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Expression and Functions of the Vascular Endothelial Growth Factors and Their Receptors in Human Basophils¹

Amato de Paulis,* Nella Prevede,* Isabella Fiorentino,* Francesca Wanda Rossi,* Stefania Staibano,[†] Nunzia Montuori,[‡] Pia Ragno,[‡] Amelia Longobardi,* Bianca Liccardo,* Arturo Genovese,* Domenico Ribatti,[§] Andrew F. Walls,^{||} and Gianni Marone^{2*}

Angiogenesis is a multistep complex phenomenon critical for several inflammatory and neoplastic disorders. Basophils, normally confined to peripheral blood, can infiltrate the sites of chronic inflammation. In an attempt to obtain insights into the mechanism(s) underlying human basophil chemotaxis and its role in inflammation, we have characterized the expression and function of vascular endothelial growth factors (VEGFs) and their receptors in these cells. Basophils express mRNA for three isoforms of VEGF-A (121, 165, and 189) and two isoforms of VEGF-B (167 and 186). Peripheral blood and basophils in nasal polyps contain VEGF-A localized in secretory granules. The concentration of VEGF-A in basophils was 144.4 ± 10.8 pg/10⁶ cells. Immunologic activation of basophils induced the release of VEGF-A. VEGF-A (10–500 ng/ml) induced basophil chemotaxis. Supernatants of activated basophils induced an angiogenic response in the chick embryo chorioallantoic membrane that was inhibited by an anti-VEGF-A Ab. The tyrosine kinase VEGFR-2 (VEGFR-2/KDR) mRNA was expressed in basophils. These cells also expressed mRNA for the soluble form of VEGFR-1 and neuropilin (NRP)1 and NRP2. Flow cytometric analysis indicated that basophils express epitopes recognized by mAbs against the extracellular domains of VEGFR-2, NRP1, and NRP2. Our data suggest that basophils could play a role in angiogenesis and inflammation through the expression of several forms of VEGF and their receptors. *The Journal of Immunology*, 2006, 177: 7322–7331.

Angiogenesis is a complex multistep phenomenon that begins with degradation of the basement membrane by cellular proteases and culminates in the growth of new blood vessels from the microcirculation (1, 2). Angiogenesis is critical for such chronic inflammatory disorders as rheumatoid arthritis (3), bronchial asthma (4, 5), and tumor growth (6). Vascular endothelial growth factor (VEGF)³ is the most potent proangiogenic mediator known so far. Originally identified from its ability to induce vascular permeability (6), VEGF is a potent inducer of endothelial proliferation, migration, and survival. It also acts as a proinflammatory cytokine (1, 2). The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) (7). VEGF-A and -B are key regulators of blood vessel growth, whereas VEGF-C and -D regulate lymphatic angiogenesis

(8). Some components of the VEGF family have differentially spliced forms that differ in their ability to enhance angiogenesis. For example, human VEGF-A has at least six isoforms: 121, 145, 165, 183, 189, and 204 (9). Of these, VEGF-A₁₆₅ and VEGF-A₁₂₁ are the most potent proangiogenic isoforms (10).

VEGF-A signals through VEGFR-1 (VEGFR-1/Flt-1) and VEGFR-2/KDR, which are expressed on endothelial cells (1, 2). Each of these VEGFRs has seven Ig-like domains in their extracellular regions and two intracellular tyrosine kinase domains. VEGF-A binds at the second and third Ig-like domain in both VEGFR-1 and VEGFR-2 (11). VEGF functions are also regulated through the production of an alternatively mRNA variant of VEGFR-1, namely soluble VEGFR-1 (sVEGFR-1) (12). sVEGFR-1 is similar to membrane-bound VEGFR-1, except it lacks the transmembrane region necessary to attach the receptor to the cell membrane and is devoid of kinase activity. Since receptor dimerization is essential for signaling through VEGFRs, sVEGFR-1 prevents VEGF from activating VEGFR-1 or VEGFR-2 by inhibiting dimerization of VEGFRs (13).

Neuropilins (NRPs) are cell surface glycoproteins that mediate neuronal guidance and angiogenesis (14). NRP1, initially described as a cell surface glycoprotein expressed on axons (15), is a receptor for semaphorins (16, 17). In the vascular system, NRP1 interacts via its b1b2 domain with VEGF-A₁₆₅ but not VEGF-A₁₂₁ (18). Therefore, NRP1 acts as a coreceptor for VEGFR-2/KDR and enhances VEGFR-2-induced responses (19).

Increasing evidence implicates angiogenesis not only in tumor growth but also in several chronic inflammatory disorders (3–5). Peripheral blood basophils express the tetrameric ($\alpha\beta\gamma_2$) structure of the Fc ϵ RI and synthesize proinflammatory mediators and a restricted Th2-like cytokine profile (IL-4 and IL-13) (20). Although basophils are normally confined to peripheral blood and are not found in normal tissue, they can infiltrate the sites of chronic inflammation (21, 22), which in certain conditions can lead to cancer

*Divisione di Immunologia Clinica ed Allergologia e Centro Interdipartimentale di Ricerca di Scienze Immunologiche di Base e Cliniche, [†]Dipartimento di Scienze Biomorfologiche e Funzionali—Sezione di Anatomia Patologica, and [‡]Istituto di Endocrinologia ed Oncologia Sperimentale, Università di Napoli Federico II, Naples, Italy; [§]Dipartimento di Anatomia Umana ed Istologia, Università di Bari, Bari, Italy; and ^{||}Immunopharmacology Group, Southampton General Hospital, Southampton, United Kingdom

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² Address correspondence and reprint requests to Dr. Gianni Marone, Department of Clinical Immunology and Allergy, University of Naples Federico II, Via Sergio Pansini 5, 80131 Napoli, Italy. E-mail address: marone@unina.it

³ Abbreviations used in this paper: VEGF, vascular endothelial growth factor; CAM, chorioallantoic membrane; FPRL1, formyl peptide receptor like-1; FPRL2, FPR like-2; HSA, human serum albumin; NRP, neuropilin; PlGF, placental growth factor; PMN, polymorphonuclear cell; sVEGFR-1, soluble VEGFR-1; TRITC, tetramethylrhodamine isothiocyanate.

(23, 24). These findings indicate that basophils might be a component of the complex network involving chronic inflammation and cancer.

In this study, we have characterized the VEGF/VEGF receptor system and its functions in human basophils. We found that basophils express mRNA for various members of the VEGF family. VEGF-A was detected in secretory granules of peripheral blood basophils and in basophils infiltrating the site of chronic inflammation. Basophils immunologically activated in vitro-released VEGF-A, which induced basophil chemotaxis. These cells expressed the tyrosine kinase VEGFR-2 (VEGFR-2/KDR), sVEGFR-1, and NRP1 and NRP2. Supernatants of immunologically activated basophils induced an angiogenic response in vivo in the chick embryo chorioallantoic membrane (CAM). Our results suggest that basophils could play a role in angiogenesis through the expression of several forms of VEGF and their receptors.

