The Journal of Immunology

RESEARCH ARTICLE | JANUARY 15 2004

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J Immunol (2004) 172 (2): 1227–1236.
https://doi.org/10.4049/jimmunol.172.2.1227

Prostaglandin E_2 Induces Degranulation-Independent Production of Vascular Endothelial Growth Factor by Human Mast Cells[1]

Raja M. Abdel-Majid and Jean S. Marshall[2]

Mast cells accumulate in large numbers at angiogenic sites, where they have been shown to express a number of proangiogenic factors, including vascular endothelial growth factor (VEGF-A). PGE_2 is known to strongly promote angiogenesis and is found in increased levels at sites of chronic inflammation and around solid tumors. The expression pattern of VEGF and the regulation of VEGF-A by PGE_2 were examined in cord blood-derived human mast cells (CBMC). CBMC expressed mRNA for five isoforms of VEGF-A and other members of the VEGF family (VEGF-B, VEGF-C, and VEGF-D) with strong expression of the most potent secretory isoforms. PGE_2 was a very strong inducer of VEGF-$A_{121/165}$ production by CBMC and also elevated VEGF-A mRNA expression. The amount of VEGF-$A_{121/165}$ protein production induced by PGE_2 was 4-fold greater than that induced by IgE-mediated activation of CBMC. Moreover, the response to PGE_2 as well as to other cAMP-elevating agents such as forskolin and salbutamol was observed under conditions that were not associated with mast cell degranulation. CBMC expressed substantial levels of the EP_2 receptor, but not the EP_4 receptor, when examined by flow cytometry. In contrast to other reported PGE_2-mediated effects on mast cells, VEGF-$A_{121/165}$ production occurred via activation of the EP_2 receptor. These data suggest a role for human mast cells as a potent source of $VEGF_{121/165}$ in the absence of degranulation, and may provide new opportunities to regulate angiogenesis at mast cell-rich sites. ***The Journal of Immunology*****, 2004, 172: 1227–1236.**

Mast cells are closely associated with blood vessels and are found in large numbers at sites of angiogenesis (1, 2). Such mast cell-rich sites include the periphery of solid tumors (3, 4), areas of wound healing, and the synovium in rheumatoid arthritis (RA)[3] (5, 6). Increased angiogenesis has also been reported in the asthmatic airway (7, 8), where mast cells play an important role in the disease. A number of in vitro and in vivo studies have demonstrated that mast cells can contribute substantially to the angiogenic process (1, 2, 9–11). More recently, it has been shown that mast cells play an important role in the development of joint inflammation (12), and mast cell-deficient mice were shown to be resistant to the development of inflammatory arthritis. Much of the mast cell's proangiogenic activity is thought to result from mast cell production of proangiogenic products, including matrix metalloproteinase 9 (9), fibroblast growth factor-2 (13), IL-8 (14), and the most potent proangiogenic mediator vascular endothelial growth factor (VEGF) (15). In some tumor sites, it has been demonstrated that mast cells are the major cell type containing immunoreactive VEGF (3, 16). At inflammatory sites and areas of wound healing, mast cells are thought to also contribute to other aspects of tissue remodeling.

The VEGF family consists of several members, including placental growth factor, VEGF-A, VEGF-B, VEGF-C, and VEGF-D (17), with VEGF-A being one of the most potent proangiogenic factors in vivo (18, 19). Some of these family members have differentially spliced forms with a range of abilities to enhance angiogenesis and to bind heparin and other extracellular matrix components. For example, human VEGF-A has six isoforms: 121, 145 165, 183, 189, and 204 (20). Of these, $VEGFA_{121}$ and $VEGFA_{165}$ have been shown to be the most potent proangiogenic isoforms (21). Other isoforms with greater ability to bind to extracellular matrix may provide long-term sources of VEGF to sustain vessel development. Most tumor cells have been demonstrated to exhibit dysregulated expression of VEGF family members. Previous in vivo (22) and in vitro (23) studies have demonstrated that VEGF-A stimulated mast cell migration. In vitro, VEGF-A, in picomolar concentrations, was very potent in stimulating mouse mast cell migration; however, it is not known yet whether VEGF-A is a chemoattractant to human mast cells. Examination of VEGF expression by primary human cells that are found at angiogenic sites is of particular importance to understanding the mechanisms that regulate angiogenesis.

Previous studies of primary human and mouse mast cells have suggested that IgE-mediated mast cell activation is an effective inducer of VEGF-A production (15). These results are consistent with observations that some VEGF-A is localized to mast cell granules (15). It is possible that mast cells will also synthesize and release VEGF-A by a degranulation-independent mechanism, as is the case with other mediators (24–26). In nongranulated cell types, such as macrophages, cAMP-elevating agents, inflammatory cytokines, estradiol, and prostanoids such as PGE_2 have been shown to elevate VEGF-A production (27–29).

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Received for publication February 24, 2003. Accepted for publication November 7, 2003.

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[1] A portion of this work was presented in abstract form by R.M.A.-M. and J.S.M. at the Canadian Society for Immunology annual meeting held in Collingwood, Ontario, Canada, on April 5–8, 2002.

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[3] Abbreviations used in this paper: RA, rheumatoid arthritis; BMMC, mouse bone marrow-derived cultured mast cell; CBMC, cord blood-derived human mast cell; EP, PGE_2 receptor; HMC-1, human mast cell line Butterfield; SCF, stem cell factor; VEGF, vascular endothelial growth factor.

0022-1767/04/$02.00

Prostanoid regulation of VEGF-A production is of particular interest, in view of a number of reports that cyclooxygenase inhibitors can inhibit tumor growth in some systems (30) in which elevated PGE_2 levels were observed (31). It has also been demonstrated that local PGE_2 levels are elevated in several chronic inflammatory conditions in which angiogenesis is an important feature, including allergic asthma (32), and in the synovial fluid of individuals with RA (33). PGE_2 exerts its effects through activation of four subtype receptors, termed EP_1, EP_2, EP_3, and EP_4 (34). Coupling to EP_1 receptor elevates intracellular Ca^{2+} levels, while activation of the EP_2 and EP_4 receptors will increase cAMP levels through activation of the adenylyl cyclase enzyme. Activation of the EP_3 receptor can increase or decrease cAMP levels depending on the splice variant expressed by the cell. Our laboratory has demonstrated previously that bone marrow-derived cultured mouse mast cells (BMMC) express transcripts for the EP_1, EP_3, and EP_4 subtype receptors, but not the EP_2 subtype receptor (25); however, the human mast cell expression and function of receptors have not been previously examined in detail. Our previous work, in the murine system, suggested that mast cell's response to PGE_2 stimulation is dependent on the activation of the EP_1/EP_3 receptor system. More recently, it has been shown that selective induction of IL-6 by mouse mast cell is dependent on the EP_3 subtype receptor (26). The above studies indicate that murine mast cells use the Ca^{2+} signaling pathway, rather than a cAMP-descendant signaling pathway, in mediating their response to PGE_2 activation.

In the current study, the ability of primary human mast cells to express mRNA for a number of VEGF family members was examined. We also evaluated the ability to increase mast cell secretion of the most proangiogenic isoforms of VEGF-A by a number of potential VEGF-inducing agents that do not induce mast cell degranulation. Our results indicate that PGE_2 and other cAMP-elevating agents are very potent inducers of VEGF-$A_{121/165}$ by human mast cells. This enhanced VEGF-$A_{121/165}$ production was observed under conditions that are not associated with mast cell degranulation. The mechanism of VEGF-$A_{121/165}$ induction in response to PGE_2 activation was also investigated.

