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Increased Stem Cell Factor Release by Hemangioma-Derived Endothelial Cells

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BACKGROUND: Capillary hemangiomas, the most common tumors in young children, consist of proliferating capillary vessels and endothelial cells. These tumors also contain large numbers of mast cells, compared with the normal surrounding skin or tissue. We have recently shown that stem cell factor (SCF), the gene product of the murine steel locus, can act as a chemoattractant for mast cells. In this study, we investigated whether SCF might be involved in the recruitment and maintenance of mast cells in hemangiomas.

EXPERIMENTAL DESIGN: Cultured endothelial cells derived from a murine hemangioma were compared with normal vascular endothelial cells for the ability to produce and release SCF, a mitogen for mast cells.

RESULTS: Conditioned medium from hemangioma-derived endothelial cells stimulated the proliferation of cultured mast cells. This proliferative activity was potentiated by interleukin-3. The same conditioned medium was unable to stimulate proliferation of mast cells expressing a defective receptor for SCF. The medium was also unable to stimulate proliferation when it was preincubated with neutralizing antibodies specific for SCF. Immunoprecipitation and Western blot analysis of the conditioned media from hemangioma cells and normal endothelial cells demonstrated the 31,000 molecular weight SCF in hemangioma-conditioned medium only. In addition, proliferative activity for mast cells could not be demonstrated in the conditioned medium of the normal endothelial cells, although Northern blot analysis indicated that both normal and hemangioma-derived endothelial cells express SCF mRNA. Reverse transcriptase-polymerase chain reaction techniques were used to amplify the DNA sequence coding for the proteolytic cleavage site used for release of SCF. Results indicated that both normal and hemangioma-derived endothelial cells express the same transcript for SCF.

CONCLUSIONS: Our data suggest that increased release of SCF is a property of hemangioma-derived endothelial cells that may account for the high numbers of mast cells observed in hemangioma tissue. This increased release of SCF is not due to alternate splicing of SCF transcripts by hemangioma cells.

Additional key words: Mast cells, Steel factor, *kit* ligand, Mast cell growth factor, Angiogenesis.

Hemangiomas, the most common tumors of infancy, vary from small, innocuous marks to mutilating, and in some cases life-threatening, lesions. Small hemangiomas may impinge on vital structures, such as the cornea or upper airways, whereas hemangiomas that grow to a large size may actually distort adjacent structures and cause functional impairment. Congestive heart failure often occurs with hepatic hemangiomatosis (1), and acute respiratory distress is a complication of subglottic hemangiomas (2). Gastrointestinal bleeding and obstruction can occur as well as perforation of the small intestine (3). A life-threatening thrombocytopenia with widespread hemorrhage (Kasabach-Merritt syndrome) may occur in association with a single enlarging hemangioma or diffuse hemangiomatosis (4).

Hemangiomas consist of an irregular proliferation of capillary vessels and/or endothelial cells. They characteristically exhibit a period of rapid proliferation followed by slow spontaneous involution and disappearance. Interesting to note, the presence of mast cells correlates with the growth of this tumor. The density of mast cells in rapidly growing hemangiomas is at least five times that of normal skin, and the density returns to normal at the time of tumor regression (5, 6). The signal(s) responsible for these changes in mast cell density have not been explored.

Mast cells can exhibit directed motility (*i.e.*, chemotaxis). Mast cells migrate in response to tumor implantation (7), tumor-derived peptides (8), matrix components, such as laminin (9), and interleukin-3 (IL-3) (10).

We have also found that mast cells migrate in response to stem cell factor, a hematopoietic growth factor (11). Stem cell factor (SCF) (12), also known as mast cell growth factor (MCGF) (13) or *kit* ligand (14), is a potent stimulator of mast cell proliferation and can potentiate IL-3-induced proliferation. Using cells derived from a murine hemangioendothelioma (15), we investigated whether altered production or release of SCF might be a property of hemangiomas by comparing the amount of SCF produced and released by hemangioma-derived endothelial cells relative to normal endothelial cells.

EXPERIMENTAL DESIGN

Cultures of endothelial cells derived from murine hemangiomas, murine aorta, and murine lung and brain microvessels were established. Growth media conditioned by these cells were tested for the ability to stimulate bone marrow-derived mast cell proliferation, a characteristic of SCF. Confirmation of SCF identity was achieved by antibody neutralization of mitogenic activity as well as by immunoprecipitation followed by Western blot analysis. Northern analysis was used to indicate steady state levels of mRNA for SCF. Reverse transcriptase-polymerase chain reaction techniques were used to determine if the release of SCF was a function of alternative splicing of mRNA transcripts produced in these cells.

