Am J Physiol Cell Physiol 287: C866–C873, 2004.
First published May 26, 2004; 10.1152/ajpcell.00238.2003.

A key angiogenic role of monocyte chemoattractant protein-1 in hemangioendothelioma proliferation

Gayle M. Gordillo, Duygu Onat, Michael Stockinger, Sashwati Roy, Mustafa Atalay, F. Michael Beck, and Chandan K. Sen

Laboratory of Molecular Medicine, Department of Surgery, Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University Medical Center, Columbus, Ohio 43210

Submitted 9 January 2003; accepted in final form 18 May 2004

Gordillo, Gayle M., Duygu Onat, Michael Stockinger, Sashwati Roy, Mustafa Atalay, F. Michael Beck, and Chandan K. Sen. A key angiogenic role of monocyte chemoattractant protein-1 in hemangioendothelioma proliferation. *Am J Physiol Cell Physiol* 287: C866–C873, 2004. First published May 26, 2004; 10.1152/ajpcell.00238.2003.—Angiomatous lesions are common in infants and children. Hemangioendotheliomas (HE) represent one type of these lesions. Endothelial cell proliferation and the development of vascular/blood cell-filled spaces are inherent in the growth of HE. Therefore, understanding mechanisms that regulate the proliferation of these lesions should provide key insight into mechanisms regulating angiogenesis. A murine model was used to test the significance of monocyte chemoattractant protein (MCP)-1 in HE proliferation. EOMA cells, a cell line derived from a spontaneously arising murine HE, generate these lesions with 100% efficiency when injected subcutaneously into syngeneic mice. MCP-1 produced by EOMA cells recruit macrophages, which were shown to induce angiogenic behavior in EOMA cells by stimulating transwell migration and inducing sprout formation on type I collagen gels. When EOMA cells were injected into MCP-$1^{-/-}$ mice, only 50% of the mice developed tumors, presumably because the low levels of MCP-1 expressed by the injected EOMA cells were enough to overcome any host deficits of this chemokine. When EOMA cells were coinjected with a neutralizing antibody to MCP-1, tumors failed to develop in any of the treated mice, including syngeneic 129P3, C57Bl/6 (wild type), and MCP-$1^{-/-}$. These results present the first evidence that MCP-1 is required for HE proliferation and may promote the growth of these lesions by stimulating angiogenic behavior of endothelial cells. This study has produced the first in vivo evidence of a complete response for any neoplasm, specifically a vascular proliferative lesion, to anti-MCP-1 therapy in animals with intact immune systems.

endothelium; vascular; macrophage; redox; angiogenesis

ANGIOMATOUS DISEASES AFFECT up to 3% of all children (31). Included in the scope of these pathological conditions are vascular neoplasms ranging in severity from benign infantile hemangiomas to malignant angiosarcomas. Hemangioendothelioma (HE) represents a vascular neoplasm of borderline or intermediate malignancy (8). It does not metastasize; however, in humans, the mortality rate for HE ranges from 12 to 24%. This lesion is associated with the development of Kasabach-Merritt syndrome (36, 47), a consumptive coagulopathy with a 20–30% mortality rate (9). Vascular neoplasms are highly angiogenic because the growth of these lesions entails endothelial cell proliferation with the development of perfused vascular spaces. The fact that humans with proliferating hemangiomas can have urinary bFGF levels elevated 25- to 50-fold higher than those of healthy volunteers (37) attests to the degree of angiogenic activity associated with vascular neoplasms. Investigators at our laboratory (3) recently demonstrated that halting the growth of HE arrests angiogenesis that is inherent in this process and that these events are associated with an inhibition of inducible monocyte chemoattractant protein (MCP)-1 expression.

MCP-1 is known to participate in angiogenic events under many conditions. Neovascularization is a significant component of chronic inflammatory conditions such as rheumatoid arthritis, psoriasis, and even atherosclerosis. Monocyte recruitment by MCP-1 is known to contribute to the progression of atherosclerosis (13). Work conducted at the Folkman laboratory (27) has shown that the extent of neovascularization in atherosclerotic lesions correlates highly with the extent of macrophage infiltration. Treatment with antiangiogenic agents reduced the extent of macrophage infiltration and plaque formation in apolipoprotein $E^{-/-}$ mice that developed atherosclerotic aortas. MCP-1 also has a significant role in wound healing. Macrophages are essential for normal wound repair (7, 22), which is an angiogenesis-dependent process. In human wounds, there are considerable levels of MCP-1 expression by basal keratinocytes, endothelial cells, and infiltrating mononuclear cells. MCP-1 is expressed almost exclusively in the first 7 days after wounding, but other chemokines for monocytes are not expressed at significant levels (11). Finally, MCP-1 is also implicated in the angiogenic capacity of tumors to support their growth. The correlation between MCP-1 expression and tumor growth has been documented for breast (20, 34, 41), ovarian (14), and bladder cancers (2). An association between the presence of tumor-associated macrophages and poor prognosis has also been reported for several other tumor types (6, 29, 40). It is surmised that the tumor-associated macrophages facilitate angiogenesis and that the consequences of this aggressive growth are reflected in higher mortality rates. Despite the established correlation between MCP-1 levels, tumor-associated macrophages, and mortality, few attempts have been made to demonstrate causality between MCP-1 expression and tumor growth in vivo. Demonstrating the biological significance of MCP-1 expression in a model of angiogenesis may have therapeutic implications for a number of important disease states.

There are two murine models of vascular neoplasms. One uses endothelial cells transformed with the middle T antigen of

Address for reprint requests and other correspondence: G. M. Gordillo, Division of Plastic Surgery, The Ohio State Univ. Medical Center, 410 W. 10th Ave., Columbus, OH 43210 (E-mail: gordillo-1@medctr.osu.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

the murine polyoma virus (4, 26, 38), and the other uses cells derived from a spontaneously arising HE (15, 44). The endothelial cells that are virally transformed are on a mixed major histocompatibility complex (MHC) background (H-2^d/H-2^b), making them suitable for use only in severe combined immunodeficiency (SCID) mice (4). The EOMA cells derived from the spontaneously arising HE are from the 129/J strain, which is commercially available (now called 129P3/J) with a defined H-2^b MHC background. EOMA cells also have been well characterized with regard to endothelial cell phenotype (28), protein expression (10, 30, 46), response to angiogenesis inhibitors (19, 31), and development of the Kasabach-Merritt syndrome (15, 42, 44). The fact that mice into which EOMA cells are injected develop Kasabach-Merritt syndrome is a good indicator of how closely this model mimics the human condition. It was previously demonstrated that EOMA cells express relatively high levels of MCP-1 in vitro (12) and that HE proliferation after EOMA cell injection is associated with macrophage infiltration (3). It is not known how MCP-1 expression affects the biology of EOMA cells. Part of deciphering the role of MCP-1 is determining whether angiogenic effects are mediated directly via an autocrine effect on EOMA cells or indirectly via recruitment of macrophages. In this study, we sought to determine the significance of MCP-1 expression in supporting HE proliferation.

