Mast Cells Can Secrete Vascular Permeability Factor/Vascular Endothelial Cell Growth Factor and Exhibit Enhanced Release after Immunoglobulin E–dependent Upregulation of Fcε Receptor I Expression

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Summary

Vascular permeability factor/vascular endothelial cell growth factor (VPF/VEGF) can both potently enhance vascular permeability and induce proliferation of vascular endothelial cells. We report here that mouse or human mast cells can produce and secrete VPF/VEGF. Mouse mast cells release VPF/VEGF upon stimulation through Fcε receptor I (FcεRI) or c-kit, or after challenge with the protein kinase C activator, phorbol myristate acetate, or the calcium ionophore, A23187; such mast cells can rapidly release VPF/VEGF, apparently from a preformed pool, and can then sustain release by secreting newly synthesized protein. Notably, the FcεRI-dependent secretion of VPF/VEGF by either mouse or human mast cells can be significantly increased in cells which have undergone upregulation of FcεRI surface expression by a 4-d preincubation with immunoglobulin E. These findings establish that at least one cell type, the mast cell, can be stimulated to secrete VPF/VEGF upon immunologically specific activation via a member of the multichain immune recognition receptor family. Our observations also identify a new mechanism by which mast cells can contribute to enhanced vascular permeability and/or angiogenesis, in both allergic diseases and other settings.

Key words: allergy • angiogenesis • c-kit • stem cell factor • vascular permeability

Mast cells are widely distributed in the connective tissues of mammals and other vertebrates, where they are frequently located in close proximity to blood vessels (1, 2). Several lines of evidence, many of them derived from morphological studies, have implicated mast cells in the regulation of physiological or pathological examples of angiogenesis, including that associated with hemangiomas (3) and other neoplasms (4, 5), rheumatoid arthritis (6), wound healing (7), and ovulation (8). Although the importance of mast cells as a source of proangiogenic factors in such settings largely remains to be determined, mast cells have been shown to secrete several mediators which might contribute to various aspects of neovascularization, including heparin (9, 10), TNF-α (11), TGF-β (12), basic fibroblast growth factor (13), and tryptase (14).

In this study, we evaluated whether mast cells can secrete the multifunctional cytokine vascular permeability factor/vascular endothelial cell growth factor (VPF/VEGF).[1] VPF/VEGF, which can be expressed by many types of normal or transformed cells (15, 16), is required for embryonic and physiological vascular development (15–17) and is thought to contribute significantly to tumor neovascularization and other pathological forms of angiogenesis (15, 16, 18). VPF/VEGF can also potently increase venular permeability to small and large molecules, both in the context of angiogen-

[1]*Abbreviations used in this paper:* Act D, Actinomycin D; BMCMC, mouse bone marrow–derived cultured mast cell(s); FBS, fetal bovine serum; HSA, human serum albumin; HMC, umbilical cord blood–derived human mast cell(s); 5-HT, 5-hydroxytryptamine; ISH, in situ hybridization; PMC, peritoneal mast cell(s); rhSCF, recombinant-methionyl human stem cell factor; rrSCF, recombinant rat stem cell factor[164]; SCF, stem cell factor; VPF/VEGF, vascular permeability factor/vascular endothelial cell growth factor.

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esis and in other settings (15, 19–21). Stimulation of certain cells via cytokine receptors can enhance expression of VPF/VEGF (15, 16). However, there has been no report of any cell type in which VPF/VEGF secretion can be initiated upon immunologically specific activation of the cells. In this study, we found that mouse or human mast cells can produce and secrete VPF/VEGF, and can secrete VPF/VEGF upon activation via the Fcε receptor I (FcεRI). Moreover, we found that the IgE-dependent secretion of VPF/VEGF can be significantly increased in mast cells that have undergone IgE-dependent upregulation of their surface expression of FcεRI (22, 23).

Materials and Methods

Mast Cells. Bone marrow–derived cultured mouse mast cells (BMCMCs) were obtained by maintaining the femoral bone marrow cells of 4–6-wk-old BALB/c mice (Charles River Laboratories, Wilmington, MA) in suspension in IL-3–containing conditioned complete medium, consisting of 10% heat-inactivated fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO), 50 μM β-mercaptoethanol (Sigma Chemical Co.), and 2 mM l-glutamine (GIBCO BRL, Gaithersburg, MD) in DMEM (GIBCO BRL) supplemented with 20% (vol/vol) of supernatants from WEHI-3 cell–conditioned medium; the cells were resuspended in fresh conditioned medium one to two times per week (24). After 4–5 wk, at least 95% of cells that remained in the cultures were identifiable as mast cells, as determined by toluidine blue or May-Grünwald/Giemsa staining. Cl.MC/C57.1 mast cells, a cloned, growth factor–independent mouse mast cell line of BALB/c origin (25, 26), were maintained as described (27). Rat peritoneal mast cells (rat PMCs) were purified from adult (175–200 g) male Wistar rats (Charles River Laboratories) exactly as described (28).

Mouse PMCs were purified from female retired breeder BALB/c mice (11). In brief, groups of 60 mice were killed by CO_2 inhalation, the abdominal skin was washed with 70% ethanol, the peritoneum was exposed by a 1–2 cm midline abdominal incision, and 5.0 ml of sterile, pyrogen-free 0.9% NaCl and 5.0 ml of air were injected into the peritoneal cavity via a 25-gauge needle. The abdomen was massaged gently for ~3 min, and the peritoneal fluid was recovered in a syringe via a 22-gauge needle, centrifuged at 1,000 rpm (~209 *g*) for 10 min at room temperature, resuspended in standard medium (DMEM supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, and 50 μM β-mercaptoethanol), at which time ~0.9% of the ~2.8×10^8 peritoneal cells were mast cells according to Kimura stain. The cells were washed once and then resuspended in 5.0 ml of standard medium, layered onto 5.0 ml of 23% metrizamide (Accurate Chemical and Science Corp., Westbury, NY) in standard medium, centrifuged at 1,500 rpm (~469 *g*) for 15 min at room temperature, and resuspended in standard medium (at which time 4.2% of the cells were mast cells), then layered in 2.5 ml over 2.5 ml of 23% metrizamide in standard medium, centrifuged at 1,500 rpm (~469 *g*) for 15 min at room temperature, and resuspended in standard medium, at which time mast cells represented 95–99% of the 3.6–5.4 $\times 10^5$ cells recovered.

Umbilical cord blood–derived human mast cells (HMCs) were generated as described elsewhere (23), in medium containing recombinant human stem cell factor (rhSCF, 80 ng/ml) and rhIL-6 (50 ng/ml; both from Amgen, Inc., Thousand Oaks, CA), and 1 μM PGE_2 (Cayman Chemical Co., Inc., Ann Arbor, MI).

