Fetal Stromal–Dependent Paracrine and Intracrine Vascular Endothelial Growth Factor-A/Vascular Endothelial Growth Factor Receptor-1 Signaling Promotes Proliferation and Motility of Human Primary Myeloma Cells

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Abstract

Induction of neoangiogenesis plays an important role in the pathogenesis of multiple myeloma. However, the mechanism by which expression of vascular endothelial growth factor (VEGF)-A and its receptors modulate the interaction of multiple myeloma cells with stromal cells is not known. Here, we describe a novel in vitro coculture system using fetal bone stromal cells as a feeder layer, which facilitates the survival and growth of human primary multiple myeloma cells. We show that stromal-dependent paracrine VEGF-A signaling promotes proliferation of human primary multiple myeloma cells. Primary multiple myeloma cells only expressed functional VEGF receptor (VEGFR)-1, but not VEGFR-2 or VEGFR-3. VEGFR-1 expression was detected in the cytoplasm and the nuclei of proliferating multiple myeloma cells. Inhibition of VEGFR-1 abrogated multiple myeloma cell proliferation and motility, suggesting that the functional interaction of VEGF-A with its cognate receptor is essential for the growth of primary multiple myeloma cells. Collectively, our results suggest that stromal-dependent paracrine and intracrine VEGF-A/VEGFR-1 signaling contributes to human primary multiple myeloma cell growth and therefore, VEGFR-1 blockade is a potential therapeutic strategy for the treatment of multiple myeloma. (Cancer Res 2005; 65(8): 3185-92)

Introduction

Multiple myeloma is a clonal B lymphocyte malignancy mainly characterized by the accumulation of terminally differentiated antibody-producing cells in the bone marrow. Multiple myeloma cells reside in close association with the stromal elements within the bone marrow. As the disease progresses, multiple myeloma cells may proliferate in the extramedullary areas (1, 2). Current treatments for multiple myeloma offer only a median survival of 3 years as investigators continue to search for novel therapeutic targets to combat the disease. One of the strategies is to target the multiple myeloma cells as well as the bone marrow microenvironment (3). It has been shown that molecules designed to bind specifically to the tyrosine kinase domain of vascular endothelial growth factor receptors (VEGFR) can efficiently block the growth of multiple myeloma cells (4, 5). VEGF-A and its receptor tyrosine kinases, namely VEGFR-1 (Flt-1) as well as VEGFR-2 (KDR, Flk-1), play a crucial role in neo-vascularization (6, 7).

Functional VEGFRs are also expressed on subsets of leukemias, resulting in autocrine loops that sustain leukemia migration and proliferation (8). VEGF-A is a potent activator of multiple myeloma cell proliferation and migration (9, 10). Elevated plasma levels of VEGF-A have been reported in patients with multiple myeloma (11) and correlated with increased angiogenesis in multiple myeloma bone marrow (12–15). Moreover, binding of multiple myeloma cells to bone marrow stromal cells markedly stimulates VEGF-A secretion (16). This triggers interleukin-6 (IL-6) production from bone marrow stromal cells (17), thereby augmenting multiple myeloma cell proliferation in a paracrine fashion. Thus, VEGF-A plays an important role in both autocrine and paracrine control of multiple myeloma cell growth. Moreover, intracellular trafficking and nuclear localization of VEGF-R-2 can promote leukemic cell proliferation (18). However, whether VEGF-A/VEGFR intracrine signaling also supports the proliferation of primary multiple myeloma cells is not known.

Prior studies have shown that immortalized and primary multiple myeloma cells express high-affinity VEGF-R-1, but not VEGFR-2 (4, 5, 19). However, the precise functional role of VEGFRs, in particular VEGFR-1, in the regulation of proliferation and interaction with the stromal cells is not known. In addition, studies of multiple myeloma biology have been limited by the lack of a coculture model for cultivating human primary multiple myeloma cells in vitro in order to study their behavior for sufficiently long-periods of time.

