Activation of Vascular Endothelial Growth Factor Receptor-2 in Bone Marrow Leads to Accumulation of Myeloid Cells: Role of Granulocyte-Macrophage Colony-Stimulating Factor[1]

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Vascular endothelial growth factor (VEGF) is a secreted cytokine that plays a major role in the formation and maintenance of the hemopoietic and vascular compartments. VEGF and its receptors, VEGFR-1 and VEGFR-2, have been found to be expressed on subsets of normal and malignant hemopoietic cells, but the role of the individual receptors in hemopoiesis requires further study. Using a VEGFR-2 fusion protein that can be dimerized with a synthetic drug, we were able to specifically examine the effects of VEGFR-2 signaling in hemopoietic cells in vivo. Mice transplanted with bone marrow transduced with this inducible VEGFR-2 fusion protein demonstrated expansion of myeloid cells (Gr-1$^+$, CD11b$^+$). Levels of myeloid progenitors were also increased following VEGFR-2 activation, through autocrine and paracrine mechanisms, as measured by clonogenic progenitor assays. VEGFR-2 activation induced expression of GM-CSF and increased serum levels in vivo. Abrogation of GM-CSF activity, either with neutralizing Abs or by using GM-CSF-null hemopoietic cells, inhibited VEGFR-2-mediated myeloid progenitor activity. Our findings indicate that VEGF signaling through VEGFR-2 promotes myelopoiesis through GM-CSF-dependent and -independent mechanisms. ***The Journal of Immunology,*** **2005, 175: 3015–3024.**

Vascular endothelial growth factor (VEGF)[3] is a secreted cytokine that stimulates the proliferation and migration of endothelial cells, and increases the permeability of blood vessels to plasma proteins (1, 2). VEGF and its receptors have been shown to be critical players in the embryonic development of blood vessels and blood cells. VEGF$^{+/-}$ embryos are devoid of most endothelial and hemopoietic cells in the blood islands, and die between embryonic day (E) 8.5 and E9.5 of embryonic development, because of defects in blood island formation (3, 4). Because of the contribution of blood islands to both vascular and blood cells, it remains unclear whether the absence of hemopoietic cells is a secondary event caused by the absence of endothelial cells lining the blood island structures or a direct effect on hemopoietic cells.

VEGF is also expressed by subsets of adult hemopoietic cells. In adult bone marrow, VEGF is secreted by hemopoietic stem cells after stimulation with cytokines (5). VEGF has been shown to inhibit the activation of the transcription factor NF-κB in hemopoietic progenitor cells, accompanied by alterations in the development of multiple lineages of hemopoietic cells (6). Moreover, chronic administration of rVEGF in mice results in inhibition of dendritic cell development and an increased production of B cells and immature Gr-1$^+$ myeloid cells (7). VEGF gene ablation experiments have demonstrated that VEGF-deficient bone marrow fails to repopulate lethally irradiated hosts, suggesting that VEGF can control hemopoietic stem cell survival during hemopoietic repopulation (8).

VEGF interacts with two tyrosine kinase receptors, VEGFR-1 (flt-1) and VEGFR-2 (flk-1, KDR). VEGFR-2-deficient embryos die at mid-gestation (E9.5) because of the absence of blood islands (9). However, VEGFR-2$^{-/-}$ embryonic stem cells retain the capacity to produce hemopoietic cells when differentiated in vitro (10, 11), but fail to contribute to primitive and definitive hemopoiesis in chimeric mice. Instead, they accumulate on the surface of the amnion, which suggests that VEGF may be involved in the migration of VEGFR-2$^+$ precursors from the mesoderm to sites of primitive hemopoiesis (12).

VEGFR-2 is expressed on 0.5–1.5% adult human CD34$^+$ cells, and it has been reported that pluripotent hemopoietic stem cells are restricted to the CD34$^+$VEGFR-2$^+$ cell fraction in humans (13). By contrast, it has also been reported that neither VEGFR-2$^+$CD34$^{low/-}$ cells nor VEGFR-2$^+$CD34$^+$ cells have long-term reconstitution capacity in mice (14). VEGFR-2 expression has also been demonstrated in neutrophils, macrophages, and some malignant hemopoietic cell lines (15–18). However, much remains to be learned regarding the role of VEGF and its receptors in postnatal hemopoiesis.

Most groups studying the effects of VEGF signaling in normal and malignant hemopoietic cells have used strategies to inhibit VEGF signaling by using either blocking Abs or small molecule kinase inhibitors. In the present study, we used an alternate approach by activating VEGFR-2 in normal bone marrow cells, and investigating the effects triggered by VEGFR-2 signaling in murine bone marrow in vivo. We have designed a strategy that allows us to specifically study the unique effects of VEGFR-2 signaling in

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Received for publication January 27, 2005. Accepted for publication June 15, 2005.

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[1] This work was supported by the National Cancer Institute of Canada with funds from the Canadian Cancer Society, and a grant-in-aid from the Heart and Stroke Foundation of British Columbia and the Yukon. B.L. was supported by a Doctoral Research Award from the Heart and Stroke Foundation of Canada. A.K. is a scholar of the Michael Smith Foundation for Health Research.

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[3] Abbreviations used in this paper: VEGF, vascular endothelial growth factor; BMP, bone morphogenetic protein; FKBP, FK506-binding protein; MIG, MSCV-IRES-GFP; SCF, stem cell factor.

0022-1767/05/$02.00

hemopoietic cells in vivo. By using a VEGFR-2 fusion protein that can be dimerized with a synthetic drug, we were able to specifically study the effects of VEGFR-2 signaling without the interference of endogenous VEGFRs. Using this strategy, we have previously demonstrated that VEGFR-2 can promote the survival of hemopoietic progenitors in vitro in the absence of hemopoietic cytokines through the activation of a PI3K-dependent pathway (19). In the present study, we demonstrate that VEGFR-2 can elicit expansion of a population of myeloid progenitor cells (Gr-1^+, CD11b^+) in bone marrow through autocrine and paracrine mechanisms. VEGFR-2 induces expression of GM-CSF, and blocking of GM-CSF inhibits VEGFR-2 autocrine and paracrine effects on hemopoietic progenitors. Our findings suggest that VEGFR-2 signaling directly promotes myeloid differentiation, but that GM-CSF is required for the increased myeloid progenitor activity.

Materials and Methods

Retroviral vectors and packaging cell lines

The intracellular domain of VEGFR-2 was fused to a modified FK506-binding protein 12 (FKBP12) domain that can dimerize in response to an analog of FK1012, AP20187 (Ariad Pharmaceuticals). The construct used in this study contained a myristoylation sequence, two modified FKBP12 domains, the signaling domain of VEGFR-2, and a C-terminal hemagglutinin epitope tag, and was cloned into a MSCV-IRES-GFP (MIG) retroviral vector, as previously reported (19). Ecotropic packaged virus was generated using the following procedure: Phoenix-AMPHO cells (G. Nolan, Department of Microbiology and Immunology, Stanford University, Palo Alto, CA) were lipofected with the vector plasmids using Fugene (Roche), according to the instructions of the manufacturer. Medium was changed after 24 h, and transfected cells were cultured for another 24 h in DMEM supplemented with 10% FBS. Supernatant was then harvested, filtered, and used for repeated infections of GP+E86 ecotropic packaging cells in the presence of 8 μg/ml polybrene (Sigma-Aldrich). After sorting for GFP expression, transduced GP+E86 cells were plated at limiting dilution. Individual clones were tested, and the highest titer clone was selected by titration of supernatants on NIH 3T3 cells.