MATERIALS AND METHODS

MCP-1 ELISA

MCP-1 expression levels for in vitro EOMA samples were determined as previously described (12). For tumor samples, residual blood was removed by rinsing the samples in ice-cold PBS, blotting them onto paper, and incubating them in ACK lysis buffer [8.29 g of NH_4Cl, 0.07 g of K_2CO_3, and 2 ml of 0.5 M Na_2EDTA (pH 8) per liter of double-distilled H_2O, pH 7.4], 10 ml/sample, for 10 min in a 37°C water bath. The tissue was rinsed in ice-cold PBS, blotted on filter paper, and snap frozen in liquid nitrogen. Frozen samples were ground, and the powder was transferred to an Eppendorf tube and resuspended in homogenization buffer [10 μl of protease inhibitor cocktail (Sigma, St. Louis, MO), 5 μl of PMSF (100 mM), 125 μl of 20% SDS, and 860 μl of PBS] at 100 mg/ml powder of homogenization buffer. The tissue was homogenized on ice four times for 20 s each with 5- to 10-s breaks. The homogenate was centrifuged at 3,500 rpm for 20 min at 4°C. The supernatants were collected and stored at −80°C until ELISA was performed. Bicinchoninic acid protein assay (Pierce, Rockford, IL) was performed according to the manufacturer's instructions to standardize MCP-1 values per milligram of protein.

BrdU Assay

Cell proliferation was assayed using bromodeoxyuridine (BrdU) ELISA (Roche, Indianapolis, IN) according to the manufacturer's instructions. EOMA cells were plated on flat-bottom 96-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) at 10,000 cells/cm^2 in normal growth medium (NGM) [Dulbecco's modified Eagle's medium (DMEM; Invitrogen/GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA) and 100 U/ml penicillin-100 μg/ml streptomycin (1% P/S; Invitrogen/GIBCO)]. Incubation conditions were 37°C, 5% CO_2, and 100% relative humidity. After cells were allowed to adhere and recover overnight, medium was removed and cells were washed twice with PBS and then twice with very low-serum medium (VLSM; DMEM, 0.2% FBS, and 1% P/S). Cells were allowed to synchronize their cell cycle in VLSM for 32 h. After serum starvation, medium was removed and NGM containing BrdU and challenges was added to wells. Challenges consisted of goat anti-mouse MCP-1 neutralizing antibody (AF-479-NA; R&D Systems, Minneapolis, MN) at 500 and 50 ng/10^6 cells in NGM, isotype control antibody (goat IgG AB-108-C; R&D Systems) at 500 and 50 ng/10^6 cells in NGM, NGM alone (reference control), mouse MCP-1 protein (479-JE; R&D Systems) at 500, 1,000, and 2,000 ng/10^6 cells in NGM, and VLSM alone (negative control). After overnight incubation for 16 h, cell growth was arrested, cells were fixed, and BrdU incorporation was assayed via colorimetric detection using a plate reader (model ELx808; Bio-Tek Instruments, Winooski, VT) at 450 nm.

Transwell Migration Assays

EOMA migration. RAW 264.7 macrophage cells (American Type Culture Collection, Manassas, VA) were seeded (2.77×10^6 cells/0.6 ml) in NGM (DMEM, 10% FBS, and 1% P/S) in 24-well tissue culture plates and incubated for 1 h at 37°C and 5% CO_2 to allow RAW cells to adhere. Wells not seeded with RAW cells had an equal volume of NGM added to them. Wells were rinsed with PBS, and 0.6 ml/well LSM (DMEM, 0.5% FBS, and 1% P/S) was added. Transwell inserts with 8-μm pore size (Costar, Corning, NY) were equilibrated by incubation in LSM for 1–2 h at 37°C and 5% CO_2 and placed in the 24-well plates, and then EOMA cells (10^5 cells/100 μl LSM) were seeded in the upper chamber. Where indicated, 100 ng/ml recombinant murine VEGF, 25 ng/ml recombinant murine MCP-1, 250 ng/ml goat anti-mouse MCP-1 monoclonal antibody, or 250 ng/ml goat IgG isotype control antibody were included in the LSM containing the EOMA cells at the time of seeding.

RAW cell migration. EOMA cells (3×10^5 cells/600 μl NGM) were seeded in the lower chamber of the 24-well plates, incubated for 1 h at 37°C and 5% CO_2 to allow adherence, and rinsed with PBS, and then 600 μl of low-serum medium (LSM) were added. Equilibrated transwell inserts were placed and seeded with RAW cells (5×10^5 cells/100 μl LSM). Where indicated, 25 ng/ml recombinant murine MCP-1, 250 ng/ml goat anti-mouse MCP-1 monoclonal antibody, or 250 ng/ml goat IgG isotype control antibody were included in the LSM containing the EOMA cells or LSM alone at the time of lower well seeding. Migration was measured after incubation at 37°C and 5% CO_2 for 5 h. Cells on the upper surface of the transwell membrane were removed by rubbing with a sterile cotton swab, and cells on the lower surface were fixed and visualized using the Hema 3 stain set (Fisher Diagnostics, Middletown, VA). Stained membranes were digitally imaged while overlaid on a hemocytometer, and the number of cells per square millimeter was determined at three different locations on each membrane.

In Vitro Assay for Sprout Formation by EOMA Cells

Collagen gels were prepared by adding 0.25 ml of collagen solution [25 μl of 10× PBS, 137.41 μl of 0.02 N acetic acid, 4.55 mg/ml solubilized type 1 rat-tail collagen solution (Upstate Biotechnology, Waltham, MA), 0.023 μl of 1 M NaOH, and 87.577 μl of H_2O] to each well of a 24-well plate and were incubated at 37°C for 30 min. Gels were washed thoroughly with PBS and then equilibrated for 4 h with 1 ml NGM/well. EOMA cells (1 ml; 1.6×10^5/ml) were seeded onto each gel in NGM and incubated at 37°C and 5% CO_2 until they were ~80% confluent. Treatments were performed in LSM. Cells were treated for 48 h with either 1 ml LSM alone, 100 ng/ml recombinant murine VEGF (Biovision, Mountain View, CA), 2.5 or 100 ng/ml recombinant MCP-1 (R&D Systems), 250 ng/ml goat anti-mouse MCP-1 monoclonal antibody (R&D Systems), 250 ng/ml goat IgG isotype control antibody (R&D Systems), or 1 ml RAW conditioned medium. RAW conditioned medium was prepared by incubating 10^6 RAW cells/ml in LSM for 16 h. Collected medium was centrifuged at 1,200 rpm for 7 min at 4°C, and the supernatant was applied to collagen gels.

