

Expression and function of the angiopoietin receptor Tie-2 in human eosinophils

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Background: Increased vascularity of bronchial mucosa is closely related to the expression of angiogenic factors, which contribute to the pathogenesis of diseases such as asthma bronchiale.

Objective: Here we examine the effects of the angiogenic growth factors angiopoietin 1 and angiopoietin 2 on eosinophil function *in vitro* and possible involvement of the angiopoietin receptor Tie-2.

Methods: Eosinophil migration was studied by micropore filter assays. Signaling mechanisms required for angiopoietin-dependent migration were tested by using signaling enzyme blockers. Tie-2 mRNA and receptor expression on the cell surface of eosinophils was demonstrated in RT-PCR and by fluorescence-activated cell sorting analysis.

Results: Angiopoietin 1 significantly stimulated eosinophil chemotaxis via activation of phosphodiesterase, phosphatidylinositol 3'-kinase, and tyrosine kinases. The effect on eosinophil migration of angiopoietin 1 was reversed by an antibody against the Tie-2 receptor and by angiopoietin 2. Incubation of eosinophils with angiopoietin 1 abolished the chemotactic effects of vascular endothelial growth factor on human eosinophils via the Tie-2 receptor. Finally, Tie-2 expression by human eosinophils was demonstrated on the transcriptional and protein level.

Conclusions: Data suggest that angiopoietin 1 stimulates directed migration and possibly inhibits vascular endothelial growth factor-induced eosinophil chemotaxis via its Tie-2 receptor, which is expressed by eosinophils. Thus, angiopoietin 1 may play an important role in the modulation of eosinophilic inflammation. (J Allergy Clin Immunol 2004;114:1077-84.)

Key words: Eosinophils, vascular endothelial growth factor, angiopoietin, asthma

Structural alterations in the airway walls of chronic inflammatory diseases include fibrosis, smooth muscle hypertrophy, hyperplasia of mucus-secreting cells, and

Abbreviations used

GFX: Bisindolylmaleimide I GF 109203X
FACS: Fluorescence-activated cell sorting
HUVEC: Human umbilical vein endothelial cells
IBMX: Isobutylmethylxanthine
PI3K: Phosphatidylinositol 3'-kinase
PKC: Protein kinase C
VEGF: Vascular endothelial growth factor.

new vessel formation.¹ Increased vascularity of bronchial mucosa is closely related to the expression of angiogenic factors, which contribute to the pathogenesis of diseases such as asthma bronchiale.² The specific growth factors vascular endothelial growth factor (VEGF) and angiopoietin 1 have clinically relevant actions on the microvasculature,³ and both of these growth factors play essential but distinct roles in vascular development. VEGF, as the key to the formation of the vascular plexus in early development,⁴ is highly expressed in the airways of subjects with asthma and correlates with mucosal vascularity.^{2,5} In contrast, angiopoietin 1 acts during the subsequent remodeling of the vasculature into a network of mature cells.^{6,7} In addition to being an angiogenic factor, VEGF is also a proinflammatory cytokine that enhances leukocyte rolling and adhesion, vascular leakage, and, therefore, inflammation, whereas angiopoietin 1 seems to have antileakage effects.^{8,9}

In allergic and parasitic diseases, cellular infiltrates consist mainly of eosinophils. Selective migration of circulating eosinophils into tissues plays an important role and is under tight regulation by a number of chemoattractants.¹⁰ Eosinophils are inflammatory cells that secrete lipid mediators and cytokines relevant to the pathophysiology of asthma bronchiale.^{10,11}

In this study we investigated the effects of angiopoietin 1 and the possible involvement of the angiopoietin Tie-2 receptor in the chemotaxis of human eosinophils. Expression of the angiopoietin Tie-2 receptor mRNA and synthesis of the cell-surface receptor were detected.

METHODS

Cells

Eosinophils were enriched from the peripheral blood of healthy donors with no history of atopic or hypereosinophilic conditions by magnetic-activated cell sorting (Miltenyi Biotec, Bergisch

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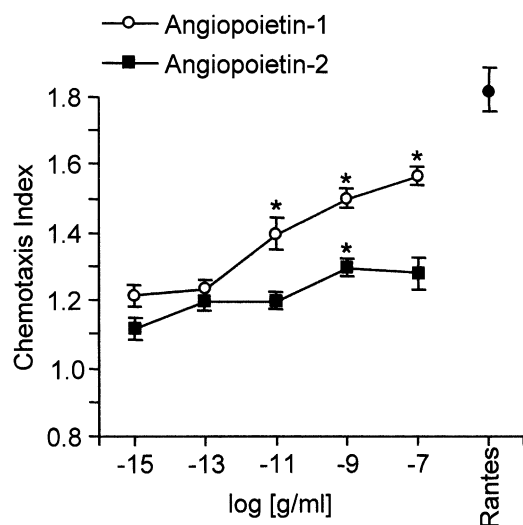


FIG 1. Effects of angiopoietin 1 and angiopoietin 2 on migration of human eosinophils. Human eosinophils were allowed to migrate toward increasing concentrations of angiopoietin 1 and angiopoietin 2. RANTES (10 ng/mL) served as a positive control. Results are means $\pm$ SEMs of the chemotaxis index; the mean distance of random migration was $64.3 \pm 3.6 \mu\text{m}$ ($n = 4$). * $P < .05$.