RESULTS AND DISCUSSION

To determine whether hemangioma cells produce and release a factor(s) that stimulate(s) the proliferation of mast cells, we tested the ability of medium conditioned by cells of a spontaneous hemangioma (EOMA cells) to stimulate the proliferation of cultured mast cells (Fig. 1*A*). Undiluted conditioned medium produced a two to three-fold increase in mast cell proliferation relative to unstimulated controls. Dilution of the conditioned medium reduced the proliferative response, indicating that the response was dose-dependent. The response to undiluted conditioned medium was equivalent to the response of the mast cells to 10 ng/ml recombinant rat SCF (rrSCF). The proliferative response to conditioned medium was potentiated by the presence of 10 U/ml IL-3, as has been shown previously for purified SCF (12).

W^{42} mast cells possess a defective c-*kit* receptor tyrosine kinase and do not grow or migrate in response to SCF. As shown in Figure 1*B*, these cells failed to proliferate in the presence of EOMA-conditioned medium, suggesting that the proliferative activity is mediated *via* activity of the c-*kit* receptor. The result was not due to a general loss of proliferative potential in these cells; they demonstrated a normal proliferative response to IL-3.

To verify that the proliferative activity in the conditioned medium from the EOMA cells was due to the presence of SCF, the conditioned medium was preincubated with antibody capable of neutralizing the activity of rrSCF. One hour of incubation with antibody was sufficient to completely abolish the proliferative activity in the conditioned medium or in control medium containing 50 ng/ml rrSCF (Fig. 2). These results suggest that all of the mast cell stimulatory activity present in hemangioma cell-conditioned medium can be attributed to SCF.

To determine whether normal endothelial cells also release SCF, conditioned media from the hemangioma-derived (EOMA) and aortic (MAEC) endothelial cells were compared. Significant proliferative activity for cultured mast cells was noted only in the EOMA-conditioned medium (Fig. 3). The proliferative response of the mast cells to EOMA-conditioned medium supplemented with 10 U/ml of IL-3 was significantly higher than the proliferative response to 10 U/ml of IL-3 alone, indicating that the proliferative response was not due merely to the presence of IL-3. No statistically significant proliferative activity was noted in the conditioned medium from MAEC when tested alone or when supplemented with IL-3.

Murine stem cell factor (*kit* ligand-1 [KL-1]) is synthesized as a 45 kD (248 amino acid)-membrane protein (13, 14, 16, 17). The membrane-bound form is processed by a serine protease at the cell surface (18). Proteolytic cleavage releases a soluble, biologically active form of stem cell factor with a molecular weight of approximately 31 kD. This soluble protein is analogous to the isolated natural rat protein that has been completely sequenced and that contains 164 or 165 amino acids (19). Both rat

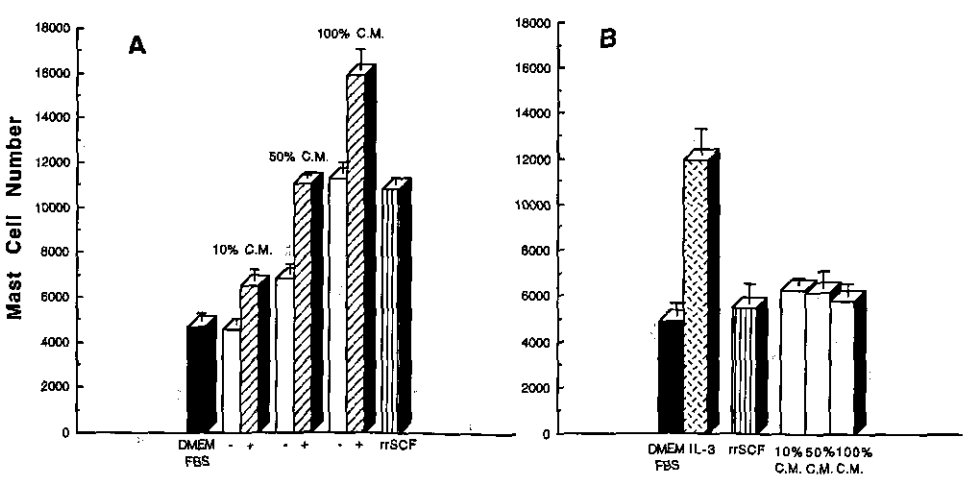

FIG. 1. A Dose-dependent stimulation of mast cell proliferation by EOMA conditioned medium. Cultured bone marrow-derived mast cells were incubated with basal medium alone, basal medium containing 10 ng/ml rrSCF, or conditioned medium (C.M.) from the hemangioendothelioma cells with (+) or without (−) 10 U/ml IL-3. Cell numbers were determined after 72 hours of incubation. Data represent means ± SEM. *B* Failure of mutant mast cells to respond to EOMA conditioned medium. W^{42} mast cells were incubated with basal medium, with basal medium containing high levels of IL-3, with basal medium containing 100 ng/ml rrSCF and in various concentrations of EOMA conditioned medium. Cell numbers were determined after 72 hours of incubation. Data represent means ± SEM.

