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Vascular Endothelial Growth Factor Receptor-2 Induces Survival of Hematopoietic Progenitor Cells*

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Vascular endothelial growth factor (VEGF) and its receptors play an essential role in the formation and maintenance of the hematopoietic and vascular compartments. The VEGF receptor-2 (VEGFR-2) is expressed on a population of hematopoietic cells, although its role in hematopoiesis is still unclear. In this report, we have utilized a strategy to selectively activate VEGFR-2 and study its effects in primary bone marrow cells. We found that VEGFR-2 can maintain the hematopoietic progenitor population in mouse bone marrow cultured in the absence of exogenous cytokines. Maintenance of the hematopoietic progenitor population is due to increased cell survival with minimal effect on proliferation. Progenitor survival is mainly mediated by activation of the phosphatidylinositol 3′-kinase/Akt pathway. Although VEGFR-2 also activated Erk1/2 mitogen-activated protein kinase, it did not induce cell proliferation, and blockade of this pathway only partially decreased VEGFR-2-mediated survival of hematopoietic progenitors. Thus, the role of VEGFR-2 in hematopoiesis is likely to maintain survival of hematopoietic progenitors through the activation of antiapoptotic pathways.

Vascular endothelial growth factor (VEGF)[1] and its two receptors, VEGFR-1 (Flt-1) and VEGFR-2 (kinase domain region/fetal liver kinase) are key regulators of vascular and hematopoietic development (1–3). Deletion of a single VEGF allele results in abnormal blood vessel development and embryonic lethality, indicating a critical dose-dependent embryonic requirement for VEGF (3, 4). In VEGFR-2 knockout embryos, there are critical defects in both hematopoiesis and vasculogenesis that mirror those found in VEGF-deficient embryos (5, 6). However, the mechanisms by which VEGFR-2 affects hematopoiesis and vasculogenesis remain unclear. Although VEGFR-2 is essential for the generation of endothelial and hematopoietic cells *in vivo* (6), these cell populations can arise *in vitro* in embryonic stem cells that are deficient for VEGFR-2 (7, 8), indicating that this receptor might play a conditional role in the generation of cells of the hematopoietic and endothelial lineage. This has led to the hypothesis that VEGFR-2 might exert its effects by promoting the survival, proliferation, and migration of the hemangioblast, precursor of both the hematopoietic and endothelial lineages, rather than acting as a switch that activates differentiation of the hemangioblast (8). This hypothesis is also supported by the fact that VEGF is known to promote proliferation and survival of endothelial cells and that most of its effects appear to be mediated through VEGFR-2 (9).

VEGF and its receptors have also been shown to play an important role in adult hematopoiesis. VEGFR-2 has been found to be expressed on a subset of hematopoietic stem cells that can differentiate into hematopoietic or vascular endothelial cells, depending on the culture conditions (10–12). Recent studies have shown that VEGF can recruit both hematopoietic cells (mainly through VEGFR-1) and endothelial progenitors (through VEGFR-2) to distant sites *in vivo*. This recruitment of marrow precursors may be critical in tumor angiogenesis (13). Furthermore, inhibition of VEGF and/or its receptors has recently been shown to reduce the number of hematopoietic progenitors *in vivo* (14). However, because of the numerous members of the VEGF ligand and receptor family, it is difficult to study the specific effects of VEGFR-2 signaling without the interference of other VEGF receptors such as VEGFR-1, VEGFR-3 (Flt-4), and the neuropilins (15).

Recently, the unique signaling effects of some hematopoietic receptors (Flt-3, Mpl, granulocyte-colony stimulating factor receptor, c-Kit) have been studied by fusing the signaling domain of these receptors to an FK506-binding protein (FKBP) that can be specifically activated using synthetic FKBP ligands (16–22). This system has permitted the demonstration that the self-renewal and differentiation of hematopoietic progenitors can be influenced through distinct, receptor-initiated signaling pathways (23).

In this study, we used this inducible dimerization strategy to specifically study the effects of VEGFR-2 signaling on hematopoietic progenitors. It has been shown that neuropilin-1 is a

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[1] The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; PI 3-kinase, phosphatidylinositol 3′-kinase; MAP, mitogen-activated protein kinase; Erk, extracellular-regulated kinase; Flt, Fms-like tyrosine kinase; FKBP, FK506-binding protein; FBS, fetal bovine serum; EGF, epidermal growth factor; GFP, green fluorescent protein; SCF, stem cell factor; IL, interleukin; IMDM, Iscove's modified Eagle's medium; CFC, colony-forming cell; CFU-S_{12}, colony-forming unit-spleen; BrdUrd, bromodeoxyuridine; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; HA, hemagglutinin; MIG, murine stem cell virus-internal ribosome entry site-enhanced green fluorescent protein.

receptor for VEGF and acts as a co-receptor that enhances the function of VEGF through VEGFR-2 (24). Furthermore, VEGFR-2 has been shown to heterodimerize with VEGFR-1 (25). The strategy we used allows us to study the unique signaling properties of VEGFR-2, without any interference from other VEGF receptors, allowing us to exclude the effects of neuropilin, or heterodimerization with VEGFR-1. To specifically study the unique signaling effects of VEGFR-2, we fused the cytoplasmic domain of this receptor, which contains the split tyrosine kinase domain, to a mutated FKBP12 domain that harbors a phenylalanine to valine mutation at amino acid 36. Although other studies have shown the signaling effects of VEGFR-2 by using VEGFR-2-specific ligands, such as VEGF-E (26), the use of a nontoxic chemical inducer of dimerization, AP20187 (Ariad Pharmaceuticals), allows us to study with high specificity VEGFR-2 signaling pathways in a cell autonomous manner. This strategy also allows us to rule out any potential signaling effects that could be triggered by neuropilin-1, which acts as a co-receptor for VEGF, enhancing its binding to VEGFR-2 (15). Moreover, AP20187 is well tolerated *in vivo*, which allows its use in studying specific signaling pathways *in vivo* and evaluation of its potential use in therapeutic strategies. Our studies show that VEGFR-2 activation results in maintenance of the hematopoietic progenitor population in conditions of cytokine starvation. This effect is mainly due to increased survival of hematopoietic progenitors through the PI 3-kinase/Akt pathway, although the Erk1/2 MAP kinase pathway may also be involved. Our results suggest that VEGFR-2 may be important in maintaining hematopoiesis by promoting the survival of hematopoietic progenitors, through the activation of PI 3-kinase, and possibly through Erk1/2 MAP kinases.

