Hypoxia-induced Up-Regulation of Angiogenin in Human Malignant Melanoma

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ABSTRACT

Angiogenesis is essential for tumor progression and metastasis, however, the angiogenesis regulators that are biologically relevant for human melanoma are still unknown. In this study, we analyzed the expression of the potent angiogenic factor angiogenin (ANG) in human melanoma in vitro and in vivo. Four different human melanoma cell lines and two normal melanocytes were kept either under normoxic or hypoxic conditions. After 24 h of hypoxic culture conditions, ANG was up-regulated in the melanoma cell lines but not in normal melanocytes. Induction levels correlated with the metastatic potential of the cell lines. These data were confirmed by Northern blot analysis.

In contrast, induction of vascular endothelial growth factor by hypoxia was equally strong in the examined highly aggressive melanoma cell lines and in one nonaggressive cell line. Other angiogenic factors tested as well as the melanoma growth stimulatory activity (Gro-α) showed no up-regulation. Thus, in the present study, hypoxia-induced up-regulation in melanoma cells was only observed for ANG and vascular endothelial growth factor.

Immunohistochemical studies showed that 8 of 10 melanomas and all 15 metastases were positive for ANG, particularly in the vicinity of small vessels, whereas all benign nevi were negative. Reverse transcription-PCR detected only weak ANG mRNA in nevi but strong signals in primary melanomas and metastases. In conclusion, we demonstrate for the first time enhanced expression of ANG in highly metastatic cell lines as well as in melanomas and metastases in vivo, suggesting that ANG expression is associated with the metastatic potential.

INTRODUCTION

Over the past decade, there has been considerable clinical and experimental evidence that tumor growth is dependent on neovascularization, a process called angiogenesis (1, 2). The induction of new capillary blood vessels allows further tumor growth and enables tumor cells to enter the circulation (3).

The first evidence that the degree of angiogenesis in a human tumor could predict the probability of metastasis came from studies of cutaneous malignant melanoma (4, 5). The mechanisms underlying the induction of angiogenesis are still poorly understood. A decrease in oxygen concentration and nutrient supply due to irregular and inadequate tumor vascularization has been considered the leading cause for angiogenesis (6). It is reasonable to assume that tumor neovascularization is achieved by the secretion of hypoxia-induced growth factors, leading to the formation of new blood vessels in undervascularized areas (1). Recently, several endothelial growth factors with angiogenic activity have been described, including the FGFs, VEGF, PDGF, and ANG (7).

\[ \text{ANG, a } M_r 14,200 \text{ protein originally isolated from medium conditioned by HT-29 human colon adenocarcinoma cells (8, 9) and subsequently from normal serum (10), is one of the most potent angiogenic factors in vivo (8, 11–13). Although its physicochemical properties and mechanisms of receptor binding have been characterized in detail, little is known about its role in neovascularization, particularly in tumor growth and metastasis. Previous experiments in which a monoclonal antibody against human ANG was shown to suppress the growth of human adenocarcinoma in nude mice by reducing tumor neovascularization support the idea of an important function of ANG in angiogenesis (14, 15). Nothing is known, however, about ANG expression and function during melanoma development.} \]

VEGF, also known as vascular permeability factor (16, 17), an endothelial cell-specific mitogen in vitro (18, 19), is considered to be an important regulator of both physiological and pathological neovascularization (20–22). De novo VEGF expression has been shown to increase tumor growth, angiogenesis, and experimental metastasis in nude mice (23). Its expression has been shown to be hypoxia inducible in different cell lines (24–27). In melanoma cell lines, VEGF is the only hypoxia-inducible factor up to now.

In this study, we compared the regulation of ANG synthesis in human malignant melanoma cell lines under hypoxic conditions with that of other angiogenic factors including VEGF and Gro-α, an autocrine growth factor for melanoma cells (28). Furthermore, we investigated its expression in benign and malignant melanocytic lesions in vivo.

