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Analysis of the Tumor Vasculature and Metastatic Behavior of Xenografts of Human Melanoma Cell Lines Transfected with Vascular Permeability Factor

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Vascular permeability factor (VPF) is an important mediator of vascular development in tumors. Some human melanoma cell lines have a low VPF expression level in culture, but this level is upregulated when growing as a tumor in nude mice. Other melanoma lines have a constitutively high VPF expression. To compare the biological behavior of tumors with these two expression patterns, a human melanoma cell line with an inducible VPF expression was transfected with VPF expression constructs. In this way, several lines were obtained that constitutively produce either the soluble VPF_{121} or the matrix-associated VPF_{189} variant at levels of 4 to 30 times the VPF level in mature tumors derived from the parental line. The recombinant VPF RNA, which lacks most of the 5′ noncoding sequences present in the endogenous VPF mRNA, was much more efficiently translated than the endogenous messenger. Upon injection in nude mice, all VPF-transfected lines developed tumors with aberrations in vascularization and in distribution of matrix components. In these tumors the blood vessels were hyperpermeable for an i.v. injected protein tracer. Transfection did not influence the in vitro growth rate of the cell lines, but the tumors from the VPF-transfected lines had higher growth rates in vivo than tumors from the parental line or the vector-transfected line. Although the incidence of lung metastasis was similar in all lines, the number of metastases per affected lung was significantly increased in mice carrying VPF-transfectant tumors. We conclude that the pattern and the level of VPF expression in a tumor are important determinants of the architecture and functionality of the vascular bed, but that overexpression of VPF does not necessarily lead to an increase of microvascular density or metastatic spread. The role of VPF in melanoma progression is obviously complex and may be difficult to derive in its generality from a single experimental model. (Am J Pathol 1996, 148:1203–1217)

Angiogenesis, the formation of new blood vessels from existing vessels, is a process essential for tumor growth. Tumors without a vascular bed do not grow beyond a diameter of 1 to 2 mm^3.[1] In addition, tumor metastasis is dependent on angiogenesis, as blood vessels provide the most important escape route for disseminating tumor cells.[1–3] In recent studies a correlation between tumor vascularization and metastatic risk has indeed been demonstrated.[4–8] Outgrowth and malignant behavior of a tumor therefore is accompanied by rapid development of the tumor vasculature, and, perhaps because of this, tumor blood vessels often have a chaotic architecture besides other characteristics that are distinct from normal blood vessels (reviewed in refs. 9 and 10). The permeability of the tumor vasculature for

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macromolecules is often higher than in normal blood vessels,[9,10] although this permeability differs significantly between various tumor types.[11] Vascular permeability has also been shown to be lower in tumor-penetrating vessels than in a vascular plexus at the tumor-host interface.[12,13] Tumor blood vessels are often prone to intravascular coagulation, leading to deposition of fibrin and the occurrence of intravascular thrombi.[14]

The development of the tumor vascular bed is thought to be mediated by a number of tumor-derived angiogenic growth factors and cytokines (reviewed in refs. 2 and 15; see also refs. 16 and 17). Among these factors, vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) is gaining attention, as it has become clear that it is an essential factor in tumor angiogenesis and tumor growth.[18–20] The main targets of VPF are endothelial cells, which are almost the only cells expressing VPF receptors.[21–25] The capacity of VPF to induce angiogenesis is probably due to the induction of a variety of functions in the endothelial cell, including proteolytic activity, chemotaxis, and proliferation.[21,26–28] VPF may also be responsible for other characteristics of the tumor vasculature, such as vascular hyperpermeability[29,30] and intravascular coagulation (via the induction of tissue factor expression on endothelial cells[31]). By enhancing tumor vessel permeability, VPF may contribute indirectly to angiogenesis, as fibrinogen leaking from the vessels can form an extravascular fibrin matrix serving as a substrate for endothelial outgrowth.[32]

In a previous report we described that a human melanoma cell line, Mel57, expressed low VPF messenger and protein levels in culture, but contained elevated levels of VPF mRNA after injection into nude mice and the formation of a tumor.[33] Hypoxia was shown to be a likely mediator of this VPF upregulation. The tumors from this melanoma line had a characteristic vascular pattern, with blood vessels penetrating the tumor parenchyma in an apparently random fashion. This melanoma line was engineered into a line constitutively expressing VPF by transfection. Xenografted to nude mice, this line developed into tumors with a vascular pattern distinct from parental line tumors; these tumors consisted of several tumor cell nodules devoid of blood vessels and separated by robust stromal septa very rich in blood vessels.[33]

In the present study we have extended these findings by generating a panel of VPF-transfected melanoma lines expressing different molecular variants of VPF at different levels. The recombinant VPF RNA, lacking most of the 5′ noncoding sequences of the *VPF* gene, was translated into protein much more efficiently than the endogenous VPF messenger. We show that the altered VPF expression pattern increased the permeability of the tumor blood vessels for macromolecules, the growth rate of the tumors *in vivo*, and the metastatic burden in the lungs.

Materials and Methods

Cell Culture

The human melanoma cell line Mel57 was cultured as previously described.[34] Transfected Mel57 cells were cultured in the same medium supplemented with 200 μg/ml hygromycin B (Boehringer Mannheim, Mannheim, Germany).

In Vitro *Growth Assay*

The growth of the Mel57 melanoma line and the transfectant lines was monitored using a non-radioactive colorimetric assay based on a tetrazolium salt reaction as described originally by Mosman.[35] On day 0, parental and transfected melanoma cells were seeded in 96-well tissue culture plates at a concentration of 500 cells/well in 100 μl culture medium and allowed to adhere overnight. On day 1, the colorimetric signal was determined in an enzyme-linked immunosorbent assay (ELISA) reader to compare the activity of the different cell types. In a number of wells, different amounts of recombinant human VPF/VEGF (Peprotech/Sanvertech, Breda, The Netherlands) were added on day 3. Medium was exchanged for fresh medium without additional VPF on days 5 and 8. Colorimetric activity was followed until day 11. In the experiments in which VPF was added, the results are expressed as the mean value ± SD of four different readings. For the determination of the *in vitro* growth curves, the mean value ± SD of 24 different measurements was calculated. For statistical analysis a Student's *t*-test for unpaired values was used.

Production of Stable Transfectants

The protein coding regions of VPF_{121} and VPF_{189} were obtained by reverse transcription polymerase chain reaction on RNA from cell line U937 and were subsequently cloned into vector pUC19, as described elsewhere.[33] After sequence analysis, the inserts were recloned between the *Xba*I and *Kpn*I sites of the plasmid EBOpLPP.[36] Plasmids with VPF_{121} and VPF_{189} inserts, as well as the empty vector, were linearized with *Apa*I, and the 7 to 8 kb

fragments were isolated from low melting point agarose gel slices and were transfected into Mel57 cells by calcium phosphate precipitation.[37] Transfected clones were selected in medium with 200 μg/ml hygromycin B, removed by scraping, and expanded until analysis of the clones and storage of stocks in liquid nitrogen was possible (see also ref. 33).

Analysis of VPF Expression on the RNA and Protein Level

RNA from cell lines was isolated using guanidinium chloride according to Peek et al,[37] and RNA from tumor xenografts was isolated by disruption in guanidinium isothiocyanate and CsCl centrifugation, as described in Sambrook et al.[38] Northern analysis and hybridizations with VPF and ubiquitin probes were performed as described earlier.[33] To monitor VPF protein secretion, cells were cultured for 24 hours in serum-free medium lacking hygromycin, containing 100 μg/ml heparin. Conditioned media were cleared by centrifugation and used for VPF protein analysis. To correct for differences in cellular density, cells were scraped and lysed, and the cellular protein content was determined with a standard protein assay (Biorad, Veenendaal, The Netherlands). Based on this protein determination, samples of conditioned media derived from equal amounts of cell material were taken and tested in a procoagulant assay.[31,39] Similarly, samples were concentrated fivefold, run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by Western blotting and staining with a polyclonal antiserum raised against *Escherichia coli*-produced VPF, as described elsewhere.[40] Staining was visualized by chemiluminescence on X-ray film and quantitated by densitometry. Levels of human VPF protein in culture supernatant were measured in an ELISA (R&D Systems, Inc., Minneapolis, MN), according to the manufacturer's specifications.

Xenografts from Line Mel57 and Transfectants in Nude Mice

About 2.5 × 10^6 cells were injected s.c. into BALB/c *nu/nu* mice as described in ref. 34. Every week tumor volumes were estimated. Tumors of between 300 and 1200 mm^3 were harvested and cut into three fragments. One part was formalin-fixed, stained with hematoxylin and eosin (H&E), and used to study overall tumor morphology and to assess the percentage of necrosis. The other parts were snap-frozen in liquid nitrogen; one was used for RNA isolations, the other to study the vascular patterns, the organization of extracellular matrix components, and the spatial distribution of VPF within the tumors (see below). To study vascular morphology in the early stages of tumor development, for some lines also smaller tumors were taken (within 1 week after the occurrence of a palpable tumor), snap-frozen, and stained immunohistochemically. Acetone-fixed 4 μm cryosections were stained with a rat monoclonal antibody (Ab), MEC 7.46, directed against mouse vascular endothelium (a gift from Dr. A. Vecchi, Milano, Italy[41]), a rabbit polyclonal Ab against mouse laminin (a gift from Dr. J. van den Born, Department of Nephrology, University Hospital Nijmegen), a goat polyclonal Ab against (human) heparan-sulfate proteoglycan (HSPG, a gift from Dr. L. van den Heuvel, Department of Pediatrics, University Hospital Nijmegen), or with a rabbit polyclonal Ab against human VPF/VEGF (Santa Cruz Biotechnology, Santa Cruz, CA; Sanvertech, Breda, The Netherlands). For visualization of the immune reactions, alkaline phosphatase-conjugated secondary Ab were used, except for the anti-HSPG reaction, which was visualized using a peroxidase-conjugated donkey anti-goat Ab. All sections were counterstained with hematoxylin.

