

# Concurrent Induction of Lymphangiogenesis, Angiogenesis, and Macrophage Recruitment by Vascular Endothelial Growth Factor-C in Melanoma

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**Interactions of tumor cells with lymphatic vessels are of paramount importance for tumor progression, however, the underlying molecular mechanisms are poorly understood. Whereas enlarged lymphatic vessels are frequently observed at the periphery of malignant melanomas, it has remained unclear whether intratumoral lymphangiogenesis occurs within these tumors. Here, we demonstrate the presence of intratumoral lymphatics and enlargement of lymphatic vessels at the tumor periphery in vascular endothelial growth factor (VEGF)-C-overexpressing human melanomas transplanted onto nude mice. VEGF-C expression also resulted in enhanced tumor angiogenesis, indicating a coordinated regulation of lymphangiogenesis and angiogenesis in melanoma progression. The specific biological effects of VEGF-C were critically dependent on its proteolytic processing *in vivo*. Furthermore, VEGF-C induced chemotaxis of macrophages *in vitro* and *in vivo*, revealing a potential function of VEGF-C as an immunomodulator. Taken together, our results identify VEGF-C as multifunctional factor involved in regulating tumor lymphangiogenesis, angiogenesis, and immune response. (Am J Pathol 2001, 159:893–903)**

The spread of tumor cells via lymphatic vessels to the regional lymph nodes is one of the most important indicators of tumor aggressiveness, and the extent of lymph node involvement is a major determinant for the staging and the prognosis of most human malignancies.<sup>1–3</sup> Abundant, rearranged, and often dilated lymphatic vessels containing clusters of tumor cells are commonly observed at the periphery of many tumors, including malignant melanomas.<sup>1,4–7</sup> However, the presence of lymphatic vessels within tumors and the ability of tumor

cells to induce lymphangiogenesis have remained controversial.<sup>8,9</sup>

Whereas tumor angiogenesis involving the formation of new blood vessels has been studied extensively, the role of lymphatic vessels in tumor pathology and the molecules regulating lymphangiogenesis have remained mostly unknown. Recently, a novel member of the vascular endothelial growth factor (VEGF) family has been identified that is distinguished by its capacity to stimulate lymphangiogenesis.<sup>10,11</sup> Vascular endothelial growth factor-C (VEGF-C) stimulated lymphangiogenesis in the avian chorioallantoic membrane assay,<sup>12</sup> and transgenic mice overexpressing VEGF-C in the skin were characterized by specific hyperplasia of the lymphatic network without obvious effects on blood vessels.<sup>13</sup> In normal adult human tissues, the VEGF-C receptor VEGFR-3 (Flt-4) has been found predominantly expressed by lymphatic endothelium.<sup>7,14</sup> However, VEGF-C also binds VEGFR-2 (KDR), that is mainly expressed by activated endothelium of blood vessels, suggesting a potential function of VEGF-C in the induction of angiogenesis.<sup>10,15</sup> Indeed, VEGF-C stimulates the proliferation and migration of blood vascular endothelial cells *in vitro* and has been shown to increase vascular permeability in the Miles assay,<sup>10,11,16,17</sup> and to induce angiogenesis in the mouse corneal micropocket assay and in the rabbit ischemic hind limb model.<sup>18,19</sup>

VEGF-C mRNA expression has been demonstrated in a large number of human tumors, including malignant melanomas.<sup>20–32</sup> Expression of its receptor VEGFR-3 has been detected in lymphatic vessels and occasionally also in blood vessels adjacent to cancer cells.<sup>7,14,21</sup> Most recently, we have shown that VEGF-C overexpression in experimental breast cancer selectively induced intratumoral lymphangiogenesis, leading to increased tumor metastasis, without obvious effects on angiogenesis.<sup>33</sup>

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In the present study we demonstrate the occurrence of intratumoral lymphangiogenesis in VEGF-C-overexpressing human melanomas transplanted onto nude mice. Moreover, VEGF-C also induced tumor angiogenesis and recruitment of macrophages. Our results indicate that the distinct biological effects of VEGF-C are critically dependent on its proteolytic processing *in vivo*.

## Materials and Methods

### Cell Culture

The human malignant melanoma cell lines MeWo,<sup>34,35</sup> WM9, and WM239 were provided by Dr. Robert S. Kerbel (Sunnybrook Health Science Center, Toronto, Canada). Human melanoma cell lines PM-WK, RPM-MC, RPM-EP, and MM-LH<sup>36</sup> were obtained from Dr. Randy Byers (Boston University Medical School, Boston, MA). The human melanoma cell lines SK-MEL-5, SK-MEL-25, SK-MEL-28, MelJuso, Colo 38, MML-1, HS695T, and MELKL-2 were obtained from the tumor bank of the German Cancer Research Center in Heidelberg, Germany. All melanoma cell lines were maintained in RPMI 1640 medium with 5% fetal bovine serum; human prostatic adenocarcinoma PC-3 cells (American Type Culture Collection, Rockville, MD) in Ham's F12 medium with 5% fetal bovine serum. All media were purchased from Life Technologies, Inc., Grand Island, NY.

### Cell Transfection and Selection

A human VEGF-C cDNA comprising the complete coding sequence (GenBank accession number X94216)<sup>10</sup> was cloned into a pcDNA3.1/Zeo expression vector (Invitrogen, San Diego, CA) that contains a CMV-enhancer promoter and a Zeocin selection cassette. The sequence and the orientation of the VEGF-C gene in the construct were verified by restriction mapping and direct sequencing. Human MeWo malignant melanoma cells were transfected either with the human VEGF-C cDNA cloned into a pcDNA3.1/Zeo vector or with the vector alone using the Superfect transfection reagent (Qiagen, Chatsworth, CA). Transfected cells were selected and maintained in growth medium containing 50  $\mu$ g/ml Zeocin. Stably transfected cell clones were individually expanded and analyzed for VEGF-C mRNA expression and protein secretion.

### RNA Isolation and Northern Analysis

Total cellular RNA was isolated from cultured cells and from tumors using the RNeasy kit (Qiagen). The isolated RNA was subjected to electrophoresis (15  $\mu$ g per lane) and transferred to Hybond-N<sup>+</sup> membranes (Amersham, Arlington Heights, IL). <sup>32</sup>P-radiolabeled DNA probes were labeled by the random priming method (Multiprime labeling kit; Amersham, Arlington Heights, IL). The VEGF-C probe used was a fragment containing nucleotides 581 to 1634 of human VEGF-C cDNA. The probe for human VEGF hybridizes with a region of VEGF mRNA common to all known VEGF splice variants.<sup>37</sup> A human  $\beta$ -actin cDNA probe (Clontech, Palo Alto, CA) was used

as a control for equal RNA loading. Blots were hybridized at 65°C for 24 hours, washed at high stringency, and exposed to X-OMAT MR film (Kodak, Rochester, NY).

