ABSTRACT

Angiogenesis is a significant prognostic factor in melanoma, but the angiogenic factors controlling the neovascularization are not well defined. The purpose of this study was to investigate whether the angiogenesis and metastasis of melanoma are promoted by vascular endothelial growth factor (VEGF), interleukin 8 (IL-8), platelet-derived endothelial cell growth factor (PD-ECGF), and/or basic fibroblast growth factor (bFGF). Cells from human melanoma lines (A-07, D-12, R-18, and U-25) transplanted to BALB/c nu/nu mice were used as tumor models. Expression of angiogenic factors was studied by ELISA, Western blotting, and immunohistochemistry. Angiogenesis was assessed by using an intradermal angiogenesis assay. Lung colonization and spontaneous lung metastasis were determined after i.v. and intradermal inoculation of tumor cells, respectively. The specific roles of VEGF, IL-8, PD-ECGF, and bFGF in tumor angiogenesis, lung colonization, and spontaneous metastasis were assessed in mice treated with neutralizing antibody. The melanoma lines expressed multiple angiogenic factors, and each line showed a unique expression pattern. Multiple angiogenic factors promoted angiogenesis in the most angiogenic melanoma lines, whereas angiogenesis in the least angiogenic melanoma lines was possibly promoted solely by VEGF. Tumor growth, lung colonization, and spontaneous metastasis were controlled by the rate of angiogenesis and hence by the angiogenic factors promoting the angiogenesis. Lung colonization and spontaneous metastasis in A-07 were inhibited by treatment with neutralizing antibody against VEGF, IL-8, PD-ECGF, or bFGF. Each of these angiogenic factors may promote metastasis in melanoma, because inhibition of one of them could not be compensated for by the others. Our observations suggest that efficient antiangiogenic treatment of melanoma may require identification and blocking of common functional features of several angiogenic factors.

INTRODUCTION

Malignant melanoma is a highly metastatic tumor type of increasing incidence, poor prognosis, and high resistance to treatment (1–3). The progression of melanoma is characterized by distinct sequential steps, from common acquired melanocytic nevus via dysplastic nevus, radial growth phase primary melanoma, and vertical growth phase primary melanoma to melanoma metastasis (4, 5). There is increasing evidence that melanoma progression requires induction of new capillary blood vessels, a process called angiogenesis (6–12). Thus, the transition from the radial to the vertical growth phase, which represents a significant worsening of the prognosis, has been shown to be dependent on neovascularization (7–9). Moreover, the probability of metastasis has been found to increase with increasing microvessel density of the primary tumor (10–12).

Melanoma cells have been shown to produce and secrete a wide variety of angiogenic factors, including VEGF (13–16), IL-8 (17–19), PD-ECGF (20, 21), and bFGF (22–24). VEGF, also known as vascular permeability factor, is a strong specific mitogen for endothelial cells and may also stimulate endothelial cell migration and reorganization (25, 26). IL-8, which belongs to the superfamily of CXC chemokines, is a multifunctional cytokine that exhibits potent angiogenic activities both in vitro and in vivo (27, 28). PD-ECGF, also known as thymidine phosphorylase and giotistatin, stimulates endothelial cell mitogenesis and chemotaxis in vitro and is strongly angiogenic in vivo, possibly through modulation of nucleotide metabolism (29). bFGF, which belongs to the family of heparin-binding growth factors, is a multifunctional protein having a well-established key role in tumor angiogenesis (30–32).

There is significant experimental evidence that these angiogenic factors are involved in the growth and metastasis of malignant melanoma. Inoculation of human melanoma cells transfected with the gene encoding VEGF into immune-deficient mice results in tumors with increased vascularization (13–15), microvessel permeability (14, 15, 33), and volumetric growth rate (14, 33). The constitutive expression of IL-8 in human melanoma cells is correlated with their lung colonization efficiency after i.v. inoculation in immune-deficient mice (17). Human melanoma cells show increased lung colonization efficiency after transfection with the gene encoding VEGF (15) or IL-8 (18). Moreover, xenografted tumors initiated from human melanoma cell lines transfected with antisense-VEGF (14, 15) or antisense-bFGF (24) show reduced vascularization and growth.

