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GM-CSF Induces Expression of Soluble VEGF Receptor-1 from Human Monocytes and Inhibits Angiogenesis in Mice

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Summary

GM-CSF promotes homeostasis of myeloid cells. We report that GM-CSF upregulates mRNA and protein production of the soluble form of membrane bound VEGF receptor-1 (sVEGFR-1) in human monocytes. This sVEGFR-1 was biologically active, as cell-free supernatants from GM-CSF-stimulated monocytes blocked detection of endogenously expressed VEGF and inhibited endothelial cell migration and tube formation, even in the presence of exogenous rhVEGF. VEGF activity was recovered by neutralizing sVEGFR-1. To determine whether these events were important in vivo, Matrigel plugs were incubated with rhVEGF, rhGM-CSF, or rhGM-CSF/rhVEGF and injected into mice. Plugs containing GM-CSF or GM-CSF/VEGF had less endothelial cell invasion than plugs containing rhVEGF and were similar to plugs incubated with PBS alone. Neutralizing antibodies specific for sVEGFR-1 injected in these plugs reversed the effects of GM-CSF or GM-CSF/VEGF, while an isogenic antibody did not. Thus, GM-CSF and monocytes play a vital role in angiogenesis through the regulation of VEGF and sVEGFR-1.

Introduction

GM-CSF drives hematopoietic precursor cells to mature granulocytes, macrophages, or dendritic cells (Wognum et al., 1994) and is used clinically to accelerate bone marrow recovery and increase the production of white blood cells to facilitate host defense (Bleharski et al., 2003).

We reported that M-CSF induces human monocytes to produce and release biologically active VEGF (Eubank et al., 2003). VEGF (isoform-A) is an angiogenic factor that promotes blood vessel formation in human cancer (Leung et al., 1989) and plays a dominant role in human health and disease as a regulator of new blood vessel growth and an inducer of vascular permeability (Dvorak, 2000). VEGF-A is also highly expressed during episodes of hypoxia (Cao et al., 1998).

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VEGF-A signals through VEGF receptor 1 (VEGFR-1) (*Flt-1*) and VEGFR-2 (*KDR*) (Matsumoto and Claesson-Welsh, 2001). Structurally, both VEGF receptors have seven immunoglobulin (Ig)-like domains in their extracellular regions and two tyrosine kinase domains, and VEGF binds at the second and third Ig-like domain in both VEGFR-1 and VEGFR-2 (Wiesmann et al., 1997). In an adult, monocytes express solely VEGFR-1 (Barleon et al., 1996), which is responsible for relaying VEGF signals (Neufeld et al., 1999). Endothelial cells express both VEGFR-1 and VEGFR-2 (Neufeld et al., 1999), and while VEGFR-2 activates cellular signaling, VEGFR-1 acts as a "sink" on these cells to sequester VEGF from VEGFR-2. Mice expressing the extracellular domain of VEGFR-1 lacking a functional tyrosine kinase domain develop normal blood vessels and survive. In contrast, null mutations of the VEGFR-1 gene results in death early in embryogenesis due to a disorganization of blood vessels. These data suggest an important regulatory role in vivo for VEGFR-1 (Risau, 1997; Ferrara et al., 1996). Other mechanisms used by endothelial cells to regulate VEGF include the production of an alternatively spliced mRNA variant of VEGFR-1, soluble VEGFR-1 (sVEGFR-1) (Kendall et al., 1996). Soluble VEGFR-1 is identical to membrane bound VEGFR-1, except it lacks the transmembrane region necessary to attach the receptor to the cell membrane (Kendall et al., 1996) and any kinase activity. Since receptor dimerization is essential for signaling through VEGF receptors, sVEGFR-1 sequesters VEGF from activating either VEGFR-1 (on monocytes) or VEGFR-2 (on endothelial cells) by inhibiting dimerization of VEGFRs (Roeckl et al., 1998). Once VEGFRs become activated, signaling follows classical receptor tyrosine kinase activating pathways (Kliche and Waltenberger, 2001).

A relationship between VEGF and GM-CSF has yet to be elucidated. Under immune stress, growth factors like M-CSF and GM-CSF stimulate differentiation of hematopoietic progenitor stem cells in the bone marrow to the myeloid compartment and influence their movement into the bloodstream (Wognum et al., 1994). In response to an infectious challenge, monocyte and macrophage recruitment and accumulation at involved sites is advantageous for host defense; however, in alternative settings like breast cancer, growth factors like M-CSF induce the release of VEGF by these monocytes (Eubank et al., 2003) and stimulate tumor metastases (Lin et al., 2001). Data presented in our current study suggest that GM-CSF reduces VEGF activity by inducing secretion of the soluble form of VEGFR-1 by human monocytes and thus by reducing biologically active VEGF available for angiogenesis.

This study demonstrates that recombinant GM-CSF stimulates human monocytes to transcribe and translate the alternatively spliced and soluble form of VEGF receptor-1. By utilizing both in vitro angiogenesis assays (endothelial cell tube formation and migration) and in vivo Matrigel plug assays in mice, we demonstrate that sVEGFR-1 sequesters VEGF from endothelial cells and interrupts angiogenesis. Our model for the inhibition of

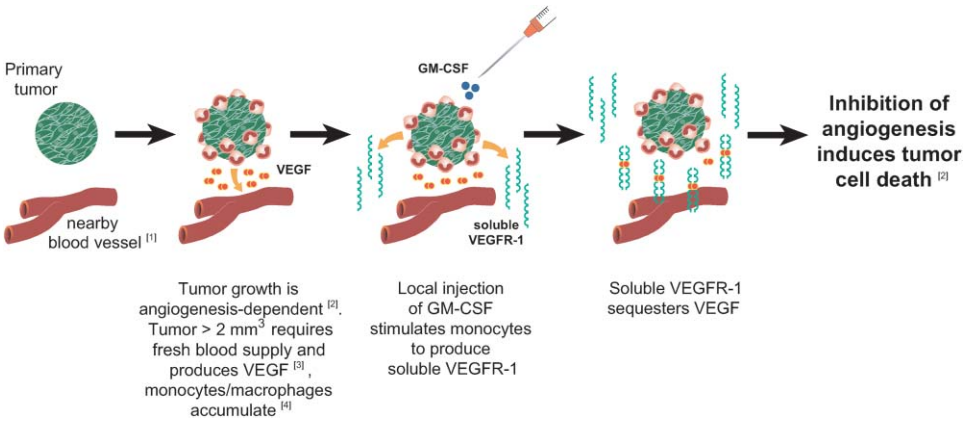


Figure 1. Proposed Model for the Inhibition of Pathological Angiogenesis

This figure was based on models published by Scalerandi et al. (2002), Heuser et al. (1984), Shweiki et al. (1995), and Clarijs et al. (2003).

angiogenesis via sequestration of VEGF by sVEGFR-1 (Figure 1) involves stimulation of local monocytes/macrophages by *direct* GM-CSF administration at the site of the primary tumor.

Results

GM-CSF Reduces VEGF Detection in the Supernatants of Stimulated Monocytes

Previously, we reported that M-CSF induces human monocytes to upregulate both VEGF mRNA transcription and protein production (Eubank et al., 2003). Since GM-CSF is also a survival factor for monocytes, we compared the levels of VEGF in the supernatants from nonstimulated and rhGM-CSF (100 ng/ml)-stimulated monocytes and expressed these values per viable monocyte. The data showed that GM-CSF-stimulated monocytes had significantly less VEGF than nonstimulated samples, as detected by VEGF ELISA at both 24 and 48 hr (Figure 2A).

The Reduction in VEGF Detection from GM-CSF-Stimulated Monocytes Not Due to Sequestration of VEGF within the Cells

Because there was reduced VEGF in the supernatants of GM-CSF-stimulated monocytes compared to nonstimulated cells, we considered that this loss in VEGF detection might be from VEGF sequestration intracellularly in GM-CSF-treated cells. Monocytes were left untreated or treated with rhGM-CSF (100 ng/ml) for 2 and 24 hr and were assayed for VEGF in monocyte whole-cell lysates. The data indicate that at 24 hr, there was significantly less VEGF sequestered within monocytes treated with GM-CSF than in nontreated samples (Figure 2B).

Because VEGF levels were insufficient in the supernatants of nonstimulated or GM-CSF-stimulated cells for immunoprecipitation studies, and because the primary structure of VEGF contains 11% methionines and cysteines, we chose to metabolically label VEGF by using ^{35}S-methionine and ^{35}S-cysteine to determine differences in VEGF protein production. Freshly isolated monocytes were either left nonstimulated or were stimulated with rhGM-CSF (100 ng/ml) or rhM-CSF (100 ng/ml) (positive control) for 36 hr, followed by the addition of both ^{35}S-methionine and ^{35}S-cysteine. Our results indicate that there is no significant difference in VEGF protein production between untreated and GM-CSF-stimulated monocytes (Figure 2C). Furthermore, real-time PCR analysis showed no difference in VEGF mRNA transcription (data not shown) or cell toxicity due to GM-CSF stimulation after 24 hr (data not shown) to explain the observed differences.