For the analysis of tumor vessel permeability, mice bearing tumors with sizes between 100 and 700 mm^3 were i.v. injected with 0.1 ml of a 3% solution of fluorescein isothiocyanate-bovine serum albumin (FITC-BSA) (11 mol FITC/mol BSA; Sigma, Brunschwig, Amsterdam, The Netherlands). Tumors were excised 2, 10, or 45 minutes after the injection of tracer, and snap-frozen in liquid nitrogen. Cryosections of these tumors, 4 μm thick, were immediately fixed in 2% formaldehyde solution for 10 minutes. The distribution of the tracer was shown by fluorescence microscopy. The deposition of extravasated fibrin was analyzed in parallel acetone-fixed tumor sections with a goat polyclonal Ab against mouse fibrinogen/fibrin (Nordic Immunological Laboratories, Tilburg, The Netherlands), using a peroxidase-conjugated donkey anti-goat secondary Ab. Other parallel sections of these tumors were stained for endothelium or HSPG (see above).

Metastasis of Transfectant Xenografts

To study the growth rate of transfected xenografts, and to estimate the frequency of metastasis and the number of metastases per mouse, two VPF-transfected lines (I-3 and III-8), and one vector-trans-

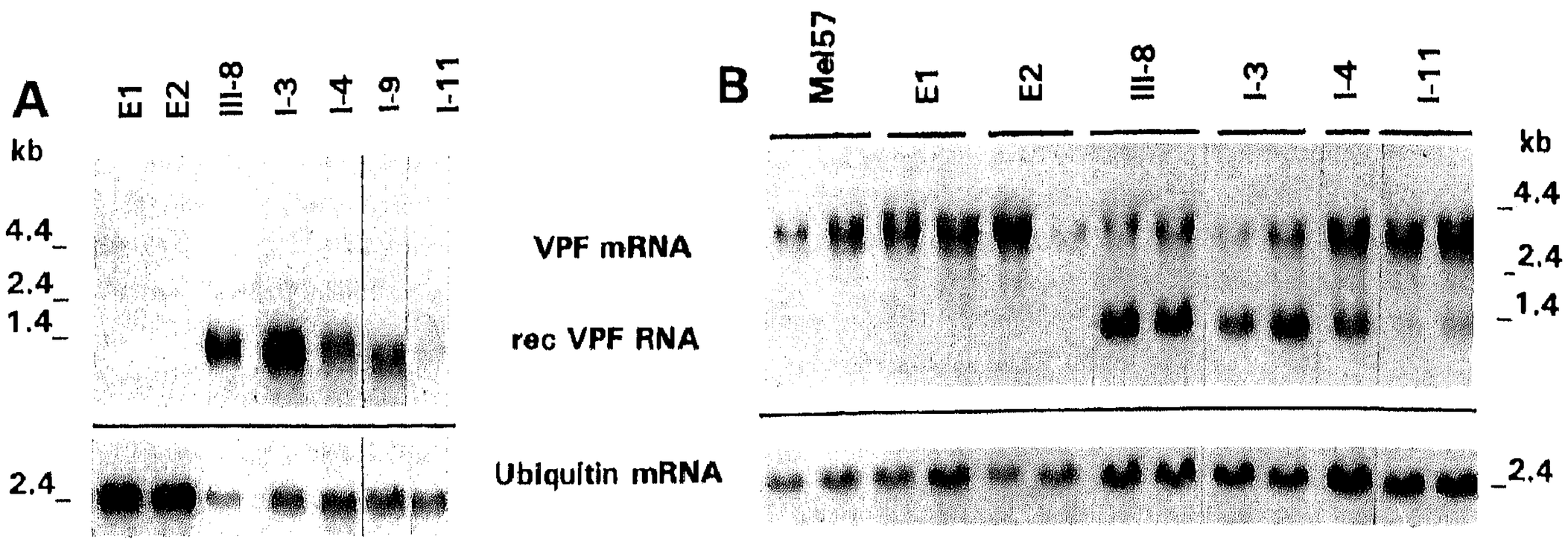


Figure 1. *Expression of endogenous and recombinant VPF RNA by melanoma cell transfectants. Northern blots containing RNA from cell lines* (A) *or xenografts in nude mice* (B) *were hybridized with a VPF probe* (upper portions) *and with a ubiquitin probe* (lower portions). *Endogenous VPF mRNA is visible as multiple bands around 3.7 kb in tumor RNA only, and recombinant VPF RNA is apparent in VPF-transfected lines and their tumors as a band of about 1.0 kb. Mel57: parental melanoma line; E1, E2: lines transfected with vector DNA only; III-8: line transfected with VPF_{189} cDNA; I-3, I-4, I-9, I-11: lines transfected with VPF_{121} cDNA. Positions of molecular mass standards are indicated on both sides.*

fected line (E2) were injected s.c. in 20 mice per group (4×10^6 cells/mouse). This regimen yielded a take rate of 100%. The tumor volume was monitored weekly and calculated as the product of three perpendicular measurements. Growth rates were plotted as the mean of 20 measurements ± SD. Mice were sacrificed after 7 weeks, unless overt illness developed or the tumor grew excessively. In these cases (n = 11) mice were sacrificed at earlier time points. Mel57 tumor growth was determined by measurements in a parallel group of 50 mice, where the mice were sacrificed at different time points after tumor take. In this group, tumor volume was determined at the time of tumor excision and expressed as the mean value of measurements in 4 to 10 mice. The transfectant tumors were excised, weighed, and divided in three parts for storage in liquid nitrogen and formalin (see above). Through the trachea, the lungs were filled with 1:4 diluted mounting solution, taken out, and divided in three parts. Two parts were formalin-fixed, and cross sections were H&E-stained and analyzed for the occurrence of metastases as described in ref. 34. Scoring was performed by three independent observers. The number of metastases found in two cross sections of both lungs was determined and the metastatic burden calculated as the mean number of metastases per cross section. The metastatic frequency was defined as the number of mice having one or more lung metastases divided by the number of mice in the group. For statistical analysis a Student's *t*-test for unpaired values was used.

Results

Selection of Melanoma Cell Transfectants

Mel57 is a melanoma line with a low level of VPF expression in culture, but an elevated level when growing as a tumor in nude mice. A transfectant of this line, producing VPF_{121} constitutively, had quite a different vascular pattern in mouse xenografts compared with the parental line tumors.[34] To determine whether the level of recombinant VPF expression, or the molecular variant expressed, is of influence on

Table 1. *Relative Levels of Recombinant (rec) VPF RNA and Endogenous (end) VPF mRNA in (Transfected) Melanoma Lines* in vivo, *and Predicted Expression Levels of VPF Protein* in vivo.

Line	rec VPF RNA *in vivo*	end VPF mRNA *in vivo*	predicted VPF expression level
Mel57		1 (0.5–1.2)	1
E1		0.7 (0.6–0.8)	0.7
E2		2.1 (0.7–3.8)	2.1
I-11	0.19 (0.15–0.25)	1.1 (0.9–1.2)	4–9
I-4	0.27 (0.17–0.38)	0.58 (0.5–0.7)	5–11
I-3	0.82 (0.53–0.97)	0.47 (0.2–0.6)	14–33
III-8	0.78 (0.74–0.80)	0.44 (0.3–0.5)	13–32

VPF band intensities after Northern hybridizations were quantified by densitometry. Values were corrected for the amount loaded by dividing the VPF intensities by the ubiquitin intensities. The average value for VPF mRNA calculated for Mel57 tumors was defined as 1. The ranges of values obtained with different tumors from one line are given in parentheses. Estimates of VPF protein expression levels in the tumors were made by considering the contribution from endogenous VPF mRNA and from recombinant VPF RNA in the tumors, assuming a 16- to 40-fold higher translation efficiency of the latter RNA. The predicted VPF protein expression level in Mel57 tumors was defined as 1.

the arrangement and the functionality of the tumor vasculature, we selected several other VPF_{121}- and VPF_{189}-transfected melanoma lines.

The expression levels of the transfectants were determined by Northern blotting. In RNA from the VPF-transfected cell lines a band of approximately 1.0 kb was seen upon hybridization with a VPF probe, representing the recombinant VPF RNA (see Figure 1A). The highest levels of recombinant VPF RNA were produced by lines I-3 (expressing VPF_{121}, described before in ref. 33) and III-8 (expressing VPF_{189}). Lines I-4, I-9, and I-11 contained lower but still detectable levels of VPF_{121}-encoding recombinant VPF RNA (see also Table 1). The major endogenous VPF transcript is 3.7 kb in length, but this was undetectable in RNA from cell line Mel57 or its derivatives. Southern blots of genomic DNA from transfected lines, digested with *Hin*dIII, showed multiple bands upon hybridization with a VPF probe, in addition to the bands deriving from the endogenous *VPF* gene (not shown), indicating that multiple copies of the transfected construct had been integrated.