We found that both ANG mRNA and protein levels are up-regulated in human melanoma cell lines under hypoxic conditions. The degree of up-regulation correlated with the metastatic potential. Immunohistochemical studies and RT-PCR analysis of tissue sections revealed that ANG is either absent or only moderately expressed in benign nevi but is abundantly present in primary malignant melanoma and melanoma metastases. Thus, our results identify ANG as an potentially important angiogenic factor for melanoma progression.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The four human melanoma cell lines MV3, BLM, IF6, and 530 (kindly provided by Dieter Kaufmann, Department of Genetics, University of Ulm, Ulm, Germany) were maintained in RPMI 1640 (Linaris, Bettingen, Darmstadt, Germany) supplemented with 10% FCS (Linaris), 2 mm L-glutamine, 100 units/ml penicillin/streptomycin, and 1% nonessential amino acids.

Normal melanocyte cultures (passages 15–20; a kind gift from Dieter Kaufmann, Department of Genetics, University of Ulm, Ulm, Germany) were maintained in HAM’s F10 medium, supplemented with 10% FCS (Linaris), 10% Nu-Serum, 2 mm L-glutamine, 100 units/ml penicillin/streptomycin, 68 ng/ml 12-O-tetradecanoylphorbol-13-acetate (Serva, Heidelberg, FRG), 68 ng/ml cholera toxin (Sigma Chemical Co., Deisenhofen, Germany), 28 μg/ml 3-isodontyl-1-methylxanthine (Sigma), 10 ng/ml bFGF (R&D Systems, Wiesbaden, Germany), and 10 ng/ml epidermal growth factor (R&D Systems). Cells were cultured in a well humidified incubator (37°C in 5% CO2, 95% air) and passaged when confluent. Cells (2 × 106) per 100-mm dishes of each cell line were cultured under standard conditions for 24 h. Supernatants of parallel cultures, kept either under normoxic or hypoxic conditions for 24 h, were collected for analysis of cytokine expression by solid phase ELISA. Cultures were further analyzed after 24 h of reoxygenation and compared with parallel cultures kept under normoxic conditions (48 h). For hypoxic treatment, cul-
tures were put in an anaerobic culture chamber (Anaerochult A; Merck, Darmstadt, Germany), and for reoxygenciation, cultures were kept again under standard conditions. Cell number was determined at the time of harvesting supernatants, and data are presented as pg/ml cells. Three independent experiments were performed.

Cytokine-ELISA. Commercial ELISA kits for human ANG, VEGF, bFGF, PDGF, TGF-β1, TGF-β2, and Gro-α (Quantikine, R&D Systems) were used, and assays performed according to the manufacturer’s specifications.

RNA Extraction from in Vitro Cell Cultures and Northern Blot Analysis. Total cytoplasmatic RNA was isolated by using the total RNeasy kit (Qiagen, Hilden, Germany). RNA concentration was determined spectrophotometrically at 260 nm. Twenty µg of RNA/sample were denatured in 50% formamide in formaldehyde buffer [0.1 m 3-(N-morpholino)propane sulfonic acid (pH 7.0), 40 mM sodium acetate, and 5 mM EDTA (pH 8.0)] for 15 min at 65°C, fractionated on a 1% agarose gel in formaldehyde buffer, and subsequently transferred to a nylon membrane (Hybond N+; Amersham, Braunschweig, Germany) in 20× SSC (1× SSC = 1× sodium chloride/sodium citrate). As a probe for ANG mRNA, a 402-bp fragment was used corresponding to the peptide-coding region. To isolate this fragment, a RT-PCR was performed on total RNA from MV3 human melanoma cells, using murine leukemia virus reverse transcriptase (Perkin-Elmer, Weiterstadt, Germany) and oligo-d(T)16 (Amersham, Braunschweig, Germany) in 20 µl reaction mixture containing 5 µM of the appropriate primer pair for ANG cDNA (see above). The PCR products were cloned into pCR II vector DNA (TA cloning kit; Invitrogen, Leek, The Netherlands) and sequenced.