EXPERIMENTAL PROCEDURES

Retroviral Vectors and Packaging Cell Lines—The intracellular domain of VEGFR-2, which exhibits tyrosine kinase activity, was fused to a modified FKBP12 domain that can dimerize in response to an analog of FK1012, AP20187 (27) (Ariad Pharmaceuticals, Inc., Cambridge, MA). The construct we used in this study contained a myristoylation sequence, two modified FKBP12 domains, the signaling domain of VEGFR-2, and a C-terminal hemagglutinin (HA) epitope tag (Fig. 1*A*). A chimeric fusion protein containing an amino-terminal myristoylation signal, two copies of a mutated FKBP12, followed by a carboxyl-terminal HA epitope tag, was released from the PC4M-Fv2E vector (Ariad) using *Eco*RI and *Bam*HI and inserted into the pEGFP-C1 plasmid (Clontech, Mississauga, Canada). An *Spe*I-linked fragment encoding the intracellular domain of human VEGFR-2 was PCR-amplified from the full-length cDNA (gift of C. Patterson) using the following primer pairs: 5′-GACTAGTAAGCGGGCCAATGGAGGG-3′ and 5′-GACTAGTAACAGGAGGAGAGCTCAGTG-3′. The amplicon was digested with *Spe*I, gel-purified, and subcloned into *Spe*I-digested pBluescript. After sequence confirmation, the fragment was released from pBluescript by *Spe*I digestion, gel-purified, and subcloned into the *Spe*I site of the pEGFPC1-FKBP12 plasmid. The FKBP-VEGFR-2 fragment was released using *Hin*dIII-*Xba*I digestion, overhanging ends filled in with Klenow fragment of DNA polymerase I, and cloned into the *Hpa*I site of a previously described murine stem cell virus-internal ribosome entry site-enhanced green fluorescent protein (MIG) vector based on an original vector kindly provided by R. Hawley (28).

Ecotropic packaged virus was generated using the following procedure. Phoenix-AMPHO cells (R. Nolan) were transfected with the vector plasmids using Fugene (Roche Applied Science, Laval, Canada) according to the instructions of the manufacturer. Medium was changed after 24 h, and transfected cells were cultured for another 24 h in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (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). 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.

Cell Lines—HMEC-1 endothelial cells (Center for Disease Control and Prevention, Atlanta, GA) were cultured in MCDB medium (Invitrogen) supplemented with 10% FBS and 10 μg/ml epidermal growth factor (EGF) (Sigma). HMEC-1 cells were retrovirally transduced using amphotropic packaged virus obtained by harvesting the supernatant of Phoenix-AMPHO cells transfected with vector plasmids 48 h prior to supernatant collection. Hematopoietic progenitors were extracted from the femurs and tibias of C3Pep mice (cross between C3H/HeJ and Pep3b) treated 4 days previously with 150 mg/kg 5-fluorouracil (Amersham Biosciences) and cultured for 48 h in Iscove's modified Dulbecco's medium (IMDM) supplemented with a serum substitute (BIT (Stem Cell Technologies Ltd., Vancouver, Canada)), 10^{-4} M 2-mercaptoethanol, 40 μg/ml low density lipoproteins (Sigma), 1 ng/ml Flt3-ligand, 300 ng/ml stem cell factor (SCF), and 20 ng/ml interleukin-11 (Stem Cell Technologies). After stimulation, cells were harvested and infected by either cocultivation with irradiated (1500 centigrays, x-ray) GP+E86 viral producer cells or by 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). Following infection, bone marrow cells were plated in the same medium for another 2 days. Cells were then sorted for GFP expression (FACS 440; Becton Dickinson).

Viability Assays—Sorted bone marrow cells were plated in IMDM supplemented with 10% FBS with or without the addition of 100 nM AP20187. We found that this dose-induced maximal survival effect on hematopoietic progenitors (data not shown). Cells were harvested at various times and counted on a hemacytometer.

CFC Assay—Transduced GFP-positive bone marrow cells were grown in IMDM supplemented with 10% FBS, with or without 100 nM AP20187, for 7 and 14 days. At these time points, hematopoietic 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 units/ml erythropoietin (Methocult GF M3434; Stem Cell Technologies). Resultant colonies were scored after 10 days of incubation.

CFU-Spleen (CFU-S_{12}) Assay—Transduced GFP-positive bone marrow cells were cultured in IMDM supplemented with 10% FBS with or without 100 nM AP20187 for 7 days. 25,000 cells were injected in the tail vein of lethally irradiated (900 centigrays, using a ^{137}Cs source) B6C3 mice (cross between C3H/HeJ and C57Bl/6J). 12 days later, mice were sacrificed, spleens were harvested and fixed in Telleyesniczky's solution, and hematopoietic colonies were counted.

Immunofluorescence—For BrdUrd staining, sorted GFP-positive bone marrow cells were cultured in IMDM supplemented with 10% FBS for 2 days and then treated for 2 h with 10 μM BrdUrd with or without 100 nM AP20187. Cytospin preparations of bone marrow cells were fixed with 4% paraformaldehyde for 5 min, washed with phosphate-buffered saline, and permeabilized with ice-cold methanol for 1 min. Slides were then incubated for 20 min at 37 °C with 2 N HCl to denature DNA. Slides were blocked in phosphate-buffered saline, 5% goat serum, 0.1% Triton X-100 for 10 min, followed by a 1-h incubation with primary antibody (anti-BrdUrd conjugated with AlexaFluor 594 (Molecular Probes, Inc., Eugene, OR), 1:50 dilution in phosphate-buffered saline, 5% goat serum, 0.1% Triton X-100). After washing, nuclear DNA was stained with 4′,6-diamidino-2-phenylindole (1 μg/ml), and slides were mounted in anti-fading solution. For activated caspase 3 staining, cytospin preparations of hematopoietic progenitors grown in culture for 14 days in IMDM containing 10% FBS were stained using the same protocol as above (the DNA denaturation step was omitted), and the following antibodies were used: anti-activated caspase 3 (BD Pharmingen, San Diego, CA) and goat anti-rabbit Ig conjugated with Texas Red (Molecular Probes).

Immunoblotting—Proteins from total cellular extracts were separated by SDS-PAGE and assessed by immunoblotting as previously described (29). Antibodies against phosphorylated VEGFR-2 and total and phosphorylated Akt and Erk MAP kinase were obtained from Cell Signaling Technology (Mississauga, Canada). Anti-HA antibody was obtained from Babco (Richmond, CA). The kinase inhibitors LY294002 and U0126 were obtained from Calbiochem.