A panel of angiogenic factors may thus be involved in the angiogenesis, growth, and metastasis of melanomas, and the panel may differ among tumors differing in angiogenic activity (34–36). However, angiogenic factors that show sufficiently high constitutive expression to promote spontaneous metastasis have not been identified conclusively thus far. The work reported here was aimed at investigating whether the angiogenesis and metastasis of melanoma may be promoted by VEGF, IL-8, PD-ECGF, and/or bFGF. Four human melanoma xenograft lines showing widely different constitutive expression of these angiogenic factors were included in the study. The specific role of each of the factors was investigated by assessing tumor angiogenesis and metastasis in mice treated with neutralizing antibody.

MATERIALS AND METHODS

Mice. Adult (8–10 weeks of age) female BALB/c nu/nu mice were used to assess tumor angiogenesis, lung colonization, and spontaneous metastasis. The mice were bred at our institute and maintained under specific pathogen-free conditions at constant temperature (24–26°C) and humidity (30–50%). Sterilized food and tap water were given ad libitum.

Cell Lines. Four human melanoma cell lines (A-07, D-12, R-18, and U-25) were included in the study (37). They were maintained in monolayer culture in RPMI 1640 (25 mM HEPES and L-glutamine) supplemented with 13% bovine calf serum, 250 mg/l penicillin, and 50 mg/l streptomycin. The cultures were
incubated at 37°C in a humidified atmosphere of 5% CO₂ in air and subcultured twice a week. The cell lines were verified to be free from Mycoplasma contamination.

ELISA. Commercial ELISA kits (Quantikine; R&D Systems, Abingdon, United Kingdom) were used according to the instructions of the manufacturer to measure VEGF, IL-8, and bFGF concentrations in culture medium. Medium samples from cultures in exponential growth were collected 24 h after change of medium. Cell numbers at the time of medium change and at the time of medium collection were determined by using a hemocytometer and a phase contrast microscope. The medium samples were centrifuged to remove particles, diluted to appropriate concentrations, and analyzed in duplicate at several dilutions. Absorbances were determined at 450 nm. Readings at 570 nm were subtracted from the readings at 450 nm to correct for optical imperfections. A standard curve was obtained by linear regression analysis of protein concentration versus absorbance in a double logarithmic plot. The rate of protein secretion ($R_{sec}$) was calculated as:

$$R_{sec} = \frac{V \Delta C}{\Delta t} \times \frac{\ln(N_f/N_i)}{(N_f/N_i - 1)}$$

where $\Delta C$ is the increase in protein concentration during the time interval $\Delta t$. $N_i$ and $N_f$ are the initial and final cell numbers, and $V$ is the volume of medium. Replica cultures were used to determine $N_i$. The second factor of the product is based on the assumption that the cell number increased exponentially with time during $\Delta t$, an assumption that was verified to be valid.

Western Blotting. Cells from cultures in exponential growth were washed in PBS and boiled in Laemmli lysis buffer for 5 min (38). Proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Membranes were incubated with antihuman VEGF rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), antihuman IL-8 mouse monoclonal antibody (R&D Systems), antihuman PD-ECGF goat polyclonal antibody (R&D Systems), or antihuman bFGF rabbit polyclonal antibody (Oncogene Science, Cambridge, MA) for 60 min. Bound antibody was detected by using a biotin-streptavidin alkaline phosphatase staining procedure. Recombinant human VEGF, IL-8, PD-ECGF, or bFGF (R&D Systems) was used as positive control. The specificity of the antibody-antigen interactions was confirmed by peptide competition studies and by incubation of membranes in solutions without primary antibody. Protein molecular weights were estimated by using broad range or high range prestained standards according to the instructions of the manufacturer (SDS-PAGE standards; Bio-Rad Laboratories, Hercules, CA).