To confirm that the recombinant VPF RNA levels found in the lines described above correlate with the amounts of active VPF protein secreted, conditioned serum-free media of these lines were harvested. The supernatants were Western blotted and stained with a polyclonal antiserum against VPF. In conditioned media of the parental line Mel57 and the vector-transfected lines E1 and E2, no VPF protein could be detected. Lines I-3 and I-4, expressing the RNA encoding recombinant VPF_{121}, secreted detectable amounts of 16 and 20 kd proteins (Figure 2), being the expected molecular masses of the nonglycosylated and glycosylated forms of VPF_{121}.[42] Line III-8, expressing the RNA for recombinant VPF_{189}, secreted the expected VPF proteins of a molecular mass of 28 and 31 kd (Figure 2) but these proteins needed heparin to be released from extracellular

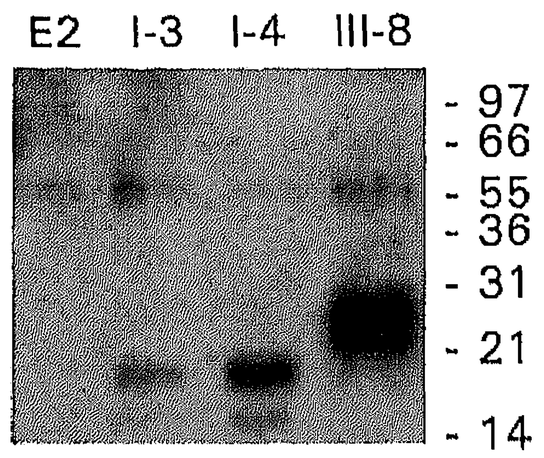


Figure 2. *VPF protein expression by Mel57 transfectants. Proteins were concentrated fivefold from 10 ml aliquots of serum-free conditioned media of melanoma cell transfectants, electrophoresed under reducing conditions, electroblotted, and stained with a polyclonal antiserum against human VPF. E2: line transfected with vector DNA only; I-3, I-4: lines transfected with VPF_{121} DNA; III-8: line transfected with VPF_{189} cDNA. Conditioned media contained 100 μg/ml heparin. Positions of molecular mass standards are indicated at the right.*

matrix.[42,43] The relative amounts of glycosylated and nonglycosylated protein were determined by densitometry of the Western blots. The absolute levels of VPF protein were determined by ELISA (Table 2). VPF_{121} appeared predominantly in the glycosylated form. A significant amount of VPF_{189} proved to be nonglycosylated (Figure 2), although the intensity of the VPF_{189} signal might have exceeded the maximal level. The ELISA analysis showed that the VPF-transfected lines I-3 and I-4 secreted 30 to 50 times more VPF than the nontransfected lines BLM and MV3. A trace of VPF was found in the Mel57 supernatant. Unfortunately, the ELISA appeared to be insensitive for VPF_{189}; therefore, the VPF levels of line III-8 could not be measured. The reason for this insensitivity is unclear. Nonetheless, it could be derived from the densitometry results that line III-8 produced VPF at a much higher level than I-3 or I-4, probably exceeding a final concentration of 1 μg/ml under the test conditions. The biological activity of the recombinant VPF was tested in a procoagulant assay, measuring the capacity to induce tissue factor expression on endothelial cells. No significant activity was present in the conditioned media of Mel57 and control trans-

Table 2. *Quantification of VPF Protein Levels in Conditioned Culture Medium*

Line	Type of VPF expressed	% glycosylation[1]	VPF concentration[1] (ng/ml supernatant)
Mel 57			0.25
E2			0.0
I-3	VPF_{121}	80	320
I-4	VPF_{121}	89	380
III-8	VPF_{189}	59	Not detectable[2]
BLM	VPF_{121} and VPF_{189}		9.6
MV3	VPF_{121} and VPF_{189}		7.2

[*] 10 ml of serum free culture medium containing 100 μg/ml heparin was conditioned on 2×10^6 cells in a 150 cm^2 flask for 24 hours.

[1] VPF protein was visualized by chemiluminescence on Western blot. VPF band intensity after autoradiography was measured on a densitometer. The figures represent the density of the band of glycosylated VPF expressed as a percentage of total VPF signal in that lane.

[2] VPF protein levels were measured in an ELISA.

[3] VPF_{189} could not be detected in ELISA.

fectants, but the lines that produced recombinant VPF were all positive in this assay (not shown). The highest tissue factor level was induced by conditioned medium from line I-3. Although no VPF_{189} was detectable in the heparin-free conditioned medium of line III-8 by Western blotting (not shown), sufficient VPF activity was present in this supernatant to cause a significant increase in the tissue factor level.

The Efficiency of Translation of Recombinant VPF RNA

The endogenous VPF mRNA has a 1 kb 5′ noncoding sequence in which a very GC-rich region and several alternative translational start sites are present.[44] These features may inhibit the efficiency of translation of the VPF mRNA. The recombinant VPF RNA lacks these sequences; only a stretch of 21 bases of the 5′ noncoding sequence of the *VPF* gene is contained in the recombinant RNA. The efficiency of translation of the recombinant VPF RNA in Mel57 transfectants was compared with that of the endogenous VPF messenger in melanoma line BLM, which has a constitutively high VPF expression.[33]

In transfectant cell line I-9, no endogenous VPF mRNA was detectable; its level of recombinant VPF_{121}-encoding RNA (1 kb) was about the same (in a molar ratio) as the level of endogenous VPF mRNA in melanoma line BLM (3.7 kb, Figure 3A). The VPF mRNA in line BLM was previously shown to encode predominantly VPF_{121} along with some VPF_{165}.[33] The VPF protein secreted by cell line BLM was barely detectable on Western blots (bands representing VPF_{121} and VPF_{165} can be seen between 16 and 24 kd), whereas the VPF_{121} bands produced by line I-9 are clearly visible (Figure 3B). Scanning of bands on autoradiographs from several Northern and Western blots revealed that VPF is 16- to 40-fold more efficiently produced from recombinant VPF RNA in line I-9 than from the endogenous VPF messenger in line BLM. In estimating the expression levels of recombinant VPF protein in tumors, we have taken this higher efficiency of translation into account (see below, and Table 1).