Gladbach, Germany) as previously described.¹² The purity of sorted eosinophils was greater than 98%, as determined by 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.

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh placenta cords and cultured as recently described.¹³ HUVECs of passage 2 were used for RNA extraction.

Eosinophil migration assay

Migration assays were performed in a modified 48-well Boyden microchemotaxis chamber (Neuroprobe, Bethesda, Md) in which a 5- μm pore size cellulose nitrate filter separated the upper and the lower chambers.^{14,15} Migration toward various concentrations of angiopoietin 1 and angiopoietin 2 (1 fg/mL to 100 ng/mL; both from Sigma, St Louis, Mo) was tested. RANTES (10 ng/mL; PeproTech, Rocky Hills, NJ) was used as a positive control. The migration depth of the cells into the filters was quantified by means of microscopy, by 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 of eosinophils into the nitrocellulose filters.

In some experiments, eosinophils were preincubated with angiopoietin 1, angiopoietin 2, a function-blocking mouse anti-human Tie-2 antibody (R&D Systems, Minneapolis, Minn), or a combination of angiopoietin 1 and the antibody for 30 minutes (4°C or 37°C) before cell migration toward angiopoietin 1, VEGF (Sigma), or RANTES was measured. Nonimmune mouse IgG (eBioscience, San Diego, Calif) served as control.

Intracellular signaling of angiopoietin on eosinophils was tested by preincubation of the cells with the intracellular enzyme blockers bisindolylmaleimide I GF 109203X (GFX; 500 nmol/L; Boehringer Ingelheim, Ingelheim am Rhein, Germany), staurosporine (10 ng/mL; from *Streptomyces* species), wortmannin (10 nmol/L; from *Penicillium funiculosum*), isobutylmethylxanthine (IBMX; 10 ng/mL), tyrphostin 23 (10 ng/mL), cholera or pertussis toxin

TABLE I. Effect of concentration gradients of angiopoietin 1 on eosinophil migration

Angiopoietin 1 (g/mL)	Upper chamber			
	Medium	10^{-11}	10^{-9}	10^{-7}
Lower chamber				
Medium	1.00 ± 0.00	1.02 ± 0.05	1.02 ± 0.03	1.03 ± 0.03
10^{-11}	$1.26 \pm 0.05^*$	1.06 ± 0.04	1.02 ± 0.02	1.00 ± 0.05
10^{-9}	$1.35 \pm 0.08^*$	$1.26 \pm 0.05^*$	1.06 ± 0.04	1.05 ± 0.05
10^{-7}	$1.50 \pm 0.04^*$	$1.40 \pm 0.04^*$	1.10 ± 0.05	1.00 ± 0.04

Different concentrations of angiopoietin 1 were added to the upper and/or lower wells of the chemotaxis chamber before cell migration. Results are given as means $\pm$ SEMs of the chemotaxis index. The mean distance of random migration was $65.3 \pm 2.7 \mu\text{m}$ ($n = 4$).

* $P < .05$.

(50 ng/mL; all from Sigma), and PP1 (1 $\mu\text{mol/L}$; an Src kinase inhibitor), PP2 (1 $\mu\text{mol/L}$; inhibits tyrosine kinases lck, fyn, and hck), or PP3 (1 $\mu\text{mol/L}$; inactive PP1 analog; all 3 from Calbiochem, San Diego, Calif) for 30 minutes. To further confirm data of the enzyme blocker experiments, dose-response studies with several key agents were performed. The cells were then washed twice, resuspended in RPMI/0.5% BSA, and tested in the migration assay toward angiopoietin 1 and RANTES.

Checkerboard analysis

To ensure that the effect observed was true chemotaxis, checkerboard analyses were performed. Eosinophils were resuspended in RPMI 1640/0.5% BSA containing various concentrations of angiopoietin 1 just before they were transferred to the upper wells of the Boyden chamber. The same concentrations of angiopoietin 1 were put in the lower wells beneath the filter of the Boyden chamber, thus causing distinct concentration gradients to form.

Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from 10^7 cells by using RNA-Bee (Tel Test Inc, Friendswood, Tex). A reverse transcriptase reaction was performed on 1 μg of RNA by using random hexamer primers. To exclude genomic DNA contamination, in control experiments the transcription reaction was performed in the absence of reverse transcriptase. The cDNA product was amplified by 38 cycles of 60 seconds at 95°C, 60 seconds at 58°C, and a 60-second extension at 72°C by using specific primer pairs (MWG Biotech, Ebersdorf, Germany; Tie-2 forward, 5'-GGA TAC GAA CCA TGA AGA TGC G-3'; reverse, 5'-CCA AAC GTG TGC AGT TCA CAA G-3'). The RT-PCR products (448 bp) were separated in 1.5% (wt/vol) agarose gels. HUVECs were used as a positive control, because Tie-2 expression on endothelial cells has previously been described.¹⁶

FACS analysis

A total of 5×10^5 eosinophils were incubated with 150 $\mu\text{g/mL}$ of human IgG for 20 minutes at 4°C. After pelleting, cells were first incubated for 30 min with specific antibodies against Tie-2 (R&D Systems) or VEGF receptor flt-1 (Abcam, Cambridge, United Kingdom) or with control antibody and, after washing, with 10 $\mu\text{g/mL}$ biotinylated goat anti-mouse IgG (eBioscience) for another 30 minutes at 4°C. Cells were stained with phycoerythrin-conjugated streptavidin (BD, San Jose, Calif) for subsequent FACS analyses on a FACScan (BD). Because the VEGF receptor flt-1 has

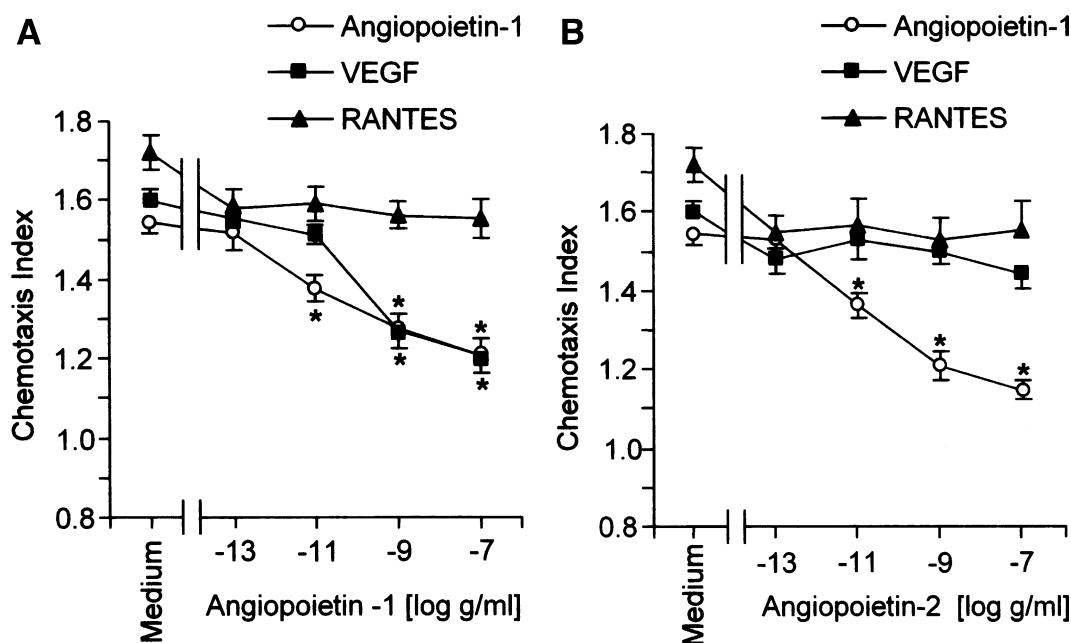


FIG 2. Effect of angiopoietin 1 and angiopoietin 2 pretreatment on eosinophil chemotaxis in response to angiopoietin 1, VEGF, and RANTES. Eosinophils were incubated with different concentrations of angiopoietin 1 (**A**) and angiopoietin 2 (**B**) for 30 minutes; thereafter, chemotaxis toward angiopoietin 1 (100 ng/mL), VEGF (10 ng/mL), and RANTES (10 ng/mL) was measured. Results are means $\pm$ SEMs of the chemotaxis index; the mean distance of random migration was $61.7 \pm 3.4 \mu\text{m}$ ($n = 4$). * $P < .05$.

previously been detected in eosinophils,¹⁷ cells were incubated with angiopoietin 1 and VEGF for 30 minutes, and receptor expression of Tie-2 and flt-1 was tested as described previously.