Immunohistochemistry. Immunohistochemical staining of tumor tissue was performed by using an indirect immunoperoxidase method. Tumors were fixed in phosphate-buffered 4% paraformaldehyde or snap-frozen in liquid nitrogen. Antihuman VEGF rabbit polyclonal antibody (Santa Cruz Biotechnology), antihuman IL-8 mouse monoclonal antibody (R&D Systems), antihuman PD-ECGF goat polyclonal antibody (R&D Systems), or antihuman bFGF rabbit polyclonal antibody (Santa Cruz Biotechnology) was used as primary antibody. Controls included omission of the primary antibody, incubation with normal rabbit immunoglobulin or normal rabbit serum, and incubation with blocking peptides before staining. The sections were counterstained with hematoxylin.

Angiogenesis Assay. Tumor angiogenesis was assessed by using an intradermal angiogenesis assay (39). A 100-µl Hamilton syringe was used to inoculate aliquots of 10 µl of cell suspension into the flanks of mice. The inoculation point lay above the s.c. muscle tissue in the deeper part of the dermis. The number of cells per inoculum was 1.0 × 10⁶. The mice were killed on day 7 after the inoculation. Small vascularized tumors had developed in the inoculation sites at that time. The skin around the inoculation sites was surgically removed, and the tumors were located with a dissecting microscope. The medium samples were centrifuged to remove particulate matter, and the tumors were located with a dissecting microscope. The medium samples were centrifuged to remove particulate matter, and the tumors were located with a dissecting microscope.

Spontaneous Metastasis Assay. Aliquots of 3.5 × 10⁴ A-07 or D-12 cells suspended in 10 µl of HBSS were inoculated intradermally in the left flank of mice, using the same procedure as used in the angiogenesis assay described above. The inoculations were performed 24 h after the mice had been immunosuppressed by 5.0 Gy of whole-body irradiation. The whole-body irradiation was performed by using a Siemens Stabilipan X-ray unit, operated at 220 kV, 19–20 mA, and with 0.5-mm copper filtration (40). The mice were removed surgically when the largest diameter had attained ~10 mm, and the wounds were closed with surgical clips. The mice were examined for clinical signs of metastases twice a week. They were killed and autopsied 3 months after the primary tumor was removed or when they were moribund. The lungs were examined for the presence of macroscopic metastases as described above.

Treatment with Neutralizing Antibody in Vivo. The specific roles of VEGF, IL-8, PD-ECGF, and bFGF in tumor angiogenesis, lung colonization, and spontaneous metastasis were investigated by treating host mice with neutralizing antibody against these angiogenic factors. The antibodies used for treatment were antihuman VEGF mouse monoclonal antibody (R&D Systems), antihuman IL-8 mouse monoclonal antibody (R&D Systems), antihuman PD-ECGF goat polyclonal antibody (R&D Systems), and antihuman bFGF goat polyclonal antibody (R&D Systems). The antibody solutions were diluted in PBS and administered to the mice in volumes of 0.25 ml by i.p. injection. In the angiogenesis and lung colonization experiments, the treatments consisted of four doses of 25 µg (VEGF and bFGF) or 100 µg (IL-8 and PD-ECGF) of antibody given in intervals of 24 h. The first dose was given 1 h before the tumor cell inoculation. In the spontaneous metastasis experiments, the treatments consisted of eight doses of antibody given daily the last 8 days before the primary tumor was removed. The treatments with Neutralizing Antibody in Vivo. Possible cytotoxic or antiproliferative effects of the neutralizing antibodies described above were investigated in vitro. A-07, D-12, R-18, or U-25 cells were cultured in RPMI 1640 (25 mM HEPES and L-glutamine) supplemented with 1% bovine calf serum, 250 µg/ml penicillin, and 50 µg/ml streptomycin in the absence or presence of 5 µg/ml of antibody for up to 8 days. The number of cells in the cultures was determined 2, 4, 6, or 8 days after the cultures were initiated by counting cells in a hemocytometer.