Activation of Mast Cells. BMCMCs or Cl.MC/C57.1 cells were incubated for 4 d either with no IgE or with 5 μg/ml of a mouse IgE anti-DNP mAb (29; added as an aliquot from a stock solution of ascites containing 1.7 mg IgE/ml). The cells were then sedimented and resuspended at 3×10^6 cells/ml of complete medium (BMCMCs), or for Cl.MC/C57.1 cells, in the same medium that was not supplemented with IL-3, and then passively sensitized with 10 μg/ml mouse IgE anti-DNP for 50 min at 4°C. Sensitized cells were washed three times in DMEM containing 5% FBS and resuspended at 4×10^6 cells/ml (for time course experiments) or 6×10^6 cells/ml (for dose response experiments and experiments using inhibitors), in standard medium. Mast cell activation was induced in multiwell plates by mixing equal volumes of cell suspension and standard medium containing twice the desired final concentration of challenging agents: DNP–human serum albumin (DNP-HSA; Sigma Chemical Co.), the protein kinase C activator PMA (Sigma Chemical Co.), the calcium ionophore A23187 (Sigma Chemical Co.), or the c-kit ligand, *Escherichia coli*–derived recombinant rat stem cell factor^{164} (rrSCF; Amgen, Inc. [2]). HMCs were incubated with or without human myeloma IgE for 4 d, then passively sensitized with human myeloma IgE and challenged with anti–human IgE exactly as described (23). Mouse PMCs were stimulated for 2 h with PMA (50 ng/ml in standard medium) or, as a control, with standard medium (vehicle) alone.

RNA Isolation and Northern Blot Analysis. Total cellular RNA was isolated as described (30), and Northern blot analyses were performed using BioTrans nylon supported membranes (ICN Biomedicals Inc., Irvine, CA) as described by the manufacturer. The mouse $VPF/VEGF_{164}$ cDNA probe was the 980-bp fragment described previously (30), and a G3PDH cDNA probe (24) was used in all experiments to control for RNA loading, blotting, and hybridization. cDNA fragments were radiolabeled with a random-primed synthesis kit (Multi-Prime; Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Blots were washed at high stringency (0.1× SSC, 1% SDS, 65°C) and exposed on X-OMAT film (Eastman Kodak Co., Rochester, NY).

Measurement of Mediators. For measurements of serotonin (5-hydroxytryptamine [5-HT]) release, BMCMCs or Cl.MC/C57.1 cells were incubated with ascites IgE at 10 μg/ml and [^{3}H]5-HT (New England Nuclear, Boston, MA) at 2 μCi/ml for 2 h at 37°C, washed, and stimulated at 37°C for 10 min, or, as specified, for 10, 60, or 120 min with or without inhibitors. Histamine release from HMCs was measured in supernatant and cell fractions, in an aliquot taken 1 h after challenge, using a radioimmunoassay kit (Immunotech, Inc., Westbrook, ME). The percentage of histamine or 5-HT released was calculated according to the following formula: % histamine or 5-HT release = [(histamine or [^{3}H]5-HT in the supernatant)/(histamine or [^{3}H]5-HT in the supernatant + histamine or [^{3}H]5-HT in the pellet)] × 100. VPF/VEGF was measured using ELISA kits for mouse or human VPF/VEGF (R&D Systems, Inc., Minneapolis, MN).

In Situ Hybridization. Cytospins of freshly purified mouse PMCs were fixed in 4% paraformaldehyde for 15 min and then stored in 70% ethanol before processing for in situ hybridization (ISH) with ^{35}S-labeled rat VPF/VEGF antisense and sense riboprobes (31) exactly as described previously (32). The antisense riboprobe hybridizes specifically with a region of VPF/VEGF mRNA common to all known VPF/VEGF splicing variants (31). The slides were examined by a single observer (L.F. Brown) who was unaware of the identity of the individual specimens, and at least 400 cells in each slide were scored as having ≥10, 5–9, or <5 associated silver grains. One or two different slides for each

condition were examined in two different experiments which gave very similar results; accordingly, the results were pooled for statistical analysis.

Immunocytochemistry. Cytospins of purified mouse PMCs were fixed in 4% paraformaldehyde in PBS for 15 min and washed three times in PBS. The cells were permeabilized with 70% ethanol at 4°C for 1 h and then rehydrated in PBS for 1 h. Slides were blocked in 1% BSA in PBS for 2 h at room temperature and then incubated at 4°C for 18 h in 1% BSA in PBS containing ~0.1 mg/ml of either a control purified rabbit IgG or an affinity-purified rabbit anti–mouse NH_2-terminal VPF/VEGF antibody (33). The slides were washed three times for 15 min in PBS, incubated with an FITC-goat anti–rabbit IgG conjugate (Sigma Chemical Co.) at 1:1,000 dilution for 1 h, then washed five times for 10 min in PBS. The slides were mounted in aqueous mounting medium (Gel/Mount; Biomeda, Foster City, CA) and visualized with a confocal microscope (model MRC1024; Bio-Rad Laboratories, Hercules, CA). Identical power and gain levels were used to capture each image for comparison. Positive cells were enumerated in five different fields in each specimen (n = 28–40 cells/field) by a single observer (K.P. Claffey) who was unaware of the identity of the individual specimens.

Statistics. Unless otherwise specified, all data are expressed as mean ± SEM, and all differences between values were compared using the two-tailed Student's t test.

Results and Discussion

Mouse Mast Cells Exhibit Increased Levels of VPF/VEGF mRNA after Stimulation via FcεRI or with PMA. We used reverse transcription PCR to search for expression of VPF/VEGF mRNA in Cl.MC/C57.1 cloned mouse mast cells of BALB/c origin (25, 26), mouse BMCMCs (>95% pure), mouse PMCs (95–99% pure), and rat PMCs (>99% pure); we used the two primer sequences reported by Cullinan-Bove and Koos (34) after two mismatches in the 5′ primer had been corrected to obtain a full match to the rat and mouse VPF/VEGF sequences (35). These primers can amplify three forms of VPF/VEGF (VPF/$VEGF_{120}$, VPF/$VEGF_{164}$, and VPF/$VEGF_{188\ and/or\ 206}$; references 15 and 16), and the resulting PCR products exhibited different sizes for each of the three forms. All of the rodent mast cell populations tested constitutively expressed mRNA for VPF/$VEGF_{120}$ and VPF/$VEGF_{164}$ and, in tests of both mouse BMCMCs and rat PMCs, the signals were enhanced in mRNA extracted from cells 4 h after they had been stimulated by FcεRI cross-linking (data not shown). Using agarose gel electrophoresis and ethidium bromide staining, we also detected, in some of these specimens, very weak signals which probably represented expression of VPF/$VEGF_{188\ and/or\ 206}$. Northern analysis of mRNA isolated from Cl.MC/C57.1 cells using a mouse VPF/$VEGF_{164}$ cDNA probe (30) showed a low level of constitutive expression of VPF/VEGF mRNA in unstimulated cells and increased levels during the first 2 h after stimulation (Fig. 1). However, levels of VPF/VEGF mRNA were much higher 2 h after stimulation with PMA (50 ng/ml) than after sensitization with IgE (10 μg/ml for 30 min) and 2 h of stimulation with antigen (DNP-HSA at 50 ng/ml); in either case, mRNA levels declined markedly by 8–24 h after stimulation (Fig. 1).