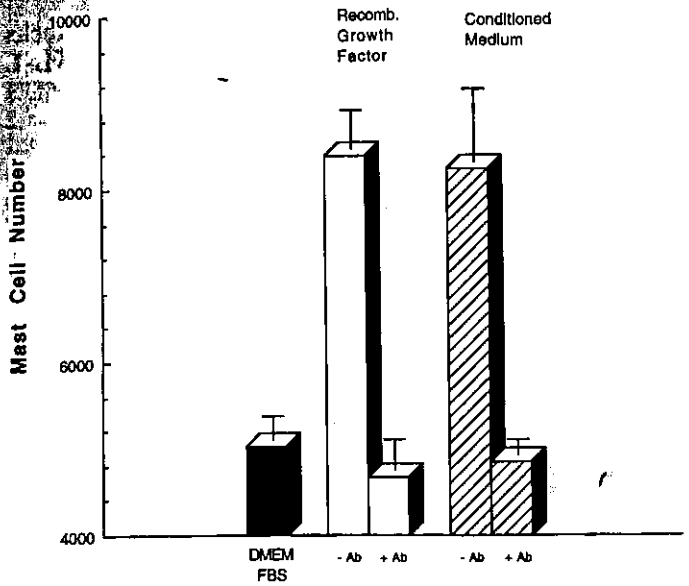

FIG. 2. Effect of anti-SCF antibodies on the mast cell proliferative activity in EOMA conditioned medium. Cultured bone marrow-derived mast cells were incubated with basal medium alone, or in basal medium containing 50 ng/ml rrSCF and EOMA conditioned medium preincubated with (+) or without (−) polyclonal antibodies against rat SCF. Cell numbers were determined after 72 hours of incubation. Data represent means ± SEM.

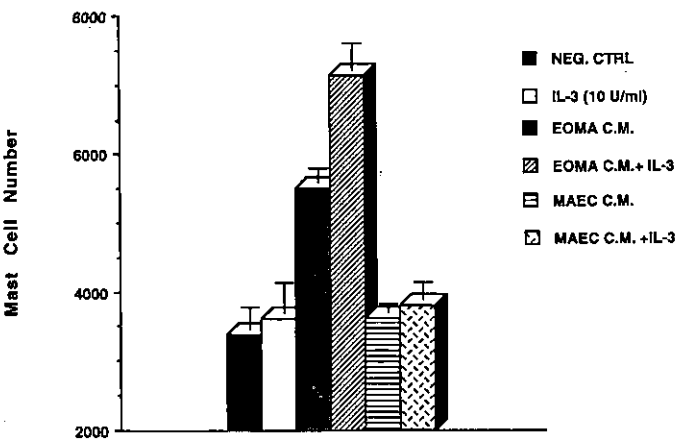

FIG. 3. Comparison of the mast cell proliferative activity in EOMA conditioned medium and medium conditioned by normal endothelial cells. Cultured bone marrow-derived mast cells were incubated with basal medium alone, basal medium containing 10 U/ml IL-3, or undiluted conditioned medium from EOMA or MAEC with or without 10 U/ml IL-3. Cell numbers were determined after 72 hours of incubation. Data represent means ± SEM.

and human SCF migrate over a range of molecular weight of 28,000 to 35,000 on sodium dodecyl sulfate (SDS) polyacrylamide gels because of extensive and heterogeneous glycosylation of the protein (19, 20).

A protein with a molecular weight of approximately 31,000 was precipitated from the conditioned medium of the EOMA cells (Fig. 4, *lane 3*). This same protein could not be precipitated from the conditioned medium of the MAEC (Fig. 4, *lane 2*), even though it was prepared from three times the number of cells used to generate conditioned medium from the EOMA cells. The major bands at approximately 25 and 50 kD represent the light and heavy chains of the rabbit immunoglobulin that were dissociated from the Protein-A Sepharose (as visualized in Fig. 4, *lane 1*, containing fresh, nonconditioned medium subjected to immunoprecipitation). A second band appearing at approximately 45 kD in the EOMA-conditioned medium has not been conclusively identified but may represent full-length SCF.

Northern blot hybridization using a ^{32}P-labeled cDNA probe specific for murine SCF revealed a single band of radioactivity at approximately 7.5 kb (Fig. 5). This mRNA for SCF was present in both the MAEC and EOMA cells. This suggests that both endothelial cell types have the ability to make SCF mRNA and, presumably, SCF protein. Human umbilical vein endothelial cells have recently been shown to express SCF mRNA, although the existence of the protein in these cells was not demonstrated (21).