Recombinant VEGF Added to the Supernatants of GM-CSF-Stimulated Monocytes Cannot Be Detected by ELISA

We next speculated that GM-CSF-stimulated monocytes released an inhibitory factor into the supernatant that blocked antigenic detection of VEGF by ELISA. We added recombinant human (rh)VEGF into supernatants generated by untreated, rhM-CSF-, and rhGM-CSF-stimulated monocytes and incubated these samples at 37°C for 30 min. While the supernatants of monocytes stimulated with GM-CSF blocked detection of rhVEGF, supernatants from non- or M-CSF-stimulated samples did not (Figure 3A). These data suggest that GM-CSF stimulated the release of a *neutralizing factor* in these supernatants that sequestered rhVEGF from antigenic detection by ELISA. We hypothesized that the VEGF inhibitory factor present in the supernatants was the alternatively spliced sVEGFR-1. Thus, we assayed the ability of recombinant sVEGFR-1 to mask rhVEGF from detection by VEGF ELISA. 1, 8, and 16 ng/ml sVEGFR-1 was incubated with 600 pg/ml rhVEGF at 37°C for 30 min and subjected to VEGF ELISA. There was a significant, dose-dependent reduction in the detection of rhVEGF due to the presence of recombinant sVEGFR-1 (Figure 3B).

Both sVEGFR-1 mRNA and Protein Levels Are Significantly Increased in Response to GM-CSF Treatment

Since rhVEGFR-1 induced a dose-dependent reduction in rhVEGF detection, we assayed each supernatant of untreated, M-CSF-, and GM-CSF-stimulated monocytes

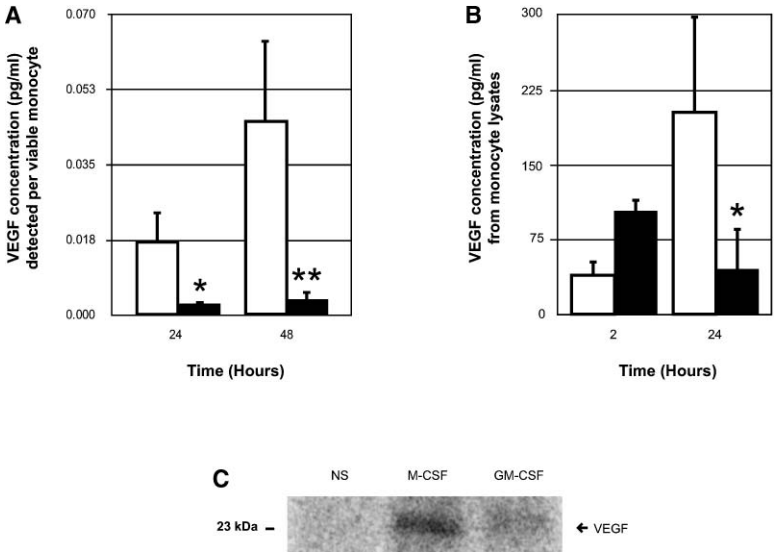


Figure 2. The Reduction in VEGF Detection from GM-CSF-Stimulated Monocytes Was Not Due to Sequestration of VEGF within the Cells

(A) Monocytes were left nonstimulated (white) or were stimulated with GM-CSF (100 ng/ml) (black) for 24 and 48 hr, and cell-free supernatants were subjected to VEGF ELISA. There was less VEGF detected by ELISA per monocyte in the GM-CSF-stimulated samples compared to nonstimulated samples (*$p < 0.01$ and **$p < 0.001$). These data represent the mean ± SEM calculated from three independent experiments.

(B) Monocytes were left untreated (white) or were stimulated with GM-CSF (100 ng/ml) (black) for 2 and 24 hr. Cell lysates were assayed for VEGF by ELISA. At 24 hr, there is significantly less VEGF sequestered within monocytes due to GM-CSF treatment relative to untreated samples (*$p < 0.05$). These data represent the mean ± SEM calculated from four independent experiments.

(C) Monocytes were left nonstimulated (NS) or were stimulated with M-CSF (100 ng/ml) (M-CSF) or GM-CSF (100 ng/ml) (GM-CSF) and were labeled with ^{35}S-Methionine/Cysteine. VEGF was purified from the cell-free supernatants by using heparin-agarose beads and separated on a SDS-PAGE gel, and densitometry was performed by using a phosphorimager. The VEGF band on the PAGE gel was clarified by detection of the band at the predicted size and was compared to the band at the same size purified from the supernatants of M-CSF-stimulated monocytes, used as a positive control. The photograph is representative of three independent experiments.

for endogenously expressed sVEGFR-1. An ELISA selective for human sVEGFR-1 showed that rhGM-CSF (100 ng/ml)-treated monocytes produce a significant amount of sVEGFR-1 compared to both non- and M-CSF-stimulated cells (Figure 4A).

To determine if GM-CSF induced transcription of sVEGFR-1 in human monocytes, primers and a probe specific for sVEGFR-1 mRNA were designed, and real-time PCR analysis was performed. After stimulation of monocytes with rhGM-CSF, the sVEGFR-1 mRNA peak was detected at 24–48 hr, and this peak was followed by a decline in production at 72 hr (Figure 4B). These data indicate a significant increase in the transcription of sVEGFR-1 mRNA in response to GM-CSF. These data led us to hypothesize that GM-CSF induced monocytes to *release* sVEGFR-1 into their supernatants.

Soluble VEGFR-1 Production Correlates to Loss in VEGF Detection

To evaluate the possibility that preformed VEGF:sVEGFR-1 complexes existed within the cell, we either left monocytes untreated or treated them with rhGM-CSF (100 ng/ml) for 24 hr and assayed both supernatant and cell lysate for the presence of sVEGFR-1. Significant concentrations of sVEGFR-1 were released into the supernatant compared to that remaining within the cell; thus, the availability of sVEGFR-1 for preexisting complex formation was limited (Figure 4C).

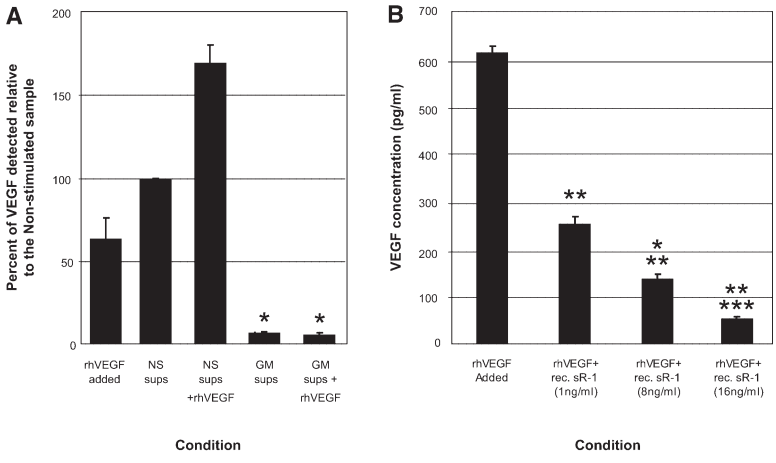


Figure 3. Recombinant sVEGFR-1 Masks VEGF from ELISA Detection

(A) Monocytes were left untreated (NS) or were treated with GM-CSF (100 ng/ml) (GM) for 24 hr. Supernatants were collected and subjected to VEGF ELISA. 450 pg/ml recombinant VEGF (rhVEGF added) was incubated with these supernatants at 37°C for 30 min, and supernatants were subjected to VEGF ELISA (NS sups + rhVEGF) and (GM sups + rhVEGF). There are significant differences in rhVEGF concentrations in both GM-CSF-stimulated samples compared to nonstimulated samples (*$p < 0.001$), but there is no difference between the GM-CSF-stimulated samples (GM sups) alone versus GM-CSF-stimulated supernatants supplemented with rhVEGF (GM sups + rhVEGF) ($p > 0.85$). These data represent the mean ± SEM calculated from three independent experiments.

(B) Increasing concentrations of recombinant sVEGFR-1 (1, 8, and 16 ng/ml) were incubated with 600 pg/ml rhVEGF, and VEGF ELISA was performed to investigate the ability of sVEGFR-1 to mask VEGF from antigenic detection of the ELISA. There is a significant dose-dependent decrease in the detection of rhVEGF due to the presence of sVEGFR-1 (*$p < 0.01$ versus VEGF + sVEGFR-1 [1 ng/ml]; **$p < 0.001$ versus VEGF added; ***$p < 0.001$ versus VEGF + sVEGFR-1 [1 ng/ml]). These data represent the mean ± SEM calculated from three independent experiments.