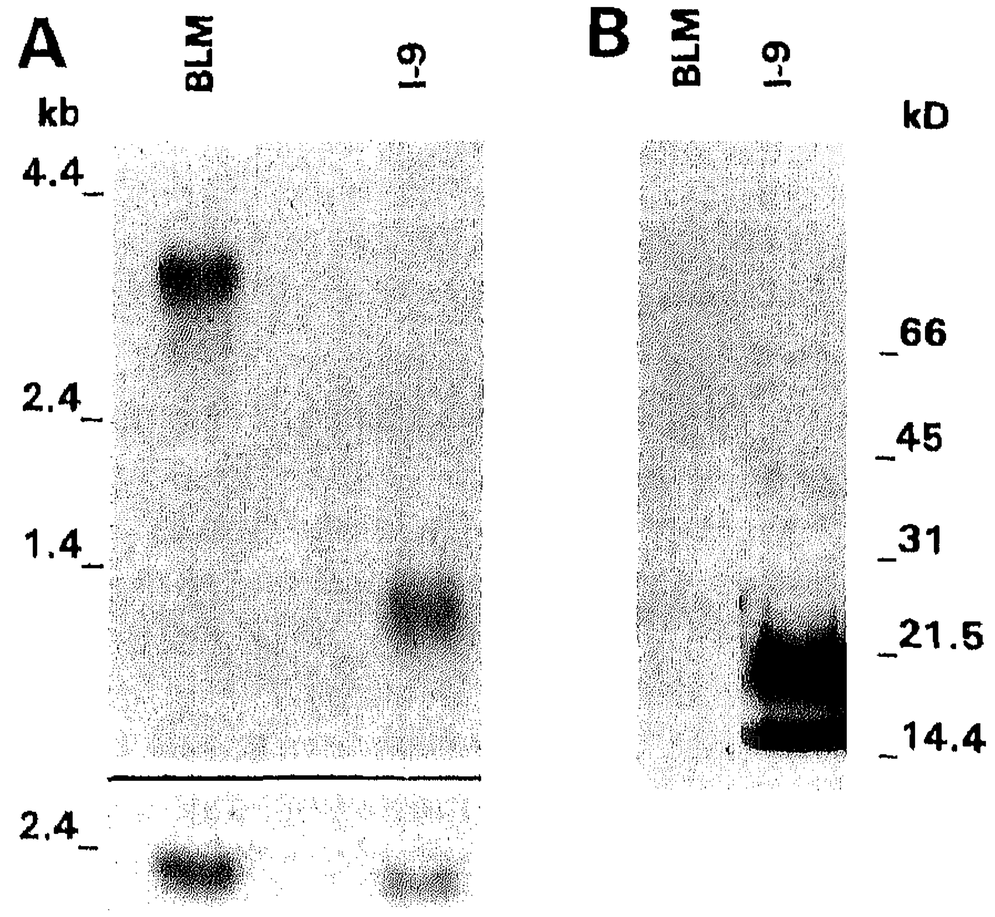


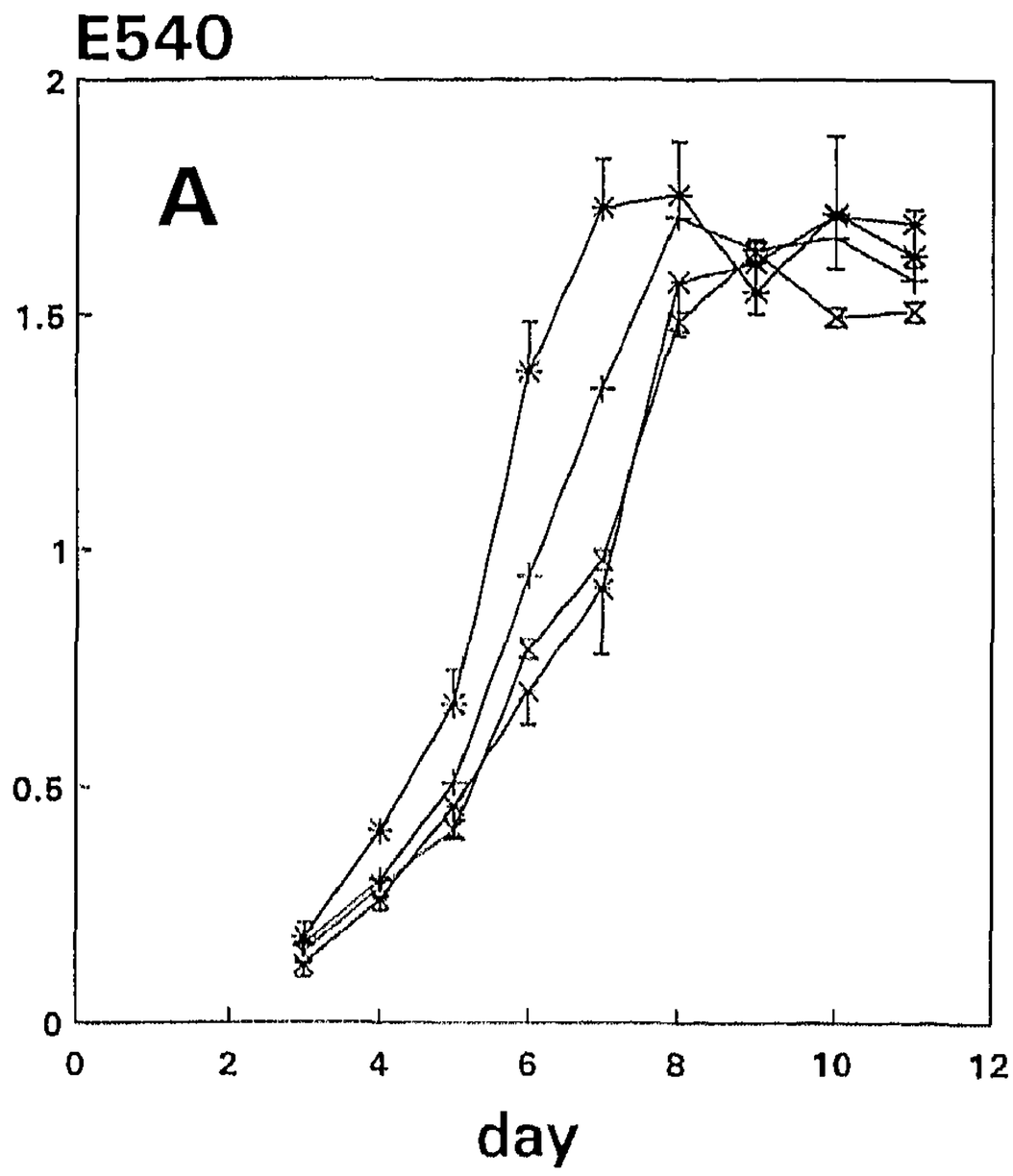
Figure 3. *Analysis of the relative translation efficiency of recombinant VPF RNA.* (**A**) *Northern blot with RNA from melanoma cell line BLM and from* VPF_{121}*-transfected cell line I-9, hybridized with a VPF probe* (upper panel) *and a ubiquitin probe* (lower panel). *Length markers are indicated on the left.* (**B**) *Serum-free conditioned media containing 100 μg/ml heparin from melanoma cell line BLM and transfectant cell line I-9, were electrophoresed on SDS-PAGE under reducing conditions, electroblotted, and stained with polyclonal antiserum against VPF (blocked before use with three volumes of heat-denatured fetal calf serum (FCS) to reduce nonspecific background). Loaded were 285 μl of line I-9-conditioned medium (as in Figure 2) and 500 μl of BLM-conditioned medium (derived from 3 times more cells than the sample from line I-9). Molecular mass standards are indicated on the right.* VPF_{121} *bands are visible at 16 and 20 kd; cell line BLM produces VPF variants of 121 and 165 amino acids, which are faintly visible as bands between 24 and 16 kd.*

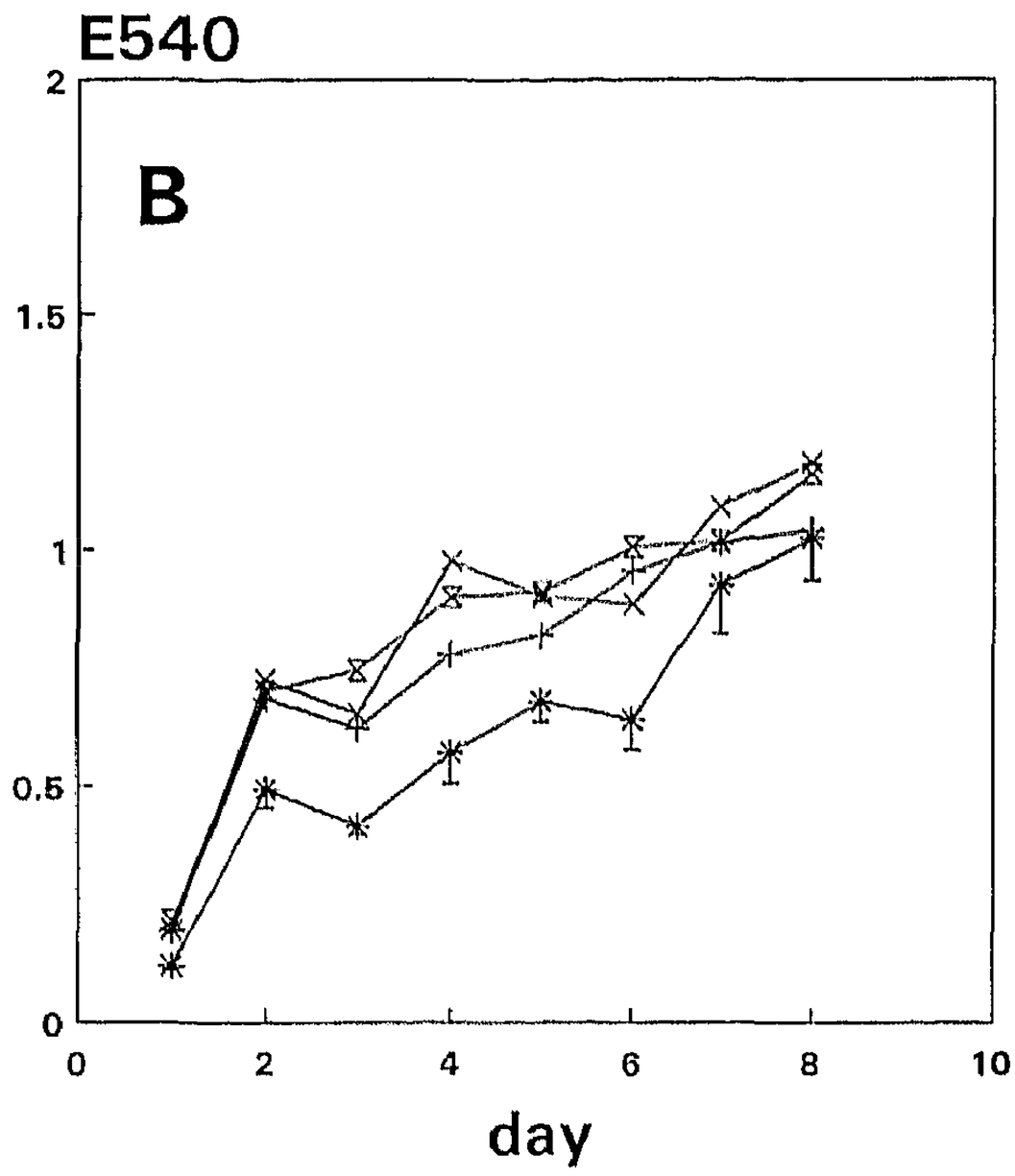
Expression of Recombinant VPF in Xenografts

Transfected melanoma lines positive for recombinant VPF expression, as well as the parental line Mel57 and vector-transfected lines E1 and E2, were injected into nude mice, and from the resulting tumors RNA was isolated. Northern blotting of these RNAs showed that recombinant VPF RNA was present in tumors from the VPF_{121}-producing lines I-3, I-4, and I-11, and from the VPF_{189}-producing line III-8 (Figure 1, B). In these tumors, as well as in the tumors from vector-transfected lines E1 and E2, the vector-encoded RNA for hygromycin phosphotransferase was also still expressed (not shown). Line I-9 produced only one small tumor after the first series of four injections, and was not included in further experiments.

In all xenografts, the levels of endogenous VPF mRNA were increased, as expected.[33] However, in tumors from the lines expressing the highest levels of recombinant VPF RNA (I-3 and III-8), the levels of endogenous VPF mRNA were about half of the level in line Mel57 tumors. This suggests that VPF might, possibly through inducing angiogenesis and preventing hypoxia, inhibit the expression of its own gene *in vivo*. The relative levels of recombinant VPF RNA and endogenous VPF mRNA in the various transfected cell lines *in vivo* are summarized in Table 1.