Statistical Analysis—A two-tailed Student *t* test was used to determine differences between treated and untreated cultures. p values < 0.05 were considered statistically significant.

RESULTS

Activation of VEGFR-2 Delays Loss of Murine Hematopoietic Progenitors—To study the effect of VEGFR-2 signaling in primary bone marrow cells independently of other VEGF receptors, including VEGFR-1, VEGFR-3, neuropilin-1, or neuropilin-2 (15), or the presence of endogenous VEGF, we used a

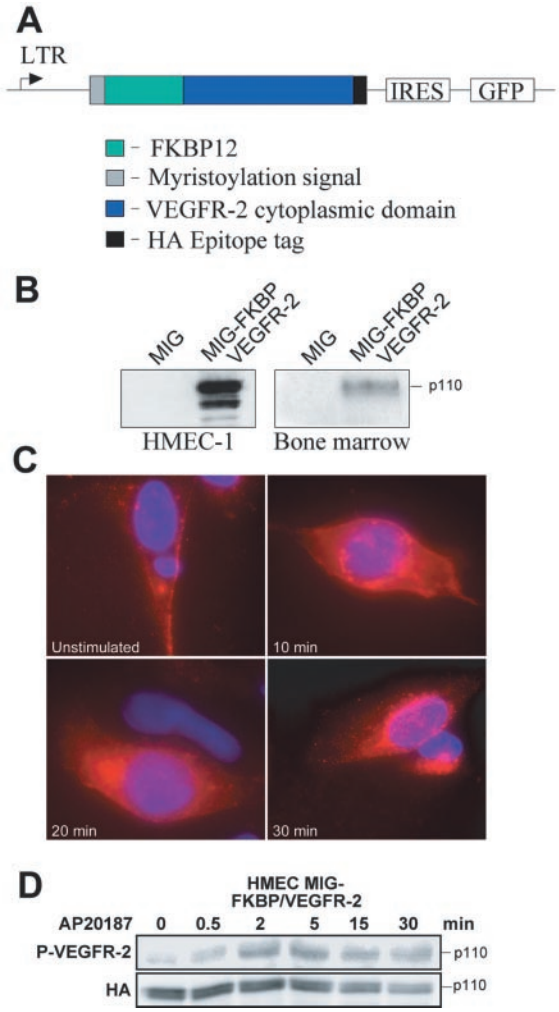

FIG. 1. **MIG-FKBP/VEGFR-2 fusion construct.** *A*, the construct includes the intracellular domain of VEGFR-2 fused to a modified FKBP12 domain. An HA epitope tag was included at the COOH terminus, and a myristoylation sequence was included at the NH_2 terminus. *B*, immunoblotting in HMEC-1 and bone marrow cells demonstrates expression of a 110-kDa protein. *C*, immunofluorescence in HMEC-1 cells reveals that the fusion protein localizes mainly to the cytoplasmic membrane when unstimulated. Stimulation with 10 nM AP20187 over a period of 30 min resulted in partial translocation of the fusion protein to the cytoplasm. *D*, HMEC-1 cells were incubated with 10 nM AP20187 for 0–30 min. Phosphorylation of FKBP/VEGFR-2 fusion protein was determined by immunoblotting using an antibody specific to phosphorylated VEGFR-2 (Tyr^{951}). The membrane was reprobed with anti-HA antibody as loading control. The fusion protein was detected in HMEC-1 cells using an anti-HA monoclonal antibody for both immunoblotting and immunofluorescence.

strategy to dimerize the intracellular domain of VEGFR-2 with a chemical inducer, AP20187. This strategy has previously been used to study the functional role of hematopoietic receptors such as Mpl, Flt-3, and c-Kit (21, 22). We cloned the intracellular domain of VEGFR-2 and fused it to a modified FKBP domain that can be specifically dimerized with a chemical inducer, AP20187 (Fig. 1*A*). When transduced into HMEC-1 cells or murine bone marrow cells, this construct gave a 110-kDa protein (Fig. 1*B*), which mainly localized to the cytoplasmic membrane when unstimulated (Fig. 1*C*). Stimulation of HMEC-1 cells with 10 nM AP20187 for 0–30 min, resulted in progressive translocation of the fused VEGFR-2 construct from the cytoplasmic membrane to the cytoplasm. Phosphorylation of the construct was observed as soon as 30 s after stimulation with 10 nM AP20187 in HMEC cells and remained over a period of at least 30 min (Fig. 1*D*).

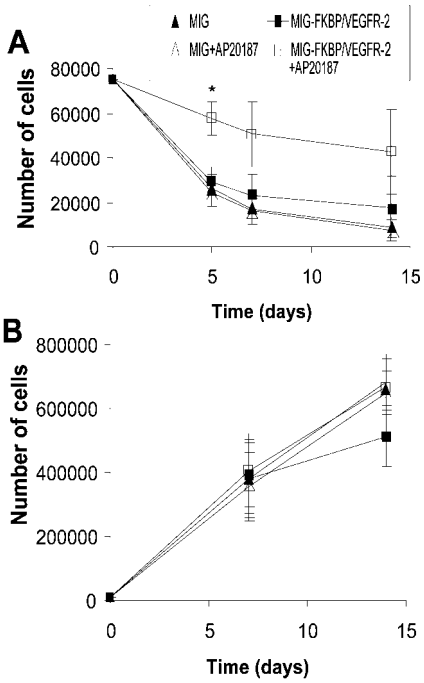

FIG. 2. **VEGFR-2 maintains hematopoietic cell number.** MIG- or MIG-FKBP/VEGFR-2-transduced bone marrow cells were incubated in IMDM supplemented with 10% FBS with or without 100 nM AP20187 in the absence (*A*) or the presence (*B*) of hematopoietic cytokines (IL-3, IL-6, and SCF) for 0–14 days. Cells were harvested at specific time points as indicated, and cell number was determined. Data represent the mean ± S.E. of three independent experiments. An *asterisk* indicates a significant difference ($p < 0.05$) between AP20187-treated cells and untreated cells.