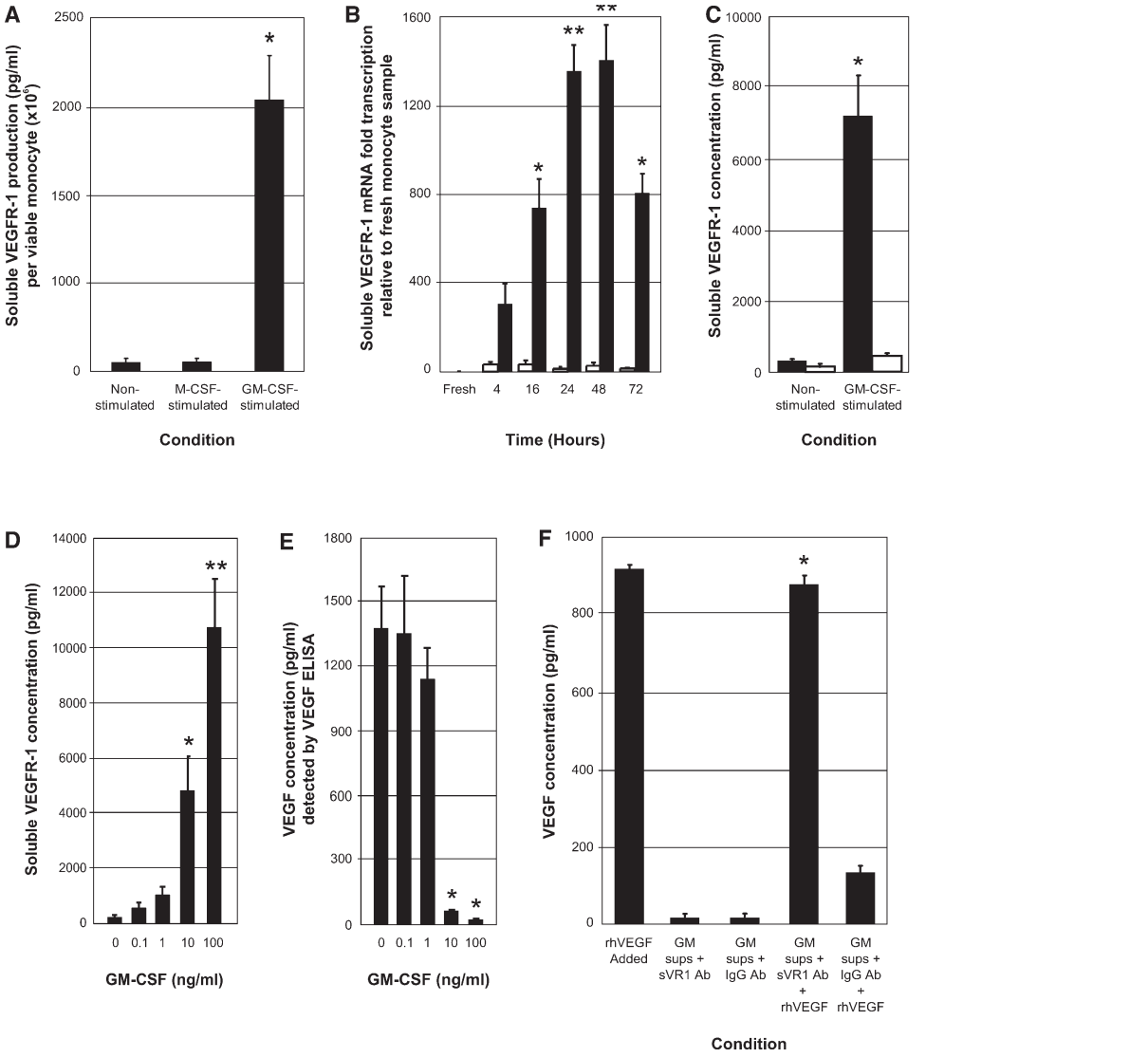


Figure 4. GM-CSF Augments sVEGFR-1 mRNA Transcription and Protein Production While Concomitantly Reducing the Detection of VEGF by ELISA

(A) Monocytes were left nonstimulated or were stimulated with M-CSF (100 ng/ml) or GM-CSF (100 ng/ml) for 24 hr, and the supernatants were subjected to sVEGFR-1 ELISA. GM-CSF-stimulated supernatants have significantly increased levels of sVEGFR-1 compared to M-CSF-stimulated or nonstimulated controls (*$p < 0.001$). These data represent the mean ± SEM calculated from seven independent monocyte donors.

(B) Monocytes were left nonstimulated (white) or were stimulated with GM-CSF (100 ng/ml) (black) for 4, 16, 24, 48, or 72 hr, and total cellular RNA was subjected to real-time PCR. sVEGFR-1 mRNA levels were significantly higher at both 24 and 48 hr versus nonstimulated samples at the same time points (*$p < 0.01$ and **$p < 0.001$). These data represent the mean ± SEM calculated from three independent experiments.

(C) Monocytes were left nonstimulated or were stimulated with GM-CSF (100 ng/ml) for 24 hr. Supernatants (black) and cell lysates (white) were subjected to sVEGFR-1 ELISA. There is significantly more sVEGFR-1 released into the supernatant than what remains within the cells (*$p < 0.001$). These data represent the mean ± SEM calculated from six individual monocyte donors.

(D) Monocytes were left nonstimulated or were stimulated with 0.1, 1, 10, or 100 ng/ml GM-CSF for 48 hr, and supernatants were subjected to sVEGFR-1 ELISA. There is a significant increase in sVEGFR-1 in both the 10 ng/ml and 100 ng/ml GM-CSF-treated samples compared to the 0, 0.1, and 1 ng/ml GM-CSF-stimulated samples (*$p < 0.01$ versus 0, 0.1, and 1 ng/ml GM-CSF-treated samples; **$p < 0.05$ versus 0, 0.1, 1, and 10 ng/ml GM-CSF-treated samples). These data represent the mean ± the SEM from three independent monocyte donors.

(E) Supernatants from the GM-CSF dose-dependent trials in (D) were concomitantly assayed by VEGF ELISA to analyze the concentration of GM-CSF that reduced VEGF antigenic detection by VEGF ELISA. GM-CSF at both 10 ng/ml and 100 ng/ml stimulates significant reduction of VEGF in a dose-dependent manner compared to 0, 0.1, and 1 ng/ml GM-CSF-stimulated samples (*$p < 0.01$ versus 0, 0.1, and 1 ng/ml GM-CSF-treated samples). These data represent the mean ± SEM from three independent monocyte donors.

(F) Monocytes were stimulated with GM-CSF (100 ng/ml) and were incubated for 24 hr. Supernatants were incubated with an antibody specific for sVEGFR-1 (sVR1 Ab) (2.5 μg/ml) or an isogenic IgG control antibody (IgG Ab) (2.5 μg/ml) for 30 min at 37°C and were then removed by using protein G beads. Supernatants were subjected to VEGF ELISA ([GM sups + sVR1 Ab] and [GM sups + IgG Ab]) for the presence of VEGF. rhVEGF (900 pg/ml) (rhVEGF Added) was incubated with some samples ([rhVEGF Added], [GM sups + sVR1 Ab + rhVEGF], and [GM sups + IgG Ab + rhVEGF]) for 30 min and subjected to VEGF ELISA. Supernatants from GM-CSF-stimulated monocytes incubated with the antibody for sVEGFR-1 allowed rhVEGF detection by ELISA, while the supernatants from GM-CSF-stimulated monocytes incubated with the isogenic IgG antibody did not (*$p < 0.001$ for VEGF detection versus GM sups + IgG Ab sample and no significant difference compared to rhVEGF Added sample). These data represent the mean ± SEM calculated from three individual monocyte donors.

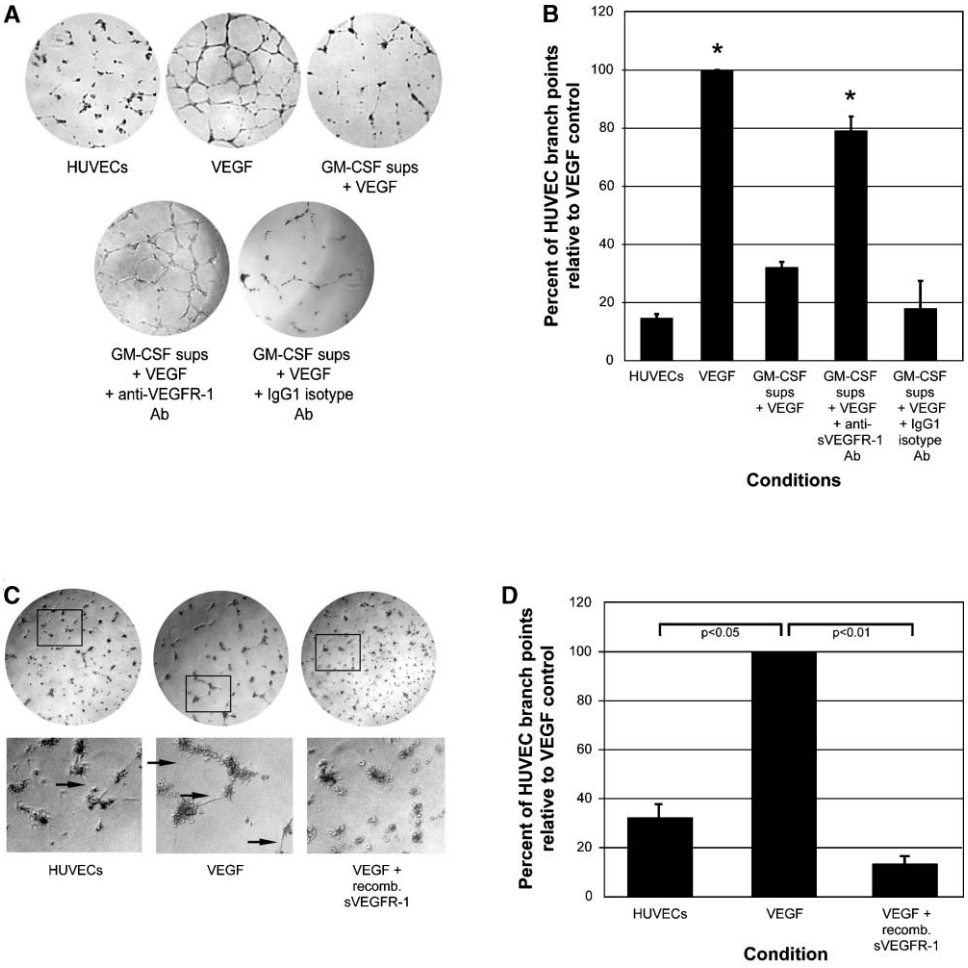


Figure 5. Supernatants from rhGM-CSF-Stimulated Monocytes Inhibit Angiogenesis Effects In Vitro