Peritoneal Macrophage Collection

Macrophages were obtained by intraperitoneal injection of 1.0 ml of 3% Brewer thioglycolate (Fisher Scientific Products, Pittsburgh, PA) as described previously (25). C57Bl/6 SCID mice (Jackson Laboratories, Bar Harbor, ME) were used to obtain a pure population of macrophages from the peritoneal exudate, which was collected 4 days after thioglycolate injection. Macrophages were collected by injecting 10 ml of PBS and aspirating the peritoneal fluid back into the syringe using a 19-gauge needle. Cells were spun down, counted using a hemocytometer, tested by trypan blue exclusion for viability, and resuspended in PBS at 2×10^7 cells/ml.

Hemangioendothelioma Production

EOMA cells were prepared for injection by harvesting them from culture with trypsin-EDTA, washing them three times in PBS, and loading them into a 1-ml tuberculin syringe. Mice (6–8 wk old) received 5×10^6 EOMA cells (5×10^7 cells/ml PBS) by subcutaneous injection in the dorsal midline. Where indicated, mice received 2.5 μg of goat anti-mouse MCP-1 monoclonal antibody (0.5 μg/10^6 cells), goat IgG isotype control antibody (0.5 μg/10^6 cells), or 5×10^5 peritoneal macrophages, all of which were contained within a 25-μl aliquot that was added to the EOMA cell suspensions in the syringes. MCP-$1^{-/-}$ mice (gift of Dr. Barrett Rollins, Dana-Farber/Harvard Cancer Center, Boston, MA) were generated by targeted disruption of MCP-1 gene as described by Lu et al. (23). These mice are on a C57Bl/6 MHC background, so wild-type C57Bl/6b mice were used as controls. All mice were euthanized 7 days after injection for HE specimen harvest.

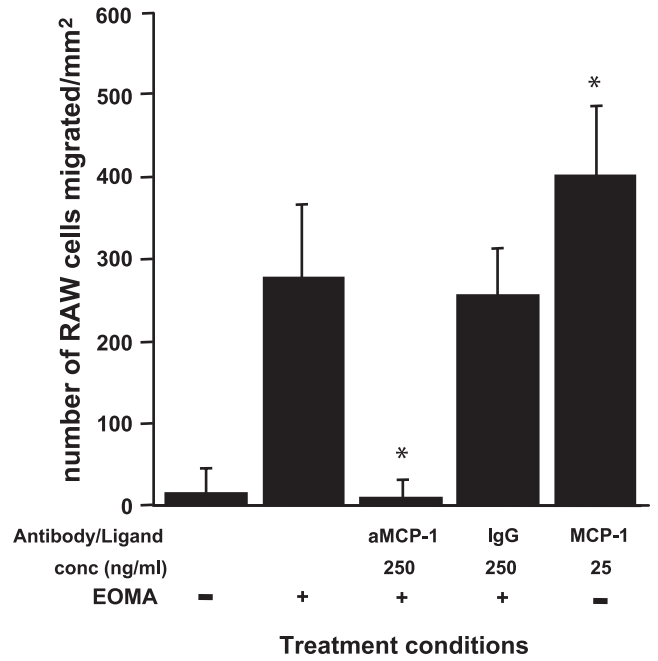

Fig. 1. EOMA cells express biologically active monocyte chemoattractant protein (MCP)-1. Determination of the number of macrophages migrating across each transwell membrane in response to EOMA cells in the lower chamber is depicted. Treatment with MCP-1 protein was used as a positive control, and untreated RAW cells with EOMA cells absent from the lower chamber were used as a negative control. RAW cell migration in response to EOMA cells was significantly inhibited by anti-MCP-1 (aMCP-1) neutralizing antibodies. RAW cell migration was also significantly stimulated by the addition of MCP-1 compared with levels observed with EOMA cell stimulation alone. Data are means ± SD of 3 experiments, with each sample run in triplicate. *$P < 0.01$. conc, Concentration.

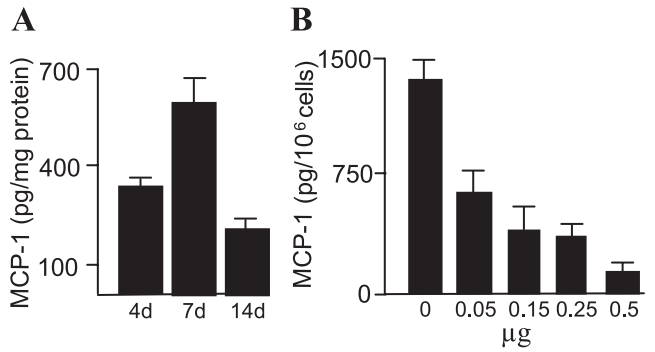

Fig. 2. MCP-1 in hemangioendotheliomas (HE) and EOMA cells. *A*: HE generated by subcutaneous injection of EOMA cells express MCP-1 as documented by ELISA. Peak levels of MCP-1 expression are shown 7 days (d) after EOMA cell inoculation. *B*: MCP-1 neutralizing antibody dose (in μg)-response curve generated for EOMA cells grown in vitro. A 10-fold excess of antibody relative to protein expression resulted in a 90% reduction in MCP-1 levels. Data are means ± SD of 3 experiments, with each sample run in triplicate.

Immunohistochemistry

Tissue specimens were snap frozen in optimum cutting temperature (OCT) compound (Miles, Elkhart, IN), supercooled in isopentane, and stored in liquid nitrogen. Frozen tissues were sectioned at 6-μm thickness, fixed for 5 min in acetone at 4°C, and stained using routine immunoperoxidase methods. The primary antibody used for macrophage identification was F4/80, a rat anti-mouse IgG_{2b} monoclonal antibody (Serotec, Raleigh, NC) used at 1:50 dilution. The secondary antibody used was mouse anti-rat IgG_{2b} alkaline phosphatase-conjugated antibody. Control sections were generated using a rat IgG_2 isotype control antibody to test primary antibody specificity and rat serum to test secondary antibody specificity. Tissue sections were counterstained with hematoxylin.

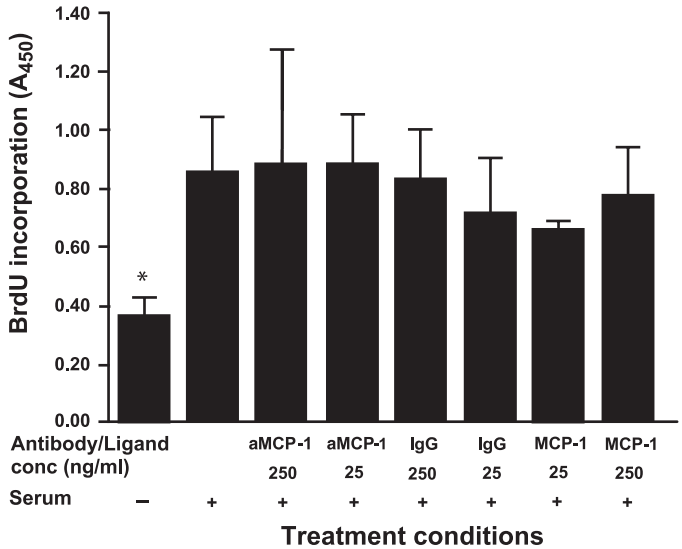

Fig. 3. MCP-1 does not stimulate EOMA cell proliferation. Bromodeoxyuridine (BrdU) assay was performed on EOMA cells treated with exogenous recombinant MCP-1 protein, neutralizing antibody to MCP-1, or isotype control antibody. None of the treatment groups affected EOMA cell proliferation. Serum starvation was used as a negative control and had a statistically significant inhibitory effect on EOMA cell proliferation. Data are means ± SD of 2 experiments, with each sample run in quadruplicate.