Antibodies

Abs used for analysis were anti-CD11b (monocytes), anti-Gr-1 (granulocytes), anti-B220 (B lymphocytes), anti-CD5 (T lymphocytes), and anti-Ter^{119} (erythroid progenitors) (BD Pharmingen). Isotype Ig was used as control (BD Pharmingen). For blocking studies, a GM-CSF-blocking Ab was obtained from R&D Systems.

Isolation of murine bone marrow cells

GM-$CSF^{-/-}$ mice (20), were a kind gift of J. Whitsett (University of Cincinatti, OH). GM-$CSF^{-/-}$ (Cincinnati Children's Hospital Medical Center), C3Pep (Ly-5.1/Ly-5.2, cross between C3H/HeJ and Pep3b), and B6C3 mice (Ly-5.2, cross between C3H/HeJ and C57BL/6J) were maintained by the animal facility of the British Columbia Cancer Research Centre in accordance with regulations approved by the University of British Columbia animal care committee. Bone marrow cells were extracted from the femurs and tibias of mice treated 4 days previously with 150 mg/kg 5-fluorouracil (Pharmacia & Upjohn). Cells were cultured for 48 h in IMDM supplemented with a serum substitute (BSA, insulin, transferrin) (StemCell Technologies), 10^{-4} M 2-ME, 40 μg/ml low-density lipoproteins (Sigma-Aldrich), 1 ng/ml recombinant human Flt3 ligand, 300 ng/ml recombinant mouse stem cell factor (SCF), and 20 ng/ml human rIL-11 (StemCell Technologies).

Gene transfer

Bone marrow cells were harvested and infected by either cocultivation with irradiated (1500 cGy, x-ray from Philips Orthovoltage Therapy Unit RT 250; Philips Medical Systems) GP+E86 viral producer cells or the addition of virus-containing supernatant from the GP+E86 producer cells in fibronectin-coated dishes. Both infection protocols involved 48-h growth on tissue culture plates with the above cytokine combination and with the addition of 5 μg/ml protamine sulfate (Sigma-Aldrich). Following infection, bone marrow cells were plated in the same medium for another 48 h. Cells were then sorted for GFP expression (FACS 440; BD Biosciences).

Bone marrow transplantation

Previously transduced and sorted bone marrow cells (1×10^6 GFP^+ cells/animal) were injected into the tail vein of lethally irradiated (900 cGy, using a ^{137}Cs source from a Sheperd irradiator model 81-14R; JL Sheperd and Associates) B6C3 mice within 24 h of irradiation. Mice were housed in microisolator units and provided with sterilized food, water, and bedding. Irradiated animals were additionally provided with acidified water (pH 3.0) and 100 mg/L ciprofloxacin. Transplanted animals were allowed to reconstitute their bone marrow for 4–8 wk before any experiments were performed. Peripheral blood and bone marrow cells were harvested from the mice to check for GFP engraftment. For bone marrow collection, mice were anesthetized using isoflurane gas (Associated Veterinary Purchasing) supplied by a vaporizer (Ohio Medical Products), and a 22-gauge needle was inserted into the femoral shaft. Bone marrow was aspirated into a syringe containing phenol-free medium supplemented with 2% FBS. Blood was collected by making a small incision on the tail and harvesting into a capillary tube coated with heparin. RBC were lysed by hypotonic shock using 0.8% ammonium chloride/0.1 mM EDTA. Both bone marrow and blood mononuclear cells were then processed for flow cytometry analysis. Mice were then treated with AP20187 or vehicle for 10 days, after which they were sacrificed by CO_2 inhalation. Peripheral blood was obtained by cardiac puncture, while bone marrow was obtained by flushing the four long bones of the limbs with IMDM supplemented with 2% FBS.

AP20187 formulation

AP20187 was a gift from Ariad Pharmaceuticals. Lyophilized AP20187 was dissolved in 100% ethanol at a concentration of 62.5 mg/ml and stored at −20°C. For in vitro use, the ethanol stock was diluted in complete culture medium to the desired concentration immediately before use. The final concentration of ethanol in the culture medium was below 0.5%. For in vivo use, the AP20187 ethanol stock solution was diluted to 2.5 mg/ml using 4% ethanol, 10% PEG-400, and 1.7% Tween 20 in water. All injections were administered to mice within 30 min of dilution into the injection solution. The dose volume was adjusted according to mouse body weight to deliver 10 mg/kg AP20187. The average injection volume was 100 μl per mouse.

Flow cytometry

Cells from bone marrow and blood were washed and resuspended in PBS containing 4% goat serum (Sigma-Aldrich), followed by primary mAb staining for 1 h at room temperature. Cells were then washed in PBS containing 4% goat serum and incubated for 30 min with R-PE-conjugated secondary Ab. After subsequent washes, cells were resuspended in PBS for flow cytometry. Samples were run on an EPICS ELITE-ESP flow cytometer (Beckman Coulter), and data were analyzed using FCS Express V2 (De Novo Software).

Preparation of cDNA and RT-PCR

Total cellular RNA was extracted from murine bone marrow using Qiagen RNeasy Quick spin columns, as described by the manufacturer. The purified total RNA preparation was used as a template to generate first strand cDNA synthesis using SuperScript II (Invitrogen Life Technologies). No PCR product was detected in the negative control reactions performed without reverse transcriptase. PCR was performed using the following primer pairs: M-CSF, 5′-ctctgtcaacggcctgtctgttat-3′, 5′-agctgcttcaccaag gactatgag-3′; thrombopoietin, 5′-tgtggactttagcctgggagaatg-3′, 5′-ttgactct gaatccctgaagcctg-3′; SCF, 5′-ctgcgggaatcctgtgactgataa-3′, 5′-cgggacctaat gttgaagagagca-3′; Flt3 ligand, 5′-gacacctgactgttacttcagcca-3′, 5′-acga atcgcagacattctggtagg-3′; GM-CSF, 5′-cttggaagcatgtagaggccatca-3′, 5′-ctt gtgtttcacagtccgtttccg-3′; bone morphogenetic protein-4 (BMP-4), 5′-ca gaaatggttcctggacacctca-3′, 5′-cacaatccaatcattccagcccac-3′; VEGF, 5′-gc tttactgctgtacctccaccat-3′, 5′-atctctcctatgtgctggctttgg-3′; IL-6, 5′-gttctctgg gaaatcgtgga-3′, 5′-tgtactccaggtagctatgg-3′; Jagged-1, 5′-aatggagactccttc acctgt-3′, 5′-cgtccattcaggcactgg-3′; Delta-1, 5′-tggttctctcagagttagcagag-3′, 5′-agacccgaagtgcctttgta-3′; Delta-4, 5′-gcattgtttacattgcatcctg-3′, 5′-gtag ctcctgcttaatgccaaa-3′; GAPDH, 5′-gcatggccttccgtgt-3′, 5′-gggccgagttggg atagg-3′.