(A) HUVECs were cultured in Matrigel as follows: HUVECs with EBM media (1 ml) (HUVECs); cells + rhVEGF (2.5 ng/ml) (VEGF); cells + 1 ml supernatants from rhGM-CSF (100 ng/ml)-stimulated monocytes + VEGF (2.5 ng/ml) (GM-CSF sups + VEGF); cells + 1 ml supernatants from rhGM-CSF (100 ng/ml)-stimulated monocytes + VEGF (2.5 ng/ml) + α-sVEGFR-1 neutralizing antibodies (2 μg/ml) (GM-CSF sups + VEGF + anti-VEGFR-1 Ab); or cells + 1 ml supernatants from rhGM-CSF (100 ng/ml)-stimulated monocytes + VEGF (2.5 ng/ml) + IgG_1 isotype antibodies (2 μg/ml) (GM-CSF sups + VEGF + IgG_1 isotype Ab). Pictures are representative of three independent trials.

(B) Tubule branch points from HUVECs stimulated as indicated above were counted, and the sum of three different fields for each condition was averaged. Supernatants from monocytes stimulated with GM-CSF supernatants + rhVEGF (2.5 ng/ml) (GM-CSF sups + VEGF) for 24 hr significantly inhibited endothelial cells from forming tube branch points compared to the rhVEGF (2.5 ng/ml) control (VEGF), and inhibition was not statistically different from cells in EBM alone (HUVECs). GM-CSF supernatants incubated with rhVEGF (2.5 ng/ml) and neutralizing antibodies specific for sVEGFR-1 (2 μg/ml) (GM-CSF sups + VEGF + α-sVEGFR-1 Ab) rescued HUVEC tube formation and restored it to levels similar to rhVEGF control levels; these levels were significantly different from levels of GM-CSF supernatants incubated with rhVEGF (2.5 ng/ml) and IgG_1 isotype antibodies (2 μg/ml) (GM-CSF sups + VEGF + IgG_1 isotype Ab) (*$p < 0.05$ versus HUVECs, GM-CSF sups + VEGF, and GM-CSF sups + VEGF + IgG_1 isotype Ab). Error bars represent the mean $\pm$ SEM calculated from three independent studies.

(C) HUVECs were grown on Matrigel as follows: HUVECs with EBM media (1 ml) (HUVECs); cells + rhVEGF (5 ng/ml) (VEGF); or cells + rhVEGF (5 ng/ml) + rh sVEGFR-1 (50 ng/ml) (VEGF+ recomb. sVEGFR-1). The circular photographs are shown at 40$\times$ magnification. Black boxes within the panels indicate areas at 200$\times$ magnification, which are shown below each panel. Arrows denote tubule formation. Photos are representative of two independent trials.

(D) Quantification of tubule branch points from the photographs in (C). There are significantly more tubule branch points in the VEGF condition versus media alone and VEGF + recombinant sVEGFR-1 ($p < 0.05$ and $p < 0.01$, respectively).

To determine the concentration of GM-CSF needed to induce monocyte production of sVEGFR-1, monocytes were left nonstimulated or stimulated with 0.1, 1, 10, or 100 ng/ml rhGM-CSF for 48 hr, and the supernatants were evaluated for sVEGFR-1 and VEGF by respective ELISAs. GM-CSF induced a dose-dependent increase in sVEGFR-1 production (Figure 4D) and a concomitant dose-dependent decrease in VEGF detection (Figure 4E).

Antigenic Detection of rhVEGF by ELISA Is Rescued with Neutralizing Antibodies Specific for sVEGFR-1

To ensure that sVEGFR-1 was responsible for masking antigenic detection of VEGF, sVEGFR-1 was immunode-

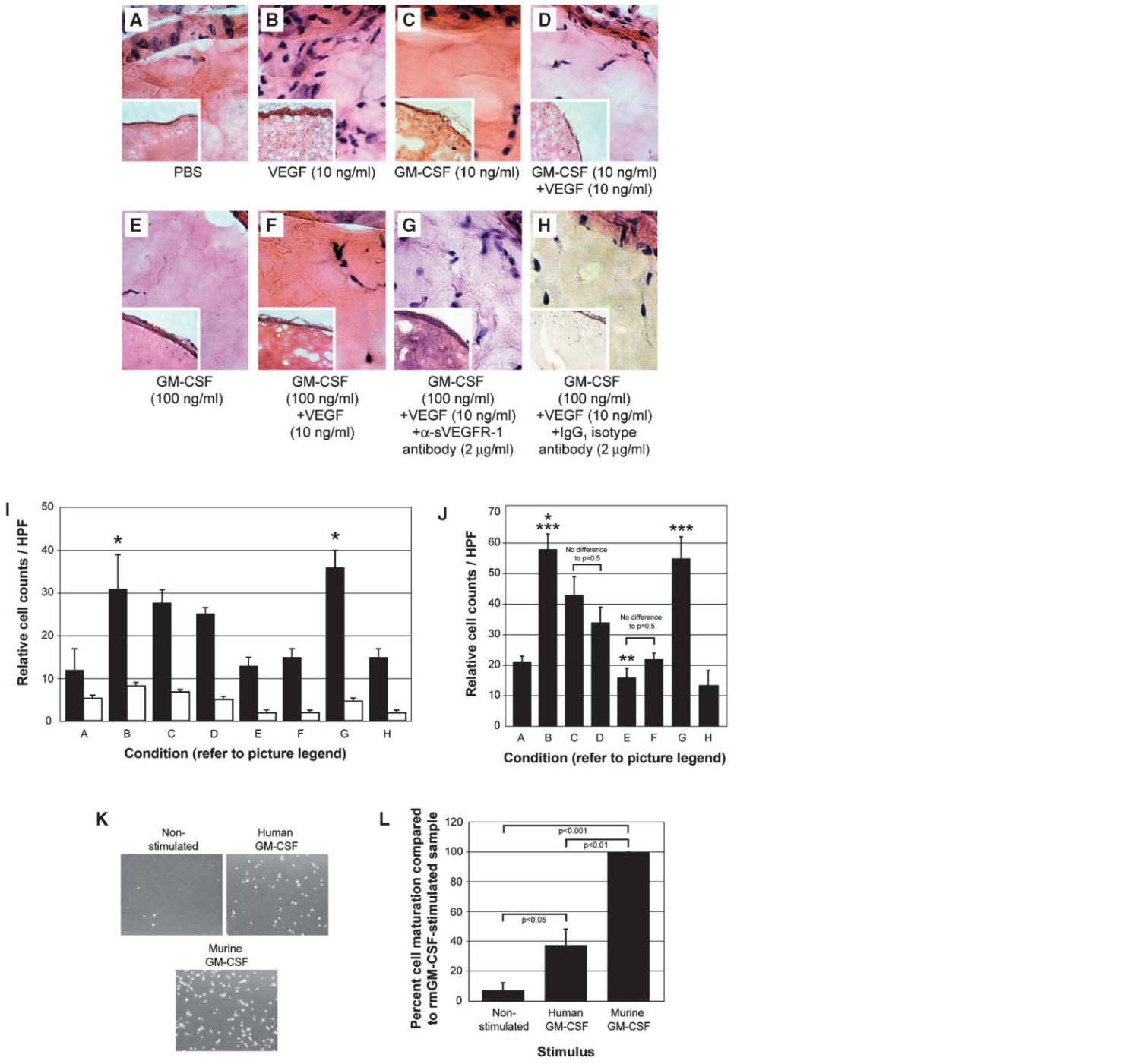


Figure 6. rhGM-CSF Treatment Inhibits Angiogenesis in a Matrigel Plug Assay in Mice

