Human Skin-Derived Mast Cells Spontaneously Secrete Several Angiogenesis-Related Factors

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Mast cells are classically recognized as cells that cause IgE-mediated allergic reactions. However, their ability to store and secrete vascular endothelial growth factor (VEGF) suggests a role in vascular development and tumorigenesis. The current study sought to determine if other angiogenesis-related factors, in addition to VEGF, were also secreted by human tissue-derived mast cells. Using proteome array analysis and ELISA, we found that human skin-derived mast cells spontaneously secrete CXCL16, DPPIV, Endothelin-1, GM-CSF, IL-8, MCP-1, Pentraxin 3, Serpin E1, Serpin F1, TIMP-1, Thrombospondin-1, and uPA. We identified three groups based on their dependency for stem cell factor (SCF), which is required for mast cell survival: Endothelin-1, GM-CSF, IL-8, MCP-1, and VEGF (dependent); Pentraxin 3, Serpin E1, Serpin F1, TIMP-1, and Thrombospondin-1 (partly dependent); and CXCL16, DPPIV, and uPA (independent). Crosslinking of FcεRI with multivalent antigen enhanced the secretion of GM-CSF, Serpin E1, IL-8, and VEGF, and induced Amphiregulin and MMP-8 expression. Interestingly, FcεRI signals inhibited the spontaneous secretion of CXCL16, Endothelin-1, Serpin F1, Thrombospondin-1, MCP-1 and Pentraxin-3. Furthermore, IL-6, which we previously showed could induce VEGF, significantly enhanced MCP-1 secretion. Overall, this study identified several angiogenesis-related proteins that, in addition to VEGF, are spontaneously secreted at high concentrations from human skin-derived mast cells. These findings provide further evidence supporting an intrinsic role for mast cells in blood vessel formation.

Keywords: mast cells, angiogenesis, tumorigenesis, VEGF, stem cell factor, FcεRI

INTRODUCTION

Mast cells are hematopoietic tissue resident immune cells that are classically recognized as the main effector cell type of Immunoglobulin E (IgE)-mediated immediate hypersensitivity reactions (1, 2). In addition to their classical role in allergy, mast cells are frequently associated with tumors in humans, and are implicated in tumorigenesis (3). The exact role of mast cells in tumor formation is not known. However, tumor-associated mast cells (TAMCs) have been shown to have both pro- and anti-tumorigenic effects in human tumors (3), and it appears that the overall impact of mast cells on tumor formation is tissue dependent (4). For example, mast cells have pro-tumor effects in human cancers of the thyroid (5, 6), stomach (7–9), and bladder (10). In contrast, mast cells appear to protect against breast cancer (11–13). In most cases, however, both pro- and anti-tumorigenic
effects of mast cells have been reported, such as in cancers of lung, colon, pancreas, and prostate. Thus, mast cells appear to have both positive and negative effects on tumor formation.

Mast cells are a major source of cytokines, chemokines, and growth factors that can contribute to tumor development (14, 15). It has been known for some time that mast cells contain VEGF within their cytoplasmic granules, and can secrete this growth factor both spontaneously and following activation (16, 17). These early observations were made in murine bone marrow-derived mast cells (BMMCs), human cord blood-derived mast cells (CBMCs), mast cells from human foreskin, and the HMC-1 cell line. In addition, we recently demonstrated the spontaneous, IL-6- and FcεRI signals, IL-6 (18), IL-9 (22), PGE2 (20), Cordicotropin Releasing Hormone (23), and adenosine (24) can also induce VEGF secretion from mast cells. Mast cell-derived VEGF was shown to induce proliferation and migration of human endothelial cells (16), and to induce angiogenesis in the chick embryo chorioallantoic membrane assay (19). Other factors, including Angiopoietin-1 (21), have also been implicated in mast cell-mediated angiogenesis and tumor growth (3, 15, 25). Nevertheless, most studies have focused on VEGF as the primary promoter of tumorigenesis from mast cells.