ELISA

Murine GM-CSF levels were determined by standard sandwich ELISA, according to the instructions of the manufacturer (R&D Systems; limit of detection: 1 pg/ml).

Clonogenic myeloid progenitor assay

Hemopoietic clonogenic progenitor frequencies were determined by plating 20,000 bone marrow cells in methylcellulose medium containing 50

ng/ml SCF, 10 ng/ml IL-3, 10 ng/ml IL-6, and 3 U/ml erythropoietin (Methocult GF M3434; StemCell Technologies). Resultant colonies were scored after 10 days of culture.

Statistical analysis

Results were analyzed by ANOVA to ascertain differences between groups, followed by a Tukey test for multiple comparisons. A Student *t* test was used in cases in which only two groups were compared.

Results

Activation of VEGFR-2 induces expansion of bone marrow myeloid cells

Although VEGFR-2 plays a critical role in the formation of the hemopoietic system, its role in adult hemopoiesis remains unclear (21). VEGF and its receptors have been reported to be expressed in a variety of hemopoietic malignancies, and such expression is usually associated with poor prognosis (22). We have previously demonstrated that VEGFR-2 dimerization in murine bone marrow mononuclear cells results in hemopoietic progenitor survival in vitro (19).

To investigate the role of VEGFR-2 activation in vivo, murine bone marrow mononuclear cells were transduced with a MSCV-IRES-GFP-FKBP-VEGFR-$2_{intracellular}$ (MIG-FKBP-VEGFR-2) construct, which can be dimerized with a chemical inducer of dimerization (AP20187). The empty vector MIG was used as control. We have previously demonstrated that the MIG-FKBP-VEGFR-2 construct is signaling competent when stimulated with AP20187 in vitro, and can induce biological responses in murine bone marrow cells (19). Transduced bone marrow was used to transplant lethally irradiated B6C3 mice. Four to 6 wk after transplantation, bone marrow and peripheral blood were obtained from mice to determine baseline engraftment of transduced cells (based on GFP expression). Following this, mice were injected in the peritoneal cavity with 10 mg/kg/day AP20187 or vehicle (1.7% Tween 20 in water) for 10 days, after which they were sacrificed. Peripheral blood and bone marrow were harvested, and the proportion of GFP^+ cells was determined by flow cytometry. We observed, on average, a 3.5-fold increase in the proportion of GFP^+ cells in the bone marrow of the mice in which the VEGFR-2 construct was dimerized with AP20187 (Fig. 1). By contrast, no significant changes were observed in the proportion of GFP^+ cells in the peripheral blood of these mice (data not shown). Mice in which VEGFR-2 was not dimerized (mice that were transplanted with empty vector bone marrow or received vehicle) did not show any changes in the proportion of GFP^+ cells in either bone marrow or peripheral blood. This result indicates that VEGFR-2 activation can elicit a marked expansion of transduced hemopoietic cells in the bone marrow, but interestingly, this expansion of GFP^+ cells in the bone marrow does not translate into an increase in the proportion of GFP^+ cells in the peripheral blood during the time frame of the experiment.

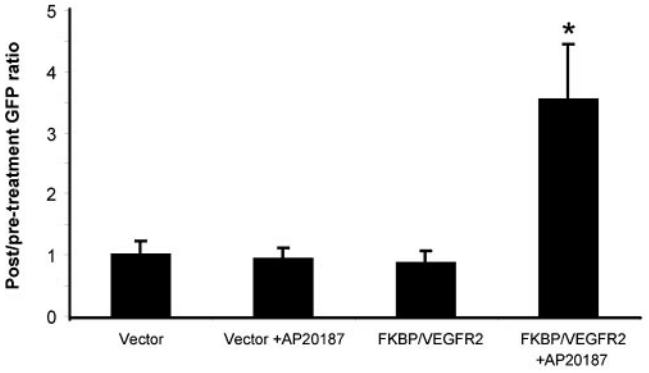

FIGURE 1. VEGFR-2 dimerization induces expansion of hemopoietic cells in vivo. B6C3 mice transplanted with MIG or MIG-FKBP-VEGFR-2 bone marrow 6 wk earlier were injected with AP20187 or vehicle for 10 days. The proportion of GFP^+ cells before and after AP20187 treatment was determined by flow cytometry. Ratios of the proportion of GFP^+ cells posttreatment relative to pretreatment are shown for bone marrow. Data ± SEM shows average of 8–10 mice per group. *, $p < 0.01$.

We next determined the phenotype of cells responding to AP20187 in mice transplanted with MIG-FKBP-VEGFR-2-transduced marrow cells. AP20187 treatment did not result in significantly increased absolute numbers of leukocytes or erythrocytes in the peripheral blood of mice transplanted with MIG-FKBP-VEGFR-2 (data not shown). Cells from bone marrow (Fig. 2) and peripheral blood (Fig. 3) of MIG- or MIG-FKBP-VEGFR-2-transplanted mice injected with AP20187 or vehicle for 10 days were stained for myeloid markers CD11b (monocytes/macrophages) and Gr-1 (granulocytes), lymphoid markers CD5 (T cells), and B220 (B cells) or an erythroid marker (Ter^{119}, bone marrow only) to determine whether VEGFR-2 activation affects the proportion of specific hemopoietic populations. VEGFR-2 dimerization induced a significant increase in the proportion of GFP^+ myeloid cells ($CD11b^+$ and $Gr\text{-}1^+$; 1.5-fold increase on average) in the bone marrow of transplanted mice (Fig. 2). It is interesting to note that when the percentage of cells of different subsets of the bone marrow (lymphoid, myeloid, and erythroid) is added, the total exceeds 100% in the FKBP-VEGFR-2-transplanted mice stimulated with AP20187. This was not observed for any of the other subgroups tested. One possible explanation for this phenomenon lies in the fact that expression of some of the markers used is not absolutely restricted to the specific lineage for which it was tested. For example, CD11b is expressed on subsets of activated B and T lymphocytes, eosinophils, and NK cells (23). Moreover, B220 is expressed on dendritic cell precursors (24) and monocyte precursors (25) in addition to B lymphocytes. This could indicate that VEGFR-2 activation can affect, to a certain extent, the differentiation and expansion of immature cells, which have been shown to coexpress lymphoid and myeloid markers (26).

We also observed a moderate increase in the GFP^+ proportion of myeloid cells compared with the GFP^- population in the peripheral blood (Fig. 3), but this increase was not as marked as observed in the bone marrow. In contrast, VEGFR-2 activation did not affect the lymphoid or erythroid populations in the bone marrow or the lymphoid cells in the peripheral blood, nor did it affect the levels of cells expressing markers of endothelial progenitors (endogenous VEGFR-2, VE-cadherin) (72). These results suggest that VEGF-elicited myeloid expansion can be mediated solely through VEGFR-2 (7, 27).

