Expression of Angiogenic Factors Cyr61 and Tissue Factor in Uveal Melanoma

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Objective: To study the expression of angiogenic factors Cyr61 and tissue factor (TF) in uveal melanoma and its correlation with blood vessel density.

Methods: Suppression subtractive hybridization was used to identify genes that are differentially expressed between cell lines of uveal melanoma and normal uveal melanocytes. Expression of these genes was subsequently verified in primary uveal melanomas and correlated with the number of blood vessels in archival specimens by immunohistochemical analysis.

Results: Cyr61 and TF are expressed at elevated levels in cell lines of uveal melanoma compared with normal uveal melanocytes. Duplication of a region of chromosome arm 1p, encompassing the genes encoding Cyr61 and TF, was observed in the melanoma cell line used in the initial subtractive hybridization. Both genes are also expressed in primary uveal melanomas, and a correlation was found between expression of TF and the number of blood vessels in archival specimens.

Conclusions: Cyr61 and TF may contribute to the angiogenic phenotype associated with uveal melanoma. A region of chromosome arm 1p also may contain oncogenes or tumor suppressor genes pertinent to the origins of this type of ocular tumor.

Clinical Relevance: New immunotherapies have been devised for the treatment of cancer based on the expression of TF. Similar approaches may be effective in treating uveal melanoma.

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further study offers therapeutic opportunities for the treatment of this blinding and life-threatening disease.

Cyr61 is a member of an emerging family of immediate-early genes that includes transcription factors, proto-oncogenes, and cytokines.18 Cyr61 can be induced by growth factors (platelet-derived growth factor and fibroblast growth factor) or oncogenes (v-src), and, once induced, grows in two cell cultures. Cyr61 is secreted as a cysteine-rich, 40-kd protein that associates with the extracellular matrix.19 Cyr61 secretion increases the growth of endothelial cell DNA and endothelial cell migration through the integrin αvβ3 and αvβ5-dependent pathways, respectively.20 Cyr61 also induces neovascularization when implanted in rat corneas, and its expression can increase the tumorigenicity of cells.19

Tissue factor is also an immediate-early gene product induced by growth factors and cytokines. Although best known for its role as the primary initiator of blood coagulation, TF is also involved in vasculogenesis, probably through intracellular signaling via its cytoplasmic domain, whereas activation of the coagulation pathway requires only the membrane anchoring of the extracellular portion of the molecule.21

Inactivation of the TF gene in knockout mice leads to embryonic lethality owing to impaired vascular development.22 These animals lack a developed periendothelial muscular wall, as seen in animals missing other angiogenic modulators, such as angiopoietin 1. This phenotype is distinct from animals deficient in the coagulation pathway, which die of hemostatic bleeding shortly after birth.23

Tissue factor is expressed in a variety of cancer cells that are more malignant and metastatic, and its expression correlates with the angiogenic phenotype and poorer prognosis.24-26 Tissue factor promotes metastasis in transfected human cutaneous melanoma cells,27 and animals expressing TF have larger and more vascularized tumors.28

The finding of TF and Cyr61 in uveal melanoma is a unique observation; expression of these angiogenic factors along with vascular endothelial growth factor (VEGF) may indicate that multiple growth factors contribute to the malignant and metastatic phenotype of ocular melanoma.

**METHODS**

**CELL CULTURE**

Normal uveal melanocytes were obtained from human donor eyes and kept in culture according to published procedures.29 Mel 270 and Mel 290 were established from biopsy samples of human uveal melanoma following published procedures29; Mel 270 is composed primarily of spindle cells, and Mel 290 is composed primarily of epithelioid cells. Tumor cell cultures were maintained at 37°C with 5% carbon dioxide in complete RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.01M HEPES, penicillin (100 U/mL), streptomycin (100 µg/mL), and 0.1% amphotericin B.

**SUPPRESSION SUBTRACTION HYBRIDIZATION**

Methods essentially followed the procedures of Diatchenko et al30 using 2 µg of poly A+ RNA derived from normal uveal melanocytes and from Mel 290 to amplify differentially expressed sequences.

**RNASE PROTECTION ASSAY**

Cyr61 and TF transcript levels were compared in uveal melanocytes Mel 270 and Mel 290 by RNase protection assay using a 249-base antisense Cyr61 riboprobe (nt539-808 of the human Cyr61 complementary DNA [cDNA] sequence), a 254-base TF riboprobe (nt765-1019 of the human TF cDNA sequence), and a 177-base antisense glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobe (nt634-811 of the human GAPDH cDNA sequence) as an internal standard. Labeled riboprobes were generated with either T3 or T7 RNA polymerase, and RNase protection assays were performed as described elsewhere,31 with some modifications. (α-[32P]Juridine trithiophosphate–radiolabeled riboprobes were hybridized to 20 µg of total RNA in solution, followed by RNase digestion with RNase A (50 µg/mL) (Sigma–Aldrich Corp, St Louis, Mo) and RNase T1 (750 U/mL) (Boehringer Mannheim Biochemicals, Indianapolis, Ind). Transcipt levels were compared between samples as a ratio to GAPDH.