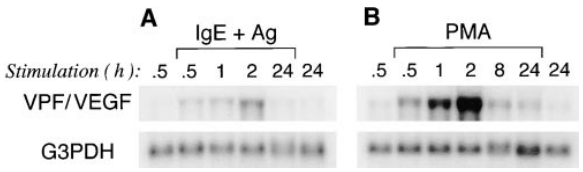

Figure 1. Northern blot analysis of total RNA from C1.MC/C57.1 mast cells stimulated through FcεRI (*A*) or with PMA (*B*). C1.MC/C57.1 cells were sensitized with IgE (10 μg IgE/ml) for 30 min, washed, and then stimulated with either 50 ng/ml DNP-HSA or 50 ng/ml PMA. Control cells (at 0.5 and 24 h) were challenged with medium (in *A*) or vehicle (0.005% DMSO, in *B*). Blotted total RNA from cells harvested at various times after stimulation was probed for VPF/VEGF and G3PDH (to demonstrate equal RNA loading).

Mouse PMCs Express VPF/VEGF mRNA and Protein. To assess whether freshly isolated mouse mast cells can express VPF/VEGF, we performed two experiments in which PMCs were purified from groups of 60 BALB/c mice, and then the PMCs (purities of 95 and 99%) were stimulated with PMA (50 μg/ml) for 2 h at 37°C. In both experiments, ISH analysis of cytocentrifuge preparations showed that PMA treatment increased expression of VPF/VEGF mRNA in mouse PMCs (compare Fig. 2 *A*, showing PMA-treated cells hybridized to the VPF/VEGF antisense riboprobe, with either Fig. 2 *B*, vehicle-treated cells hybridized to the VPF/VEGF antisense riboprobe, or Fig. 2 *C*, PMA-treated cells hybridized to the VPF/VEGF sense probe). Silver grain count analysis confirmed that PMA treatment enhanced VPF/VEGF mRNA expression in mouse PMCs. Both experiments gave very similar results, which were pooled for presentation in Fig. 2 *D*. PMA stimulation resulted in percentages of cells that gave strongly positive (≥10 grains/cell) or moderately positive (5–9 grains/cell) signals for VPF/VEGF that were threefold (6 versus 2%) or more than threefold (18 versus 5%), respectively, those for vehicle-treated control cells. Similar results were also obtained in an analysis of mouse PMCs that had been incubated with PMA (50 ng/ml) for 2 h after overnight culture at 37°C in standard medium (data not shown).

Aliquots of the same mouse PMC preparations were analyzed in cytospins by immunocytochemistry. As shown in Fig. 3, VPF/VEGF immunoreactivity was detected in some of the mouse PMCs that had been incubated with medium (vehicle) alone (14.2 ± 7.3% [mean ± SD] positive cells with the anti-VPF/VEGF antibody, Fig. 3 *C*, versus 3.9 ± 1.1% positive cells with the control antibody, Fig. 3 *A*; $P < 0.035$). However, enhanced VPF/VEGF immunoreactivity ($P < 0.043$ versus results for the vehicle-incubated cells stained with the anti-VPF/VEGF antibody) was observed in the cells that had been stimulated with PMA for 2 h (26.5 ± 8.4% positive cells with the anti-VPF/VEGF antibody, Fig. 3 *D*, versus 4.7 ± 1.4% positive cells with the control antibody, Fig. 3 *B*; $P < 0.005$). Giemsa-stained cytospin preparations (Fig. 3, *E* and *F*) show the purity of the mouse PMC populations analyzed.

