RAPID COMMUNICATION

Angiogenic Factors Stimulate Mast-Cell Migration

By Barry L. Gruber, Mary J. Marchese, and Richard Kew

Mast cells accumulate at sites of angiogenesis. The factor(s) that control mast-cell recruitment at these sites have yet to be defined. We sought to determine if angiogenic factors result in mast-cell chemotaxis. In this study, we observed that platelet-derived growth factor-AB (PDGF-AB), vascular endothelial cell growth factor (VEGF), and basic fibroblast growth factor (bFGF) each cause directed migration of murine mast cells at picomolar concentrations, with a typical bell-shaped dose-response curve. Another potent angiogenic factor, platelet-derived endothelial cell growth factor (PD-ECGF), appears to promote chemokinesis of mast cells, whereas tumor necrosis factor-α, a weak angiogenic factor, is less robust but still functions as a mast cell chemotactic factor. Epidermal growth factor (EGF), a growth factor with minimal angiogenic properties, was ineffective as a mast cell chemotactic factor. A checkerboard analysis confirmed the directional chemotactic response of PDGF-AB, VEGF, and bFGF, while indicating the chemokinetic response induced by PD-ECGF. Cross-desensitization of growth-factor-induced directed migration was observed between PDGF-AB and bFGF, and also between PDGF-AB and PD-ECGF. Tyrosine kinase-inhibitor genistein effectively dampened the chemotactic responses, whereas pertussis toxin had no effect. In summary, our findings suggest that factors known to act on endothelial cells and stimulate neovascularization may simultaneously serve to recruit mast cells to these sites. The local accumulation of mast cells is believed to facilitate new vessel formation through complex cell-cell interactions.

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the lower compartment of a 48 well micro-chemotaxis chamber (Neuroprobe, Cabin John, MD). The lower compartments were covered with a two-filter sandwich, a lower 5-µm cellulose nitrate filter and an upper 8-µm polycarbonate filter (both purchased from Neuroprobe), and then 50 µL of the cell suspensions (4 × 10⁶/mL) were pipetted into the upper compartments. The chemotaxis chamber was then incubated for 3 hours in a humidified incubator with 5% CO₂.

After the incubation period, the upper polycarbonate filters were discarded while the lower cellulose nitrate filters were fixed in 2-propanol, stained with acid hematoxylin, cleared with xylenes, and then mounted on a microscope slide with Permount (Fisher Scientific, Springfield, NJ). Mast-cell chemotaxis was quantitated microscopically by counting the number of cells that had traversed the upper 8-µm polycarbonate filter and were attached to the surface of the 5-µm cellulose nitrate filter. In each experiment, five fields per duplicate filter were measured at 400 × magnification (high-power field, HPF) and data are presented as the mean number of cells/HPF. A modified checkerboard analysis was performed to ascertain if the migration observed was chemotactic or chemokinetic and to determine the effect of one factor on the response to a second angiogenic factor. The concentration of each factor that induced maximal migration was incubated with the cells for 15 minutes before adding the cells to the chemotaxis chamber. Mast cells pretreated with the various angiogenic factors were then allowed to migrate toward control buffer or peak concentrations of the factors for 3 hours at 37°C. The results were compared with buffer controls. For certain experiments, cells were preincubated with 0.2 µg/mL PT or 0.25 ng/mL genestein (100 µmol/L) for 4 hours at 37°C and the effect on migration in response to optimal concentrations of PDGF or VEGF was determined.

Histamine assay. To determine if the angiogenic factors induced mast-cell degranulation, an enzyme immunoassay (Immunootech, AMAC Inc, Westbrook, ME) was used to quantitate released histamine, which is acylated as per the manufacturer's directions before performing the assay. The mast cells (10⁶ cells/0.1 mL) were incubated with either DMEM media alone or with the addition of individual angiogenic factors for 40 minutes at 37°C, over a range of concentrations from 3 to 300 ng/mL. A standard curve was generated for each assay between 10⁻¹⁰ and 10⁻⁷ mol/L histamine and sample results were extrapolated from this standard curve. Total histamine release was obtained by boiling the cells for 20 minutes and the results are expressed as the following:

Net Histamine Release

\[\text{Net Histamine Release} = \frac{\text{Sample} - \text{Spontaneous Release}}{\text{Total Release}} \times 100\%\]

All the assays were performed with duplicate samples and each experiment was repeated at least twice.

