Modulation of Angiogenesis and Tumorigenicity of Human Melanocytic Cells by Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor

Ulrich Graeven, Ulrich Rodeck, Sonja Karpinski, Monika Jost, Statthis Philippou, and Wolff Schmiegel

Department of Medicine, Ruhr Universitat Bochum, Knappschaftskrankenhaus, 44892 Bochum, Germany [U. G., S. K., W. S.]; Department of Pathology Augustia Krankenhaus, 44892 Bochum, Germany [S. P.]; and Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 [U. R., M. J.]

ABSTRACT

Human melanoma cells express two prominent angiogenic factors, e.g., vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF/fibroblast growth factor-2). In this study, we report on the relative contribution of these two factors to in vitro and in vivo growth of a tumorigenic melanoma cell line (WM164) and nontumorigenic, immortalized melanocytes (FM516SV). Overexpression of either cytokine significantly boosted tumorigenicity of WM164 cells in immunodeficient SCID mice. Attempting to overexpress bFGF antisense sequences produced no viable clones confirming earlier reports that autocrine bFGF is obligatory to melanoma cell survival and growth. By contrast, down-regulation of endogenous VEGF production did not affect growth of WM164 cells in vitro. In vivo expansion of WM164 cells expressing VEGF antisense was delayed but not abrogated. Forced expression of either bFGF or VEGF in immortalized but nontumorigenic melanocytes did not induce sustained tumor growth in vivo highlighting that neither of the two factors is sufficient for induction of tumorigenicity in this model system. Overexpression of either cytokine in WM164 cells led to the development of atypical large vessels but not to an increase in microvessel density. Taken together our results confirm an essential autocrine role of bFGF in human melanoma and indicate a beneficial but nonessential role of VEGF in the tumorigenic phenotype of human melanoma cells.

INTRODUCTION

Human melanoma cells produce a plethora of angiogenic cytokines including VEGF-A (1), bFGF/FGF2 (2, 3) placenta-derived growth factor; (4), platelet-derived growth factor (5), transforming growth factor-β (6). In addition, melanoma cells produce extracellular matrix components and metalloproteases, which are relevant to angiogenesis, including tenasin (7), components of the plasminogen activator system (8–10), gelatinases (11), and CD44 (12, 13).

The multitude of angiogenic factors produced by this tumor type raises the question of whether any of them is rate-limiting in supporting melanoma-derived vessel formation and/or tumorigenicity in vivo. In this study, we focused on the relative contribution of two potent melanoma-derived angiogenic cytokines, VEGF and bFGF, to these processes. Selection of these two factors was guided by their documented importance to the angiogenic process in other experimental systems and the observation that both are frequently overexpressed in transformed as compared with normal melanocytes. Specifically, VEGF is perceived to be one of the most important mediators of tumor-associated angiogenesis (reviewed in Ref. 14). Initially named vascular permeability factor, it was renamed VEGF to highlight its mitogenic effects on endothelial cells. Recent work demonstrated that cultured human melanoma cells frequently and constitutively overexpress VEGF when compared with normal neonatal melanocytes (1, 15–17). Like VEGF, bFGF was initially identified as a growth factor for endothelial cells (18). In endothelial cells, bFGF has been shown to induce VEGF expression (19), and bFGF and VEGF synergize in inducing endothelial cell growth (20–23). bFGF also exerts essential autocrine functions in supporting melanoma cell growth in vitro and in vivo (2, 3, 24, 25). Melanoma cells in vitro and in situ frequently coexpress bFGF and VEGF (17).

These findings prompted us to investigate the relative importance of tumor-derived VEGF and bFGF to the tumorigenic/angiogenic phenotype in a human melanoma cell line (WM164) that coexpresses both growth factors. In addition, we investigated whether forced expression of either VEGF or bFGF could induce tumorigenicity in an immortalized but nontumorigenic human melanocyte line.

MATERIALS AND METHODS

Origin and Culture of Human Cells. Normal human melanocytes were obtained from neonatal foreskins. WM164 melanoma cells originated from a metastatic lesion and were established in the laboratory of Dr. M. Herlyn at the Wistar Institute, Philadelphia, PA. HM164 melanocytes expressing the SV40 T antigen were described previously (26). Both cell lines were free of contaminating normal cells such as endothelial cells, immune/inflammatory cells, or keratinocytes.