Materials and Methods

Mast cells

After informed consent of the donors, highly purified cord blood-derived mast cells (CBMC) were obtained by long-term culture of cord blood progenitor cells, as previously described (35). Briefly, cord blood mononuclear cells were cultured for 6–8 wk at 37°C in 5% CO_2 in RPMI 1640 medium supplemented with L-glutamine, 1 μM HEPES, 0.1 U/ml penicillin, 100 μg/ml streptomycin, and 20% FCS (all from Invitrogen, Burlington, Ontario, Canada); 100 ng/ml human recombinant stem cell factor (SCF; PeproTech, Rocky Hill, NJ); 3×10^{-7} M PGE_2; and 20% CCL-204 (American Type Culture Collection, Manassas, VA) normal human skin fibroblast supernatant as a source of IL-6. The medium was renewed every 7 days. The purity of mast cells was assessed by toluidine blue staining (pH 1.0) of cytocentrifuge preparations. Mature mast cells were identified by their morphological features and the presence of metachromatic granules. Only those preparations containing >96% mast cells were used in our studies.

The human mast cell line HMC-1 (36) and its subclone HMC-1 5C6 (37) were grown in Iscove's medium supplemented with 0.1 U/ml penicillin, 100 μg/ml streptomycin, 10% FCS, and 1 μM HEPES. The human basophilic mast cell line, KU812, was maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 μM HEPES, 10% FCS, 0.1 U/ml penicillin, and 100 μg/ml streptomycin.

RT-PCR of human mast cell RNA

Total RNA was extracted from HMC-1, HMC-1 5C6, KU812, CBMC (purity 98–100%), and lung tissue using TRIzol reagent, according to manufacturer's instructions (Invitrogen). One microgram of total RNA was reverse transcribed using random primers and Moloney murine leukemia virus transcriptase (Invitrogen), and the gene of interest was amplified using RT-PCR and specific primers and 35 cycles.

Primer sequences used for RT-PCR amplification of the VEGF family members were as follows: 1) forward VEGF-A primer F1, 5′-GAGTACCC TGATGAGATCGAG-3′ (nt 206–227, accession NM 003376.1), and forward VEGF-A primer F2, 5′-GGAGAGATGAGCTTCCTACAG-3′ (nt 368–388); reverse VEGF-A primer R1, 5′-TCACCGCCTCGGCTTGT CACA-3′ (nt 572–592); forward VEGF-A primers were prepared, as mentioned previously (38), and sequences are as follows: forward VEGF-A primer F3, 5′-GAGAATTCGGCCTCCGAAACCATGAACTTTCTGCT 3′; reverse VEGF-A R2, 5′-GAGCATGCCCTCCTGCCCGGCTCAC CGC-3′; forward VEGF-A primer F4, 5′-TTGCCTTGCTGCTCTAC CTCC-3′ (nt 51–71); reverse VEGF-A R1. The above primer pairs amplify the region between exons 1 and 8, and therefore, detect each of the five isoforms and distinguish between them based on the presence or absence of exons 6 and 7. Two additional oligonucleotides were prepared to span exons 2 or 3 to exon 7 and thus detect the longer isoforms (VEGF-A165, VEGF-A189, and VEGF-A206) based on the presence or absence of exons 6a and/or 6b. Forward VEGF-A primer F4, 5′-TTGCCTTGCTGCTCTAC CTCC-3′ (nt 51–71); reverse VEGF-A R3, 5-TGCAACGCGAGTCTGT GTTTT-3′ (nt 513–533); forward VEGF-A primer F6, 5′-CTATCAGCG CAGCTACTGCCA-3′ (nt 154–174); and reverse VEGF-A R3. 2) VEGF-B primer pairs were prepared, as described previously (39). Forward VEGF-B primer hp1, 5′-CCTGACGATGGCCTGGAGTGT-3′ (nt 251–271); forward VEGF-B primer hp2, 5′-TGTCCCT GGAAGAACACAGCC-3′ corresponding to mRNA nt 345–365; reverse VEGF-B primer hp3, 5′-GCCATGTGTCACCTTCGCAG-3′ (nt 661–680). 3) Two primer pair sets were used for amplification of VEGF-C: forward VEGF-C primer F2, 5′-ATGTTTTCCTCGGATGCTGGA-3′ (nt 1158–1168, accession NM 005429.1); reverse VEGF-C primer R2, 5′-CATTGGCT GGGGAAGAGTTTG-3′ (nt 1334–1354); the second set of oligonucleotides was prepared, as mentioned previously (40), and sequences were as follows: forward VEGF-C primer F3, 5′-TGCCGATGCATGTCTAA-3′ (nt 996-1012); reverse VEGF-C primer R3, 5′-TGAACAGGTCTCTTC-3′ (nt 1233–1246). 4) VEGF-D primer pair was prepared, as mentioned previously (40): forward VEGF-D primer, 5′-GTATGGACTCTCGCTCAGCAT-3′; reverse VEGF-D primer, 5′AGGCTCTCTTCATTGCAACAG-3′ (nt 736–756 and 941–961), respectively. Specificity of primers for each of the genes was verified using BLASTN at the National Center for Biotechnology Information. Primers from the human β-actin gene were used as internal controls. Negative controls included RT-PCR without cDNA or omission of the reverse-transcriptase reaction.

Primer pairs for the PGE_2 receptor subtypes EP_1 and EP_3 were prepared, as reported previously (41). EP_2 and EP_4 primers were also prepared according to a previously published report (42).

Real-time quantitative PCR

CBMC (100% purity) were activated, as previously mentioned, for 45 min, or 1.5, 3, 4.5, or 24 h in medium alone or following the addition of PGE_2 at 10^{-6} M concentration. Two-step RT-PCR was performed in which total RNA was extracted and 1 μg was reverse transcribed, as detailed above. The second step of quantitative real-time PCR was performed using a 96-well optic tray on the ABI PRISM 7000 Sequence Detection System by using TaqMan Universal PCR Master Mix, No AmpErase UNG, Assays-on-Demand Gene Expression oligonucleotide primers and TaqMan probes (Applied Biosystems, Foster City, CA), and cDNA. VEGF-A-specific primers span exons 1 and 2 and amplify a 150-bp product. Human GAPDH-specific oligonucleotide primers were used to normalize for the expression of VEGF-A. The PCR thermal cycle conditions were as follows: initial step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was run in triplicates in two separate determinations. Data collection and analysis were performed with the SDS software (Applied Biosystems), after which data were exported and further analyzed. PCR efficiency was determined empirically using a standard curve with serial dilutions of cDNA from HMC-1 cell line. Once PCR efficiency was determined (>95%), the comparative cycle threshold method was used and results were calculated according to manufacturer's instructions. In the above set of experiments, the amount of VEGF-$A_{121/165}$ released in supernatants was also determined using VEGF-A ELISA, as described below.

Activation of mast cells with various activating agents

Before CBMC activation, mast cells were placed in a culture medium devoid of PGE_2 for a period of 24–48 h, after which mast cells were washed twice by centrifugation in RPMI 1640 containing 1% FCS. Mast cells were then suspended in RPMI 1640 containing 1% FCS, 0.1 U/ml penicillin, 100 mg/ml streptomycin, 1 μM HEPES, 10 ng/ml SCF, and 100 μg/ml soybean trypsin inhibitor (Sigma-Aldrich, Oakville, Ontario, Canada), denoted hereafter as activation medium. Mast cells were incubated at

0.5×10^6 cells/ml for 5, 24, 48, or 72 h at 37°C in activation medium alone, or with the addition of the following potential activating agents: forskolin, pentoxifylline, salbutamol, 17 β-estradiol, PGE_2 (all from Sigma-Aldrich), IL-1β (PeproTech), IL-6 (PeproTech), PGE_1, 1-OH-PGE_1, 17-phenyl-ω-trinor-PGE_2, sulprostone, butaprost, and misoprostol (all purchased from Cayman Chemicals, Ann Arbor, MI). All experiments were performed in triplicates, and samples were stored at –20°C until assay. HMC-1 cells were suspended in Iscove's medium containing 1% FCS, 0.1 U/ml penicillin, and 100 mg/ml streptomycin, and were otherwise activated under similar conditions.