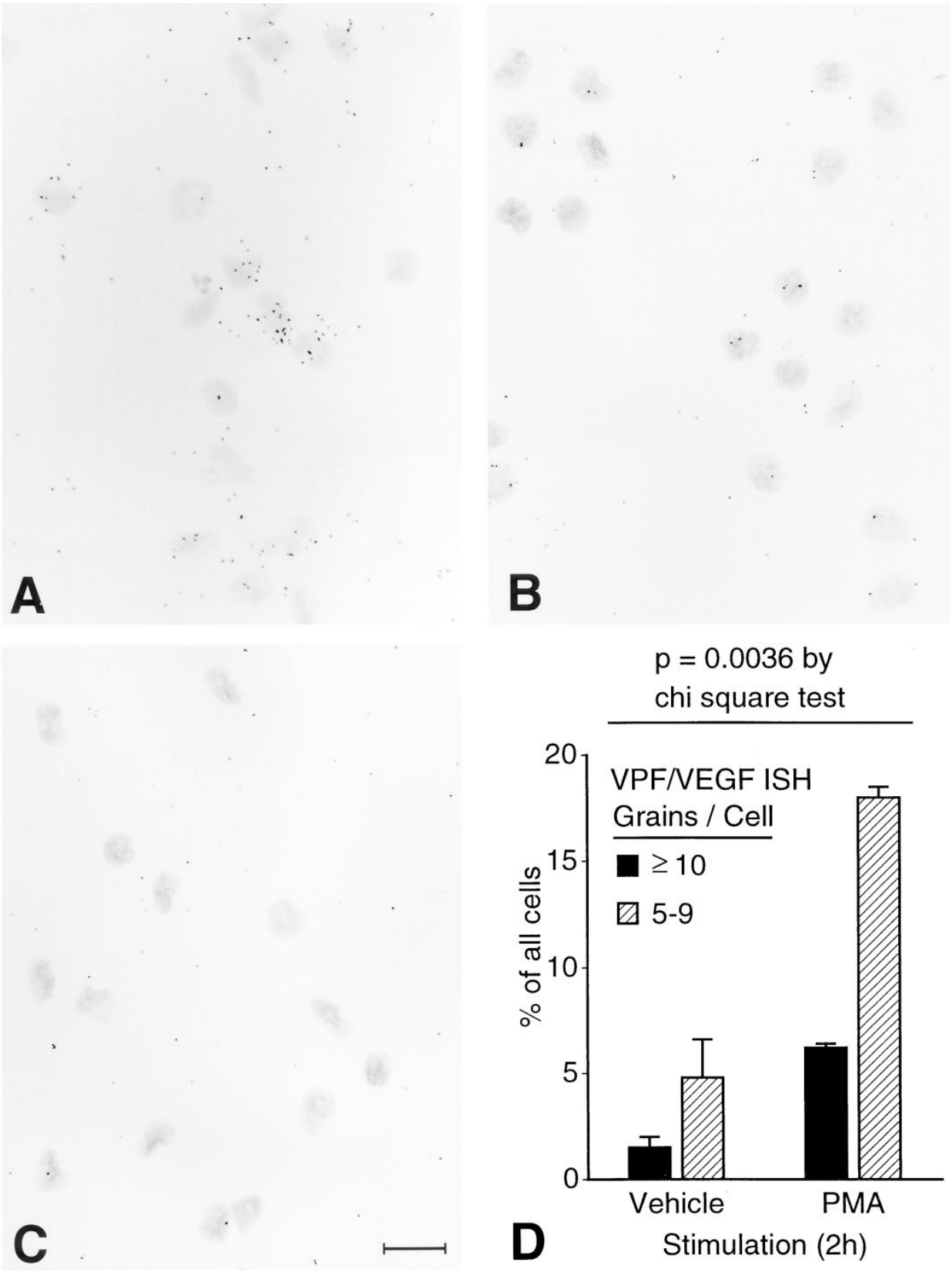

Figure 2. ISH studies of freshly isolated mouse PMCs. Expression of VPF/VEGF mRNA is higher in mast cells that had been treated with PMA (50 ng/ml) for 2 h before hybridization to an ^{35}S-labeled antisense riboprobe (*A*) than in vehicle-treated (control) mast cells that had been hybridized to the same antisense riboprobe (*B*). No specific signal is seen in PMA-stimulated mast cells hybridized to the ^{35}S-labeled sense (control) riboprobe (*C*). Bar = 16 μm. (*D*) Graphic depiction of VPF/VEGF expression by PMA- versus vehicle-treated mast cells.

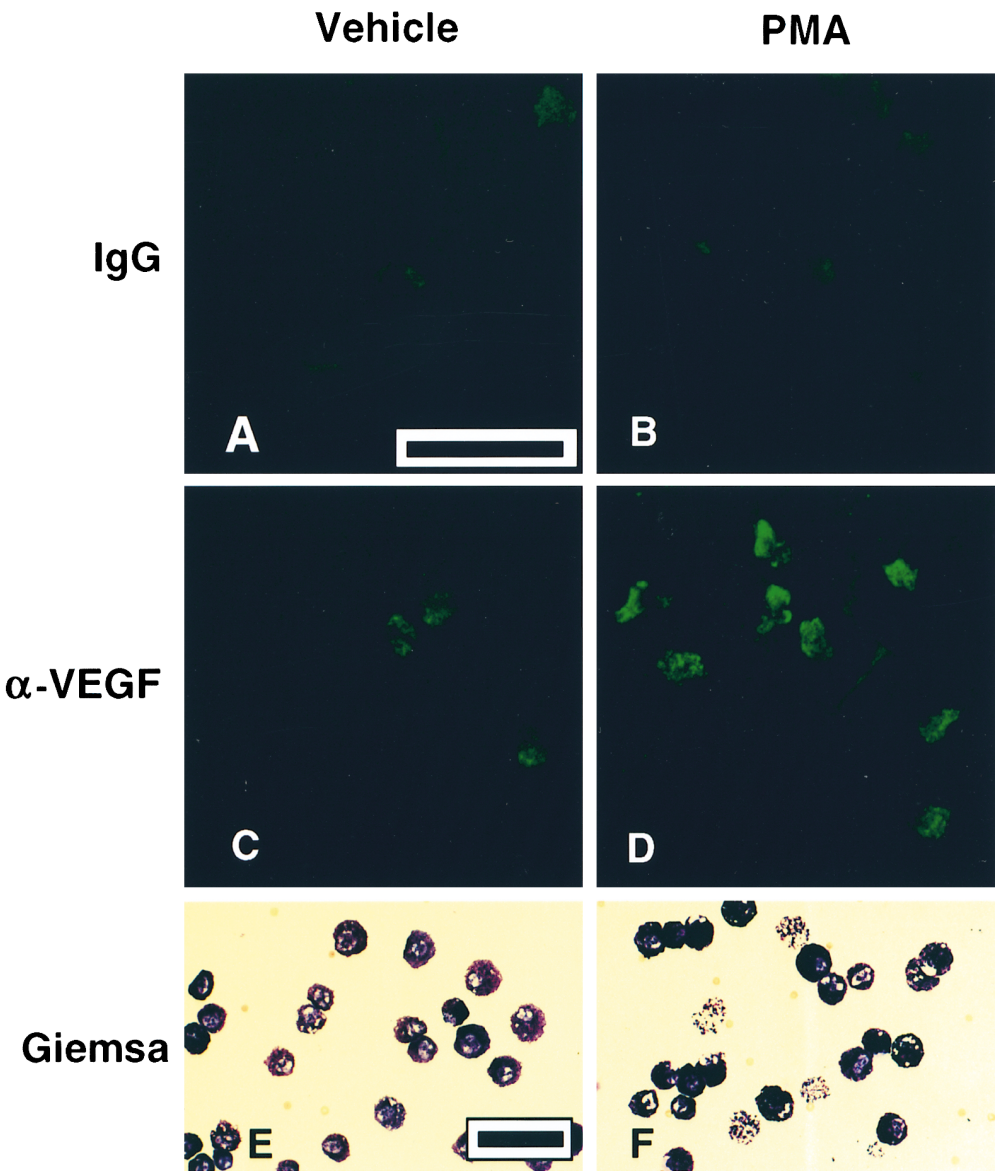

Figure 3. Immunofluorescent detection of VPF/VEGF immunoreactivity in a highly purified (99% pure) population of freshly isolated mouse PMCs. (*A* and *B*) Little or no staining of PMCs that had been incubated for 2 h with vehicle alone (*A*) or PMA (50 ng/ml; *B*) before incubation with a control rabbit IgG preparation (*IgG*). (*C* and *D*) VPF/VEGF immunoreactivity was detected with a rabbit anti-VPF/VEGF (*α-VEGF*) antibody in some PMCs that had been incubated for 2 h with either vehicle alone (*C*) or PMA (50 ng/ml; *D*). Black bar in *A* (for *A–D*) = 50 μm. (*E* and *F*) Giemsa-stained cytospin preparations of the same vehicle- (*E*) or PMA- (*F*) stimulated purified PMCs that were used for immunofluorescent detection of immunoreactivity (*A–D*). Black bar in *E* (for *E* and *F*) = 50 μm.