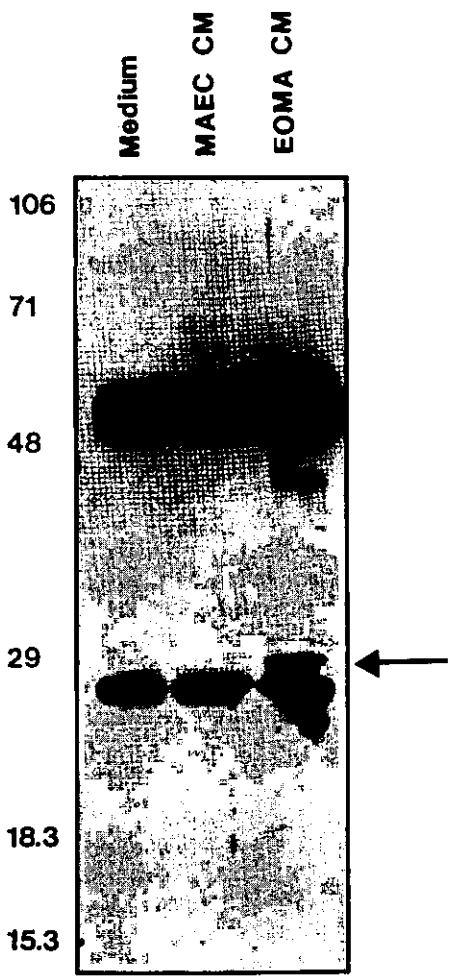

FIG. 4. Demonstration of stem cell factor in conditioned media of EOMA and normal endothelial cells. Conditioned media from EOMA and MAEC as well as fresh, nonconditioned medium were immunoprecipitated with rabbit anti-murine SCF and Protein-A Sepharose, separated by SDS polyacrylamide gel electrophoresis, blotted to nitrocellulose, and probed with the same polyclonal antibody specific for SCF. Immunodetected proteins were visualized using a chemiluminescence detection system. Soluble SCF (molecular weight ≈ 31,000) is indicated by the *arrow*. Molecular weight standards (in kilodaltons) are also indicated at the left. *Lane 1*, fresh medium; *lane 2*, MAEC-conditioned medium; *lane 3*, EOMA-conditioned medium.

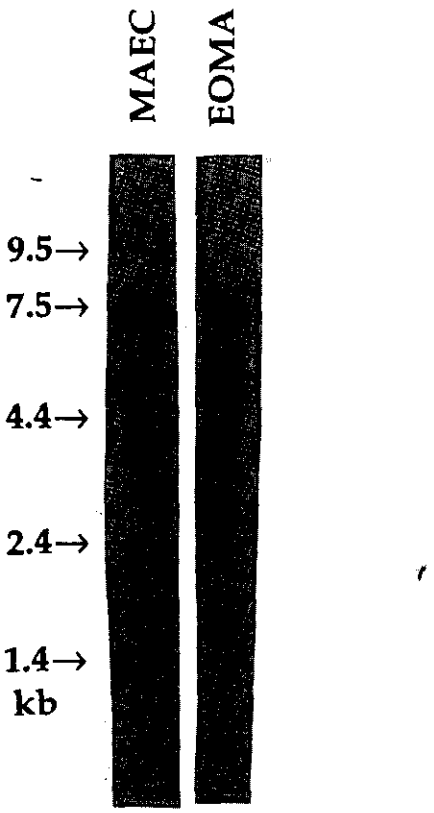

FIG. 5. Northern blot analysis of mRNA from EOMA and MAEC. Three micrograms of mRNA from EOMA and MAEC were separated on an agarose-formaldehyde gel, transferred to nylon membranes, hybridized with a ^{32}P-labeled cDNA probe specific for murineSCF, and processed for autoradiography.

Several growth factors are synthesized as transmembrane precursors that are proteolytically processed to generate soluble factors that function in juxtacrine and/or paracrine stimulation. Colony stimulating factor-1 (CSF-1), which is genetically and structurally related to SCF (22), has been shown to be cleaved proteolytically to produce a soluble growth factor (23, 24). Other membrane growth factors include tumor necrosis factor α (TNFα) (25) as well as transforming growth factor α (TGFα) and several other members of the epidermal growth factor family (18).

Little work has been done to examine the regulation of SCF expression and processing. Huang *et al.* (17) demonstrated that proteolytic cleavage of the membrane-bound SCF in COS-1 transfected cells was stimulated by the protein kinase C inducer phorbol 12-myristate 13-acetate and the calcium ionophore A23187, suggesting differential regulation of this process. The data presented here indicate an inherent difference between the endothelial cells of the hemangioma and those found in the normal vasculature. The data suggest that hemangioma-derived endothelial cells are able to release soluble SCF whereas normal endothelial cells are not. Both cell types make SCF and transport it to the cell surface, but only the hemangioma-derived cells appear to be able to release significant quantities of the soluble protein.