Here we describe a novel in vitro coculture system using fetal bone stromal cells as a feeder layer, which facilitates the growth and survival of human primary multiple myeloma cells. This provides an opportunity to study the role of the VEGF-A/VEGFR axis which accounts for the multiple myeloma cell growth. Remarkably, we show that VEGFR-1 is present in the cytoplasm and the nuclei of proliferating multiple myeloma cells. Blockade of VEGFR-1 by a neutralizing monoclonal antibody (mAb) maintains VEGFR-1 in a membrane-bound localization preventing its nuclear translocation, and therefore blocks proliferation and migration of primary multiple myeloma cells. Collectively, our results suggest that stromal-dependent paracrine and intracrine VEGF-A/VEGFR-1 signaling contributes to human primary multiple myeloma cell survival, proliferation, and migration.
Materials and Methods

Cytokine and antibodies. Recombinant VEGF-A was obtained from R&D Systems (Minneapolis, MN). The expression profile of VEGFRs in multiple myeloma cells was determined by immunohistochemical staining and flow cytometry using 1 μg/mL of the following mAbs: anti-Flt-1 (clone FB5), anti-KDR (clone 1121) and anti-Flt-4 antibodies (clone 3C5). Neutralizing mAb (10 μg/mL) to VEGF was used to study the role of each receptor in multiple myeloma cells: anti-Flt-1 (clone 6.12), anti-KDR (clone 1C11) and anti-Flt-4 antibodies (clone 3C5). All of these mAbs were kindly provided by ImClone Systems, Inc (New York, NY).

Bone marrow specimens and culture of multiple myeloma cells. Heparinized bone marrow aspirates were freshly obtained from multiple myeloma patients after appropriate informed consent approved by the Institutional Review Board at Cornell University Medical College. The bone marrow specimens were mixed to disaggregate cell clumps. Excess tissue and clumps were removed by filtering the cell suspension through a 70-μm nylon tissue strainer (BD Falcon, Bedford, MA). The cell filtrate was subjected to RBC lysis buffer (1:5, v/v, Roche, Indianapolis, IN). After incubation at room temperature for 10 minutes, cells were pelleted by centrifugation, and washed once with serum-free X-VIVO 20 medium (Bio-Whittaker, Walkersville, CA). Multiple myeloma cells were then purified using magnetic cell sorting CD138 Microbeads (clone B-B4; Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturers’ instructions. Cells were positively selected over a magnetic column with 95% to 99% purity.

Fetal bone stroma isolation and culture. The Institutional Review Board of Cornell University Medical College approved the use of fetal tissue. Femur from one 8- to 12-week-old human fetus from spontaneous abortion was mechanically dissociated and the cell suspension was filtered with a syringe through a 40-μm nylon mesh (Millipore, Billerica, MA). Fetal bone stromal cells were cultured in M199 medium containing 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin.

Characterization of fetal bone stromal cells. Fetal bone stromal cells were grown on glass slides and fixed with 10% paraformaldehyde. For immunohistochemistry, fetal bone stromal cells were subjected to primary antibodies raised against platelet endothelial cell adhesion molecule-1 (1:100, Dako, Glstrup, Denmark), vimentin (1:50, Dako, Carpinteria, CA), and α-smooth muscle actin (1:800, Sigma, St. Louis, MO). Detection of bound primary antibody was done with the Dako Envision horseradish peroxidase/3,3′-diaminobenzidine staining kit (Dako). Fetal bone stromal cells were also stained for VEGFR-1, VEGFR-2, and VEGFR-3. Fetal bone stromal cells were incubated with the mAb or an unspecific, isotype-matched murine antibody as a control, washed and then incubated with secondary FITC-conjugated antibodies. Cells were washed and analyzed using a Coulter FC-500 Flow Cytometer.

Angiogenesis array of fetal bone stromal cells. Fetal bone stromal cells were grown to 50% confluence, washed with serum-free X-VIVO 20 medium, and then incubated with X-VIVO 20 medium. After 72 hours, supernatant was collected and analyzed for soluble angiogenic factors with an angiogenesis antibody array (Panomics, Redwood City, CA) as per the manufacturer’s instructions.

Multiple myeloma cell growth on fetal bone stromal cell monolayer. Fetal bone stromal cells (5 × 10^6 cells per well) were seeded in 12-well plates and left to adhere before a 24-hour starvation, and multiple myeloma cells (3 × 10^4/mL) were then added to the stromal feeder layer in serum-free medium. The physical interaction between fetal bone stromal and multiple myeloma cells was further shown using PKH2 (green) and PKH26 (red) fluorescent cell linkers, respectively, according to the manufacturer’s instructions (Sigma). The growth of cocultured multiple myeloma cells was compared with that without stromal feeder layer. Proliferation of the multiple myeloma cells was evaluated by first collecting the nonattached multiple myeloma cells, and second by detachting the residual attached multiple myeloma cells using a cell dissociation solution (Sigma). The viability of the combined collected fractions of multiple myeloma cells was determined by trypan blue exclusion after 7, 14, and 21 days in the coculture system. Some functional studies to investigate the role of VEGFRs were designed in which neutralizing mAb to VEGF were added every 2 days in the culture medium. These experiments were done on four multiple myeloma samples and each condition was done in triplicate.