Statistical methods

Data are expressed as means $\pm$ SEMs. Means were compared by the Mann-Whitney *U* test and Kruskal-Wallis ANOVA. A difference with $P < .05$ was considered to be significant. Statistical analyses were performed with the StatView software package (Abacus Concepts, Berkeley, Calif).

RESULTS

Effects of angiopoietin 1 and angiopoietin 2 on eosinophil migration

To investigate the chemotactic properties of angiopoietin 1 and angiopoietin 2 in the absence of chemoattractants, freshly prepared eosinophils were allowed to migrate toward different concentrations of angiopoietin 1 and angiopoietin 2 (1 fg/mL to 100 ng/mL). RANTES (10 ng/mL) was used as a positive control. Concentrations of angiopoietin 1 ranging from 100 ng/mL to 10 pg/mL profoundly increased migration in a dose-dependent manner, whereas angiopoietin 2 showed only weak effects on eosinophil chemotaxis, with a maximal response that was not more than 50% of that observed with angiopoietin 1 (Fig 1).

In checkerboard analysis the migratory response toward angiopoietin 1 was confirmed as true chemo-

taxis. Eosinophil migration occurred only in the presence of a positive concentration gradient (Table I).

Effects of incubation with angiopoietin 1 and 2 on the chemotactic response of eosinophils to angiopoietin 1 and VEGF

Angiopoietin 2 acts as a natural antagonist of angiopoietin 1 by blocking Tie-2 activation in endothelial cells,¹⁸ whereas angiopoietin 1 itself might antagonize the effect of VEGF on vessel wall permeability.⁸ We tested those proposed interactions in our functional assay. Eosinophils were preincubated with different concentrations of angiopoietin 1 and angiopoietin 2 for 30 minutes at 37°C in a humidified atmosphere (5% CO₂), and migration toward angiopoietin 1 (100 ng/mL), VEGF (10 ng/mL), or RANTES (10 ng/mL) was measured. Whereas angiopoietin 1 was able to diminish the effects of angiopoietin 1 and VEGF (Fig 2 A), angiopoietin 2 abolished eosinophil migration toward angiopoietin 1 but did not affect VEGF-induced eosinophil migration. Pretreatment of either angiopoietin 1 or angiopoietin 2 did not inhibit the migratory response toward RANTES (Fig 2). This reaction pattern was temperature independent, because pretreatment of cells at 4°C revealed an equal result (not shown) but was time dependent, and significantly reduced migration toward VEGF was observed after an angiopoietin 1 pretreatment period of 5 minutes or longer (Fig 3).

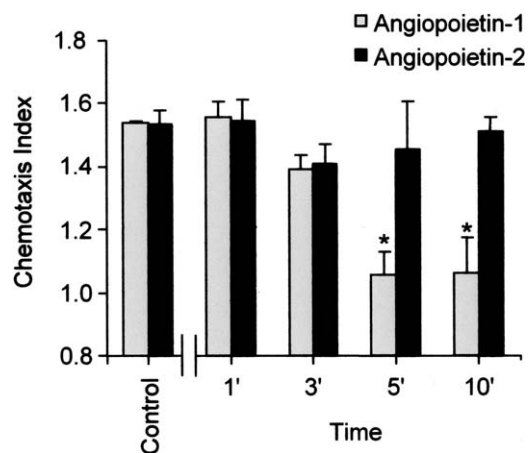


FIG 3. Time-dependent inhibition of VEGF-induced eosinophil migration by angiopoietin 1. Eosinophils were incubated with angiopoietin 1 or angiopoietin 2 (both at 100 ng/mL) for various time periods. After washing of cells, chemotaxis toward VEGF (10 ng/mL) was measured. Results are given as the means $\pm$ SEMs of the chemotaxis index; the mean distance of random migration was $44.6 \pm 2.3 \mu\text{m}$ ($n = 4$). * $P < .05$.

Effects of antibodies to Tie-2 receptor on the chemotactic response of eosinophils to angiopoietin 1

Because it is known that angiopoietin 1 mediates its effects in endothelial cells and hematopoietic cells primarily via its specific angiopoietin receptor Tie-2,^{16,19} chemotaxis experiments with angiopoietin 1 were performed in the presence of antibodies to the Tie-2 receptor. Cells were pretreated with different concentrations of an anti-Tie-2 mAb or an isotype control antibody and were then allowed to migrate toward angiopoietin 1 (100 ng/mL) or RANTES (10 ng/mL). Anti-Tie-2 significantly inhibited directed migration of eosinophils toward angiopoietin 1, but not toward RANTES (Fig 4, A). To further elucidate the involvement of Tie-2 in angiopoietin 1-mediated inhibition of VEGF-induced eosinophil chemotaxis, cells were coincubated with different concentrations of the Tie-2 mAb and angiopoietin 1 (100 ng/mL) before the migratory response toward VEGF was tested. Blocking Tie-2 with the antibody (100 ng/mL to 10 $\mu\text{g/mL}$) antagonized the inhibitory effect of angiopoietin 1 and restored VEGF-induced chemotaxis. This was not seen with control nonimmune IgG. RANTES-induced chemotaxis was not affected by this treatment (Fig 4, B).