Materials and Methods

Reagents

The following reagents were used: 60% HClO₄ (Baker Chemical); human serum albumin (HSA), PIPES, protease inhibitors, cycloheximide, and anti-VEGFR-2/KDR mAb (Sigma-Aldrich); HBSS, FCS, and RNAqueous-4PCR (Ambion); Superscript III and TRIzol reagent (Invitrogen Life Technologies); RPMI 1640 (Invitrogen Life Technologies) with 25 mM HEPES buffer and Eagle's MEM (Flow Laboratories); Dextran 70 and Percoll (Pharmacia Fine Chemicals); HRP-conjugated anti-mouse IgG (Amersham Biosciences) and protein colorimetric assay (Bio-Rad); anti-VEGFR-2/KDR mAb (Sigma-Aldrich) and FITC- and PE-labeled anti-IgE Abs (Caltag Laboratories); PE-labeled anti-VEGFR-2/KDR mAb, VEGF-A₁₆₅ and VEGF-A₁₂₁, and polyclonal goat anti-human VEGF-A (R&D Systems); human recombinant VEGF-A (R&D Systems); isotype control goat IgG Ab (Chemicon International); rabbit polyclonal anti-NRP1 and anti-NRP2 and rabbit polyclonal anti-VEGF-A (Santa Cruz Biotechnology); FITC-conjugated rabbit anti-mouse secondary Ab, tetramethylrhodamine isothiocyanate (TRITC)-conjugated swine anti-rabbit secondary Ab (DakoCytomation); FITC-labeled goat anti-rabbit IgG, FITC-, and PE-labeled isotype control goat IgG Abs (BD Biosciences); FITC-labeled goat anti-rabbit IgG (Abcam); nonimmune normal horse serum (Vector Laboratories); and 4',6'-diamidino-2-phenylindole (Roche).

Buffers

The PIPES buffer used in these experiments was made up of 25 mM PIPES (pH 7.4), 110 mM NaCl, and 5 mM KCl. The mixture is referred to as "P." PCGM contains, in addition to P, 5 mM CaCl₂ and 1 g/L D-glucose (25). PACGM contains, in addition to P, 3% HSA, 1 mM CaCl₂, 1 g/L dextrose, and 0.25 g/L MgCl₂·6H₂O (pH 7.4); PGMD contains 0.25 g/L MgCl₂·6H₂O, 10 mg/L DNase, and 1 g/L gelatin in addition to P (pH 7.4). PBS contains 8 g/L NaCl, 1.15 g/L Na₂HPO₄, 200 mg/L KCl, and 200 mg/L KH₂PO₄ (pH 7.4).

Purification of peripheral blood basophils

Basophils were purified from the peripheral blood of healthy volunteers, aged from 20 to 39 years, negative for HIV-1, HIV-2, hepatitis C virus, and hepatitis B virus Abs. Buffy coat cell packs were provided by the Immunohematology Service (University of Naples Federico II). Informed consent, according to the guidelines of the University of Naples Federico II institutional review board for the use of humans in research, was obtained. Cells were reconstituted in PBS containing 0.5 g/L HSA and 3.42 g/L sodium citrate and loaded onto a counter-current elutriator (Beckman Coulter). Several fractions were collected, and fractions with >20 × 10⁶ basophils and a good purity (>15%) were enriched by discontinuous Percoll gradients. Basophils were further purified to near homogeneity (>99%) by depleting B cells, monocytes, NK cells, dendritic cells, erythrocytes, platelets, neutrophils, eosinophils and T cells, using a mixture of hapten-conjugated CD3, CD7, CD14, CD15, CD16, CD36, CD45RA, and anti-HLA-DR Abs and MACS MicroBeads coupled to an anti-hapten mAb (26). The magnetically labeled cells were depleted by retaining them on a MACS column in the magnetic field of the MidiMACS (Miltenyi Biotec). Yields ranged from 3 to 10 × 10⁶ basophils with purity >99%, assessed by basophil staining with Alcian blue, and counting in a Spiers-Levy eosinophil counter (26).

Flow cytometric analysis of surface molecules

Flow cytometric analysis of cell surface molecules was performed as described previously (27). Briefly, after saturation of nonspecific binding sites with total rabbit IgG, cells were incubated for 20 min at +4°C with specific or isotype control Abs. For indirect staining, this step was followed by a second incubation for 20 min at +4°C with an appropriate anti-isotype-conjugated Ab. Finally, cells were washed and analyzed with a FACSCalibur Cytofluorometer using CellQuest software (BD Biosciences). A total of 10⁴ events for each sample was acquired in all cytometric analyses.

Reverse transcriptase-PCR

Total cellular RNA was isolated by RNAqueous-4PCR (Ambion) or TRIzol reagent (Invitrogen Life Technologies), according to the supplier's protocol. RNA was quantified by spectroscopy. Two micrograms of total RNA was reversely transcribed with random hexamer primers and 200 U of Superscript III Reverse Transcriptase (Invitrogen Life Technologies) (27). Two microliters of reversely transcribed DNA was then amplified, using VEGF-A₁₂₁₋₁₆₅-specific 5' sense (GTGAATGCAGACCAAA GAAAG) and 3' antisense (AAACCCTGAGGGAGGCTC) primers, VEGF-A₁₈₉-specific 5' sense (GTATAAGTCTGGAGCGT) and 3' antisense (AAACCCTGAGGGAGGCTC) primers, VEGF-B-specific 5' sense (TGTCCTGGAAGAACACAGCC) and 3' antisense (GCCATGTGT CACCTTCGCA) primers, VEGF-C-specific 5' sense (ATGTTTTCTCG GATGCTGGA) and 3' antisense (CATTGGCTGGGGAAGAGTTT) primers, VEGF-D-specific 5' sense (GTATGGACTCTCGCTCAGCAT) and 3' antisense (AGGCTCTCTTCATTGCAACAG) primers, VEGFR-2/ KDR-specific 5' sense (GACTTCACTGAGGAATACCC) and 3' antisense (CATGGCCCTGACAAATGTG) primers, sVEGFR-1-specific 5' sense (ACAATCAGAGGTGAGCACTG) and 3' antisense (CTGCTAT CATCTCCGAAGCT) primers, NRP-1-specific 5' sense (ATGGATAT GTTTCCTCTCACC) and 3' antisense (CTGGAGATACCTCTGTGG) primers, NRP2-specific 5' sense (CCCCGAACCCAACCAGAAGA) and 3' antisense (GAATGCCATCCCAGATGTCCA) primers, and GAPDH-specific 5' sense (GCCAAGGGTCCATCATCTC) and 3' antisense (GTA GAGGCAGGATGATGTTTC) primers, as a control. Two sets of primers were used for VEGFR-1/Flt-1: 1) specific 5' sense (ATCAGAGATCAG GAAGACC) and 3' antisense (GGAACCTCATCTGGTCCAT) primers; and 2) specific 5' sense (CTATGGAAGATCTGATTTCTT) and 3' antisense (GGTATAAATACACATGTGCTT) primers. The reaction products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide, followed by photography under UV illumination (27).

Chemotaxis assay

Modified Boyden chambers were used for chemotaxis assays. Twenty-five microliters of PACGM buffer with or without the indicated concentrations of chemoattractants were loaded in the lower compartments of a 48-well microchemotaxis chamber (NeuroProbe). The lower compartments were covered with 5-μm-pore polyvinylpyrrolidone-free polycarbonate membranes. Fifty microliters of the cell suspension (5 × 10⁴/well), resuspended in PACGM, was pipetted in the upper compartment. The chemotactic chamber was incubated for 1 h at 37°C in a incubator with 5% CO₂ (Automatic CO₂ Incubator, model I60IR; ICN Flow). At the end of incubation, the membrane was removed; the upper side was washed with PBS, and the filter was fixed, stained with May-Grunwald/Giemsa, and mounted on a microscope slide with Cytoseal (Stephens Scientific). Basophil chemotaxis was quantitated microscopically by counting the number of cells attached to the surface of a 5-μm cellulose nitrate filter (28). In each experiment, 10 fields per triplicate filter were measured at a magnification of ×40. The results were compared with buffer controls. Checkboard analysis was used to discriminate chemotaxis and nondirect migration (chemokinesis) of basophils. In these experiments, basophils were placed in the upper chemotactic chambers, and various concentrations of stimuli or buffer were added to the upper or lower wells or to both. Spontaneous migration (chemokinesis) was determined in the absence of chemoattractants or when stimuli were added to either the lower or upper chambers.