To investigate the effect of VEGFR-2 in hematopoietic cells, bone marrow from mice treated with 5-fluorouracil to activate bone marrow precursor cells was harvested and transduced with the VEGFR-2 fusion construct. As a control, the empty MIG vector was used. After sorting, transduced GFP-positive cells were plated in IMDM supplemented with 10% FBS with or without 100 nM AP20187, and cell number was counted at days 5, 7, and 14. We found that cell number decreased rapidly, indicating the necessity of cytokines for the survival of bone marrow cells. However, in marrow cells in which the VEGFR-2 construct was dimerized by the addition of AP20187, we observed a smaller decrease in cell number (Fig. 2*A*). After two weeks in culture, cell numbers in bone marrow control cultures were 2.5-fold lower than the ones in which VEGFR-2 was dimerized. This effect was not observed in VEGFR-2-transduced cells that did not receive AP20187, indicating that dimerization of VEGFR-2 is required for maintaining hematopoietic cell numbers. We next tested whether dimerization of VEGFR-2 has an additive effect on medium supplemented with hematopoietic cytokines that provide optimal growth conditions (30). Transduced bone marrow cells were cultured in medium containing cytokines that are known to induce hematopoietic cell proliferation (IL-3, IL-6, and SCF), with or without 100 nM AP20187 (Fig. 2*B*). With these growth conditions, we did not observe any significant change when VEGFR-2 was dimerized in comparison with the control cells, suggesting that VEGFR-2 does not signal a proliferative effect that is synergistic with these hematopoietic cytokines.

To test whether VEGFR-2 can preserve the viability and activity of hematopoietic progenitors in the absence of hematopoietic cytokines, VEGFR-2 and control cells were cultured in cytokine-free medium for 7 and 14 days with or without the addition of dimerizer, after which cells were plated in methyl-

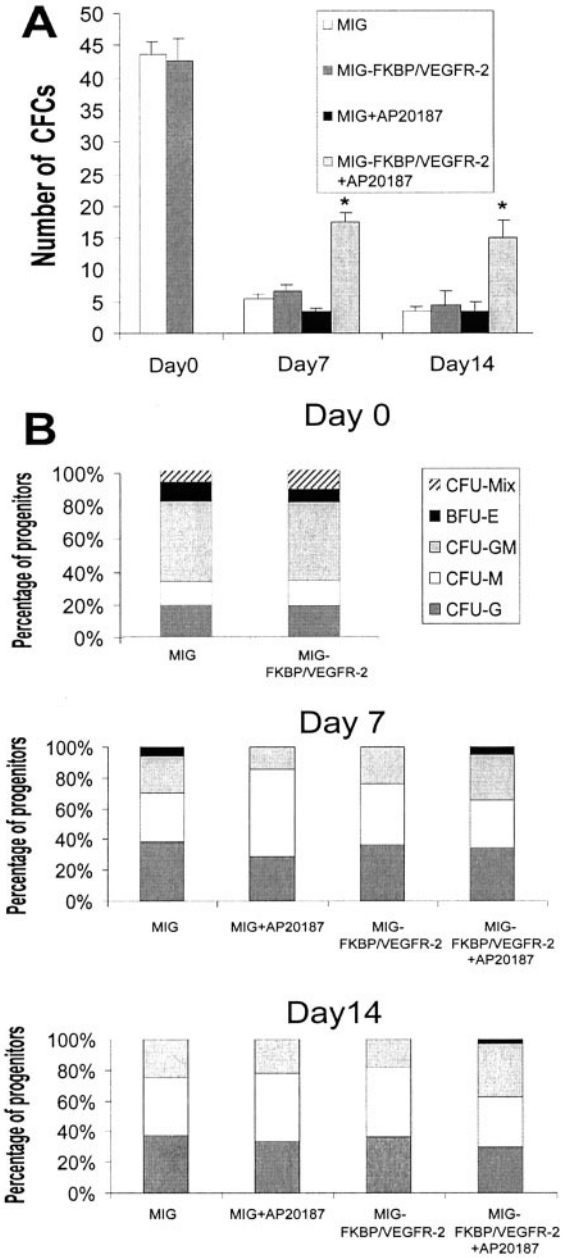

FIG. 3. **VEGFR-2 delays the loss of CFCs.** Hematopoietic progenitors transduced with MIG- or MIG-FKBP/VEGFR-2 were grown in IMDM containing 10% FBS for 7 or 14 days and then plated in complete methylcellulose medium to test for progenitor activity as measured by CFC number after 10 days (*A*). Dimerization of VEGFR-2 did not affect the proportion of different progenitors over time as measured by scoring for the type of colonies formed in the CFC assay (*B*). Data represent the mean ± S.E. of three independent experiments. An *asterisk* indicates a significant difference ($p < 0.05$) between AP20187-treated cells and untreated cells.

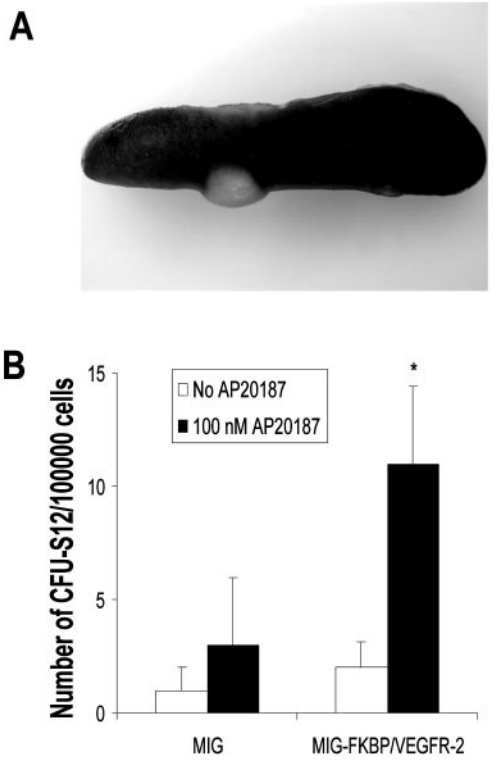

FIG. 4. **VEGFR-2 dimerization maintains the number of multipotential bone marrow progenitors.** Hematopoietic primitive myeloid progenitors cultured for 7 days in IMDM plus 10% FBS, with or without AP20187, were injected into lethally irradiated B6C3 mice, and spleen colonies were counted after 12 days (*A* and *B*). Data represent the mean ± S.E. of three independent experiments. An *asterisk* indicates a significant difference ($p < 0.05$) between AP20187-treated cells and untreated cells.

cellulose medium to assay for hematopoietic progenitors. We found that dimerization of VEGFR-2 maintained hematopoietic progenitor potential in liquid culture. Over a 2-week period in culture, we observed an 8-fold decrease in the number of progenitors in control bone marrow cultures. In contrast, when the FKBP-VEGFR-2 construct was dimerized with AP20187, we observed a 3-fold increase in the maintenance of progenitors over control cultures, consistent with the findings in Fig. 2 (Fig. 3*A*). Although VEGFR-2 dimerization maintained the hematopoietic progenitor population for a period of 2 weeks in the absence of other cytokines, we did not observe a significant change in the proportion of different hematopoietic progenitors as measured by the CFC assay (Fig. 3*B*). This result suggests that VEGFR-2 promotes hematopoietic cell survival and/or proliferation but does not affect differentiation of hematopoietic progenitors.