Blocking of intracellular signaling enzymes in angiopoietin 1-induced chemotaxis of eosinophils

To elucidate signaling pathways involved in transmitting angiopoietin 1 effects in eosinophil migration, different intracellular enzyme blockers were used and compared with effects in RANTES-induced migration

of eosinophils. The blockers staurosporine, GFX, IBMX, wortmannin, and tyrphostin 23 were used at established signal transduction-blocking concentrations at which random migration of the cells is not influenced. The specific protein kinase C (PKC) inhibitor GFX and staurosporine, a nonspecific inhibitor of PKC that also affects protein kinase A signaling, did not affect angiopoietin 1-induced eosinophil migration, whereas the phosphodiesterase inhibitor IBMX and wortmannin, a specific inhibitor of phosphatidylinositol 3'-kinase (PI3K), decreased angiopoietin 1-induced chemotaxis in eosinophils. Furthermore, the tyrosine kinase inhibitor tyrphostin 23 significantly inhibits the migration of eosinophils toward angiopoietin 1. In subsequent experiments, selective inhibitors of Src kinases, PP1 and PP2, were used. Both blocked the migratory response of eosinophils toward angiopoietin 1, suggesting the involvement of Src tyrosine kinases. Dose-response studies with staurosporine, IBMX, and the Src tyrosine kinase inhibitors confirmed the involvement of PI3K and the phosphodiesterase and tyrosine kinases in angiopoietin-mediated eosinophil migration (data not shown). Furthermore, the chemotactic response was inhibited by G protein affecting cholera toxin, but not pertussis toxin (Table II).

Expression of the Tie-2 receptor in eosinophils

The surface expression of the Tie-2 receptor on these cells and the mRNA content of eosinophils were tested. In FACS analyses, intact eosinophils from different donors stained slightly positive with an anti-Tie-2 mAb, compared with control IgG, thus indicating cell-surface expression of the angiopoietin/Tie-2 receptor protein. Incubation with angiopoietin 1 or VEGF did not affect the expression of Tie-2 or of the VEGF receptor flt-1 (data not shown). To determine whether Tie-2 receptor mRNA is expressed in eosinophils, RT-PCR analysis was performed and confirmed the presence of Tie-2 receptor mRNA in human peripheral blood eosinophils from different healthy donors (Fig 5).

DISCUSSION

The presence of angiogenesis in asthma is being documented by an increasing number of studies (see review²⁰). Eosinophil infiltration is a characteristic feature of allergic inflammation, and levels of VEGF correlate well with the percentage of eosinophils and eosinophil cationic protein in induced sputum of patients with asthma.²¹ To date, no studies have been performed on the interactions of angiopoietins and eosinophils. Therefore, effects of angiopoietins on eosinophils were explored *in vitro* in more detail. We also looked at interactions of angiopoietins with VEGF and a possible involvement of the Tie-2 receptor.

In a previous study, angiopoietin 1, but not angiopoietin 2, led to a directed migration of HUVECs.²² The