EBV analysis. To rule out the possibility that multiple myeloma cell growth is secondary to EBV transformation, bone marrow aspirate and cultured multiple myeloma cells were examined for the presence of EBV by in situ hybridization for EBER (EBV-encoded small RNAs, Dako Corporation, Via Real, CA).

Apoptosis. Multiple Myeloma cells cocultured on fetal bone stromal cells or alone in serum-free X-VIVO 20 medium were collected and analyzed for the presence of apoptotic cells using the ApoAlert Annexin V-FITC propidium iodide (PI) Apoptosis Kit (Becton Dickinson, Palo Alto, CA), following the manufacturer’s instructions. Flow cytometry analysis was performed using a Coulter FC-500 Cytometer. Results were shown as the percentage of live cells (annexin V- PI-), early apoptotic cells (annexin V+ PI-), late apoptotic cells (annexin V+ PI+), and dead cells (annexin V+ PI+). Each set of experiments was done in triplicate.

Immunofluorescence microscopy. After coculturing with fetal bone stromal cells for 21 days, multiple myeloma cells were spun onto glass microscope slides and VEGFR-1, VEGFR-2, and VEGFR-3 expression was detected by immunofluorescent technique. Multiple myeloma cells were fixed in 3.7% paraformaldehyde and washed in PBS. After permeabilization with methanol (90%), multiple myeloma cells were incubated with the primary antibodies, washed and then incubated with secondary FITC-conjugated antibodies (1:1,000, Vector Laboratories, Burlingame, CA). After washing, samples were mounted in Vectashield containing 4′,6-diamidino-2-phenylindole (DAPI) and analyzed by fluorescence microscopy at 40× magnification (Olympus, NJ).

Flow cytometry. The multiple myeloma cells were collected following the 21-day coculture with fetal bone stromal cells and stained for VEGFR-1, VEGFR-2 and VEGFR-3. The multiple myeloma cells were fixed in 4% paraformaldehyde and permeabilized in methanol (90%). The multiple myeloma cells were incubated with the specific neutralizing mAb or an unspecific, isotype-matched murine antibody as a control, washed, and then incubated with secondary phycoerythrin (PE)- or FITC-conjugated antibodies. Cells were washed and analyzed using a Coulter FC-500 Cytometer. VEGF expression was analyzed in four primary multiple myeloma samples.

Transwell migration assay. Primary multiple myeloma cell migration was assayed using a Boyden chamber system with 8 μm pore size inserts (Costar, NY). Multiple myeloma cells were starved in X-VIVO 20 for 6 hours prior incubation with neutralizing mAb to VEGFR-1, VEGFR-2, or VEGFR-3 for 4 hours. Multiple myeloma cells (1.5 × 10^5 per insert) were then placed into the upper chamber of the transwell system in X-VIVO 20 supplemented with 0.2 mg/mL bovine serum albumin. The lower chamber was filled with X-VIVO 20 (2 mg/mL bovine serum albumin) in the presence or absence of 50 ng/mL of VEGF-A. A fetal bone stromal cell monolayer cultured in serum-free X-VIVO 20 for 2 days was also used as a chemoattractant. After a 6-hour incubation, the number of viable migrated cells was counted in the lower chamber by trypan blue exclusion. The migration index, defined as the number of viable migrated cells in the sample divided by the number of viable migrated cells in the control (no cytokine or fetal bone stromal cells), was calculated to compare motility of cells relative to control. The transwell migration assay was repeated thrice in triplicate.