### Western Analysis

Cells grown to 80% confluency were lysed with Izuhara buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 1% Triton X-100, 30 mmol/L Na-pyrophosphate, 50 mmol/L Na-fluoride, 1 mmol/L Na-orthovanadate, pH 7.4) containing protease inhibitors (phenylmethylsulfonyl fluoride, 0.02 mol/L; leupeptin, 50  $\mu$ g/ml; aprotinin, 50  $\mu$ g/ml). The amount of protein was determined with the BioRad protein assay (BioRad, Hercules, CA), and 15  $\mu$ g were analyzed on the gel. Conditioned media were obtained from subconfluent cells grown for 60 hours in serum-free media, concentrated 100-fold using Centricon-10 columns (Amicon, Beverly, MA), and 15  $\mu$ g of protein were loaded on the gel. Tumors were snap-frozen in liquid nitrogen, lysed by homogenizing the tissue (0.5 g) in 2 ml of a buffer containing 2% sodium dodecyl sulfate, 50 mmol/L Tris (pH 7.4), and protease inhibitors as above, and 10  $\mu$ l were loaded onto the gel. All samples were boiled in denaturing Laemmli sample buffer (BioRad), analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and blotted onto polyvinylidene difluoride membranes (BioRad). Filters were blocked overnight with 5% nonfat milk in phosphate-buffered saline (PBS)/0.1% Tween 20 and were incubated with a rabbit antiserum against human VEGF-C (1:1000).<sup>16</sup> After washes, membranes were incubated with horseradish-peroxidase-conjugated anti-rabbit IgG (1:2000; Amersham), washed, and analyzed using the Amersham ECL+ chemiluminescence reagents. Receptor phosphorylation assays were performed as described,<sup>33</sup> using antibodies against mouse VEGFR-2 (Santa Cruz Biotechnology, Santa Cruz, California) and phosphotyrosine (PY-20; ICN Biomedicals, Aurora, Ohio).

### Tumorigenicity Assay

Stably transfected MeWo cells ( $2 \times 10^6$  in 100  $\mu$ l of serum-free culture medium) were injected intradermally on each side of the back of 8-week-old female Swiss/c (nu/nu) nude mice (five mice for each clone). Three clones were analyzed for each construct. Tumor growth was measured weekly using a digital caliper. Tumors were harvested when they reached a size of  $>1200$  mm<sup>3</sup> or after 6 to 8 weeks, and were embedded in OCT compound and frozen in liquid nitrogen or were fixed in 4% paraformaldehyde/PBS and processed for routine histology. For RNA or protein extractions, tumors were snap-frozen in liquid nitrogen. Two independent experiments with five mice in each group were performed.

### Generation of a Polyclonal Anti-Mouse VEGFR-3 Antibody

The 19-amino acid synthetic peptide CYPGKQAERAK-WVPERRSQ, corresponding to amino acid residues 265 to 285 in the Ig-like domain 3 of the mouse VEGFR-3

extracellular domain, was used to immunize rabbits with standard techniques. The antisera were affinity purified and specific staining of lymphatic vessels was confirmed in adult mouse tissues (heart, lung, spleen, and liver).

### *Indirect Immunofluorescence*

Cryosections were stained as previously described,<sup>38</sup> using antibodies against mouse CD31 (dilution 1/30; Pharmingen, San Diego, CA), LYVE-1 (1/300),<sup>33,39</sup> VEGFR-3 (1/50; rabbit polyclonal; 1/30; R&D Systems, Minneapolis, MN), CD11b/Mac-1 (1/200; Pharmingen), or F4/80 (1/200; Serotec, Raleigh, NC). The secondary antibodies, labeled with either Texas Red or fluorescein isothiocyanate (Jackson ImmunoResearch, West Grove, PA) were used at the dilution 1/50. Cell nuclei were counterstained with Hoechst bisbenzimidazole (Sigma, St. Louis, MO) at 20  $\mu\text{g}/\text{ml}$ . Specimens were examined by using a Nikon E-600 microscope (Nikon, Melville, NY).

### *Computer-Assisted Morphometric Analysis*

For analysis of tumor vascularization, immunohistochemical stainings were performed on 6- $\mu\text{m}$  frozen sections of tumor xenografts using an antibody against mouse CD31.<sup>40</sup> Tissue sections were viewed using a Nikon E-600 microscope and images were captured with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI). Two MeWo/control and two MeWo/VEGF-C clones were analyzed, five tumors each. On each tumor section, three randomly chosen fields were evaluated at  $\times 60$  magnification. Morphometric analysis was performed using the IPLab software (Scanalytics, Fairfax, VA) to determine vessel density, size distribution, and the relative tumor area covered by vessels.<sup>33</sup> For analysis of macrophage densities, sections were stained with CD11b/Mac-1 antibody as described above. Three MeWo/control and three MeWo/VEGF-C clones were analyzed, three tumors each. For each tumor, the total area of the adjacent overlying skin was analyzed, and the relative area of the skin occupied by macrophages was determined using the IPLab software. The unpaired *t*-test was used for statistical analysis.

### *Computed Tomography*

Computed tomography was performed to determine the clearance rate of a lymphographic contrast agent from the tumors. Mice were imaged 5 weeks after tumor cell inoculation. Mice bearing MeWo/control tumors were additionally analyzed when the tumor size equaled that of the MeWo/VEGF-C tumors at 5 weeks ( $\sim 1000 \text{ mm}^3$ ). Three mice carrying two tumors each were analyzed in both groups. After anesthesia with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), mice were injected with an iodinated lymphographic contrast agent,<sup>41</sup> and imaging was performed by using a helical computed tomography scanner (TCT900S/xII; Toshiba, Tokyo, Japan). The contrast agent was administered very slowly by injection into the center of each tumor (20  $\mu\text{l}$  per tumor)

by using a 30-gauge needle attached to a Hamilton syringe. Mice were imaged before, immediately after, 6 hours, 24 hours, 48 hours, and 72 hours after the injection of the contrast agent. Each imaging study consisted of a helical data acquisition through the tumor and the axillary region for the assessment of axillary lymph nodes. The imaging parameters were 120-kV tube voltage, 150-mA tube current, 2-mm slice thickness, and a high-resolution imaging mode with an 80-mm field of view. Images were reconstructed onto a  $512 \times 512$  image matrix yielding  $0.05 \text{ mm}^3$  voxels. Data analysis was performed with the image analysis program DIP Station (HIPG, Boulder, CO) to obtain the average signal intensity value for the tumor volume. Signal intensity values in computed tomography are expressed as Hounsfield units that change linearly as a function of contrast agent concentration in the tissue.<sup>42</sup> A signal-time curve was created for each tumor and the rate constant was calculated using a mono-exponential model as a first approximation. The average clearance rate was then calculated for each group of animals.

### *Isolation of Mouse Macrophages, Flow Cytometry, and Chemotaxis Assay*

Macrophage accumulation was induced in Swiss/c nu/nu mice by intraperitoneal injection of 1 ml of 3% Brewer thioglycollate medium and macrophages were harvested 5 days after injection by lavage with Hanks' balanced salt solution.<sup>43</sup> For flow cytometry, macrophages were detached from the tissue culture dish by scraping, incubated for 15 minutes at  $4^\circ\text{C}$  with antibodies to CD11b or mouse VEGFR-3 and with fluorescein isothiocyanate-labeled corresponding secondary antibodies. Chemotaxis was examined using 24-well Transwell migration chambers (8  $\mu\text{m}$  pore size; Costar). Inserts were coated on the underside with 10  $\mu\text{g}/\text{ml}$  of collagen type I (Collagen Corp., Palo Alto, CA) and  $4 \times 10^5$  cells were added to the upper compartment in 100  $\mu\text{l}$  of serum-free Dulbecco's modified Eagle's medium. VEGF-C (1 to 100 ng/ml) or the macrophage chemoattractant *N*-formyl-met-leu-phe (fMLP;  $10^{-6}$  mol/L) were added to 600  $\mu\text{l}$  of serum-free media in the lower compartment only. After 3 hours, inserts were fixed and washed as described<sup>44</sup> and cell nuclei were stained with propidium iodide. The number of migrated cells was determined by using the IPLab software. For every insert, three fields were evaluated at  $\times 120$  magnification. All assays were performed in triplicate.