**WESTERN BLOTTING**

Normal uveal melanocytes and melanoma cells were lysed in 10mM Tris buffer, pH 6.8, in the presence of protease inhibitors, sonicated, and then boiled in a solubilization buffer containing sodium dodecyl sulfate and β-mercaptoethanol. Proteins were resolved using a sodium dodecyl sulfate–15% polyacrylamide gel. (Protein derived from 100000 cells was loaded per lane.) Nonspecific sites on blots were saturated by sequential incubation with 5% wt/vol nonfat dry milk and 5% wt/vol bovine serum albumin in TBST (10mM Tris-Cl, pH 8.0; 150mM sodium chloride; and 0.05% vol/vol Tween-20). Blots were incubated with primary antibodies (mouse anti–TF IgG [No. 4509; American Diagnostica, Greenwich, Conn], rabbit anti–Cyr61 antisemur [Mumin Corp, Chicago, Ill]; and mouse anti–GAPDH IgG [BioGenes, Inc, Kingston, NJ]) for 1 hour. After washing, blots were incubated for 1 hour with a horseradish peroxidase–conjugated goat anti-IgG (Jackson Laboratories, West Grove, Pa), diluted 1:10000. Antibody binding was detected by chemiluminescence according to the manufacturer’s procedures (Amersham Pharmacia Biotech, Piscataway, NJ). Glyceraldehyde-3-phosphate dehydrogenase immunostaining served as an internal control to verify uniform sample loading.

**CYTOGENETIC STUDIES**

Chromosome studies were performed by exposing proliferating cells for 30 minutes to colcemide (0.1 µg/mL final concentration). The cultures were then treated with a hypotonic solution of 0.075M potassium chloride for 20 minutes, followed by fixation in 3:1 methanol glacial acetic acid. The slides were banded by immersion in a 1% trypsin solution (Sigma–Aldrich Corp) for 25 seconds at room temperature, rinsed in tap water, air dried, and immersed in Leishman stain for 2 minutes, rinsed, and air dried. Twenty well-banded metaphase cells were examined under a microscope, photographed, and karyotyped.

Fluorescent in situ hybridization analysis, including controls, was performed essentially following published procedures32 using paraffin sections of the original Mel 290 tumor specimen and a digoxigenin-labeled 1.8-kilobase TF cDNA probe. Binding was detected using a fluorescein isothiocyanate conjugated–labeled antidigoxigenin antibody (Ventana Medical Systems, Tuscon, Ariz).

**REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION**

RNA was isolated from biopsy samples of uveal melanoma obtained through the oculoplastics service of the Department of
Ophthalmology and Visual Sciences, University of Wisconsin Hospital. (Portions of the tumor also were processed for routine pathological examination.) The RNA was treated with DNase I and reverse transcribed with oligo(dt) primers using MMLV reverse transcription followed by polymerase chain reaction (RT-PCR) amplification using specific primers for TF (forward, 5'-TAA CGC GAG TAC AGA CAG-3'; reverse, 5'-AAG TCC TCG GTC ACA GTG CA-3'), Cyr61 (forward, 5'-GTT TCC AGC CCA ACT GTA AAC ATC-3'; reverse, 5'-TTT CTC GTC AAC TCC ACC TCG GAG-3'), VEGF (forward, 5'-TCA CGA AGT GGT GAA GTT CAT GG-3'; reverse, 5'-AAG CTC ATC TCT CCT ATG TGC TGG-3'), and GAPDH (forward, 5'-TCC GGG AAA CTG TGG GTG CAT-3'; reverse, 5'-TTT CTA GAC GGC AGG TCA GTG-3'). Aliquots were removed at 15, 20, 25, and 30 cycles and subjected to electrophoresis. The relative amount of each cDNA was compared between cell lines in a linear range using GAPDH to standardize.

Archival Tissue

Recent studies demonstrate that RNA can be extracted from paraffin-embedded tissue samples for subsequent PCR amplification.34-37 Briefly, uveal tumor tissue was excised from an alternate hematoxylin-eosin-stained section for orientation. Procedures essentially followed those of Stanta and Schneider.35 Primers specific for Cyr61 (forward, same as for fresh tissue; reverse, 5'-TAA CTT TGA CCA GCC GAG GGT T-3'), TF (forward, 5'-GAA CCC AAA CCC GTC AAT CAA-3'; reverse, 5'-GGT GAG GAG ACA CTC TGT GT-3'), VEGF (same as for fresh tissue), and GAPDH (forward, 5'-ACC ATG GGG AAG GTG AAG G-3'; reverse, 5'-CAT TGA TGG CAA CAA TAT CCA C-3') were chosen that spanned a region of each gene separated by an intron.