Extrapolation of the higher efficiency of VPF production from the recombinant VPF RNA than from the endogenous messenger (see above) to the *in vivo* situation suggests that the levels of recombinant VPF protein expressed by the VPF-transfected tumors greatly exceed the VPF levels expressed by the pa-





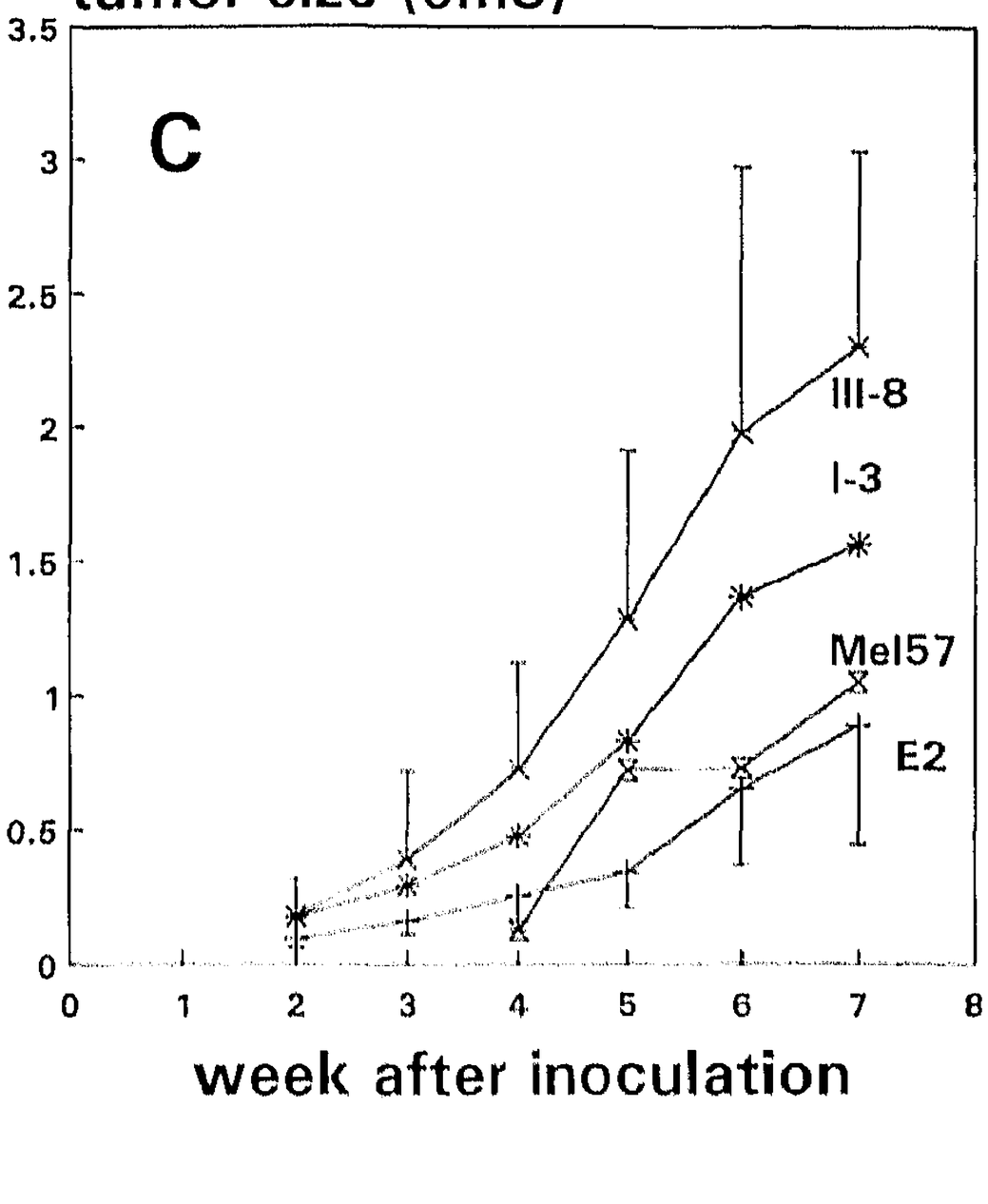


Figure 4. *Growth characteristics of the VPF-transfected melanoma cell lines III-8 and I-3, the vector-transfected line E2, and the parental line Mel57.* (A) In vitro *growth curves of the different melanoma lines at a serum concentration of 10% FCS, determined in a colorimetric assay.* (B) In vitro *growth at a serum concentration of 2% FCS.* (C) In vivo *growth curves of the different melanoma lines. In* C *the symbols used in both panels are identified. Bars indicate half the SD range. From several curves the error bars have been omitted for clarity, but are of comparable dimensions.*

rental line tumors. The level of recombinant VPF RNA in line I-3 *in vivo* is only 1.8-fold its endogenous messenger level. The amount of recombinant VPF protein produced would be, however, 13- to 33-fold the amount of VPF produced by Mel57 tumors. Tumors from the lower expressing lines I-4 and I-11, are still predicted to express 4- to 10-fold the VPF levels of Mel57 tumors (Table 1).

Growth of Transfected Melanoma Cells and Melanoma Xenografts

The *in vitro* growth curves of melanoma line Mel57, of the vector-transfected line E2, and the transfectant lines I-3 and III-8 were determined in a colorimetric assay. All melanoma lines had growth rates comparable to the parental line with slightly higher growth

rates for I-3 and E2 at a serum concentration of 10% (Figure 4a), but a slightly lower rate for I-3 at 2% serum (Figure 4b). VPF expression did not influence growth *in vitro* in an autocrine way, since addition of exogenous recombinant VPF protein did not alter the growth rate of the Mel57 line (data not shown). Moreover, hybridization of Northern blots using probes for murine *flt* and *flk*-1 showed that transcripts coding for these VPF receptors were not detectable in the cultured melanoma cell lines (data not shown), suggesting that functional VPF receptors were not present. There was no overall relation between the growth rates *in vitro* and tumor growth *in vivo*. Tumors from line III-8 had a significantly higher growth rate *in vivo* than E2 or Mel57 line tumors, both of which lines had identical growth curves (Figure 4c). Line I-3 tumors had an intermediate growth rate.

The percentages of tumor necrosis were determined from formalin-fixed and H&E-stained sections. No significant differences between the lines were observed (data not shown). The patterns of necrosis were rather variable; all lines produced some tumors with extensive necrosis (up to 60 to 80% of cross sections), while most lines (I-11, I-4, III-8, E1, and E2) also developed tumors with low necrosis percentages (10 to 40%). Although the higher levels of VPF expression in the VPF-transfected lines caused faster tumor growth, this did not result in a higher ratio of viable cells, which might have been expected if the increased level of VPF had increased the density of tumor blood vessels.

Immunohistological Analysis of VPF-Transfected Melanomas

Staining of xenograft sections from VPF-transfected lines with MEC 7.46 and with Ab against laminin or HSPG showed that all transfected tumors developed the aberrant vascular pattern and extracellular matrix component distribution described previously. (Figure 7)[33]

To study the distribution of VPF in the melanoma xenografts, sections were stained with a polyclonal anti-VPF Ab. In general, low staining intensities were found, especially in control tumors (from lines Mel57, E1, or E2). Diffuse VPF staining throughout the tumors was found in cross sections of line I-3, I-4, and III-8 tumors, indicative of the fact that VPF is expressed constitutively by these tumors. More intense VPF staining was often seen in the stromal compartments of VPF-transfected tumors (Figure 5, d and e) as well as of control tumors (Figure 5b). VPF staining colocalized, in part, with endothelial cells, confirming the finding that VPF accumulates on tumor blood vessels, but it was also seen on stromal cells that did not stain with the endothelial marker (compare Figures 5b and 5c). Although especially VPF_{189} has a high affinity for heparin, VPF staining in tumors from the line expressing this variant, III-8, or from other lines, did not colocalize with HSPG staining (not shown).

Hypoxia was suggested to be a cause for the observed upregulation of VPF expression in Mel57 xenografts.[33] This hypothesis predicts that higher levels of VPF should be expressed around necrotic areas in such tumors. In some sections of line Mel57, E1, and E2 tumors, staining for VPF was indeed most intense in cells lining necrotic areas (Figure 5a), suggesting an influence of hypoxia on the induction of VPF expression in the control tumors. However, there was not always an intense VPF staining seen adjacent to necrosis, whereas in a very small line E1 tumor without any necrosis VPF staining was already visible in the stroma (Figure 5b). Therefore, tumor necrosis appears to be neither necessary nor sufficient for the induction of VPF expression, and mechanisms other than hypoxia must be involved in this induction as well.

To test the expression of VPF protein in human patient melanoma lesions, cryosections of primary melanoma and melanoma metastasis were stained with polyclonal anti-VPF Ab. In all cases the presence of VPF could be detected, although the levels varied considerably. Figure 6 shows VPF staining in two different metastatic lesions. Figure 6a shows a pigmented lesion with a nodular growth pattern that contained a subpopulation of tumor cells that stained intensely for VPF. A metastatic lesion with a diffuse architecture showed diffuse positivity in the tumor cells and a marked staining of the vascular bed (Figure 6b).

Examination of Vascular Permeability in the Transfectant Xenografts

Since VPF is an inducer of vascular permeability,[29] the permeability of the blood vessels for proteins may be enhanced in tumors derived from the VPF-transfected lines. This was tested by i.v. injections of fluorescein-conjugated albumin (FITC-BSA), and subsequent examination of the tumors.

