**VEGF₁₆₅, but not VEGF₁₈₉, Stimulates Vasculogenesis and Bone Marrow Cell Migration into Ewing’s Sarcoma Tumors In vivo**

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**Abstract**

We previously showed that bone marrow stem cells participate in the tumor vessel expansion that supports the growth of Ewing’s sarcoma tumors in vivo. The purpose of this study was to determine the relative importance of two isoforms of vascular endothelial growth factor (VEGF) in tumor vessel expansion and recruitment of bone marrow–derived cells during tumor growth. We injected VEGF₁₆₅-siRNA–transfected cells (TCsi/7-1), control siRNA–transfected cells (TC/si-control), or TC71 parental Ewing’s sarcoma cells into nude mice. The TCsi/7-1 tumors were then treated with adenoviral vectors expressing VEGF₁₆₅ (Ad-VEGF₁₆₅), VEGF₁₈₉ (Ad-VEGF₁₈₉), or β-galactosidase (Ad-β-gal). Bone marrow cells labeled with fluorescent tracker dye were injected into the mice 3 weeks later. The TCsi/7-1 tumors were significantly smaller (P < 0.05), had decreased vessel density, and showed significantly lower bone marrow cell migration than did TC71 parental and TC/si-control tumors. Treatment with Ad-VEGF₁₆₅, but not Ad-VEGF₁₈₉ or Ad-β-gal, resulted in a significant increase in bone marrow cell infiltration, tumor vessel density, and tumor growth. Immunohistochemical staining revealed that the injected bone marrow cells migrated to and incorporated into the expanding CD31⁺ tumor vessel network. Taken together, these data show that VEGF₁₆₅ is a chemotactrant that recruits bone marrow cells into the tumor area. These data provide a mechanism by which Ewing’s sarcoma cells induce vasculogenesis.


**Introduction**

Ewing’s sarcoma is the second most common malignant bone tumor in children and young adults (1). It is a highly vascular tumor, with elevated levels of vascular endothelial growth factor (VEGF) shown in both Ewing’s cell lines and primary tumor specimens (2, 3). Several findings suggest that VEGF plays an important role in the pathogenesis of Ewing’s sarcoma. For example, serum VEGF levels are elevated in Ewing’s patients compared with those in healthy controls and decline after tumor regression (4, 5). In addition, positive staining for VEGF at diagnosis is correlated with a poor prognosis (3). Finally, EWS-ETS, the specific Ewing’s sarcoma oncprotein, was found to be a transcription factor for VEGF (3). Unfortunately, the long-term survival of patients with Ewing’s sarcoma remains disappointingly short despite the use of aggressive multimodal therapy (6, 7). Understanding the molecular and biological properties that contribute to and support the growth of this tumor may offer insights into specific targeted therapy.

The expansion of the tumor vascular network is crucial for tumor growth. VEGF has a central role in both normal and tumor angiogenesis and vasculogenesis. Binding of VEGF to its receptor induces mitogenesis and chemotaxis of normal endothelial cells, which contribute to new vessel formation. VEGF has several different isoforms resulting from alternative splicing of the gene (8). VEGF₁₆₅ and VEGF₁₂₁ are freely secreted, whereas VEGF₁₈₉ is sequestered in the extracellular matrix and requires cleavage by proteases for activation. The dysregulation of VEGF contributes to the pathogenesis of a variety of diseases, including macular degeneration, rheumatoid arthritis, and tumor growth (9). We have previously shown that Ewing’s sarcoma cells overexpress the VEGF₁₆₅ isoform, but not VEGF₁₈₉ or VEGF₁₂₁, compared with normal osteoblasts (10). It is presumed that this switch from the 189 to the 165 isoform must be beneficial to the tumor. However, the exact activity that VEGF₁₆₅ induces, which VEGF₁₈₉ cannot achieve, is unclear as both induce local angiogenesis.