Short-term activation experiments

CBMC were rested out from PGE_2 for at least 24 h and then were washed twice by centrifugation in RPMI 1640 medium containing 1% FCS. CBMC were resuspended in activation medium alone or with the addition of PGE_2 at 10^{-6} M concentration at 37°C for 1, 4.5, or 48 h. At the end of the 1- and 4.5-h incubation, CBMC were collected by centrifugation at $200 \times g$ for 20 min, after which cells were washed twice with RPMI 1640 containing 1% FCS and then resuspended in activation medium alone for the rest of the 48-h incubation period. Supernatants were collected at 1- and 47-h, 4.5- and 43-h, and 48-h time points, and VEGF-A121/165 production by CBMC was determined, as mentioned below.

VEGF-A ELISA

VEGF-$A_{121/165}$ production (secreted or cell associated) by mast cells was measured using an optimized sandwich ELISA (R&D Systems, Minneapolis, MN), according to manufacturer's instructions, with some modifications. Briefly, Maxisorp ELISA plates (Nunc/Inter Med, Montreal, Quebec, Canada) were coated with 0.4 μg/ml goat anti-human VEGF-$A_{121/165}$ capture Ab that detects VEGF-A_{121} and VEGF-A_{165} isoforms and blocked with 1% BSA and 5% sucrose in PBS, pH 7.2–7.4. The standards and samples were added to the plate in duplicates, and biotinylated anti-VEGF-A was used for detection at 25 ng/ml. Ab binding was visualized using streptavidin-alkaline phosphatase (Invitrogen) and an ELISA amplification system (Invitrogen). The lower detection limit for VEGF-$A_{121/165}$ was 1 pg/ml.

IgE-mediated mast cell activation

CBMC were incubated for 48–72 h at 37°C in culture medium without PGE_2 containing 10 μg/ml human myeloma IgE (Chemicon International, Temecula, CA). Sensitized cells were washed three times by centrifugation to remove unbound IgE and were used immediately in experiments. Sensitized cells were suspended in RPMI 1640 containing 1% FCS, 0.1 U/ml penicillin, 100 mg/ml streptomycin, 1 μM HEPES, 10 ng/ml SCF, and 100 μg/ml soybean trypsin inhibitor (Sigma-Aldrich), and were challenged with one of the following: pentoxifylline (1 mg/ml), PGE_2 (10^{-6} M), rabbit anti-human IgE (Chemicon International), or control rabbit IgG (Invitrogen) at two different concentrations (35 and 18 ng/ml) for 48 h at 37°C, and GM-CSF and VEGF-$A_{121/165}$ content of cell-free supernatants was determined.

GM-CSF ELISA

GM-CSF was assayed using optimized in-house ELISA. Briefly, Maxisorp ELISA plates (Nunc/Inter Med) were coated at room temperature with GM-CSF human mAb (Genzyme, Cambridge, MA) at 1 μg/ml and were blocked for 1 h at 37°C with 2% BSA and 0.05% Tween 20 in PBS, pH 7.2–7.4. Samples and standard (Genzyme) were added to the plate, after which biotinylated anti-human GM-CSF detection Ab (Endogen, Woburn, MA) was added at 0.1 μg/ml and plates were incubated at 37°C for 1 h. Binding of detection Ab was visualized using streptavidin-alkaline phosphatase and an ELISA amplification system (Invitrogen).

β-Hexosaminidase release assay

A total of 0.25×10^6 cells/ml was incubated for 20 min at 37°C in HEPES-Tyrodes buffer (137 mM Na, 5.6 mM glucose, 2.7 mM KCl, 0.5 mM NaH_2PO_4, 1 mM HEPES, 0.1% BSA, pH 7.3 at 300 mOsm/kg) with either buffer or any of the following agents: Ca^{+2} ionophore A23187 (10^{-6} M), forskolin (10^{-5} M), salbutamol (1 mg/ml), pentoxifylline (1 mg/ml), and PGE_2 (10^{-6}–10^{-8} M). β-Hexosaminidase release was stopped by pelleting the cells at $140 \times g$ for 10 min at 4°C. β-Hexosaminidase content in the supernatants and pellets was determined using a previously reported method (43).

Flow cytometric analysis

The expression of the EP_2 and EP_4 subtype receptors by the mast cell-like cell line KU812 and by CBMC was examined using flow cytometry. The macrophage cell line U937 was used as a positive control in this part of the study. Briefly, 0.5×10^6 cells/well were incubated with the primary Ab (Cayman Chemicals) in immunofluorescence buffer (1% BSA, 0.2% NaN_3 in 1× PBS) for 1 h at 4°C at a final concentration of 0.5 and 2 μg/ml of EP_2 and EP_4, respectively. After washing, cells were incubated for 1 h at 4°C with the secondary Ab, FITC-conjugated anti-rabbit IgG (Sigma-Aldrich). Following three washes with immunofluorescence buffer, cells were resuspended in 400 μl of 1% Formalin, and 10,000 cells were analyzed on a FACSCalibur. The results with the specific Abs were compared with those using rabbit IgG control Ab.

Statistical analysis

All data are expressed as the mean ± SEM. Statistical analysis was performed using either a nonparametric approach including Freidmans' test, followed by Wilcoxon signed-ranks test, or a parametric test using repeated measures ANOVA, followed by Dunnett's test. The choice of test was based upon the degree of normality of the data distribution.

Results

Expression of VEGF-A, VEGF-B, VEGF-C, and VEGF-D by human mast cells

The expression pattern for VEGF was determined using RT-PCR analysis of the mast cell line HMC-1 and its subclone 5C6, the basophil/mast cell line KU812, and primary cultures of CBMC from three separate donors, with normal lung tissue used as a positive control. Both human mast cell lines and CBMC expressed transcripts for several members of the VEGF family (Fig. 1). Transcripts for VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{165}, VEGF-A_{189}, and VEGF-A_{206} were detected in all mast cell lines with strong expression of the secretory isoforms VEGF-A_{121} and VEGF-A_{165} (Fig. 1*A*). Amplification of CBMC mRNA derived from three donors confirmed the expression pattern observed in human mast cell lines with predominant expression of VEGF-A_{121} and VEGF-A_{165} isoforms and a weaker expression of the membrane-bound isoforms, VEGF-A_{189} and VEGF-A_{206} (Fig. 1*B*). VEGF-B was expressed by both human mast cell lines and lung tissue with VEGF-B_{167} as the predominant isoform. A weak expression of the longer isoform, VEGF-B_{186}, was detected by human mast cell lines (Fig. 1*A*). Transcripts for only the shorter isoform of VEGF-B_{167} were detected in CBMC (Fig. 1*B*). Transcripts for VEGF-C and VEGF-D were detected in mast cell lines, lung tissue (Fig. 1*A*), and CBMC (Fig. 1*B*).

Activation of CBMC with cAMP-elevating agents

The ability of cAMP-elevating agents to enhance the production of VEGF-A by human mast cells following activation for up to 48 h was examined. The amount of VEGF-A secreted by human mast cells was determined using an optimized ELISA that uses a polyclonal anti-human VEGF-A Ab that recognizes the secretory isoforms VEGF-A_{121} and VEGF-A_{165}. In view of high constitutive levels of VEGF-$A_{121/165}$ secretion by HMC-1 cells (38.24 ± 3.39 pg/ml, $n = 3$) as compared with primary cultures of cord blood mast cells (9.4 ± 2.4 pg/ml, $n = 12$), the regulation of VEGF-$A_{121/165}$ secretion was examined in primary cultures of CBMC.