Vector Construction and Transfection of Melanocytic Cells. Full-length VEGF cDNA encoding VEGF165 was amplified by reverse transcription-PCR from a melanoma-derived RNA preparation (WM164) using VEGF sense and antisense primers described earlier (1). The VEGF primers correspond to sequences in the untranslated 5′ and 3′ regions of the VEGF gene resulting in amplification of four splice variants of 516, 648, 720, and 771 bp. The PCR product corresponding to VEGF165 was recovered from agarose gels and cloned into the pCRII vector using a TA cloning kit (Invitrogen BV, Holland). After verification of VEGF sequences by direct sequencing, the purified VEGF 165 cDNA fragment was subcloned in sense and antisense direction into the polyclinicker region of pCB6+ (27) containing the cytomegalovirus promoter/enhancer to drive expression of the transgene and a neomycin selection cassette driven by the SV40 promoter. bFGF cDNA encoding the M,.17,800 form of bFGF (kindly provided by A. Sommer, Synergen Inc. Boulder, CO) was first subcloned into the pCRII vector and then subcloned in sense and antisense direction into the polyclinicker region of pCB6+. WM164 melanoma cells and FM516V melanocytes were transfected as described previously (28) either with the empty pCB6+ vector for control purposes or the bFGF and VEGF sense and antisense vector constructs. The cells were passaged 48 h after transfection and maintained in the presence of 800 μg/ml G418 for selection. Individual colonies as well as pooled colonies were expanded and tested for transgene expression as described below.

RNA Isolation and Northern Blot Analysis. Total cellular RNA was prepared from cultured cells or melanocytic tumors recovered from inoculated mice using the RNAeasy RNA kit (Quiagen, Hilden, Germany). Total cellular RNA (6 μg/lane) was separated on formaldehyde-containing agarose gels and transferred to nylon membranes. VEGF and bFGF RNA sense and antisense probes were prepared by in vitro transcription from the VEGFpCRII or bFGFpCRII vector by use of the SP6 and SP7 DNA polymerases, respectively. Probes were labeled with digoxigenin using a commercially available kit.
(Boehringer Mannheim, Mannheim, Germany) and used for hybridization analyses according to instructions provided by the manufacturer.

Preparation of CM. Melanoma cells and FM516SV cells were maintained in W489 base medium consisting of four parts MCDB153 and one part L15 supplemented with 2% FCS and insulin (5 μg/ml). To prepare CM, cells were plated at 3 × 10^4/well in six-well 35-mm culture plates in 3 ml of W489 supplemented with 0.2% bovine serum albumin, fatty acid free. After 3 days supernatants were collected and centrifuged at 500 × g to remove cellular debris. Cell numbers were assessed after collection of CM using a Coulter Counter.

Determination of in Vitro VEGF, bFGF, and TSP-1 Protein Production. Secretion of VEGF into tissue culture supernatants was measured after preparation of CM using a VEGF ELISA (R&D Systems, Rüsselsheim, Germany) according to the manufacturer’s instructions. bFGF production was measured in cell extracts using a bFGF ELISA (R&D Systems) according to the manufacturer’s instructions. For bFGF Western blot analysis, cells were grown in complete medium for 72 h and then lysed in radioimmunoprecipitation assay buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, Aprotinin 30 μg/ml, sodium orthovanadate 10 μM, 10 mg/ml phenylmethylsulfonfylfluoride). Samples in Laemml loading buffer [1 ml of glycerol, 0.5 ml of β-mercaptoethanol, 3 ml of 10% SDS, 1.25 ml of 1 M Tris–HCl (pH 6.7), and 1–2 mg of bromophenol blue] were separated on a 15% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were probed with an anti-bFGF antibody (Santa Cruz Biotechnology, Heidelberg, Germany). After incubation with a secondary goat antirabbit antibody linked to phosphatase (Sigma Chemical Co., Taufkirchen, Germany), blots were developed using the nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate chromogen system according to manufacturer’s instructions. 