VEGFR-2 increases the proportion of myeloid progenitors in bone marrow

To assess whether VEGFR-2 induces expansion of myeloid progenitors, bone marrow mononuclear cells were harvested from AP20187- or vehicle-treated mice, and the GFP^+ and GFP^- populations were collected separately and plated in methylcellulose to quantitate myeloid progenitors. AP20187-induced VEGFR-2 dimerization produced an increase in bone marrow myeloid progenitors in both the GFP^+ and GFP^- populations (Fig. 4). The fact that we did not observe any increase in the proportion of $Gr\text{-}1^+$ or $CD11b^+$ cells in the GFP^- population, whereas we observed an increase in myeloid progenitors, which are $Gr\text{-}1^+$ and/or $CD11b^+$, may be due to the low proportion of progenitors in the bone marrow. Therefore, an increase of progenitors would not be reflected in the proportion of $CD11b^+$ and $Gr\text{-}1^+$ cells in the bone marrow. Surprisingly, the expansion of GFP^- progenitors was even greater than the one observed in the GFP^+ population. Because GFP^-

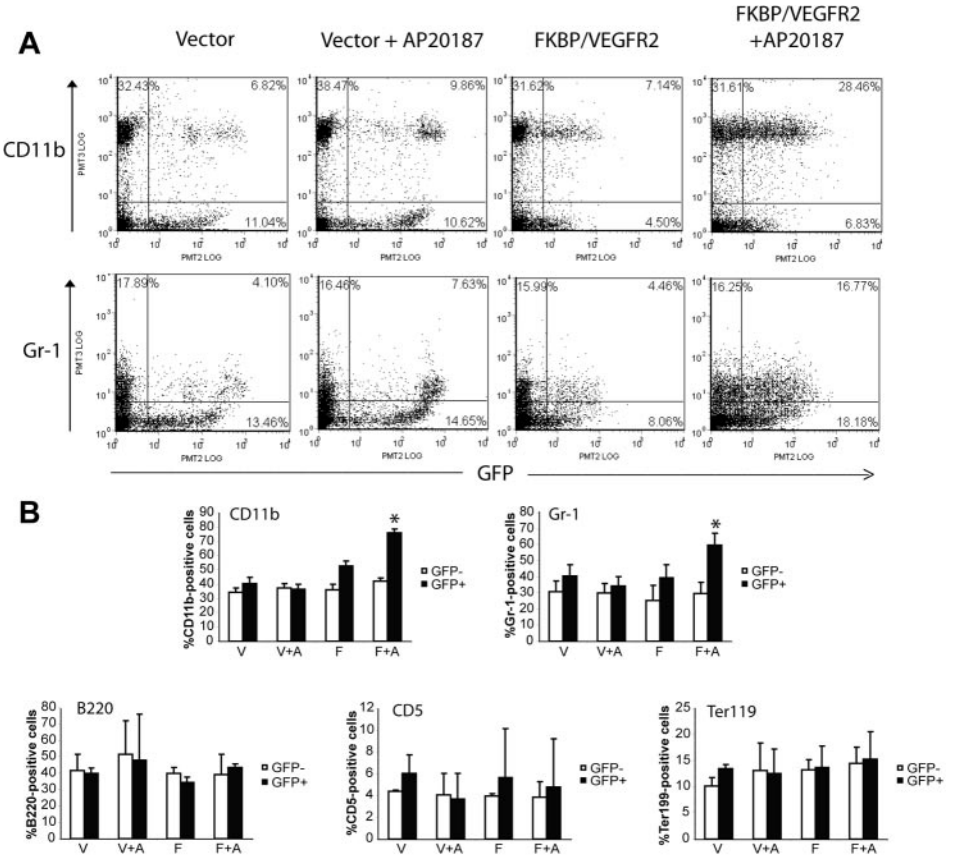

FIGURE 2. VEGFR-2 activation induces expansion of myeloid, but not erythroid and lymphoid, populations in the bone marrow. B6C3 mice transplanted with bone marrow transduced with either MIG or MIG-FKBP-VEGFR-2 were injected with vehicle or AP20187 for 10 days. Cells were harvested from bone marrow and labeled for specific myeloid (Gr-1, CD11b), lymphoid (CD5, B220), or erythroid markers (Ter^{119}). *A*, Representative flow cytometry dot plots of myeloid markers. *B*, Average proportion of specific markers in the GFP^+ and GFP^- bone marrow populations post-AP20187 treatment. Data ± SEM represents average of at least five mice per group. V, Vector; F, FKBP-VEGFR/2; A, AP20187. ∗, $p < 0.05$.

cells do not express the FKBP-VEGFR-2 construct (>85% of GFP^- cells were Ly-5.1^- in a typical experiment, and therefore recipient derived; data not shown) and do not respond to AP20187, it is likely that VEGFR-2 dimerization in the GFP^+ cells induced expression of a factor that can positively modulate myeloid progenitor expansion in the GFP^- population, indicating that VEGFR-2 activation can promote myelopoiesis in part through a paracrine mechanism.

VEGFR-2 activation induces GM-CSF expression and secretion

To identify the potential paracrine factor in VEGFR-2-mediated hemopoietic effects, semiquantitative RT-PCR was performed on murine bone marrow cells to detect the effect of VEGFR-2 activation on the mRNA levels of various growth factors and cytokines known to modulate hemopoiesis. Among these, we assayed for expression of cytokines such as SCF, Flt3 ligand, IL-3, IL-6, M-CSF, GM-CSF, thrombopoietin, and VEGF. We also examined the expression of Notch ligands, Delta-1, Delta-4, and Jagged-1, and BMP-2 and BMP-4, which are known to modulate myelopoiesis. After retroviral transduction, bone marrow cells were sorted for GFP expression, plated in cytokine-free medium, and stimulated with 100 nM AP20187 for 1 h, after which RNA was harvested and processed for RT-PCR (Fig. 5).

VEGFR-2 activation did not affect the levels of expression of the Notch ligands or BMP-4. BMP-2 and IL-3 could not be detected in any of the bone marrow RNA samples, although we were able to detect them in spleen and liver tissues, respectively. However, among the cytokines tested, we found that VEGFR-2 dimerization induced a significant increase of GM-CSF at the mRNA level (2.5-fold), whereas it did not affect the levels of the other cytokines tested. To determine whether GM-CSF was also upregulated at the protein level, we collected supernatant from transduced bone marrow mononuclear cells stimulated with AP20187 for 48 h in vitro. Protein concentration determined by ELISA demonstrated a significant increase of GM-CSF protein in the medium of cultured bone marrow cells when VEGFR-2 is activated (Fig. 6*A*). To determine whether AP20187-induced VEGFR-2 activation can increase levels of GM-CSF in vivo, we assayed the serum of mice transplanted with marrow expressing the VEGFR2 fusion construct and found GM-CSF to be significantly increased when VEGFR-2 was dimerized in vivo compared with control mice (Fig. 6*B*). However, such measurements can grossly underestimate local production because GM-CSF is very rapidly consumed by cells such as polymorphonuclear cells (28). These results confirm that VEGFR-2 dimerization is able to stimulate cells in the bone marrow to up-regulate GM-CSF.