We have shown that Ewing’s tumors recruit bone marrow–derived progenitor cells to participate in tumor neoangiogenesis (10, 11). Inhibition of VEGF₁₆₅ using specific siRNA technology reduced the migration of stem cells into the growing tumor, decreased tumor vessel expansion, and inhibited tumor growth (10). The purpose of this study was to determine the relative importance of VEGF₁₆₅ and VEGF₁₈₉ in tumor vasculogenesis and the recruitment of bone marrow cells to the tumor area. To that end, we constructed adenoviral vectors containing the gene encoding either VEGF₁₆₅ gene (Ad-VEGF₁₆₅) or VEGF₁₈₉ (Ad-VEGF₁₈₉). Using our Ewing’s sarcoma mouse model and siRNA inhibition of VEGF, we showed that VEGF₁₆₅, but not VEGF₁₈₉, was responsible for the chemotraction of bone marrow cells to the tumor area. VEGF₁₈₉ was unable to compensate for the loss of VEGF₁₆₅ production and could not rescue tumor growth, restore bone marrow cell migration into the tumor area, or enhance vessel density in tumors in which VEGF₁₆₅ was inhibited.
Results
VEGF<sub>189</sub> Does Not Compensate for Loss of VEGF<sub>165</sub>

We have previously shown that Ewing's sarcoma cell lines and primary tumors overexpress VEGF with a shift in isoform expression from VEGF<sub>189</sub> to VEGF<sub>165</sub> compared with normal osteoblast cells (10). We also showed that bone marrow cells participate in the expansion of the Ewing's tumor vasculature (11). Reduction of the VEGF<sub>165</sub> isoform using either siRNA or antisense technology inhibited Ewing's sarcoma growth in vivo and decreased the recruitment of progenitor cells to the tumor area and the number of tumor vessels (10, 12). To compare the effects of VEGF<sub>165</sub> and VEGF<sub>189</sub> on tumor growth, migration of bone marrow cells to the tumor, and development of tumor vascularization, we injected TC71 parental, TC/si-control, and VEGF<sub>165</sub>-siRNA–inhibited TCsi/7-1 cells into mice. TCsi/7-1 tumors were treated with Ad-VEGF<sub>165</sub>, Ad-VEGF<sub>189</sub>, Ad-β-gal, or PBS starting on day 5 after injection of tumor cells (Fig. 1).

Total RNA was extracted from the different tumor tissues and assayed for VEGF isoform expression by reverse transcription-PCR. Parental TC71 and TC/si-control tumors expressed more VEGF<sub>165</sub> than did the TCsi/7-1 tumors, whereas the expression of VEGF<sub>189</sub> in all three groups was approximately equal. Treatment of TCsi/7-1 tumors with Ad-VEGF<sub>165</sub> increased the expression of VEGF<sub>165</sub> only (Fig. 2A and B); the expression of VEGF<sub>189</sub> was not altered. By contrast, the expression of VEGF<sub>189</sub>, but not VEGF<sub>165</sub>, dramatically increased after treatment of tumors with VEGF<sub>189</sub>. Ad-β-gal did not alter either VEGF<sub>165</sub> or VEGF<sub>189</sub> expression (Fig. 2A and B). These results show that intratumoral injection of Ad-VEGF<sub>165</sub> or Ad-VEGF<sub>189</sub> specifically enhanced the expression of that particular isoform only. Real-time PCR results indicated that β-gal expression was very high only in the tumor treated with Ad-β-gal, suggesting that Ad-β-gal control tumors were equally well treated (data not shown). VEGF antibodies are unable to distinguish between VEGF<sub>165</sub> and VEGF<sub>189</sub> proteins and can therefore be used only to assess total VEGF protein. Total VEGF protein expression was reduced in TCsi/7-1 tumors compared with TC71 parental tumors (Fig. 2C). VEGF protein expression increased following injection of either Ad-VEGF<sub>165</sub> or Ad-VEGF<sub>189</sub>. Whereas we could not distinguish which isoform was produced, these results show that total VEGF protein and RNA levels were restored in TCsi/7-1 tumors treated with Ad-VEGF<sub>165</sub> or Ad-VEGF<sub>189</sub> (Fig. 2B and C).

We next investigated the effects of VEGF<sub>165</sub> or VEGF<sub>189</sub> restoration on tumor growth and vessel density to determine whether VEGF<sub>189</sub> can compensate for the loss of VEGF<sub>165</sub>. The mean tumor size was significantly smaller in the VEGF<sub>165</sub>-siRNA–inhibited TCsi/7-1 tumors compared with the TC71 parental or TC/si-control tumors (Fig. 3; P < 0.05). Intratumoral injection of TCsi/7-1 tumors with Ad-VEGF<sub>165</sub> significantly enhanced tumor growth compared with tumors injected with Ad-β-gal (P < 0.05). By contrast, tumor growth was not significantly changed following injection of Ad-VEGF<sub>189</sub> (P > 0.05). These data suggest that VEGF<sub>165</sub>, but not VEGF<sub>189</sub>, is able to rescue tumor growth.