For Northern hybridization, the purified membranes were labeled to high specific activity with [32P]dATP using a random primer labeling system (Boehringer Mannheim, Mannheim, Germany) and sequencing. Membranes were UV crosslinked and prehybridized in a dextran sulfate buffer (100 g/dl dextran sulfate, 0.6 M NaCl, 0.2 M NaHPO4, 6 mM EDTA, 1.75% laurylsarcosinate, and 50 µg/ml salmon sperm DNA, pH 6.2) for 1 h at 65°C. Hybridization was carried out in the same prehybridization solution containing 5 × 106 cpm/ml of labeled probe. After hybridization for 16 h at 65°C and 6 h at 60°C, membranes were washed twice with 2 × SSPE/0.1% SDS at room temperature, once with 1 × SSPE/0.1% SDS at 60°C, and once again with 0.2 × SSPE/0.1% SDS. Membranes were then exposed to Kodak XAR-5 films with intensifying screens at −80°C for 20 days. Fold increase of hybridization signals was determined by phosphoimaging (Phosphorimager; Fuji Bas-2000, Fuji). Ethidium bromide-stained agarose gel was used as control for equal RNA loading.

Immunohistochemistry. Five µm paraffin-embedded sections of 10 primary human malignant melanomas (Clark levels II–V), 15 cutaneous metastases with histopathologically confirmed necrotic areas, and 8 benign nevi were cut into primary antibodies were used as controls.

RNA Extraction from Biopsy Specimens and RT-PCR. Biopsies of eight primary melanomas, eight metastases, and eight nevi were cut into 25-µm cryostat sections and homogenized. Total cellular RNA was extracted by using the total RNeasy kit (Qiagen, Hilden, Germany) and analyzed for integrity by ethidium bromide agarose gel electrophoresis. cDNA was generated from 100 ng of total RNA from each biopsy specimen by using murine leukemia virus reverse transcriptase (Perkin-Elmer, Weiterstadt, Germany) in the presence of oligo-d(T)16, deoxynucleotide triphosphates, RNase inhibitor, and MgCl2, in 20-µl volumes, according to the manufacturer’s instructions. The mixture was incubated at 42°C for 15 min, at 99°C for 5 min, and then 10 min at 4°C. Subsequently, CDNA was used as template for 35 cycles PCR in the presence of 80 µl of reaction mixture containing MgCl2 and AmpliTaq DNA polymerase (Perkin-Elmer), according to the manufacturer’s instructions, and 0.15 µM of the appropriate primer pair for ANG cDNA (see above).

Each cycle profile consisted of 5 min of denaturation at 94°C, 30 s at 58°C for annealing, and 1 min at 72°C for primer extension with a final extension step of 10 min at 72°C. The PCR products were electrophoresed in 1% agarose gels in the presence of ethidium bromide and photographed under UV light.

Statistics. For each cytokine, data are given as mean value ± SD of three independent experiments. Statistical analysis was performed using the Student’s t test in the case of the ELISA data.

For each tumor specimen, vWF-positive blood vessels were counted in three high power fields of areas with strong ANG immunoreactivity, compared with tumor areas with no ANG immunoreactivity. Blood vessel number is given as mean ± SD for primary melanoma and melanoma metastases, respectively. For statistical analysis, the Wilcoxon test was used. The level of significance was chosen as P ≤ 0.05. The association of ANG expression with the proliferative index (Ki-67) was investigated using the same method.