RESULTS

Mast-cell chemotaxis to angiogenic factors. When cultured murine mast cells were placed in a Boyden-type microchamber, they migrated across an 8-µm polycarbonate filter toward a gradient of extremely low concentrations of PDGF, VEGF, PD-ECGF, and bFGF as shown in Fig 1. The most potent chemotactic responses were observed for PDGF, VEGF, and PD-ECGF, with the latter growth factor showing a narrow range of responsiveness peaking at 1 pmol/L (Fig 1). PDGF and VEGF were slightly broader in their activity with peak responsiveness between 100 fmol/L and 1 pmol/L (Fig 1). In contrast, the mast cells were considerably less sensitive to TNF-α and essentially unresponsive to EGF (Fig 1). The dose response of mast cells to each angiogenic factor followed a bell-shaped curve with higher concentrations (nanomolar) resulting in a loss directed migration, a characteristic of most chemotactic factors.

Checkerboard analysis of mast-cell movement to angiogenic factors. The mast-cell migratory response to all of the angiogenic factors tested except PD-ECGF was largely caused by chemotaxis and not chemokinesis because a checkerboard analysis, whereupon the concentration of chemotactant above and below the filter was varied, demonstrated that significant migration was observed only when there was a gradient of the factor below the filters (Fig 2). In the case of PD-ECGF, the analysis showed mostly chemokinetic-type responses. A complete dose-response checkerboard analysis confirmed that PD-ECGF activity was chemokinetic rather than chemotactic (data not shown).

Cross-desensitization of chemotactic responses. The modified checkerboard analysis, using peak concentrations of different factors in the upper and lower chambers, was performed to determine if any individual factor was capable of desensitizing the cells from responding to a different factor. The results as shown in Fig 2 suggest that several factors markedly cross-desensitize and reduce the mast-cell responses. In particular, incubation of the mast cells with PDGF diminishes subsequent responsiveness to PD-ECGF and bFGF. To a certain degree, the converse was also observed. Thus, incubation with PD-ECGF, or especially bFGF, decreased subsequent responsiveness to PDGF. In contrast, the responsiveness to VEGF, was not affected by other angiogenic factors and did not lower responsiveness to other factors.

Effect of receptor inhibitors on chemotactic responses. The effect of inhibiting G-protein-coupled responses or tyrosine-kinase–coupled responses was determined by the addition of PT or genestein, respectively. The results, as shown in Fig 3, indicate that PDGF and VEGF stimulation of chemotaxis could be dampened by nearly 50% (P < .01) with the tyrosine kinase inhibitor, whereas PT had essentially no effect. These inhibitors had no deleterious effect on cell viability at the concentrations used, as confirmed by greater than 98% viability by trypan blue exclusion.

Histamine assay. The effect of the angiogenic factors on histamine release was determined. No histamine was released in response to incubation of PDGF-AB, VEGF, EGF, bFGF, or TGF-β at the concentrations used. However, PD-ECGF (1 nmol/L) consistently resulted in a net histamine release, mean 18% ± 1%.

DISCUSSION

We have previously reported that TGF-β is the most potent chemotactic factor yet to be described for mast cells, causing mast cells to rapidly migrate in vitro against a chemical gradient of TGF-β. Mast cells express classical TGF-β receptors and ligation of these receptors results in a dramatic and rapid shape change to a polarized morphology, typical of activated cells preparing to migrate. Because TGF-β is a potent angiogenic factor, we speculated that the accumula-
tion of mast cells at sites of newly formed granulation tissue is partly a result of the multifunctional effects of this cytokine on different cells. In the current investigation, we sought to determine if other angiogenic factors could likewise act in a bifunctional manner—i.e., stimulate neovascularization by activating endothelial cells while simultaneously recruiting mast cells. The capacity of previously described angiogenic factors, including PDGF-AB, bFGF, VEGF, PD-ECGF, and TNF-α, to stimulate mast-cell migration was studied. We found that, similar to TGF-β, each of these factors induced directed migration in vitro with a typical bell-shaped dose-response curve, although checkerboard analysis showed that PD-ECGF was more chemokinetic in its activity. In contrast, EGF, a factor with minimal angiogenic properties, was essentially ineffective in mobilizing mast cells. The only factor that, in addition to chemotaxis, stimulated histamine release was PD-ECGF.