To confirm that VEGFR-2 can independently maintain the multipotent hematopoietic progenitor population, we utilized the CFU-S_{12} assay following liquid culture of bone marrow cells for 7 days in cytokine-free medium. Colonies were enumerated in each of the spleens harvested 12 days following injection of bone marrow cells (Fig. 4*A*). As seen in Fig. 4*B*, VEGFR-2 dimerization resulted in a 5-fold increase in the proportion of CFU-S_{12} cells, compared with bone control marrow cultures. These results suggest that VEGFR-2 can maintain the activity and viability of primitive hematopoietic progenitors in the absence of other exogenous cytokines.

VEGFR-2 Does Not Increase S-phase Entry in Hematopoietic Precursors—It is known that, in endothelial cells, VEGF can induce cell proliferation. It has been suggested that this effect is mainly mediated through VEGFR-2 (9). We tested whether dimerization of VEGFR-2 also resulted in bone marrow cell proliferation, which could account in part for the delay in the loss of hematopoietic progenitors that we observed. Bone marrow cells were grown in cytokine-free medium for 2 days, then treated with BrdUrd with or without AP20187 for 2 h. Cytospins of cells were then labeled with an anti-BrdUrd antibody (Fig. 5*A*). We found that dimerization of VEGFR-2 did not result in a greater proportion of cells which incorporated BrdUrd, indicating that VEGFR-2 signaling alone may not be sufficient to induce proliferation of hematopoietic progenitors (Fig. 5*B*).

VEGFR-2 Activation Reduces the Number of Apoptotic Cells in Hematopoietic Precursors—It has also been shown that VEGF can induce antiapoptotic signaling through phosphatidylinositol 3′-kinase (PI 3-kinase) in endothelial cells subjected to serum deprivation (31). Since we observed a delay in loss of progenitors when VEGFR-2 is dimerized, we postulated that this effect was caused by an inhibition of apoptosis, since VEGFR-2 dimerization alone did not affect proliferation of hematopoietic progenitors. It has been shown that caspase 3 is present in hematopoietic precursor cells and is activated during apoptosis (32, 33). To test whether VEGFR-2 inhibits hematopoietic cell apoptosis, transduced bone marrow cells were subjected to cytokine deprivation and incubated with or without AP20187 for 14 days. At this point, cytospins were made and stained for the activated form of caspase 3 (Fig. 6*A*). We found that the proportion of apoptotic cells was 2-fold lower in bone marrow cells in which VEGFR-2 was dimerized compared with bone marrow control cultures (Fig. 6*B*). Hence, inhibition of apoptosis through VEGFR-2 signaling would explain in part the maintenance of hematopoietic progenitors observed.

VEGFR-2 Activates the PI 3-Kinase and Erk MAP Kinase Pathways—Since VEGFR-2 dimerization reduces the amount of apoptotic cells, we examined signaling pathways known to be induced by VEGF in endothelial cells. In particular, the PI 3-kinase/Akt and the MAP kinase pathways are both implicated in VEGF signaling and have potential roles in cell survival (31, 34). To determine the kinetics of activation of Akt and Erk1/2 by VEGFR-2, endothelial cells transduced with MIG or MIG-FKBP/VEGFR-2 were starved overnight in medium supplemented with 5% FBS and then treated with AP20187 for 0–60 min. Membranes were reprobed with total Akt or Erk as a loading control. Following dimerization of VEGFR-2, we found that both Akt and Erk1/2 were activated. Akt phosphorylation peaked between 10 and 20 min (Fig. 7*A*), whereas maximum Erk1/2 phosphorylation was observed between 20 and 30 min (Fig. 7*B*). Activation of Akt was biphasic, with a second peak of phosphorylation after 60 min (Fig. 7*A*). This biphasic activation of Akt in response to VEGFR-2 dimerization was observed in three independent experiments. We next checked whether the Akt and Erk1/2 pathways were also induced in murine bone marrow cells. Transduced GFP-positive bone marrow cells were incubated for 2 days in cytokine-free medium and then stimulated for 20 min with 100 nM AP20187. As with endothelial cells, we also observed activation of Akt (Fig. 7*C*) and Erk1/2 (Fig. 7*D*) in bone marrow following VEGFR-2 dimerization. Either of these kinases could account, at least in part, for the survival that we observed in response to VEGFR-2 dimerization, since induction of these signaling pathways by other hematopoietic cytokines, such as SCF and erythropoietin, has been implicated in hematopoietic cell survival (35-37).

To specifically study the above signaling pathways in mediating hematopoietic progenitor survival, we used specific inhibitors of each signaling pathway. LY294002 is an inhibitor of PI 3-kinase, which activates Akt, whereas U0126 has been shown to block Erk1/2 MAP kinase phosphorylation by specific inhibition of MEK (38). To determine whether VEGFR-2 mediated survival was mediated through Akt and/or Erk1/2, transduced GFP-positive bone marrow cells were incubated with or without 100 nM AP20187 in the presence of the PI 3-kinase inhibitor LY294002 or the MEK inhibitor U0126 at concentrations that blocked each of these kinases (Fig. 7, *C* and *D*). Cell number was monitored over a period of 14 days. We found that inhibition of PI 3-kinase blocked the antiapoptotic effect of VEGFR-2 dimerization induced by cytokine deprivation, indicating the essential role of this pathway in VEGFR-2-mediated survival