ELISA. Following a 72 hour-incubation of multiple myeloma cells and fetal bone stromal cells cultured in serum-free condition (X-VIVO 20), VEGF-A and placental growth factor levels were measured in the supernatants using commercially available ELISA kits (R&D Systems). Results are shown as picograms per milliliter per 1 × 10^6 cells of VEGF-A or placental growth factor in culture supernatants, and each sample was done independently in triplicate.
**Results**

**Characterization of fetal bone stromal cells.** The difficulty of cultivating multiple myeloma cells *in vitro* has limited the studies of multiple myeloma cell biology. In this study, we used a fetal bone stromal cell feeder layer to support the long-term propagation of primary human multiple myeloma cells. Using immunohistochemistry, we showed that fetal bone stromal cells expressed the mesenchymal antigen vimentin (Fig. 1A) and the α-smooth muscle actin (Fig. 1B), but were negative for the expression of endothelial cell marker platelet endothelial cell adhesion molecule-1 and VEGF receptors (data not shown). The fully confluent cells secrete VEGF-A and IL-6 (Fig. 1C). As shown in Fig. 1D, multiple myeloma cells (yellow arrows) were cocultured on a semiconfluent fetal bone stromal cell monolayer (black arrow). Using fluorescent red and green dye, respectively, to label the multiple myeloma cells growing on fetal bone stromal cells, the multiple myeloma cells were detected to closely interact with the fetal bone stromal cells (Fig. 1D). After a 21-day coculture, clusters of multiple myeloma cells could easily be identified as cellular clusters attached to stromal cells (Fig. 1E).

**Fetal bone stromal cells support multiple myeloma growth.**

To assess the effect of the fetal bone stromal cell on the multiple myeloma cell proliferation in serum-free condition, the multiple myeloma cells were collected every 7 days until day 21 after the coculture started, and the number of multiple myeloma cells was compared with the cells grown in the absence of a stromal layer. Without fetal bone stromal cells, the percentage of dead multiple myeloma cells increased to 78.78 ± 13.33% and 97.77 ± 2.10% after 7 and 14 days of culture, respectively (Fig. 2A). After a 21-day culture, all the multiple myeloma cells underwent apoptosis. In sharp contrast, the number of viable multiple myeloma cells grown in coculture with the fetal bone stroma increased to 337.77 ± 29.63%, which represents a 3-fold expansion at the end of experiment. In the four specimens tested, all multiple myeloma cells were EBER-negative excluding the possibility that the cultured multiple myeloma cells were EBV-transformed (data not shown).

**Fetal bone stromal cells support multiple myeloma survival.**

At day 21 of coculture, the collected multiple myeloma cells were subjected to Annexin V and PI staining to assess the effect of fetal bone stromal cells on multiple myeloma cell survival. The majority

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**Figure 1.** The fetal bone stromal cells support multiple myeloma cell growth. A and B, immunohistochemical staining of fetal bone stromal cells. The cells were stained with vimentin (A) and α-smooth muscle actin (B). Fetal bone stromal cells were positive for the mesenchymal antigen vimentin and α-smooth muscle actin consistent with an immunophenotype of activated fibroblasts. Magnification (×400); C, soluble factors expressed by fetal bone stromal cells. Fetal bone stromal cells were grown in serum-free medium for 72 hours and supernatant was analyzed for soluble factors with an antibody array. Duplicate black dots show the presence of soluble factors; D, association of fetal bone stromal and multiple myeloma cells. Multiple myeloma cells (yellow arrow) were seeded on a semiconfluent fetal bone stromal cell monolayer (black arrow). The interaction between fetal bone stromal and multiple myeloma cells was highlighted using PKH2 green and PKH26 red fluorescent cell linkers, respectively. Magnification (×400); E, sustained growth of multiple myeloma cells on fetal bone stromal cell monolayer. Multiple myeloma cells were cocultured with fetal bone stromal cells in serum-free condition. Robust multiple myeloma cell proliferation was observed after 21 days. Magnification (×400).
of the primary multiple myeloma cells (98% of the total cells) were alive (Annexin V− PI−) when they were seeded in serum-free condition (Fig. 2B). Without fetal bone stromal cells to support the multiple myeloma cell growth, 97.8% of cells underwent apoptosis after 21 days. In contrast, the fetal bone stromal cells significantly supported the survival of the multiple myeloma cells (74.1% Annexin V− PI−, P < 0.05), although 25.6% of cells were in early and late apoptosis. The data are representative of three independent experiments.