Qualitative representation of angiogenesis in mice in response to Matrigel plugs treated with the following conditions:
(A) Matrigel supplemented with PBS.
(B) Matrigel supplemented with rhVEGF (10 ng/ml).
(C) Matrigel supplemented with rhGM-CSF (10 ng/ml).
(D) Matrigel supplemented with rhGM-CSF (10 ng/ml) + rhVEGF (10 ng/ml).
(E) Matrigel supplemented with rhGM-CSF (100 ng/ml).
(F) Matrigel supplemented with rhGM-CSF (100 ng/ml) + rhVEGF (10 ng/ml).
(G) Matrigel supplemented with rhGM-CSF (100 ng/ml) + rhVEGF (10 ng/ml) + α-sVEGFR-1 antibodies (2 μg/ml).
(H) Matrigel supplemented with rhGM-CSF (100 ng/ml) + rhVEGF (10 ng/ml) + IgG_1 isotype antibodies (2 μg/ml).
(I) Relative count of CD31 (+) cells (black) and CD68 (+) cells (mononuclear cells) (white) that penetrate the Matrigel plugs in response to stimuli; cells were observed by using a 40× objective. There was significantly more CD31 (+) cells in Matrigel plugs treated with (B) rhVEGF (10 ng/ml) alone and (G) GM-CSF (100 ng/ml) + rhVEGF (10 ng/ml) + α-sVEGFR-1 antibodies (2 μg/ml) than in plugs treated with (E) GM-CSF

pleted from the supernatants of 24 hr, rhGM-CSF (100 ng/ml)-stimulated monocytes by using specific neutralizing antibodies targeting the extracellular domain of the sVEGFR-1. Next, rhVEGF (900 pg/ml) was added to these sVEGFR-1-depleted supernatants, and supernatants were assayed for VEGF by ELISA. Depletion of sVEGFR-1 from these supernatants recovered the detection of added rhVEGF. There was no statistical difference between rhVEGF (900 pg/ml) alone and sVEGFR-1-depleted supernatants + rhVEGF (900 pg/ml). In contrast, the detection of rhVEGF was blocked in supernatants immunodepleted by using an isotype IgG_1 antibody (Figure 4F).

Angiogenic Activity of VEGF on Endothelial Cells Is Inhibited by the Presence of sVEGFR-1 Secreted by Monocytes

Endothelial cell tube formation and migration are two in vitro methods to measure the angiogenic effects on cells with growth factors like VEGF (Yahata et al., 2003). To analyze the antiangiogenic activity of sVEGFR-1 produced by GM-CSF-stimulated monocytes, we cultured human umbilical vein endothelial cells (HUVECs) in cell-free supernatants of non- or rhGM-CSF (100 ng/ml)-stimulated monocytes (24 hr incubation) and incubated these HUVECs on growth factor-reduced Matrigel matrix for 20 hr. Through qualitative observation (Figure 5A) and quantitative analysis of tube formation (Figure 5B), the data indicate that GM-CSF-stimulated, monocyte-expressed sVEGFR-1 inhibited tube formation in HUVECs *in combination with* added rhVEGF (2.5 ng/ml) compared to that induced by rhVEGF (2.5 ng/ml) alone (positive control). To assure that sVEGFR-1 was responsible for the reduction in HUVEC capillary-like formation, these supernatants were incubated with neutralizing antibodies specific for sVEGFR-1 or equal amounts of isogenic IgG antibodies. As expected, antibodies to sVEGFR-1 restored tube formation to that induced by rhVEGF (2.5 ng/ml) alone. In contrast, HUVEC samples incubated with GM-CSF-stimulated supernatants or GM-CSF-stimulated supernatants + rhVEGF (2.5 ng/ml) + isogenic IgG antibodies had substantially less tube formation than samples treated with antibodies to sVEGFR-1 (Figures 5A and 5B). To verify that sVEGFR-1 can prevent tube formation of endothelial cells, we cultured HUVECs alone, with rhVEGF (5 ng/ml), or with VEGF (5 ng/ml) + recombinant sVEGFR-1 (50 ng/ml) (Figure 5C). We speculate that any tube formation of HUVECs in the basal media alone arises from VEGF produced by the endothelial cells themselves (Uchida et al., 1994). Indeed, recombinant sVEGFR-1 significantly inhibited endothelial cell tube formation compared to media alone and VEGF control samples (Figure 5D). In addition, to corroborate the HUVEC tube formation assay, we assayed the ability of GM-CSF-stimulated supernatants from monocytes to reduce HUVEC migration through a porous filter disk by using rhVEGF as the chemoattractant. These results were similar to those from the tube formation assay and demonstrated reduced endothelial cell migration in response to conditioned supernatants from GM-CSF-stimulated cells (data not shown).

GM-CSF Treatment Inhibits Angiogenesis in a Matrigel Plug Assay in Mice

Our next objective was to determine if these in vitro observations correlated to in vivo effects of GM-CSF on angiogenesis; thus, we used the Matrigel plug assay in mice. Prior to injection, unpolymerized growth factor-reduced Matrigel matrix was supplemented with PBS alone, with rhVEGF (10 ng/ml), or with rhVEGF (10 ng/ml) + recombinant human sVEGFR-1 (160 ng/ml) to assess if recombinant sVEGFR-1 could inhibit angiogenesis and von Willebrand factor (vWf) (+) cell recruitment within the Matrigel plugs augmented by VEGF as the positive control. After 10 days, the mice were sacrificed, and the plugs were removed, sectioned, and analyzed for vWf (+) cells to identify endothelial cell recruitment. VEGF-treated plugs significantly increased migration of vWf (+) cells and blood vessel formation within the Matrigel plugs compared to PBS-treated plugs (mean values: 28 $\pm$ 1 vWf [+] cells per HPF for VEGF; 15 $\pm$ 1 vWf (+) cells per HPF for PBS plugs) ($p < 0.001$ for VEGF-treated plugs versus PBS plugs). Additionally, those plugs incubated with VEGF (10 ng/ml) + recombinant sVEGFR-1 (160 ng/ml) had significantly less vWf (+) cells (mean value: 20 $\pm$ 1 vWf [+] cells per HPF) than the VEGF (10 ng/ml)-treated plugs ($p < 0.001$). These data suggest that recombinant human sVEGFR-1 inhibited VEGF activity within these plugs.

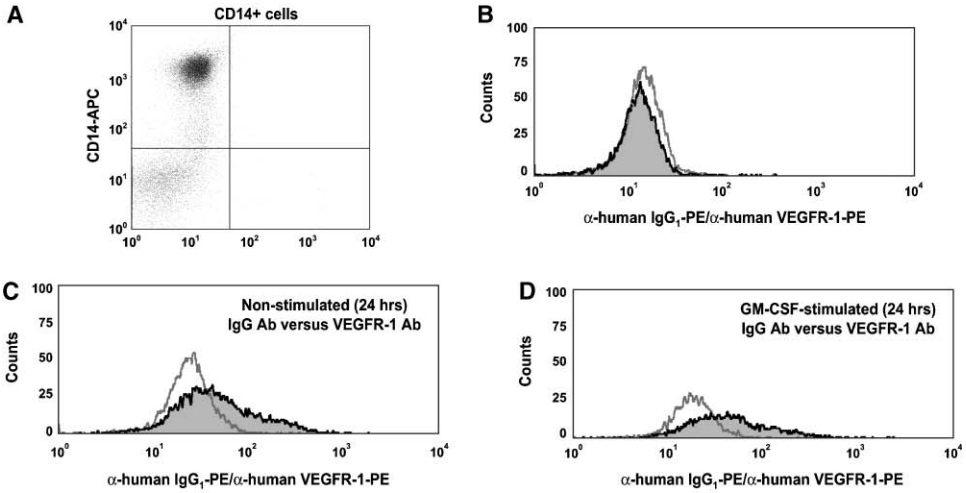
We next incubated Matrigel with rhVEGF (10 ng/ml), rhGM-CSF (10 ng/ml), rhGM-CSF (10 ng/ml) + rhVEGF (10 ng/ml), rhGM-CSF (100 ng/ml), rhGM-CSF (100

(100 ng/ml), (F) GM-CSF (100 ng/ml) + rhVEGF (10 ng/ml), or (H) GM-CSF (100 ng/ml) + rhVEGF (10 ng/ml) + IgG isotype antibody (2 μg/ml) (*$p < 0.05$).

(J) Total cell counts identified by H&E staining for cell nuclei that penetrated the Matrigel plugs in response to stimuli; cells were observed by using a 40$\times$ objective. (E) GM-CSF (100 ng/ml)-treated plugs displayed reduced blood vessel presence within the plugs compared to (B) rhVEGF (10 ng/ml) alone and those plugs containing (G) rhGM-CSF + VEGF + α-sVEGFR-1 neutralizing antibodies. The (E) GM-CSF (100 ng/ml)- and (C) GM-CSF (10 ng/ml)-treated plugs had less cells than plugs treated with (B) VEGF (10 ng/ml) or with (G) sVEGF-R Ab (*$p < 0.003$ versus the GM-CSF (10 ng/ml) + VEGF (10 ng/ml) sample, **$p < 0.003$ versus the GM-CSF [10 ng/ml] sample, and ***$p < 0.001$ versus each of the PBS, GM-CSF [100 ng/ml], and GM-CSF [100 ng/ml] + VEGF [10 ng/ml] samples). Values represent the number of total cell nuclei per 40$\times$ field. At least three mice were tested per group. Note: Large pictures are shown at 400$\times$ magnification, and insets are shown at 100$\times$ magnification for all samples.