Results indicated that CBMC constitutively produce very small amounts of VEGF-$A_{121/165}$, a result that is consistent with the low expression in BMMC (15). However, following activation with the cAMP-elevating agents forskolin ($n = 8$–11) and pentoxifylline ($n = 6$), a dose-dependent increase in VEGF-$A_{121/165}$ production was observed (Fig. 2*A*). Higher doses of forskolin and pentoxifylline induced a very significant VEGF-$A_{121/165}$ response from human mast cells ($p < 0.01$).

VEGF-$A_{121/165}$ production by CBMC in response to salbutamol

Salbutamol is a β-agonist widely used as a short-acting bronchodilator in the treatment of asthma, which is known to elevate

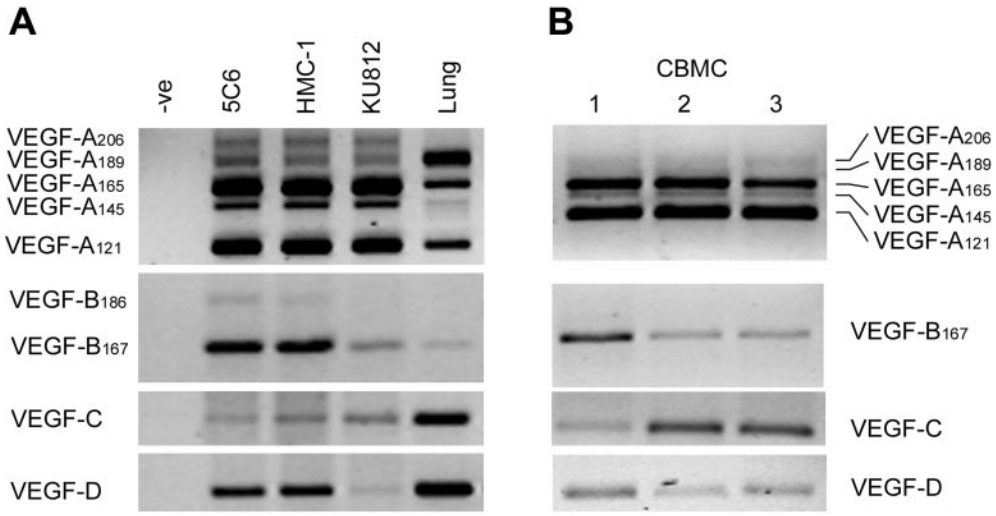

FIGURE 1. RT-PCR analysis of VEGF-A, VEGF-B, VEGF-C, and VEGF-D transcripts. *A*, Expression of members of the VEGF family in human mast cell/basophil lines HMC-1 5C6 (*lane 2*), HMC-1 (*lane 3*), KU812 (*lane 4*), human lung tissue (*lane 5*), and *B*, in primary cultures of CBMC from three different donors. Amplification of the VEGF-A gene using primer pair F1/R1 and 35 cycles resulted in the presence of five bands, 254, 326, 386, 458, and 509 bp, corresponding to VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{165}, VEGF-A_{189}, and VEGF-A_{206}, respectively. Note that the secretory isoforms VEGF-A_{121} and VEGF-A_{165} are strongly expressed in human mast cell lines. The expression pattern of cord blood mast cells was similar to that of the human mast cell lines with strong expression of the VEGF-A_{121} and VEGF-A_{165} isoforms. VEGF-B gene amplification results in the visualization of two bands at 429 bp corresponding to VEGF-B_{186}, and 328 bp corresponding to the VEGF-B_{167} isoform. Amplification of the VEGF-C gene revealed the presence of a strong band at 176 bp corresponding to VEGF-C. Agarose gel electrophoresis visualized the presence of a 225-bp band corresponding to VEGF-D. Primers from the human β-actin gene were used as internal controls (data not shown). Other controls included RT-PCR without cDNA (*lane 1*) or reverse-transcriptase reaction without the reverse-transcriptase enzyme.

cAMP levels. Salbutamol also induced an increase in VEGF-$A_{121/165}$ production by CBMC in a dose-dependent manner ($n = 6$), eliciting maximal effects at 1 mg/ml ($p < 0.01$; Fig. 2*B*). In additional experiments ($n = 6$), CBMC were activated with salbutamol at doses between 10^{-3} and 10^{-6} M. Similar results were obtained with statistically significant induction at the 10^{-3} M concentration (data not shown).

Activation of CBMC with 17 β-estradiol or proinflammatory cytokines

Angiogenesis plays a major role in the ovaries, where new blood vessels are formed during the oestrus cycle. The ability of mast cells to secrete VEGF-$A_{121/165}$ in response to activation with β-estradiol was therefore examined. Results indicated that activation with β-estradiol ($n = 6$–9) did not have an effect on VEGF-$A_{121/165}$ production at physiologically relevant concentrations; however, a small, but significant induction ($p < 0.05$) was observed at the highest concentration used in this study (10^{-4} M) (Fig. 2*C*). CBMC ($n =$ 9–11) treated with a range of concentrations of IL-1β or IL-6 (0.1–10 ng/ml) did not produce significantly elevated levels of VEGF-$A_{121/165}$ (7.44 $\pm$ 2.08; 7.03 $\pm$ 1.89, respectively) as compared with medium control (6.4 $\pm$ 1.06).

VEGF-$A_{121/165}$ production by CBMC in response to activation with PGE_2

Lipid mediators such as PGs and leukotrienes play a critical role in the regulation of inflammation and immune responses. One of these mediators, PGE_2, is known to be elevated in inflammation and in the microenvironment of tumors and has been shown to enhance angiogenesis (31). PGE_2 treatment of CBMC ($n = 12$) resulted in a potent, significant ($p < 0.001$), and dose-dependent production of VEGF-$A_{121/165}$ (Fig. 2*D*). Both PGE_2 and salbutamol induced VEGF-$A_{121/165}$ production by CBMC and both agents are present at high concentrations in a mast cell-rich environment in the lung of patients with asthma. The effects of combined PGE_2 and salbutamol treatment were therefore examined ($n = 6$). Both optimal (10^{-6} M) and suboptimal (10^{-8} M) concentrations of PGE_2 in the presence of various concentrations of salbutamol (10^{-3}–10^{-6} M) were used. Activation of CBMC in the presence of optimal concentrations of PGE_2 and 10^{-3} M concentration of salbutamol had an additive, but not synergistic, effect on VEGF-$A_{121/165}$ production as compared with treatment with PGE_2 alone (data not shown).

We have also examined the ability of PGE_2 at a concentration of 10^{-6} M to induce VEGF-$A_{121/165}$ production after treatment of CBMC for a period of 1 or 4.5 h, followed by washing and incubation in the activation medium alone for the rest of the 48 h. Our results indicate that short-term incubation of CBMC with PGE_2 leads to a trend toward an increase in the amounts of VEGF-$A_{121/165}$ produced by human mast cells; however, this increase was not significant (data not shown, $n = 4$). In contrast to the above results, continuous 48-h PGE_2 treatment of the same cells led to a significant ($p < 0.005$) increase in VEGF-$A_{121/165}$ production.