Huang *et al.* (17) showed that the soluble form of SCF is generated by efficient proteolytic cleavage from a transmembrane precursor termed *kit* ligand-1 or KL-1. KL-2, an alternatively spliced version of KL-1, lacks exon 6, which codes for the cleavage site found in KL-1. An alternative but much less efficient cleavage site exists in KL-2. As a consequence, the protein encoded by KL-2 tends to remain cell-associated. Flanagan *et al.* (26) reported that the ratio of KL-1 to KL-2 mRNAs varied in different tissues, indicating that the alternative splicing event that creates them is regulated in a tissue-specific manner. They suggested that regulated alternative splicing may provide a mechanism for the differential expression of the cell surface or soluble forms of SCF in different cells. To determine whether differential release of soluble SCF by hemangioma-derived and normal endothelial cells was due to alternative splicing, reverse transcriptase-polymerase chain reaction (RT-PCR) techniques were used to identify the transcripts in these cells. Primers were designed to amplify a portion of the DNA sequence that included exon 6. The amplified segment of DNA was predicted to be 185 base pairs in length if exon 6 was present, and 101 base pairs in length if exon 6 was absent.

Figure 6 illustrates the PCR products obtained by amplification of cDNA from BALB/c-3T3 fibroblasts (*lane 1*) as well as several hemangioma-derived endothelial cells (*lanes 2–4*) and normal endothelial cells (*lanes 5* and *6*). BALB/c-3T3 fibroblasts are known to express relatively large amounts of KL-1 (26) and were included as a positive control. Sl/Sl^d fibroblasts have a deletion in the SCF gene that removes the transmembrane and intracellular domains (26). These cells only produce a secreted form of SCF, which lacks a portion of the sequence amplified by our primers. Therefore, cDNA from these cells was included as a negative control (*lane 7*). A 185-base pair DNA fragment was amplified in 3T3 cells and represents the KL-1 transcript. No 101-base pair fragment was amplified, indicating the absence of the the KL-2 transcript. This finding supports the Northern analysis data that demonstrated only a single SCF mRNA (Fig. 5). Sl/Sl^d fibroblasts did not exhibit either PCR product as expected. The larger molecular weight bands in *lane 8* represent genomic DNA evident in control tubes containing no reverse transcriptase (*i.e.*, samples in which cDNA could not be synthesized from the RNA [data not shown]). The same 185-base pair fragment amplified in the 3T3 fibroblasts was amplified in three different hemangioma-derived endothelial cell lines: the spontaneous EOMA cells (*lane 2*) as well as the retrovirus-induced Py4–1 (*lane 3*) and bEnd cells (*lane 4*). These data suggest that the production of SCF is a general property of hemangiomas and not just a property of the EOMA cells. In addition, the same transcript was identified in aortic (*lane 5*), lung (*lane 6*), and brain (data not shown) endothelial cells, indicating that this is a general property of endothelial cells, both large vessel and microvascular in origin. Finally, RT-PCR techniques were also used with human microvascular endothelial cells and primers specific for the human SCF sequence. The larger KL-1 sequence was amplified in these cells as well (data not shown), indicating that production of SCF is not just a property of murine endothelial cells. However, a small amount of the KL-2 cDNA was also amplified in the human cells, which was not evident in the murine endothelial cells.