(K) C57BL/6 mice were sacrificed, and bone marrow from the femur was collected. After washing in RPMI-1640, the bone marrow was cultured and either left nonstimulated, stimulated with hGM-CSF (100 ng/ml), or stimulated with mGM-CSF (100 ng/ml). Every 2 days, the media was collected and fresh media and GM-CSF treatment was administered. After 10 days, the plates were washed with PBS and pictures taken (5 per well).

(L) Cell counts represent the percentage of mGM-CSF activity (ability to induce murine bone marrow cell maturation) from Figure 7K. Human GM-CSF induces murine bone marrow cell maturation significantly more than PBS treatment (nonstimulated) ($p < 0.05$).



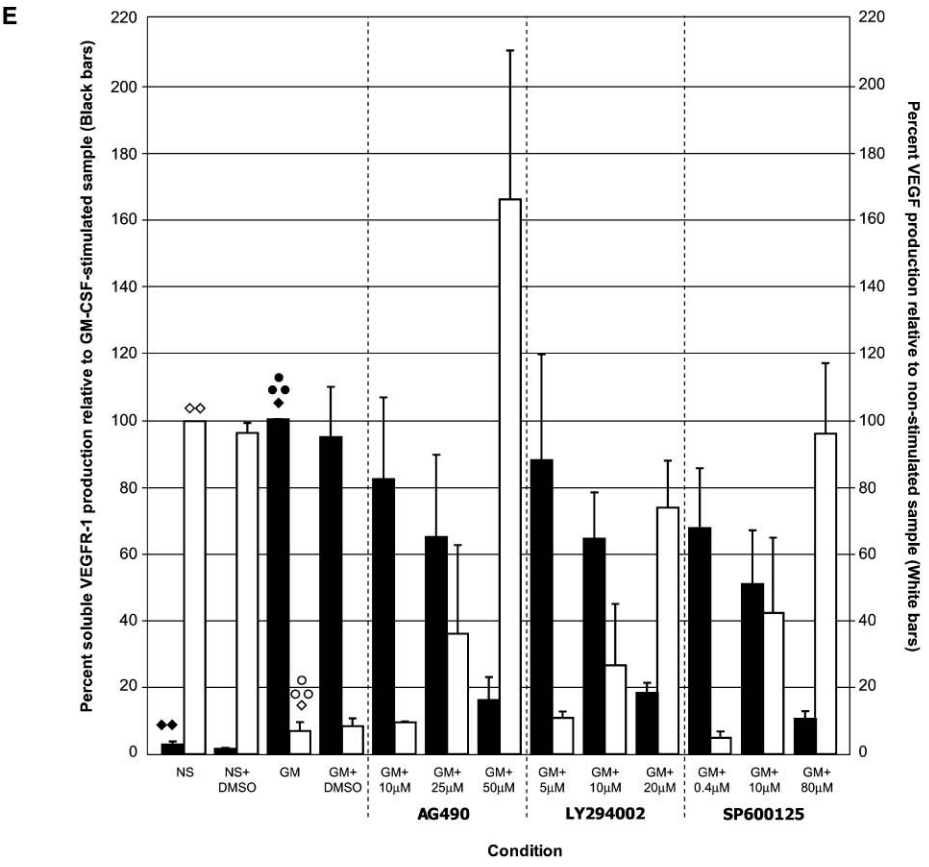


Figure 7. Investigation of the Mechanism of VEGFR-1 Expression and sVEGFR-1 Secretion from Human Monocytes

(A) A population of CD14 (+) cells isolated by flow cytometry by using α-human CD14-phycoerythrin antibodies.

(B) Fresh CD14 (+) cells were analyzed for VEGFR-1 expression by using mouse α-human VEGFR-1-phycoerythrin (shaded) and mouse α-human IgG_1-phycoerythrin (open) antibodies. There is no significant difference between the isotype and VEGFR-1 antibody ($p > 0.5$). These data represent the mean ± SEM calculated from three independent monocyte donors.

(C) Nonstimulated CD14 (+) cells were cultured for 24 hr and were analyzed for VEGFR-1 expression (shaded) and IgG_1 (open) compared to fresh cells (B). There is a significant difference between fresh and nonstimulated monocytes after 24 hr for VEGFR-1 expression ($p < 0.05$). These data represent the mean ± SEM calculated from three independent monocyte donors.

(D) GM-CSF (100 ng/ml)-stimulated CD14 (+) cells were cultured for 24 hr and were analyzed for VEGFR-1 expression (shaded) and IgG_1 (open) compared to fresh cells (B). There is a significant difference in VEGFR-1 expression between fresh and GM-CSF-stimulated monocytes after 24 hr ($p < 0.05$), and there is no significant difference in VEGFR-1 expression between nonstimulated and GM-CSF-stimulated monocytes at 24 hr in culture ($p > 0.2$). These data represent the mean ± SEM calculated from three independent monocyte donors.

ng/ml) + rhVEGF (10 ng/ml), rhGM-CSF (100 ng/ml) + rhVEGF (10 ng/ml) + neutralizing antibodies specific for sVEGFR-1 (2 μg/ml), or rhGM-CSF (100 ng/ml) + rhVEGF (10 ng/ml) + isotype IgG_1 antibodies (2 μg/ml) and then subcutaneously injected the mixture into C57BL/6 female mice. Qualitative observation of these slides suggested that plugs incubated with both rhGM-CSF (100 ng/ml) or rhGM-CSF (100 ng/ml) + rhVEGF (10 ng/ml) (Figures 6E and 6F) had less CD31 (+) cells compared to plugs treated with rhVEGF (10 ng/ml) (Figure 6B). Importantly, plugs supplemented with rhGM-CSF (100 ng/ml) + rhVEGF (10 ng/ml) + neutralizing antibodies for sVEGFR-1 (Figure 6G) had significantly more CD31 (+) cells than plugs treated with PBS, rhGM-CSF (100 ng/ml), rhGM-CSF (100 ng/ml) + rhVEGF (10 ng/ml), and rhGM-CSF (100 ng/ml) + rhVEGF (10 ng/ml) + isogenic antibodies of the same isotype (mouse IgG_1) (Figures 6A, 6E, 6F, and 6H, respectively). When considering CD31 (+) and CD68 (+) cell recruitment, CD68 (+) cell migration was less affected by the addition of GM-CSF than CD31 (+) cell migration.

To quantify differences, digital photographs of the H&E-, CD31-, and CD68-stained slides were taken by using an inverted, phase-contrast microscope at 400× magnification for each sample. Figures 6I illustrates a significant reduction in the number of CD31 (+) cells invading the plugs treated with PBS, rhGM-CSF (100 ng/ml), rhGM-CSF (100 ng/ml) + rhVEGF (10 ng/ml), or rhGM-CSF + rhVEGF + IgG_1 isotype antibodies compared to plugs treated with rhVEGF (10 ng/ml) or rhGM-CSF (100 ng/ml) + rhVEGF (10 ng/ml) + α-VEGFR-1 antibodies. Of interest, plugs treated with rhGM-CSF had a dose-dependent reduction in relative cell counts (Figure 6J). In addition, there was no statistical difference between plugs injected with rhGM-CSF (10 ng/ml) or rhGM-CSF (100 ng/ml) compared to rhGM-CSF (10 ng/ml) + rhVEGF (10 ng/ml) or rhGM-CSF (100 ng/ml) + rhVEGF (10 ng/ml), respectively.

Because there is confusion as to whether recombinant human GM-CSF can stimulate the maturation of bone marrow-derived murine macrophages, we compared the ability of rhGM-CSF and rmGM-CSF to induce macrophage maturation at 10 days. Using bone marrow obtained from normal C57BL/6 mice, we found that both rhGM-CSF and rmGM-CSF induced more macrophage maturation than nonstimulated cells (Figure 6K). Of note, rmGM-CSF at equal concentration was more potent than rhGM-CSF in promoting maturation (Figure 6L).

Membrane Bound VEGFR -1 Expression Is Unchanged Due to GM-CSF Stimulation

Because sVEGFR-1 is an alternatively spliced variant of the membrane bound form of the same gene product (*flt-1*), it is important to understand if the ratio of soluble to membrane bound VEGFR is altered by GM-CSF stimulation in monocytes. After isolating a fresh population of CD14 (+) cells (monocytes) from whole blood (Figure 7A), relative expression of membrane bound VEGFR-1 was investigated (Figure 7B). By standardizing the expression of VEGFR-1 using an antibody specific for the receptor and subtracting the relative value of isotype IgG_1, our data show no significant difference in VEGFR-1 expression at the membrane surface at 24 hr between nonstimulated and GM-CSF-stimulated cells ($p > 0.2$) (Figures 7C and 7D).