Mouse Mast Cells Release VPF/VEGF in Response to IgE and Specific Antigen, PMA, A23187, or SCF, and Antigen-dependent Release Is Markedly Enhanced after IgE-dependent Up-regulation of Surface Expression of FcεRI. Because of the large numbers of mice required to generate highly purified preparations of PMCs (60 mice yield ~3–6 × 10^5 mast cells of 95–99% purity) and the sensitivity of the VPF/VEGF ELISA assay, we used in vitro–derived mast cells for studies of VPF/VEGF secretion. To examine whether mouse mast cells can secrete VPF/VEGF protein in response to various types of stimuli, we tested BMCMCs which either had or had not undergone upregulation of FcεRI surface expression by a 4-d preincubation with IgE at 5.0 μg/ml (22). Mouse BMCMCs that were not preincubated for 4 d with IgE, but were sensitized with IgE for only 2 h before antigen challenge, gave ~10% specific release of 5-HT (Fig. 4 *B*) in response to DNP-HSA and, by 6 h after stimulation with antigen at 10 or 50 ng/ml, released a small but statistically significant amount of VPF/VEGF (~2.5 times levels in control cells that were not stimulated with antigen; $P < 0.005$, Fig. 4 *A*). However, in accord with our previous findings (22), preincubation of BMCMCs with 5 μg IgE/ml for 4 d before further sensitization and antigen stimulation resulted in an approximately twofold increase in specific release of 5-HT compared with cells not preincubated with IgE for 4 d (Fig. 4 *B*). The enhancement of VPF/VEGF secretion was even more striking (at 100 ng antigen/ml, to approximately nine times the levels produced by cells that had not been preincubated with IgE for 4 d; Fig. 4 *A*).

The markedly enhanced IgE-dependent release of VPF/VEGF from BMCMCs after a 4-d preincubation with IgE is in accord with our finding that such BMCMCs also exhibited greatly enhanced IgE- and antigen-dependent secretion of IL-4 and IL-6 (22). However, stimuli other than IgE and antigen gave very different patterns of 5-HT and

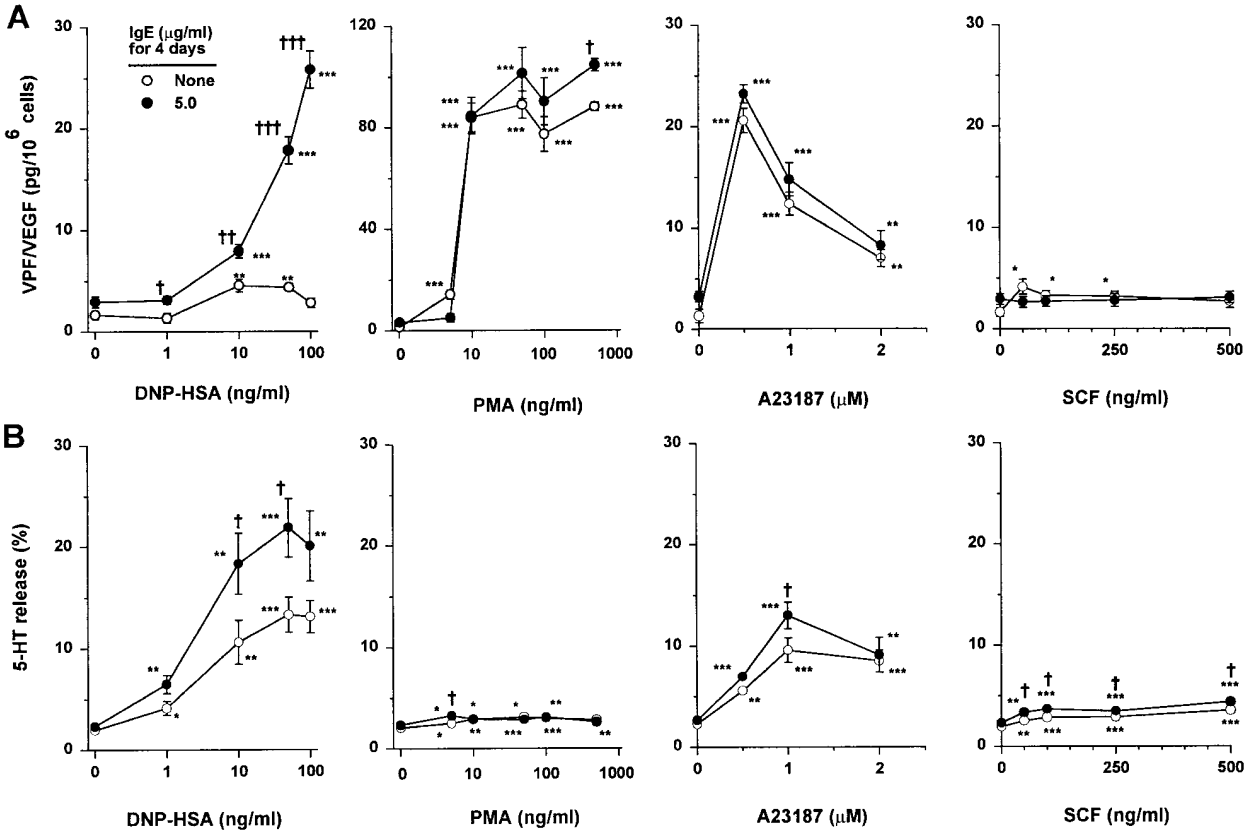

Figure 4. Release of (*A*) VPF/VEGF (in 6 h) and (*B*) serotonin (5-HT, in 10 min) from BMCMCs that exhibit different levels of surface expression of FcεRI, after challenge with various concentrations of either specific antigen (DNP-HSA), the protein kinase C activator PMA, the calcium ionophore A23187, or SCF. BALB/c BMCMCs were cultured without IgE or with IgE at 5.0 μg/ml for 4 d before passive sensitization and antigen challenge or challenge with A23187, PMA, or SCF for 6 h. 0 in the PMA and A23187 experiments refers to cells incubated without PMA or A23187, respectively, but in the highest concentration of vehicle used for these studies (DMSO at 0.5%). All values are mean ± SEM ($n = 8$/point) of data pooled from two experiments with different batches of BMCMCs (each $n = 4$/point) that gave very similar results. $^{*}P < 0.05$, $^{**}P < 0.005$, or $^{***}P < 0.0001$ versus corresponding control values for unstimulated or vehicle-treated cells. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.005$, or $^{\dagger\dagger\dagger}P < 0.0001$ versus corresponding values for cells cultured without IgE for 4 d. Note the different VPF/VEGF scale for PMA stimulation.

VPF/VEGF release by BMCMCs, and these responses were not substantially affected by preincubation of the cells in IgE for 4 d. PMA induced levels of VPF/VEGF secretion that were approximately four times those of the highest levels of FcεRI-dependent release (Fig. 4 *A*), but this was associated with minimal release of 5-HT (Fig. 4 *B*). The calcium ionophore A23187 induced release of both 5-HT and VPF/VEGF, whereas the c-kit ligand SCF induced little or no VPF/VEGF release in 6 h, and a minimal amount of 5-HT release (Fig. 4, *A* and *B*).

The kinetics of VPF/VEGF release from BMCMCs that were stimulated with fixed concentrations of DNP-HSA (50 ng/ml), PMA (50 ng/ml), A23187 (1 μM), or SCF (500 ng/ml) are shown in Fig. 5. We controlled the experimental conditions to exclude hypoxia (which can enhance VPF/VEGF production in other cell types [36]) by using a maximum final concentration of 2×10^6 cells/ml, by stimulating the cells in multiwell plates filled to <40%, and by mixing the cell suspension carefully every 2 h.