Statistical Analysis. Results are presented as arithmetic mean ± SE. Statistical comparisons of data were performed by one-way ANOVA. When significant differences were found, the Dunnett’s method was used to identify the data sets that differed from the control data. Probability values of $P < 0.05$ were considered significant. All Ps were determined from two-sided tests. The statistical analysis was performed by using SigmaStat statistical software (Jandel Scientific GmbH, Erkrath, Germany).

RESULTS

Significant secretion of VEGF and IL-8 was seen in all cell lines, whereas A-07 was the only line that showed detectable secretion of bFGF (Table 1). A-07 showed higher VEGF secretion than U-25 ($P < 0.05$), which in turn showed higher VEGF secretion than D-12 and R-18 ($P < 0.05$). The IL-8 secretion was also higher in A-07 than in the other lines ($P < 0.05$). Moreover, D-12 showed higher IL-8 secretion than U-25 ($P < 0.05$), which in turn showed higher IL-8 secretion than R-18 ($P < 0.05$).

All cell lines showed significant expression of VEGF, IL-8, and bFGF, whereas A-07 was the only line that showed significant ex-

Table 1 Rate of secretion of angiogenesis factors of human melanoma cell lines

<table>
<thead>
<tr>
<th>Melanoma</th>
<th>VEGF</th>
<th>IL-8</th>
<th>bFGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-07</td>
<td>714 ± 140</td>
<td>571 ± 153</td>
<td>1.9 ± 0.8</td>
</tr>
<tr>
<td>D-12</td>
<td>18 ± 1</td>
<td>291 ± 86</td>
<td>0</td>
</tr>
<tr>
<td>R-18</td>
<td>16 ± 2</td>
<td>4 ± 1</td>
<td>0</td>
</tr>
<tr>
<td>U-25</td>
<td>85 ± 8</td>
<td>109 ± 27</td>
<td>0</td>
</tr>
</tbody>
</table>

*Mean ± SE of five independent experiments.
pression of PD-ECGF, as revealed by Western blot analysis (Fig. 1). A-07 showed substantially higher VEGF, IL-8, and bFGF expression than the other lines. Moreover, six specific bFGF bands were seen in A-07, whereas D-12, R-18, and U-25 showed only 2–4 specific bFGF bands.

A-07 tumors showed significant expression of VEGF, IL-8, PD-ECGF, and bFGF, as revealed by immunohistochemistry (Fig. 2). Also D-12, R-18, and U-25 stained positive for VEGF, IL-8, and bFGF in vivo, but the staining was weaker than that in A-07 tumors for all proteins. Significant expression of PD-ECGF could not be detected in D-12, R-18, or U-25 tumors.

Intradermal inoculation of A-07, D-12, R-18, or U-25 cells in mice evoked a strong angiogenic response (Fig. 3). The angiogenic response, measured as number of tumor-oriented capillaries per tumor at day 7 after the inoculation of $1.0 \times 10^6$ cells, differed substantially among the lines. A-07 evoked the strongest angiogenic response, followed by D-12, R-18, and U-25. The differences in number of tumor-oriented capillaries between A-07 and D-12, D-12 and R-18, and R-18 and U-25 were all statistically significant ($P < 0.05$). The angiogenic response evoked by A-07 was inhibited in mice treated with neutralizing antibody against VEGF, IL-8, PD-ECGF, or bFGF ($P < 0.05$). Treatment with neutralizing antibody against VEGF or IL-8 also reduced the angiogenic response evoked by D-12 ($P < 0.05$) but not treatment with neutralizing antibody against PD-ECGF or bFGF ($P > 0.05$). The angiogenic response evoked by R-18 and U-25 was inhibited in mice treated with neutralizing antibody against VEGF ($P < 0.05$) but not in mice treated with neutralizing antibody against IL-8, PD-ECGF, or bFGF ($P > 0.05$). Similar results and identical conclusions were achieved by expressing angiogenic response as number of tumor-oriented capillaries per mm of tumor circumference (data not shown).