Histamine release

Basophils (~6 × 10⁴ basophils/tube) were resuspended in PCG, and 0.1 ml of the cell suspension was placed in 12 × 75-mm polyethylene tubes (Sars-tadt) and warmed to 37°C; 0.1 ml of each prewarmed releasing stimulus was added, and incubation was continued at 37°C (25). The reactions were stopped by centrifugation (1000 × g, 22°C, 2 min), and the cell-free supernatants were assayed for histamine content with an automated fluorometric technique (29). Total histamine content was assessed by lysis induced by incubating the cells with 2% HClO₄ before centrifugation. To

calculate histamine release as a percentage of total cellular histamine, the spontaneous release of histamine from basophils (2–12% of the total cellular histamine) was subtracted from both the numerator and denominator (25). All values are based on the means of duplicate determinations. Replicates differed in histamine content by <10%.

VEGF-A ELISA

VEGF-A release in the culture supernatants of basophils was measured in duplicate determinations with a commercially available ELISA (R&D Systems).

Chick embryo chorioallantoic membrane assay

Fertilized White Leghorn chicken eggs were incubated under constant humidity at 37°C (30). On the third day of incubation, a square window was opened in the shell after removal of 2–3 ml of albumen so as to detach the developing CAMs from the shell. The window was sealed with a glass of the same dimension, and the eggs were returned to the incubator. CAMs were treated at day 8 with supernatants of anti-IgE-activated (0.3 µg/ml) basophils dissolved in 3 µl of DMEM and adsorbed on 1-mm³ sterilized gelatin sponges (Gelfoam; Upjohn). Sponges containing vehicle alone were used as negative controls, whereas sponges containing 500 ng/embryo of human recombinant VEGF-A (R&D Systems) were used as positive controls. In some experiments, supernatants of anti-IgE-activated basophils were preincubated with polyclonal goat anti-VEGF-A-neutralizing Ab (500 ng/embryo) (R&D Systems) or with the isotype control goat IgG (500 ng/ml) (Chemicon International) before implantation. CAMs were examined daily until day 12 and photographed in ovo with a stereomicroscope SR equipped with the Zeiss Camera System MC63. In some experiments, blood vessels entering the sponge within the focal plane of the CAM were counted by two observers in a double-blind fashion at ×50 magnification.

Double-immunofluorescence staining on cytopsin

Basophils purified as previously described were left to adhere on glass precoated with 1% poly-L-lysine (Menzel-Glaser) for 1 h at 22°C. Then cells were fixed in 4% paraformaldehyde. Nonspecific bonds were blocked by preincubating fixed basophils with nonimmune normal horse serum (Vector Laboratories) for 30 min at 22°C (31). Double-immunofluorescence staining was performed by incubation overnight at 4°C with the primary Abs BB1 (1/100) and rabbit polyclonal anti-VEGF-A (1/300) (A-20; Santa Cruz Biotechnology). Cells were then incubated with FITC-conjugated rabbit anti-mouse secondary Ab (DakoCytomation) (1/50) for 1 h at 22°C and with TRITC-conjugated swine anti-rabbit secondary Ab (DakoCytomation) (1/50) for 1 h at 22°C. The nuclear counterstaining was performed with 4',6'-diamidino-2-phenylindole (Roche) for 15 min at 22°C. Finally, the glasses were mounted with coverslips using a synthetic mounting medium (DakoCytomation). Basophils were observed under a Zeiss Axiovert 100 M microscope adapted with a LSM 510 confocal system. Images were recorded with LSM 510 software (Zeiss) and exported as a JPG.

Double-immunofluorescence staining on cryostat sections of nasal polyps

Nasal polyp specimens were obtained from patients undergoing polypectomy. These patients did not assume anti-inflammatory steroids for at least 15 days before surgery. Surgically removed specimens were immediately frozen at –25°C, and 5-µm-thick cryostat sections were cut from frozen tissue blocks by a motor-driven cryostat (31). Sections were picked up on clean glass slides, fixed in acetone for 10 min at 22°C, and stored at –25°C until use. Nonspecific bonds were blocked by preincubating fixed tissue with nonimmune normal horse serum (Vector Laboratories) for 30 min at 22°C. Double-immunofluorescence staining was performed by incubation overnight at 4°C with the primary Abs BB1 (1/100) and rabbit polyclonal anti-VEGF-A (1/300) (A-20; Santa Cruz Biotechnology). Sections were then incubated with FITC-conjugated rabbit anti-mouse secondary Ab (DakoCytomation) (1/50) for 1 h at 22°C and with TRITC-conjugated swine anti-rabbit secondary Ab (DakoCytomation) (1/50) for 1 h at 22°C. Finally, the glasses were mounted with coverslips using the Dako fluorescent mounting medium. Sections were analyzed under a Zeiss Axiovert 100 M microscope adapted with a LSM 510 confocal system. Images were recorded with LSM 510 software (Zeiss) and exported as a JPG.

Lactate dehydrogenase assay

Lactate dehydrogenase release at the end of the incubations served as an index of cytotoxicity. It was measured in cell-free supernatants using a commercially available kit (Sigma-Aldrich) (27).

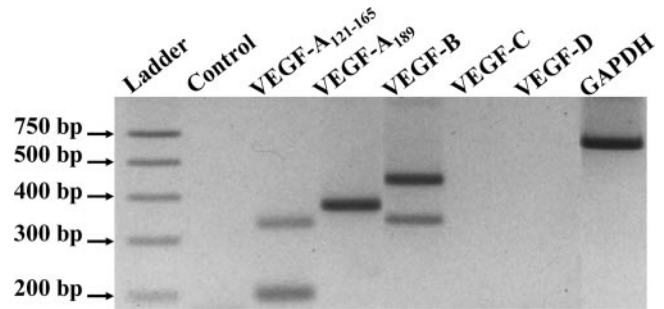


FIGURE 1. Expression of different forms of VEGF mRNA in human basophils. Purified basophils were lysed in lysis buffer to obtain a total RNA preparation. Total RNA was reverse-transcribed and amplified by 40 PCR cycles in the presence of VEGF-specific primers and of GAPDH primers as loading control. PCR amplification of buffer represented the negative control. PCR products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide, followed by photography under UV illumination.

Statistical analysis

The results are expressed as the mean ± SEM. Values from groups were compared using paired Student's *t* test or ANOVA and then by Duncan's new multiple range test when appropriate (32). Significance was defined as *p* < 0.05.

Results

Expression of mRNA for the components of the VEGF family in human basophils

The human VEGF-A gene encodes major peptides of 189, 165, and 121 aa as a result of alternative splicing. VEGF-A₁₆₅, the predominant isoform in several normal and tumor cells, lacks the residues encoded by exon 6, whereas VEGF-A₁₂₁ lacks the residues encoded by exons 6 and 7 (2). We investigated the expression of VEGF mRNA in basophils purified (>99%) from peripheral blood of normal donors. The analysis of PCR products by electrophoresis in agarose gel revealed three VEGF-A isoforms (VEGF-A₁₂₁, VEGF-A₁₆₅, and VEGF-A₁₈₉) and two VEGF-B isoforms (VEGF-B₁₆₇ and VEGF-B₁₈₆) (Fig. 1). By contrast, VEGF-C and VEGF-D mRNA, two angiogenic mediators of lymphatic development (8), were not detected in human basophils.

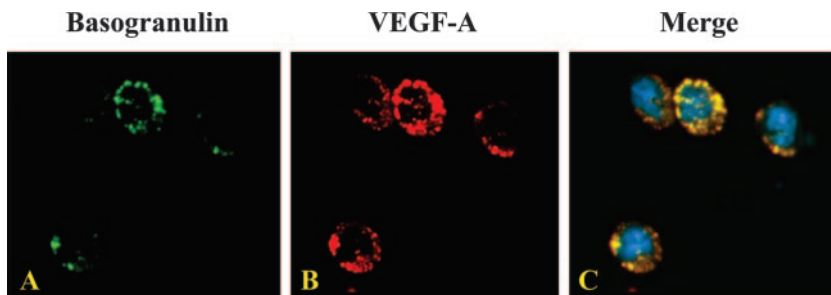
Detection of VEGF-A in human basophils

We then investigated VEGF-A expression in basophils at protein level. Basophils were lysed in 1% Triton X-100/PBS in the presence of protease inhibitors, and the total content of immunoreactive VEGF-A was measured by specific ELISA. In a series of eight experiments, the concentration of VEGF-A in basophils ranged from 50 to 160 pg/10⁶ basophils with a mean value of 144 ± 10.8 pg/10⁶ cells.