RESULTS

ANG mRNA and Protein Are Strongly Up-Regulated in Human Melanoma Cell Lines under Hypoxic Conditions. To assess whether the expression of angiogenic factors is differentially regulated in melanocytic cells, we studied two highly metastatic human melanoma cell lines, two melanoma cell lines with low metastatic potential (29, 30), and two normal melanocyte cultures for expression of different angiogenic factors, including ANG and VEGF, compared with the melanoma growth stimulatory activity factor Gro-α. Fig. 1 shows mean values ± SD of three independent experiments for each cytokine. High induction of ANG protein levels was found in the highly aggressive melanoma cell lines MV3 and BLM under hypoxic culture conditions (Fig. 1A). The cell lines IF6 and 530, having low metastatic potential, expressed significantly lower levels of ANG protein under hypoxia. Differences between baseline and corresponding induction levels within each cell line were statistically significant (P ≤ 0.05). Differences of absolute values of induction levels between highly and low aggressive cell lines, but not fold increase, were statistically significant (P ≤ 0.05). Release of ANG into the supernatant persisted and further increased during 24 h of reoxygenation but returned to normal levels after 48–72 h (data not shown).

For VEGF, the highly aggressive cell lines (MV3 and BLM) showed similar baseline and induction levels (3-fold up-regulation; Fig. 1B). However, in contrast to ANG, VEGF displayed the strongest induction in the low aggressive cell line IF6 (8-fold up-regulation). Thus, VEGF production under hypoxic stress shows no clear correlation with the metastatic potential. Differences between the baseline and the corresponding induction level of each cell line were statistically significant (P ≤ 0.05). The 530 cell line is much better. Expression of Gro-α chemokine, known to be involved in the growth of melanoma, was not affected by hypoxia in any of the four melanoma cell lines tested (Fig. 1C). Other angiogenic factors, like bFGF, PDGF, TGF-β1, and TGF-β2, which were tested in parallel, showed no or only minimal up-regulation (data not shown).

Normal human melanocytes showed similar constitutive cytokine levels for ANG (Fig. 1D). The differences between baseline levels of melanocytes and melanoma cell lines were not statistically significant. However, in contrast to melanoma cell lines, ANG was down-regulated in melanocytes under hypoxic conditions. VEGF and Gro-α in melanocytes showed low constitutive as well as induction levels. To exclude the possibility that cytokine release by cell damage contributed to the effects seen, the total amount of nonviable cells was estimated by trypan blue staining. The total amount of nonviable cells in supernatants and cell monolayer under hypoxic conditions did not
ANG mRNA expression under normoxic, hypoxic, and reoxygenation conditions was studied by Northern blot hybridization. ANG mRNA was detectable as a transcript of 700 bp in all four melanoma cell lines (Fig. 2). Under normoxia, low ANG mRNA levels were found in the highly metastatic cell lines MV3 and BLM. The cell lines IF6 and 530 gave no detectable hybridization signals. Under hypoxic conditions, ANG mRNA levels were elevated 10-fold in the highly metastatic cell lines. Compared with MV3 and BLM, the low metastatic cell lines IF6 and 530 showed significantly lower signals under hypoxia. Upon reexposure of cells to normal oxygen tension, ANG expression returned to its low constitutive level. Cultured normal melanocytes constitutively expressed very low levels of ANG mRNA and showed reversible, although weak, ANG mRNA up-regulation under hypoxic conditions (data not shown).

ANG Immunoreactivity and ANG mRNA Can Be Detected in Tissues of Primary and Metastatic Melanoma but not in Benign Melanocytic Lesions. To investigate whether ANG protein can be detected in human melanocytic lesions in vivo, its expression was studied by immunohistochemical staining. Paraffin-embedded tissue sections of 10 primary melanomas (Clark levels II–V), 15 melanoma metastases, and 8 nevocellular nevi (all from different individuals) were stained using a polyclonal antiserum directed against human recombinant ANG. ANG protein distribution was compared with the expression of vWF, an endothelial cell marker, with that of Ki-67, a histochemical marker for proliferating cells.