### *Cell Growth Assays*

*In vitro* proliferation rates of transfected MeWo cell clones were determined by using the BrdU labeling and detection kit (Boehringer Mannheim, Mannheim, Germany). Cells were plated in 96-well plates ( $5 \times 10^3$  cells/well; 8 wells/cell clone) and were allowed to proliferate for 24 hours before addition of BrdU (10  $\mu\text{mol}/\text{L}$ ) for 6 hours. The absorbance was determined at 405 nm using a microtiter plate reader (Titertek, Huntsville, AL). To assess the effect of VEGF or VEGF-C on MeWo cell proliferation, MeWo/control clone 5 was seeded into a 96-well



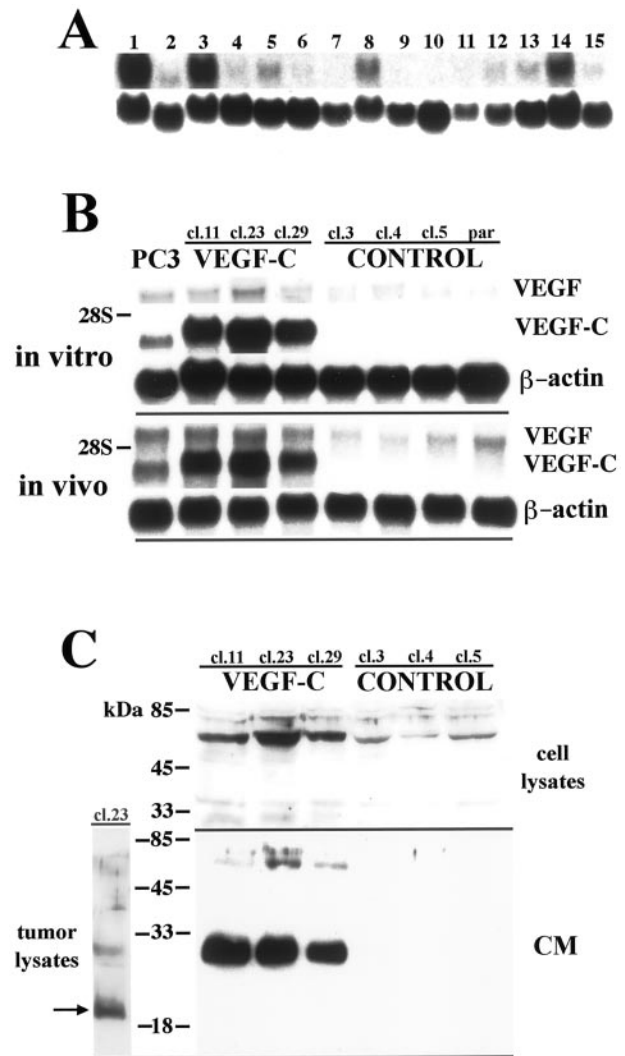
plate at a density of  $2.5 \times 10^3$  cells/well, and cells were treated for 5 days with 20 ng/ml of recombinant human VEGF-C<sup>16</sup> or VEGF (R&D Systems), six wells each. Cell proliferation was determined by using the BrdU kit. In additional experiment, MeWo/control clones were plated in 12-well plate inserts (1  $\mu$ m pore size;  $1 \times 10^4$  cells/insert; four inserts each), and were co-cultured for 7 days with either MeWo/VEGF-C cl. 23 or MeWo/control cl. 5 ( $1 \times 10^4$  cells/well). Total cell nuclei were stained with the Hoechst bisbenzimidazole DNA stain and cells that had incorporated BrdU (10  $\mu$ mol/L, 2 hours) were visualized by immunofluorescent staining using an anti-BrdU antibody<sup>38</sup> (Pharmingen, San Diego, CA). Inserts were mounted on slides and the percentage of proliferating cells was determined by using the IPLab software. For every insert, three randomly chosen fields were evaluated at  $\times 120$  magnification. All experiments were repeated twice. The unpaired *t*-test was used for statistical analysis.

## Results

### Overexpression of VEGF-C in the Human Melanoma Cell Line MeWo

We first examined the expression of VEGF-C in several human melanoma cell lines by Northern analysis and found that VEGF-C mRNA was expressed in most (10 of 15) melanoma cell lines studied, as a single band of 2.4 kb (Figure 1A). In contrast, normal human melanocytes expressed little or no VEGF-C mRNA *in vitro* (data not shown). To examine the role of VEGF-C in tumorigenesis, we transfected the melanoma cell line MeWo, established from a lymph node metastasis of a nodular malignant melanoma,<sup>34,35</sup> devoid of VEGF-C expression, to constitutively overexpress VEGF-C. As determined by Northern analysis, the parental MeWo cell line and three vector-transfected control clones (MeWo/control) did not express any detectable amounts of VEGF-C mRNA *in vitro* or *in vivo* (Figure 1B). Three VEGF-C-transfected cell clones (MeWo/VEGF-C) expressed high levels of VEGF-C mRNA in culture, as well as in tumors that reached the size of  $\sim 1200$  mm<sup>3</sup> (Figure 1B). To exclude the possibility of altered production of VEGF or other angiogenic factors by MeWo/VEGF-C cells, we examined the expression of VEGF, basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF)-B in MeWo transfectants by Northern analysis. Parental, control, and VEGF-C-transfected MeWo cells expressed comparable, low levels of endogenous human VEGF mRNA *in vitro* and *in vivo* (Figure 1B), and little or no bFGF or PDGF-B mRNA (data not shown).