Localization of VEGF-A in human peripheral blood basophils

To verify the intracellular localization of VEGF-A, we analyzed cytopsin of enriched preparations of peripheral blood basophils by confocal microscopy. We used a mAb BB1 that specifically recognizes basogranulin in secretory granules of human basophils but not in neutrophils, lymphocytes, and monocytes (22, 33). We also used a rabbit polyclonal Ab raised against aa 1–140 of human VEGF-A. Fig. 2A shows the localization of basogranulin in secretory granules of the cells. Fig. 2B shows the localization of VEGF-A in the cytoplasm of basophils. Last, Fig. 2C shows the colocalization of BB1⁺ secretory granules with VEGF-A immunoreactivity. Staining of basophils with irrelevant isotype controls was negative (data not shown). Similar results were obtained in 10

FIGURE 2. Intracellular localization of VEGF-A in human basophils. Double immunofluorescence staining of an enriched preparation of peripheral blood basophils examined by confocal microscopy. *A*, The intracellular localization of basogranulin by using the BB1 mAb (green); *B*, the cells immunostained for VEGF-A (red); and *C*, the colocalization of basogranulin and VEGF-A in human basophils (yellow).



additional preparations of basophils purified from peripheral blood of healthy volunteers. These results are compatible with the hypothesis that VEGF-A is stored in secretory granules of peripheral blood basophils.

Localization of VEGF-A in basophils in nasal polyps

Nasal polyps are characterized by hyperplasia of the mucosal epithelium and submucosal mucous glands with underlying areas of infiltrating inflammatory cells and proliferating blood vessels (34). VEGF and its receptors have been implicated in nasal polyposis (35). Nasal polyp tissues obtained from four patients undergoing polypectomy were examined by immunohistochemistry using the mAb BB1 that specifically recognizes human basophils also in tissue (22, 33) and a rabbit polyclonal anti-VEGF-A. Fig. 3 shows BB1⁺ basophils in the perivascular area. These cells stained positive also for VEGF-A. Fig. 3 also shows the colocalization of BB1 and VEGF-A. Staining of tissues with irrelevant isotype controls was negative. No basophils were detected in normal nasal mucosa obtained from the middle turbinate (data not shown).

Kinetics of VEGF-A and histamine release from human basophils

In a series of five experiments, we evaluated the kinetics of VEGF-A and histamine release in unstimulated basophils and in cells immunologically activated with anti-IgE. Basophils activated with anti-IgE rapidly released histamine, which peaked at 30 min. Fig. 4 shows that stimulation of basophils with anti-IgE caused a small increase in VEGF-A secretion that was detectable after 30 min. The release of VEGF-A induced by anti-IgE reached a plateau after 2 h of incubation and remained stable up to 4 h. Basophils kept in culture for up to 3–4 h had a small spontaneous release of histamine (~10%) and of VEGF-A (~20 pg/10⁶ basophils).

The release of VEGF-A induced by anti-IgE from basophils that required hours of incubation suggested that protein synthesis may be necessary for this phase. Thus, we examined the effect of cycloheximide (protein synthesis inhibitor) on both the early (30 min) and late (2 h) release of VEGF-A induced by anti-IgE. In three experiments, preincubation (30 min) of basophils with cycloheximide (10 μM) did not modify the early (30 min) release of VEGF-A

induced by anti-IgE (24.7 ± 2.0 vs 25.3 ± 3.4 pg/10⁶ basophils). In contrast, the production of VEGF-A after 2 h of incubation with anti-IgE was reduced significantly by cycloheximide (58.0 ± 3.7 vs 34.3 ± 4.7 pg/10⁶ basophils; *p* < 0.01). Cycloheximide did not affect basophils viability (data not shown).

Recently, Abdel-Majid and Marshall (36) demonstrated that PGE₂ is a potent inducer of VEGF-A secretion by human mast cells. Therefore, in three experiments, we investigated the possibility that a wide spectrum of PGE₂ concentrations (10⁻⁹–10⁻⁵ M) induces the release of VEGF-A from human basophils. In none of these experiments did PGE₂ cause the release of VEGF-A (data not shown).

Expression of mRNA for VEGFRs in human basophils

Two VEGFRs, VEGFR-1/Flt-1 and VEGFR-2/KDR, are expressed on endothelial cells (37) and on some immune cells (38–43). These VEGFRs are structurally highly homologous; however, their biochemical features are quite distinct. Although VEGFR-1/Flt-1 has a greater affinity for VEGF-A, VEGFR-2/KDR is tyrosine-phosphorylated more efficiently upon ligand binding (44). A soluble truncated form of VEGFR-1 (sVEGFR-1) that contains only the first six Ig-like domains (12) binds to VEGF-A as strongly as does full-length VEGFR-1 and inhibits VEGF-A activity by sequestering it from signaling receptors and by forming non-signaling heterodimers with VEGFR-2. We analyzed the mRNA expression of VEGFR-1/Flt-1, VEGFR-2/KDR, and sVEGFR-1 in human basophils. Fig. 5A shows the results of a typical experiment demonstrating that VEGFR-2/KDR, but not VEGFR-1/Flt-1, is expressed in basophils. Interestingly, basophils also constitutively expressed sVEGFR-1. Similar results were obtained in seven experiments using mRNA purified from basophils of different donors. In other experiments, we used two different sets of primers to identify VEGFR-1/Flt-1 mRNA in basophils (see *Materials and Methods*). As a positive control, we used human polymorphonuclear cells (PMNs) and PBMCs that express VEGFR-1/Flt-1 (38, 39, 41, 43). Fig. 5B shows the results of one of three typical experiments demonstrating that VEGFR-1/Flt-1 was expressed in both PMNs and PBMCs but not in basophils.

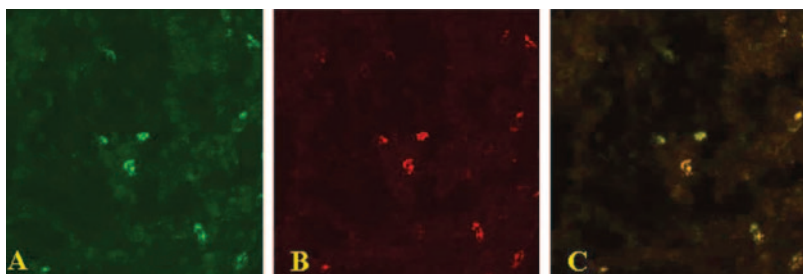


FIGURE 3. Confocal micrographs of nasal polyps stained for basophils (green) and VEGF-A (red). *A*, A cluster of BB1-immunoreactive basophils. *B*, VEGF-A-positive cells. *C*, The colocalization of basogranulin and VEGF-A in basophils in nasal polyps.

