of platelets in bronchial asthma. *Chest* 1998;113:452-8.
22. Leavell KJ, Peterson MW, Gross TJ. The role of fibrin degradation products in neutrophil recruitment to the lung. *Am J Respir Cell Mol Biol* 1996;14:53-60.
23. Kobayashi H, Gabazza EC, Taguchi O, Wada H, Takeya H, Nishioka J, et al. Protein C anticoagulant system in patients with interstitial lung disease. *Am J Respir Crit Care Med* 1998;157:1850-4.
24. Yan SB, Dhainaut JF. Activated protein C versus protein C in severe sepsis. *Crit Care Med* 2001;29(suppl 7):S69-74.
25. White B, Schmidt M, Murphy C, Livingstone W, O'Toole D, Lawler M, et al. Activated protein C inhibits lipopolysaccharide-induced nuclear translocation of nuclear factor kappaB (NF-kappaB) and tumour necrosis factor alpha (TNF-alpha) production in the THP-1 monocytic cell line. *Br J Haematol* 2000;110:130-4.
26. Coughlin SR. Sol Sherry lecture in thrombosis: how thrombin 'talks' to cells: molecular mechanisms and roles in vivo. *Arterioscler Thromb Vasc Biol* 1998;18:514-8.
27. Coughlin SR, Vu TK, Hung DT, Wheaton VI. Characterization of a functional thrombin receptor. Issues and opportunities. *J Clin Invest* 1992;89:351-5.
28. Coughlin SR. Protease-activated receptors in vascular biology. *Thromb Haemost* 2001;86:298-307.
29. Miike S, McWilliam AS, Kita H. Trypsin induces activation and inflammatory mediator release from human eosinophils through protease-activated receptor-2. *J Immunol* 2001;167:6615-22.
30. Ye X, Fukudome K, Tsuneyoshi N, Satoh T, Tokunaga O, Sugawara K, et al. The endothelial cell protein C receptor (EPCR) functions as a primary receptor for protein C activation on endothelial cells in arteries, veins, and capillaries. *Biochem Biophys Res Commun* 1999;259:671-7.
31. Fukudome K, Esmon CT. Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor. *J Biol Chem* 1994;269:26486-91.
32. Joyce DE, Gelbert L, Ciaccia A, DeHoff B, Grinnell BW. Gene expression profile of antithrombotic protein c defines new mechanisms modulating inflammation and apoptosis. *J Biol Chem* 2001;276:11199-203.
33. Vignola AM, Chanez P, Chiappara G, Siena L, Merendino A, Reina C, et al. Evaluation of apoptosis of eosinophils, macrophages, and T lymphocytes in mucosal biopsy specimens of patients with asthma and chronic bronchitis. *J Allergy Clin Immunol* 1999;103:563-73.