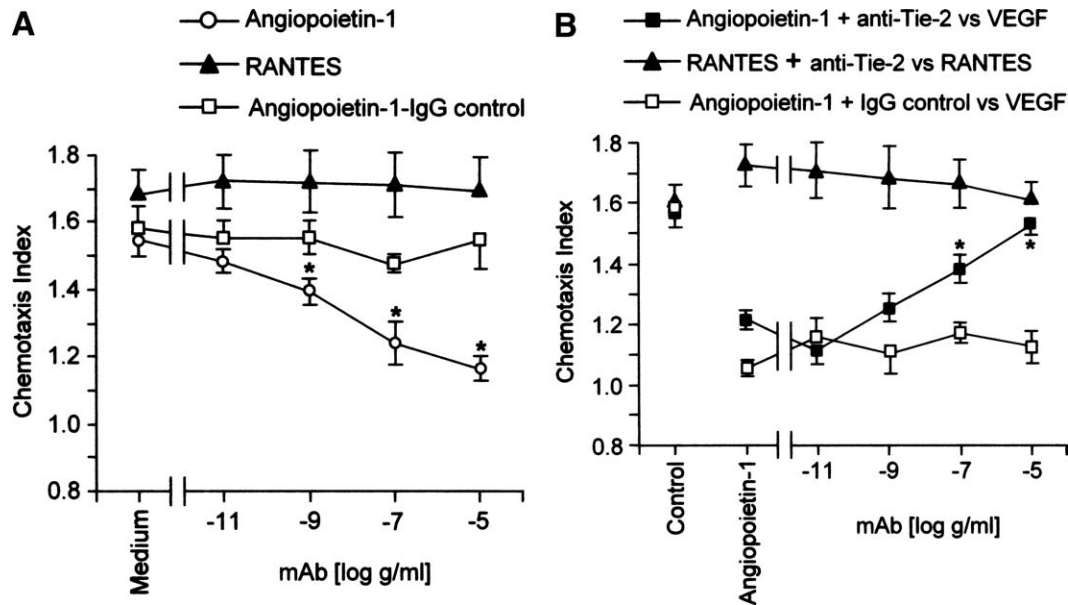


FIG 4. Effects of angiopoietin Tie-2 mAb on angiopoietin 1-induced eosinophil migration (A). Eosinophils were preincubated with different concentrations of angiopoietin Tie-2 mAb for 30 minutes, and migration toward angiopoietin 1 (100 ng/mL) and RANTES (10 ng/mL) was tested. Migration of cells incubated with nonimmune IgG toward angiopoietin 1 was used as a control (*Angiopoietin-1-IgG control*). Cells were incubated with angiopoietin 1 (100 ng/mL) and different concentrations of anti-Tie-2 mAb for 30 minutes, and chemotaxis toward VEGF (10 ng/mL) or RANTES (10 ng/mL) was performed (B). Migration toward VEGF after incubation with angiopoietin 1 and IgG served as control. Results are given as means $\pm$ SEMs; the mean distance of random migration was $65.6 \pm 1.48 \mu\text{m}$ ($n = 4$). * $P < .05$.

present study is the first description of a chemotactic effect of angiopoietin 1 on leukocytes. Angiopoietin 1 was able to enhance eosinophil migration in a dose-dependent manner with a maximal response in the range of 100 ng/mL to 10 pg/mL, which is the range of angiopoietin 1 found in plasma of human subjects.²³ In contrast, angiopoietin 2 exerts only minor chemotactic properties on eosinophils (Fig 1). Checkerboard assays confirmed that angiopoietin 1 acts directly chemotactically: eosinophil migration depended on the presence of an angiopoietin 1 concentration gradient (Table I). Effective concentrations are in good correlation with angiopoietin 1-induced migration of HUVECs.²²

Several studies provide evidence that VEGF, which has the ability to induce transient vascular leakage, vasodilation, and endothelial cell migration,²⁴ may play an important role in the early stages of asthma bronchiale.^{2,5,21,25-28} VEGF expression patterns coincide spatially and temporally with blood vessel growth under physiological and pathologic conditions. In contrast, angiopoietins do not participate in the initial vasculogenic phase, but rather play critical roles in angiogenic outgrowth, vessel remodeling, and maturation.^{6,29} Various studies have confirmed the profound permeability effects of VEGF and have shown that transgenic angiopoietin 1 acts reciprocally as an antipermeability factor when provided chronically during vessel formation.^{8,30} Even

TABLE II. Effect of blocking of signaling enzymes on eosinophil migration toward angiopoietin 1 and RANTES

Treatment	Eosinophil migration: chemotaxis index (mean $\pm$ SEM)	
	Angiopoietin 1 (100 ng/mL)	RANTES (10 ng/mL)
Medium	1.55 $\pm$ 0.02	1.77 $\pm$ 0.06
Wortmannin (10 nmol/mL)	1.27 $\pm$ 0.05*	1.33 $\pm$ 0.11*
Tyrphostin (10 $\mu\text{mol/mL}$)	1.39 $\pm$ 0.07*	1.43 $\pm$ 0.06*
GFX (500 nmol/L)	1.49 $\pm$ 0.04	1.44 $\pm$ 0.09*
Staurosporine (10 ng/mL)	1.47 $\pm$ 0.08	1.14 $\pm$ 0.02*
IBMX (100 $\mu\text{mol/L}$)	1.22 $\pm$ 0.09*	1.51 $\pm$ 0.08
PP1 (1 $\mu\text{mol/L}$)	1.18 $\pm$ 0.01*	1.29 $\pm$ 0.06*
PP2 (1 $\mu\text{mol/L}$)	1.25 $\pm$ 0.05*	1.33 $\pm$ 0.06*
PP3 (1 $\mu\text{mol/L}$)	1.45 $\pm$ 0.03	1.63 $\pm$ 0.07
Pertussis toxin (50 ng/mL)	1.45 $\pm$ 0.09	1.37 $\pm$ 0.07*
Cholera toxin (50 ng/mL)	1.19 $\pm$ 0.04*	1.57 $\pm$ 0.12