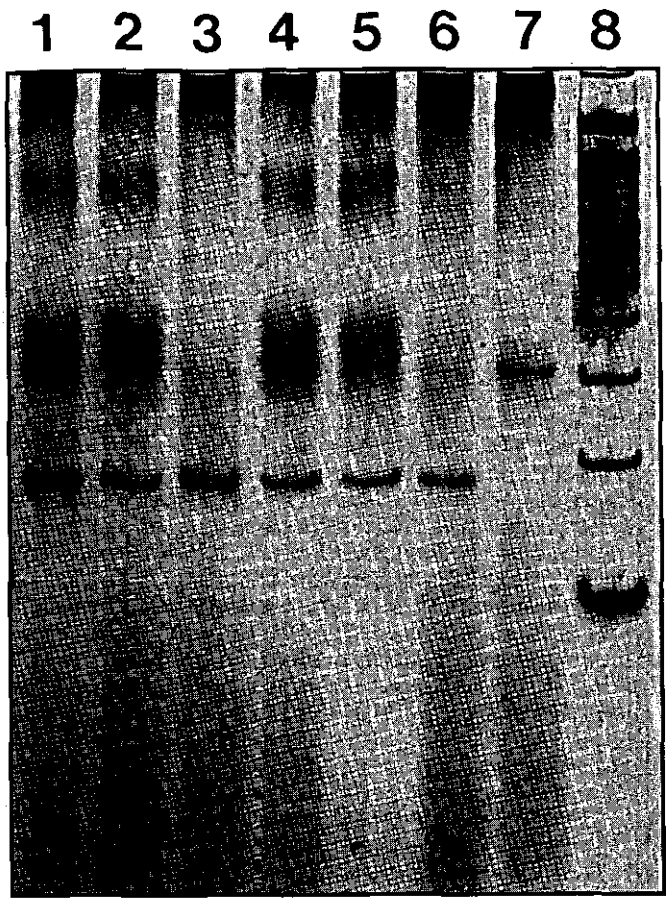

FIG. 6. Polymerase chain reaction (PCR) amplification of SCF cDNAs from hemangioma-derived and normal endothelial cells. PCR was performed on first strand cDNA prepared using reverse transcriptase and total RNA from (a) BALB/c-3T3 fibroblasts, (b) EOMA cells, (c) Py4-1 cells, (d) bEnd cells, (e) murine aortic endothelial cells, (f) murine lung microvascular endothelial cells, and (g) Sl/Sl^d fibroblasts. The PCR products were separated on an 8% polyacrylamide gel under nondenaturing conditions. DNA bands were demonstrated by silver staining. A DNA ladder, included in *lane 8*, indicates the migration of DNA fragments in 100-base pair increments (100, 200, 300 base pairs, etc.)

Angiogenesis is the process by which new capillaries are formed from preexisting blood vessels. Endothelial cells respond to angiogenic stimuli by producing proteases to degrade their investing basement membrane and surrounding matrix. The endothelial cells then migrate into the interstitium and proliferate to form sprouts. The anastomosis of two sprouts generates new capillary loops through which blood may flow. Expression of the polyoma virus middle T (mT) oncogene severely perturbs the ability of endothelial cells to form normal vascular structures. Infection of mice with a retrovirus carrying the mT oncogene results in the rapid formation of cavernous hemangiomas (27). These hemangiomas provide a source of stable endothelioma (End) cell lines that retain specific endothelial cell markers and express mT antigen. Injection of these cells into mice results in the rapid formation of hemangiomas, which demonstrates that these cells are the primary cause of the lesions (28). Montesano *et al.* (29) demonstrated that the brain End (bEnd) hemangioma cells exhibited increased production of urokinase-type plasminogen activator and decreased production of plasminogen activator inhibitors. If this excess proteolytic activity was neutralized by adding exogenous serine protease inhibitors, normal capillary-like tubules were formed by the cells. These findings suggest that the difference between hemangioma-forming endothelial cells and normal endothelial cells lies in their protease production. Increased protease production would provide the means for release of SCF by the hemangioma-derived endothelial cells that was diminished or absent in normal endothelial cells. Western blotting techniques were used to demonstrate the presence of SCF in all endothelial cells tested in this study, whereas immunoprecipitation followed by Western blotting confirmed the release of SCF into the medium of the three different hemangioma-derived endothelial cells and confirmed the absence of release into the medium of normal brain microvascular endothelial cells (data not shown).

Hemangiomas are benign tumors made up of proliferating endothelial cells and capillaries and are found in children. Mast cells are observed in large numbers in these proliferating hemangiomas both in humans and in mice injected with EOMA cells (personal communication, Dr. Michael O'Reilly, Children's Hospital, Harvard Medical School, Boston). The presence of mast cells in hemangiomas has never been completely understood. The data presented in this study indicate that endothelial cells in the hemangioma release soluble SCF, which may act to recruit mast cells into the hemangioma (ll). Once inside the tumor mass, the mast cells may also proliferate and degranulate in response to SCF (13, 30). Degranulation with subsequent release of mast cell products, such as heparin, histamine, or tumor necrosis factor α may then stimulate the growth of the endothelial cells of the hemangioma (5). Thus, a two-way interaction between endothelial and mast cell products may account for the rapid growth seen with this tumor.

METHODS

REAGENTS

Recombinant rat stem cell factor (rrSCF) and neutralizing antibodies for rrSCF were generously provided by Dr. Krisztina Zsebo, Amgen Inc., Thousand Oaks, CA. Polyclonal rabbit anti-murine stem cell factor as well as recombinant murine interleukin-3 (IL-3) were obtained from Genzyme (Cambridge, MA). One unit of IL-3 is defined as the amount required to support half-maximal tritiated thymidine incorporation by 2.5×10^3 FDC-P2 cells in 100-μl cultures as described by Prestidge *et al.* (31).

CELLS

The spontaneous hemangioma, or EOMA, cells were the kind gift of Dr. Robert Auerbach, University of Wisconsin, Madison, WI. These endothelial cells (15) were originally derived by Hoak *et al.* (32) from a mixed hemangioendothelioma arising spontaneously in a mouse. Py4-1 cells were generously provided by Dr. Victoria Bautch, University of North Carolina, Chapel Hill, NC. These endothelial cells were isolated from hemangiomas induced by expression of the polyoma early region gene in transgenic mice (33). They exhibit properties of differentiated endothelial cells but are tumorigenic when reintroduced into mice. Endothelioma cells isolated from primary murine brain endothelial cells (bEnd) infected with a middle T

antigen-expressing retrovirus (34) were obtained from Dr. Werner Risau, Max-Planck Institute, Bad Nauheim, Germany. These cells also represent a stable cell line exhibiting specific endothelial cell markers. In contrast to the Py4-1 cells, they express only polyoma middle T antigen. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, and 100 μg/ml streptomycin. BALB/c-3T3 fibroblasts (obtained from the American Type Culture Collection, Rockville, MD) and Sl/Sl^d fibroblasts (kindly provided by Dr. Alan Bernstein, Mount Sinai Hospital, Toronto, Canada) were cultured in the same medium. MAEC as well as brain and lung microvessel endothelial cells (also provided by Dr. Auerbach) were cultured under similar conditions, although the microvascular cells required 20% FBS.