VEGF<sub>165</sub>, but not VEGF<sub>189</sub>, Augments Tumor Vessel Density

The mean vessel density was significantly reduced in the TCsi/7-1 tumors compared with the TC71 tumors (Fig. 4A). Intratumoral injection of Ad-VEGF<sub>165</sub>, but not Ad-VEGF<sub>189</sub>, enhanced tumor vessel formation and brought the mean vessel density to the level seen in the TC71 parental tumors (Fig. 4A and B). Again, these data suggest that VEGF<sub>165</sub> is a more potent stimulus than VEGF<sub>189</sub> for vessel formation in Ewing’s tumors.

VEGF<sub>165</sub>, but not VEGF<sub>189</sub>, Stimulates Migration of Bone Marrow Cells to Tumors

Bone marrow cells labeled with the CM-Dil red fluorescent tracker dye were used to investigate the chemotactic abilities of VEGF<sub>165</sub> and VEGF<sub>189</sub>. There was a robust migration of bone marrow cells into TC71 parental tumors following their intravenous injection (Fig. 5A). By contrast, the number of migrated bone marrow cells was significantly lower in the TCsi/7-1 tumors. Treatment of TCsi/7-1 tumors with Ad-VEGF<sub>165</sub> not only restored VEGF<sub>165</sub> tumor expression (Fig. 2A and B) but also increased the migration of bone marrow cells into the tumor area (Fig. 5A). By contrast, enhanced VEGF<sub>189</sub> expression following treatment of the TCsi/7-1 tumors with Ad-VEGF<sub>189</sub> did not result in enhanced migration of bone marrow cells, despite the increase in VEGF<sub>189</sub> expression (Figs. 2 and 5A). Taken together, these data suggest that VEGF<sub>165</sub>, but not VEGF<sub>189</sub>, is responsible for the chemotraction of bone marrow cells to the tumor area.

Migrated Bone Marrow Cells Participate in Tumor Vessel Formation

To confirm that migrated bone marrow cells participate in tumor vessel formation, tumor sections were subjected to CD31

Intratumor injection of Ad-VEGF<sub>165</sub>, Ad-VEGF<sub>189</sub>, Ad-β-gal, or PBS

Injection of 2 X 10<sup>6</sup> tumor cells subcutaneously

**FIGURE 1.** The scheme for animal experiments. Six groups of mice were used: TC71 cells treated with PBS; TCsi/7-1 cells treated with PBS; TCsi/7-1 cells treated with Ad-VEGF<sub>165</sub>; TCsi/7-1 cells treated with Ad-VEGF<sub>189</sub>; TCsi/7-1 cells treated with Ad-β-gal; and TC/si-control cells treated with PBS.
FIGURE 2. Expression of VEGF<sub>165</sub> and VEGF<sub>189</sub> following intratumoral injection of adenoviral vectors. TC71-parental, TC/si-control, or TCsi/7-1 tumors were treated with or without Ad-VEGF<sub>165</sub>, Ad-VEGF<sub>189</sub>, or Ad-h-gal. A. Total RNA was extracted and analyzed by reverse transcription-PCR. 18S was used as the internal control. B. Densitometric analysis of relative VEGF isoform expression, adjusted to 18S internal control. C. Immunohistochemical staining for total VEGF protein in tumor tissues.
in the TCsi/7-1 group. Determined by comparing tumor size in indicated group with the tumor size in each mouse group was quantified weekly. Bars, SD. The mean tumor size in TCsi/7-1 mouse group was significantly smaller than that in TC71 group or TC71/si-control group (*, P < 0.05), suggesting that inhibition of VEGF165 expression decreased tumor growth. Treatment of TCsi/7-1 tumors with Ad-VEGF165 significantly stimulated tumor growth compared with untreated TCsi/7-1 tumors (P < 0.05), whereas treatment with Ad-VEGF189 induced bigger tumors but did not have significant difference compared with the tumor size in TCsi/7-1 group (P > 0.05). *, P value was determined by comparing tumor size in indicated group with the tumor size in the TCsi/7-1 group.