To determine whether GM-CSF was primarily responsible for the VEGFR-2-induced paracrine effects on hemopoietic progenitors, we tested whether blocking the activity of GM-CSF was sufficient to abolish the VEGFR-2-induced paracrine effects on hemopoietic progenitor activity. GM-CSF signaling was inhibited using a GM-CSF-blocking Ab. At a dose of 1 μg/ml, the blocking Ab completely inhibited GM-CSF-induced phosphorylation of Erk1/2 MAPK in bone marrow cells, as determined by Western blot (data not shown). Bone marrow cells transduced with either MIG or MIG-FKBP-VEGFR-2 were cocultured with untransduced primary bone marrow at a 1:1 ratio, with or without AP20187, in the presence or absence of a blocking GM-CSF Ab (1 μg/ml) and the following cytokine combination: Flt3 ligand (1 ng/ml), SCF (10 ng/ml), and IL-11 (1 ng/ml). After 10 days, cells were sorted based on GFP expression. For each culture condition, the sorted GFP^+ and GFP^- populations were plated in methylcellulose to assay progenitor activity. VEGFR-2 dimerization resulted in an increase in the number of progenitors in both the GFP^+ and GFP^-

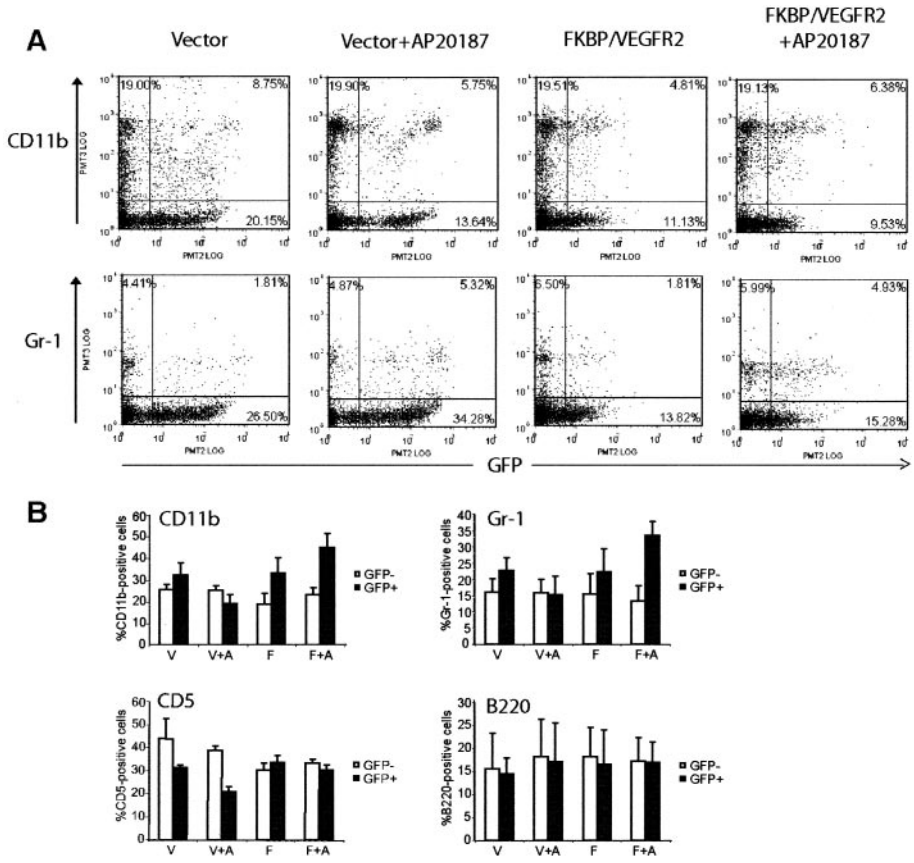

FIGURE 3. VEGFR-2 activation increases the Gr-1$^+$ and CD11b$^+$ subpopulations in the peripheral blood. B6C3 mice transplanted with bone marrow transduced with either MIG or MIG-FKBP-VEGFR-2 were injected with vehicle or AP20187 for 10 days. Cells were harvested from peripheral blood and labeled for specific myeloid (Gr-1, CD11b) or lymphoid markers (CD5, B220). *A*, Representative flow cytometry dot plots of myeloid markers. *B*, Average proportion of specific markers in the GFP$^+$ and GFP$^-$ peripheral blood populations post-AP20187 treatment. Data ± SEM represents average of at least five mice per group. V, Vector; F, FKBP-VEGFR/2; A, AP20187. ∗, A significant difference ($p < 0.05$) between the GFP$^+$ and the GFP$^-$ populations.

populations of bone marrow cells in the presence of the previously mentioned cytokines, indicating that VEGFR-2 signaling is not redundant with that of the specific cytokines in the assay medium (Fig. 7*A*). However, addition of the GM-CSF-blocking Ab resulted in a significant reduction ($p = 0.031$) of the number of progenitors in the GFP$^-$ population cocultured with VEGFR-2-transduced cells in the presence of AP20187, indicating that GM-CSF is responsible at least in part for the increase in the number of progenitors observed when VEGFR-2 is dimerized. Although blocking GM-CSF also decreased the number of progenitors in the GFP$^+$ population when VEGFR-2 was dimerized, this decrease was not as marked as the one observed in the GFP$^-$ population and was not found to be statistically significant ($p = 0.17$). To confirm the role of GM-CSF in the VEGFR-2-mediated expansion of myeloid progenitors and to determine the existence of a GM-CSF internal autocrine loop, which could account for the activity of VEGFR-2 on progenitors, bone marrow harvested from GM-CSF$^{-/-}$ mice was used. GM-CSF$^{-/-}$ bone marrow cells transduced with either FKBP-VEGFR-2 or MIG were cocultured with untransduced wild-type bone marrow cells, as previously described for Fig. 7*A*. After coculture, GFP$^+$ and GFP$^-$ cells were analyzed by flow cytometry for expression of myeloid markers, sorted for GFP expression, and plated in methylcellulose for progenitor assays. The proportion of cells expressing either Gr-1 or CD11b on GM-CSF$^{-/-}$ cells was not significantly different from that observed in bone marrow from wild-type mice (Table I). In both GM-CSF$^{-/-}$ and wild-type cells, we also observed a small increase in both Gr-1$^+$ and CD11b$^+$ cells in the cocultures in which VEGFR-2 was activated, although this increase was not statistically significant (Table I). Lack of GM-CSF resulted in abrogation of VEGFR-2-induced expansion of myeloid progenitors in both the GFP$^+$ and GFP$^-$ populations (Fig. 7*B*), which not only confirms that GM-CSF is responsible for the increase of myeloid progenitors driven by VEGFR-2 in untransduced cells through a paracrine mechanism, but also highlights a role for an internal autocrine mechanism in transduced cells (29, 30).

Discussion

VEGF and its receptors are expressed in both normal and malignant hemopoietic cells (13–15, 17, 18, 22). Although the mechanisms by which VEGF regulates hemopoiesis remain to be further elucidated, recent data have shown that VEGF can have profound effects on hemopoiesis. VEGF can inhibit dendritic cell development and increase the production of B cells and the generation of immature myeloid cells (7, 31). Moreover, it has been shown that VEGF-deficient bone marrow cells fail to repopulate lethally irradiated hosts, and also fail to form colonies in vitro (8). Because subsets of hemopoietic cells express VEGFR-1 and VEGFR-2, it can be difficult to identify the respective role of each receptor, and how they specifically mediate VEGF effects.