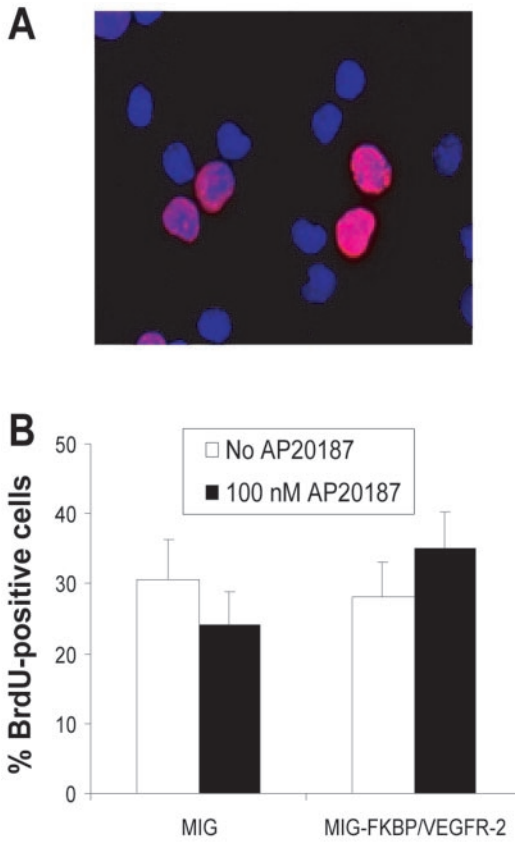

FIG. 5. **VEGFR-2 dimerization does not increase BrdUrd uptake in hematopoietic progenitors.** MIG- or MIG-FKBP/VEGFR-2-transduced bone marrow cells cultured for 2 days in IMDM supplemented with 10% FBS were treated for 2 h with 10 nM BrdUrd with or without 100 nM AP20187, and cytospin preparations were stained with an anti-BrdUrd antibody conjugated with Alexa 594. 4′,6-Diamidino-2-phenylindole staining was used to identify nuclei (*A*). The number of BrdUrd-positive cells was quantitated and expressed as a percentage of total nuclei counted (*B*). 200–300 cells were counted per cytospin preparation. Data represent the mean ± S.E. of three independent experiments.

in bone marrow cells (Fig. 8*A*). Blockade of PI 3-kinase also inhibited the survival of hematopoietic progenitors induced by VEGFR-2 dimerization (Fig. 8*B*), further demonstrating the critical role of this pathway in survival of hematopoietic progenitors. In contrast, blockade of the MAP kinase pathway with U0126 did not inhibit VEGFR-2-induced cell survival (Fig. 8*C*). Interestingly, despite minimal effect on cell survival, inhibition of Erk1/2 partially inhibited hematopoietic progenitor activity mediated by VEGFR-2 (Fig. 8*D*). This discrepancy suggests that hematopoietic progenitors are more dependent on the Erk1/2 MAP kinase pathway for survival than more mature/differentiated cells, which constitute the majority of cells present after 14 days in liquid culture (data not shown).

DISCUSSION

It is known that VEGF is essential, in a dose-dependent manner, for the formation of both the vascular and hematopoietic systems (4). Moreover, studies have shown that the absence of VEGFR-2 in mouse embryos causes defects in the development of endothelial and hematopoietic cells, a phenotype also observed in heterozygous VEGF-deficient mice (5, 6), suggesting that many of the effects of VEGF on early hematopoiesis and vasculogenesis might be mediated by VEGFR-2. It has been postulated that VEGF might act on embryonic hematopoiesis/vasculogenesis by promoting the survival/proliferation and migration of a population of ventral mesodermal cells

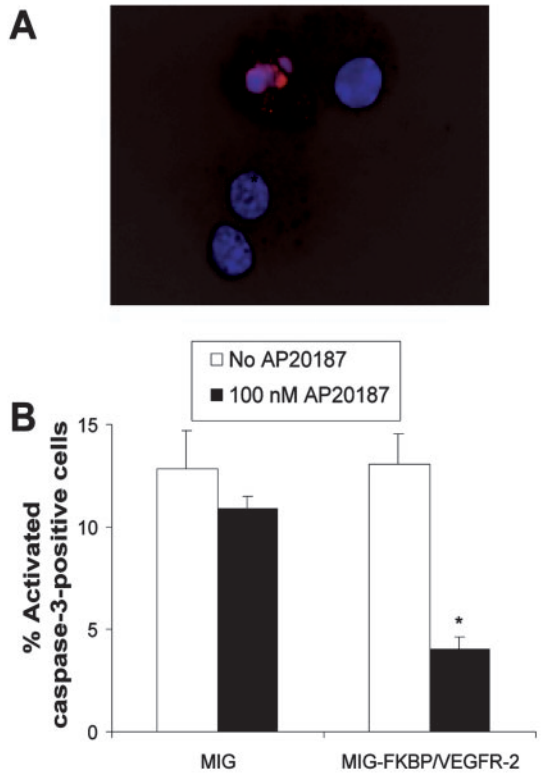

FIG. 6. **VEGFR-2 dimerization inhibits apoptosis of cytokine-starved hematopoietic progenitors.** Cytospin preparations of MIG- or MIG-FKBP/VEGFR-2-transduced bone marrow cells cultured for 14 days in IMDM containing 10% FBS with or without 100 nM AP20187 were stained with an anti-activated caspase 3 antibody. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (*A*). Cells with activated caspase 3 were quantitated and expressed as a percentage of total cells counted (*B*). 200–300 cells were counted per cytospin preparation. Data represent the mean ± S.E. of four independent experiments. An *asterisk* indicates a significant difference ($p < 0.05$) between AP20187-treated cells and untreated cells.