In tumors from line Mel57 or from vector-transfected lines, 2 minutes and 10 minutes after injection very little extravasation of tracer had occurred: fluorescence was almost exclusively seen within the vas-

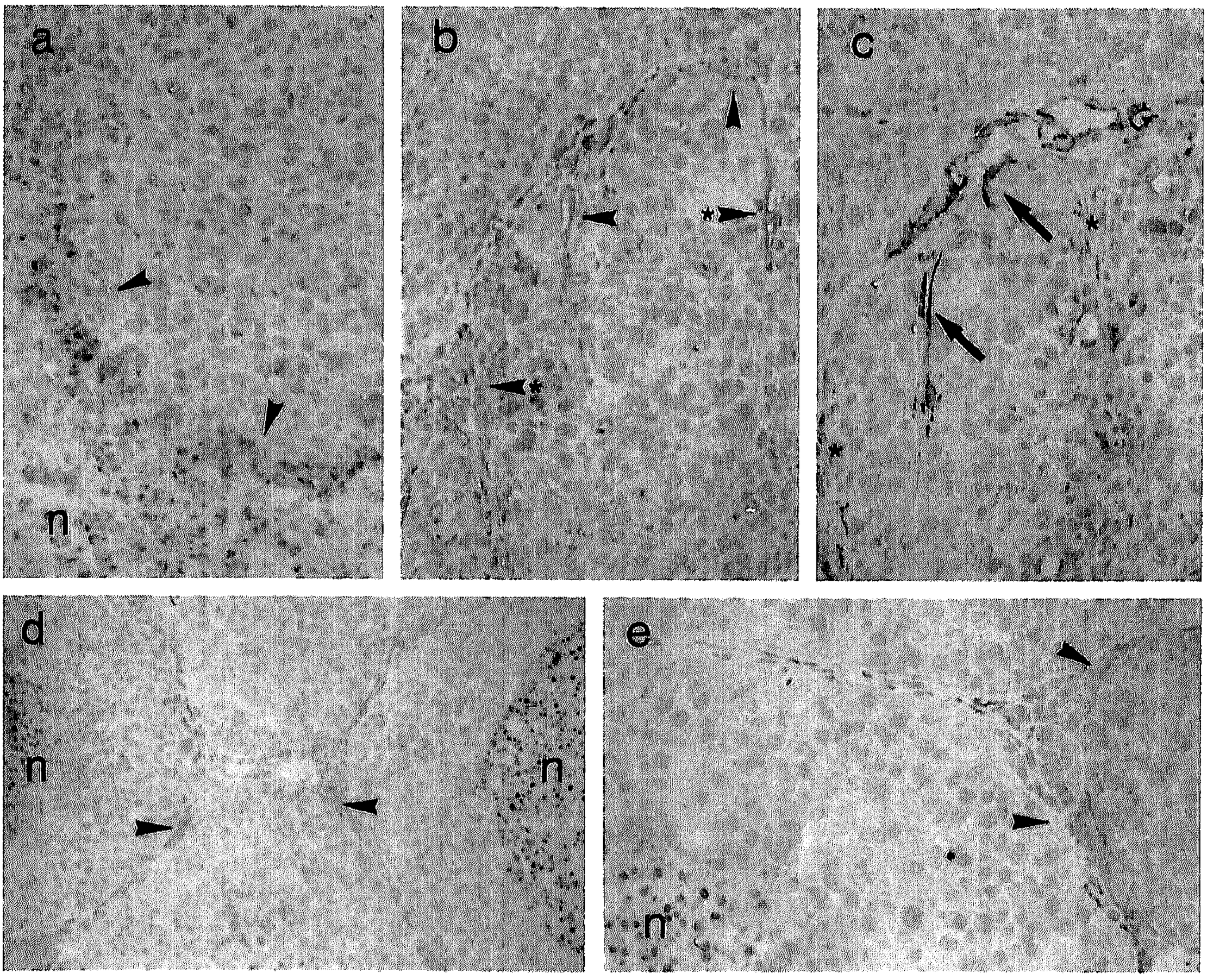


Figure 5. *Localization of VPF protein in tumors from nontransfected and transfected melanoma lines. Sections of tumors were stained with a polyclonal Ab anti-VPF VEGF* (a, b, d, *and* e) *or with the anti-endothelial Ab* (c). *Tumors were from the following lines:* (a) *Mel57, parental line;* (b *and* c) *E1, serial sections showing the same region of a small tumor (1.5 × 0.8 mm) stained for VPF and endothelium, respectively;* (d) *L3,* (e) *III-8. Necrotic areas are marked "n." Delicate VPF staining is visible in red. Therefore, the regions staining for VPF are also marked by arrowheads. In* c, *endothelial staining is marked by arrows. Asterisks indicate nonendothelial stromal structures in panel* c *that do stain for VPF in panel* b. *Magnifications:* a–c *and* e, *× 200;* d: *× 100.*

cular walls (Figure 7a). After 45 minutes, however, small amounts of tracer had leaked from the vessels into the tumor mass, visible as short protrusions coming out of the vessels (Figure 7b). In all VPF-transfected tumors, 2 minutes after injection, tracer was found not only in the walls of what appeared to be functional blood vessels (Figure 7f), but part of it was also found in the rest of the stromal septa. These septa, therefore, seemed to contain channels connecting the blood vessels. These channels partly colocalized with endothelial cells, and thus they may be part of the vascular system. Very extensive leakage from the vascular system into the tumor mass was evident 10 minutes and 45 minutes after injection, as tracer was not only seen in the stromal compartments rich in endothelial cells, but had also spread through parts of the tumor parenchyma (Figure 7g). Still, the tracer was retained within parts of the tumor cell mass rich in matrix proteins (Figure 7, g and i, right side), which apparently formed a fine network of interstitial channels. In the control tumors a similar or even more extensive network of matrix proteins was present (Figure 7d), but tracer leakage was much less intense (see above). The centers of the tumor nodules, poor in matrix proteins, were not reached by the protein tracer (Figure 7g), in accordance with earlier reports on protein leakage from tumor blood vessels.[13,45] These data thus show that in VPF-overproducing tumors protein leaks more extensively from the tumor vessels into the tumor cell compartment than in control tumors, and that the distribution of the extravasated protein in the tumor parenchyma colocalizes with a network of extracellular matrix.

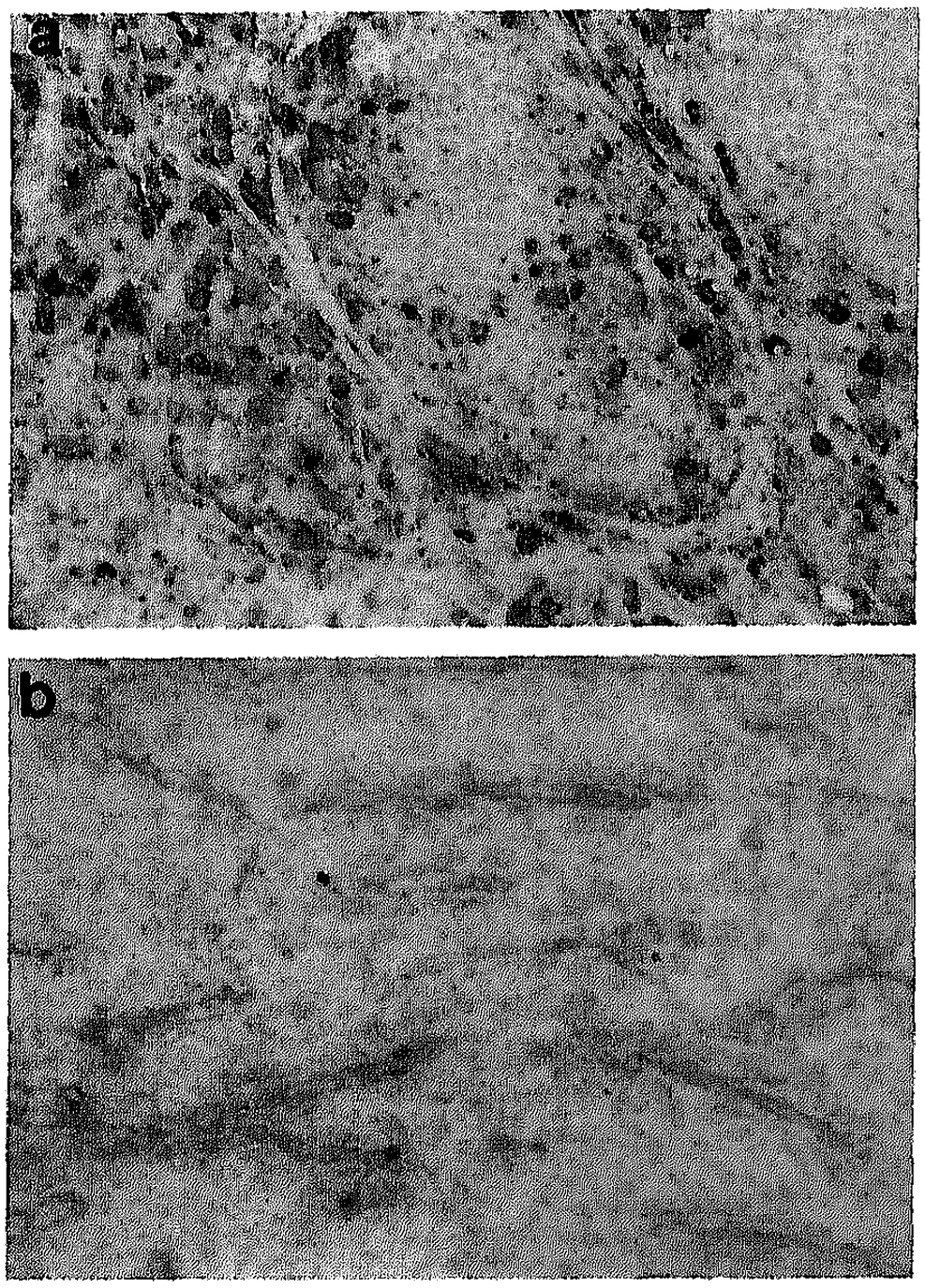


Figure 6. *Localization of VPF protein in human patient melanoma metastasis lesions. Cryosections were stained with a polyclonal anti-VPF Ab.* a: *Pigmented* (brown) *lesion shows focal intense intracellular staining* (red) *for VPF protein.* b: *Lesion with a diffuse growth pattern shows diffuse weak staining for VPF, whereas vascular cells are more prominently stained. Magnification,* × *100.*

As a consequence of this higher vascular permeability, the formation of an extravascular fibrin matrix may be favored, facilitating the growth of new blood vessels,[32] or directing the pattern in which angiogenesis takes place. Staining for fibrinogen/fibrin in cross sections of these tumors did not show significant differences in the distribution of fibrin, however. Fibrin staining was most intense in the vascular walls, but some fibrin deposition was also visible as short protrusions from blood vessels in all types of tumors (Figure 7, e and j). The network of extravascular fibrin was certainly not more extensive in VPF-overproducing tumors. Apparently, extravasated fibrin was deposited close to the blood vessels, and protein leakage from tumor vessels was not rate-limiting for the development of an extravascular fibrin matrix.