To characterize the sections were examined at low magnification (×100) without additional staining using an inverted light microscope.

RESULTS

Characterization of Cell Lines Transfected with VEGF and bFGF Constructs. We chose two cell lines representing different stages of melanoma development to evaluate cytokine effects on tumorigenicity and angiogenesis. WM164 cells were derived from a metastatic lesion, proliferate in serum-free medium, grow anchorage-independent in semisolid medium, and are tumorigenic when inoculated into immunodeficient mice (8). FM516SV cells were derived from a normal neonatal melanocyte strain by transfection with a plasmid encoding the SV40T antigen. These cells are postcrisis and immortalized but do not form tumors when inoculated into nude or SCID mice (26). On the basis of these criteria, we consider them to represent an intermediate stage of melanoma development. Both cell lines were stably transfected with plasmids encoding either full-length bFGF or VEGF165 cDNA. In addition, WM164 cells were transfected with bFGF and VEGF sequences in antisense orientation. Expression of transgenes was confirmed by Northern blot analyses using appropriate sense and antisense riboprobes as shown in Fig. 1, A and B. Both VEGF sense-transfected WM164 and FM516SV cells secreted ~100-fold higher VEGF levels when compared with mock-transfected cells (Fig. 2A). Conversely, WM164 cells transfected with antisense VEGF secreted significantly less VEGF (2–4% of mock-transfected controls) depending on the clone (Vas2 versus Vas4) investigated. Similarly, transfection of bFGF sense cDNA into WM164 and FM516SV cells led to robust expression both on the mRNA (Fig. 1B) and the protein levels (Fig. 3). By contrast, forced expression of bFGF antisense sequences produced no viable clones consistent with the critical role of bFGF in melanoma cell growth described previously (2, 3).

Earlier studies have demonstrated that bFGF can induce VEGF production in glioma cells (29). Thus, we determined whether WM164 or FM516SV cells overexpressing bFGF secreted VEGF at higher levels than mock-transfected cells. As shown in Fig. 2B, forced

[Image 308x144 to 561x256]
expression of bFGF did not increase VEGF secretion in these cells. For WM164 cells overexpression of bFGF even led to a slight decrease of VEGF production. Similarly, overexpression of VEGF did not affect bFGF expression in WM164 and FM516SV cells as determined by Western blot (Fig. 3). Because constitutive bFGF protein levels in melanoma cells were barely detectable by Western blot analyses (Fig. 3) we also used a bFGF ELISA to test for potential changes in bFGF production that could not be detected by Western blotting. The bFGF ELISA was performed on cell extracts because of the fact that bFGF does not have a signal sequence for secretion. As shown in Fig. 2C, bFGF protein levels in VEGF sense or antisense mRNA-transfected cells were comparable with those of mock-transfected cells.

To additionally investigate functional aspects of melanoma-derived VEGF, we selected clone WM164-Vas2 based on the dramatic reduction (>98%) of endogenous VEGF production observed for this clone. In the case of VEGF- and bFGF-overexpressing cells we used pools of transfected cells for additional analysis.

**In Vitro Growth and Phenotype of Transfectants.** Both bFGF (2, 3) and VEGF (30, 31) have been reported previously to support melanoma growth in an autocrine manner. To account for possible autocrine effects of these factors we first determined growth characteristics of all of the transfected cells in serum-free medium (Fig. 4). No significant differences in cell growth were observed between mock-transfected and bFGF- or VEGF-overexpressing cells. However, it should be noted that bFGF-overexpressing FM516SV cells accumulated slightly but consistently higher cell numbers than controls during the observation period of 10 days. Similarly, down-regulation of VEGF expression in WM164-Vas2 cells had no discernible effect on in vitro cell growth. (Fig. 4; Ref. 1) These results indicate that endogenous VEGF has no obvious autocrine role as it relates to melanoma proliferation in vitro. None of the transfected cells demonstrated changes in cell morphology, shape, or attachment as observed by light microscopy (results not shown).