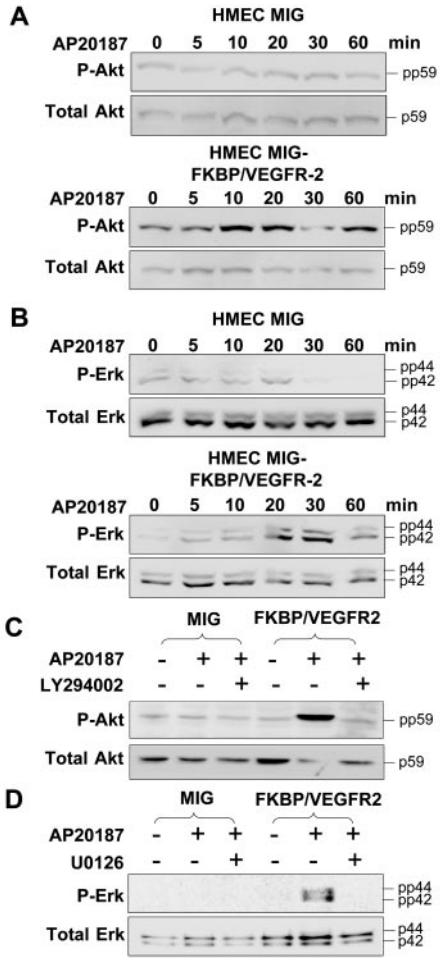

FIG. 7. **VEGFR-2 dimerization activates Akt and Erk1/2 MAP kinases in hematopoietic progenitors.** Quiescent HMEC-1 cells were incubated with 10 nM AP20187 for 0–60 min as indicated. Cytokine-starved MIG- or MIG-FKBP/VEGFR-2-transduced bone marrow progenitors were treated for 20 min with 100 nM AP20187. Phosphorylation of Akt (*A* and *C*) or Erk1/2 (*B* and *D*) was determined by immunoblotting using antibodies specific to phosphorylated Akt or Erk1/2. *Lanes 1* and *4*, untreated; *lanes 2* and *5*, 100 nM AP20187 for 20 min; *lanes 3* and *6*, pretreatment with 20 μM LY294002 (Akt) or 10 μM U0126 (Erk) for 90 min followed by treatment with 100 nM AP20187 for 20 min. Membranes were reprobed with anti-Akt or anti-Erk1/2 antibodies as loading controls. Data represent one experiment of three independent experiments showing similar findings.

with hemangioblastic activity (39, 40). In vitro studies with embryonic stem cells also suggest that cells of both the hematopoietic and endothelial lineages can originate from a single cell (41). However, in contrast to the findings in vivo, both endothelial and hematopoietic cells can originate from embryonic stem cells that are VEGFR-2$^{-/-}$ (8). This has led to the theory that the hemangioblast originates independently of VEGF or VEGFR-2, although the survival, migration, and proliferation of this cell are dependent on the action of VEGF (42).

Little is known about the effect of VEGF on adult hematopoiesis, although both VEGFR-1 and VEGFR-2 have been described in hematopoietic precursors (10, 43). Recent reports demonstrate that VEGF, in addition to being a critical regulator of vasculogenesis and hematopoiesis in the embryo, can promote the survival of adult hematopoietic stem cells (14, 44).

Although many studies postulate that VEGF mediates most of its effects through VEGFR-2, it is difficult to make a clear statement about this because of the complexity of the VEGF-VEGFR system. To specifically study the effects of VEGFR-2 signaling, we used a strategy that allowed the specific activation of the VEGFR-2 signaling domain by using a chemical inducer of dimerization. This strategy has been successfully used before to specifically study the effects of hematopoietic receptors such as Mpl, Flt-3, granulocyte colony-stimulating factor receptor, and c-Kit on cell proliferation and differentiation (21–23). These studies have shown that Mpl can induce long term proliferation of murine hematopoietic progenitors, whereas the effects of Flt-3 and granulocyte colony-stimulating factor receptor are much more modest (23). We therefore used a construct containing the intracellular domain of VEGFR-2, which includes the tyrosine kinase domain, fused to domains that can dimerize in response to the chemical inducer of dimerization, AP20187. This construct was localized to the cytoplasmic membrane in unstimulated HMEC-1 cells and translocated into the cytosol with concomitant phosphorylation following stimulation with AP20187, a phenomenon observed with many endogenous hematopoietic receptors. Interestingly, however, Otto *et al.* (45) have shown that membrane localization of the thrombopoietin receptor, Mpl, is not required for the full range of Mpl function in hematopoietic cells.

We were interested in the role of VEGFR-2 in hematopoietic progenitors, since little is known about the effects of this receptor on hematopoiesis. In murine bone marrow, our results indicate that VEGFR-2 can maintain hematopoietic progenitor potential following dimerization, since cells fail to survive in the absence of AP20187. It is interesting to note that when hematopoietic progenitors were cultured in the presence of hematopoietic cytokines (IL-3, IL-6, and SCF), there was no