**Vascular endothelial growth factor receptor-1 is localized to the nucleus of the proliferating multiple myeloma cells.** We further evaluated the presence of VEGFR-1, VEGFR-2, and VEGFR-3 in primary human multiple myeloma cells. Using flow cytometry on three multiple myeloma samples, we were unable to detect expression of VEGFR-1, VEGFR-2, and VEGFR-3 on the surface of the multiple myeloma cells (data not shown). However, VEGFR-1 expression was detected within the permeabilized multiple myeloma cells from all the studied bone marrow patients (4 out of 10) and the DAPI staining showed that VEGFR-1 was predominantly present in the nuclei of the multiple myeloma cells (Fig. 3A). In contrast, neither VEGFR-2 nor VEGFR-3 staining was detectable in the four patient samples. The intracellular expression of VEGFR-1 was confirmed by flow cytometry on four permeabilized multiple myeloma cells (Fig. 3B). The absence of VEGFR-2 and VEGFR-3 was also confirmed by immunohistochemical staining.

**The blockade of vascular endothelial growth factor receptor-1 inhibits multiple myeloma growth.** Based on the nuclear presence of VEGFR-1 and the absence of VEGFR-2 and VEGFR-3 expression in the multiple myeloma cells, we hypothesized that the expression of VEGFR-1 may play a critical role in multiple myeloma biology. The multiple myeloma cell growth assay, in which the multiple myeloma cells were cocultured with a semiconfluent fetal bone stromal cell monolayer in serum-free conditions, was done in the presence or absence of various neutralizing mAb to VEGFRs. The neutralizing mAb against either VEGFR-2 or VEGFR-3 did not interfere with the multiple myeloma cell growth after 21 days in the coculture system as compared with the isotype IgG control (266.67 ± 16% and 280 ± 30% versus 253.34 ± 32.84%, respectively; Fig. 4). In contrast, the neutralizing mAb to VEGFR-1 markedly suppressed the growth of the multiple myeloma cells as compared with the number of multiple myeloma cells initially placed in the coculture system (47.66 ± 16%, P < 0.001, n = 4), suggesting that the expression of VEGFR-1 plays a key role in the survival and proliferation of human primary multiple myeloma cells. In addition, VEGFR-1 expression was localized to the cytoplasm but not the nucleus, when multiple myeloma cells were treated with neutralizing mAb against VEGFR-1 (4 out of 10 bone marrow samples; Fig. 5). The immunofluorescent staining for VEGFR-2 and VEGFR-3 was negative (data not shown).

**The blockade of vascular endothelial growth factor receptor-1 inhibits multiple myeloma motility.** Having shown that VEGFR-1 supports multiple myeloma cell growth, we next investigated whether VEGFR-1 blockade could interfere with multiple myeloma cell motility and invasive potential. Cell motility was assayed by measuring the transmembrane migration activity of multiple myeloma cells (3 multiple myeloma samples out of 10) seeded in transwell inserts. As shown in Fig. 6, VEGF-A induced an increase in multiple myeloma cell migration (2.46 ± 0.08-fold, P < 0.001, n = 3; Fig. 6). Neutralizing mAb against

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**Figure 2.** Fetal bone stromal cells support multiple myeloma cell growth and survival. A, multiple myeloma cell growth on fetal bone stromal cells. Multiple myeloma cells were seeded in serum-free condition in the presence or absence of a semiconfluent fetal bone stromal monolayer for 21 days. Multiple myeloma cells were collected every 7 days for assessment for viability by trypan blue exclusion. After 21 days in culture, all the multiple myeloma cells without fetal bone stromal cells were dead, whereas sustained growth of multiple myeloma cells was observed in the coculture system (**P < 0.001** as compared with the number of cells at day 0, n = 4); B, multiple myeloma cell survival on fetal bone stromal cells. The multiple myeloma cells were stained with Annexin V/PI at days 0 and 21 of the culture in the presence (+FBS) or absence (−FBS) of the fetal bone stromal cells. Results are shown as the percentage of viable cells (Annexin V− PI−), early apoptotic cells (Annexin V+ PI−), late apoptotic cells (Annexin V+ PI+), and dead cells (Annexin V− PI+). Without fetal bone stromal cells to support the multiple myeloma cell growth, the multiple myeloma cells were prone to cell death (PI+). In contrast, the fetal bone stromal cells significantly supported the survival of the multiple myeloma cells (74.1% Annexin V− PI−; P < 0.05, n = 3). The data presented are representative of three independent experiments.
VEGFR-1 decreased the VEGF-A-induced transmigration of multiple myeloma cells (1.48 ± 0.21-fold, \(P < 0.001, n = 3\)). The presence of fetal bone stromal cells conferred a stronger chemotactic effect on the multiple myeloma cells (4.09 ± 0.60-fold, \(P < 0.001, n = 3\)) as compared with that by VEGF-A. Neutralizing mAb against VEGFR-1 also decreased the fetal bone stromal cell–induced multiple myeloma cell migration (2.49 ± 0.10-fold, \(P < 0.01, n = 3\)). However, neutralizing mAb against VEGFR-1 did not completely block multiple myeloma cell transmigration, suggesting that other factors besides VEGF-A may support the migration of the multiple myeloma cells. Blockade of VEGFR-2 and VEGFR-3 had no significant effect on multiple myeloma cell motility.