**In Vivo Tumorigenicity of Transfectants.** Next, we determined tumorigenic properties in athymic mice of VEGF and bFGF overexpressing WM164 and FM516SV cells and of WM164 cells in which VEGF production was ablated. As shown in Fig. 5A, forced expression of bFGF significantly accelerated tumor development of WM164 cells inoculated into SCID mice. Overexpression of VEGF had an...
even more dramatic effect on tumorigenicity of WM164 cells leading to rapid development of large tumors at the inoculation site (Fig. 5B). At autopsy, the internal organs of tumor-bearing mice were scanned by inspection and palpation for the presence of tumor nodules; no evidence for macroscopic metastases was found in any of the transfectants investigated. Expression of the VEGF antisense transgene in WM164-Vas2 cells resulted in significant delay in tumor formation relative to mock-transfected cells (Fig. 5B). For ~4 weeks after inoculation no tumor masses were palpable. However, ~50 days after inoculation palpable tumor masses appeared in all of the mice inoculated and subsequently expanded at rates comparable with those observed for mock-transfected WM164 cells.

Overexpression of VEGF in FM516SV was not associated with tumor formation during the observation time of 3 months. By contrast, FM516SV cells overexpressing bFGF revealed transient and reproducible tumorigenicity within 4–10 days after inoculation (Fig. 6). However, after 8 days, tumor volumes decreased progressively, and residual small palpable masses disappeared gradually over a time period of 2 weeks. Additional observation did not reveal any recurrence of tumors within 3 months after inoculation. Results described below on FM516SV tumors relate to tumors excised at day 7, i.e., at the peak of tumor formation.

Transgene Expression in Vivo. Expression of transgenes in tumors was monitored by in situ immunohistochemical analyses using antibodies to bFGF and VEGF, respectively. Results on staining for CD31 are presented in the “tumor vascularity in vivo” section. Immunohistochemistry for VEGF demonstrated moderate staining of mock-transfected WM164 cells for VEGF consistent with the expression pattern observed in vitro (Fig. 7a). bFGF staining in tumors from WM164 mock-transfected cells was barely detectable (Fig. 7b), which is consistent with low bFGF expression as determined by Western blot and ELISA analysis (Figs. 2c and 3). As expected, in tumors formed by VEGF-overexpressing clones, the cells reacted strongly with a VEGF specific antibody (Fig. 7d). bFGF expression in VEGF-overexpressing tumors was not detectably different from that observed in mock-transfected cells (Fig. 7, b and e). Importantly, WM164-Vas2 tumors did not react with VEGF antibodies demonstrating that these tumors had not regained expression of VEGF (Fig. 7g).

Fig. 4. Effect of VEGF sense or antisense mRNA expression or bFGF sense mRNA expression on in vitro growth of WM164 or FM516SV cells. A. WM164 cells transfected with the empty vector (WM164-M), stably expressing VEGF sense (WM164-Vs), or antisense mRNA (WM164-Va2), bars, ± SE. B. WM164 cells transfected with the empty vector (WM164-M) or stably expressing bFGF sense mRNA (WM164-Fs), bars, ± SE. C. FM516SV cells transfected with the empty vector (FM516SV-M) or stably expressing VEGF sense mRNA (FM516SV-Vs), or D. stably expressing bFGF sense mRNA (FM516SV-Fs), bars, ± SE. All cells were seeded at 0.5 x 10^3/well (six-well plates) in W489 base medium (total volume of 2 ml) supplemented with 0.2% BSA. Cell growth was assayed after 3 and 7 or 10 days by counting the number of viable cells using a Coulter counter.

bFGF expression in 164-Vas2 tumors was comparable with controls in SCID mice indicating that delayed tumor growth was not contributed to up-regulated bFGF expression (Fig. 7, b and h). VEGF expression in bFGF-overexpressing WM164-Fs tumors was comparable with that observed in mock-transfected cells (Fig. 7, a and j). Thus, no evidence of in vitro cross-induction between melanoma-derived VEGF and bFGF was found. As expected, tumors formed by bFGF-overexpressing cells (WM164-Fs) revealed intense bFGF immunoreactivity (Fig. 7k).

Expression of VEGF sense and antisense transgenes in tumor tissues was additionally confirmed by Northern blot analysis using strand-specific probes (Fig. 8). We observed robust expression of VEGF antisense sequences in WM164-Vas2 tumors consistent with the immunostaining results shown in Fig. 7g.