Here, we demonstrate that tissue-derived mast cells from human skin spontaneously secrete several angiogenesis-related factors, in addition to VEGF, at high concentrations. Specifically, we identified CXCL16, DPPIV, Endothelin-1, GM-CSF, IL-8, MCP-1, Pentraxin 3, Serpin E1, Serpin F1, TIMP-1, Thrombospondin-1, and uPA as being spontaneously secreted at levels greater than VEGF. Secretion of some factors required stem cell factor (SCF) whereas others were secreted completely independent of the survival factor. We show that FcεRI crosslinking enhances, but also inhibits, the secretion of several factors. Lastly, we show that IL-6 induced the secretion of MCP-1. Together, these data demonstrate that mast cells are a major source of several different angiogenesis-regulating factors. Thus, supporting the notion of an intrinsic role for mast cells as regulators of blood vessel formation (15).

MATERIALS AND METHODS

Isolation, Purification, and Culture of Human Skin Mast Cells

Mast cells were isolated and purified from fresh surgical specimens of human skin tissues that were purchased from the Cooperative Human Tissue Network (CHTN) of the National Cancer Institute. These studies were approved by the human studies Internal Review Board (IRB) of University of South Carolina. The tissues, which were received within 24 h post-surgery, were mechanically minced with surgical scissors, and then digested with collagenase type II, hyaluronidase, and DNase I in HBSS buffer (1X HBSS, 0.04% NaHCO3, 1% fetal bovine serum, 1% HEPES, 0.1% CaCl2) containing Amphotericin B and Antibiotic/Antimycotic solution. A total of 3 × 1 h digestions at 37°C were performed. The samples were filtered through 40 µm nylon cell strainers after each digestion, and the dispersed cells were collected by centrifugation. The cells were separated on a Percoll cushion by density centrifugation. The cells at the interface of buffer and Percoll layers were collected, washed and re-suspended at 5 × 106 cells/ml in serum-free X-VIVO 15TM media (Lonza) supplemented only with recombinant human stem cell factor (SCF, 100 ng/ml) (PeproTech). A small aliquot is usually stained with Toluidine Blue in order to get a rough idea of the starting number of mast cells obtained, which is ~5–7% of the total population based on our extensive experience. Total cells were transferred onto 24-well plates and maintained under standard culture conditions (37°C, 5% CO2) with weekly media changes and transfer to new plates as necessary. At ~4–6 weeks of culture, most of the non-mast cells have died-off leaving a majority of mast cells. Purity was assessed by metachromatic staining with acidic toluidine blue, and by immunofluorescence staining for FcεRI expression with PE-labeled anti-human FcεRI antibody [clone AER-37 (CRA)] and mouse IgG2bx isotype control (BioLegend). The mast cells were used only when >95% of the cells were FcεRI+ (~8 weeks of culture). A published diagram of the isolation procedure with representative figures can be found in Troupin et al. (26).

Proteome Arrays

Cell-free supernatants were profiled with the membrane-based Human Angiogenesis Proteome ProfilerTM Array (R&D Systems) according to the manufacturers’ instructions. The blots were scanned on an Odyssey® CLx Infrared Imaging System (LI-COR Biosciences).

Cytokine Measurements

Cytokines in cell-free medium were measured with enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) according to the manufacturers’ instructions. Absorbance values were obtained with a BioTek Synergy HT microplate reader, and cytokine concentrations were determined using Gen5 Data Analysis Software.

IgE Sensitization and FcεRI Crosslinking

Mast cells were incubated in X-VIVO 15TM media containing SCF (100 ng/ml) and chimeric human IgE anti-NP (1 µg/106 cells) (clone JW8/1; AbD Serotec) overnight at 37°C, 5% CO2. The cells were washed and re-suspended at 106 cells/ml in X-VIVO 15TM media and activated with the hapten 4-hydroxy-3-nitrophenylacetyl conjugated to bovine serum albumin at a 16:1 molar ratio (NP-BSA; Biosearch Technologies) at the indicated concentration at 37°C for the indicated amount of time.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 6.0c for Mac OS X, GraphPad Software, La Jolla California USA, www.graphpad.com.
RESULTS