IMMUNOHISTOCHEMICAL ANALYSIS

Sections (5 µm) of uveal melanoma were deparaffinized with xylene, rehydrated in a graded series of ethanol, and endogenous peroxidase activity reduced by washing with 0.3% methanolic hydrogen peroxide. In some experiments, duplicate slides were incubated overnight with primary antibody, 1 µg/mL (enzyme substrate reaction (EPR) was performed using superpredominant dilution of antibody). Typically, Vector VIP (Vector Laboratories, Burlingame, Calif) and streptavidin-HRP complex, 1 µg/mL (Amersham Pharmacia Biotech). Typically, Vector VIP (Vector Laboratories) was used as the substrate to optimize differences between the reaction product (blue/purple) and background melanin. Tissue factor immunostaining was scored as high or low by 2 observers (R.L.G., H.A.), and the number of blood vessels in alternate sections was counted in 3 separate fields (original magnification ×20) per duplicate tumor section. The evaluation was masked, whereby specimens were coded separately for TF immunostaining and blood vessel counting. Duplicate sections from 9 tumor specimens were evaluated in this manner. The protocol for accessing tumor specimens was approved by the institutional review board at the University of Wisconsin.

RESULTS

To identify differentially expressed genes that may contribute to the malignant and metastatic phenotypes associated with human uveal melanoma, the method of suppression subtractive hybridization was initially used to compare messenger RNA patterns between normal uveal melanocytes and Mel 290, an established epithelioid cell line derived from a biopsy specimen of human uveal melanoma. Two genes identified by this approach, Cyr61 and TF, are known to be expressed in a variety of cancer cells, and their expression correlates with the angiogenic phenotype and poorer prognosis.19,24,25

The differential expression of Cyr61 and TF was confirmed initially by Western blot analysis (Figure 1). An immunoreactive band corresponding to TF was detected at the appropriate relative molecular mass (47 kd) in Mel 290 but not in lysates of normal uveal melanocytes and Mel 270 spindle cells. Similar results were obtained with antibodies to Cyr61 (relative molecular mass, 41 kd [Figure 1]). GAPDH immunostaining served as an internal control to verify uniform sample loading in the same series of immunoblots.

To determine whether the differential expression of Cyr61 and TF could be explained by a difference in the relative amounts of transcript, an RNase protection assay was performed using RNA obtained from normal uveal melanocytes, Mel 270 spindle cells, and Mel 290 epithelioid cells (Figure 2). The Cyr61 and TF transcript levels were compared between samples as a ratio to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).
munoblotting analysis and the suppression subtractive hybridization experiment, which selected for elevated gene expression in Mel 290.

The cytogenetic analysis of Mel 290 revealed an altered female karyotype (Figure 3): 46,XX,del(1)(p22.3p34.3), der(8)t(1;8)(p13;q24.3). Mel 290 was initially thought to display a terminal deletion of chromosome 1 at p32 and the translocation of the entire short arm of chromosome 1 to the terminal long arm of chromosome 8. However, use of fluorescent in situ hybridization with a probe for 1p36 demonstrated that the terminal region was present and, therefore, that the chromosome 1 deletion was interstitial, involving loss of 1p22.3→p34.3. Therefore, the translocation of the entire chromosome 1 short arm to chromosome 8 resulted in duplication of 1p13.1→1p22.3 and 1p35→pter. Other than this region, the karyotype was balanced, all of the cells demonstrated the same karyotype, and the cell line remained relatively stable.

Tissue factor maps to band 1p21-1p22, and Cyr61 similarly maps to 1p22.3. Duplicate of these genes due to a chromosomal imbalance, therefore, may explain the elevated levels of Cyr61 and TF measured in the epithelioid cells of Mel 290. Alternatively, genetic events not directly arising in this region of chromosome 1 may nonetheless induce expression of Cyr61 and TF. These structural changes in the Mel 290 cell line reflect alterations in the original patient tumor from which the cell line was derived. Fluorescent in situ hybridization analysis demonstrated 3 copies of the TF gene in a population of tumor cells (Figure 3, inset), confirming the duplication of this region of chromosome arm 1p in the original patient specimen. Some cells in the tumor specimen, however, had fewer TF fluorescent signals, indicating either heterogeneity in the tumor or, more likely, that chromosomes in these cells were confined to a different plane of section.

The independent cytogenetic findings from Mel 290 are consistent with the outcome of the suppression subtractive hybridization experiment and the data obtained from the RNase protection assays, RT-PCR, and Western blot analysis that expression of Cyr61 and TF is enhanced in Mel 290.