A number of polypeptide growth factors have been shown to influence vascular growth. We selected several of the better-characterized factors to further study their effect on mast cells. To our knowledge, no previous data concerning the effects of these growth factors on mast-cell migration have been published. FGF is perhaps best known for its effect on endothelial cell growth. Both acidic and basic FGF are potent mitogens and chemotaxants for both small- and large-vessel endothelial cells, induce protease production, and are angiogenic in vivo. PDGF exists in two major isoforms; we chose to study the heterodimer to maximize any biologic effects. PDGF acts as a mitogen and chemotaxant for fibroblasts, pericytes, and smooth-muscle cells. However, only the PDGF-BB isoform appears to stimulate chemotaxis of these cells, perhaps by its selective action on β-type receptors. Whether this observation can be extended to mast cells was not explored in this study. PDGF is also angiogenic in vivo. A related peptide is VEGF, essentially identical to the previously described vascular permeability factor (VPF), both containing eight conserved cysteine residues similar to PDGF A and B chains. VEGF is believed to be largely specific for endothelial cells, acting as a cell mitogen and inducing increased permeability. However, VEGF also stimulates the migration of monocytes across endothelial cell monolayers, and recep-
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PD-ECGF has endothelial cell chemotactic activity in vitro

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nor 3 hours at 37°C + 5% CO2. Numbers represent mean ± SEM (n = 3) of total cells/high power field (400×).

Fig 2. Modified checkerboard analysis of mast-cell migration to angiogenic factors. An optimal chemotactic concentration of angiogenic factors (0.1 pmol/L PDGF-AB, 0.1 pmol/L VEGF, 1 pmol/L PD-ECGF, 10 pmol/L bFGF) were placed in the upper (cell) and/or lower (stimulus) compartments as indicated. Cells that were co-incubated with the factors were preincubated for 10 minutes at 22°C before being added to the assay. Cell migration was measured for 3 hours

Receptors for VEGF have been described in both endothelial cells and monocytes. VEGF is one of the most potent angiogenic factors in vivo. PD-ECGF was isolated by its capacity to act as an endothelial cell mitogen, although it does not stimulate cell proliferation, and sequencing has shown that this angiogenic factor is identical to the human enzyme, thymidine phosphorylase. In addition to mitogenic activity, PD-ECGF has endothelial cell chemotactic activity in vitro and angiogenic activity in vivo.

Fig 3. Effect of genistein or PT pretreatment on mast-cell migration to PDGF-AB and VEGF. Mast cells (4 million/mL) were pretreated with either buffer (control) 0.2 µg/mL PT or 0.25 mg/mL genistein for 4 hours at 37°C and then placed directly into the chemotaxis chamber and allowed to migrate toward 1 pmol/L VEGF, 1 pmol/L PDGF-AB, or buffer for 3 hours at 37°C + 5% CO2. Numbers represent mean ± SD (n = 2) of total mast cells/high power field (400×). Asterisk indicates that the value is significantly less (P < .01) than corresponding control value.