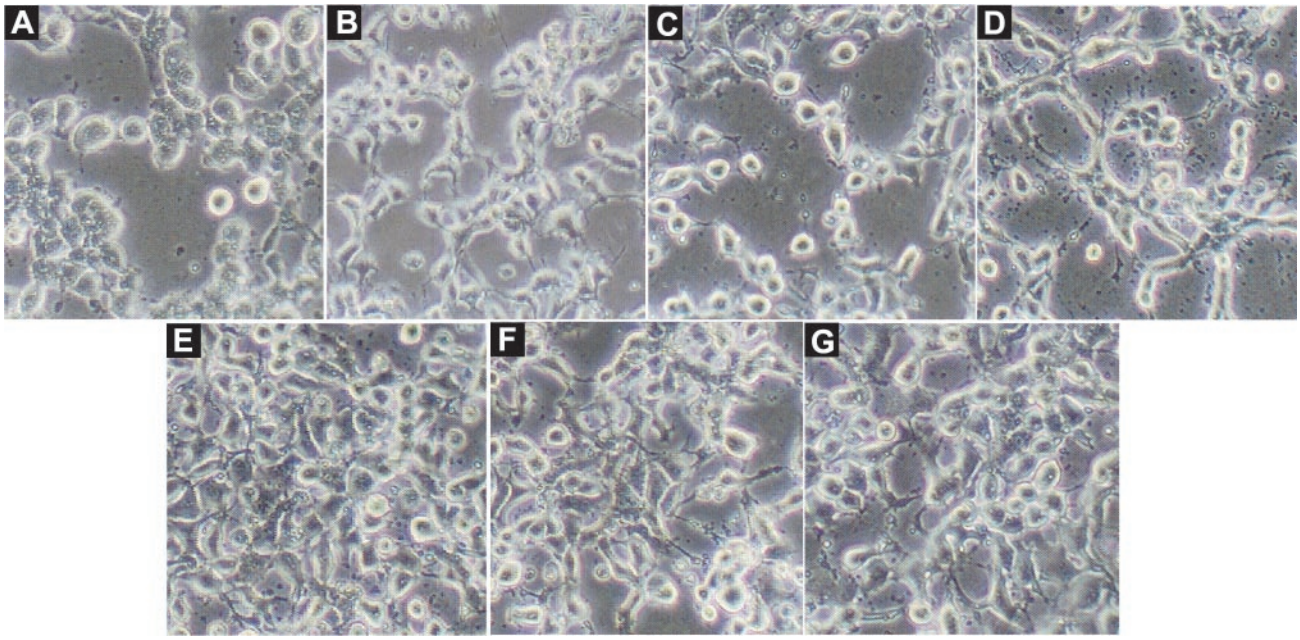

Fig. 4. EOMA cell sprout formation on type I collagen gels. *A*: VEGF (100 ng/ml) positive control. *B*: recombinant MCP-1 (2.5 ng/ml). *C*: recombinant MCP-1 (100 ng/ml). *D*: macrophage-conditioned media. *E*: untreated EOMA cells. *F*: neutralizing antibody to MCP-1 (250 ng/ml). *G*: IgG control antibody (250 ng/ml). No sprouting is seen with untreated EOMA cells, significant sprouting occurs with VEGF and macrophage-conditioned media, and modest sprouting occurs with MCP-1. No effect on sprouting occurs with neutralizing antibody to MCP-1. Experiment was performed 3 times with samples in triplicate, and representative findings are shown. Giemsa stain, ×200 magnification.

Statistical Analyses

For in vitro experiments, data are reported as means ± SD of three experiments, with each sample run in triplicate. Means were compared using an independent samples *t*-test, and $P < 0.05$ was considered statistically significant. For in vivo data, statistical analysis was done using multiple Fisher's exact tests, with *P* values adjusted using the step-down Bonferroni method of Holm. Comparisons between treatment groups were performed within the same mouse strain, and $P < 0.05$ was considered statistically significant.

RESULTS

We (12) previously demonstrated that EOMA cells have a high basal level of MCP-1 expression in vitro. Transwell experiments were performed to document that the MCP-1 expressed by EOMA cells was biologically active. Murine RAW 264.7 macrophages were placed in the upper chamber, and EOMA cells were placed in the lower chamber. Macrophage migration across the transwell membrane was observed in the absence of EOMA cells in the lower chamber and represented basal transwell migration levels. The results were normalized to these basal levels. The presence of EOMA cells in the lower chamber resulted in a statistically significant increase in transwell migration above basal levels, and equivalent levels of transwell migration were observed in macrophages treated with MCP-1 alone. The addition of MCP-1 neutralizing antibody to EOMA-stimulated macrophages abrogated any inducible increases in transwell migration (Fig. 1). Thus EOMA cells can stimulate macrophage migration via the expression of MCP-1.

To determine the in vivo significance of MCP-1, we confirmed that HE specimens express MCP-1 by performing ELISA on tumor extracts at 4, 7, and 14 days after EOMA cell

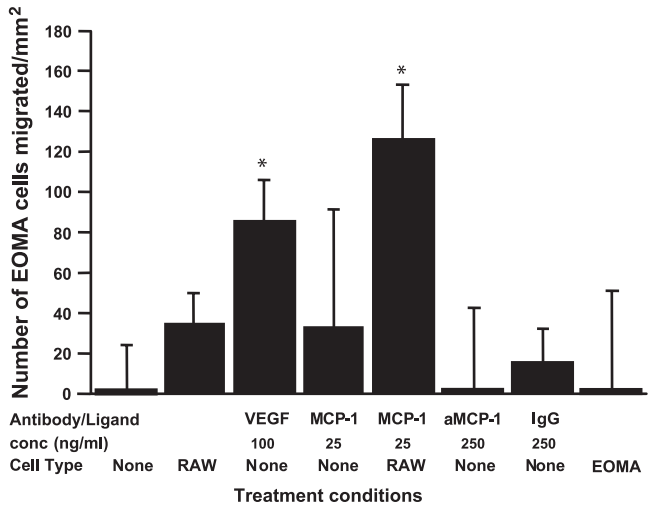

Fig. 5. Macrophages stimulate EOMA cell chemotaxis in response to MCP-1. Quantitative determination of the number of EOMA cells migrating across each transwell membrane in response to macrophages in the lower chamber is depicted. Treatment with VEGF was used as a positive control, and untreated EOMA cells with RAW macrophages absent from the lower chamber were used as a negative control. A statistically significant increase in migration is shown in VEGF-treated vs. untreated EOMA cells. The addition of macrophages or MCP-1 both increased EOMA cell migration, but only the combination of macrophages and MCP-1 resulted in a statistically significant increase. The presence of EOMA cells in the lower chamber did not stimulate migration of EOMA cells in the upper chamber. Data are means ± SD of 3 experiments, with each sample run in triplicate. *$P < 0.05$.