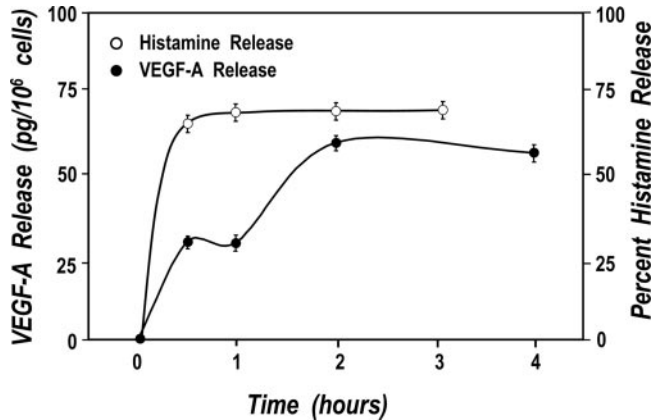


FIGURE 4. Kinetics of VEGF-A and histamine release from human basophils. Basophils were incubated with buffer (spontaneous release) or with anti-IgE (0.3 μ g/ml). At each time point, supernatants were collected and centrifuged (1000 \times g, 4°C, 5 min). The release of VEGF-A from basophils induced by anti-IgE is indicated by \bullet . The release of histamine from basophils induced by anti-IgE is indicated by \circ . VEGF-A and histamine release in the supernatants were determined by ELISA and fluorometric techniques, respectively. The values are expressed as the mean \pm SEM of five experiments.

Expression of mRNA for NRP1 and NRP2 in human basophils

Both NRP1 and NRP2 are expressed by endothelial cells (45, 46). Increasing evidence suggests that NRPs are required to initiate

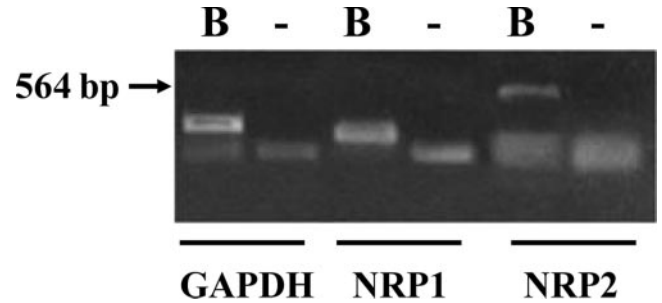


FIGURE 6. Expression of NRP1 and NRP2 mRNA in human basophils (B). Purified basophils were lysed in TRIzol reagent to obtain a total RNA preparation. Total RNA was reverse-transcribed and amplified by 40 PCR cycles in the presence of NRP1- and NRP2-specific primers and GAPDH primers as loading control. PCR amplification of buffer represented the negative controls (-). PCR products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide, followed by photography under UV illumination.

immune responses. For instance, NRP1 expression is observed in human CD4⁺CD25⁺ Treg cells (47) and rodent B lymphocytes, monocytes (19), T cells, and dendritic cells (40). Fig. 6 shows the result of a typical experiment demonstrating that NRP1 and NRP2 mRNA were expressed in basophils. Similar results were obtained in two experiments using mRNA purified from basophils of different donors.

Flow cytometry of VEGFR-2/KDR, NRP1, and NRP2 expression on human basophils

We then investigated the expression of VEGFRs at protein level by flow cytometry. Purified basophils (>99%) were incubated with a PE-labeled anti-VEGFR-2/KDR mAb and a FITC-labeled anti-IgE mAb or with purified control IgG. The results presented in Fig. 7 indicate that the vast majority (~80%) of peripheral basophils express on their surface epitopes recognized by a mAb against the extracellular domains of VEGFR-2. Fig. 8 shows that NRP1 and NRP2 were expressed on the vast majority (>80%) of basophils of normal donors.

Effects of VEGF-A₁₆₅ and VEGF-A₁₂₁ on chemotaxis of human basophils

VEGF-A stimulates endothelial cell migration (48), and it is chemotactic for certain human immune cells (38, 39, 42, 43). We evaluated the in vitro effects of a wide range of concentrations (5–500 ng/ml) of VEGF-A₁₆₅ on basophil chemotaxis. Fig. 9 shows the results of seven experiments demonstrating that low

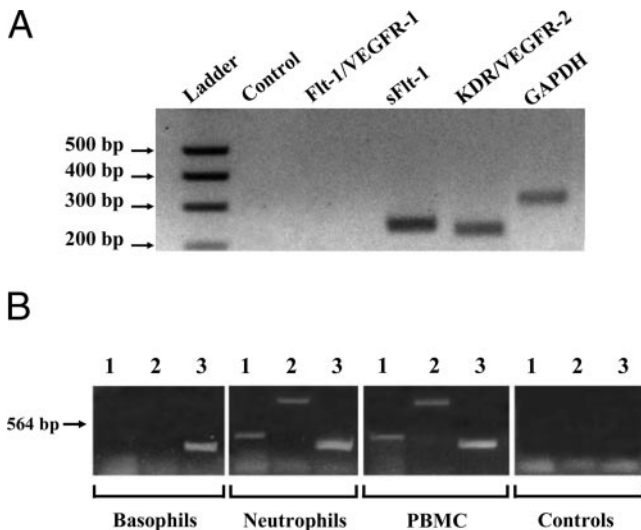


FIGURE 5. A, Expression of VEGFR mRNA in human basophils. Purified basophils were lysed in lysis buffer to obtain a total RNA preparation. Total RNA was reverse-transcribed and amplified by 40 PCR cycles in the presence of VEGFR-1/Flt-1-, VEGFR-2/KDR-, and sVEGFR-1-specific primers and GAPDH primers as loading control. PCR amplification of buffer represented the negative control. PCR products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide, followed by photography under UV illumination. B, Expression of VEGFR1/Flt-1 receptor mRNA in human basophils, PMNs, and PBMCs. Purified basophils, PMNs, or PBMCs were lysed in TRIzol reagent to obtain a total RNA preparation. Total RNA was reverse-transcribed and amplified by 40 PCR cycles in the presence of two sets of specific VEGFR-1/Flt-1 primers and GAPDH primers as loading control. PCR amplification of buffer represented the negative controls. Lanes 1 and 2 represent the results obtained with primers 1 or primers 2 for VEGFR-1/Flt-1 (see *Materials and Methods*). Lane 3 represents results obtained with primers for GAPDH. PCR products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide, followed by photography under UV illumination.

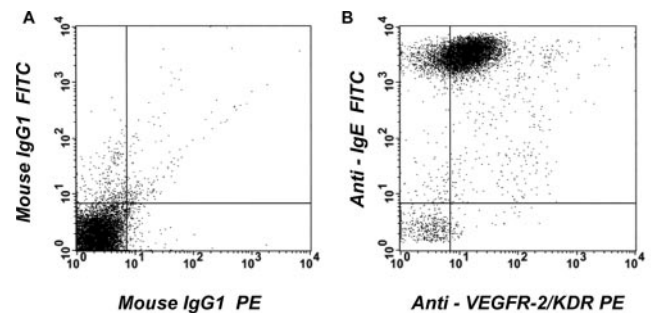


FIGURE 7. Cytofluorimetric analysis of VEGFR2/KDR expression by human basophils. Basophils were incubated (4°C, 20 min) with monoclonal anti-VEGFR-2/KDR PE-labeled and anti-IgE FITC-labeled (B) or isotype-matched Abs (A).