Cells were incubated with enzyme blockers for 30 minutes. After washing, cells migrated toward angiopoietin 1 (100 ng/mL) and RANTES (10 ng/mL). The mean distance of random migration was $65.3 \pm 4.28 \mu\text{m}$ ($n = 4$). * $P < .05$.

short-term administration of angiopoietin 1 is reported to protect the adult vasculature from leaking by counteracting actions of VEGF and inflammatory agents.³¹ Angiopoietin 1 and VEGF can also act cooperatively

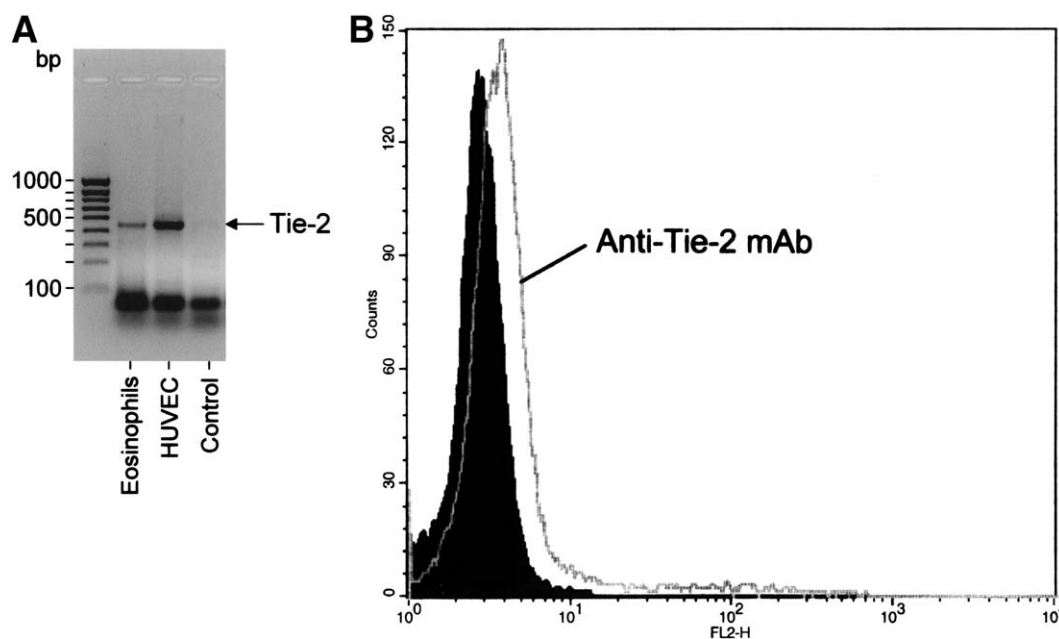


FIG 5. RT-PCR and FACS analysis of Tie-2 receptor in eosinophils. Detection of angiopoietin receptor Tie-2 mRNA in eosinophils and HUVECs by RT-PCR (**A**). Tie-2 is represented by the 448-bp product. FACS analysis of surface Tie-2 on eosinophils (**B**). Cells were incubated with either isotype-matched control IgG or anti-Tie-2 mAb and stained with phycoerythrin-conjugated streptavidin.

and interactively during vascular development³; however, a recent study showed that angiopoietin 1 may counteract VEGF-induced inflammation by reducing VEGF-induced endothelial adhesiveness for leukocytes.³² Therefore, it is possible that the effects of VEGF on the early stages of asthma bronchiale are counteracted by angiopoietin 1 in chronic airway inflammation.

Our group recently demonstrated that VEGF is a potent chemoattractant for human eosinophils and, thus, may be a modulator of eosinophilic inflammation.¹⁷ Therefore, we wondered whether angiopoietin 1 may interact with VEGF in effects on human eosinophils. Incubation with angiopoietin 1, but not with angiopoietin 2, resulted in an abrogation of VEGF-stimulated migration of eosinophils (Fig 2B). Time-response experiments further revealed that a significant inhibition of the VEGF-induced migration by angiopoietin 1 appeared after an incubation period of 5 minutes, which is comparable to the timing of its effects on Akt and Erk phosphorylation in endothelial cells.³³ Besides the inhibition of the proinflammatory-acting and VEGF-induced leukocyte adhesion to endothelial cells, our results suggest that angiopoietin 1 might directly modulate VEGF-driven eosinophil inflammation.