Eight of 10 primary melanomas showed focal or widespread strong tumor cell immunoreactivity for ANG (Fig. 3). Blood vessels and sweat glands displayed weak positive staining (data not shown). Significant staining of melanocytic cells could not be observed in any of the benign nevi studied (data not shown). All 15 melanoma metastases expressed strong ANG immunoreactivity, particularly at their margins and in proximity to necrotic and presumably hypoxic areas (Fig. 4a). Compared with parallel tissue sections stained with a monoclonal antibody against vWF (Fig. 4c), ANG protein was predominantly localized in the vicinity of strongly vascularized tumor areas. The mean value of vessel density per high power field in ANG-positive tumor areas for all primary melanomas was 19.6 ± 11.3 and in melanoma metastases 29.6 ± 7.7. The vessel excess 20% in all cell cultures. Furthermore, it did not correlate with the cytokine levels (not shown).

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density in ANG-negative areas was 10.4 ± 3.4 in primary melanomas and 4.8 ± 2.4 in melanoma metastases. Differences between both values of ANG-positive and ANG-negative areas were statistically significant (P < 0.01) for melanoma metastases but not for primary melanomas. In 8 of 15 metastases, ANG-positive tumor cells were preferentially localized in areas of Ki-67 immunoreactivity. However, in seven metastases, there was no clear correlation. No immunoreactivity was detectable in tissue sections stained with normal serum and antigen-blocked primary antibodies (data not shown).

Twenty-four human melanocytic lesions were assessed for ANG mRNA expression by RT-PCR. Total RNA was extracted from eight primary melanomas, eight metastases, and eight benign nevi. RNA was reverse transcribed into cDNA and then amplified using an ANG-specific primer pair. To confirm the identity of the cDNA product generated by sequence-specific primers, the PCR-amplified ANG cDNA was analyzed by agarose gel electrophoresis and compared with the fragment size of 402 bp predicted by the location of the primers used. All samples expressed mRNA for β-actin, indicating the integrity of the cDNAs and their ability to serve as templates for amplification (data not shown). PCR-amplified DNA was sequenced, and the results are compared to the known DNA sequence of the ANG gene (data not shown). All primary melanomas and all cutaneous metastases showed distinct expression of ANG mRNA transcripts (Fig. 5), whereas six of eight of the benign nevocellular nevi failed to express ANG mRNA.

DISCUSSION

The process of angiogenesis is considered to be critical for the growth of primary solid tumors but appears to be also important for the development of metastasis (2, 31). Angiogenesis is regulated by positive and negative factors, which are secreted and/or deposited by tumor or stroma cells, which thus act on endothelial cells in a paracrine manner. Positive factors induce quiescent endothelial cells to proliferate and to invade the tumor tissue.

Malignant melanoma, a tumor with increasing incidence and poor prognosis in high-risk cases, has long been the focus of intense clinical and laboratory investigation. For skin melanoma of thin and intermediate thickness, it has been shown that a high blood vessel density is an independent risk factor associated with negative out-

Fig. 3. a, immunohistochemistry of a human primary malignant melanoma stained with a polyclonal antibody against ANG (×50). b, higher power view of a (×200). Tumor cells, which were indicated by arrows in the figure, show strong cytoplasmatic staining for ANG.
and not to the release of preexisting cytokine from cellular stores (e.g., caused by cell damage). ANG protein levels were decreased under hypoxic conditions in melanocytes, whereas mRNA was slightly up-regulated. Therefore, expression of ANG mRNA is not absolutely concordant with protein synthesis, as already suggested by Weiner et al. (38). This might further indicate that ANG is regulated, at least in part, at the posttranscriptional level.