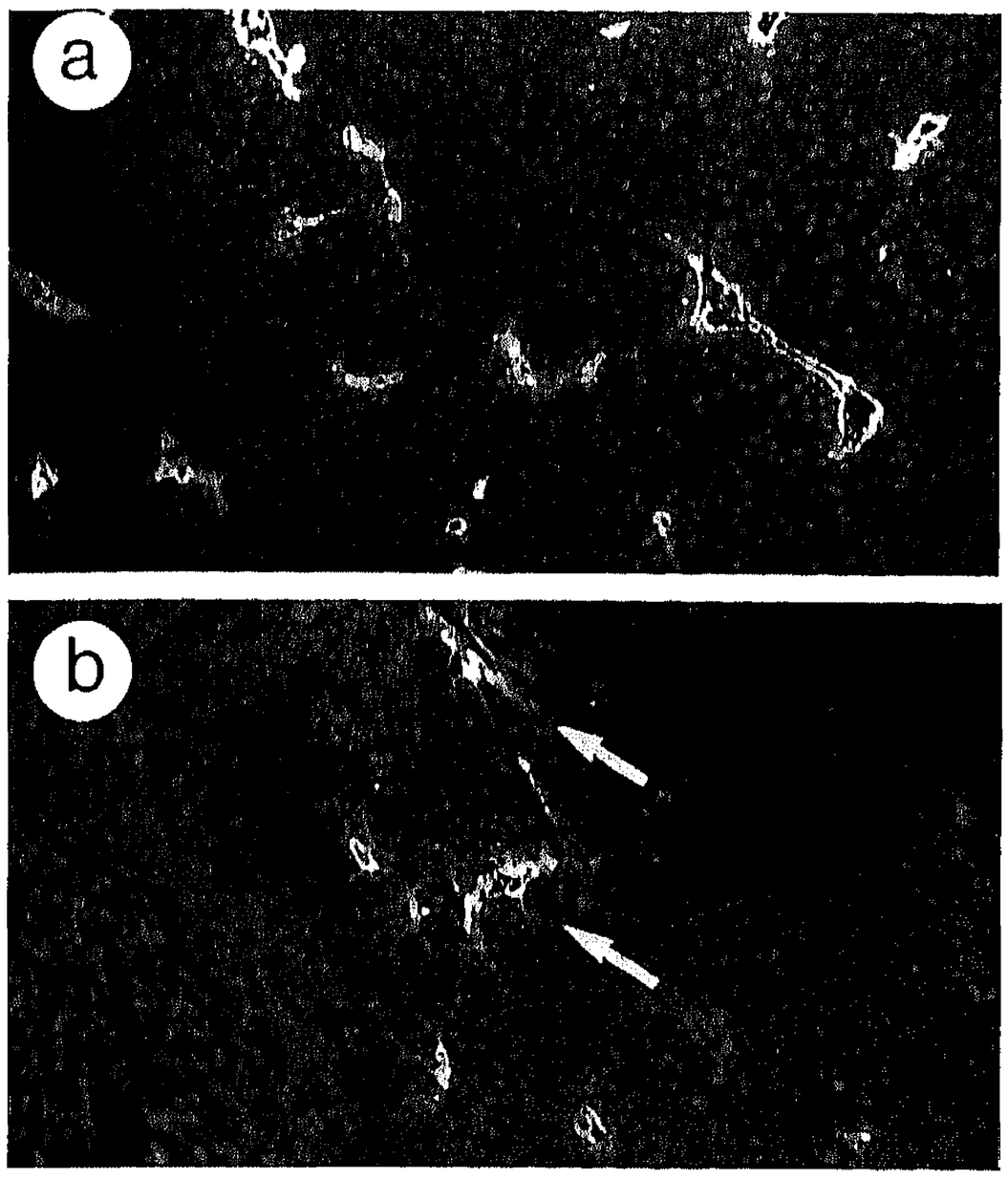
Metastatic Properties of the Transfectant Xenografts

Because tumor angiogenesis is believed to be essential for tumor growth and metastasis,[1–3] the altered vascular phenotype caused by a different VPF expression pattern may also change the growth rate and the metastatic potential of the tumors. To test this, cells of the lines E2, I-3, and III-8 were injected s.c. in groups of 20 mice each. The parental melanoma line Mel57 is of an earlier passage than all the transfected lines at the time of injection, since the latter lines have all been cloned and selected in hygromycin-containing medium for a prolonged period (see Materials and Methods). Therefore, vector-transfected line E2 is probably a better control in this experiment than parental cell line Mel57. Weekly measurements of the tumor volumes showed that the growth rates of the VPF-transfected xenografts were significantly increased compared with that of the E2 line (see above, and Figure 4c). After 7 weeks, the mice were sacrificed. Previous experiments had indicated that the incidence of metastasis in tumors from the maternal line approached a value of 50% at that time, so that this time schedule would allow an estimate of both an increase and a decrease in the metastasis frequency. The tumors were excised and weighed and the numbers of lung metastases estimated. Table 3 shows that the VPF-transfected xenografts have comparable densities but a significantly higher weight than the control E2 tumors. Similar frequencies of metastasis were found, but the metastatic burden, ie, the number of metastases per affected lung, was significantly higher in the VPF transfectants. A correlation between the number of metastases in a lung section and the size of the corresponding primary tumor was not found (not shown).

Discussion

The aim of this study was to test whether a change from an inducible to a constitutive VPF expression pattern in melanoma cells affects the development of the tumor vascular bed, and if so, whether the level of VPF expression influences the vascular pattern. This question was inspired by the observation that

Figure 7. *Leakage of macromolecular tracer from blood vessels and deposition of extravascular fibrin. FITC-labeled BSA was i.v. injected in mice bearing line Mel57 tumors* (a–e) *or line I-4 tumors* (f–j). *Sections shown in* a *and* f *are from tumors removed 2 minutes after injection;* b *and* g *show tumors removed 45 minutes after injection. Tracer was visualized by fluorescence microscopy. Leakage of tracer is indicated by arrows.* (c–e) *Serial sections corresponding with* b. (h–j) *Serial sections corresponding with panel* g. (c *and* h) *Staining with the anti-endothelial Ab, showing the distribution of blood vessels.* (d *and* i) *staining with an anti-HSPG Ab, showing the distribution of matrix proteins.* (e *and* j) *Staining with an antifibrinogen/fibrin Ab, showing the deposition of (extravascular) fibrin. Necrotic areas are marked "n." Magnification,* × *100.*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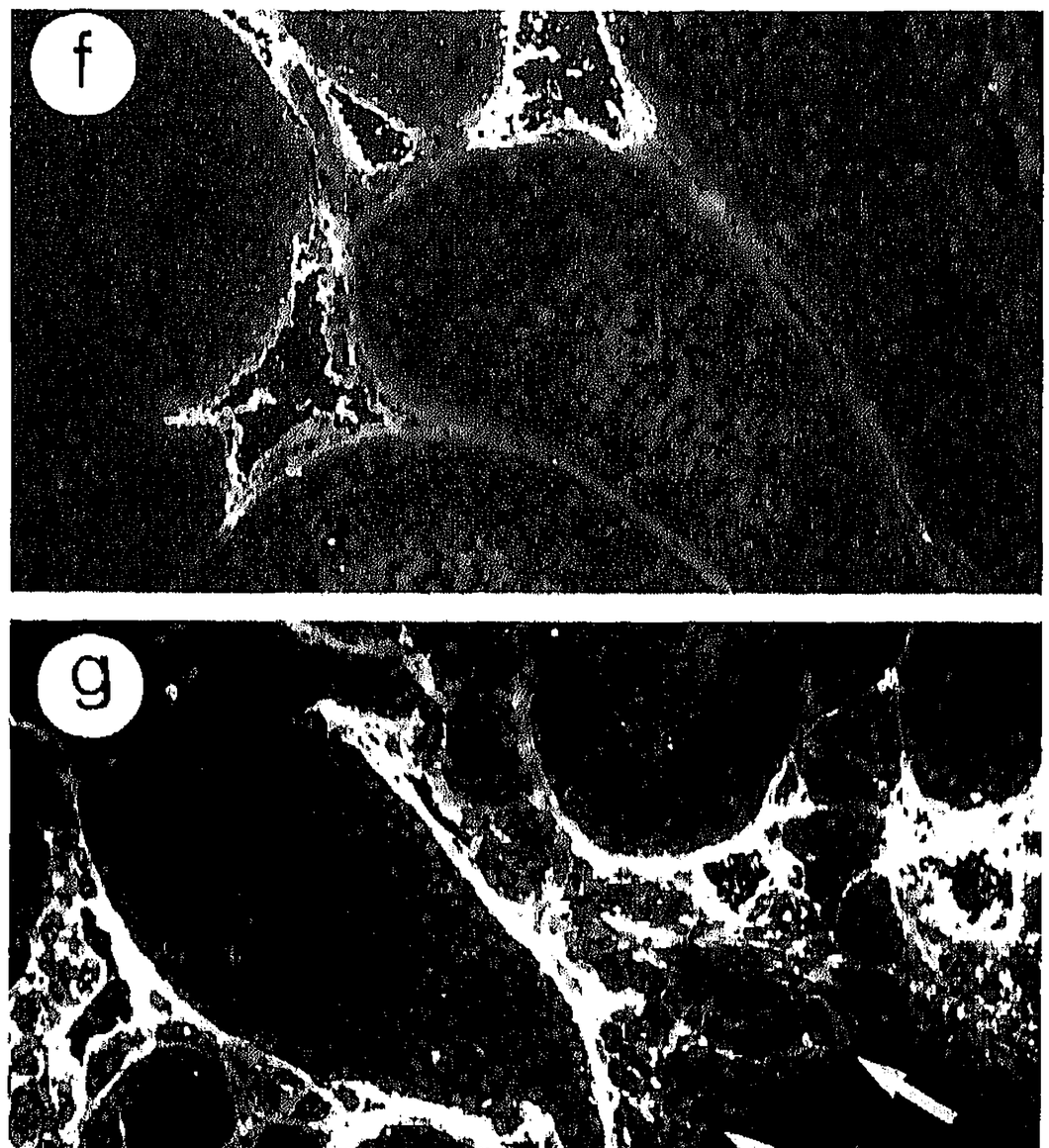
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Table 3. *Biological Behavior of Xenografts of VPF- or Vector-Transfected Melanoma Lines*