Using a strategy that allowed us to selectively activate VEGFR-2, we have previously demonstrated that VEGFR-2 is able to mediate hemopoietic progenitor survival in vitro by activating the PI3K pathway (19). In this study, we demonstrate that VEGFR-2 activation in bone marrow mononuclear cells elicits the expansion of myeloid cells in vivo. After a 10-day regimen of AP20187, there was a net increase in the proportion of CD11b$^+$

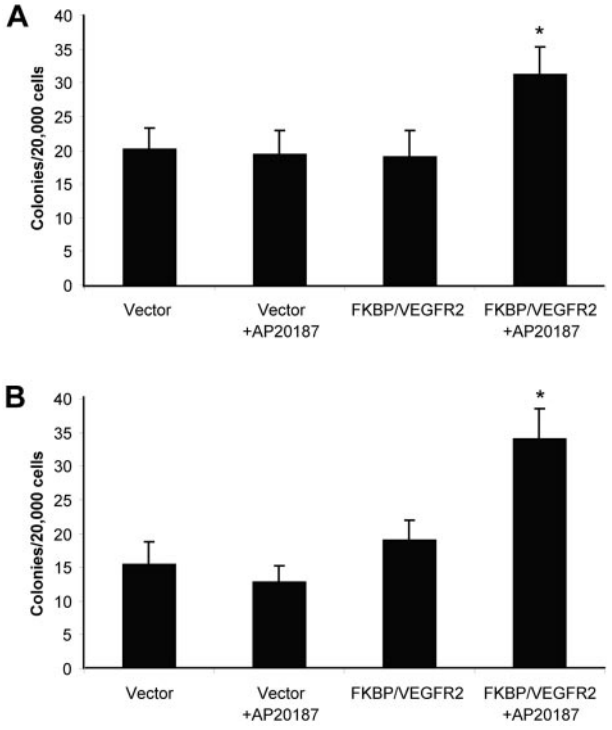

FIGURE 4. VEGFR-2 induces expansion of bone marrow myeloid progenitors in vivo. B6C3 mice transplanted with bone marrow transduced with either MIG or MIG-FKBP-VEGFR-2 were injected with vehicle or AP20187 for 10 days. Bone marrow was harvested and sorted for GFP expression, and the GFP^+ (*A*) and GFP^- (*B*) cells were plated in methylcellulose supplemented with hemopoietic cytokines for colony assays. Colonies were enumerated after 10 days. Data ± SEM represents the average of six independent experiments. *, $p < 0.05$.

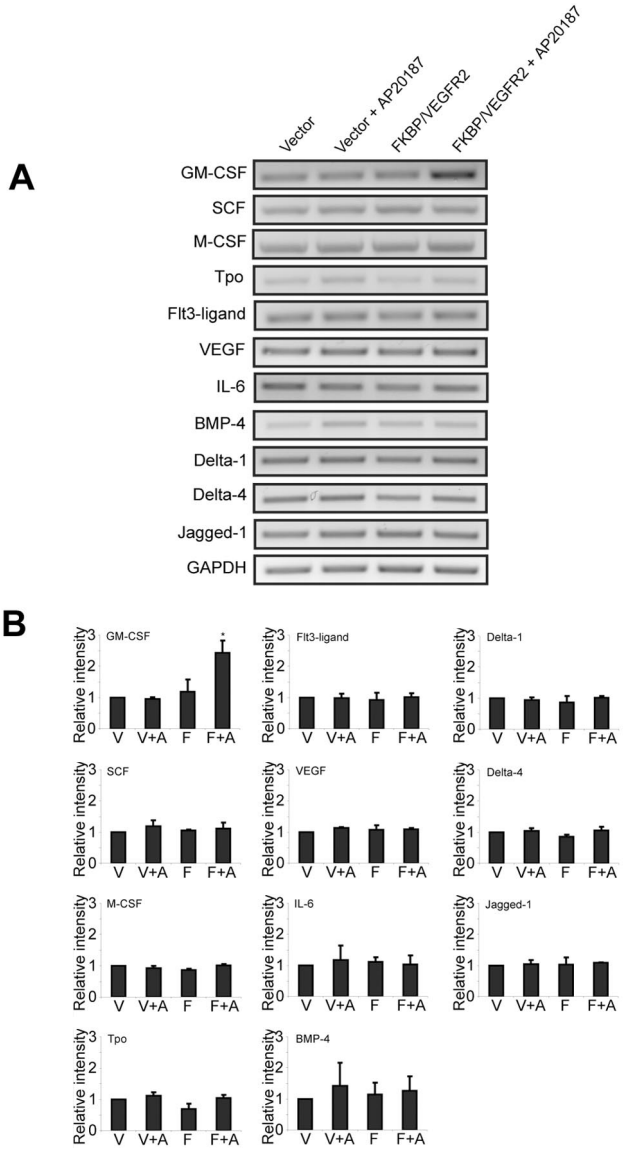

FIGURE 5. VEGFR-2 activation induces mRNA expression of GM-CSF. *A*, Bone marrow mononuclear cells transduced with MIG or MIG-FKBP-VEGFR-2 were incubated with or without 100 nM AP20187 for 60 min. RNA was harvested, and RT-PCR was performed using primers specific for mouse hemopoietic factors. *B*, Densitometric analysis of RT-PCR results, normalized to GAPDH. Data ± SEM represents average of three independent experiments. V, Vector; F, FKBP-VEGFR/2; A, AP20187. *, $p < 0.05$.

and $Gr\text{-}1^+$ cells in the GFP^+ population of the bone marrow of FKBP-VEGFR-2-transduced mice. It is noteworthy that this increase was not as marked in the peripheral blood. The lack of mobilization of $CD11b^+/Gr\text{-}1^+$ cells could be a result of their immature state, as immature myeloid cells are retained within the bone marrow microenvironment. A variety of adhesion molecules (cadherins, VCAM-1) and chemokine receptors (CXCR4) is expressed on either stromal cells or immature progenitors and prevents their release from the bone marrow microenvironment until full maturation has been achieved (32, 33). It is therefore possible that VEGFR-2 activation alone is not sufficient to promote full differentiation of progenitors that remain in an immature state, and they are therefore retained within the bone marrow. However, activation by additional cytokines has the potential to release these cells from the bone marrow. Cytokines such as G-CSF are widely used to promote the mobilization of hemopoietic progenitors: a gradual increase in progenitor numbers in the blood, peaking between 4 and 7 days following G-CSF administration, is observed. VEGF has also been reported to promote the mobilization of bone marrow-derived cells. It can mobilize hemopoietic stem cells through VEGFR-1 and endothelial progenitor cells through VEGFR-2 (34). Furthermore, VEGF can promote the migration of leukemic cells in vitro through VEGFR-2 (35), but little is known regarding the effects of VEGFR-2 on hemopoietic cell migration in vivo. Our results indicate clearly that VEGFR-2 signaling alone does not promote peripheral blood mobilization of myeloid cells, suggesting that additional signals, provided by other VEGFRs, may also be required. Alternatively, this could be a result of the inability of AP20187 to induce cell mobilization because, as opposed to VEGF, it is an intracellular ligand, and cannot reproduce the extracellular concentration gradient found to promote cell migration (31).

Although the GFP^- population of transplanted mice did not display changes in the proportion of $CD11b^+$ and $Gr\text{-}1^+$ cells in the bone marrow or peripheral blood following VEGFR-2 activation, the cells of the GFP^- population had a marked increase in progenitor activity when VEGFR-2 was dimerized. Proteins that could potentially act in a paracrine manner to promote myelopoiesis in the GFP^--untransduced cells include ligands that activate the Notch pathway, the bone morphogenetic proteins, and the hemopoietic cytokines SCF, Flt3 ligand, IL-6, M-CSF, and GM-CSF (36–40). Of all the hemopoietic factors examined, we found that VEGFR-2 only up-regulated GM-CSF in bone marrow cells.