Western blot analyses confirmed that high VEGF-C mRNA levels correlated with high amounts of VEGF-C protein expression (Figure 1C). We detected a strong band of  $\sim 60$  kd in cell lysates of VEGF-C transfectants, corresponding to VEGF-C precursor,<sup>16</sup> and only trace amounts in control cells. Large amounts of the secreted 31-kd form that activates VEGFR-3<sup>16</sup> were selectively found in culture supernatants of VEGF-C-transfected clones. None of the clones expressed any detectable levels of the 21-kd form *in vitro*. In VEGF-C-overexpress-

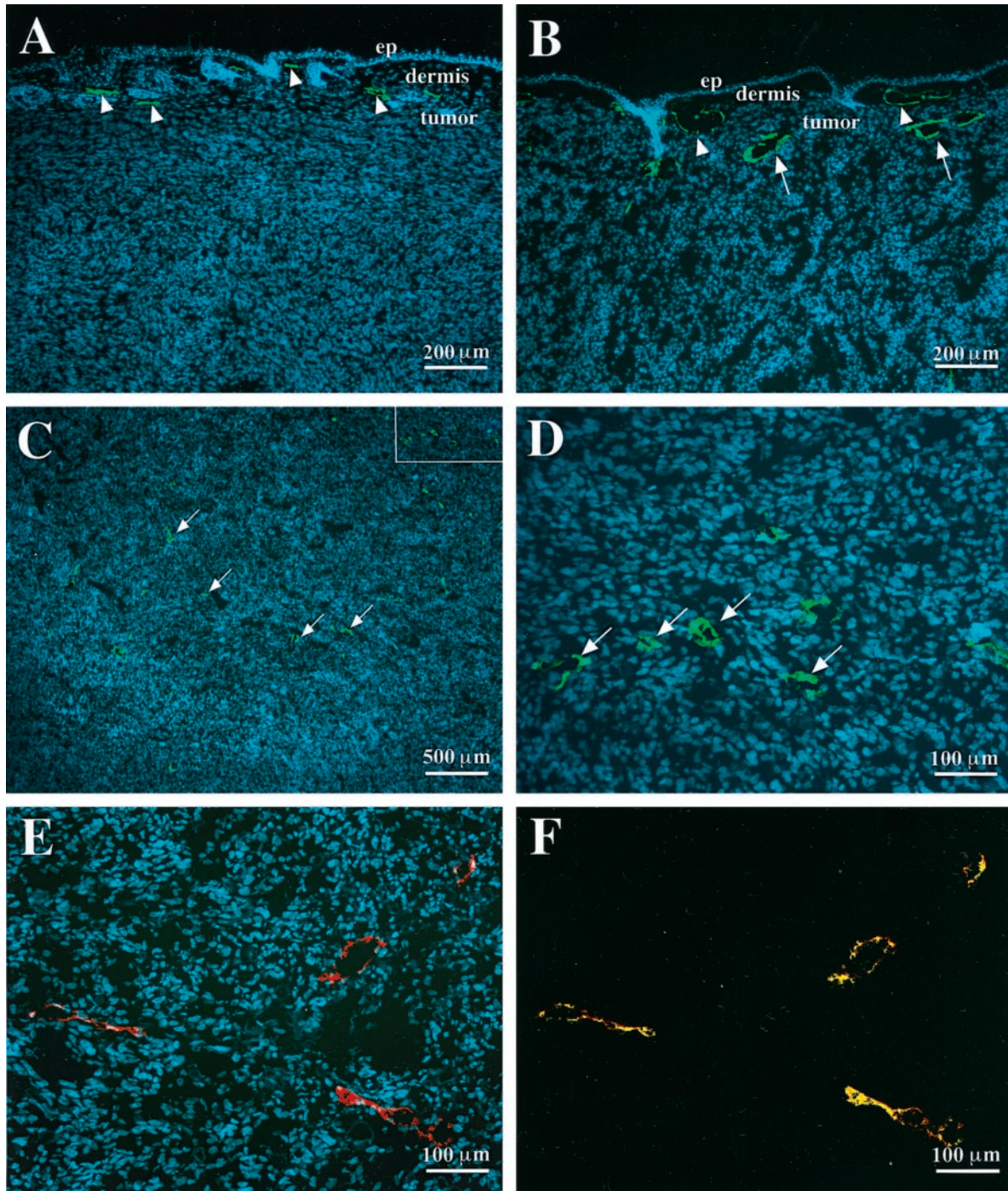


**Figure 1.** Expression of VEGF-C in melanoma cell lines and overexpression of VEGF-C in stably transfected MeWo cell clones. **A:** Northern blot analysis of the expression of VEGF-C mRNA (2.4 kb) in cultured melanoma cell lines. 1, positive control PC-3 mRNA; 2, SK-MEL-5; 3, SK-MEL-25; 4, SK-MEL-28; 5, WM9; 6, WM 239; 7, Colo; 8, MelJuso; 9, MML-1; 10, MS695T; 11, Melk12; 12, PM-WK; 13, RPM-EP; 14, RPM-MC; 15, MM-LH. Hybridization with  $\beta$ -actin was performed as a loading control (**bottom panel**). **B:** Northern blot analysis of cultured MeWo cell clones and extracts of MeWo cell clones grown as tumors *in vivo* demonstrates strong overexpression of VEGF-C in VEGF-C-transfected clones. VEGF-C mRNA was not detectable in clones transfected with the control vector or in the parental cell line. The prostatic carcinoma cell line PC-3 served as a positive control. Note that VEGF-C expression did not alter VEGF mRNA expression in transfected clones. **C:** Western blot analysis of cultured cells demonstrates increased amounts of VEGF-C precursor protein ( $\sim 60$  kd) in cell lysates and secretion of large amounts of the partially processed VEGF-C ( $\sim 31$  kd) into the media by MeWo/VEGF-C cells, as compared to control transfectants. Western blot of VEGF-C-overexpressing tumor lysates shows that VEGF-C is fully processed *in vivo* into the mature, 21-kd form (**arrow**).

ing tumors however, we predominantly detected the mature 21-kd VEGF-C form, a high-affinity ligand for both VEGFR-2 and VEGFR-3, demonstrating that complete processing of VEGF-C occurred *in vivo* (Figure 1C).

### VEGF-C Induces Tumor Lymphangiogenesis

The VEGF-C-overexpressing MeWo cell lines were injected intradermally into immunodeficient mice and tu-



**Figure 2.** VEGF-C-overexpressing tumors are infiltrated with lymphatic vessels. **A:** Immunofluorescent staining for LYVE-1 (green) demonstrates compressed lymphatic vessels (**arrowheads**) in the dermis overlying MeWo/control tumor. Note the absence of lymphatic vessels within the tumor mass. **B:** Enlarged lymphatic vessels in the overlying dermis (**arrowheads**) and within the peripheral rim of the MeWo/VEGF-C tumor (**arrows**). **C** and **D:** Numerous lymphatic vessels in the central areas of the MeWo/VEGF-C tumor (**arrows**). Expression of VEGFR-3 (**E**) (red) and co-localization of VEGFR-3 with LYVE-1 (**F**) (green) on intratumoral lymphatic vessels. Cell nuclei are counterstained blue with Hoechst. ep, epidermis.

mors were harvested 6 weeks after the injection. To visualize tumor-associated lymphatic vessels we used an antibody against LYVE-1, a recently discovered specific marker of lymphatic vessels in normal tissues<sup>39,45</sup> and in tumors.<sup>33</sup> We detected lymphatic vessels with mostly compressed lumina in the skin surrounding control tumors (Figure 2A, arrowheads). In contrast, lymphatic ves-

sels were frequently enlarged in the skin surrounding VEGF-C-overexpressing tumors (Figure 2B, arrowheads) and also within the peripheral areas of the tumors (Figure 2B, arrows). Importantly, numerous small lymphatic vessels were observed within VEGF-C-producing tumors (Figure 2, C and D; arrows), but not in control tumors (Figure 2A), demonstrating that VEGF-C induced tumor



lymphangiogenesis. All LYVE-1-positive lymphatic vessels also expressed VEGFR-3 (Figure 2, E and F). The effects of VEGF-C overexpression on lymphatic vessels were not associated with a modulation of the expression of the related lymphangiogenesis factor, VEGF-D,<sup>46,47</sup> because, as determined by Northern analyses, little or no VEGF-D mRNA was detected in cultured cells and in tumor extracts of control and VEGF-C-overexpressing cells (data not shown).