To ensure that expression of Cyr61 and TF is not the result of in vitro culture conditions but rather reflects actual occurrences in primary tumor tissues, fresh biopsy samples and archival specimens of uveal melanoma were analyzed for expression of Cyr61 and TF. Figure 4 illustrates the results from RT-PCR experiments using 9 different archival specimens of uveal melanoma that had been fixed in formalin and embedded in paraffin. Cyr61 and TF were detected in all 9 samples; thus, expression of these 2 factors occurs in primary tumors and is not simply the result of culture conditions.

Expression of VEGF was measured in each specimen as well, but the detection was less pronounced. In addition to archival specimens, expression of each of these angiogenic factors was demonstrated by RT-PCR using RNA obtained from fresh biopsy samples of uveal melanoma (Figure 4B). Glyceraldehyde-3-phosphate dehydrogenase amplification from each sample served as an internal control to verify uniform sample loading.
In further immunohistochemical experiments, duplicate sections from 9 tumor specimens were stained with antibodies specific for TF, and alternate sections from the same specimens were stained with CD34 antibodies to help delineate blood vessels. First, the immunostaining associated with TF was uniformly associated with tumor cells and not with a subpopulation of other cell types (Figure 5A, purple stain). Next, TF immunostaining was rated as high or low by 2 independent observers (R.L.G., H.A.), the number of blood vessels was counted, and the 2 variables were compared (Figure 5). Five specimens were rated high for TF immunoreactivity and had a mean ± SD blood vessel count of 35.0 ± 16.8, and 4 specimens with low TF immunoreactivity had a mean ± SD blood vessel count of 1.5 ± 1.4. These data demonstrate a correlation between TF and the number of blood vessels. A larger sampling of specimens is now being studied to corroborate these initial findings, and similar studies with Cyr61 are also being conducted.

**COMMENT**

Like most solid tumors, uveal melanomas may require a new blood supply to grow beyond 1 to 2 mm. Because the eye lacks lymphatics, permeable blood vessels also form the primary route by which tumor cells enter the circulation, escape the eye, and disseminate to distant regions of the body. Most research has focused on VEGF as the principal angiogenic factor contributing to tumor neovascularization in the eye. More extensive investigations have been conducted on the role of VEGF in blinding diseases with associated neovascularization, as in diabetic retinopathy, retinopathy of prematurity, and age-related macular degeneration (for a review, see Aiello). However, it is unclear whether uveal melanoma tumor
cells express VEGF in a manner that can fully explain the proliferation and morphogenesis of vascular endothelial cells requisite for new blood vessel formation extending throughout the tumor mass. Instead, these tumor cells may express multiple angiogenic factors that independently or together with VEGF regulate new vessel formation. In addition to conventional angiogenesis, these same factors may support vascular mimicry, an alternative source of tumor circulation.

In this article we demonstrate expression of Cyr61 and TF, 2 angiogenic factors that contribute to tumor growth and patient prognosis in other types of cancer. These factors have not been studied extensively in the eye. Neither factor is expressed in normal uveal melanocytes, but several independent measurements reported herein demonstrate their simultaneous expression in the more malignant epithelioid cell line used in the initial subtractive hybridization experiments that revealed their identity. In addition, genes encoding these 2 factors map to a region of chromosome arm 1p that is duplicated in the same epithelioid cell line, possibly explaining their elevated expression. This region of 1p may also harbor genes related to the initial events of transformation, since no other structural mutations thus far have been associated with Mel 290. Newer techniques, such as comparative genomic hybridization, however, have further resolving power and may detect additional mutations. Of further significance, Cyr61 and TF can be detected in fresh biopsy samples and archival specimens of uveal melanoma by molecular methods and by immunohistochemical techniques, reducing concerns about artifacts from culture conditions. Although RNA protection assays demonstrated higher levels of expression of Cyr61 and TF in epithelioid cell lines than did spindle cells, more extensive amplification by RT-PCR revealed expression of both factors in archival specimens of spindle cell and epithelioid cell uveal melanomas. More quantitative methods need to be applied to archival specimens to determine whether there is a precise correlation between the level of these specific transcripts and the tumor cell type.

Interest recently has been raised in the selective expression of TF in tumor blood vessels and not mature vessels, since TF can be used as a target for immunotherapy. A similar approach could be applied to the treatment of uveal melanoma, since we demonstrated its expression in tumor cells but not in melanocytes. Immunotherapies directed toward the tumor circulation also could augment other types of treatment.

Ultimately, to determine whether TF, Cyr61, and VEGF contribute to new vessel formation, it will be necessary to alter the expression of each of these factors alone or in combination with one another in cell lines of uveal melanoma and then to evaluate vascular density and tumor growth in animals inoculated with these transfected cell lines. Elucidation of the pathways involving Cyr61, TF, and other angiogenic factors will provide new opportunities for intervening in the growth and progression of uveal melanoma.

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