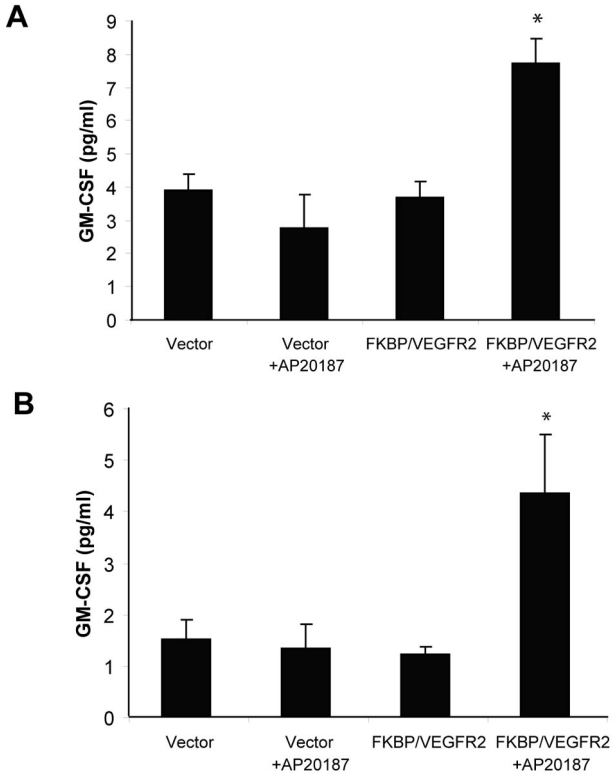

FIGURE 6. VEGFR-2 activation induces expression of GM-CSF in vitro and in vivo. *A*, Bone marrow cells transduced with either MIG or MIG-FKBP-VEGFR-2 were incubated in the presence of 100 nM AP20187. Cell supernatant was harvested after 48 h, and GM-CSF concentrations were quantitated by ELISA. *B*, Serum was collected from mice stimulated for 10 days with or without AP20187 (10 mg/kg/day) following transplantation with MIG- or MIG-FKBP-VEGFR-2-transduced bone marrow, and GM-CSF concentrations were quantitated by ELISA. Data ± SEM represents average of three independent experiments (*A*) or five mice per group (*B*). *, $p < 0.05$.

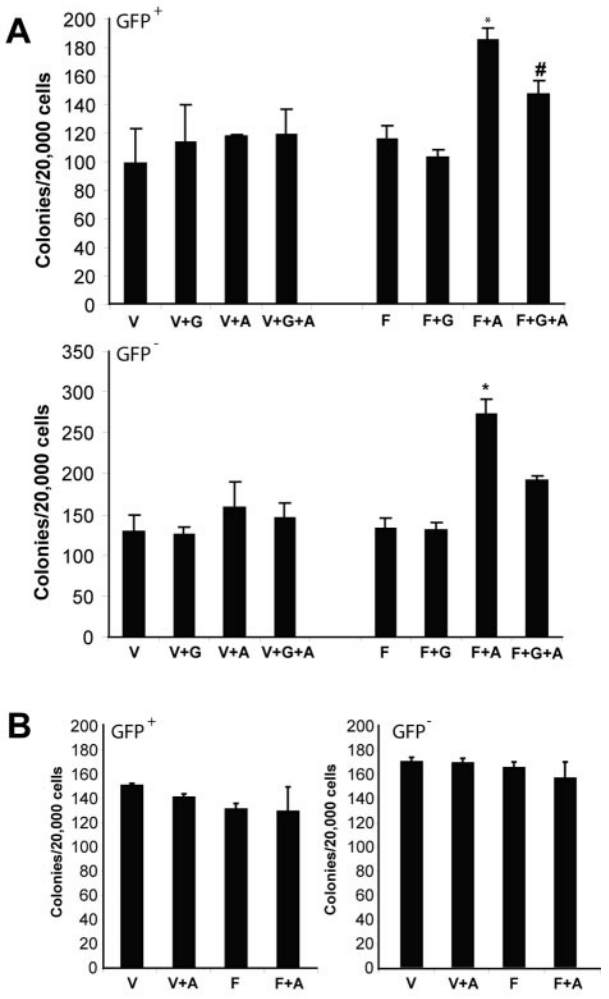

FIGURE 7. Blocking of GM-CSF inhibits VEGFR-2-induced expansion of myeloid progenitors. *A*, Transduced (GFP^+) and untransduced (GFP^-) bone marrow cells were cocultured for 10 days with or without AP20187 (100 nM) and/or GM-CSF-blocking Ab (1 μg/ml) or isotype control. After 10 days, GFP^+ and GFP^- cells were separated by flow sorting and plated in methylcellulose medium for progenitor assays. Data ± SEM represents average of three independent experiments. *B*, Transduced GM-$CSF^{-/-}$ bone marrow cells (GFP^+) were cocultured with untransduced bone marrow cells (GFP^-) from wild-type mice for 10 days with or without AP20187 (100 nM). After 10 days, GFP^+ and GFP^- cells were separated by flow sorting and plated in methylcellulose medium for progenitor assays. Bars represent the mean ± SD of two independent experiments. V, Vector; F, FKBP-VEGFR/2; G, GM-CSF-blocking Ab; A, AP20187. *, A significant difference with other treated cultures ($p < 0.05$), except for the one marked with # ($p = 0.17$). Group marked with # is not significantly different from other treated cultures.

Dimerization of the FKBP-VEGFR-2 construct in murine endothelial cells also up-regulated GM-CSF (data not shown), suggesting that the hemopoietic effects of VEGF in vivo may include stromal regulation of cytokines. GM-CSF has pleiotropic and widespread effects on hemopoietic cells, and exhibits overlapping activities on hemopoietic progenitors with other cytokines, including M-CSF, G-CSF, IL-3, IL-6, and SCF. Our data indicate that GM-CSF is an important mediator of VEGFR-2-induced expansion of myeloid progenitors.

It is worthwhile mentioning that VEGFR-2-induced induction of GM-CSF may also occur through an indirect mechanism. GM-CSF is normally secreted by subsets of T lymphocytes, fibroblasts, vascular endothelial cells, and mast cells (41). Cytokines such as IL-12 (42), VEGF (43), TNF-α, and IL-1β (44) have also been shown to up-regulate the expression of GM-CSF in T lymphocytes (IL-12) and endothelial cells of the bone marrow stroma (VEGF, TNF-α, and IL-1β). Therefore, if VEGFR-2 activation increases production of these cytokines, it is possible that cytokine-stimulated hemopoietic cells (T lymphocytes) or bone marrow stromal cells may account at least in part for the increased levels of GM-CSF. Although we confirmed that VEGFR-2 activation did not up-regulate VEGF, further testing will be required to determine whether VEGFR-2 can up-regulate other cytokines that may be implicated in the indirect expression of GM-CSF by bone marrow stroma cells.