FIGURE 3. Effect of VEGF165 on tumor growth in vivo. Mean tumor size in each mouse group was quantified weekly. Bars, SD. The mean tumor size in TCsi/7-1 mouse group was significantly smaller than that in TC71 group or TC71/si-control group (P < 0.05), suggesting that inhibition of VEGF165 expression decreased tumor growth. Treatment of TCsi/7-1 tumors with Ad-VEGF165 significantly stimulated tumor growth compared with untreated TCsi/7-1 tumors (P < 0.05), whereas treatment with Ad-VEGF189 induced bigger tumors but did not have significant difference compared with the tumor size in TCsi/7-1 group (P > 0.05). * , P value was determined by comparing tumor size in indicated group with the tumor size in the TCsi/7-1 group.

immunofluorescence staining. The colocalization of migrated bone marrow cells and CD31+ tumor vessels (Fig. 5B) indicates that vasculogenesis, in addition to angiogenesis, contributes to the tumor vessel expansion.

Human Microvascular Endothelial Cell Migration to Cultured Supernatants

The data shown in Fig. 5 suggest that VEGF165 plays a significant role in the ability of tumor cells to chemoattract bone marrow cells. To assess the effects of VEGF165 and VEGF189 on endothelial cell migration, we used an in vitro chemotaxis assay with human microvascular endothelial cells (HMVEC) to quantify the chemotactic ability of cultured supernatants from TC71-parental and TCsi/7-1 cells. Inhibiting VEGF165 production reduced the chemotactic ability of supernatants, as shown by the reduction in HMVEC migration (Fig. 6). Chemotactic activity was restored after transfection of TCsi/7-1 cells with Ad-VEGF165 but not Ad-VEGF189 (Fig. 6).

Discussion

Despite numerous therapeutic trials, there has been no change in the 2-year metastasis-free survival of patients with Ewing’s sarcoma in more than 15 years (1, 6, 7). A better understanding of the mechanisms that regulate the growth and progression of Ewing’s sarcoma may help in the development of novel therapeutic approaches for patients with this disease. The data presented here show that VEGF165 is critical for the growth and development of human TC71 Ewing’s tumors in vivo. We previously showed that human TC71 cells express VEGF with a shift in isoform production from the membrane-bound VEGF189 to the soluble VEGF165 (10). Here, using siRNA that specifically blocked VEGF165, we showed that VEGF165-inhibited TCsi/7-1 cells grow poorly in vivo, and TCsi/7-1 tumors have decreased vascularity and mean vessel density. This decreased in vivo growth was not secondary to a decrease in cell doubling time because the in vitro growth of TCsi/7-1 cells is similar to that of TCsi-control cells (12). Restoration of VEGF165 expression using an adenoviral vector to deliver the VEGF165 gene in vivo restored tumor growth and mean vessel density (Figs. 3 and 4). By contrast, TCsi/7-1 tumors treated with Ad-VEGF189 showed increased VEGF189 expression and had increased VEGF protein (Fig. 2), but the growth rate and mean vessel density were unchanged compared with control or Ad-β-gal-treated tumors (Figs. 3 and 4), indicating that VEGF189 did not compensate for the loss of VEGF165. Taken together, these data suggest that VEGF165 has an additional mechanism for stimulating tumor vascular expansion, one that VEGF189 cannot achieve.

VEGF165 is diffusible, unlike VEGF189, and is therefore able to affect cells well beyond the local tumor microenvironment. Indeed, VEGF165 has been shown to be a chemoattractant for bone marrow stem cells, which express VEGF receptor 1 and VEGF receptor 2 (13, 14). We previously showed that bone marrow stem cells migrate to the growing Ewing’s sarcoma tumor in vivo, differentiate into endothelial cells, and contribute to the vascular expansion that is needed as the Ewing’s tumor grows (10, 11). The data presented in Fig. 5A and B confirm that bone marrow cells migrate from the vasculature into the tumor and participate in tumor vessel formation. Our data further showed that VEGF165 is the chemotactic stimulus controlling bone marrow cell migration into the tumor. Selective inhibition of VEGF165 using siRNA technology reduced the number of bone marrow cells that migrated into the tumor (Fig. 5A). Similar to what we observed for tumor growth and vessel density, bone marrow cell migration into the tumor was restored following intratumoral injection of Ad-VEGF165 but not Ad-VEGF189 (Figs. 3 and 4). Our data therefore suggest that VEGF165 production affects the distal mobilization and recruitment of stem cells into the tumor area.