Tumors developing after inoculation of FM516SV bFGF-overexpressing cells were removed on day 7, because spontaneous regression occurred beyond this time point as shown in Fig. 6. The majority of cells in these tumors stained strongly with an antibody to Melan A (Fig. 9b), a marker for human melanocytic cells. They also expressed high levels of bFGF (Fig. 9d) and moderate levels of VEGF comparable with the in vitro data (Fig. 9c).

Tumor Vascularity in Vivo. To assess the effects of VEGF and bFGF overexpression on tumor-associated neovascularization we stained intratumoral vessels with CD31/PECAM-1-specific antibodies. Representative results of this analysis are shown in Figs. 7g and 9, and quantitative results of the microvessel density analysis are given in Fig. 10. VEGF overexpression in WM164 was associated with the formation of large vessels lined with CD31+ cells, which dominated the appearance of the tumors (Fig. 7f). To a lesser extent large lacunar vessels were also observed in tumors obtained by inoculation with bFGF overexpressing WM164 cells (Fig. 7i). These lacunar vessels caused a net increase in vascular surface area in these tumors when compared with mock-transfected controls similar to results recently described by Grunstein et al. (32) for VEGF-transfected fibrosarcoma cells. By contrast, tumors formed by either WM164 mock control or WM164-Vas cells did not contain these large vessels (Fig. 7c and i). When microvessel density was determined according to Weidner (33) we observed no relationship between VEGF or bFGF expression and
and j) and WM164-Vs (Fig. 12, i and j) and, to a lesser extent, WM164-M cells (Fig. 12, a and b), showed a tendency to form tube-like structures albeit not as delineated as HUVEC cells. Especially after 72 h the majority of melanoma cells remain poorly organized in irregular-shaped cell clusters. By contrast, the VEGF AS mRNA-transfected WM164-Vas2 cells formed loose cell aggregates with no evidence of tube formation indicative for an endothelial-like phenotype or vascular mimicry (Fig. 12, c and d).

**DISCUSSION**

This study demonstrates that both VEGF and bFGF, when overexpressed by melanoma cells, support melanoma cell growth in vivo. These results are consistent with previous studies in which associations between VEGF (36) and/or bFGF (37) production and biological aggressiveness of human melanomas have been described. However, our results also indicate that of these two melanoma-derived factors only bFGF is critically important for tumor formation in vivo, whereas VEGF is dispensable. These conclusions are based on the findings that WM164-Vas cells formed rapidly growing tumors after a lag period of several weeks as compared with controls (1), and we could not establish a WM164 variant in which bFGF protein production was successfully and consistently ablated (2). The critical importance of autocrine bFGF production by melanoma cells has been demonstrated earlier (2, 3, 25, 38). By contrast, it was surprising that VEGF production was not essential to either growth of WM164 cells in vitro through an autocrine mechanism or WM164 tumor formation through paracrine mechanisms in vivo. Although suppression of VEGF production significantly delayed tumorigencity of WM164 cells in mice, the tumors that eventually formed expanded at rates that were comparable with those achieved by control mock-transfected cells. These tumors continued to express VEGF antisense transcripts and produced no VEGF protein attesting to the fact that they did not rely on VEGF production for tumor formation. Our results extend earlier studies, which reported stunted tumor development in melanoma cells with down-regulated VEGF expression (39, 40). As in the present study, this earlier report also demonstrated similar in vitro growth rates for...
mock-transfected control and for VEGF sense and antisense-transfected human melanoma cells. However, some differences between these earlier studies and the results presented here deserve attention.