injection. Expression of MCP-1 protein was observed at all three time points, with peak expression occurring at 7 days after EOMA cell injection (Fig. 2*A*). A dose-response curve for neutralizing antibody to MCP-1 was then generated using ELISA measurements of MCP-1 from EOMA cell extracts. Untreated EOMA cells expressed 1.5 ng of MCP-1 protein/10^6 cells, and addition of 500 ng of neutralizing antibody/10^6 cells resulted in a 90% decrease in MCP-1 levels (Fig. 2*B*). This information was used to estimate basal levels of MCP-1 (1.5 ng/10^6 cells) for in vitro experiments involving EOMA cells and to calculate the dose of neutralizing antibody for in vivo experiments.

Because EOMA cells constitutively express MCP-1 and MCP-1 has been shown to be proangiogenic (35, 45), the possibility of proangiogenic responses through direct positive feedback independent of macrophage recruitment must be considered. Angiogenic responses of EOMA cells in vitro were evaluated on the basis of the following criteria: proliferation, sprout formation, and migration. To determine whether MCP-1 could independently stimulate EOMA cell proliferation, a BrDU assay was performed. The ability of MCP-1 protein to stimulate proliferation or neutralizing antibody to MCP-1 (anti-MCP-1) to inhibit proliferation was compared with basal untreated rates of EOMA proliferation. Serum starvation was used as a negative control, and a goat IgG isotype antibody was used as a treatment control. EOMA cell proliferation was significantly inhibited by serum starvation, but there was no statistically significant difference in the rates of proliferation observed between untreated, MCP-1-treated, and anti-MCP-1-treated EOMA cells (Fig. 3). MCP-1 does not have a mitogenic effect on EOMA cells.

Next, we sought to determine whether MCP-1, or the macrophages recruited as a result of MCP-1, promoted sprout formation by EOMA cells grown on type I collagen gels or transwell migration. Responses to macrophage-conditioned media, recombinant murine MCP-1, and neutralizing antibody to MCP-1 were compared to clarify the extent of angiogenic contributions through direct (autocrine) or indirect (macrophage recruitment) mechanisms. Recombinant murine VEGF was used as a positive control for both assay systems.

In the collagen gel assay, there was no spontaneous induction of sprouting from basal levels of MCP-1 expression, as shown in the untreated EOMA cells (Fig. 4*E*). Sprout formation was clearly triggered by VEGF, macrophage-conditioned media, and MCP-1 (Fig. 4, *A–D*). Treatment with MCP-1 neutralizing antibody or IgG control antibody had no effect on

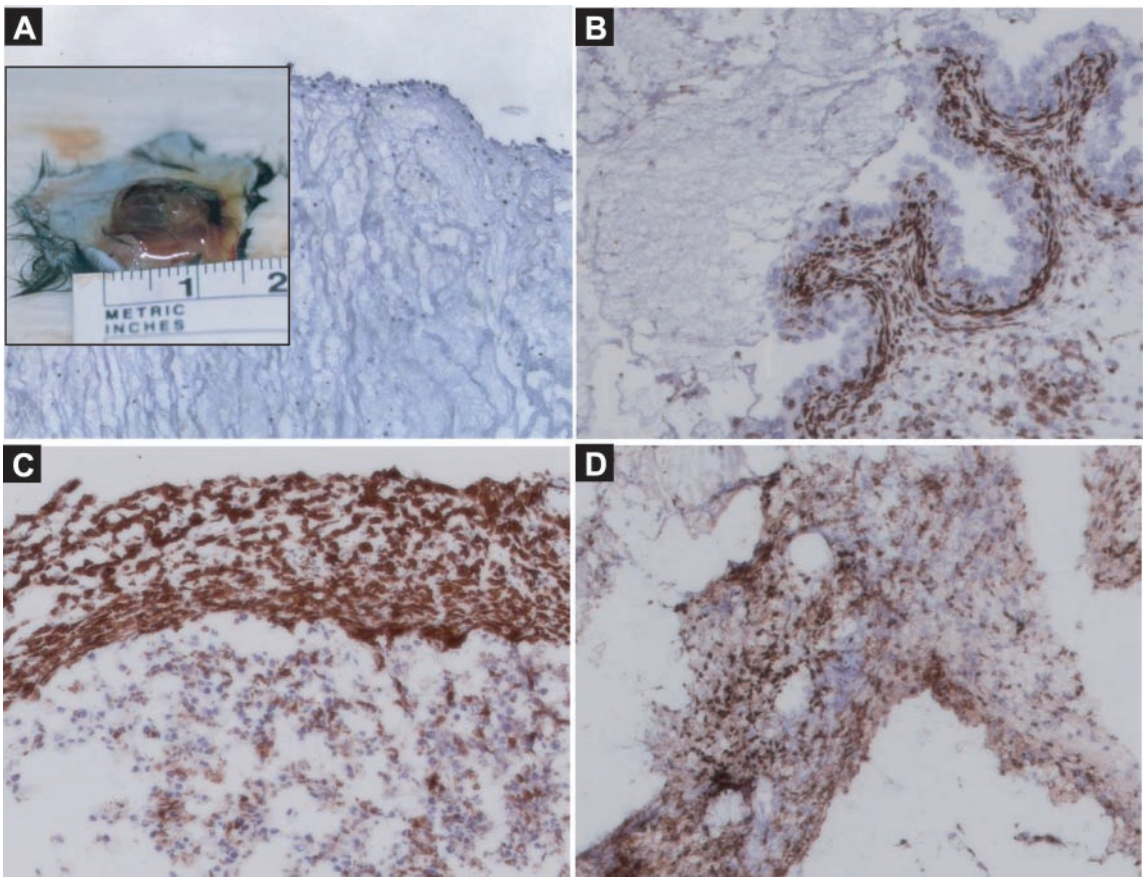

Fig. 6. MCP-$1^{-/-}$ mice that develop HE have macrophage infiltrate. Macrophages were identified on the basis of F4/80 reactivity. The absence of HE development was determined as the absence of a visible lesion on gross inspection and necropsy (see *inset*). The absence of reactivity with a rat IgG_2 isotype control antibody (*A*) or with rat serum (not shown) confirmed that nonspecific binding of the primary or secondary antibody was not observed in HE sections. Despite the absence of MCP-1 in the knockout mice, there was no difference in the extent of macrophage infiltration between syngeneic 129P3 mice (*B*) and MCP-$1^{-/-}$ mice (*D*). Extensive macrophage infiltration was seen in the capsule of HE lesions from wild-type C57Bl6 mice (*C*), which may reflect the minor histocompatibility differences between 129P3 and C57Bl6 mice. Magnification, ×100.

sprouting (Fig. 4, *F* and *G*). While augmenting MCP-1 levels did induce a sprout response, it suggests that contributions from basal levels of MCP-1 expression toward sprout formation are minimal and that angiogenic responses may be enhanced by macrophage recruitment based on the levels of sprouting seen with macrophage-conditioned media.