The findings that VEGF165 stimulated the migration of bone marrow cells to the tumor area, that inhibiting VEGF165 suppressed bone marrow cell migration and tumor vessel development, and that VEGF189 could not restore vessel development, bone marrow cell migration, or tumor growth support our hypothesis that vasculogenesis is an important component in Ewing’s sarcoma growth and development. Our data also suggest that the decreased tumor growth seen in the TCsi/7-1 tumors is both the result of an effect on local endothelial cells (angiogenesis) and a secondary consequence of the inhibition of bone marrow cell chemotaxis into the tumor area (vasculogenesis).

Several questions about vasculogenesis in Ewing’s sarcoma growth remain unanswered. The exact nature of the stem/progenitor cells that participate in the expansion of the tumor vascular network is unclear. We used CM-Dil–labeled whole-mouse bone marrow cells as the source of stem/progenitor cells. Diverse cell types are contained within this bone marrow cell population. We are in the process of identifying the bone marrow stem/progenitor cell populations that participate in Ewing’s sarcoma vasculogenesis. Our recent data suggested that bone marrow stem/progenitor cells play diverse roles within the Ewing’s sarcoma tumor microenvironment. The tumor microenvironment may play a role in regulating the...
differentiation of recruited stem/progenitor cells. It is unclear at this time whether vasculogenesis is more important in the early establishment of the tumor or whether the migration of stem cells is also required for maintaining and increasing the tumor vasculature when the tumor is large and has well-established vessels.

In summary, we have shown that VEGF<sub>165</sub> is crucial for the recruitment of bone marrow cells to the tumor area, tumor vascular expansion, and tumor growth. These data support our hypothesis that interfering or supressing the migration of bone marrow cells into the tumor can severely affect the expansion and growth of tumor vessels, resulting in suppression of tumor growth. Bone marrow cells have been shown to be important in wound healing and repair of damaged vessels. Understanding the mechanisms that support and contribute to the growth and development of Ewing’s sarcoma may allow the identification of new targeted therapeutic approaches that not only affect local angiogenesis but also interfere with vasculogenesis. Bone marrow progenitor cells may also serve to rescue chemotherapy- or radiation-induced tumor vessel damage by providing cells that can form new tumor vessels. Therefore, blocking angiogenesis and vasculogenesis may not only inhibit tumor initiation or early tumor expansion at metastatic sites but perhaps also block tumor relapse by interfering with tumor vessel repair. It behooves us to consider such approaches.
because the survival rates for this cancer have remained stagnant at 40% to 50% over the past 20 years.

Materials and Methods

Cell Lines

HMVECs were purchased from Cambrex and grown in microvascular endothelial cell medium with 5% fetal bovine serum, 12 μg/mL bovine brain extract, 10 μg/mL human epidermal growth factor, 1 μg/mL hydrocortisone, and 1 μg/mL GA-1000. HMVECs (passage 3 or 4) were no more than 80% confluent when used for these experiments. TC71 human Ewing’s sarcoma cells (10, 11) were cultured in Eagle’s modified essential medium with 10% fetal bovine serum, 2 mmol/L l-glutamine, 1 mmol/L sodium pyruvate, 1 × nonessential amino acids, and 2 × minimal essential medium vitamin solution (Life Technologies). TC-71 cells stably transfected with VEGF165-siRNA (TCsi/7-1) and TC/si-control cells were maintained in the TC71 medium with 200 μg/mL hygromycin B (Invitrogen Life Technologies). All of the cells were negative for Mycoplasma, as determined by a Mycoplasma Plus PCR primer set (Stratagene).