Interesting was the observation that VEGFR-2 activation increased the proportion of Gr-1^+ and $CD11b^+$ cells in the GFP^+ population of the bone marrow, but not in the GFP^- population, whereas the increase in progenitors was more marked in the GFP^- population than the GFP^+ population. Because there is no translatable expansion of untransduced myeloid ($CD11b^+$ and Gr-1^+) cells in the bone marrow or periphery of mice in which VEGFR-2 is activated, even though there is a significant increase of progenitors in the bone marrow, we conclude that the autocrine effects of VEGFR-2 and GM-CSF are prevalent over the paracrine effect. Even though GM-CSF can stimulate the proliferation of myeloid colony-forming cells (45), this cytokine alone is not sufficient to induce a marked expansion of $CD11b^+$ and Gr-1^+ cells in the time

Table I. *Expression of Gr-1 and CD11b in transduced bone marrow cells*[a]

	Wild-type		GM-CSF$^{-/-}$	
	CD11b	Gr-1	CD11b	Gr-1
MIG	82.2 ± 3.0%	40.0 ± 2.3%	82.6 ± 1.6%	35.2 ± 4.8%
MIG + AP20187	81.6 ± 1.7%	36.9 ± 0.4%	86.4 ± 1.1%	39.9 ± 4.0%
FKBP-VEGFR-2	81.5 ± 4.5%	41.9 ± 1.9%	85.1 ± 3.5%	43.5 ± 3.2%
FKBP-VEGFR-2 + AP20187	83.9 ± 3.9%	43.3 ± 6.2%	89.2 ± 2.9%	47.8 ± 6.9%

[a] Expression of Gr-1 and CD11b in transduced bone marrow cells from wild-type and GM-CSF$^{-/-}$ mice following 10 days of incubation with or without AP20187 in the presence of Flt3 ligand, IL-11, and SCF

frame of the experiment. By contrast, VEGFR-2 signaling by itself can induce expansion of differentiated cells, but cannot increase the number of myeloid colony-forming cells without the action of GM-CSF (Fig. 7*B*). Only when VEGFR-2 signaling is combined with that of GM-CSF can we observe a marked expansion of both progenitors and differentiated myeloid cells in the bone marrow. It therefore appears that, in the GFP$^+$ population, VEGFR-2 signaling in combination with GM-CSF drives the rapid expansion and differentiation of myeloid progenitors, whereas in the GFP$^-$ population, there is expansion of myeloid progenitors, but minimal differentiation. Because we have previously shown that VEGFR-2 by itself cannot induce cell proliferation of hemopoietic cells, it is likely that VEGF acts synergistically with other hemopoietic growth factors to promote cell proliferation (19). Such molecules may include other hemopoietic cytokines (IL-1β, IL-5, IL-11) (46–48) or signaling molecules (sonic hedgehog, wnt) (49, 50). Up-regulation of some of these molecules could potentially account for some of the VEGFR-2-induced effects on myeloid cell expansion.

Although no data exist on synergistic signaling between GM-CSF and VEGF, both VEGF and GM-CSF have been shown to synergize with other cytokines (51–55). GM-CSF signals through the recruitment and the activation of JAK-2, STAT-3, and STAT-5 (56, 57). The JAK/STAT pathway is involved in embryonic stem cell self-renewal and has been hypothesized to be an important hallmark of self-renewal capabilities in general (58, 59). GM-CSF can act synergistically with SCF, whose receptor c-Kit is part of the same receptor family as VEGFR-2, to promote the growth and differentiation of primitive hemopoietic cells (60). It is therefore possible that the signals provided by VEGFR-2, which activate the MAPK and PI3K pathways, complement those generated by GM-CSF to drive the rapid expansion and differentiation of myeloid progenitors. This could account, at least in part, for the differences observed in the proportion of bone marrow Gr-1$^+$ and CD11b$^+$ cells observed between the GFP$^+$ population and the GFP$^-$ population.

We also demonstrate that VEGFR-2 can expand myeloid progenitors in vitro even in the presence of hemopoietic cytokines (Fig. 7), indicating that VEGFR-2 signaling is not redundant and can potentiate the action of other cytokines. We show in this study that VEGFR-2 has the potential to expand hemopoietic progenitors in the presence of Flt3 ligand, SCF, and IL-11. This effect was not observed in the presence of IL-3, IL-6, and SCF (19). The redundancy between IL-3 and GM-CSF signaling, which share the common β-chain receptor signaling unit (61), may help explain why VEGFR-2 does not promote further expansion of progenitors when IL-3 is present, while it could potentiate progenitor expansion in the absence of IL-3, through the release of GM-CSF. Activation of the β-chain receptor signaling unit by IL-3 or GM-CSF may therefore be necessary for VEGFR-2 to induce progenitor expansion.

Increased accumulation of immature myeloid cells and CD11b$^+$ macrophages in the bone marrow, lymphoid organs, and spleens of mice implanted with tumors that secrete VEGF has previously been reported (27, 62). More recently, increased production of a more defined population of Gr-1$^+$/CD11b$^+$ immature myeloid cells has been described in several mouse tumor models (63, 64). Increased production of these cells might be triggered by different soluble tumor-derived factors such as VEGF, GM-CSF, M-CSF, IL-6, and IL-10 (65). Treatment of mice with VEGF resulted in dramatic accumulation of Gr-1$^+$ cells in peripheral lymphoid organs (7). Similarly, we observed that VEGFR-2 induced a marked increase in the proportion of Gr-1$^+$ and CD11b$^+$ cells in the bone marrow (Fig. 2) and spleen (data not shown) of transplanted mice. These effects are likely to be the result of VEGFR-2 signaling in hemopoietic cells and the subsequent increase in production of GM-CSF. The contribution of GM-CSF might be essential in this process, because blocking GM-CSF signaling inhibited the expansion of myeloid progenitors in cells not expressing VEGFR-2.

VEGF and its receptors are also expressed in cells and cell lines derived from various hematological malignancies (66). mRNA for both VEGFRs has been detected in cells from various hemopoietic malignancies, such as acute myeloid leukemias and myelodysplastic syndromes (15, 67–69). In addition, several reports have documented inhibitory effects by various classes of small molecule inhibitors targeting VEGFR-1 and VEGFR-2 on the growth of human myeloid leukemia cell lines and in acute myeloid leukemia blasts independently of their effects on angiogenesis (70). Moreover, neutralization of human VEGFR-2 with specific mAbs prolonged survival in mice xenotransplanted with human VEGFR-2$^+$ leukemic cell lines (71). In light of the results we present in this work, it is possible that VEGF-induced autocrine and paracrine stimulation has the potential to induce rapid expansion of leukemic blasts expressing VEGFR-2. VEGFR-2 activation in leukemic cells could induce up-regulation of GM-CSF, which could in turn synergize with VEGF signaling to drive rapid leukemic cell expansion. Whether this process is also important for the expansion of normal myeloid cells is unclear, because VEGFR-2 is expressed at much lower levels and in a smaller proportion of cells in physiological settings. It will therefore be of interest to further study the effects of VEGFR-2 in both normal and malignant hemopoietic cells.

Acknowledgments

We thank Ariad Pharmaceuticals for providing AP20187 and the cloning vector PC4M-Fv2E (⟨www.ariad.com/regulationkits⟩). We also thank Fred Wong for assistance with flow cytometry.

Disclosures

The authors have no financial conflict of interest.

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