Effects of IgE-mediated mast cell activation

Murine and human mast cells have been previously shown to secrete increased amounts of VEGF-$A_{121/165}$ following IgE-mediated activation (15). To examine the relative ability of IgE-mediated activation to induce VEGF-$A_{121/165}$, CBMC were sensitized with human IgE at 10 μg/ml for 48–72 h and then challenged with an optimal dose of rabbit anti-human IgE, rabbit IgG control, PGE_2 (10^{-6} M), or pentoxifylline (1 mg/ml). The effectiveness of IgE-mediated activation was confirmed by measuring GM-CSF production by CBMC. Anti-IgE treatment resulted in a small, but significant induction of VEGF-$A_{121/165}$ secretion by CBMC as compared with control rabbit IgG treatment (Fig. 3*A*). Activation of CBMC for 20 min, followed by measurements of β-hexosaminidase release indicated that PGE_2, forskolin, and pentoxifylline did not induce significant CBMC degranulation, indicating that

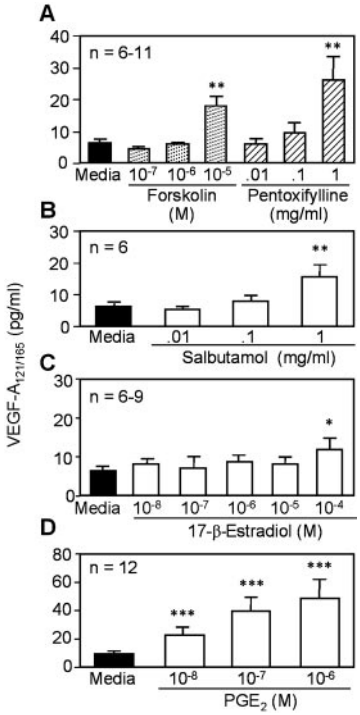

FIGURE 2. VEGF-$A_{121/165}$ secretion by CBMC in response to activation with various stimuli for 48 h. *A*, Both forskolin and pentoxifylline induced an increase in VEGF-$A_{121/165}$ production in a dose-dependent manner. High doses induced significant VEGF-$A_{121/165}$ production by mast cells (10^{-5} M forskolin and 3.6×10^{-3} M pentoxifylline). *B*, Treatment of human mast cells with salbutamol resulted in increased VEGF-$A_{121/165}$ production in a concentration-dependent manner with maximal effect at 1 mg/ml (4×10^{-3} M). *C*, Significant induction of VEGF-$A_{121/165}$ was only observed at the highest concentration of 17 β-estradiol (10^{-4} M). *D*, VEGF-A-enhanced release was observed following activation of CBMC with various concentrations of PGE_2. Results are presented as mean ± SEM of 6–12 independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with medium control values for unstimulated CBMC.

VEGF-$A_{121/165}$ production occurs via a degranulation-independent mechanism in response to these stimuli (Fig. 3*B*).

Time course production of VEGF-$A_{121/165}$ in response to various stimuli

The ability of mast cells to up-regulate the secretion of VEGF-$A_{121/165}$ in response to various stimuli following activation for up to 72 h was investigated (Fig. 4). All VEGF-$A_{121/165}$ secretion inducers showed a similar time course in which VEGF-$A_{121/165}$ production was readily detected at 5 h and increased by 24 h. Supernatant VEGF-$A_{121/165}$ levels continued to increase at 48 h, and further induction was observed at 72 h. These results indicate that mast cells can produce a sustained VEGF-$A_{121/165}$ response following activation. As shown in Fig. 4, PGE_2 was the strongest inducer of VEGF-$A_{121/165}$ secretion by CBMC at all times. A 3-fold increase in VEGF-$A_{121/165}$ secretion (16.2 ± 6.19, $n = 3$) was detected at 5 h as compared with medium controls (5.04 ± 1.6, $n = 3$). A 6-fold increase in VEGF-$A_{121/165}$ secretion was detected at 24 and 48 h (42.6 ± 11.9, $n = 3$ and 57.16 ± 15.9, $n = 4$, respectively) as compared with medium control (7.02 ± 2.9 and 9.8 ± 1.3). This increase in VEGF-$A_{121/165}$ secretion was sustained at 72 h (89.1 ± 39.6 PGE_2 vs 18.3 ± 3.5 medium, $n = 3$).

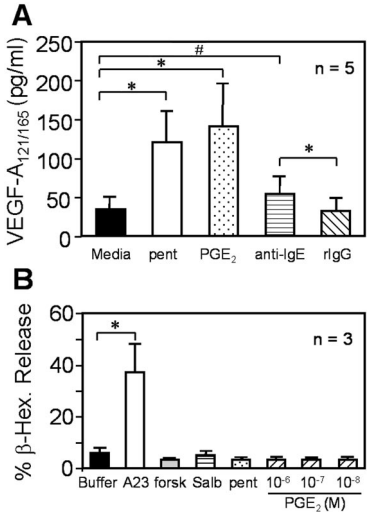

FIGURE 3. Relationship between degranulation and VEGF-$A_{121/165}$ secretion by CBMC. *A*, Pentoxifylline and PGE_2 potentiate a strong VEGF-$A_{121/165}$ response in IgE-sensitized CBMC as compared with medium control. Treatment with anti-IgE, a strong inducer of degranulation in mast cells, resulted in a small, but significant increase in VEGF-$A_{121/165}$ production as compared with its corresponding rabbit IgG (*, $p < 0.05$) control and with medium control (#, $p < 0.05$). *B*, Measurements of β-hexosaminidase release by CBMC following activation for 20 min at 37°C. Activation of CBMC with Ca^{2+} ionophore, A23187, resulted in a significant release of β-hexosaminidase as compared with buffer control (*, $p < 0.05$). Neither PGE_2 nor other cAMP-elevating agents caused mast cell degranulation as compared with buffer control, suggesting that these stimuli enhanced VEGF-$A_{121/165}$ secretion via a degranulation-independent mechanism. *, $p < 0.05$ as compared with the corresponding control value. Symbols: pent, pentoxifylline; forsk, forskolin; Salb, salbutamol; and A23, Ca^{2+} ionophore A23187.

Mechanism of VEGF-$A_{121/165}$ up-regulation by CBMC in response to PGE_2 activation

In light of the above data, we sought to investigate the mechanism by which mast cells up-regulate VEGF-$A_{121/165}$ production in response to PGE_2 activation. The CBMC EP receptor expression pattern, at the mRNA and protein levels, and the usage of such PGE_2 receptors were examined. RT-PCR analysis indicated that both human mast cell lines and primary cultures of CBMC express transcripts for each of the PGE_2 subtype receptors EP_1, EP_2, EP_3, and EP_4 (Fig. 5*A*). We further investigated the expression of the EP_2 and EP_4 receptors by human mast cells at the protein level using flow cytometric analysis. Our results indicate that the mast cell-like cell line, KU812, expresses moderate levels of EP_2 subtype receptor (Fig. 5*B*) with a mean fluorescence intensity of 14.21 for KU812 cells as compared with 5.09 for the corresponding control. Low levels of EP_4 subtype receptor protein were detected on KU812 cells (10.11 and 6.82 for anti-EP_4- and rIgG-treated control KU812 cells, respectively). CBMC, in contrast, expressed low levels of the EP_2 subtype receptor (48.54 and 24.81 for anti-EP_2 and control rIgG, respectively) and had no detectable EP_4 subtype receptor (Fig. 5*B*).