	Tumor weight* (n)	Tumor volume† (n)	Tumor density‡ (n)	Metastatic incidence§	Metastatic burden‖ (n)
E2	0.88 ± 0.36 (17)	0.89 ± 0.44 (17)	1.1 ± 0.2 (17)	11/20 (55%)	3.1 ± 2.8 (11)
I-3	1.50 ± 1.08 (14)	1.56 ± 1.36 (14)	1.1 ± 0.2 (16)	9/17 (53%)	8.0 ± 10.8 (9)
III-8	2.16 ± 0.77 (15)	2.31 ± 0.73 (15)	0.9 ± 0.1 (17)	8/18 (44%)	9.1 ± 5.8 (8)

* Weight (g) ± SD of tumors excised 7 weeks after inoculation. Statistical level of significance of the difference between the tumor weight of E2 and I-3, $p < 0.02$, and of E2 and III-8, $p < 0.001$.
† Volume (cm^3) ± SD of tumors measured 7 weeks after inoculation.
‡ Density (g/cm^3) ± SD of all measured tumors.
§ Number of cases with one or more lung metastases/total number of mice in the group.
‖ Mean ± SD of the number of metastases counted in cross-sections of the lungs containing tumor cells. In the III-8 group one case with an excessive amount of metastases (~100) was omitted. Statistical level of significance of the difference between the metastatic burden of line E2 and I-3, $p < 0.08$, and E2 and III-8, $p < 0.01$.

human melanoma lines differed in their pattern of VPF expression. Some lines, such as BLM, had a constitutively high level of VPF expression, whereas other melanoma lines with a somewhat different vasculature, such as Mel57, had a much lower expression level of VPF in culture, which *in vivo* was upregulated to the same level as that found in line BLM.[33] By transfection of line Mel57, several lines with a high basal level of VPF expression were obtained. These lines provided a model to test the influence of the VPF expression pattern *in vivo*.

An intriguing observation using the transfectant lines *in vitro* was the higher efficiency of translation of the recombinant VPF RNA compared with that of the endogenous VPF mRNA. In the 5′ noncoding sequence of the endogenous VPF messenger, a GC-rich region is present as well as several AUG and GUG alternative initiation codons.[44] Such features have also been found in the 5′ noncoding region of the PDGF-B chain transcript, where they have a profound inhibitory effect on the translation efficiency.[46,47] The 5′ region of the recombinant VPF RNA contained a vector-encoded SV40-derived leader and only a 21 base stretch of the 5′ noncoding sequence of the *VPF* gene. The higher translation efficiency of this recombinant transcript indicates that also in the 5′ noncoding region of the VPF mRNA sequences are present that inhibit translation.

When studying and interpreting the differences in biological behavior induced by the transfection of VPF expression constructs, one should be aware of unexpected genetic changes that can occur during the transfection and selection procedure. Mutations may yield a selective advantage *in vivo* and cause heterogeneity of the tumor cell population. However, both the vascular patterns, the vascular permeability, and the matrix distribution were phenotypically stable within the parental line Mel57, the vector-transfected lines, and the various VPF-transfected lines. Also, morphological heterogeneity within tumors was rarely seen. Therefore, there is no reason to assume that the observed differences in tumor morphology and physiology are caused by unexpected genetic changes; they are much more likely to be caused by the experimentally intended changes in the VPF expression pattern.

The lines transfected with VPF expression constructs indeed produced tumors with a strikingly different vascular pattern from the parental line or vector-transfected lines. The vascular pattern seen in tumors from the VPF-transfected lines was, unexpectedly, also very different from that in other melanoma lines constitutively expressing VPF, such as BLM.[33] This comparison is not quite appropriate, however, as VPF is probably not the only determinant of the vascular phenotype. Furthermore, the VPF expression levels of the transfectant lines *in vivo* are presumably an order of magnitude higher than the expression level of line BLM.

The aberrant vascular phenotype induced by the VPF-transfected lines in the present studies was not dependent on the level of recombinant VPF expression. It should be noted, however, that even in the line with the lowest expression level (I-11), VPF was probably still overexpressed by a factor of 4 to 9 compared with normal Mel57 xenografts. Transfectant lines with even lower expression levels than line I-11 would have been useful, but were difficult to identify by Northern blotting because of their extremely low levels of recombinant VPF RNA.

The vascular pattern in tumors from line III-8, which expresses the heparin-binding VPF_{189} variant, was not significantly different from the pattern in the tumors producing the diffusible variant VPF_{121}. This indicates that this molecular variant is biologically active and that it must, at least in part, be released from the producing cells. Proteases such as plasmin may be present in the tumor parenchyma, which can release a biologically active part of VPF_{189}.[43]

It is difficult to give an explanation for the development of the aberrant vascular phenotype in the

tumors from VPF-transfected lines. A suitable explanation for the absence of tumor-penetrating vessels in the VPF-overproducing tumors could be the absence of a VPF concentration gradient that would develop in control tumors as a result of hypoxia-induced VPF expression. However, the correlation between tumor necrosis and VPF staining was not as strong as might have been expected if hypoxia were the only inductive factor. Also, the fact that different vascular patterns had already evolved in very small tumors from lines E1 and I-3, without any signs of necrosis, makes this hypothesis unlikely.

Varying degrees of extravascular fibrin deposition could influence the patterns of neovascularization in the tumors as well.[32] Although the permeability of the tumor vessels for proteins was, as expected, chronically increased in the tumors producing recombinant VPF, it did not lead to a more extensive fibrin network in these tumors. Extravasated fibrinogen may be quickly converted into fibrin and be deposited close to the blood vessels from which it originated. Furthermore, along with larger amounts of extravasating fibrinogen, also larger amounts of plasminogen may leak from the vessels. This may be activated by plasminogen activators, which are probably expressed in increased amounts by endothelial cells because of the action of VPF,[26] and which may also be expressed by the tumor cells. Plasmin could then remove most of the deposited fibrin. An enhanced activity of proteinases in the tumors from VPF-transfected lines could also explain the low amounts of matrix proteins detected within their tumor cell nests. It is therefore of great interest to investigate in future studies the presence and distribution of specific proteases in these tumors.

Although changing VPF expression in a melanoma line from inducible to constitutive does change the pattern of tumor vascularization qualitatively, the mechanism by which VPF influences this pattern is still unclear. The determination of the vascular pattern is clearly dependent upon a number of parameters that are not quite understood, of which VPF expression is one. Initially, we expected that overexpression of VPF would increase the number of tumor blood vessels, a finding that was very recently reported in a tumor xenograft model of human breast carcinoma.[33] However, because of the dramatic change in architecture, it was impossible to estimate the density of functional blood vessels in the VPF-transfectant tumors. Hence, our model appears not to be a suitable system to test whether the number of tumor blood vessels is rate-limiting to the process of metastasis, in contrast to the described breast carcinoma model.[33] However, it was not tested in this latter model whether *in vivo* upregulation of VPF expression occurred. Because of this, the differences in VPF expression *in vivo* between transfected and non-transfected tumors were unknown. Therefore, further studies on the role of VPF in angiogenesis and tumor vessel function are required.

Immunostaining of human melanoma lesions showed that VPF protein is expressed in patient material. Both nodular and diffuse growth patterns were observed, which seemed to be accompanied by a different staining pattern for VPF. However, the consistence of this finding will have to be addressed in a more extensive study.

The notion that the tumor vasculature is important for metastasis urged us to study the consequences of a change in vascular type for the metastatic potential of the tumors. The vector-transfected E2 line was used as a control, since the parental line Mel57 was of a lower passage at the time of injection into mice and had not undergone the transfection and selection procedures, and therefore may not be a fair control line in these experiments. On the other hand, unexpected events may have genetically altered line E2 (as discussed above). This possibility is hard to verify, but it does limit the reliability of line E2 as a control in the metastasis experiment. Although the VPF-transfected xenografts had increased growth rates compared with the control, we could not demonstrate significant differences in the metastatic incidence between these lines, ie, the percentage of mice that had one or more metastases in the examined lung sections. A remarkable finding was, however, that the number of metastases per affected lung was considerably higher in mice carrying VPF-transfected tumors than in the controls. It is unclear at what stage of the multi-step process of metastasis this effect is caused. A correlation with growth rate or size of the tumors was not found, but there might be a relation with the increased vascular permeability in the VPF-transfected xenografts.

The presence of laminin and proteoglycans in the stromal septa of VPF-overproducing melanomas suggested that these matrix proteins act as a basal membrane, forming a barrier between tumor cells and the highly vascular stroma. Of course, the presence of such a membrane may counteract metastatic spread and would hamper a straightforward interpretation of the findings obtained in this study.

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