Human Skin-Derived Mast Cells Constitutively Secrete Several Angiogenesis-Related Factors

It is well-established that mast cells store and spontaneously secrete VEGF (16, 17). In our studies, VEGF was consistently detected at high levels in supernatants from non-activated human skin mast cells (18). Therefore, we sought to determine if other angiogenesis-related proteins, in addition to VEGF, were also secreted constitutively from tissue-derived mast cells. To do so, human skin-derived mast cells were washed and cultured in serum-free medium containing only stem cell factor (SCF), which is required for differentiation and survival of mast cells (27–31), and soybean trypsin inhibitor (SBTI) to prevent proteolysis of secreted proteins by endogenous proteases (32). After a 24 h incubation period, the cell-free medium was analyzed with the Human Angiogenesis Proteome Profiler™ Array (R&D Systems), which enabled us to detect 55 different angiogenesis-related proteins. To our surprise, several proteins—CXCL16, DPPIV, Endothelin-1, GM-CSF, IL-8, MCP-1, Pentraxin 3, Serpin E1, Serpin F1, TIMP-1, Thrombospondin-1, and uPA—were detected at surprisingly high levels with this proteome array analysis (Figure 1). It was particularly interesting that these proteins were secreted at levels much greater than VEGF.

To validate the proteome profiler array data, we cultured human skin-derived mast cells from different donor tissues in serum-free medium containing only SCF and SBTI for 24 h, and measured IL-8, VEGF, MCP-1, GM-CSF, TIMP-1, and Serpin F1 with ELISA. As shown in Figure 2A, all proteins analyzed were detected at quantifiable levels after 24 h in culture under non-stimulated conditions. Importantly, TIMP-1 and VEGF, which were detected by proteome array at high and low levels, respectively, were also detected at high and low quantities with ELISA. Thus, the ELISA data essentially mirrors the relative signal intensities of the proteome array.

In addition, we also determined the concentration of IL-8, VEGF, MCP-1, TIMP-1, and Serpin F1 in media from cultures of resting skin-derived mast cells collected during routine (every 7 days) media changes. IL-6 and TNF were also analyzed as positive and negative controls, respectively, since previous studies had shown that IL-6 but not TNF was spontaneously secreted by human skin mast cells (18, 33). As shown in Figure 2B, IL-8, VEGF, MCP-1, GM-CSF, TIMP-1, and Serpin F1 were all detected in the cell free medium. In agreement with the proteome array data, TIMP-1 and Serpin F1 were detected at extremely high concentrations, followed by MCP-1, IL-8, and VEGF. As expected, IL-6 (positive control) was detected whereas TNF (negative control) was not. It is worth noting that the time in culture, and cell densities of the different mast cell cultures was variable at the time the media was collected, and that SBTI, which is not usually added to the culture media, was not present in the media collected from the established cultures. Together, these findings demonstrate that human skin-derived mast cells spontaneously secrete a variety of angiogenesis-related proteins, in addition to VEGF, at high levels in the absence of any exogenously added stimuli.

Dependence on Stem Cell Factor

To determine if secretion of the angiogenesis-related factors was due to stimulation of c-kit by exogenously added SCF, we cultured human skin-derived mast cells with and without SCF (100 ng/ml) in serum-free media containing only SBTI for 24 h, and analyzed the cell-free medium with the Human Angiogenesis Proteome Profiler™ Array. As shown in Figure 3, there was no difference in secretion of CXCL16, DPPIV, and uPA in the presence or absence of SCF, whereas endothelin-1, GM-CSF, IL-8, MCP-1, and VEGF secretion was almost completely abolished in the absence of SCF. In addition, secretion of Pentraxin 3, Serpin E1, Serpin F1, TIMP-1, and Thrombospondin-1 was significantly reduced, but still detected at very high levels in the absence of SCF. Thus, we have identified three groups of angiogenesis-related proteins whose secretion is independent (CXCL16, DPPIV, and uPA), dependent (endothelin-1, GM-CSF, IL-8, MCP-1, and VEGF), or somewhat dependent (Pentaxin 3, Serpin E1, Serpin F1, TIMP-1, and Thrombospondin-1) on SCF.