JAK, JNK, and PI3-Kinase Pathways Are Responsible for Production of sVEGFR-1 from Monocytes

Since the known biological effects attributed to myeloid progenitor cells by GM-CSF include three known pathways; JNK (Terada et al., 1997; Nagata et al., 1997), PI3-kinase, and JAK (Wojchowski and He, 2001), we assayed the ability of three potent inhibitors of these pathways (SP600125, LY294002, and AG490, respectively) to reduce the production of sVEGFR-1 from human monocytes subsequent to stimulation by GM-CSF. Human monocytes were left untreated or were stimulated with rhGM-CSF (100 ng/ml) in the presence or absence of specific signal transduction inhibitors. Supernatants were collected and subjected to sVEGFR-1 and VEGF ELISAs. Our results indicate a dose-dependent reduction in sVEGFR-1 from AG490 (JAK), SP600125 (JNK), and LY294002 (PI3-kinase pathway) and restoration of VEGF antigenic detection within these same supernatants (Figure 7E). Trypan blue analysis was performed to ensure that changes in sVEGFR-1 and VEGF production were not due to toxicity to the cells. Of note, 50 μM AG490 restored VEGF detection to higher levels than those seen in nonstimulated cells. We are evaluating the possibility that the JAK activity is a negative regulator of native VEGF production to explain this finding.

Discussion

This paper introduces a novel, to our knowledge, role for GM-CSF in regulating VEGF activity by stimulating secretion of the *soluble* form of the membrane bound VEGFR-1 (sVEGFR-1) from human monocytes and thus inhibiting VEGF-induced angiogenesis, both in vitro and in vivo.

The impetus of this study emanated from a report showing that M-CSF (+/−) mice were protected from breast cancer metastases and that overexpressing M-CSF in the primary tumor induced metastases to a level seen in wild-type mice (Lin et al., 2001). As a potential mechanism for this effect, we reported that M-CSF

(E) Monocytes were left untreated (NS), were left untreated with DMSO (NS + DMSO), or were treated with GM-CSF (100 ng/ml) (GM) in combination with inhibitors (AG490, LY294002, or SP600125) for 24 hr. Supernatants were subjected to sVEGFR-1 (black bars) and VEGF (white bars) ELISAs. AG490 (JAK2), LY294002 (PI3-kinase), and SP600125 (JNK) reduced the production of sVEGFR-1 (one closed circle, $p < 0.05$ versus GM-CSF + 50 μM AG490); two closed circles, $p < 0.05$ versus GM-CSF + 20 μM LY294002 one closed diamond, $p < 0.03$ versus GM-CSF + 80 μM SP600125; and two closed diamonds, $p < 0.02$ versus GM-CSF alone). Concomitantly, all three inhibitors rescued the detection of VEGF (one open circle, $p < 0.05$ versus GM-CSF + 20 μM LY294002; two open circles, $p < 0.02$ versus nonstimulated; one open diamond, $p < 0.02$ versus GM-CSF + 80 μM SP600125; and two open diamonds, $p < 0.001$ versus GM-CSF + 50 μM AG490). These data represent the mean ± SEM calculated from three independent monocyte donors.

induces human monocytes to release biologically active VEGF (Eubank et al., 2003). In the performance of that study, we observed that GM-CSF-stimulated monocytes had significantly less VEGF compared to both rhM-CSF-stimulated and nonstimulated monocytes, as detected by VEGF ELISA. Now, we report that the reduction in VEGF observed in response to rhGM-CSF stimulation is due to the production of a *soluble* form of VEGF receptor-1. An ELISA specific for human sVEGFR-1 protein showed that monocytes produced significantly more sVEGFR-1 in response to GM-CSF than in response to M-CSF or those left untreated. These data were further supported by real-time PCR analysis showing that sVEGFR-1 mRNA in GM-CSF-stimulated monocytes peaked at 48 hr, while there was no increase in mRNA levels in nonstimulated cells. Likewise, GM-CSF-stimulated monocytes produced a dose-dependent increase in sVEGFR-1 mRNA transcription and subsequent protein expression, which was significantly greater than in nonstimulated samples.

To investigate the mechanism of loss in antigenic VEGF detection in the supernatants of GM-CSF-stimulated monocytes, these supernatants were incubated with neutralizing antibodies specific for sVEGFR-1 to rescue rhVEGF detection. As predicted, rhVEGF detection was restored in samples incubated with antibodies to sVEGFR-1, while incubation with isogenic IgG antibodies did not restore VEGF detection. Of note, while neutralizing antibodies to sVEGFR-1 restored detection of exogenous rhVEGF, neutralizing antibodies did not rescue detection of endogenous VEGF released in the supernatants of GM-CSF-stimulated monocytes. We speculate that the affinity of preformed protein-receptor complexes was too strong for the antibodies to disrupt, while antibodies added to these supernatants prior to adding rhVEGF allowed competitive inhibition of binding of rhVEGF to the sVEGFR-1.

Next, we wanted to know if supernatants from GM-CSF-stimulated monocytes containing sVEGFR-1 inhibited tube formation of endothelial cells in an in vitro Matrigel angiogenesis model. Cell-free supernatants from GM-CSF-stimulated monocytes inhibited HUVEC tube formation compared to supernatants from nonstimulated cells, confirming that GM-CSF induced monocytes to produce antiangiogenic molecules. More importantly, in vivo data showed that mice injected with Matrigel plugs supplemented with recombinant rhGM-CSF + rhVEGF had significantly less angiogenesis into these plugs compared to plugs supplemented with rhVEGF. Similarly, CD31 (+) cells that invaded the plugs were proportional to bioavailable antigenic VEGF. In contrast, infiltration of CD68 (+) cells within these plugs was not statistically different in any condition. These data suggest that CD68 (+) cells were responding to GM-CSF to regulate VEGF activity and inhibit recruitment of endothelial cells. The mechanism for the differences in angiogenesis seen in plugs treated with or without rhGM-CSF likely reflects the relative production of sVEGFR-1 in GM-CSF-stimulated samples, as antibodies to sVEGFR-1 in rhGM-CSF-treated Matrigel plugs restored blood vessel formation and CD31 (+) cell recruitment to levels seen in plugs incubated with rhVEGF alone.

Since it has been shown that certain tumors can metastasize in the presence of VEGF (Folkman, 1990), it is of great interest pharmacologically to understand the mechanism by which GM-CSF induces overproduction of sVEGFR-1, a molecule that can sequester VEGF and block its activity. sVEGFR-1 mRNA and protein was upregulated by GM-CSF, and since there was no change in the amount of VEGFR-1 surface expression on monocytes in GM-CSF-stimulated cells compared to cells left untreated after 24 hr, we concluded that sVEGFR-1 was transcriptionally regulated by GM-CSF. However, it is possible that GM-CSF induced the production of an intermediate factor to account for sVEGFR-1 expression. To dissect the signaling pathways involved, we used pharmacological inhibitors instead of transfection studies because of the difficulty in transfecting primary human monocytes, and we found that JAK, JNK, and PI3-kinase inhibitors reduced the production of sVEGFR-1 in GM-CSF-stimulated monocytes and recovered detection of VEGF in the samples.

The observation that rhGM-CSF stimulates human monocytes to release sVEGFR-1 and inhibit VEGF-induced angiogenesis has direct impact on solid organ tumors, in which monocyte and macrophage influx into primary tumors under the influence of M-CSF stimulation may enhance tumor metastases (Lin et al., 2001), perhaps through the production of VEGF (Eubank et al., 2003). The observation that rhGM-CSF reduces the biological activity of VEGF suggests that in addition to promoting granulocyte production after chemotherapy, rhGM-CSF may also have antitumor effects through the ability to reduce tumor metastases and angiogenesis. Pharmacologically, GM-CSF is currently used in therapy to treat a number of conditions related to neutropenia and bone marrow transplantation. Existing treatment strategies for recovery of bone marrow in transplant patients include 125–250 $\mu g/m^2$ given daily by IV infusion over 2 hr, beginning within 2 hr after allogeneic BMT (bone marrow transplantation) and continuing for up to 27 days. However, as opposed to systemic administration of rhGM-CSF to induce bone marrow recovery, our data suggest that local injection of rhGM-CSF may be needed to reduce tumor metastases through the production and release of sVEGFR-1 by monocytes.

Soluble VEGFR-1 treatment in tumors is a valid approach, as past studies with various forms of sVEGFR-1 have targeted VEGF and reduced its angiogenic effects (Goldman et al., 1998). Currently, a "decoy" soluble receptor, known as VEGF-TRAP, composed of the first three Ig-like domains of VEGFR-1 fused to the constant region (Fc) of human IgG_1 effectively suppresses tumor growth and vascularization in vivo (Holash et al., 2002). To our knowledge, this is the first report to show that monocytes are an endogenous source of sVEGFR-1 from GM-CSF treatment. At present, we are investigating the involvement of M-CSF and GM-CSF and their effects on monocytes and macrophages in both physiological and pathophysiological angiogenesis utilizing murine models.