For the transwell model, EOMA cells were placed in the upper chamber and macrophages were placed in the lower chamber. The independent contributions of MCP-1 and macrophages to EOMA cell migration are depicted in Fig. 5. Basal levels of EOMA cell migration were established in the absence of cells in the lower chamber. The results are normalized to these basal levels. Autocrine effects due to spontaneous expression of MCP-1 by EOMA cells were evaluated by placing EOMA cells in both the upper and lower chambers. There was no increase in EOMA cell migration above basal levels when EOMA cells were present in the lower well. Treatment with neutralizing antibodies to MCP-1 did not significantly inhibit basal levels of transwell migration in EOMA cells. Therefore, levels of MCP-1 spontaneously expressed by EOMA cells are not sufficient to stimulate transwell migration. In the absence of macrophages, the addition of recombinant MCP-1 beyond constitutively expressed levels did not result in a statistically significant increase in migration compared with basal levels. Transwell migration tended to increase with the addition of macrophages in the lower well compared with basal levels of EOMA cell migration, but this was not statistically significant. The amount of migration stimulated by enhancing the level of MCP-1 was not significantly different from the amount of migration observed with the addition of macrophages alone. However, there was a synergistic increase in transwell migration when EOMA cells were exposed to both macrophages and MCP-1 compared with EOMA and macrophages alone ($P = 0.02$). These findings suggest that significant angiogenic responses may occur when a threshold level of MCP-1 is achieved and macrophages are present.

The effects of MCP-1 on EOMA cell angiogenic responses in vivo were evaluated using HE proliferation as the indicator. Two different strains of mice underwent subcutaneous EOMA cell injection. One strain of mice used was 129 P/3, which is syngeneic with EOMA. The other strain of mice was C57Bl/6, which is the MHC background of the MCP-$1^{-/-}$ mice. Both 129P3 and C57Bl/6 are H-$2k^b$ and H-$2d^b$ and have only minor histocompatibility differences. The MCP-$1^{-/-}$ genotype in the knockout mice was confirmed by PCR before treatment (data not shown). When EOMA cells were injected subcutaneously, they produced large (1.0- to 2.0-cm diameter) HE within 1 wk with 100% efficiency in both the 129P/3 and the wild-type C57Bl/6 mice. However, the incidence of HE in MCP-$1^{-/-}$ mice was only 50%. The presence of a visible lesion, either upon gross inspection or after removal of dorsal skin, at 7 days was considered a positive result for HE formation. It is possible that MCP-1 expressed by EOMA cells supported HE development in half of the MCP-$1^{-/-}$ mice. A critical level of MCP-1 may be required to support HE proliferation, and that critical threshold was achieved by EOMA cell production in only half of all MCP-$1^{-/-}$ mice. In MCP-$1^{-/-}$ mice that did produce HE, abundant macrophages were detected in the specimens, as demonstrated by F4/80 immunoreactivity (Fig. 6), indicating that macrophages are tightly associated with HE proliferation. No T cells or neural tissue was detected in tissue sections as observed by staining for CD3, CD8, or s100 (not shown). When MCP-1 neutralizing antibody was used to eliminate all sources of MCP-1, HE proliferation was completely inhibited in all mice, including syngeneic 129P3 mice (Fig. 7).

To confirm that macrophages actively participate in HE proliferation, peritoneal macrophages were coinjected along with the EOMA cells and neutralizing antibody to MCP-1. Thioglycolate was injected intraperitoneally in C57Bl/6 SCID mice to obtain a homogeneous population of inflammatory exudate cells consisting only of macrophages. Peritoneal macrophages harvested after thioglycolate injection are known to be angiogenic (16, 32). By coinjecting macrophages, HE formation was restored in C57Bl/6 wild-type controls, despite the presence of neutralizing antibody to MCP-1. The incidence of HE formation in MCP-$1^{-/-}$ mice coinjected with macrophages exceeded the levels of those animals receiving EOMA cells alone. Because HE proliferation was completely inhibited by the addition of MCP-1 neutralizing antibody (Fig. 7), the restoration of HE development by the addition of macrophages establishes that macrophages do contribute the growth of these lesions.

DISCUSSION

Macrophages are recognized as having a key role in facilitating angiogenesis (17, 18, 21, 33). Our observations specifically link MCP-1 as a key and limiting contributor to HE development. Although the HE lesion is a neoplasm of endothelial cell origin, the essential role of MCP-1 in supporting HE proliferation also indicates that accessory cells such as macrophages play a significant role in facilitating the growth of this tumor. Although several investigators have used the HE model to evaluate the antiangiogenic effects of *N*-acetylcysteine,

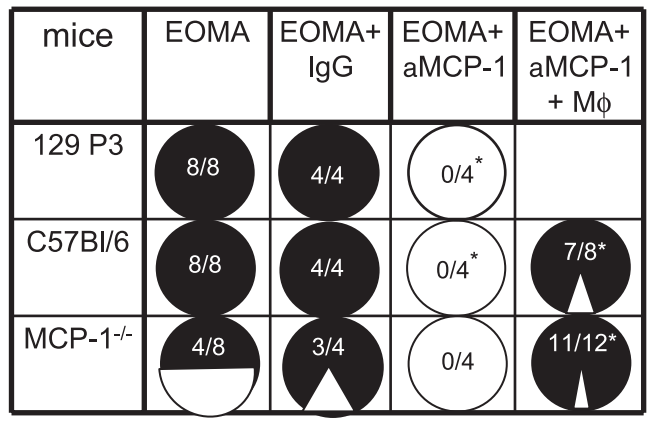

Fig. 7. Incidence of HE in 3 strains of mice that underwent subcutaneous injection of EOMA cells. The EOMA control group was injected with EOMA cells without any other additive. The EOMA + aMCP-1 group was injected with a mixture of EOMA and the MCP-1 neutralizing antibody (0.5 $\mu g/10^6$ cells). The EOMA + IgG group was injected with a mixture of EOMA and rat IgG isotype control antibody (0.5 $\mu g/10^6$ cells). The EOMA + aMCP-1 + Mϕ group was injected with EOMA cells and a mixture of MCP-1 neutralizing antibody and peritoneal macrophages (5×10^5 cells). All mice received a total injection volume of 150 μl. Differences were statistically significant when comparing the incidence of HE in mice receiving EOMA cells alone vs. anti-MCP-1 treatment or IgG vs. anti-MCP-1 treatments for both 129P3 and C57Bl/6 strains. Among MCP-$1^{-/-}$ mice treated, statistically significant differences were not observed with EOMA or EOMA + aMCP-1, but there was a statistically significant difference between EOMA + aMCP-1 and EOMA + aMCP-1 + Mϕ. *$P < 0.05$.