To determine more specifically which of the EP receptors mediated VEGF-$A_{121/165}$-enhanced production by human mast cells, selective agonists that bind specifically to one or more of the EP receptors were used (34). The EP_3/EP_1 selective agonists, 17-phenyl-ω-trinor-PGE_2 ($n = 6$) and sulprostone ($n = 4$), did not potentiate the release of VEGF-$A_{121/165}$ by human mast cells (Fig. 5*C*). In contrast, misoprostol ($n = 6$), which is a selective agonist for the EP_2 and EP_3 subtype receptors, and 1-OH-PGE_1 ($n = 6$),

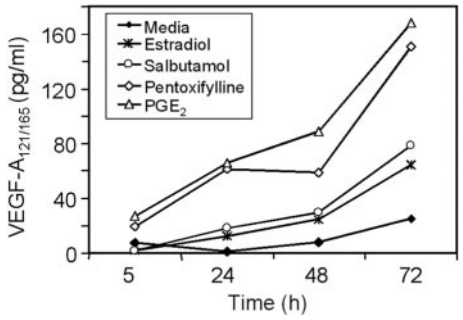

FIGURE 4. Time course of VEGF-$A_{121/165}$ secretion by CBMC. CBMC were activated for a period of 5, 24, 48, or 72 h with medium alone, 17 β-estradiol (10^{-4} M), salbutamol (1 mg/ml), pentoxifylline (1 mg/ml), or PGE_2 (10^{-6} M). Increased amounts of VEGF-$A_{121/165}$ protein were secreted in response to activation with all reagents, with the greatest response observed at 72 h. Data shown are representative of 3–12 separate independent experiments.

which binds with high affinity to the EP_2 and to a lesser extent to the EP_4 subtype receptors, were able to potentiate a significant induction of VEGF-$A_{121/165}$ at high concentrations ($p < 0.01$, Fig. 5*D*). Similarly, PGE_1 ($n = 4$), which is an analog to PGE_2 that binds preferentially to EP_2, EP_3, and EP_4 subtype receptors and with lower affinity to the EP_1 receptor, induced a significant production of VEGF-$A_{121/165}$ at higher concentrations ($p < 0.05$). The above results indicate that human mast cells most probably induce VEGF-$A_{121/165}$ through activation of the EP_2 and/or EP_4 receptor. To distinguish between the two receptors, a specific agonist for the EP_2 subtype receptor, butaprost, was used to activate CBMC. Activation of CBMC ($n = 6$) with butaprost potentiated the release of VEGF-$A_{121/165}$ in a dose-dependent manner (Fig. 6) with significant induction at 10^{-5} M ($p < 0.01$) and 10^{-6} M concentrations ($p < 0.05$). Taken together, these data along with the expression of the EP_2 subtype receptor at the protein level, but not the EP_4 subtype receptor, indicate that PGE_2 is most likely to induce VEGF-$A_{121/165}$ in CBMC via an EP_2-specific mechanism.

Mechanisms of VEGF-$A_{121/165}$ secretion

In a separate set of experiments, the amount of cell-associated VEGF-$A_{121/165}$ was determined as compared with that released in the medium following activation with PGE_2 (10^{-6} M) for 48 h. The amount of cell-associated VEGF-$A_{121/165}$ in cells that were incubated in medium alone was 151.12 ± 15.49 pg/10^6 cells ($n = 7$), while cells incubated with PGE_2 contained 124.42 ± 12.88 pg/10^6 cells. These data are in keeping with previous published studies, which reported the localization and the association of VEGF-$A_{121/165}$ with mast cell granules (15). The amount of VEGF-$A_{121/165}$ released in the supernatants following PGE_2 treatment was 73.46 ± 11.17 pg/10^6 cells; this is a 4-fold increase in VEGF-$A_{121/165}$ production into the supernatant as compared with cells activated with medium alone (16.64 ± 3.04 pg/10^6 cells, $n = 7$).

To better understand the mechanism of VEGF-$A_{121/165}$ secretion by human mast cells, the total amount of VEGF-$A_{121/165}$ produced by CBMC (secreted and cell associated) during the most active phase of production at 24 h was determined. Our results indicate that there was a 2-fold significant increase ($p < 0.05$, $n = 4$) in the total amount of VEGF-$A_{121/165}$ following activation with PGE_2 (318.22 ± 24.54 pg/10^6 cells) as compared with medium control (177.18 ± 11.66 pg/10^6 cells). This increase was due to increased VEGF-$A_{121/165}$ secretion following PGE_2 activation (103.08 ± 13.1 pg/10^6 cells) as compared with medium control (7.1 ± 1.77 pg/10^6 cells) as well as due to increased amounts of cell-associated VEGF-$A_{121/165}$ (215.16 ± 18.29 and 167.78 ± 10.3 pg/10^6 cells, respectively). The above data indicate that the sustained release of VEGF-$A_{121/165}$ in response to activation is due to de novo synthesis rather than release from granules because activation of CBMC with Ca^{2+} ionophore A_{23187} (a strong inducer of degranulation) did not result in the significant release of VEGF-$A_{121/165}$ (26.48 ± 3.57 pg/10^6 cells) as compared with medium control (18.4 ± 2.34 pg/10^6 cells, $n = 4$). Further evidence to support de novo synthesis was obtained from real-time PCR, in which VEGF-A gene regulation was examined following activation with PGE_2 at a concentration of 10^{-6} M for 1.5, 3, or 24 h. Real-time results indicate that CBMC up-regulated VEGF-A mRNA by 2.42 ± 0.15- and 2.73 ± 0.44-fold compared with medium control-treated cells by 1.5 and 3 h, respectively, and returned back to normal levels by 24 h. We have also examined up-regulation of VEGF-A mRNA at 45 min and at 4.5 h.

Discussion

This study demonstrates that primary human mast cells express transcripts for several members of the VEGF family, including VEGF-A, VEGF-B, VEGF-C, and VEGF-D, each of which has been shown to play an important role in angiogenesis and lymphogenesis. PGE_2, an important inflammatory mediator, was the most potent inducer of the potent proangiogenic VEGF-$A_{121/165}$ isoforms by human mast cells. PGE_2 induced VEGF-$A_{121/165}$ production in primary cultures of CBMC via activation of the EP_2 subtype receptor.

Previous studies have reported the expression of VEGF-A isoforms in BMMC and rat peritoneal mast cells (15), and in the human mast cell leukemic cell line (HMC-1) (44); however, this is the first report to define the VEGF-A isoforms expressed by primary cultures of human mast cells. Interestingly, human mast cells express mRNA for the less common VEGF-A_{189} and the rare VEGF-A_{145} and VEGF-A_{206} splice variants. The expression of both the secretory and heparin-binding isoforms of VEGF-A suggests that mast cells can provide these growth factors for endothelial cells for both short- and long-term usage. Mast cell granules are known to be rich in heparin and other highly sulfated proteoglycans, which may provide a storage site for members of the VEGF family (15).

Expression analysis revealed that human mast cells also express two transcripts for VEGF-B, with strong expression of the shorter heparin-bound isoform VEGF-B_{167} and one transcript for each VEGF-C and VEGF-D. This is the first study, to our knowledge, to describe the expression of VEGF-B, -C, and -D members of the family by mast cells. Coexpression of different members of the VEGF family by human mast cells is potentially important in situations in which members of the VEGF family may be differentially regulated, such as the case in certain kinds of tumors (45, 46) and in the ovaries (47). Mast cells might play a broad role in angiogenesis by producing a range of angiogenic growth factors that, in combination with VEGF-A, can form heterodimers as well as homodimers, which have differing abilities to bind heparin and extracellular matrix components.