Cultured bone marrow-derived mast cells (CMC) were isolated from marrow washed from femurs of WBB6 mice. Briefly, femurs were dissected free of muscle and connective tissue, and the marrow cavity was rinsed two times with 1 ml of sterile Minimal Essential Medium α (MEMα, GIBCO Laboratories, Grand Island, NY). The cells were pooled, centrifuged (200 g for 10 minutes), and resuspended at a concentration of 1 to 5 × 10^6 cells/ml in MEMα containing 10% FBS, 10% pokeweed mitogen-stimulated spleen cell conditioned medium as a source of IL-3 (35), 7 × 10^{-5} M 2-mercaptoethanol (2-ME), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. One half of the medium was replaced with fresh medium every 7 days. Only mast cells remain in culture after 30 days, as determined by positive staining with alcian blue.

Homozygous mutant W^{42}/W^{42} mast cells were the generous gift of Dr. Alan Bernstein. These cells express a c-*kit* receptor that is deficient in tyrosine kinase activity. They were maintained in the same culture medium as the CMC and were passaged every 3 to 4 days to keep the cell density at approximately 5 × 10^5 cells/ml.

Conditioned Medium

Conditioned medium used to stimulate mast cell proliferation was prepared by incubating cultures of EOMA or MAEC in serum-free MEMα for 24 hours. These conditioned media were collected, filtered, supplemented with FBS (to a final concentration of 10%) and 2-ME (to 7 × 10^6 M) and added to target mast cells for proliferation assays as described below. For immunoprecipitation and Western analysis, the cells were grown in DMEM and supplemented as described above for culture. The media were collected, filtered, and immunoprecipitated with a polyclonal rabbit anti-murine SCF (see below).

Proliferation Assays

Four to 5000 CMC or W^{42} mutant mast cells were resuspended in fresh or conditioned medium (both containing 10% FBS and 7 × 10^{-5} M 2-ME [basal medium]) and plated in 96-well tissue culture trays. In some experiments, the conditioned medium was also supplemented with murine IL-3. The mast cells were incubated for 72 hours at 37° C and counted in a hemacytometer.

In the experiments involving neutralizing antibodies, conditioned medium and medium containing rrSCF were incubated with polyclonal rabbit anti-rrSCF antibodies (Amgen, Inc.) for 1 hour at 37° C before mast cells were added. The mast cells were incubated an additional 72 hours and counted as described above.

Immunoprecipitation and Western Blot Analysis

Conditioned media were prepared and collected as described above. The conditioned media were precleared (1 hour, room temperature) using affinity purified rabbit immunoglobulin linked to Protein A-Sepharose (Pharmacia, Piscataway, NJ) and centrifuged. The samples were then immunoprecipitated overnight at 4° C using polyclonal rabbit anti-murine stem cell factor (Genzyme, Cambridge, MA) at a concentration of 2 μg/ml previously bound to Protein-A Sepharose. The Sepharose with bound SCF was collected by centrifugation, rinsed well with Tris-buffered saline (TBS, 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl) and boiled in SDS sample buffer lacking 2-ME to release the SCF. After boiling, 2-ME was added to the samples, which were loaded on a 9.5 to 16% SDS polyacrylamide gradient gel and run under standard denaturing conditions (Laemmli). Separated proteins were blotted to nitrocellulose using 25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol, and 0.02% SDS in a wet transblot system (BioRad, Hercules, CA). The nitrocellulose was blocked with 3% gelatin in TBS and incubated with rabbit anti-murine SCF at a concentration of 5 μg/ml in TBS with 0.1% Tween 20 (TTBS) and 1% gelatin for 2 hours at room temperature or overnight at 4° C. The nitrocellulose was washed six times (5 minutes each wash) in TTBS, incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and diluted 1:20,000 in TTBS with 1% gelatin for 1 hour at room temperature. The nitrocellulose was again washed six times in TTBS, drained briefly, and incubated in Enhanced Chemiluminescence (ECL, Amersham, Arlington Heights, IL) substrate reagent for 1 minute. The blot was wrapped in Saran Wrap™ and exposed to film (ECL Hyperfilm, Amersham, Arlington Heights, IL) for 1 to 2 minutes. The molecular weights of the immunodetected bands from conditioned media were compared to molecular weight standards.