FcεRI Crosslinking Augments and Inhibits Secretion of Angiogenesis-Related Proteins

To determine the effect of FcεRI crosslinking on secretion of angiogenesis-related proteins particularly those found to be spontaneously secreted, human skin-derived mast cells were sensitized with anti-DNP IgE, and then challenged with DNP-HSA (100 ng/ml) for 24 h. The cell-free medium was collected, and analyzed with the Human Angiogenesis Proteome Profiler™ Array as in the previous experiments. As shown in Figure 4A, FcεRI crosslinking increased the secretion of GM-CSF, IL-8, Serpin E1, and VEGF, and induced the secretion of Amphiregulin and MMP-8. Surprisingly, FcεRI crosslinking also resulted in significant reduction in spontaneous secretion of CXCL16, Endothelin-1, Serpin F1, Thrombospondin-1, MCP-1, and Pentraxin-3 (Figure 4B). To confirm the proteome array data, the experiment was repeated and IL-8, VEGF, TIMP-1, GM-CSF, Serpin F1, and MCP-1 in the cell-free supernatants were quantified with ELISA (Figures 4C–H). Confirming the proteome array data, IL-8, VEGF, TIMP-1, and GM-CSF were increased following FcεRI crosslinking, whereas Serpin F1 and MCP-1 secretion was inhibited.

It is well-known that FcεRI signals upregulate cytokine gene expression and secretion. Therefore, it was surprising that the spontaneous secretion of some proteins was inhibited following FcεRI crosslinking. To rule out the possibility that free NP-BSA in the sample interfered with the antigen-antibody binding in the ELISA assay, we generated standard curves of MCP-1 and Serpin F1 in the presence and absence of NP-BSA (100 ng/ml). We observed no significant shift in the curves with NP-BSA (Figure S1), indicating that the observed FcεRI-induced inhibition in secretion was not likely due to interference by free NP-BSA with the ELISA. Thus, FcεRI crosslinking augments and
inhibits the spontaneous secretion of angiogenesis-related factors from mast cells.

**IL-6 Induces MCP-1 Secretion**

Previously, we demonstrated that IL-6 could induce VEGF synthesis and secretion from human skin mast cells (18). Therefore, to determine if angiogenesis-related factors other than VEGF were also induced with IL-6, we cultured human skin-derived mast cells without and with IL-6 (100 ng/ml) for 24 h, and analyzed the cell-free medium with the Human Angiogenesis Proteome Profiler™ Array. Interestingly, among all the proteins detectable with the array, the most noteworthy observation was a robust and significant increase in MCP-1 in the presence of added IL-6 (Figure S2 and Figure 5A). To validate the array data, human skin-derived mast cells were cultured without
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FIGURE 3 | Spontaneous secretion of angiogenesis-related proteins from human mast cells is variably dependent on stem cell factor. Human skin mast cells were cultured in serum-free media containing SBTI ± SCF for 24 h, and the cell-free supernatant was analyzed with the Human Angiogenesis Proteome Profiler™ Array (R&D Systems). The graph bars represent the mean signal intensity ± S.E.M. (n = 4) obtained from membrane arrays incubated with media from individual mast cell cultures prepared from skin tissue of different donors. Significance was determined with Student’s t-test. * p < 0.05; ** p < 0.01; *** p < 0.01; # p < 0.001.

and with IL-6 (100 ng/ml) for 6 h, and MCP-1 in the cell-free medium was measured with ELISA. As shown in Figure 5B, mast cells cultured with MCP-1 secreted significantly more MCP-1 compared to those cultured without IL-6. These findings demonstrate a previously unknown ability of IL-6 to potentiate MCP-1 secretion from human skin-derived mast cells.