Oku et al. (40) used SK-Mel-2 human melanoma cells, which produce only very low levels of VEGF under normoxic or hypoxic conditions, whereas the WM164 cells used in the present study secrete VEGF constitutively at high levels (\(1000 \text{ pg/ml}\)). Thus, down-regulation of VEGF expression in WM164 was expected to have a more dramatic effect on tumorigenicity than down-regulation of VEGF production in SK-Mel-2 cells. The complete absence of WM164-Vas2 tumor masses during the first 3–4 weeks after inoculation is consistent with this prediction. However, the growth rate of tumors that eventually developed was rapid and indistinguishable from controls. WM164-Vas2 tumor development occurred despite continued expression of antisense VEGF sequences in WM164-Vas2 tumors associated with absent VEGF protein production. Thus, the fast growth rate of these tumors is likely to be attributable to the development of VEGF-independent mechanisms for sustained tumor growth and angiogenesis. The presence of at least two independent angiogenic pathways in melanoma cells mediated by either VEGF receptors or members of the Tie family has been documented very recently to contribute to melanoma-associated angiogenesis (31). Forced expression of soluble forms of either VEGFR-1 or Tie2, which acted to neutralize ambient VEGF or angiopoietin-1, respectively, were shown to inhibit in vivo expansion of A375 melanoma cells. Importantly, as in the present study, melanoma formation was not abolished. Instead, poorly vascularized, relatively small and slow growing tumor masses developed. By contrast, we describe here the in vivo emergence of fast-growing WM164 variant cells, which expanded in vivo despite sustained suppression of VEGF production by antisense sequences. Emergence of these variants was not an isolated phenomenon but occurred with similar kinetics in all of the mice inoculated with WM164-Vas2 melanoma cells. In conclusion, the delayed but rapid expansion of WM164-Vas cells in vivo must have been attributable to alternative angiogenic mechanisms that substituted for tumor-derived VEGF. Taken together, our results highlight that, at least in WM164 melanoma cells, VEGF production is beneficial but not required for tumorigenicity in vivo.

This is in contrast to bFGF, which was absolutely required for cell growth even in vitro. We were unable to establish clones that stably overexpressed antisense sequences consistent with an essential autocrine role of bFGF in melanoma development as described earlier (2, 3, 25). By contrast, WM164 cells overexpressing bFGF were established easily. Interestingly, these cells had population-doubling rates that were indistinguishable from mock-transfected or parental WM164 cells. This result indicates that the level of endogenous bFGF produced by WM164 cells is sufficient for maximal autocrine growth stimulation, at least under the in vitro conditions used here. However, bFGF-transfected WM164 cells formed tumors at a faster rate than
mock-transfected cells. This result suggests significant paracrine effects of overexpressed and, implicitly, of endogenous bFGF in WM164 cells.

Forced expression of either bFGF or VEGF did not induce sustained tumor formation of FM516SV cells in mice. Interestingly, forced expression of bFGF in FM516SV cells caused transient tumor formation associated with some degree of neovascularization. A recent report described that forced expression of bFGF in normal neonatal melanocytes induced a transformed phenotype as determined by growth factor-independent proliferation in vitro and anchorage-independence in soft agar (41). In addition, these cells formed nests of proliferating melanocytes when injected into the dermis of human skin grafts on SCID mice. Our results extend this earlier report insofar as we observed macroscopic albeit transitory tumor formation of bFGF-transduced FM516SV cells. The FM516SV bFGF tumors spontaneously and reproducibly regressed indicating that stable bFGF overexpression alone is not sufficient to support tumorigenicity of FM516SV cells defined as continuous and inexorable expansion of transformed cells in vivo. Unfortunately, no data are available for comparison relating to the long-term persistence and growth of bFGF-overexpressing melanocytes described by Nesbit et al. (41). At present, it is unclear why overexpression of bFGF or VEGF does not induce tumorigenicity in FM516SV cells. It appears possible that factors other than either bFGF or VEGF are also required for melanoma growth in vivo to occur. Conversely, the production of antiangiogenic factors by normal cell types may limit the angiogenic propensity of these cells. In support of this notion we describe here that FM516SV melanocytes produce TSP-1, whereas WM164 do not.