Using computed tomography we found that the clearance rate of a contrast agent from VEGF-C-overexpressing tumors to regional lymph nodes was considerably slower than from control tumors (Figure 3). The clearance rate was independent of tumor size. Assessment of axillary lymph nodes confirmed that the contrast agent had been transported by the lymphatic vessels to the lymph nodes (data not shown).

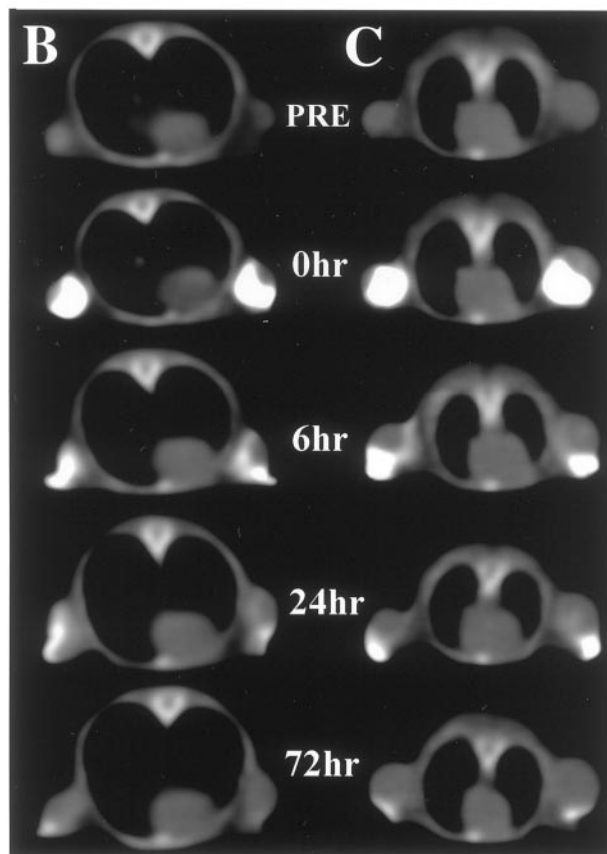
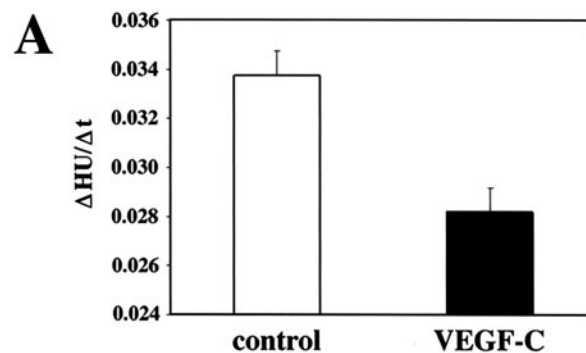
### VEGF-C Increases Tumor Angiogenesis

To analyze intratumoral angiogenesis, tumor sections were stained with an antibody against mouse CD31.<sup>48</sup> In both experimental groups, vessels were distributed rather uniformly throughout the tumors (Figure 4, A and B). Computer-assisted morphometric analysis revealed significantly increased vascular densities (Figure 4C) in 6-week-old tumors derived from VEGF-C-overexpressing clones ( $40 \pm 12$  vessels/mm<sup>2</sup>) as compared to control clones ( $23 \pm 7$  vessels/mm<sup>2</sup>;  $P < 0.0002$ ). Furthermore, the relative tumor area covered by vessels was significantly increased in MeWo/VEGF-C tumors (Figure 5D) as compared to control tumors ( $2.65 \pm 0.9\%$  versus  $1.48 \pm 0.4\%$ ;  $P < 0.0001$ ). The distribution of vessel sizes however, was not significantly different (data not shown). The majority of tumor vessels were smaller than  $800 \mu\text{m}^2$ , with approximately half of all vessels in both control and VEGF-C-overexpressing tumors smaller than  $400 \mu\text{m}^2$ .

Immunoprecipitation studies of tumor lysates revealed increased amounts of VEGFR-2 and enhanced VEGFR-2 phosphorylation levels in VEGF-C-overexpressing tumors (Figure 4E), which is in accordance with the observed production of the mature form of VEGF-C in these tumors. Taken together, these results identify the mature form of VEGF-C as a potent tumor angiogenesis factor.

### VEGF-C Stimulates Recruitment of Macrophages

Histological analysis revealed an increased inflammatory response in the skin surrounding VEGF-C-transfected melanomas, as compared to control tumors. Using an antibody to CD11b that has broad reactivity with dermal macrophages,<sup>49</sup> we observed a markedly increased density of macrophages surrounding MeWo/VEGF-C tumors (Figure 5, A and C). Immunofluorescent analysis demonstrated that CD11b-positive macrophages expressed VEGFR-3 (Figure 5, B and D). The identity of VEGFR-3-positive cells as macrophages was further confirmed by staining with an additional macrophage marker, F4/80<sup>50</sup> (Figure 5, E and F).