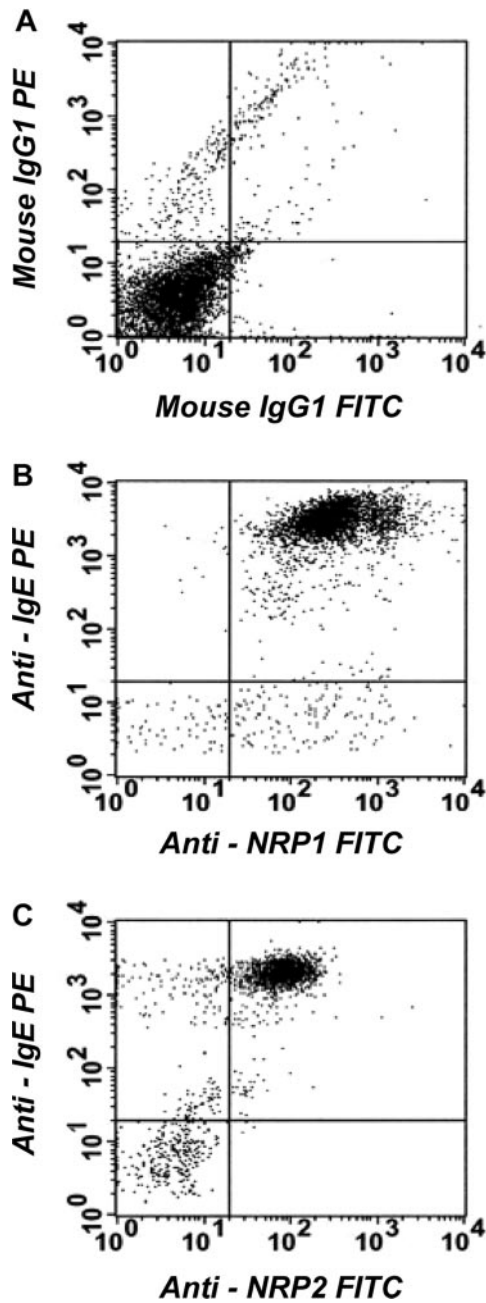


FIGURE 8. Cytofluorimetric analysis of NRP1 and NRP2 expression by human basophils. Basophils were incubated (4°C, 20 min) with monoclonal anti-NRP1 (B), anti-NRP2 (C), or isotype-matched Abs (A). Cells were stained (4°C, 20 min) with secondary Ab (FITC-conjugated goat anti-mouse).

concentrations of VEGF-A₁₆₅ caused basophil chemotaxis, which reached a plateau at 250 ng/ml.

As we demonstrate in this study, human basophils express mRNA for VEGF-A₁₆₅ and VEGF-A₁₂₁. Unlike VEGF-A₁₆₅, VEGF-A₁₂₁ does not express exon 7 (2). Although these two VEGF-A are both capable of activating VEGFR-1 and VEGFR-2, their potency in inducing biological activities is markedly different (49). When we compared the effects of increasing concentrations of VEGF-A₁₆₅ and VEGF-A₁₂₁ on basophil chemotaxis, we found that the potency of VEGF-A₁₂₁ was significantly lower than VEGF-A₁₆₅ (Fig. 9).

We did a checkerboard analysis to determine whether VEGF-A₁₆₅-induced migration of basophils resulted from chemotaxis

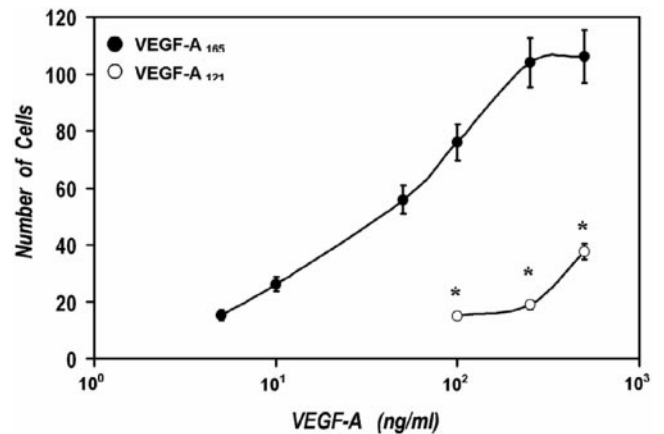


FIGURE 9. Effects of increasing concentrations of VEGF-A₁₆₅ and VEGF-A₁₂₁ on human basophil chemotaxis. Basophils were allowed to migrate with the indicated concentrations of VEGF-A₁₆₅ or VEGF-A₁₂₁ for 1 h at 37°C in a humidified (5% CO₂) incubator. Values are the mean ± SEM of six experiments with different basophil preparations. *, *p* < 0.001 when compared with the corresponding value of VEGF-A₁₆₅ (paired Student's *t* test). Bars are not indicated when they are graphically too small.

or chemokinesis. We found that VEGF-A₁₆₅ concentration-dependently induced the migration of basophils when added to the lower wells of the chemotaxis chamber. An optimal concentration of VEGF-A₁₆₅ (250 ng/ml) added to cells in the upper wells or to both compartments did not induce directional basophil migration (data not shown). Thus, VEGF-A-induced migration of basophils resulted from chemotaxis rather than from chemokinesis.

Effect of anti-VEGFR-2 Ab on basophil chemotaxis caused by VEGF-A₁₆₅

To verify whether the basophil chemotaxis caused by VEGF-A was mediated by the activation of VEGFR-2/KDR, we used an Ab against this receptor in blocking experiments. Fig. 10 shows that preincubation of basophils with an anti-VEGFR-2/KDR mAb (1–10 μg/ml) dose-dependently inhibited VEGF-A-dependent basophil chemotaxis. Preincubation of basophils with an irrelevant isotype mAb (1–10 μg/ml) did not modify basophil chemotaxis

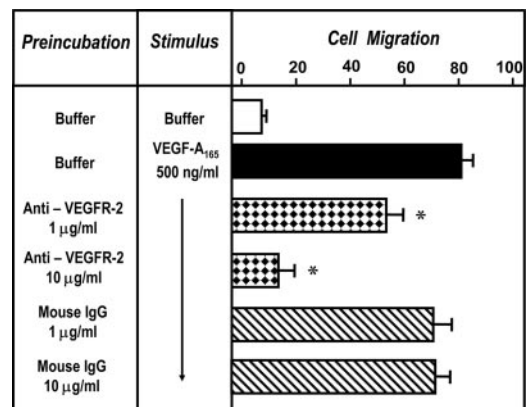


FIGURE 10. Effects of preincubation of basophils with an anti-VEGFR-2/KDR Ab (1 and 10 μg/ml) or nonimmune mouse IgG (1 and 10 μg/ml) on VEGF-A₁₆₅-induced chemotaxis. Basophils, preincubated (1 h at 37°C) with Abs, were allowed to migrate with the indicated concentration of VEGF-A for 1 h at 37°C in a humidified (5% CO₂) incubator. Values are the mean ± SEM of three experiments with different basophil preparations. *, *p* < 0.01 when compared with cells stimulated with VEGF-A₁₆₅ (Duncan's test).

caused by VEGF-A. Thus, binding of VEGF-A to VEGFR-2/KDR appears to be a requirement for VEGF-A-mediated basophil chemotaxis.

Heterologous desensitization between VEGF-A₁₆₅ and eotaxin or Hp (2–20)

Human basophils display a variety of membrane receptors whose engagement induces chemotaxis. We have demonstrated that the vast majority (~80%) of basophils express the chemokine CCR3 receptor, whose activation by eotaxin/CCL11 induces chemotaxis (28). Moreover, the *Helicobacter pylori*-derived peptide Hp (2–20) is a potent basophil chemoattractant, which exerts its effect through activation of formyl peptide receptor like-1 (FPRL1) and FPR like-2 (FPRL2) (23). Preincubation of basophils with high concentrations of the agonist is known to cause desensitization to a subsequent challenge with the same stimulus (23, 27). We examined the relationship between VEGF-A₁₆₅ and eotaxin or Hp (2–20) by evaluating the effects of heterologous desensitization between these stimuli on basophil chemotaxis. Purified basophils were preincubated with buffer or with VEGF-A₁₆₅ (500 ng/ml) or eotaxin (100 ng/ml) or Hp (2–20) (500 nM) in P-EDTA for 1 h at 37°C. At the end of incubation, cells were washed and suspended in PACGM. Fig. 11A shows the results of a typical experiment in which the response to VEGF-A₁₆₅ (500 ng/ml) was abolished by preincubation with the homologous stimulus (500 ng/ml). When basophils were desensitized by preincubation with eotaxin or Hp (2–20), which exert their effects by activating specific CCR3 and FPRL1/2 receptors, respectively (23, 27, 28), the response to the heterologous stimulus VEGF-A₁₆₅ was not affected. Similar results were obtained when basophils were challenged with a lower concentration (100 ng/ml) of VEGF-A₁₆₅ (Fig. 11B).