In endothelial cells, angiopoietin 1 activates Tie-2 receptors by rapid autophosphorylation,³⁴ whereas angiopoietin 2 does not show this activity. Moreover, in endothelial cells, but not in fibroblasts, angiopoietin 2 substantially blocks angiopoietin 1 effects, suggesting that the physiological role of angiopoietin 2 might be to antagonize angiopoietin 1 activation of Tie-2.¹⁸ Indeed, angiopoietin 2 was able to diminish the effects of angiopoietin 1 on eosinophil migration in our experiments

(Fig 2, A). Thus, the function of angiopoietin 2 as a negative regulator of Tie-2 seems not to be limited to endothelial cells.

All of the known angiopoietins bind primarily to Tie-2, and it is unclear whether there are independent ligands for another Tie receptor, Tie-1. Previous studies have demonstrated that the Tie-2 receptor is mainly expressed in the vascular endothelium and hematopoietic cells.^{16,19} Our chemotaxis data, using a function-blocking anti-Tie-2, indicate that angiopoietin 1 stimulates migration of eosinophils via Tie-2 receptors (Fig 4, A). As expected, this antibody failed to affect RANTES-induced eosinophil migration. Moreover, coinubation of angiopoietin 1 with the Tie-2 mAb abolished the inhibitory effects of angiopoietin 1 on VEGF-induced eosinophil migration (Fig 4, B). These results indicate that ligation of the angiopoietin receptor Tie-2 with angiopoietin 1 is required for the functional response to occur. To confirm functional data, we demonstrated that eosinophils express the Tie-2 gene (Fig 5) and produce the Tie-2 protein on the cell surface, as seen by FACS analyses. Incubation with neither angiopoietin 1 nor VEGF affected the expression of Tie-2 or the VEGF receptor flt-1, making receptor downregulation in the interaction of these substances less likely.

Angiopoietin-1 and VEGF cooperate in the migration and survival of endothelial cells by activation of PI3K and mitogen-activating protein kinase pathways,³⁵ whereas disruption of endothelial cell/cell interactions requires activation of PKC.³⁶ To analyze which of these pathways mediates the chemotactic response of eosinophils toward angiopoietin-1, cells were incubated with different

enzyme blockers. The specific inhibitor of PI3K—wortmannin—as well as the phosphodiesterase inhibitor IBMX and the tyrosine kinase inhibitor tyrphostin-23 significantly inhibited angiopoietin-1-induced chemotaxis in eosinophils, whereas the PKC inhibitor GFX and staurosporine did not affect angiopoietin-1-induced eosinophil migration. These results show that PI3K, phosphodiesterase, and tyrosine kinases are also involved in angiopoietin-1-induced eosinophil migration. In conjunction with our previous finding that the PI3K pathway is involved in VEGF-mediated effects on eosinophils,¹⁷ this may indicate that activation of PI3K regulates interactions of angiopoietin and VEGF on eosinophils. Because of the effects by the tyrosine kinase inhibitor tyrphostin-23, we tested the influence of specific inhibitors of the Src/tyrosine kinase family members on cell migration toward angiopoietin-1. Src kinases have been implicated in multiple signaling pathways that regulate cellular growth, migration, and cell survival.^{37,38} The specific inhibitors of the Src family tyrosine kinases PP1 and PP2, but not the inactive analog PP3, abolished angiopoietin-1-induced chemotaxis, suggesting a function of SRC tyrosine kinases in eosinophil migration toward angiopoietin 1.

Our chemotaxis experiments revealed an inhibition of eosinophil migration toward angiopoietin after incubation with cholera toxin, whereas incubation with pertussis toxin was ineffective. Cholera toxin activates G_s protein and leads to an increase in the activity of adenylate cyclase, resulting in increased levels of cyclic AMP.³⁹ This is also in agreement with our finding that the cyclic AMP-increasing IBMX inhibited angiopoietin 1-induced chemotaxis. It was previously shown that signaling of other tyrosine kinase growth factor receptors, such as the insulin-like growth factor 1 receptor, might involve G proteins.^{40,41} The results of our migration experiments indicate activation of G proteins in the Tie-2-induced effects of angiopoietin 1 on eosinophil migration. RANTES, at nanomolar concentrations, activates a heterotrimeric G_i protein-coupled signaling pathway upon binding to any one of its known 7-transmembrane-spanning and G protein-coupled receptors.⁴² This was confirmed in our functional assays, in which RANTES-induced eosinophil chemotaxis was affected by pertussis toxin, but not by cholera toxin.

Taken together, these results show angiopoietin 1-directed migration and inhibition of VEGF-induced eosinophil chemotaxis via activation of the angiopoietin receptor Tie-2. This furthermore indicates that interactions with VEGF on eosinophil function may be among the immunomodulatory effects of the angiogenic factor angiopoietin 1.

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