RNA Isolation. Poly A+ RNA (mRNA) was isolated from 1 to 2 × 10^7 cells using the Poly ATtract^R System (Promega Corp., Madison, WI). Briefly, cells were extracted in guanidine thiocyanate/SDS extraction buffer containing 2% 2-ME and sonicated for 15 to 30 seconds. Biotinylated oligo (dT) probe (2.5 μl of a 50-pmole/μl solution) was added to the RNA, mixed well, and incubated at 70° C for five minutes. The lysate was transferred to a sterile 15-ml Corex tube and centrifuged at 12,000 × g for 10 minutes at room temperature to clear the homogenate of cell debris and precipitated proteins. The supernatant was removed, added to a tube containing a suspension of streptavidin paramagenetic particles (in 0.5 mls of 0.5× (sodium chloride/sodium citrate) and mixed well. The magnetic particles with bound mRNA were immobilized with a magnet, and the supernatant was removed. The particles were washed four times with 0.1× SSC (1.5 ml per wash). After the final wash, the supernatant was removed, and the mRNA was eluted by suspending the magnetic particles in 1 ml of RNase-free water. The particles were immobilized with a magnet, and the aqueous phase containing the mRNA was transferred to a sterile RNase-free tube. The RNA was concentrated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol and by incubating overnight at −70° C. The RNA was pelleted by centrifugation at 12,000 × g for 10 minutes at 4° C and resuspended in RNase-free deionized water at 1 to 2 μg/μl.

Northern Hybridization. RNA samples were separated in 0.8% agarose-formaldehyde gels using 40 mM MOPS (pH 7.0)/10 mM sodium acetate/1 mM EDTA as the running buffer and running at 5 volts/cm for 4 hours. Three micrograms of mRNA were denatured in 2.2 M formaldehyde/50% of formamide (v/v) and loaded per lane. RNA molecular weight markers were also included on the gel. The separated nucleic acids were transferred overnight to Hybond^R nylon membranes (Amersham, Arlington Heights, IL) by wicking with northern transfer solution reagent (Biotecx, Houston, TX). After transfer, the positions of the RNA markers were marked on the membrane, and the nucleic acids were bound to the nylon by baking at 80° C for 2 hours in a vacuum oven. Membranes were

prehybridized in a low background hybridization solution (Biotecx, Houston, TX) containing 1 M NaCl, 0.1% SDS, and 1× blocking reagent (Biotecx, Houston, TX) for 1 hour at room temperature in a sealed bag. The prehybridization solution was removed and replaced with hybridization solution containing blocking reagent and a ^{32}P-labeled cDNA probe (5×10^5 dpm/ml of hybridization solution) for murine SCF (Oncogene Science, Uniondale, NY). The hybridization reaction was carried out overnight at 42° C. The membrane was removed from the bag and washed at room temperature in 1× SSC (20× SSC contains 3 M NaCl and 0.3 M sodium citrate, pH 7) and 0.1% SDS three times at room temperature for 7 minutes and three times at 55° C for 10 minutes. After washing, the filter was wrapped in Saran Wrap^R, exposed to Kodak X-Omat AR film at −70° C for 48 to 72 hours with an intensifying screen, and processed for autoradiography.

Reverse Transcriptase-Polymerase Chain Reaction Amplification. Total RNA was isolated from cells with TriSolv™ (Biotecx Laboratories, Houston, TX), and cDNA was generated using SuperScript II™ (GIBCO/BRL, Gaithersburg, MD). For cDNA synthesis, 5 μg of total RNA in 13 μl of diethylpyrocarbonate (DEPC)-treated water was incubated with 1 μl of 50 ng/μl random hexamers, heated to 70° C for 10 minutes, and incubated for 1 minute on ice. Added to this mixture was 2 μl of buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl, 25 mM $MgCl_2$, 1 μg/μl bovine serum albumin), 1 μl of dNTP mixture (10 mM each of dATP, dCTP, dGTP and dTTP), 2 μl of 100 mM DTT, and 1 μl of reverse transcriptase (200 U/μl). The total mixture was incubated at room temperature for 10 minutes, at 42° C for 50 minutes, at 70° C for 15 minutes, and then placed on ice. Two units of RNase H were added, and the tube was incubated at 37° C for 20 minutes. The first strand cDNA generated by these methods was amplified directly by adding the following to the same tube: 8 μl of buffer (as above), 68 μl of sterile distilled water, 2 μl of amplification primer 1 (5 μM), 2 μl of amplification primer 2 (5 μM), and 2 to 5 units of *Taq* polymerase (GIBCO/BRL). The primers used were: 5′-TGGTGGCATCTGACACTAGTGACTGT-3′ and 5′-TGTCAATTGTAGGCCCGAGTCTTCA-3′. The reaction mixture was heated to 94° C for 5 minutes to denature the RNA/cDNA hybrid, and polymerase chain reaction was carried out for 30 cycles. The amplified DNA fragments were separated on an 8% polyacrylamide gel under nondenaturing conditions and were visualized by staining with silver stain (BioRad, Richmond, CA).

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