Unstimulated BMCMCs constitutively released a small amount of VPF/VEGF, a process that was unaffected by a 4-d pretreatment with IgE. This constitutive release rate, as determined by linear regression analysis, was 0.18 ± 0.08 pg/10^6 cells · h^{-1} (mean ± SD; $n = 10$). Cells that had not been preincubated with IgE for 4 d released ~2.3 pg VPF/VEGF/10^6 cells in response to 50 ng/ml of DNP-HSA, primarily within the first 3 h after stimulation, whereas BMCMCs that had been pretreated with IgE at 5 μg/ml for 4 d released much larger amounts of VPF/VEGF, and release persisted for at least 24 h. Release of VPF/VEGF in response to A23187 also reached near maximal levels at 3 h. PMA stimulation resulted in the rapid induction of VPF/VEGF, which reached a plateau after ~12 h, perhaps reflecting PMA-induced degradation of certain protein kinase C isoforms (37).

rrSCF (at 500 ng/ml) significantly enhanced release of VPF/VEGF only when the BMCMCs were exposed to this growth factor for >12 h (Fig. 5). By contrast, we reported recently that rrSCF can induce such BMCMCs to release IL-6 within 30 min of stimulation (27). Because IL-6 can induce VPF/VEGF expression in several cell lines (38), we tested whether IL-6 can stimulate BMCMCs to

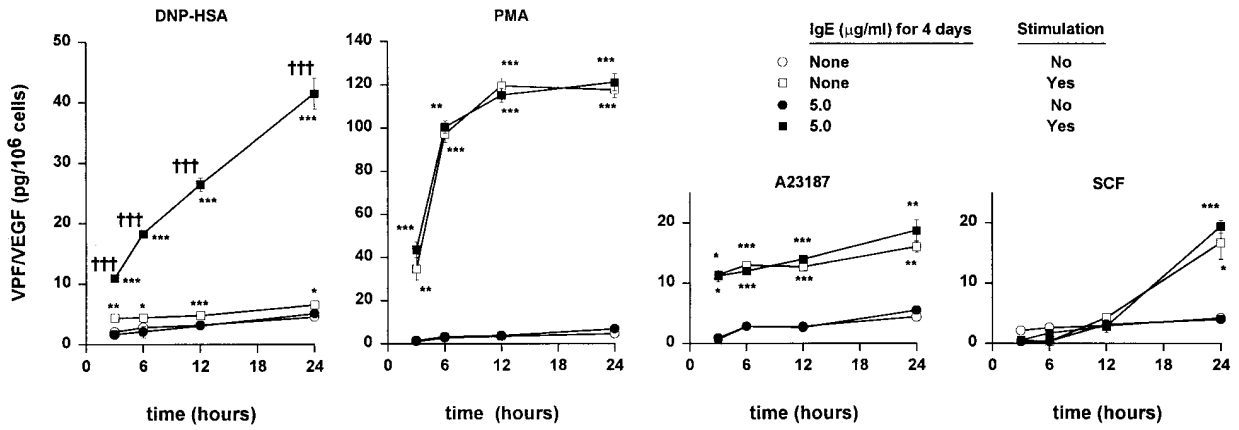

Figure 5. Kinetics of the release of VPF/VEGF from BALB/c BMCMCs that exhibit different levels of surface expression of FcεRI, after challenge with either specific antigen (DNP-HSA, at 50 ng/ml), PMA (50 ng/ml), A23187 (1 μM), or SCF (500 ng/ml). BMCMCs were cultured without IgE or with IgE at 5.0 μg/ml for 4 d before passive sensitization and antigen challenge or challenge with A23187, PMA, or SCF. Cells used as unstimulated controls for DNP-HSA– or SCF-stimulated cells were maintained in medium alone. PMA- or A23187-stimulated cells were compared with cells maintained in medium containing vehicle (DMSO at 0.1%). All values are mean ± SEM ($n = 8$/point) of data pooled from two experiments with different batches of BMCMCs (each $n = 4$/point) that gave very similar results. $^{*}P < 0.05$, $^{**}P < 0.005$, or $^{***}P < 0.0001$ versus corresponding control values for unstimulated or vehicle-treated cells. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.005$, or $^{\dagger\dagger\dagger}P < 0.0001$ versus corresponding values for cells cultured without IgE for 4 d. Note the different VPF/VEGF scale for PMA.

release VPF/VEGF. BMCMCs preincubated with either no IgE or 5 μg IgE/ml for 4 d were stimulated with recombinant mouse IL-6 (R&D Systems, Inc.) in concentrations between 10 and 500 ng/ml for 12 h, but no release of VPF/VEGF above control levels could be detected (data not shown). Similarly, exposure of BMCMCs to active recombinant TGF-β_1 (2 or 10 ng/ml; R&D Systems, Inc.), another potentially mast cell–derived cytokine (12) that can stimulate VPF/VEGF production in certain cell types (39), for 12 h did not induce detectable release of VPF/VEGF (data not shown).

FcεRI-dependent Stimulation of BMCMCs or Cl.MC/C57.1 Mast Cells Induces the Release of Both Preformed and Transcriptionally Induced VPF/VEGF. We found that the majority of VPF/VEGF released in response to stimulation with A23187, or with IgE and antigen in BMCMCs that had not been pretreated with IgE for 4 d, occurred within the first 3 h after stimulation (Fig. 5). Therefore, we assessed whether a fraction of the VPF/VEGF secreted by these cells, like mast cell–derived TNF-α (11), might be released from a preformed pool. Cl.MC/C57.1 cells and BMCMCs were sensitized for 2 h with 10 μg/ml of mouse IgE, washed, then preincubated with or without the inhibitor of intracellular protein transport, Brefeldin A (5 μg/ml; Sigma Chemical Co. [40]), or the transcription inhibitor Actinomycin D (Act D, at 10 μg/ml; Sigma Chemical Co. [11]) for 5 min. Cells were then challenged for 10, 60, or 120 min (with our without the inhibitor) with 50 ng/ml DNP-HSA or with medium alone (Fig. 6). Neither inhibitor induced a significant reduction in cell viability during the experiments, as assessed by trypan blue exclusion (22).

Stimulation with specific antigen rapidly induced degranulation of BMCMCs, as reflected in increased levels of 5-HT in the cells' supernatant and a parallel reduction in the cell-associated content of 5-HT, at 10 or 60 min after stimulation, to levels ~33 or 44% lower than those in the unstimulated cells at baseline (Fig. 6 *A*). And, in accord with previous findings with rat basophilic leukemia cells (37) or BMCMCs (11), neither Brefeldin A nor Act D had a substantial effect on 5-HT release (Fig. 6 *A*).

