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

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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 malignant potential. Hemangioendotheliomas range in severity from benign infantile hemangiomas to malignant angiosarcomas. Hemangioendothelioma proliferation 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 SV40, which results in hemangioendotheliomas. The other uses EOMA, a murine macrophage, which results in tumors that may develop into hemangioendotheliomas. EOMA cells were coinjected with a neutralizing antibody to MCP-1, tumors failed to develop in any of the treated mice. A murine model was used to test the significance 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

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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-2a/H-2b), making them suitable for use only in severe combined immunodeficiency (SCID) mice (4). The EOMA cells derived from the spontaneously arising HE are the 129/J strain, which is commercially available (now called 129P3/J) with a defined H-2b 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 NH4Cl, 0.07 g of K2CO3, and 2 ml of 0.5 M Na2EDTA (pH 8) per liter of double-distilled H2O, 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. Bicinechonic 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 assessed 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/cm2 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% CO2, 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/106 cells in NGM, isotype control antibody (goat IgG AB-108-C; R&D Systems) at 500 and 50 ng/106 cells in NGM, NGM alone (reference control), mouse MCP-1 protein (479-JE; R&D Systems) at 500, 1,000, and 2,000 ng/106 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; BioTek Instruments, Winoski, VT) at 450 nm.

Transwell Migration Assays

EOMA migration. RAW 264.7 macrophage cells (American Type Culture Collection, Manassas, VA) were seeded (2.77×106 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% CO2 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% CO2 and placed in the 24-well plates, and then EOMA cells (105 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 (3×105 cells/600 μl NGM) were seeded in the lower chamber of the 24-well plates, incubated for 1 h at 37°C and 5% CO2 to allow adherence, and rinsed with PBS, and then 600 μl of low-serum medium (LASM) were added. Equilibrated transwell inserts were placed and seeded with RAW cells (5×105 cells/100 μl LASM). 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 LASM containing the EOMA cells or LASM alone at the time of lower well seeding. Migration was measured after incubation at 37°C and 5% CO2 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 H2O] 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; 1.6×106/ml) were seeded onto each gel in NGM and incubated at 37°C and 5% CO2 until they were ∼80% confluent. Treatments were performed in LASM. Cells were treated for 48 h with either 1 ml LASM 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 106 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 x 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 x 10^6 EOMA cells (5 x 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 x 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.

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 IgG2b alkaline phosphatase-conjugated antibody. Control sections were generated using a rat IgG 2b isotype control antibody to test primary antibody specificity and rat serum to test secondary antibody specificity. Tissue sections were counterstained with hematoxylin.

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.

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.
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
Expression of MCP-1 protein was observed at all three time points, with peak expression occurring at 7 days after EOMA cell injection (Fig. 2A). 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. 2B). 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. 4E). Sprout formation was clearly triggered by VEGF, macrophage-conditioned media, and MCP-1 (Fig. 4A–D). Treatment with MCP-1 neutralizing antibody or IgG control antibody had no effect on...
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 and H-2d 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 129P3 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. Thioglycollate 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 thioglycollate 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,

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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 μg/10^6 cells). The EOMA + IgG group was injected with a mixture of EOMA and rat IgG isotype control antibody (0.5 μg/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 × 10^6 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. *$P < 0.05$. 

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anti-MCP-1 therapy for hemangioendothelioma.

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.

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