The mechanisms regulating the expression and secretion of VEGF-A are poorly understood. In this study, we examined the regulation of VEGF-$A_{121/165}$ expression at the protein level in human mast cell lines and primary cultures of CBMC using a wide range of potential VEGF-A and mast cell regulators, including cAMP-elevating agents, proinflammatory cytokines, and β-estradiol. Our results indicate that the tumorigenic mast cell line, HMC-1, produced high levels of VEGF-$A_{121/165}$ as compared with

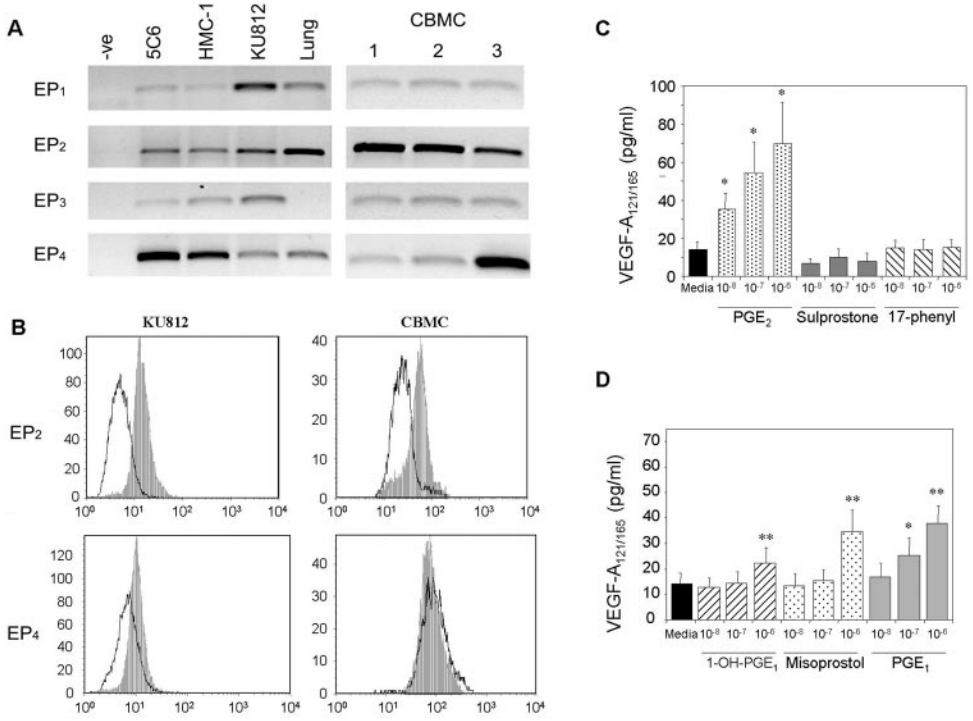

FIGURE 5. Mechanisms of VEGF-$A_{121/165}$ secretion by CBMC in response to PGE_2 activation. *A*, RT-PCR analysis of mast cell lines and CBMC revealed the presence of transcripts for each of the PGE_2 subtype receptpors. *B*, Flow cytometric analysis of KU812 revealed the presence of both EP_2 and EP_4 subtype receptors. CBMC expressed low levels of the EP_2 subtype receptor. Results are representative of four independent experiments. *C*, Effects of EP_3/EP_1 selective agonists on the release of VEGF-$A_{121/165}$ from CBMC following activation for 48 h. Activation of CBMC with various concentrations of PGE_2 ($n = 6$) resulted in an enhanced VEGF-$A_{121/165}$ secretion as compared with medium control. Activation of CBMC with the selective agonists EP_3/EP_1 receptors, sulprostone, or 17-phenyl-ω-PGE_2 (17-phenyl) did not potentiate VEGF-$A_{121/165}$ production as compared with medium control (*, $p < 0.05$). *D*, Activation of CBMC with the EP_2/EP_3 selective agonist, misoprostol, and the EP_2/EP_4 selective agonist, 1-OH-PGE_1, resulted in enhanced VEGF-$A_{121/165}$ secretion as compared with medium control (*, $p < 0.05$; **, $p < 0.01$).

primary cultures of CBMC. In addition, the amounts of VEGF-$A_{121/165}$ secreted by HMC-1 were not up-regulated following stimulation for 48 h. For example, the amounts of VEGF-A released in the supernatants following activation with forskolin and PGE_2 were 43.09 ± 1.18 and 46.83 ± 7.69 pg/ml ($n = 3$), respectively, as compared with medium control (38.24 ± 3.39 pg/ml). These results are not surprising because many tumorigenic cell lines such as the HMC-1 have been previously shown to have disregulated VEGF-A expression. This may be due to alterations in the function of the von Hippel-Lindau protein (48, 49), which are common in tumor cell lines.

Results from our primary cultures of CBMC revealed that they up-regulate VEGF-$A_{121/165}$ production (~2.5- to 4-fold) in a time- and dose-dependent manner in response to activation with a number of cAMP-elevating agents. It is noteworthy that the amount of VEGF-$A_{121/165}$ produced by mast cells in response to forskolin activation was less than that produced following PGE_2 treatment. This might be explained by the lower, but sustained duration of cAMP elevation in response to PGE_2 activation as compared with the short robust increase in cAMP levels following forskolin activation because both agents have been shown to increase cAMP in murine mast cells (25). Alternatively, other PGE_2-dependent signaling pathways may contribute to the observed response. However, as in other systems (50), cAMP-dependent pathways appear to play an important role in VEGF-$A_{121/165}$ induction by human mast cells.

A wide range of stimuli is known to elevate intracellular cAMP levels, including a number of pharmacological agents used in therapy for allergic diseases such as salbutamol. Activation of CBMC with salbutamol resulted in an enhanced production of VEGF-$A_{121/165}$. These results suggest that salbutamol, at high local concentrations, could potentially contribute to an enhancement of VEGF-$A_{121/165}$ production by resident mast cells in the lung.

Recent in vivo and in vitro studies have demonstrated the stimulation of VEGF-A production in the uterus by estradiol at 10^{-8}-10^{-9} M concentrations (51). Our results indicate that, in contrast to the above system, human mast cells up-regulate VEGF-$A_{121/165}$ secretion only at high concentrations of estradiol, suggesting that estrogens are not important inducers of VEGF-$A_{121/165}$ by human mast cells, although it has been suggested that mast cells may respond to estrogen in other ways (52, 53). In addition, our results indicate that the proinflammatory cytokines IL-1 and IL-6 are not important modulators of $VEGF_{121/165}$ production by human mast cells. This is in marked contrast to the VEGF-A response of other types of cells following activation with these stimuli (54).

PGE_2 is an important mediator of mast cell function and a powerful modulator of immune responses. PGE_2 has also been shown to be elevated in the microenvironment of tumors and during inflammation (31). Reduction of PG synthesis, through the use of nonsteroidal anti-inflammatory drugs, can reduce angiogenesis and tumor regression in some situations (30, 55). Mast cells have been shown to produce a number of mediators in response to PGE_2 without concurrent degranulation (25). Results of the current study reveal that PGE_2 is also a potent inducer of VEGF-$A_{121/165}$ production by human mast cells. Treatment with PGE_2 resulted in a 3-fold increase by 5 h, and a 6-fold increase in VEGF-$A_{121/165}$ secretion was detected at 24 h, which was persistent up to 72 h. The significant increase in VEGF-$A_{121/165}$ production by CBMC in response to PGE_2 (10^{-8} M) demonstrates that this prostanoid is

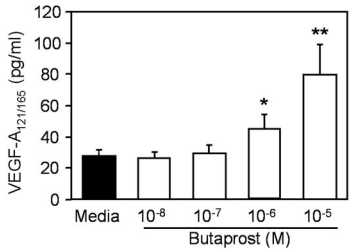

FIGURE 6. Effects of the EP_2 selective agonist, butaprost, on the release of VEGF-$A_{121/165}$ from CBMC following activation for 48 h. Activation of CBMC with various concentrations of butaprost ($n = 6$) resulted in a dose-dependent enhancement of VEGF-$A_{121/165}$ production as compared with medium control. Significant induction was observed at the highest concentrations 10^{-5} and 10^{-6} M (*, $p < 0.05$; **, $p < 0.01$). PGE_2 at a concentration of 10^{-8} M was used as a positive control in these experiments (mean 51.55 $\pm$ 10.9 pg/ml) as compared with medium control (27.27 $\pm$ 3.0 pg/ml).

a potent regulator of VEGF-$A_{121/165}$ production by human mast cells.