batimastat, IL-12, and angiostatin (1, 19, 31, 39, 43), none of these agents focus on the contributions of macrophages. By removing MCP-1 from the local tumor environment at the time of EOMA cell inoculation, the significance of macrophages in allowing HE to develop was highlighted.

Our results indicate that MCP-1 promotes HE proliferation by recruiting macrophages to stimulate proangiogenic behaviors such as sprout formation in vitro. The concept of a positive feedback loop in the stimulation of angiogenic behavior in endothelial cells is supported by the fact that endothelial cells are known to express CCR2, the sole receptor for MCP-1 (35, 45). On the basis of reverse transcription-polymerase chain reaction results with sequencing of the product, EOMA cells express CCR2 as well (data not shown). Thus they have the capacity to respond directly to MCP-1. Our in vitro observations suggest that the direct effects of MCP-1 on EOMA angiogenic responses are minimal. However, the effects of MCP-1 on EOMA angiogenic responses are enhanced in the presence of macrophages as shown by the ability of MCP-1 to stimulate endothelial cell migration in vitro and the consistent presence of macrophage infiltration in HE lesions in vivo.

It is important to note that our findings demonstrating a critical role for MCP-1 in HE proliferation are likely to have broad significance beyond the specific experimental model used in the current study. Salcedo et al. (35) evaluated the role of MCP-1 in breast cancer by injecting a human breast carcinoma cell line into SCID mice. Those mice treated with neutralizing antibody to MCP-1 demonstrated increased survival and decreased volume of lung metastases compared with control mice. Taken together, our results and those of Salcedo et al. highlight the potential of MCP-1-neutralizing approaches to limit tumor formation in vivo. The present results constitute the first in vivo evidence demonstrating a complete response for any neoplasm, and specifically a vascular proliferative lesion, to anti-MCP-1 therapy in mice with intact immune systems. It is becoming clear that multiple approaches are required to block angiogenesis (24), and manipulation of chemokine function may have merit as a new therapeutic approach (5). These results support the concept of antiangiogenic strategies that go beyond a focus on endothelial cells.

ACKNOWLEDGMENTS

G. M. Gordillo is with the Division of Plastic Surgery.

GRANTS

This work was supported by grants from the Fresh Start Surgical Gifts Award/Plastic Surgery Educational Foundation, the Medical Research and Development Fund of the Department of Surgery, and The Ohio State University, and by National Institute of General Medical Sciences Grant K08 GM-066994 (to G. M. Gordillo with C. K. Sen as mentor). This work is also partly supported by National Institute of General Medical Sciences Grant GM-27345 (to C. K. Sen).

REFERENCES

1. **Albini A, Morini M, D'Agostini F, Ferrari N, Campelli F, Arena G, Noonan DM, Pesce C, and De Flora S.** Inhibition of angiogenesis-driven Kaposi's sarcoma tumor growth in nude mice by oral *N*-acetylcysteine. *Cancer Res* 61: 8171–8178, 2001.
2. **Amann B, Pearbo F, Wirger A, Hugenschmidt H, and Schultze-Seeman W.** Urinaryl levels of monocyte chemoattractant protein-1 correlate with tumor stage and grade in patients with bladder cancer. *Br J Urol* 82: 118–121, 1998.
3. **Atalay M, Gordillo G, Roy S, Rovin B, Bagchi D, Bagchi M, and Sen CK.** Anti-angiogenic property of edible berry in a model of hemangioma. *FEBS Lett* 544: 252–257, 2003.
4. **Bautch VL, Toda S, Hassell JA, and Hanahan D.** Endothelial cell tumors develop in transgenic mice carrying polyoma virus middle T oncogene. *Cell* 51: 529–537, 1987.
5. **Bergers G, Song S, Meyer-Morse N, Bergsland E, and Hanahan D.** Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *J Clin Invest* 111: 1287–1295, 2003.
6. **Bingle L, Brown N, and Lewis C.** The role of tumour-associated macrophages in tumor progression: implications for new anticancer therapies. *J Pathol* 196: 254–265, 2002.
7. **DiPietro LA.** Wound healing: the role of the macrophage and other immune cells. *Shock* 4: 233–240, 1995.
8. **Enzinger F and Weiss S.** *Soft Tissue Tumors*. St. Louis, MO: C. V. Mosby, 1983.
9. **Esterly N.** Cutaneous hemangiomas, vascular stains and malformations, and associated syndromes. *Curr Probl Dermatol* 7: 65–108, 1995.
10. **Felbor U, Dreier L, Bryant R, Ploegh H, Olsen B, and Mothes W.** Secreted cathepsin L generates endostatin from collagen XVIII. *EMBO J* 19: 1187–1194, 2000.
11. **Gillitzer R and Goebeler M.** Chemokines in cutaneous wound healing. *J Leukoc Biol* 69: 513–521, 2001.
12. **Gordillo G, Atalay M, Roy S, and Sen C.** Hemangioma model for in vivo angiogenesis: inducible oxidative stress and MCP-1 expression in EOMA cells. *Methods Enzymol* 352: 422–432, 2002.
13. **Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, and Rollins BJ.** Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. *Mol Cell* 2: 275–281, 1998.
14. **Hefler L, Tempfer C, Heinze G, Mayerhofer K, Breitenecker G, Leodolter S, Reinthaller A, and Kainz C.** Monocyte chemoattractant protein-1 serum levels in ovarian cancer patients. *Br J Cancer* 81: 855–859, 1999.
15. **Hoak JC, Warner ED, Cheng HF, Fry GL, and Hankenson RR.** Hemangioma with thrombocytopenia and microangiopathic anemia (Kasabach-Merritt syndrome): an animal model. *J Lab Clin Med* 77: 941–950, 1971.
16. **Koch A, Cho M, Burrows J, Polverini P, and Leibovich S.** Inhibition of production of monocyte/macrophage-derived angiogenic activity by oxygen free-radical scavengers. *Cell Biol Int* 16: 415–425, 1992.
17. **Koch A, Polverini P, Kunkel S, Harlow L, Pietro LD, Elner V, Elner S, and Strieter R.** Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science* 258: 1798–1801, 1992.
18. **Koch AE, Polverini PJ, and Leibovich SJ.** Induction of neovascularization by activated human monocytes. *J Leukoc Biol* 39: 233–238, 1986.
19. **Lannutti B, Gately S, Quevedo M, Soff G, and Paller A.** Human angiostatin inhibits murine hemangioendothelioma tumor growth in vivo. *Cancer Res* 57: 5277–5280, 1997.
20. **Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J, and Harris AL.** Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Res* 56: 4625–4629, 1996.
21. **Leibovich S, Polverini P, Shepard H, Wiseman E, and Nuseir N.** Macrophage-induced angiogenesis. *Nature* 329: 630–632, 1987.
22. **Leibovich SJ and Ross R.** The role of the macrophage in wound repair: a study with hydrocortisone and antimacrophage serum. *Am J Pathol* 78: 71–100, 1975.
23. **Lu B, Rutledge BJ, Gu L, Fiorillo J, Lukacs NW, Kunkel SL, North R, Gerard C, and Rollins BJ.** Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J Exp Med* 187: 601–608, 1998.
24. **Marx J.** A boost for tumor starvation. *Science* 301: 452–454, 2003.
25. **Meltzer M.** *Methods for Studying Mononuclear Phagocytes*. New York: Academic, 1981.
26. **Montesano R, Pepper M, Mohle-Steinlein U, Risau W, Wagner E, and Orci L.** Increased proteolytic activity is responsible for the aberrant morphogenetic behavior of endothelial cells expressing middle T oncogene. *Cell* 62: 435–445, 1990.
27. **Moulton KS, Vakili K, Zurakowski D, Soliman M, Butterfield C, Sylvin E, Lo KM, Gillies S, Javaherian K, and Folkman J.** Inhibition of plaque neovascularization reduces macrophage accumulation and progression of advanced atherosclerosis. *Proc Natl Acad Sci USA* 100: 4736–4741, 2003.