i.v. inoculation of A-07 or D-12 cells in mice resulted in significant lung colonization (Fig. 4). The lung colonization efficiency of A-07 was reduced in mice treated with neutralizing antibody against VEGF, IL-8, PD-ECGF, or bFGF ($P < 0.05$). Treatment with neutralizing antibody against VEGF or IL-8 also reduced the angiogenic response evoked by D-12 ($P < 0.05$) but not treatment with neutralizing antibody against PD-ECGF or bFGF ($P > 0.05$). The angiogenic response evoked by R-18 and U-25 was inhibited in mice treated with neutralizing antibody against VEGF ($P < 0.05$) but not in mice treated with neutralizing antibody against IL-8, PD-ECGF, or bFGF ($P > 0.05$). Similar results and identical conclusions were achieved by expressing angiogenic response as number of tumor-oriented capillaries per mm of tumor circumference (data not shown).

DISCUSSION

A-07, D-12, R-18, and U-25 melanoma cells expressed multiple angiogenic factors in vitro, and each cell line showed a unique expression pattern. Thus, A-07 showed high expression and secretion of VEGF, IL-8, and bFGF and significant expression of PD-ECGF. D-12, R-18, and U-25 showed significant expression of VEGF, IL-8, and bFGF, but the expression of each of these factors was lower than that in A-07. D-12, R-18, and U-25 differed from A-07 also by showing no expression of PD-ECGF and no secretion of bFGF. D-12 differed from R-18 and U-25 by showing substantially higher secretion of IL-8. Finally, U-25 differed from R-18 by showing significantly higher secretion of VEGF and IL-8. These observations are consistent with previous observations suggesting that melanoma cell lines may differ substantially in the expression of angiogenic factors (34–36).

The differences among A-07, D-12, R-18, and U-25 cells in the expression of VEGF and IL-8 were more pronounced when the expression was measured by ELISA than when measured by Western blotting. Western blot analysis gives information on intracellular protein concentrations, whereas ELISA analysis provides information on the rate of protein secretion. There is no a priori reason to believe that the rate of protein secretion should be correlated to the intracellular concentration of protein. In fact, the present study suggests that the rate of protein secretion cannot be predicted from the intracellular protein concentration as determined by Western blot analysis. The rate of protein secretion is probably more relevant for the rate of tumor neovascularization than is the intracellular concentration of protein, because angiogenesis is induced by binding of protein to endothelial cell receptors.

A-07 cells, which evoked the strongest angiogenic response in vivo, showed substantially higher expression of bFGF than D-12, R-18, and U-25 cells. bFGF may promote tumor angiogenesis directly by acting synergistically with VEGF (42–44) and indirectly by up-regulating the synthesis and secretion of VEGF (45, 46).

The expression of VEGF, IL-8, PD-ECGF, and bFGF of A-07, D-12, R-18, and U-25 tumors, determined by immunohistochemical analysis, was in agreement with that of the corresponding cell lines in vitro, i.e., A-07 tumors showed substantially stronger staining for VEGF, IL-8, and bFGF than D-12, R-18, and U-25 tumors and were the only tumors that stained positive for PD-ECGF, consistent with the in vitro data in Table 1 and Fig. 1. This observation was not expected, because A-07, D-12, R-18, and U-25 tumors differ substantially in the fraction of hypoxic cells (47), and hypoxia may...
up-regulate the expression of VEGF (48, 49), IL-8 (19, 50), and PD-ECGF (51). The expression of VEGF has been shown to be up-regulated in A-07, D-12, R-18, and U-25 cells exposed to hypoxia in vitro (16, 52). However, hypoxia-induced up-regulation of VEGF, IL-8, or PD-ECGF was not detected in the immunohistochemical analysis. All tissue sections stained homogeneously for VEGF, IL-8, and PD-ECGF, i.e., the staining was not more intense in regions adjacent to necrosis than in regions close to functional capillaries, possibly because VEGF, IL-8, and PD-ECGF were secreted and diffused from hypoxic tissue into surrounding normoxic tissue. The staining patterns of VEGF, IL-8, and PD-ECGF differed substantially from the staining pattern of the hypoxia marker pimonidazole in A-07, D-12, R-18, and U-25 tumors (53).