We have compared ANG secretion to that of VEGF, another potent angiogenic factor, known to be induced in several human tumors by hypoxia (24). Interestingly, the melanoma cell lines showed different forms of regulation for ANG compared with VEGF. Both angiogenic factors were inducible under hypoxia; however, the strongest induction of VEGF was observed in the low aggressive cell line IF6. These findings are in accordance with previous in vitro and in vivo studies by Pötgens et al. (27). These authors found similar VEGF mRNA expression in MV3, BLM, IF6, and Mel57 cells (the latter also low metastatic) under culture conditions of low oxygen tension and after injecting these cell lines into nude mice. Thus, VEGF levels under hypoxia in vitro and in vivo do not correlate with the metastatic potential. Furthermore, a VEGF-transfected melanoma cell line neither displayed an increased growth rate nor produced tumors of higher vascularization. Consequently, they proposed that VEGF cannot represent the only factor relevant to angiogenesis and metastasis in these tumors. In the present study, we have shown that another angiogenic factor, ANG, is specifically induced in human melanoma cell lines by hypoxia and that the degree of the induction correlates with their metastatic potential.

On the basis of these in vitro data, we further analyzed ANG expression in vivo. Using RT-PCR, we could demonstrate strong signals for ANG mRNA in all primary melanomas and metastases tested, whereas benign nevi were either negative or gave weak ANG signals. By immunohistochemistry, there was focal or widespread ANG immunoreactivity in primary melanomas. In melanoma metastases, ANG was predominantly seen in tumor areas located at the border of necrotic regions and along the invasive front of the tumor. The necrotic areas themselves were negative for ANG. The presence of ANG in the periphery of necrotic tumor areas suggests its in vivo induction by hypoxia, at least in metastases. We found a significant correlation between ANG-positive tumor cells and vWF-positive capillaries, suggestive of an ANG-derived neoangiogenesis. Ongoing ANG production in the presence of newly formed capillaries might be due to nonsufficient oxygen supply by inadequate vascularization. In fact, it has been shown recently that even in highly vascularized tumors, well-perfused tumor areas were found to be hypoxic (39). In our investigations, there was no significant correlation between Ki-67 reactivity and ANG expression.

Hypoxia-induced up-regulation in melanoma cells appears to be restricted to ANG and VEGF, because other angiogenic factors, like bFGF, PDGF, TGF-β1, and TGF-β2, which were tested in parallel, showed no or only very little up-regulation (data not shown). Also, Gro-α, a cytokine highly expressed by melanoma cells with no known angiogenic activity, was not inducible by oxygen deprivation. Therefore, up-regulation of cytokines under hypoxic conditions in melanoma appears to be limited to a subset of angiogenic factors, in particular ANG and VEGF.

How ANG expression and synthesis are transcriptionally regulated...
under hypoxic conditions and which signal transduction pathways are involved are still unknown. Hypoxia-inducible factor-1 appears to be the principal transcription factor that binds to the oxygen-sensitive enhancer of the VEGF gene (40). Interestingly, the putative ANG promoter (9) carries two identical possible binding sites (5′-TCAGGGC-3′) for HIF-1 at the −463 and −330 position, which differ from the original published sequence (5′-TCAGTGGCGG-3′) (39) only in 2 bp. Thus, ANG might also be regulated by HIF-1. Additionally, there is an AP-1-like site (5′-TGACTAA-3′) at the −1072 position. AP-1 is also known to be hypoxia inducible (41).

Taken together, our data conclusively support the concept that induction of ANG in human malignant melanoma occurs in response to oxygen deprivation. Therefore, in this tumor type VEGF is not the only angiogenic factor up-regulated under hypoxic conditions. ANG could be the connecting link between tumor hypoxia and subsequent neovascularization, which allows further tumor expansion and subsequent metastasis.

Administration of a noncytotoxic neutralizing monoclonal antibody against ANG resulted in prevention and/or delay of the outgrowth of human colon adenocarcinoma, lung adenocarcinoma, and fibrosarcoma xenografts in athymic mice (14, 15). Because malignant melanoma is a well-vascularized tumor type whose switch to the angiogenic phenotype appears to occur very early in tumor development, it will be interesting to study whether inhibition of ANG binding to its receptor on endothelial cells will affect the progression of malignant melanoma.

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