DISCUSSION

Mast cells are classically known to be involved in allergic and inflammatory reactions. However, their ability to secrete VEGF, and to localize in and around solid tumors has suggested a role for mast cells in tumorigenesis. Here, we report the novel observation that human skin-derived mast cells spontaneously secrete several proteins that, in addition to VEGF, have the potential to influence angiogenesis and vascular development. Specifically, we identified CXCL16, DPPIV, Endothelin-1, GM-CSF, IL-8, MCP-1, Pentraxin 3, Serpin E1, Serpin F1, TIMP-1, Thrombospondin-1, and uPA as being spontaneously secreted at very high levels from human skin-derived mast cells. Functionally, CXCL16 (34), Endothelin-1 (35, 36), GM-CSF (37), IL-8 (38), MCP-1 (39), and uPA (40) are reportedly pro-angiogenic, whereas Serpin E1 (Pigment Epithelium-Derived Factor) and Thrombospondin-1 are considered endogenous inhibitors of angiogenesis (41). However, some studies indicate a pro-angiogenic property of Thrombospondin-1 (42). Similarly, Pentraxin-3 (43), DPPIV (44–46), TIMP-1 (36, 47), and Serpin E1 (Plasminogen Activator Inhibitor) (48, 49) have each also been implicated as having both pro- and anti-angiogenesis properties. Additional studies will determine the specific contribution of each of these mast cell-derived proteins in tumor development, but the fact that most of these factors were secreted at levels much greater than VEGF suggests that they could potentially have a greater influence on mast cell-mediated angiogenesis and tumor development than VEGF.

Our finding that Endothelin-1, GM-CSF, IL-8, MCP-1, and VEGF secretion was almost completely abolished in the absence of SCF, and that Pentraxin 3, Serpin E1, Serpin F1, TIMP-1, and Thrombospondin-1 secretion was also diminished, albeit to a much lower extent, indicates that c-kit signals play a significant role in the secretion of these factors. On the other hand, it is possible that the observed inhibition in secretion of some proteins in the absence of SCF is simply a reflection of an overall reduction in cellular function. However, this appears to be unlikely since not all proteins were equally affected with some proteins being markedly more sensitive to SCF withdrawal than others. The fact that CXCL16, DPPIV, and uPA secretion was completely unaffected by SCF withdrawal indicates that loss of kit signaling rather than a global reduction in protein secretion. Interestingly, the fact that mast cells require SCF for survival suggests that the existence of mast cells and secretion of these angiogenesis regulating factors go hand-in-hand, perhaps indicating a primordial role for mast cells in angiogenesis and neovascularization in development.

FcεRI signals are generally considered positive signals that result in the induction of cytokine gene expression and protein production, and other functional events. Therefore, it was surprising to find that secretion of CXCL16, Endothelin-1, Serpin F1, Thrombospondin-1, MCP-1 and Pentraxin-1 was significantly inhibited following FcεRI crosslinking. We ruled out the possibility that this was due to interference by unbound NP-BSA with the ELISA assay. However, another possibility is that the observed reduction was due to cleavage by a
FIGURE 4 | FcεRI crosslinking enhances and inhibits human mast cell secretion of angiogenesis-related factors. Human skin mast cells were sensitized with anti-DNP IgE and then activated with DNP-HSA (100 ng/ml) for 24 h in serum-free media containing SCF and SBTI. The cell-free supernatants were analyzed with the Human Angiogenesis Proteome Profile™ Array (R&D Systems) (A,B). The graph bars represent the mean signal intensity ± S.E.M. (n = 4) obtained from membrane arrays incubated with media from individual mast cell cultures prepared from skin tissue of different donors. In separate experiments with different mast cell cultures, media was collected and analyzed with ELISA for IL-8 (C), VEGF (D), TIMP-1 (E), GM-CSF (F), Serpin F1 (G), and MCP-1 (H). The data points in (C–H) represent values obtained from mast cells isolated from individual skin tissue of three different donors. Significance was determined with Student’s t-test. *p < 0.05; **p < 0.01; ***p < 0.001; #p < 0.01.