The VPF/VEGF content of these cells also dropped significantly by 10 or 60 min after antigen challenge (by ~25 or 36% versus initial values for unstimulated cells), with a corresponding significant increase in the levels of VPF/VEGF in the cells' supernatant (Fig. 6 *B*). The changes at 10 min were only minimally affected by Brefeldin A, whereas Act D had little or no effect on FcεRI-dependent secretion of VPF/VEGF at either 10 or 60 min (Fig. 6 *B*). By contrast, either Brefeldin A or Act D significantly reduced the levels of VPF/VEGF secreted by 2 h after stimulation, to levels which approximated those released in the first 10 min after activation (Fig. 6 *B*). Brefeldin A treatment also significantly enhanced levels of cell-associated VPF/VEGF in IgE- and antigen-activated BMCMCs by 2 h after stimulation, whereas Act D treatment had the opposite effect (Fig. 6 *B*).

The simplest explanation for these findings is that the VPF/VEGF secreted by BMCMCs at early intervals after FcεRI cross-linking includes a fraction that is derived from preformed stores. However, in accord with the findings shown in Fig. 1, the data in Fig. 6 also indicate that the VPF/VEGF released from the cells at later intervals after FcεRI-dependent stimulation reflects the de novo synthesis of VPF/VEGF through gene transcription, and the release of the protein by a Brefeldin A–sensitive mechanism.

An identically designed experiment with Cl.MC/C57.1 cells gave results that were qualitatively similar to those obtained in our two experiments with BMCMCs (data not

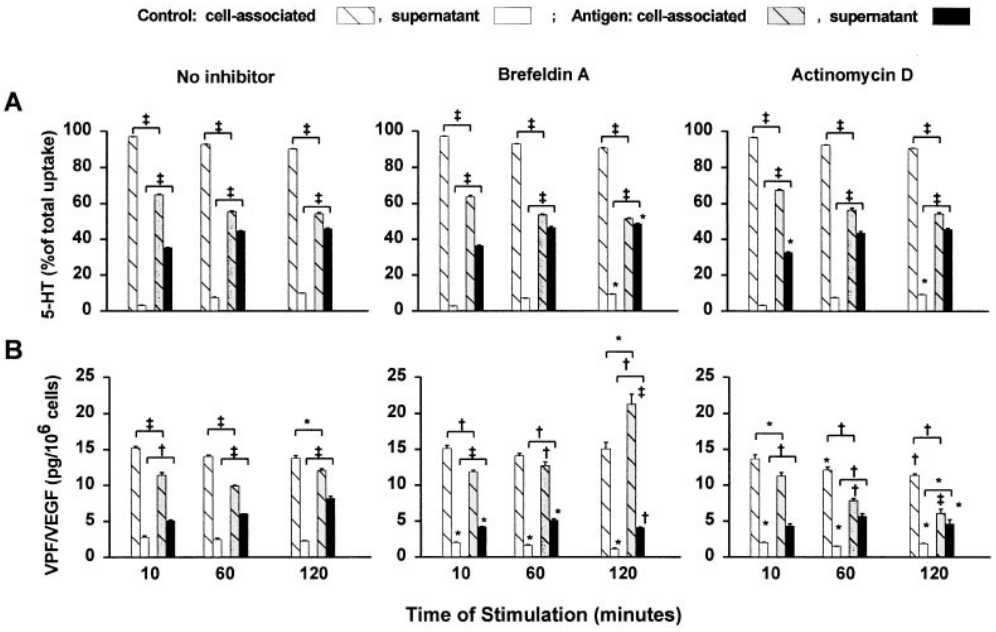

Figure 6. Kinetics of release of 5-HT (*A*) and VPF/VEGF (*B*) from BMCMCs stimulated through the FcεRI. Mast cells were sensitized with IgE (10 μg IgE/ml) for 2 h and then preincubated as indicated for 5 min at 37°C with either no inhibitor, the inhibitor of intracellular protein transport Brefeldin A (5 μg/ml), or the transcription inhibitor Act D (10 μg/ml). Cells were then challenged for various time periods with DNP-HSA (*Antigen*) or medium (*Control*). Data for VPF/VEGF are mean ± SEM (n = 3/point); data for [^{3}H]5-HT are mean ± SEM (n = 4/point). $^*P < 0.05$, $^\dagger P < 0.005$, $^\ddagger P < 0.0001$ versus values for the same time point in cells incubated without the inhibitor, or, as indicated (*brackets*), versus corresponding values for control cells that were not challenged with DNP-HSA. A second identical experiment with BMCMCs gave very similar results.

shown). However, Cl.MC/C57.1 mast cells, which are tumorigenic in vivo (26), constitutively released VPF/VEGF at a much higher rate than did BMCMCs. By linear regression analysis, the rate of constitutive release of VPF/VEGF by Cl.MC/C57.1 cells was 3.43 ± 0.49 pg/10^6 cells · h^{-1} (mean ± SD; n = 3), ~19-fold that for BMCMCs. This constitutive secretion of VPF/VEGF, but not the cells' constitutive release of 5-HT, was significantly reduced by Brefeldin A (~66% reduction at 2 h, $P < 0.005$ versus values for control cells without Brefeldin A). These findings support the conclusion that VPF/VEGF can be constitutively translocated by mast cells to the extracellular space, apparently without the involvement of the cytoplasmic granules.

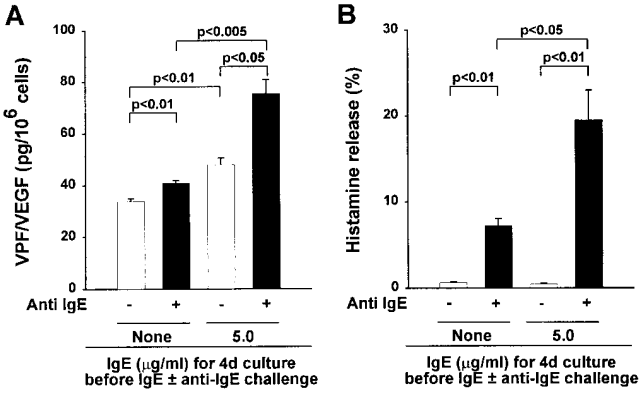

Figure 7. IgE-induced enhancement of FcεRI expression in human mast cells increases the ability of these cells to release VPF/VEGF (over a 16-h period; *A*) and histamine (over a 1-h period; *B*) in response to FcεRI cross-linking. HMCs (12 wk of culture, 85% mast cell purity) were cultured without or with human myeloma IgE at 5 μg/ml for 4 d before further passive sensitization with IgE and challenge with goat anti–human IgE antibody. The data shown (mean ± SEM; n = 3/bar) are representative of the results obtained in three separate experiments using mast cells derived from three different cord blood donors.