Experimental Procedures

Materials

Blood donors were obtained from the American Red Cross. Fetal bovine serum (FBS) (certified < 0.06 EU/ml endotoxin levels) was obtained from Hyclone Laboratories. Recombinant human (rh)GM-

CSF, rhVEGF, rh-sVEGFR-1, the human VEGF Duoset ELISA Development Kit, and the human sVEGFR-1 Quantitikine Kit were purchased from R&D Systems. α-human VEGFR-1 antibody was purchased from Sigma-Aldrich. Growth Factor-Reduced Matrigel matrix and BD Biocoat Invasion Chambers were purchased from Discovery Labware. Human Umbilical Vein Endothelial Cells (HUVECs), Endothelial Basal Medium (EBM), and EGM Singlequots were all purchased from BioWhittaker, Inc. The absolutely RN^A RT-PCR Miniprep Kit for total RNA purification was purchased from Stratagene. The SuperScript First-Strand Synthesis System for RT-PCR Kit for cDNA synthesis was purchased from GIBCO-BRL. Taqman Universal PCR Master Mix was obtained from Applied Biosystems. The human sVEGFR-1 (*sFlt-1*) probe (5′-6FAM-CTGTTTTCTCTCGGA TCT-MGB- 3′), the *sFlt-1* Forward Primer (5′-AGGTGAGCACTG CAACAAAAAG-3′), and the *sFlt-1* Reverse Primer (5′-GTGGTA CAATCATTCCTTGTGCTT-3′) were designed by using Primer Express v1.0 software (ABI Prism, Perkin-Elmer) and synthesized by Applied Biosystems. The primers and probe sequence specific for VEGF mRNA analysis by real-time PCR are as previously described (Eubank et al., 2003). C57BL/6 female mice were purchased from Jackson Laboratories. Human serum albumin (0.1%) was added to all samples to act as a carrier for recombinant VEGF. The JAK inhibitor (JAK2 and JAK3) AG490, the PI3-kinase inhibitor LY294002, and the JNK inhibitor SP600125 were purchased from Calbiochem.

Monocyte Isolation

Single donor monocytes were isolated either from source leukocyte packs obtained from the American Red Cross or by negative selection from fresh blood by using the Monocyte Negative Isolation Kit (Miltenyi Biotec). Of note, monocyte purity is >90% as per the manufacturer. For all experiments, monocytes were resuspended in either 5×10^6 or 10×10^6 cells/condition in RPMI-1640 + 0.1% human serum albumin (HSA) + 10 μg/ml polymyxin B and were left nonstimulated or stimulated with 100 ng/ml rhM-CSF or rhGM-CSF. Polymyxin B was added as further protection against endotoxin contamination in cell cultures.

VEGF and sVEGFR-1 Production Measured by ELISA

Human monocytes were stimulated immediately after isolation by 100 ng/ml rhM-CSF or rhGM-CSF, or they were left untreated and then incubated at 37°C, 5% CO_2 for the indicated time (0, 24, or 48 hr); cell-free supernatants were collected. For inhibitor studies, compounds were added to monocytes for 30 min at 37°C, and the cells were stimulated or left untreated for 24 hr.

VEGF and sVEGFR-1 Measurement in Monocytic Whole-Cell Lysates

Isolated monocytes were stimulated with 1, 10, or 100 ng/ml rhGM-CSF and were analyzed as previously described (Eubank et al., 2003).

^{35}S-Methionine/Cysteine Labeling of VEGF

Freshly isolated monocytes were either left nonstimulated or were stimulated with rhM-CSF (100 ng/ml) or rhGM-CSF (100 ng/ml) and 5% CO_2 for 36 hr at 37°C. All supernatants were aspirated, and fresh DMEM (methionine/cysteine-free) media (1 ml) was added to each sample, followed by 50 μCi/ml of both ^{35}S-methionine and ^{35}S-cysteine for 12 hr. The cultured monocytes were centrifuged at 5,000 rpm for 5 min, and the supernatants were collected and incubated with heparin agarose for 2 hr at 4°C to isolate labeled VEGF and were separated on an 8% SDS-PAGE gel. Gels were dried and subjected to densitometry by using a phosphorimager. VEGF was identified by the predicted molecular weight and by comparison to the band purified from M-CSF-stimulated monocytes, used as a positive control for VEGF production.

Total RNA Isolation from Monocytes

Monocyte total RNA was collected as previously described (Eubank et al., 2003).

Real-Time Polymerase Chain Reaction

Soluble VEGFR-1 primers and probe with MGB quencher were designed based on the human sVEGFR-1 sequence (accession U01134). 2× Universal Master Mix was used in the reaction mixture containing 0.83 μl of 12 μM each forward and reverse primers, 200 nM probe (FAM-MGB), 0.25 μl "20×" 18S internal control probe (VIC-MGB), 17.3 μl DEPC-treated water, and 4 μl cDNA from each sample for a 50 μl total reaction volume. The real-time polymerase chain reaction was completed on the ABI Prism Sequence Detector 7700 (Perkin-Elmer) by using Sequence Detector v1.7 software. Reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Fold induction or reduction of VEGF or soluble VEGFR-1 mRNA was calculated as previously described (Eubank et al., 2003).

In Vitro HUVEC Tube Formation Assay

Isolated monocytes were left nonstimulated or were stimulated with rhGM-CSF (100 ng/ml) and incubated at 37°C, in 5% CO_2, for 24 hr. Cell-free supernatants were harvested and then frozen at −80°C. Prior to using these rhGM-CSF-stimulated monocyte supernatants, all remaining rhGM-CSF was immunodepleted by using α-rhGM-CSF antibodies (2 μg/ml) at 4°C for 2 hr and was subjected to protein G agarose beads for removal. HUVECs were cultured in these supernatants by using growth factor-depleted Matrigel. Antiangiogenic activity was assessed by the inhibition of branch points from capillary-like tube structures formed between the endothelial cells. Matrigel was distributed in a 96-well plate (60 μl/well) and allowed to solidify at 37°C. HUVECs (passes 1–4) were serum starved in EBM for 2 hr. All controls and samples were resuspended in EBM and had 1.5×10^5 HUVECs/well. All components were rotated at 4°C for at least 1 hr before addition to HUVECs. The culture was incubated at 37°C for 20 hr. Tube formation was observed, and digital pictures were captured. Quantification of antiangiogenic activity was measured by counting branch points from tubes formed between discrete endothelial cells in each well relative to the positive control (2.5 ng/ml rhVEGF). Total branch points in three high-powered fields were counted per well in a blinded manner. Additionally, HUVECs were cultured in EBM alone, with rhVEGF (5 ng/ml), or with rhVEGF (5 ng/ml) + recombinant sVEGFR-1 (50 ng/ml) and were allowed to incubate at 37°C for 16 hr. Five photographs per well were taken, and the number of tube branch points were quantified as indicated above.

In Vivo Matrigel Plug Assay

Six-week-old C57BL/6 female mice were anesthetized with isoflurane and subcutaneously injected with 0.5 ml growth factor-reduced Matrigel matrix supplemented with either PBS + 0.1% HSA, 1, 10, or 100 ng/ml rhM-CSF; 10 ng/ml rhVEGF alone or in combination with 1, 10, or 100 ng/ml rhGM-CSF; or 100 ng/ml rhGM-CSF incubated with 2 μg/ml sVEGFR-1 neutralizing antibodies or isotype antibodies. All components added to the unpolymerized Matrigel were allowed to incubate at 4°C for at least 4 hr prior to injection. After 10 days, the mice were sacrificed and skinned, and the Matrigel plugs were removed and flash-frozen in liquid nitrogen. At least three mice were used per experimental group.

Histology

Total cellular influx within the plugs was determined by using H&E stain. Photographs of randomly selected high-powered fields with a 40× objective lens were captured for each sample, and they were then counted in a blinded manner and averaged. Relative cell counts per high-powered field were quantified by counting individual endothelial cells, identified by CD31 immunostaining (50:1 in PBS for 1 hr at room temperature, followed by three washes in PBS for a total of 30 min for frozen sections) or by von Willebrand factor immunostaining (by Dr. Donna Kusewitt, Veterinary Pathology, Director of Veterinary Biosciences and Histology/Immunohistochemistry, The Ohio State University, for formalin-fixed, paraffin-embedded sections), which passed inside the perimeter of the Matrigel plug and contributed to the composition of a blood vessel. Mononuclear cells were identified by CD68 immunostaining (200:1 in PBS for 1 hr at room temperature, followed by three washes in PBS for a total of 30 min). "Angiogenesis" is defined as the process of vascularization involving development of blood vessels within the Matrigel plugs.

FLOW Analysis for VEGFR-1 Expression

Human monocytes isolated from whole blood were cultured for 24 hr in EBM and were either left untreated or were treated with 100 ng/ml rhGM-CSF and were subsequently counted and stained for the expression of membrane bound VEGFR-1 by using either mouse α-human VEGFR-1-phycoerythrin mAb (R&D Systems) or mouse IgG_1-phycoerythrin isotype antibody (Pharmingen).

Statistical Analyses

Minitab statistical software utilizing a nonparametric ANOVA with Tukey's post-hoc test was performed to determine differences between groups by using MiniTab software. Groups were considered significantly different at $p < 0.05$.

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