Effect of VEGF-A₁₆₅ on histamine and cytokine release from human basophils

We evaluated the effect of increasing concentrations of VEGF-A₁₆₅ on histamine and cytokine (IL-4 and IL-13) release from

basophils purified (>99%) from healthy individuals. In five experiments, VEGF-A₁₆₅ (10–500 ng/ml) did not cause either histamine or cytokine release from basophils. In these experiments, anti-IgE (0.1 μg/ml) was a potent stimulus for the secretion of histamine and cytokines (IL-4 and IL-13) from basophils (data not shown). In three experiments, preincubation (1 h at 37°C) of basophils with VEGF-A₁₆₅ (10–100 ng/ml) did not modify the release of histamine and cytokines caused by anti-IgE from basophils (data not shown).

Basophil-derived VEGF induces an angiogenic response in the chick embryo CAM

The results of the experiments reported above demonstrate that the two forms of VEGF-A synthesized by basophils (VEGF-A₁₆₅ and VEGF-A₁₂₁) induce basophil chemotaxis in vitro. In three experiments, we next investigated the possibility that basophil supernatants of immunologically activated basophils can induce an angiogenic response in vivo. To this aim, we used the chick embryo CAM assay at day 8 of incubation implanted with gelatin sponges adsorbed with basophil supernatants. Sponges adsorbed with vehicle alone or with VEGF-A₁₆₅ were used as negative and positive controls, respectively. At day 12 of incubation, macroscopic observations of the CAMs showed that supernatants of basophils activated with anti-IgE for 2 h induced an angiogenic response characterized by the presence of allantoic vessels spreading radially toward the sponge in a spoked-wheel pattern (number of vessels at the sponge-CAM boundary = 27 ± 4). A similar macroscopic angiogenic response was observed in the implants treated with 500 ng of VEGF-A₁₆₅ (number of vessels at the sponge-CAM boundary = 30 ± 4). The number of vessels at the sponge-CAM boundary caused by supernatants of unstimulated basophils kept in culture for 2 h was 14 ± 3. No vascular reaction was detectable around the sponges treated with vehicle alone (number of vessels at the sponge-CAM boundary = 7 ± 2). To assess whether the angiogenic response induced by basophil supernatants was due in part to their content of VEGF-A, supernatants of anti-IgE-activated

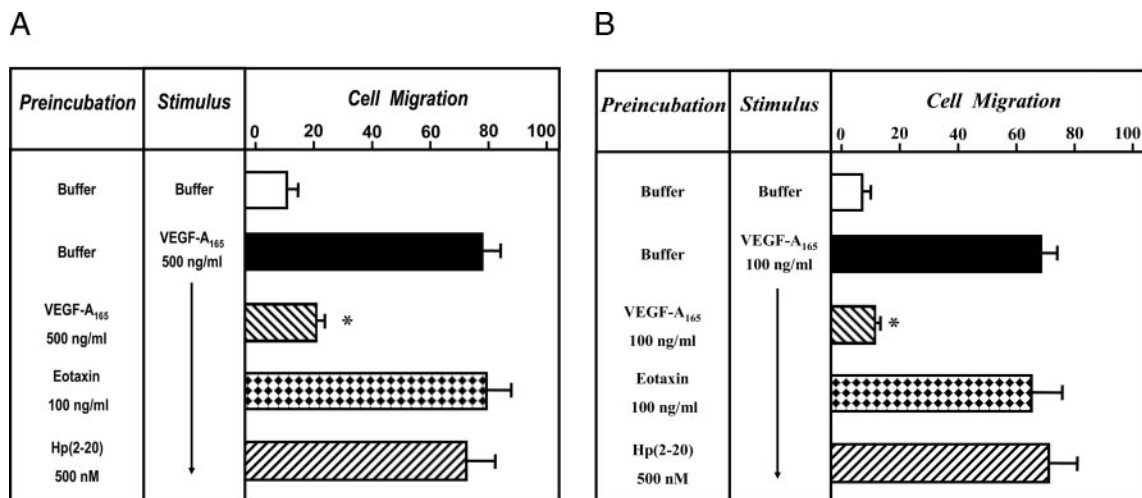


FIGURE 11. A, Effects of heterologous desensitization between VEGF-A₁₆₅ and eotaxin or Hp (2–20) on basophil chemotaxis. Basophils were incubated in PIPES buffer containing EDTA (4 mM) or VEGF-A₁₆₅ (500 ng/ml) or eotaxin (100 ng/ml) or Hp (2–20) (500 nM) for 30 min at 37°C. At the end of incubation, cells were washed (twice) and resuspended in PACGM and challenged with VEGF-A₁₆₅ (500 ng/ml). Basophils were allowed to migrate for 1 h at 37°C in a humidified (5% CO₂) incubator. Values are the mean ± SEM of three experiments with different basophil preparations. Value of $p < 0.01$ when compared with cells stimulated with VEGF-A₁₆₅ (Duncan's test). B, Effects of heterologous desensitization between VEGF-A₁₆₅ and eotaxin or Hp (2–20) on basophil chemotaxis. Basophils were incubated in PIPES buffer containing EDTA (4 mM) or VEGF-A₁₆₅ (100 ng/ml) or eotaxin (100 ng/ml) or Hp (2–20) (500 nM) for 30 min at 37°C. At the end of incubation, cells were washed (twice) and resuspended in PACGM and challenged with VEGF-A₁₆₅ (100 ng/ml). Basophils were allowed to migrate for 1 h at 37°C in a humidified (5% CO₂) incubator. Values are the mean ± SEM of three experiments with different basophil preparations. *, $p < 0.01$ when compared with cells stimulated with VEGF-A₁₆₅ (Duncan's test).

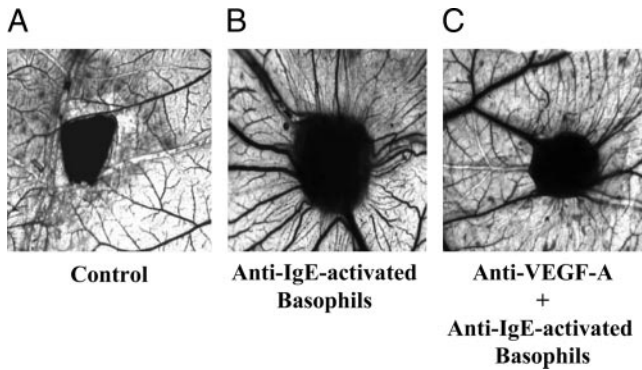


FIGURE 12. Effects of supernatants of human basophils, challenged with anti-IgE (0.3 $\mu\text{g}/\text{ml}$ for 2 h at 37°C) or with buffer, on the angiogenic response in the chick embryo CAM. Chick embryo CAMs at day 8 of incubation were implanted with gelatin sponges adsorbed with buffer alone (A) or with supernatants of basophils activated with anti-IgE for 2 h (B). At day 12 of incubation, macroscopic examination of the CAMs showed that supernatants of anti-IgE-activated basophils induced an angiogenic response characterized by the presence of allantoic vessels spreading radially toward the sponge in a spoked-wheel pattern (B). Anti-VEGF-A Ab reduced the angiogenic response of anti-IgE-activated basophil supernatants (C). This experiment is representative of three experiments with similar results.

basophils were preincubated with an anti-VEGF-A Ab and then added to the CAM. Anti-VEGF-A Ab reduced the angiogenic response of basophil supernatants (number of vessels at the sponge-CAM boundary = 11 ± 3 ; $p < 0.001$ vs anti-IgE-activated basophil supernatants) (Fig. 12). Incubation of the basophils with the isotype control did not affect this response (data not shown).