protease not susceptible to inhibition by SBTI that are released from mast cells following activation. If so, the fact that FcεRI crosslinking led to increased secretion of GM-CSF, IL-8, Serpin E1, and VEGF production, and also induced the secretion of Amphiregulin and MMP-8 indicates that not all proteins were susceptible to degradation by the putative protease. A more intriguing possibility is that FcεRI signals act to inhibit the spontaneous secretion of these proteins, perhaps by interfering with kit signaling or other unknown signals. It is interesting to speculate on whether signals from FcεRI, or other receptors, serve to negatively regulate the intrinsic spontaneous secretion of angiogenesis regulating proteins from mast cells. The current study also demonstrated that treatment with IL-6 significantly induced the secretion of pro-angiogenic MCP-1 from human skin-derived mast cells. We previously demonstrated that IL-6 could induce the production of VEGF (18), which we also observed here. Together, these data together indicate that IL-6 can indirectly induce mast cell-mediated angiogenesis by inducing the secretion of MCP-1 and VEGF.

Mast cells are heterogeneous population of cells (50). In humans, two subpopulations were identified over 30 years ago based on their expression of chymase (C) and tryptase (T) in their cytoplasmic granules: MCCT and MCT (51).
Immunochemical analysis demonstrated that the MC_{TC} type expresses both tryptase and chymase whereas the MC_{T} type expresses only tryptase. MC_{TC} type cells are the predominant type in the skin whereas MC_{T} cells make up the majority of mast cells in lung and mucosa of the small intestine. Ultrastructural analysis by immunoelectron microscopy revealed that both MC_{T} and MC_{TC} mast cells have large numbers of cytoplasmic granules. However, MC_{TC} mast cell granules are more uniformly electron dense and larger than MC_{T} granules, which are more variable in shape than MC_{TC} granules (52).

Cultured skin-derived mast cells, such as those used in this study, have been extensively studied and characterized, and are believed to be representative of in situ-matured MC_{TC} mast cells. In addition to expressing both chymase and tryptase, various studies have demonstrated that cultured skin-derived mast cells express carboxypeptidase and complement factor 5a receptor (CD88) whereas lung MC_{T} cells do not (53, 54), A3AR adenosine receptor at significantly lower levels compared with dispersed lung mast cells (55), complement factors C3a and C5a (56), FcεRIα (57), gp130, and membrane-bound IL-6Rα (18). However, whether skin-derived mast cells that have been cultured for an extensive period of time are phenotypically identical to those that were initially dispersed from the skin tissue or resident in skin tissue is not clear. It is also possible that cultured skin-derived mast cells developed in vitro from progenitors and are distinct from in situ-matured mast cells that were originally isolated. Indeed, mast cell progenitors have been found in human skin follicles (58). Therefore, longstanding question of whether cultured skin-derived mast cells are phenotypically and functionally identical to tissue resident mast cells remains to be answered.

Overall, this study shows that human skin-derived mast cells have an intrinsic ability to spontaneously secrete several proteins, in addition to VEGF, with the potential to regulate blood vessel formation. The study further suggests that extracellular signals from FcεRI and very likely other receptors expressed on the mast cell surface could serve to modulate the spontaneous secretion of some proteins. In addition to supporting the known role for mast cell in tumorigenesis, these findings raise the possibility of a critical role for mast cells in development.

**DATA AVAILABILITY**

All datasets generated for this study are included in the manuscript and/or the Supplementary Files.

**ETHICS STATEMENT**

This study has been reviewed by the Institutional Review Board (IRB) of the University of South Carolina, and has been deemed exempt from Protection of Human Subjects Research regulations. Therefore, it does not qualify as human subjects research. The skin tissues used to obtain the mast cells for this study were purchased from the NCI-sponsored Cooperative Human Tissue Network (CHTN). Subjects from whom tissues are obtained by the CHTN are consented by that organization. Subject identifiers are not provided to the investigators.

**AUTHOR CONTRIBUTIONS**

CM performed the experiments and assisted with data analysis. ZM assisted with experiments and immunoassay development. CM and ZM isolated and purified the mast cells. GG directed the project, analyzed the data, and wrote the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01445/full#supplementary-material
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