The fetal bone stromal cells secrete high levels of vascular endothelial growth factor-A. In order to determine how autocrine and/or paracrine VEGF-A and placental growth factor stimulation promote the growth and migration of multiple myeloma cells, the protein levels of VEGF-A and placental growth factor were determined in the serum-free medium obtained from fetal bone stromal cells. Fetal bone stromal cells secreted high levels of VEGF-A (2,348.2 ± 254.0 pg/mL/1 × 10^6 cells, \(n = 3\)) as compared with multiple myeloma cells (105.8 ± 34.7 pg/mL/1 × 10^6 cells, \(n = 3\)). This drastic difference in VEGF-A secretion suggests that VEGF-A through paracrine stimulation supports primary multiple myeloma cell growth and migration. In contrast, the level of placental growth factor secreted by fetal bone stromal cells and multiple myeloma cells was negligible (data not shown), suggesting VEGF-A as the primary factor released that activates VEGFR-1 signaling, thereby promoting multiple myeloma cell survival, proliferation, and migration.

Figure 3. VEGFR-1, but not VEGFR-2 and VEGFR-3, is expressed on multiple myeloma cells. A, the multiple myeloma cells were harvested following the 21-day coculture with fetal bone stromal cell monolayer and stained for VEGFRs. The multiple myeloma cells were permeabilized and subjected to immunofluorescent staining using mAb against VEGFRs (FITC) and DAPI to stain the DNA (nucleus). VEGFR-1 expression was detected inside the multiple myeloma cells and the DAPI staining showed the nuclear expression of VEGFR-1. Neither VEGFR-2 nor VEGFR-3 were detectable. Results were obtained from four different multiple myeloma samples in independent experiments. Magnification (×1,000); B, the multiple myeloma cells were collected following the 21-day coculture with fetal bone stromal cell monolayer and stained for VEGFRs. The multiple myeloma cells were permeabilized and stained using mAb against VEGFR-1 (phycoerythrin, FL2), VEGFR-2 and VEGFR-3 (FITC, FL1). The intracellular expression of VEGFR-1 was confirmed using flow cytometry. VEGFR expression was analyzed in four multiple myeloma samples. The absence of VEGFR-2 and VEGFR-3 was also confirmed.
Discussion

Accumulating evidence has shown that VEGFRs, including VEGFR-1, VEGFR-2, and VEGFR-3 are expressed in subsets of primary human multiple myeloma cells. However, the functional role of the activation of VEGF/VEGFR signaling axis on primary multiple myeloma survival, proliferation, and mobilization in the context of interaction with their microenvironment remains unclear. The majority of the in vitro observation originates from the use of established multiple myeloma cell lines due to the lack of an adequate coculture model to maintain primary multiple myeloma cells in vitro. Here, we described a coculture model involving primary human multiple myeloma cells growing on a feeder layer of fetal bone stromal cells in serum-free culture medium. Remarkably, adult stromal cells were not effective in supporting the growth of primary multiple myeloma cells in serum-free condition (data not shown). Using fetal bone stromal cells, we were able to sustain the growth of multiple myeloma cells for at least 3 weeks. This highlights a salient feature in multiple myeloma pathobiology: the interaction of multiple myeloma cells with their permissive marrow stromal microenvironment is essential for the long-term proliferation and migration of multiple myeloma cells.