A similar expression pattern of pro- and antiangiogenic factors has been described in bladder cancer and normal urothelial cells. (42). Campbell et al. (42) demonstrated that normal urothelial cells, which failed to induce endothelial growth in a rat corneal assay, expressed VEGF at the same level as bladder carcinoma cells but retained TSP-1 production, which was reduced by >94% in angiogenic bladder
carcinoma cells. Additional evidence for the significance of the differential expression of TSP-1 in FM516SV cells as opposed to WM164 cells is provided by the recent finding that TSP-1 inhibits angiogenesis in vivo by inducing apoptosis of activated endothelial cells (43). Induction of apoptosis in activated endothelial cells is one possible explanation for the spontaneous regression of tumors formed after s.c. inoculation FM516SV-Fs cells in SCID mice. Taken together our data indicate that the loss of TSP-1 might be a key event in the switch from an antiangiogenic to an angiogenic phenotype and even more critical for melanoma formation than the capacity to secrete VEGF. Future studies will determine whether forced expression of TSP-1 in malignant melanomas neutralizes the angiogenic/tumorigenic potential of these cells as well, as has been shown for cutaneous squamous cell carcinomas (35).

Of note, both VEGF- and bFGF-overexpressing WM164 cells formed rapidly expanding tumors that contained larger vessels lined with CD31-positive endothelial cells similar to those formed by Grunstein et al. (32) for VEGF-transfected fibrosarcoma cells. These lacunar endothelia were induced by transgene expression, because they were absent from tumors formed by either WM164 mock control or WM164-Vas2 cells. However, when microvessel density was determined according to Weidner (33) we observed no relationship between VEGF or bFGF expression and vessel density in WM164 cells. Notably, even WM164-Vas2 cells, which express no VEGF, formed tumors with microvessel densities comparable with controls. Furthermore, WM164-Vs cells formed tumors with the lowest microvessel density, and the highest microvessel count was observed in the spontaneously regressing tumors formed by FM516SV cells overexpressing bFGF. These results indicate induction of unusual vessels characterized by large lumina but not microvessels by bFGF or VEGF overexpression in the two melanoma cell lines tested. This result raised the issue of whether bFGF or VEGF when overexpressed elicited the formation of atypical vessels. Specifically, Maniotis et al. (44) described recently that melanoma cells are capable of “vascular mimicry,” i.e., the formation of vascular structures without endothelial lining. We addressed this possibility by testing WM164 transfectants in an in vitro angiogenic assay. The results presented here are consistent with the notion that melanoma cells may participate in vascular mimicry as described recently (44). Overexpression of bFGF or VEGF appears to induce tube formation under the assay conditions used, additionally raising the question of whether these two cytokines participate in vascular mimicry by melanoma cells. This issue is presently under investigation.

Taken together, our results highlight that of the two melanoma-derived growth factors investigated only bFGF is critically important to tumorigenicity of WM164 cells in vivo. In contrast to bFGF, VEGF serves no essential autocrine or paracrine functions although it accelerates tumor expansion in vivo dramatically when overexpressed. Furthermore, our results demonstrate the potential importance of endogenous angiogenesis inhibitors like TSP-1 because the expression of VEGF and bFGF could only sustain or accelerate tumor growth in the absence of TSP-1.

REFERENCES
2. Becker, D., Meier, C. B., and Herlyn, M. Proliferation of human malignant melanoma cells is inhibited by antisense oligodeoxynucleotides targeted against basic fibroblast growth factor. EMBO J., 8: 3693–3694, 1989.
19. Seghezzi, G., Patel, S., Ren, C. J., Gualandris, A., Pintucci, G., Robbins, E. S.,
15. Kumar, R., Kuniyasu, H., Bucana, C. D., Wilson, M. R., and Fidler, I. J. Spatial and
13. Takahashi, K., Eto, H., and Tanabe, K. K. Involvement of CD44 in matrix metallo-
5. Nip, J., Rabbanii, S. A., Shihata, H. R., and Broud, P. Coordinated expression of the
4. de Vries, T. J., Kistoo, J. L., Silvers, W. K., and Mintz, B. Expression of plasminogen
Modulation of Angiogenesis and Tumorigenicity of Human Melanocytic Cells by Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor

Ullrich Graeven, Ulrich Rodeck, Sonja Karpinski, et al.

Cancer Res 2001;61:7282-7290.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/19/7282

Cited articles
This article cites 42 articles, 23 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/19/7282.full#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/61/19/7282.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/61/19/7282.
Click on "Request Permissions" which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.