The angiogenic factors expressed in vivo were produced primarily by the parenchymal melanoma cells and were thus of human origin. The immunohistochemical analysis showed that the angiogenic factors were mainly localized intracellularly in melanoma cells and not in stromal host cells. Moreover, host cells, i.e., macrophages, leukocytes, and fibroblasts, have been shown to constitute <1% of the total number of cells in A-07, D-12, R-18, and U-25 tumors (37). Our conclusion is also supported by the observation that the expression of angiogenic factors in vivo was similar to that in vitro.

The angiogenic response after intradermal cell inoculation differed significantly among the melanoma lines. The sequence of the lines from high to low angiogenic response, measured as number of tumor-oriented capillaries per tumor or number of tumor-oriented capillaries per mm of tumor circumference, was A-07 > D-12 > R-18 > U-25. The tumor growth rate has also been shown to differ significantly among the melanoma lines (39). The sequence of the lines from high to low volumetric growth rate is identical to that for angiogenic response, i.e., A-07 > D-12 > R-18 > U-25. The close association between volumetric growth rate and angiogenic response suggests that the growth of A-07, D-12, R-18, and U-25 tumors is governed primarily by the rate of angiogenesis.

The angiogenic factors promoting angiogenesis in A-07, D-12, R-18, and U-25 tumors, identified by measuring tumor-induced angiogenic response in mice treated with neutralizing antibody, differed among the lines. The angiogenesis of A-07 tumors, which showed the highest rate of angiogenesis, was promoted by VEGF, IL-8, and PD-ECGF, as well as bFGF. VEGF and IL-8, but probably not PD-ECGF or bFGF, promoted angiogenesis in D-12 tumors, which showed the second highest rate of angiogenesis. The angiogenesis of R-18 and U-25 tumors was promoted by VEGF but probably not by IL-8, PD-ECGF, or bFGF. All lines expressed bFGF, whereas A-07 was the only line secreting bFGF, suggesting that promotion of tumor angiogenesis by bFGF may require bFGF secretion. A-07 and D-12 showed significantly higher secretion of IL-8 than did R-18 and U-25, indicating that promotion of tumor angiogenesis by IL-8 may require...
high IL-8 concentrations. Our observations suggest that multiple angiogenic factors are involved in the angiogenesis of highly angiogenic melanomas, whereas the angiogenesis in poorly angiogenic melanomas may be promoted solely by VEGF. Angiogenic factors other than those studied here may also be involved in the angiogenesis of melanoma, including angiogenin (54) and angiopoietin-1 (55).

The angiogenic factors that were found to promote tumor angiogenesis were also found to be essential for tumor metastasis. Lung colonization and spontaneous metastasis in A-07 were inhibited by treatment with neutralizing antibody against VEGF, IL-8, PD-ECGF, or bFGF, as was A-07-induced angiogenesis. Treatment with neutralizing antibody against VEGF or IL-8 inhibited lung colonization and spontaneous metastasis as well as angiogenesis in D-12, whereas none of these biological phenomena was inhibited by treatment with neutralizing antibody against PD-ECGF or bFGF. These observations suggest that spontaneous lung metastasis in A-07 and D-12 tumors is governed by the angiogenic potential of the tumor cells and hence controlled by the angiogenic factors controlling the angiogenesis.

The metastatic process is composed of a cascade of linked, sequential, and highly selective steps involving multiple host-tumor interactions. These steps include invasion of tumor cells into blood vessels, survival in the blood circulation, arrest in the capillary bed of a secondary organ, extravasation into the secondary organ interstitium and parenchyma, and tumor cell proliferation and angiogenesis in the secondary organ. The metastatic propensity of a tumor may be influenced by the angiogenic potential of the tumor cells by two independent mechanisms: high microvessel density in the primary tumor may enhance the opportunity of tumor cells to gain access to the blood circulation; and elevated capacity to induce neovascularization may increase the probability of tumor cells trapped in secondary organ capillary beds to give rise to macroscopic tumor growth (56). The probability of spontaneous lung metastasis in A-07 and D-12 tumors was probably limited by the capacity of the tumor cells to induce
angiogenesis in the lungs, because spontaneous lung metastasis and lung colonization were inhibited by treatment with neutralizing antibody against the same angiogenic factors. However, it cannot be excluded that the probability of spontaneous lung metastasis was influenced by the angiogenic potential of the tumor cells also via the microvessel density of the primary tumor. Interestingly, the microvessel density is ~2.5-fold higher in A-07 tumors than in D-12 tumors (57).