We also show that VEGF-A released from fetal bone stromal cells interacts with VEGFR-1 expressed on multiple myeloma cells in a paracrine fashion, conferring a sustained proliferative potential in vitro. This survival advantage is not mediated through viral oncogenesis (EBV), as all primary human multiple myeloma cells obtained from bone marrow aspirates tested negative for EBV. Neutralizing mAb to VEGFR-1 abrogated multiple myeloma cell proliferation through induction of apoptosis, suggesting that functional interaction of VEGF-A with its cognate receptor is essential for multiple myeloma cell growth. VEGF-A secreted by multiple myeloma cells as well as by fetal bone stromal cells acts as an autocrine and paracrine factor to stimulate multiple myeloma cell growth, in conjunction with IL-6 produced by stromal cells. Since IL-6 also enhances the production and secretion of VEGF-A by multiple myeloma cells (16, 17), VEGFR-1 blockade seems to be an effective strategy to block the growth of multiple myeloma cells, overcoming the mitogenic effect conferred by IL-6-induced VEGF-A production by stromal and multiple myeloma cells.

The intracellular trafficking and nuclear localization of VEGFR-1 suggests that a VEGF-A/VEGFR-1 intracrine signaling may be
VEGFR-1 promotes multiple myeloma growth. Multiple myeloma cell motility was assayed using a Boyden chamber system. Multiple myeloma cells were pretreated for 4 hours with neutralizing mAb against VEGFR-1, VEGFR-2, VEGFR-3, or an isotype IgG antibody as control. Then, multiple myeloma cells were plated in the upper chamber of a transwell and the lower chamber was filled with a serum-free X-VIVO 20 medium in presence or absence of 50 ng/mL of VEGF-A. A fetal bone stromal cell monolayer that was cultivated in serum-free X-VIVO 20 for 2 days was also used as a platform for chemotaxing multiple myeloma cells. After a 6-hour incubation, cells that had migrated in the lower chamber were then harvested and counted. Results are expressed as the number of migrated cells in the different conditions of incubation divided by the number of migrated cells in the control (no cytokine or fetal bone stromal cells). VEGF-A present in the lower chamber induced an increase in multiple myeloma cell motility, which was blocked by neutralizing mAb against VEGFR-1 (*P < 0.05, **P < 0.01, ***P < 0.001 as compared with unstimulated; **P < 0.01, ***P < 0.001 as compared with stimulated cell migration; n = 3).

Flow cytometric data suggests that subsets of the multiple myeloma cells have a more robust expression of VEGFR-1. The higher level of expression of VEGFR-1 may be dependent on the cell cycle of the proliferating cells or reflect subsets of multiple myeloma cells at different stages of their maturation. Whether multiple myeloma cells expressing higher levels of VEGFR-1 are more responsive to VEGF-A or placental growth factor is not known and is the subject of future studies.

Migration of primary multiple myeloma cells is necessary for the homing of tumor cells to the bone marrow niche, for expansion of malignant plasma cells within the bone marrow microenvironment, and for the egress into peripheral blood contributing to disease progression (27–30). Here, we show that neutralizing mAb to VEGFR-1 diminished multiple myeloma cell motility in vitro transwell experiments, suggesting that the functional interaction of VEGF-A with its cognate receptor is essential for multiple myeloma homing and migration. The motility of multiple myeloma cells was partially blocked by a neutralizing mAb against VEGFR-1, suggesting that the fetal bone stromal cells may secrete other cytokines and/or chemokines to promote multiple myeloma cell migration independent of VEGF-A/VEGFR-1 signaling. A prime example is the chemokine stromal cell–derived growth factor-1a, which has been implicated in multiple myeloma cell migration after binding to its receptor CXCR4 (31). Therefore, it is logical to speculate that VEGF-A and stromal cell–derived growth factor-1a could act synergistically to promote multiple myeloma cell migration.

We also show that the adult marrow stromal cells are significantly less efficient than the human fetal derived cells in supporting multiple myeloma cell growth. The precise mechanism for this intriguing functional diversity between fetal and adult stromal feeder layers is not known and is the subject of future studies. However, it is conceivable that in contrast to the adult stromal cells, fetal stromal cells are epigenetically programmed to produce proangiogenic factors, and as such, are permissive for supporting the growth of the multiple myeloma cells.

Taken together, the results shown here show the feasibility of blocking paracrine and intracrine VEGF-A/VEGFR-1 loops on multiple myeloma cells as a means of inducing multiple myeloma cell apoptosis. Alone or in combination with
chemotherapeutic agents, VEGFR-1 blockade may have clinical relevance for its therapeutic activity against multiple myeloma. Ongoing studies are in progress to further characterize the regulation of VEGFR-1 trafficking in multiple myeloma cells as well as profiling of downstream targeted genes in order to delineate distinct intracellular signaling pathways involved in VEGF-A/VEGFR-1-mediated multiple myeloma growth and migration.

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