FIGURE 5. VEGF165 stimulate bone marrow cell migration and participation in tumor vessel formation. A, TC71 or TCsi/7-1 cells were injected into nude mice. Tumors were treated on day 5 with Ad-VEGF165 or Ad-VEGF189. Red fluorescent CM-Dil–labeled syngeneic bone marrow cells were intravenously injected on week 4. Tumors were removed 2 d later and analyzed by immunohistochemistry. Red fluorescence, bone marrow cells that migrated into the tumors (top). The mean number of migrated bone marrow cells was quantified from five random microscope fields. Bars, SD (bottom). B, CD31 immunohistochemical analysis of tumors, suggesting that the migrated bone marrow cells participated in tumor vessel formation. As shown in B, the migrated bone marrow cells (red) were merged with CD31+ tumor vessel endothelial cells (green), showing CD31+ cells derived from injected bone marrow cells that migrated and incorporated into the tumor vessels (yellow; see arrow).
Construction of Adenoviral Vectors

The genes encoding human VEGF\textsubscript{165} or VEGF\textsubscript{189} were excised from the pLen-VEGF\textsubscript{165} or pLen-VEGF\textsubscript{189} vectors. The shuttle vectors pCA13-VEGF\textsubscript{165} and pCA13-VEGF\textsubscript{189} were constructed by subcloning the human VEGF\textsubscript{165} or VEGF\textsubscript{189} fragments into the pCA13 vector. The packaging vector pJM17 (Microbix Biosystems) and either shuttle vector were then cotransfected into human embryonic kidney 293 cells by FuGENE6 (Boehringer Mannheim). Infectious recombinant adenovirus plaques were collected 10 to 14 days after transfection and then propagated and screened. The viral DNA was purified and the presence of human VEGF\textsubscript{165} or VEGF\textsubscript{189} was confirmed by PCR. PCR was done using specific primers. The PCR products were resolved in 2% agarose gel stained with ethidium bromide and visualized under UV light. The control adenoviral vector, Ad-\textbeta-gal, is an adenovirus type 5–based vector that lacks E1A, E1B, and E3 but contains \textbeta-galactosidase. These recombinant replication-deficient adenoviral vectors were propagated in 293 cells as previously described (15). The viruses were purified twice using cesium chloride-gradient ultracentrifugation and then dialyzed and titrated using the standard methods. The cells were infected with adenovirus at 10 plaque-forming units per cell for 48 h and then used as indicated for various experiments.

Construction of VEGF\textsubscript{165}-siRNA Vector and Establishment of Stable Transfected Cell Line

A VEGF\textsubscript{165}-siRNA vector was constructed in our laboratory as previously described (12). This vector was shown to specifically inhibit the expression of VEGF\textsubscript{165}, but not VEGF\textsubscript{189}, following transfection into TC-71 cells (12). The resultant TCSI/7-1 cell line (clone 7-1) had significantly lower expression of VEGF\textsubscript{165} but unchanged expression of VEGF\textsubscript{189} compared with TC-71 cells transfected with the siRNA control vector (TCSI/si-control cells). VEGF protein production was also inhibited in the TCSI/7-1 cells (12).

Reverse Transcription-PCR

Total RNA was extracted from the specified cells or tissues. cDNA was synthesized using a reverse-transcription system (Promega) and amplified by PCR with specific primers for VEGF (sense, 5'-CACATAGAGATGAGCTTC-3'; antisense, 5'-CCGCTCGGCTTTGTCACAT-3'). The initial denaturation was done at 94°C for 5 min. The products were then subjected to denaturation at 94°C for 1 min, annealing at 59°C for 1 min, extension at 72°C for 1 min (30 cycles), and a final elongation at 72°C for 10 min. The PCR products were subjected to electrophoresis on a 2% agarose gel with ethidium bromide and visualized under UV light. The VEGF\textsubscript{189}, VEGF\textsubscript{165}, and VEGF\textsubscript{121} isoforms were 300, 230, and 100 bp, respectively. 18S primers and competimers (Ambion) were used as internal controls. The relative VEGF isoform expression in each band was determined by densitometry and adjusted to the 18S internal loading control.

Animal Studies

Four- to five-week-old specific pathogen-free athymic (T cell–deficient) nude mice were purchased from Charles River Breeding Laboratories. The mice were housed in an animal facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care and maintained in accordance with the current regulations and standards of the U.S. Department of Agriculture and Department of Health and Human Services and the NIH.