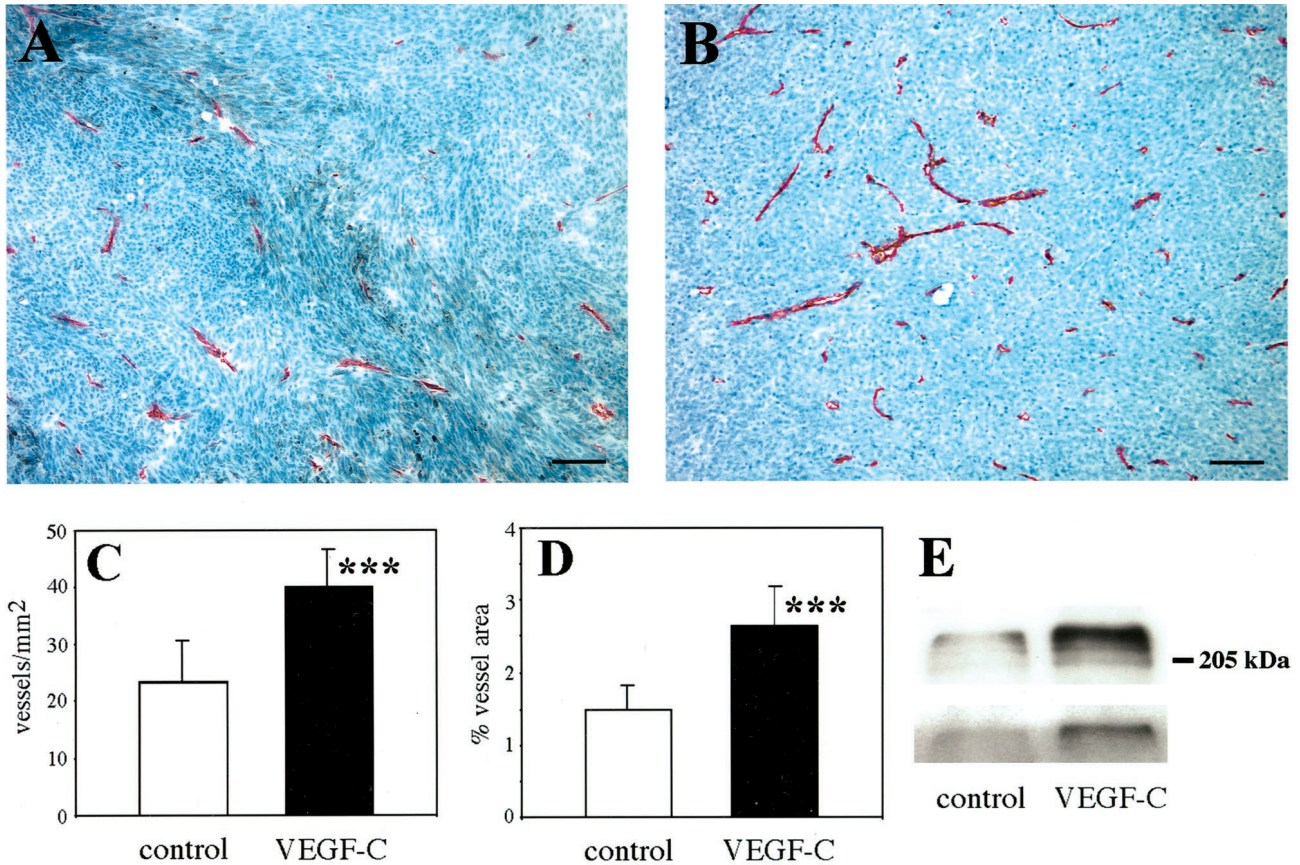


**Figure 3.** Impaired clearance of the lymphographic contrast agent from VEGF-C-overexpressing MeWo tumors. Contrast agent was injected into the tumors and the images of tumors were obtained with a computed tomography scanner for up to 72 hours after injection. **A:** Signal intensity values, expressed as Hounsfield units, were plotted against time and the clearance rate constant ( $\Delta$ Hounsfield units/ $\Delta$ t) was calculated for each tumor. The data are expressed as the mean values  $\pm$  SD of six tumors for each tumor type. Representative images of control (**B**) and VEGF-C-overexpressing tumors of comparable size (**C**), obtained before (PRE) and after injection of the contrast agent (0 to 72 hours).

Flow cytometry analysis confirmed the expression of VEGFR-3 on cultured mouse peritoneal macrophages (Figure 5G). Importantly, VEGF-C stimulated macrophage chemotaxis *in vitro* in a dose-dependent manner (Figure 5H).

### Melanoma Cell Growth in Vivo and in Vitro

After orthotopic injection into immunodeficient mice, MeWo/control tumors reached a volume of  $\sim 1200 \text{ mm}^3$



**Figure 4.** Increased vascularization of MeWo/VEGF-C-overexpressing tumors. **A** and **B**: Immunohistochemical staining with an anti-mouse CD31 antibody reveals increased vascularization in VEGF-C-overexpressing xenotransplants (**B**), as compared to control tumors (**A**). Scale bars, 50  $\mu$ m. **C**: Markedly increased vascular densities in MeWo/VEGF-C-overexpressing tumors, expressed as number of vessels per mm<sup>2</sup> tumor area ( $P < 0.0002$ ). **D**: Significant increase of the relative area covered by vessels in VEGF-C-overexpressing tumors ( $P < 0.0001$ ). Data are expressed as mean values  $\pm$  SD of two individual clones per tumor type (five tumors each). **E**: Immunoprecipitation analysis of tumor lysates revealed increased amounts of VEGFR-2 (top panel) and enhanced VEGFR-2 phosphorylation levels (bottom panel) in VEGF-C-expressing MeWo tumors.

within 5 to 6 weeks after injection. Overexpression of VEGF-C did not increase the tumor growth rate, but rather resulted in growth reduction by 40 to 80% after 6 weeks, as compared to control tumors (Figure 6A). The extent of tumor growth suppression was correlated with the macrophage densities in the peritumoral areas. In the skin surrounding control tumors, macrophages occupied  $4.0 \pm 0.8\%$  of the total skin area examined. In contrast, tumors derived from MeWo/VEGF-C cl.29 cells that exhibited a growth suppression of up to 40% at 6 weeks showed an increase of peritumoral macrophage densities to  $12 \pm 1.5\%$ . Tumors derived from MeWo/VEGF-C cl.23 cells that exhibited the most prominent growth suppression (up to 80%), showed further increase in macrophage densities to  $23 \pm 1.9\%$ .

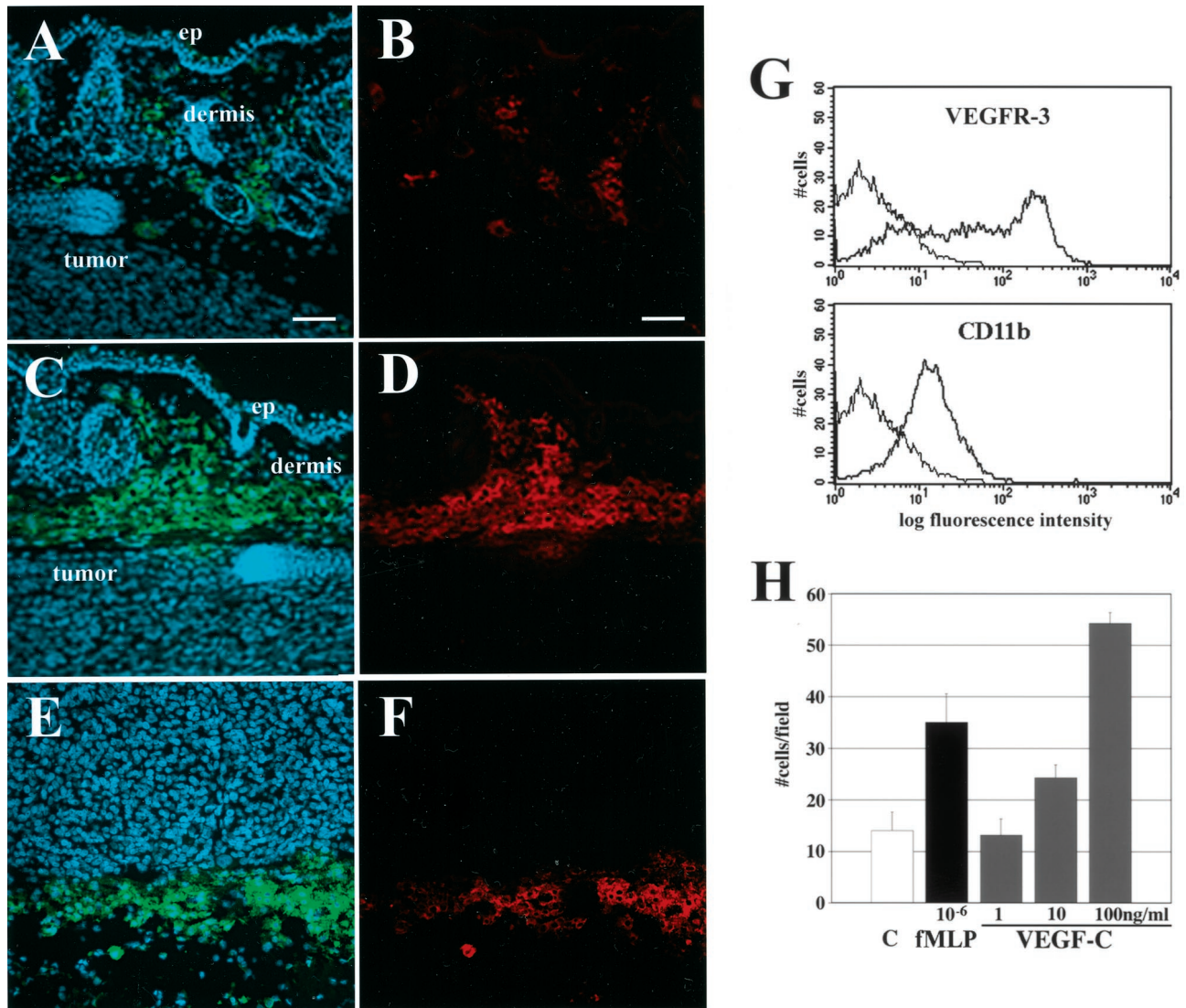
To determine whether VEGF-C expression directly affected tumor cell proliferation, MeWo transfectants were tested in several *in vitro* assays. As measured by BrdU incorporation, no significant differences in cell proliferation rates were detected between MeWo/control and MeWo/VEGF-C clones (Figure 6B). Moreover, the proliferation of control clones was not significantly altered on addition of 20 ng/ml recombinant human VEGF or VEGF-C to the culture medium (Figure 6C). Proliferation of control cells was also not altered by secretion of