28. **Obeso J, Weber J, and Auerbach R.** A hemangioendothelioma-derived cell line: its use as a model for the study of endothelial cell biology. *Lab Invest* 63: 259–269, 1990.
29. **Ono M, Torisu H, Fukushi J, Nishie A, and Kuwano M.** Biological implications of macrophage infiltration in human tumor angiogenesis. *Cancer Chemother Pharmacol* 43: S69–S71, 1999.
30. **O'Reilly M, Boehm T, Shing Y, Fukai N, Vasios G, Lane W, Flynn E, Birkhead J, Olsen B, and Folkman J.** Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 88: 277–285, 1997.
31. **O'Reilly M, Brem H, and Folkman J.** Treatment of murine hemangioendotheliomas with the angiogenesis inhibitor AGM-1470. *J Pediatr Surg* 30: 325–330, 1995.
32. **Polverini PJ, Cotran PS, Gimbrone MA, and Unanue ER.** Activated macrophages induce vascular proliferation. *Nature* 269: 804–806, 1977.
33. **Polverini PJ and Leibovich SJ.** Induction of neovascularization in vivo and endothelial proliferation in vitro by tumor-associated macrophages. *Lab Invest* 51: 635–642, 1984.
34. **Saji H, Koike M, Yamori T, Saji S, Seiki M, Matsushima K, and Toi M.** Significant correlation of monocyte chemoattractant protein-1 expression with neovascularization and progression of breast carcinoma. *Cancer* 92: 1085–1091, 2001.
35. **Salcedo R, Ponce M, Young H, Wasserman K, Ward J, Kleinman H, Oppenheim J, and Murphy W.** Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood* 96: 34–40, 2000.
36. **Sarkar M, Mulliken J, Kozakewich H, Robertson R, and Burrows P.** Thrombocytopenic coagulopathy (Kasabach-Merritt phenomenon) is associated with kaposiform hemangioendothelioma and not common infantile hemangioma. *Plast Reconstr Surg* 100: 1377–1386, 1997.
37. **Takahashi K, Mulliken J, Kozakewich H, Rogers R, Folkman J, and Ezekowitz R.** Cellular markers that distinguish the phases of hemangioma during infancy and childhood. *J Clin Invest* 93: 2357–2364, 1994.
38. **Taraboletti G, Belotti D, Dejana E, Montovani A, and Giavazzi R.** Endothelial cell migration and invasiveness are induced by a soluble factor produced by murine endothelioma cells transformed by polyoma virus middle T oncogene. *Cancer Res* 53: 3812–3816, 1993.
39. **Taraboletti G, Garofalo A, Belotti D, Drudis T, Borsotti P, Scanziani E, Brown PD, and Giavazzi R.** Inhibition of angiogenesis and murine hemangioma growth by batimastat, a synthetic inhibitor of matrix metalloproteinases. *J Natl Cancer Inst* 87: 293–298, 1995.
40. **Torisu H, Ono M, Kiryu H, Furue M, Ohmoto Y, Nakayama J, Nishioka Y, Sone S, and Kuwano M.** Macrophage infiltration correlates with tumor stage and angiogenesis in human malignant melanoma: possible involvement of TNFα and IL-1α. *Int J Cancer* 85: 182–188, 2000.
41. **Ueno T, Toi M, Saji H, Muta M, Bando H, Kuroi K, Koike M, Inadera H, and Matsushima K.** Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. *Clin Cancer Res* 6: 3282–3289, 2000.
42. **Verheul H, Panigrahy D, Flynn E, Pinedo H, and D'Amato R.** Treatment of Kasabach-Merritt syndrome with pegylated recombinant human megakaryocyte growth and development factor in mice: elevated platelet counts, prolonged survival and tumor growth inhibition. *Pediatr Res* 46: 562–565, 1999.
43. **Wang C, Quevedo ME, Lannutti BJ, Gordon KB, Guo D, Sun W, and Paller AS.** In vivo gene therapy with interleukin-12 inhibits primary vascular tumor growth and induces apoptosis in a mouse model. *J Invest Dermatol* 112: 775–781, 1999.
44. **Warner ED, Hoak JC, and Fry GL.** Hemangioma thrombocytopenia, and anemia. The Kasabach-Merritt syndrome in an animal model. *Arch Pathol* 91: 523–528, 1971.
45. **Weber K, Nelson P, Grone HJ, and Weber C.** Expression of CCR2 by endothelial cells. *Arterioscler Thromb Vasc Biol* 19: 2085–2093, 1999.
46. **Wei W, Moses M, Wiederschain D, Arbiser J, and Folkman J.** The generation of endostatin is mediated by elastase. *Cancer Res* 59: 6052–6056, 1999.
47. **Zuckerberg L, Nickoloff B, and Weiss S.** Kaposiform hemangioendothelioma of infancy and childhood: an aggressive neoplasm associated with Kasabach-Merritt syndrome and lymphangiomatosis. *Am J Surg Pathol* 17: 321–328, 1993.