HMCs Produce and Secrete VPF/VEGF. We showed recently that incubation with human myeloma IgE can upregulate HMC FcεRI expression and that this results in enhanced anti-IgE–dependent mast cell release of histamine and macrophage inflammatory protein 1α (23). HMCs were incubated with either no IgE or with 5 μg/ml of human myeloma IgE for 4 d before further passive sensitization with IgE and challenge with goat anti–human IgE antibody for up to 16 h. Fig. 7 shows the results from one of three similar experiments. We found that HMCs (which, to maintain their viability, must be maintained continuously in rhSCF-containing medium) secrete VPF/VEGF in the absence of FcεRI-dependent activation. However, significantly increased amounts of VPF/VEGF (Fig. 7 *A*) and histamine (Fig. 7 *B*) were released after cell stimulation through FcεRI cross-linking. Pretreatment of HMCs with IgE for 4 d significantly enhanced both spontaneous (by ~20%) and anti-IgE–induced (by ~57%) release of VPF/VEGF (Fig. 7 *A*), as well as anti-IgE–induced release of histamine (Fig. 7 *B*), compared with results for cells with no 4-d pretreatment with IgE. In a second experiment with a different batch of HMCs (98% purity), IgE pretreatment enhanced spontaneous release of VPF/VEGF by 38% and the anti-IgE–induced release by 51% at 4 h. In both of these experiments, as well as in a third experiment with >99% pure HMCs, release of VPF/VEGF was significantly enhanced in anti-IgE–treated as opposed to unstimulated cells (by 42, 6, and 124% for HMCs without a 4-d prein-

cubation with IgE, and by 85, 16, and 171%, respectively, with a 4-d preincubation with IgE).

These experiments reveal three similarities in the secretion of VPF/VEGF by HMCs and BMCMCs: (*a*) the cells release some VPF/VEGF constitutively, under usual conditions of cell culture; (*b*) the cells exhibit enhanced release of VPF/VEGF in response to immunologic stimulation through the FcεRI; and (*c*) IgE-induced enhancement of FcεRI expression can enhance the cells' ability to release VPF/VEGF in response to FcεRI-dependent stimulation.

Conclusions. VPF/VEGF is appropriately regarded as an important regulator of neovascularization (15–17); however, it is also one of the most potent vascular permeability factors yet described, with a molar potency in guinea pig skin that is 50,000-fold that of histamine (20). Accordingly, the finding that mast cells can produce and secrete VPF/VEGF identifies a new mechanism by which mast cells might regulate angiogenesis, vascular permeability, and/or other VPF/VEGF-mediated effects on microvessels (21).

Moreover, our finding that the stimulation of mast cells via the FcεRI can enhance the cells' ability to produce and secrete VPF/VEGF demonstrates that VPF/VEGF can be expressed in an immunologically specific, FcR-dependent manner, at least by this type of effector cell. To our knowledge, this also represents the first example of a cell which can secrete VPF/VEGF upon stimulation via any member of the multichain immune recognition family of cell surface receptors. In addition, the observation that the ability of mast cells to release VPF/VEGF in response to FcεRI-dependent activation is significantly enhanced in cells that have been exposed to high concentrations of IgE indicates that VPF/VEGF may be a particularly important mast cell–derived mediator of augmented vascular permeability at sites of acute or chronic allergic inflammation, or parasite infection, in subjects with high circulating levels of IgE (22, 23, 41).

Our data indicate that the release of VPF/VEGF by mast cells can be regulated differentially from that of preformed mediators such as 5-HT or histamine. As with TNF-α (11), some of the VPF/VEGF produced by mast cells can be rapidly secreted from an apparently preformed pool, and both VPF/VEGF and biogenic amines were secreted upon activation of mast cell degranulation via the FcεRI or by the calcium ionophore, A23187. However, PMA induced an even more substantial release of VPF/VEGF than did IgE and antigen or A23187, and such PMA-induced VPF/VEGF production was associated with minimal degranulation and release of 5-HT. The latter finding indicates that it may be of interest to search for endogenous activators of mast cell protein kinase C that can induce the release of VPF/VEGF preferentially over biogenic amines and other cytoplasmic granule–associated preformed mediators.

While this manuscript was under revision, Grützkau et al. (42) reported that the HMC-1 human mast cell leukemia cell line can constitutively express and secrete three isoforms of VPF/VEGF (of 121, 165, and 189 amino acids) and that stimulation of the cells for 24 h with PMA (25 ng/ml) and A23187 (250 nM) resulted in enhanced secretion of these three VPF/VEGF isoforms and induced the expression of $VPF/VEGF_{206}$. We have also observed that HMC-1 cells can express and constitutively secrete VPF/VEGF and can release substantially increased (~2.5-fold) amounts of VPF/VEGF upon stimulation with PMA and the calcium ionophore, A23187 (data not shown). In the experiments by Grützkau et al. (42), PMA- and A23187-stimulated HMC-1 cells released ~800 pg of VPF/VEGF per 10^6 cells over a 12-h period, an amount that is much higher (by approximately ninefold) than that produced by anti-IgE–stimulated nonneoplastic human mast cells over a period of 16 h (see Fig. 7). These findings are in accord with our results with the tumorigenic Cl.MC/C57.1 mouse mast cell line of BALB/c origin, which constitutively released VPF/VEGF at a rate that was ~19-fold that of nonneoplastic BALB/c mouse BMCMCs. Taken together, these observations indicate that the amounts of VPF/VEGF that can be secreted by neoplastic mast cell lines can greatly exceed those produced by the corresponding nonneoplastic cells. Whether this property of neoplastic mast cells can also be expressed in vivo, for example, in those patients who develop vascular abnormalities in association with mastocytosis (e.g., telangiectasia macularis eruptiva perstans [43]), remains to be determined.

Clearly, further work will also be required to assess the importance of nonneoplastic mast cells as a source of VPF/VEGF in vivo. We have found that freshly isolated mouse PMCs can exhibit enhanced expression of VPF/VEGF mRNA and protein (as detected by ISH and immunocytochemistry, respectively) after stimulation with PMA for 2 h in vitro, and Grützkau et al. (42) have detected VPF/VEGF immunoreactivity by postembedding immunoelectron microscopy in some, but not all, of the cytoplasmic granules of partially purified human skin mast cells. These findings indicate that in vivo–derived, as well as in vitro–derived, nonneoplastic mast cells can express VPF/VEGF. Moreover, Grützkau et al. (42) found that other dermal cells in their preparations, including macrophages, vascular endothelial cells, and fibroblasts, were negative for VPF/VEGF protein by immunoelectron microscopy, even though each of these cells can produce VPF/VEGF under some circumstances in vitro (15, 16). Thus, the observations of Grützkau et al. (42) are consistent with our data in indicating that, upon appropriate stimulation, mast cells may be able to release some VPF/VEGF from preformed stores. However, it remains to be determined under what circumstances mast cells represent a critical source of VPF/VEGF in vivo, and, in such instances, what signals regulate the mast cell's production and secretion of the various isoforms of this mediator.

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