The antibody treatments that inhibited lung colonization and spontaneous metastasis in A-07 and D-12 melanomas lasted 4 and 8 days, respectively. The reduced lung colonization and spontaneous metastasis in antibody-treated mice were most likely a consequence of inhibited angiogenesis rather than of cytotoxic or antiproliferative effects of the antibodies, because the growth of melanoma cells in vitro was not inhibited by antibody treatment. This interpretation is based on the assumption that neovascularization and macroscopic growth of lung colonies can be inhibited by treatment with neutralizing antibodies against angiogenic factors also when the antibody treatment is given to lung colonies consisting of less than ~50 cells, which is the maximum size of 4-day-old A-07 and D-12 lung colonies. This assumption is not consistent with the general current thinking that angiogenesis is initiated when the tumor mass reaches a diameter of ~1 mm. However, the validity of our assumption is strongly supported by recent observations by Li et al. (58) studying the initial phases of tumor cell-induced angiogenesis in skin window chambers. They observed modifications of the host vasculature when the tumor mass reached 60–80 cells and saw functional new blood vessels when the tumor mass reached 100–300 cells. Moreover, they found that ex-flk 1, a truncated soluble VEGF receptor protein known to be antiangiogenic and nontoxic, prevented angiogenesis and tumor growth in four of six window chambers when administered as a single dose at the same time as 40–50 tumor cells were inoculated into the window chambers. This observation led the authors to conclude that individual tumor cells are dependent on early angiogenic activities for both survival and proliferation in vivo (58).

Previous studies have shown that lung colonization of human melanoma cells in immune-deficient mice can be inhibited by treatment with neutralizing antibody against VEGF (52) or by treatment with agents blocking the receptor of IL-8 (59). The present study confirms these observations and shows further for the first time that treatment with neutralizing antibody against VEGF or IL-8 can inhibit spontaneous lung metastasis of human melanoma xenografts. It has been shown by others that treatment with neutralizing antibody against VEGF can inhibit spontaneous metastasis of human epidermoid carcinoma xenografts (60), human prostate carcinoma xenografts (61), and human colon and gastric carcinoma xenografts (62), and treatment with neutralizing antibody against IL-8 can inhibit spontaneous metastasis of non-small cell lung carcinoma xenografts (63). Moreover, the study reported here also shows for the first time that spontaneous lung metastasis of human melanoma xenografts can be inhibited by treatment with neutralizing antibody against PD-ECGF or bFGF.

The most important finding reported here is that the angiogenesis and spontaneous lung metastasis of A-07 melanoma xenografts can be inhibited by treatment with neutralizing antibody against VEGF, IL-8, PD-ECGF, or bFGF. Each of these angiogenic factors was probably essential for the angiogenesis and metastasis of A-07 tumors, because inhibition of one of them could not be compensated for by the others. This observation suggests that inhibition of a single angiogenic factor pathway may be beneficial in the treatment of malignant melanoma. However, it is unlikely that complete inhibition of tumor growth and metastasis of highly angiogenic melanomas would be possible by this strategy, because multiple angiogenic factors are involved. Efficient antiangiogenic treatment of malignant melanoma may require identification and blocking of common functional features of several angiogenic factors. Treatment strategies that target the common signal cascades in the end organ of angiogenesis, i.e., the endothelial cell, may be more universally effective than those targeting specific angiogenic factors or their receptors.

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Vascular Endothelial Growth Factor, Interleukin 8, Platelet-derived Endothelial Cell Growth Factor, and Basic Fibroblast Growth Factor Promote Angiogenesis and Metastasis in Human Melanoma Xenografts

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