Parental TC71, TCSI/7-1, and TC/si-control cells were collected in mid-log growth phase. Mice were subcutaneously injected with 2 × 10\textsuperscript{6} cells in 0.1-mL HBSS. Tumors were detected 5 days after injection. The TCSI/7-1 tumor–bearing mice were then intratumorally injected with Ad-VEGF\textsubscript{165}, Ad-VEGF\textsubscript{189}, Ad-\textbeta-gal, or PBS (Fig. 1). A total volume of 20 \textmuL were injected in several different sites within each tumor. Injection was done twice weekly for 5 weeks. Ten mice were used in each group. Each experiment was repeated thrice. The

**FIGURE 6.** VEGF\textsubscript{165} stimulates migration of HMVECs. In vitro migration assay was done using cultured tumor cell supernatants in Transwell chambers. The mean number of migrated HMVECs in each group was quantified from five random microscope fields. Bars, SD.
tumors were measured every week with calipers and their diameters were recorded. Tumor volumes were calculated using the formula \(ab^2/2\), where \(a\) is the longer diameter and \(b\) is the shorter diameter, expressed in cubic millimeters. Tumor tissue was collected from each group and analyzed for VEGF isoform expression by reverse transcription-PCR and for CD31 expression by immunohistochemical staining.

**Immunohistochemical Staining for CD31 and VEGF**

Tumor tissues were collected from the mice and analyzed by routine pathology by H&E staining. Frozen sections were fixed with acetone and incubated in 3% H2O2 in methanol for 10 min to block endogenous peroxidase. Nonspecific protein was blocked by incubation in 5% normal horse serum plus 1% normal goat serum in PBS for 20 min. Expression of CD31 was detected in blood vessels with rat anti-mouse CD31 primary antibody (PharMingen) and goat anti-rat horseradish peroxidase–conjugated secondary antibody, followed by incubation with the chromogen diaminobenzidine. The number of tumor blood vessels in five random microscopic fields was counted for each tumor and the mean was determined. This analysis was done in triplicate and the results were expressed as the mean of three independent experiments. VEGF expression in tumors was detected by incubating the paraffin-embedded sections with rabbit anti-human VEGF primary antibody (Santa Cruz Biotechnology) and horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G (Jackson Immunolaboratories). Gill’s hematoxylin was used as a counterstain.

**In vivo Bone Marrow Cell Migration Assay**

Mouse bone marrow cells were obtained from the femurs of nude mice. The cells were washed in sterile PBS, spun at 200 × g for 5 min, resuspended at a density of 1 × 10^6 cells/mL, and stained with CM-Dil fluorescent dye at a concentration of 5 μL/mL (Vybrant Cell-Labeling Solution, Molecular Probes). The cells were then incubated at 37°C for 15 min, washed twice with PBS, and injected intravenously into mice (3 × 10^6 stained cells in 100 μL per mouse). The mice were killed 7 days later and tumor tissues were frozen. The CM-Dil–labeled cells were detected and counted in tumor tissue samples with Zeiss fluorescence microscope (Carl Zeiss) at the same setting. The mean number of migrated bone marrow cells in each tumor area was calculated by counting the number in five random microscope fields using software Image-Pro Plus. The frozen samples were also stained with anti-CD31 primary antibody and antimouse Alexa 488–conjugated secondary antibody. The colocalization of the migrated bone marrow cells (red) and tumor vessels (green) was the yellow area.

**In vitro Vascular Endothelial Cell Migration Assay**

TC71 or TCsi/7-1 cells (3 × 10^5) were seeded in six-well plates. The medium was replaced with fresh medium the following day. TCs/7-1 cells were treated with Ad-VEGF 165, Ad-VEGF 189, or PBS for 48 h. The cultured supernatants from TC71 or TCsi/7-1 cells treated with Ad-VEGF 165, Ad-VEGF 189, or PBS were collected and used as the conditioned medium. Transwell chambers (Costar) were pretreated with serum-free medium at 37°C for 1 h before being seeded with HMVECs at 1 × 10^4 per well in 100 μL of medium with 0.1% fetal bovine serum. The chambers were then inserted into 24-well plates containing 600 μL of each conditioned medium and incubated at 37°C for 6 h to allow the HMVECs to migrate. Cells on the upper side of the filter were removed with cotton swabs. Migrated cells on the lower side of the filter were fixed, stained with H&E, and counted under a binocular microscope. The average number of migrated cells was calculated from five random microscope fields.

**Statistical Analysis**

The two-tailed Student t test was used to compare the tumor volumes and numbers of blood vessels in the treatment and control groups. \(P < 0.05\) was considered statistically significant.

**References**

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