Receptors for each of these growth factors, except PD-ECGF, have been characterized and found to contain intrinsic tyrosine kinase activity. Our experimental data, indicating that genistein was effective in dampening the chemotactic responses whereas PT had no effect, is consistent with the notion that tyrosine-kinase stimulation was involved in the chemotaxis rather than a G-protein-coupled signaling mechanism. These tyrosine-kinase receptors are classified into subclasses, based on differences in their ligand-binding extracellular domains and the presence of a kinase insert region. VEGF receptors, of which two have been described, are similar structurally to the α- and β-type PDGF receptors and to the FGF receptor family. These receptors are activated by binding to ligand dimers mediating receptor dimerization; this is in contrast to EGF, which undergoes a conformational change after binding to a ligand monomer, thus inducing receptor dimerization. Whether this difference in extracellular activation mechanisms explains the lack of responsiveness of mast cells (and endothelial cells) to EGF or merely that mast cells lack EGF receptors is not clear at present. Activation of these receptors leads to autophosphorylation of several proteins including the cytoplasmic portion of the receptor as well as other substrates. Included among these substrates are phospholipase C-γ, GTPase-activating protein, phosphotyidylinositol 3'-kinase, members of the pp60Src family, and Raf-1. Autophosphorylated receptors serve as docking sites for proteins that contain Src-homology region 2 (SH2) domains, which may serve as a link in activating downstream nuclear signals such as Ras. A number of other intermediates are rapidly being discovered, as intense efforts are underway to dissect these complex intracellular signal transduction pathways. What has become evident recently is that many different growth-factor signaling pathways apparently converge to common signals capable of modulating nuclear events. In this regard, it is interesting that our experiments indicate that different growth factors were capable of desensitizing the specific response to one another. This was most evident in the case of PDGF and FGF. Recently PDGF and FGF were both shown to stimulate production of similar intracellular tyrosine-phosphorylated proteins of approximately 145 kD, which bound to the phosphotyrosine-binding domain of SHC, a protein implicated in signaling through Ras. Although these precise activated intermediates may be unrelated to those which mediate chemotaxis, they nonetheless represent examples of convergent postreceptor cellular events that may partly explain the dampened response to a second extracellular ligand signal. Alternatively, preliminary evidence suggesting that mast cells may synthesize and release FGF allows speculation that one factor (e.g., PDGF) may induce secretion of another factor (in this case, FGF), thus causing desensitization indirectly.
The effective concentration of angiogenic factors which maximally stimulated mast cell migration was in the picomolar range, a concentration easily achieved at a local site during active angiogenesis. These factors may be synthesized and released after appropriate physiologic stimuli, such as hypoxia or invading tumor cells. Many examples of mast-cell accumulation in association with angiogenesis can be found in the literature. Early observations indicated that mast cell density and the number of blood vessels bears a direct correlation. Tumor implants, which may release many of the angiogenic factors studied in this investigation, reportedly lead to a 40-fold increase in mast-cell density in the chick embryo. Multiple reports of mast cell hyperplasia in the vicinity of tumor invasion have been recently reviewed. Mast-cell density is markedly increased in hemangiomas and returns to normal with tumor regression. Mast cells appear 24 hours after tumor implants on the chick chorioallantoic membrane and precede new vessel appearance by 2 days. This suggests that the tumors produce factors which promote mast cell chemotaxis. The fact that the mast cells then serve to facilitate angiogenesis is implicated by studies of mast-cell-deficient mice. Tumor angiogenesis occurs in these mice, but at a reduced rate and, furthermore, the local reconstitution of mast cells leads to restoration of the angiogenic response.

Compared to previously reported mast-cell chemoattractants, of which there are surprisingly few, TGF-β and the presently described angiogenic factors are by far the most potent yet described: being 10- to 100-fold more active than c-kit ligand, interleukin-3 (IL-3), or laminin. A recent report described the capacity of c-kit ligand and the intecrine RANTES to induce slight chemotactic responses in immature human mast cells and other chemokines such as MCAF, as well as RANTES, can stimulate murine mast-cell migration. It is interesting to speculate that the chemotactic response to PDGF and c-kit ligand (also known as stem-cell factor) may be similar in mechanism because both bind to structurally related tyrosine-kinase receptors. Nonetheless, the presence of tyrosine-kinase growth factor receptors on mast cells other than c-kit have not been described to date. Our data, using the tyrosine-kinase inhibitor genistein, supports the possibility that a broad family of these receptors may be expressed on mast cells. Further investigation is warranted to characterize these receptors and determine their biologic effects on mast-cell physiology. In either case, our findings should prove very useful in comprehending the long-standing observation of mast cells accumulating at sites of angiogenesis.

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