VEGF-C by MeWo/VEGF-C cells into the culture medium in a co-culture experiment (Figure 6D). In accordance with these findings, MeWo transfectants did not express any detectable levels of VEGFR-2 or VEGFR-3, as determined by reverse transcriptase-polymerase chain reaction, Northern analysis, and *in situ* hybridization (data not shown). The rate of spontaneous apoptosis *in vitro* was comparable between control and VEGF-C clones ( $1.3 \pm 0.3\%$  versus  $1.1 \pm 0.2\%$ ). Taken together, these results demonstrate that VEGF-C did not exert direct effects on the growth of MeWo cells.

### Discussion

Whereas the significance of angiogenesis in tumors has been well documented, the ability of tumor cells to stimulate lymphangiogenesis and the existence of intratumoral lymphatic vessels have remained controversial.<sup>8,9,51</sup> Expression of the lymphangiogenic factor VEGF-C has been detected in many tumor types<sup>20-32</sup> including malignant melanomas,<sup>20</sup> and our results demonstrate expression of VEGF-C in several human melanoma cell lines *in vitro*. To directly investigate the biological role of VEGF-C in tumors, we studied the effects of





**Figure 5.** VEGF-C has chemotactic activity for macrophages. **A–D:** Double immunofluorescent staining for the macrophage marker CD11b (green, **A** and **C**) and mouse VEGFR-3 (red, **B** and **D**) in the skin overlying MeWo/control (**A** and **B**) and MeWo/VEGF-C (**C** and **D**) tumors, demonstrating expression of VEGFR-3 by macrophages. Note increased number of macrophages in the skin surrounding VEGF-C-overexpressing tumors. Macrophages surrounding MeWo/VEGF-C tumors also expressed another macrophage marker, F4/80 (**E**) (green), and VEGFR-3 (**F**) (red). Cell nuclei are counterstained blue with Hoechst in **A**, **C**, and **E**. Scale bars, 50  $\mu$ m. **G:** Flow cytometry analysis reveals expression of VEGFR-3 on mouse peritoneal macrophages. The x axis values are displayed on a logarithmic scale as arbitrary fluorescence units and the y axis values are on a linear scale and represent number of cells. **H:** VEGF-C dose-dependently stimulates migration of mouse peritoneal macrophages in a chemotaxis assay. The macrophage chemoattractant *N*-formyl-met-leu-phe (fMLP) was used as a positive control. Data are expressed as the mean values  $\pm$  SD for a representative experiment.

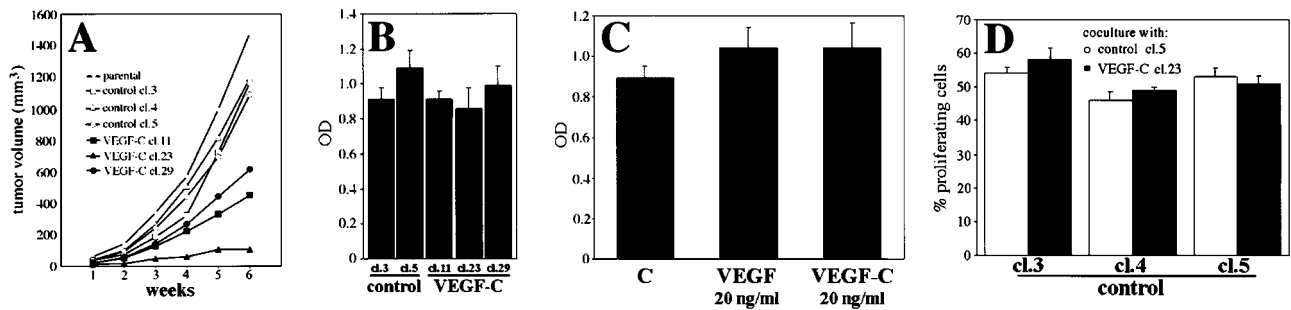
VEGF-C overexpression in an orthotopic nude mouse tumor model using the human melanoma cell line MeWo<sup>35</sup> stably transfected with VEGF-C.

Our results demonstrate infiltration of VEGF-C-overexpressing tumors with lymphatic vessels, even in the most central tumor areas. It has been suggested previously that lymphatic vessels are absent from tumors.<sup>8,52,53</sup> However, most evidence in support of this concept has been indirect and has been based on the absence of detectable perfusion of lymphatic vessels after injection of contrast agent into tumors.<sup>9,54,55</sup> Moreover, the direct visualization of lymphatic vessels in tissue sections has been problematic because of the lack of molecular markers that reliably distinguish the lymphatic from the blood vasculature.<sup>56</sup> By using a recently derived antibody to the

mouse hyaluronan receptor LYVE-1, that is selectively expressed on lymphatic vessels in normal tissues<sup>39,45</sup> and in tumors,<sup>33</sup> we were able to demonstrate intratumoral lymphatics in experimental melanomas overexpressing VEGF-C. All lymphatic vessels selectively expressed VEGFR-3. These results extend our recent findings that demonstrated, for the first time, the occurrence of intratumoral lymphangiogenesis in an orthotopic model of breast cancer.<sup>33</sup>

VEGF-C overexpression also resulted in enlargement of peritumoral lymphatic vessels, whereas intratumoral lymphatics did not appear to be enlarged and were found either with an open lumen or collapsed. Under normal circumstances lymphatic capillaries are partially or fully collapsed, because of the lack of smooth muscle cover-