The potency of PGE_2 indicates that this prostanoid might induce VEGF-$A_{121/165}$ production through activation of multiple signaling pathways, including elevation of intracellular cAMP. PGE_2 has been previously demonstrated to increase IL-6 production in rat mast cells (24) via a cAMP-dependent pathway (25). However, the lack of VEGF-$A_{121/165}$ production in response to IL-6 activation suggests that PGE_2 does not induce its effect via IL-6. Up-regulation of VEGF-$A_{121/165}$ expression in response to activation with PGE_2 has been reported previously in other systems (41), including RA synovial fibroblasts (56, 57). In chronic inflammation, such as in RA and asthma, high levels of inflammatory cytokines, growth factors including VEGF-A, and PGs are thought to play an important role in the development of angiogenesis and in the pathogenesis of the disease (58, 59). The current data demonstrate the potential of mast cells in these sites to enhance angiogenesis via increased secretion of VEGF-$A_{121/165}$ in response to locally elevated levels of PGE_2.

The potent effects of various VEGF-$A_{121/165}$ regulators, as well as PGE_2, observed in this study could not be explained by the presence of contaminating cells in our CBMC culture because mast cells were of 96% or greater purity when used in all experiments. Moreover, similar results were obtained when those preparations with homogeneous populations of mast cells (100%) were used ($n = 4$). Compared with other cell types, our results indicate that primary cultures of human mast cells were more vigorous in their response to PGE_2. Primary cultures of human fibroblasts and keratinocytes constitutively produced higher amounts of VEGF-$A_{121/165}$ at ~300 and 900 pg/ml/10^6 cells, respectively (60), as compared with primary human mast cells (18.79 pg/ml/10^6 cells) following stimulation for 48 h. CBMC showed a 6-fold increase as compared with fibroblasts, which only had a 2-fold increase, in VEGF-$A_{121/165}$ production when stimulated with PGE_2 at 10^{-6} M concentration. Primary human keratinocytes, in contrast, did not respond at all to PGE_2 activation (60). The sustained production of VEGF-$A_{121/165}$ up to 72 h by human mast cells may be of particular importance in sustaining the angiogenic response, particularly in view of the strategic location of mast cells close to blood vessels.

In this study, we have also investigated the mechanism of the sustained release of VEGF-$A_{121/165}$ from mast cells. Results of real-time PCR analysis indicate that CBMC up-regulate VEGF-A mRNA by 2.4-fold following PGE_2 treatment, which returns back to normal levels by 24 h. This induction at the mRNA level is accompanied by a significant increase in VEGF-$A_{121/165}$ secretion as well as increased amounts of cell-associated VEGF-$A_{121/165}$. This sustained increase in total VEGF-$A_{121/165}$ following PGE_2 activation is due to de novo synthesis rather than release from granules because activation of CBMC for a short period of time (1 h) with PGE_2 or treatment with a potent degranulatory agent (Ca^{2+} ionophore) does not result in significant release of VEGF-$A_{121/165}$ from cells. Collectively, these results indicate that human mast cells use complex mechanisms, including de novo synthesis and possibly a delayed mechanism of protein release in their regulation of VEGF-$A_{121/165}$.

Previous reports of VEGF-A production by mast cells have demonstrated degranulation-associated VEGF-A release as well as the presence of cell-associated VEGF-A (15). In contrast, the current study of human mast cells demonstrates sustained, degranulation-independent production of VEGF-A in response to both PGE_2 and other cAMP-elevating agents. This observation suggests that morphologically intact granulated mast cells may be a substantial source of VEGF-A under some pathological conditions. Furthermore, traditional mast cell-stabilizing agents may not be effective in preventing mast cell VEGF-A production.

The mechanism by which PGE_2 induces VEGF-$A_{121/165}$ production by mast cells was examined using a combination of expression, flow cytometry, and pharmacological studies. mRNA expression analysis revealed that human mast cells expressed four PGE_2 receptors, EP_1, EP_2, EP_3, and EP_4, while protein expression revealed that CBMC expressed the EP_2 subtype receptor. Despite the mRNA expression of EP_4 subtype receptor, there was no EP_4 detectable at the protein level on the surface of CBMC. This is in contrast to the mast cell lines, which demonstrated surface expression of this receptor using the same reagents. The lack of EP_4 expression on CBMC may relate to posttranscriptional regulation or to a requirement for additional signals for cell surface rather than intracellular expression.

To determine more precisely which of the EP receptors mast cells used, we used a pharmacological approach in which selective agonists for one or more of the EP receptor agonists were used to activate CBMC. Our studies indicated that the EP_2 selective receptor agonist, butaprost, was able to mimic PGE_2 effects by CBMC. Results from less selective EP_2 agonists supported this finding. Collectively, results presented in this work strongly indicate that enhanced VEGF-$A_{121/165}$ production by human mast cells in response to PGE_2 occurs via activation of the EP_2 subtype receptor. This is the first report to identify the expression of the EP_2 subtype receptor by human mast cells, which is in contrast to the murine BMMC system in which EP_2 expression was absent (25). We have also identified that EP_2 receptor ligation induces VEGF-$A_{121/165}$ production by human cells. These results are in marked contrast to the EP_1/EP_3-mediated activation of murine mast cells, which induced degranulation-independent GM-CSF and IL-6 production (25) and the EP_3-mediated induction of IL-6 in mouse mast cells (26). Unlike the murine system, in which mast cells use EP_1 and/or EP_3 receptors for mediation of PGE_2 effects, our results indicate that human mast cells use the EP_2 receptor system to mediate their biological effects in response to PGE_2 activation. Expression of EP_2 subtype receptor has been shown in other immune cells such as macrophages and T cells, in which it mediated the effects of PGE_2 in suppressing Ag-specific proliferation of these cells (61). The selective use of EP_2 receptor to mediate VEGF-$A_{121/165}$ production is in keeping with the known ability of this receptor system to be coupled to elevations in intracellular cAMP (34, 62).

Finally, in studies presented in this work, we have supplemented our primary cultures with PGE_2 and SCF. Both factors are necessary for optimal mast cell growth (35, 63). CBMC were cultured for at least 24 h in culture medium devoid of PGE_2 before experimentation to avoid potential interference with the experiments. It is possible that exposure to PGE_2 may influence EP receptor expression. However, in vivo, it is likely that mast cells are also frequently in contact with PGE_2 during differentiation, especially at mucosal sites such as the intestine and airways and in the microenvironment of tumors, where elevated levels of PGE_2 have been reported.

In this study, we have identified a novel mechanism by which human mast cells increase VEGF-$A_{121/165}$ secretion in response to PGE_2. Identifying the mechanism of VEGF-$A_{121/165}$ induction is of great importance in finding therapeutic agents to block the mast cell contribution to angiogenesis in chronic inflammation and at tumor sites. Antagonizing the EP_2 subtype receptor could be of great importance to block mast cell production of VEGF-$A_{121/165}$ at angiogenic sites, as well as blocking other cell types, such as fibroblasts, that have been shown to use the EP_2/cAMP signaling pathway in VEGF-$A_{121/165}$ production in response to activation with PGE_2 (56, 57).

In conclusion, this study is the first to demonstrate that human mast cells express VEGF-B, VEGF-C, and VEGF-D, which adds to the complexity of mast cell's role in angiogenesis. Moreover, we have demonstrated that human mast cells can selectively upregulate the production of the most potent isoforms of VEGF-A in response to activation with cAMP-elevating agents including PGE_2. The close association between mast cells and blood vessels (63) would be expected to enhance the importance of mast cell VEGF-A production to the angiogenic response. The production of other proangiogenic stimuli by mast cells such as matrix metalloproteinase-9, TNF-α, and IL-1β could further enhance the proangiogenic activity of this cell type. PGE_2-induced mast cell VEGF-A expression may be of particular importance within the microenvironment of solid tumors and at sites of chronic inflammation, such as the synovium in RA, in which both mast cells numbers and PGE_2 levels are elevated.

Acknowledgments

We thank Ula Kadela-Stolartz and Yi-Song Wei for excellent technical support.

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