Discussion

The VEGF/VEGFR system is emerging as a fundamental regulator of angiogenesis and tissue remodeling in inflammatory and neoplastic disorders (1, 2). Increasing evidence suggests that this complex biological system can also modulate several aspects of immune and inflammatory reactions (3–5, 38, 40, 41). In this study, we have identified a new mechanism by which human basophils might influence angiogenesis and inflammation through the synthesis and immunologic release of various isoforms of VEGF. In addition, we demonstrate that two VEGF-A isoforms function as basophil chemoattractants, presumably by interacting with VEGFRs. This novel autocrine loop involves the expression of several components of the VEGF family and their receptors on basophils.

Basophils purified from healthy donors express mRNA for three major isoforms of VEGF-A (121, 165, and 189) and two isoforms of VEGF-B (167 and 186). Interestingly, VEGF-C and -D mRNA, two mediators of lymphatic development (8), were not detected in basophils. Therefore, basophils display a selective expression of certain members of the VEGF family. It is conceivable that other components of the VEGF family could be expressed under different experimental conditions (e.g., hypoxia) or in certain pathological situations (e.g., inflammation and neoplasia).

We also demonstrate that VEGF-A is contained in the cytoplasmic secretory granules of basophils. The relevance of this finding is supported by two observations. First, VEGF-A colocalized with the basogranulin selectively present in basophil secretory granules (22, 33). More importantly, VEGF-A was localized in basophils infiltrating the sites of inflammation of biopsies of patients with nasal polyps. Thus, VEGF-A appears to be present not only in peripheral blood but more importantly at sites of chronic inflammation. The latter observation emphasizes the possibility that

VEGF-A synthesized and released from basophils plays a dual role in inflammatory angiogenesis. First, VEGF-A released from circulating basophils might activate VEGF receptors present on circulating endothelial cell precursors and immune cells; second, VEGF-A released from basophils infiltrating the sites of chronic inflammation might represent a local source of an important angiogenic and chemotactic factor.

We have found that VEGF-A is stored in secretory granules and that the immunologic release of this cytokine from basophils follows a biphasic pattern: rapid release (30 min) after the anti-IgE challenge and a second wave, 2–4 h after challenge. The first wave of release is probably due to the secretion of preformed VEGF-A because it is not inhibited by the protein synthesis inhibitor cycloheximide. The second wave of VEGF-A release presumably represents *de novo* synthesis because it is significantly inhibited by cycloheximide. Our results indicate that the kinetics of the immunologic release of VEGF-A by basophils differs from that of IL-13 synthesized by these cells. In fact, the IgE-mediated release of VEGF-A from basophils is rapid and reaches a plateau within 4 h. By contrast, the release of IL-13 from basophils reaches a plateau ~ 18 h after immunologic challenge (50–52). Interestingly, immunologically activated human basophils express a very restricted (namely IL-4 and IL-13) cytokine profile (51–53). VEGF-A is the latest addition to the series of cytokines synthesized by these immune cells.

In our experiments, we found that IgE cross-linking is a stimulus for the production of VEGF-A. Similarly, Ag and anti-IgE can induce the release of VEGF-A from mast cells (54). Abdel-Majid and Marshall (36) have demonstrated that PGE_2 is a potent inducer of VEGF-A secretion from human mast cells. By contrast, we found that PGE_2 did not induce the production of VEGF-A from human basophils. This adds to the long list of immunological and biochemical differences between these two types of immune cells (20).

Mast cells, basophils, and eosinophils are considered primary effector cells in allergic disorders (20, 55). It has been shown that human mast cells synthesize and release several isoforms of VEGF-A (36, 54, 56, 57). Also human eosinophils exert direct proangiogenic effects (58). In the present study, we demonstrate that circulating and tissue infiltrating basophils express and release VEGF-A. Taken together, these findings support the hypothesis that the release of angiogenic factors from primary effector cells of allergic inflammation could represent a relevant aspect of tissue remodeling in chronic allergic disorders.

An important finding of this study is that VEGF-A exerts an autocrine chemotactic effect on human basophils, presumably through the engagement of VEGFR-2/KDR, NRP1, and NRP2. In addition, it is conceivable that VEGF produced by other immune or neoplastic cells might contribute to basophil infiltration in a variety of chronic inflammatory and neoplastic disorders.

The expression of VEGFRs on immune cells is still under careful scrutiny. Most human monocytes express VEGFR-1/Flt-1 but not VEGFR-2/KDR (38, 39, 59, 60). However, a small fraction ($\sim 2\%$) of CD14^+ cells expresses VEGFR-2/KDR (61). Using flow cytometry we found that VEGFR-2/KDR is expressed on the surface of $\sim 70\%$ of basophils purified from healthy donors. This observation is supported by the presence of mRNA for VEGFR-2/KDR in these cells. Using two different sets of primers, we did not detect VEGFR-1/Flt-1 mRNA in basophils from healthy donors, whereas sVEGFR-1 mRNA was abundantly expressed.

We found that an Ab that blocks VEGFR-2/KDR antagonizes the chemotactic effect of VEGF-A on basophils. We also performed cross-desensitization experiments to verify the specificity

of the activation route for VEGF-A. We found that basophils pre-incubated with VEGF-A were desensitized to a subsequent challenge with the homolog stimulus. In contrast, when basophils were exposed to eotaxin, which binds to CCR3 (28), or to Hp (2–20), which binds to FPRL1/FPRL2 (23), the chemotactic response to VEGF-A was not affected. The results of these two groups of experiments are consistent with the hypothesis that VEGF-A induces basophil chemotaxis by activating VEGFR-2/KDR.

It appears that basophils, by expressing VEGF-2/KDR and not VEGFR-1/Flt-1, differ from the vast majority of monocytes (38, 39, 41, 62), eosinophils (42), and neutrophils (43) that express VEGFR-1/Flt-1, and not VEGFR-2/KDR. VEGFR-1/Flt-1 appears to mediate chemotaxis in human monocytes (38, 39), neutrophils (43), and eosinophils (42). Endothelial cells express both VEGFR-1/Flt-1 and VEGFR-2/KDR (63), and whereas VEGFR-2/KDR activates cellular signaling, VEGFR-1/Flt-1 acts as a trap to sequester VEGF from VEGFR-2/KDR. Soluble VEGFR-1, synthesized by GM-CSF-activated monocytes (41), also prevents VEGF from activating VEGFR-1/Flt-1 (on monocytes) and VEGFR-2 (on endothelial cells) by inhibiting dimerization of VEGFR. Interestingly, also human basophils express mRNA for sVEGFR-1.

We have also found that most basophils express NRP1, a coreceptor for VEGF-A₁₆₅ (18, 45), which enhances VEGFR-2/KDR-induced responses (19). We also found that basophils express NRP2 mRNA and protein. NRP1 has no known enzymatic activity and therefore participates in signal transduction events by forming a complex with tyrosine kinase receptors. However, there is evidence that NRP1 supports the autocrine functions of VEGF in cells lacking VEGFR-2 expression (64). This raises the possibility that, in certain cells, NRP1 functions either alone or in concert with other tyrosine kinase-linked receptors to transduce VEGF signaling. It has been demonstrated that NRP1 on cells other than endothelial cells can induce angiogenesis (19). Therefore, it is feasible that NRP1 highly expressed on basophils can enhance angiogenesis even when VEGF is not abundantly expressed.

The results of this study might have practical implications in several inflammatory disorders in which basophils that infiltrate sites of inflammation play a prominent role (21–23). In fact, we found that basophils in peripheral blood and tissue are a source of VEGF, which is the most potent angiogenic factor known so far. The identification of VEGFR-2/KDR and NRP1 and NRP2 on basophils has revealed a novel autocrine loop. This finding raises the possibility that basophils might modulate angiogenesis. Pharmacological manipulation of the VEGF/VEGFR network, which is already showing promise in relation to neoplastic angiogenesis (65), might also be effective in disorders associated with basophil recruitment and activation.

Acknowledgments

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Disclosures

The authors have no financial conflict of interest.

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