**Figure 6.** Growth of VEGF-C-transfected MeWo cell clones *in vivo* and *in vitro*. **A:** Markedly decreased tumor growth of VEGF-C-transfected MeWo cells. Each point represents a mean value of 10 tumors. **B:** Control-transfected and VEGF-C-transfected cell clones exhibited similar proliferation rates *in vitro*, as determined by BrdU incorporation. **C:** Proliferation of control-transfected MeWo cells (clone 5) was not altered by addition of exogenous VEGF or VEGF-C (20 ng/ml), as determined by BrdU incorporation. **D:** MeWo/control cells showed comparable proliferation rates when co-cultured with MeWo/VEGF-C or MeWo/control cells, as determined by counting BrdU-labeled cells. Data are expressed as the mean values  $\pm$  SD for a representative experiment.

age and the low pressure within the lymphatic system.<sup>57-59</sup> Increased demand for fluid transport in tissue results in widening of their lumina; however, if overdistended, lymphatics become dysfunctional.<sup>60</sup> Hence, the functional state of lymphatic vessels cannot be deduced from their morphology, because an open lumen can indicate both vessels with dysfunction as well as normally functioning vessels with increased load. Functional studies of lymphatic vessels in mice have been extremely difficult to perform because of the low spatial resolution and the insufficient signal intensity imposed by most currently used methods. By using computed tomography and a recently developed lymphographic contrast agent with an increased concentration of iodine that results in image enhancement,<sup>41</sup> we have established a simple, minimally invasive procedure to measure the efficacy of lymphatic transport in mice. In comparison to control tumors, lymphatic transport from MeWo/VEGF-C tumors to regional lymph nodes was markedly retarded, indicating functional impairment of lymphatic vessels in terms of transport or uptake of fluid or functional overload because of an increase in VEGF-C-induced vascular permeability. The significance of delayed fluid clearance from tumors for the rate of tumor cell metastasis via the lymphatics remains to be established. At present, however, it is unclear how tumor cells are transported within lymphatic vessels, and the mechanisms of fluid and particle uptake into lymphatic vessels are most likely distinct from the mechanisms of tumor cell entry into the lymphatics.

VEGF-C has been reported to increase microvascular permeability in the Miles assay, with a fourfold to fivefold lower potency than VEGF.<sup>16</sup> VEGF-C was also 50- to 100-fold less potent than VEGF in inducing proliferation of bovine capillary endothelial cells,<sup>10,16</sup> human umbilical vein endothelial cells, or lung microvascular endothelial cells *in vitro*.<sup>11,19</sup> In contrast, VEGF-C promoted proliferation of human dermal microvascular endothelial cells with a similar potency as VEGF,<sup>17</sup> suggesting that microvascular endothelial cells derived from the skin blood vessels may be more responsive to VEGF-C than endothelial cells from other tissues. Moreover, VEGF-C acted synergistically with VEGF on *in vitro* angiogenesis,<sup>17,61</sup> suggesting that in VEGF-C-expressing melanomas VEGF-C may act in cooperation with VEGF to increase vascular permeability and angiogenesis. In fact, our re-

sults demonstrate that, in addition to its role in lymphangiogenesis, VEGF-C also acted as a tumor angiogenesis factor. We detected increased microvascular densities in VEGF-C-overexpressing melanomas by morphometric analyses of CD31-stained tumor sections. In accordance with these findings, computed tomography with an intravascular contrast agent revealed increased perfusion of MeWo/VEGF-C tumors (data not shown). This was not because of up-regulation of the major angiogenic factors VEGF, bFGF, and PDGF-B, because their expression remained comparable between VEGF-C-overexpressing and control tumors. The stimulatory effect of VEGF-C on tumor angiogenesis seemed to be mediated through VEGFR-2, because we observed marked induction of VEGFR-2 expression and phosphorylation in VEGF-C-overexpressing melanomas, as compared to control tumors.

VEGF-C was originally described as a specific growth factor for lymphatic vessels<sup>12,13</sup> but was later also found to induce angiogenesis of blood vessels,<sup>18,19</sup> raising a question about the mechanisms that determine the distinct effects of VEGF-C *in vivo*. As demonstrated *in vitro*, the secreted 31-kd VEGF-C protein predominantly activates VEGFR-3 whereas the mature, fully processed 21-kd form also activates VEGFR-2, suggesting that the biological functions of VEGF-C may be regulated by differential proteolytic processing.<sup>16</sup> The major VEGF-C form produced by MeWo/VEGF-C cells *in vitro* was the partially processed 31-kd form. In tumors however, the secreted VEGF-C was processed further to the mature 21-kd form, indicating an important role of tumor-host interactions for VEGF-C processing. As predicted by *in vitro* studies, the mature 21-kd form of VEGF-C stimulated both lymphangiogenesis and angiogenesis in VEGF-C-overexpressing melanomas. In contrast, overexpression of VEGF-C in breast cancer has been recently shown to selectively induce tumor lymphangiogenesis, but not tumor angiogenesis.<sup>33</sup> In the breast cancer model, however, the predominant VEGF-C form detected was the 31-kd form that selectively activated VEGFR-3 on the lymphatic vessels. Together, these results demonstrate that the biological effects of VEGF-C in tumors are critically dependent on the *in vivo* proteolytic processing and provide an explanation for the selective induction of lymphangiogenesis in certain physiological and pathological settings.

Lymphatic vessels, serving as a pathway for trafficking of antigen-presenting cells and macrophages, are important components of the immune system; therefore, activation of the lymphatic system by VEGF-C might have an impact on immune functions. In fact, we detected expression of VEGFR-3 on mouse macrophages *in vitro* and *in vivo* by flow cytometry and immunofluorescent analysis, and VEGF-C induced macrophage chemotaxis, indicating that VEGF-C can act as a direct immunomodulator. In agreement with our results, expression of VEGFR-3 has been reported in some hematopoietic and leukemia cells.<sup>62,63</sup> Furthermore, we observed increased densities of peritumoral macrophages in the skin surrounding VEGF-C-transfected melanomas. Enhanced host-tumor defense capabilities by increased recruitment of macrophages may explain the observed growth impediment of VEGF-C-overexpressing melanomas, because increased densities of peritumoral macrophages were correlated with the extent of tumor growth suppression. VEGF-C did not exert direct effects on tumor cells, because co-culture of VEGF-C-overexpressing cells with control cells did not result in altered proliferation of control cells, and addition of recombinant VEGF-C to control cells did not affect their growth rate *in vitro*. MeWo transfectants also did not express any detectable levels of VEGFR-2 or VEGFR-3, as determined by reverse transcriptase-polymerase chain reaction, Northern analysis, and *in situ* hybridization (data not shown). Our finding that VEGF-C did not increase the growth of melanomas is in agreement with a recent report showing that VEGF-C did not promote breast cancer growth, although it promoted tumor metastasis.<sup>33</sup>

Taken together, our results provide evidence that the proteolytic processing of VEGF-C *in vivo* is critical for determining its biological function in tumors. In addition to its role as a tumor lymphangiogenesis factor, our findings identify novel functions of the mature form of VEGF-C in melanoma, acting as a tumor angiogenesis factor and as an immunomodulator.

### Note Added in Proof

While this paper was being published, three other papers also reported increased tumor lymphangiogenesis in different VEGF-C or VEGF-D overexpressing mouse tumor models.<sup>64–66</sup>

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