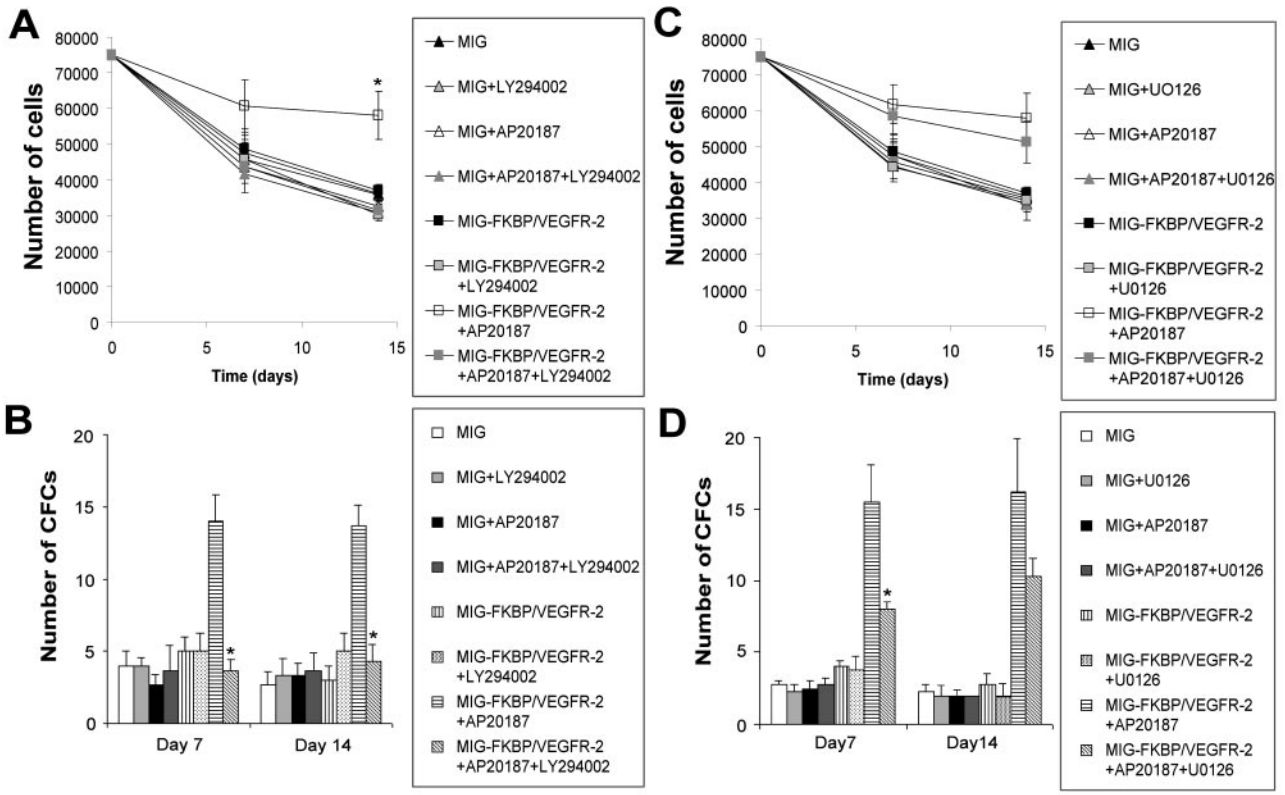

FIG. 8. **Effect of PI 3-kinase or MEK inhibition on hematopoietic progenitor survival.** MIG- or MIG-FKBP/VEGFR-2-transduced hematopoietic progenitors were cultured in IMDM supplemented with 10% FBS with or without 100 nM AP20187 in the presence or in the absence of 20 μM of the PI 3-kinase inhibitor, LY294002 (*A* and *B*), or the MEK inhibitor, U0126 (*C* and *D*), for 0–14 days. Cells were harvested at specific time points, and cell number was determined (*A* and *C*). Cells were collected after 7 and 14 days and plated in methylcellulose medium to assay for hematopoietic progenitor activity (*B* and *D*). Data represent the mean ± S.E. of three independent experiments. An *asterisk* indicates a significant difference ($p < 0.05$) between AP20187-treated cells and untreated cells.

effect of VEGFR-2 dimerization on cell number. This would suggest that VEGFR-2 does not induce a proliferative signal in hematopoietic progenitors that is distinct from IL-3, IL-6, and SCF. Although Erk1/2 MAP kinase was activated, VEGFR-2 dimerization failed to induce proliferation of bone marrow progenitors, indicating that signals provided by other factors may be necessary for the proliferation of hematopoietic cells. It is likely then that the delay in cell loss observed through VEGFR-2 dimerization reflects an effect on cell survival. This was verified when we found that VEGFR-2 decreased the fraction of apoptotic cells when hematopoietic progenitors were cultured in the absence of exogenous cytokines.

The importance of cell survival in the maintenance of hematopoietic progenitors was further demonstrated by blocking the PI 3-kinase pathway. This signaling pathway, through Akt, has been shown to play a crucial role in cytokine-mediated survival (31, 46), as well as in the self-renewal of primary multipotential hematopoietic progenitors (47). Blockade of PI 3-kinase completely abolished the maintenance of hematopoietic progenitors mediated by VEGFR-2, indicating that this pathway is critical for VEGFR-2-mediated survival in bone marrow progenitor cells. In contrast, we did not observe a significant effect on cell survival mediated by VEGFR-2 when the Erk1/2 MAP kinase pathway was blocked using the MEK inhibitor, U0126. This would imply that, although the Erk1/2 MAP kinase pathway has been shown to play a role in apoptosis prevention (48), the PI 3-kinase pathway through Akt is the main regulator of VEGFR-2-induced survival signaling in hematopoietic progenitors. However, it is noteworthy that inhibition of Erk1/2 activation did reduce the number of hematopoietic progenitors as measured by the CFC assay. This effect may be explained by the fact that most cells remaining after 14 days in culture in the absence of exogenous cytokines are differentiated, and studies have shown that the cytokine-induced survival of differentiated cells, such as macrophages, is mediated mainly by the PI 3-kinase pathway and not the Erk1/2 MAP kinase pathway (49, 50). Thus, this may indicate that hematopoietic progenitors are more dependent on the Erk1/2 MAP kinase pathway for VEGFR-2-induced survival than more mature cells.

Although VEGFR-2 signaling promoted survival of hematopoietic progenitors and maintained their progenitor potential, it did not seem to affect the differentiation of those progenitors. In contrast, other hematopoietic cytokine receptors, such as Mpl, induce a dramatic expansion of multipotential progenitors and megakaryocytes (21, 23). A recent study has demonstrated that a combination of signals, JAK2 plus either c-Kit or Flt-3 together, can support extensive hematopoietic progenitor cell self-renewal although neither of these receptors can sustain the growth of bone marrow cells alone (47). Whether VEGFR-2 requires additional signals to induce cell proliferation in hematopoietic cells remains unknown, and further studies would be needed to assess this issue.

The distinctions between the roles of the two VEGF receptors in mediating VEGF effects in hematopoiesis still need to be clarified. Both receptors are expressed on subsets of hematopoietic cells such as hematopoietic stem cells, monocytes, and platelets for VEGFR-1 (43, 51) and hematopoietic stem cells, endothelial progenitors, and platelets for VEGFR-2 (10, 51). This overlap in VEGF receptor expression makes it difficult to specifically study the role of each individual receptor. Recent reports have shed some light on the role of VEGFR-1 and its effects on processes such as angiogenesis, hematopoiesis, and

inflammation. VEGFR-1 has been shown to promote hematopoiesis by recruiting hematopoietic stem cells from the bone marrow, which favors differentiation and mobilization. Moreover, inhibition of VEGFR1 blocked hematopoietic stem cell cycling, differentiation, and hematopoietic recovery after BM suppression, revealing a function for VEGFR-1 signaling during hematopoiesis (43). VEGFR-2 is implicated in the recruitment and differentiation of endothelial progenitors (11, 52). However, despite its critical role in the development of the hematopoietic system, little is known about the role of VEGFR-2 in adult hematopoiesis. In this paper, we show that VEGFR-2 can induce a protective effect in hematopoietic progenitors. Although VEGFR-2 has been reported to be the main effector of VEGF signaling in endothelial cells, promoting proliferation, survival, and migration, it appears that both VEGF receptors play distinct and important roles in adult hematopoiesisis, and further studies will be needed to clarify this issue.

The strategy used in this study allowed us to demonstrate, for the first time, that VEGFR-2 can activate the PI 3-kinase and Erk1/2 pathways, without any interaction with other VEGF receptors such as the neuropilins or VEGFR-1, in hematopoietic progenitors. Our results show that VEGFR-2 can induce maintenance of hematopoietic progenitors in the absence of exogenous hematopoietic cytokines. This may help to explain, at least in part, the critical role of VEGFR-2 in embryonic hematopoiesis, in which this receptor may promote the survival of early hematopoietic precursors.

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