Abstract

We recently identified genes encoding tumor endothelial markers (TEMs) that displayed elevated expression during tumor angiogenesis. From both biological and clinical points of view, TEMs associated with the cell surface membrane are of particular interest. Accordingly, we have further characterized four such genes, TEM1, TEM5, TEM7, and TEM8, all of which contain putative transmembrane domains. TEM5 appears to be a seven-pass transmembrane receptor, whereas TEM1, TEM7, and TEM8 span the membrane once. We identified mouse counterparts of each of these genes, designated mTEM1, mTEM5, mTEM7, and mTEM8. Examination of these mTEMs in mouse tumors, embryos, and adult tissues demonstrated that three of them (mTEM1, mTEM5, and mTEM8) were abundantly expressed in tumor vessels as well as in the vasculature of the developing embryo. Importantly, expression of these mTEMs in normal adult mouse tissues was either undetectable or detected only in a small fraction of the vessels. These results demonstrate conservation of human and mouse tumor angiogenesis at the molecular level and support the idea that tumor angiogenesis largely reflects normal physiological neovascularization. The coordinate expression of TEM1, TEM5, and TEM8 on tumor endothelium in humans and mice makes these genes attractive targets for the development of antiangiogenic therapies.

Introduction

Inhibition of tumor angiogenesis as an anticancer strategy has generated much excitement among cancer researchers and clinicians. This enthusiasm stems from several theoretical advantages of targeting the endothelial cells that line tumor vessels rather than the tumor cells themselves (reviewed in Refs. 1 and 2): (a) targeting endothelial cells rather than tumor cells obviates many of the pharmacokinetic problems associated with drug delivery (3); (b) a significant bystander effect can also be expected because each endothelial cell supports the growth of many tumor cells; and (c) targeting the genetically stable endothelial cells should reduce the likelihood of developing resistant disease and should be applicable to a wide variety of tumor types (4, 5). However, realization of the full potential of antiangiogenic approaches will require a better understanding of the molecular differences between normal and tumor vessels, effective strategies to exploit these differences, and model systems in which to evaluate them.

To further understand human tumor angiogenesis, we recently conducted an unbiased gene expression analysis of endothelial cells isolated from normal human colonic tissue or from human colorectal cancers (6). This analysis identified 46 transcripts, named TEMs,3 which were significantly up-regulated in tumor compared with normal endothelium. The majority of these genes had not been characterized previously. Expression of several of these TEMs in tumor endothelium was confirmed by reverse transcription-PCR and in situ hybridization. TEMs localized on the cell surface and conserved across species are of particular interest for future therapeutic approaches for two reasons: (a) cell surface targets are directly accessible via the bloodstream, facilitating detection as well as intervention with both small molecules and macromolecular compounds; and (b) conservation of such cell surface proteins allows the establishment of model systems that are required for preclinical development and testing. In this regard, rodent tumor models are the gold standard, but the extent of overlap between tumor angiogenesis in rodents and humans is unclear.

With these principles in mind, we set out to identify a series of cell surface TEMs that were structurally and functionally conserved in mouse and human tumor endothelium. To find such genes, we expanded the sequence of the most differentially expressed novel TEMs and used hydrophobicity plots to predict cell surface localization. We then identified and determined the sequence of the mouse counterparts of these genes and used in situ hybridization to examine mRNA expression patterns in murine tumors, embryos, and adult tissues. These studies identified three cell surface TEMs that had similar properties in both species and provide new evidence that angiogenesis in tumors is similar to normal developmental angiogenesis.

Materials and Methods

Identification of Human TEMs. Partial sequences were completed by performing 5' RACE with the Marathon cDNA Amplification kit (Clontech, Palo Alto, CA) according to the manufacturer’s protocol with Marathon-Ready cDNA from human fetal brain (Clontech) as a template. RACE products were sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) and analyzed in an ABI 3700 automated sequencer. Extending the EST sequences identified previously by the TEM3 tag revealed this to be a longer version of the TEM7 transcript, derived from a second polyadenylation site. TEM7 EST sequences were identified by homology to TEM7 using tBLASTn of the National Center for Biotechnology Information database, and the complete sequence was identified using RACE.

Identification of Mouse TEMs. Mouse ESTs homologous to human TEM1, TEM5, TEM7, and TEM8 were identified using BLASTn and tBLASTn searches. Extensive 5' and 3' RACE analysis was performed using the Marathon cDNA Amplification kit (Clontech) to identify the complete sequences. RACE was performed using mouse specific internal primers and Marathon-Ready cDNA prepared from a xenografted human lung cancer (Clontech).
buffer [10 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 1 mM EDTA], sections were incubated at 37 °C with RNase mixture (Ambion, Austin, TX) diluted 1:35 in TNE.

Results

Identification of Cell Surface TEMs. Using SAGE technology, we previously identified partial cDNAs corresponding to several novel genes (TEM1–8) that were expressed in tumor endothelial cells (6). Because many cell surface proteins have their signal sequences at the NH2 terminus, it was important to obtain full-length clones to determine which of these would likely be located on the cell surface. The 15-bp SAGE tags from the eight most abundantly expressed novel TEMs were used to identify corresponding 3' EST clones, and then a combination of in silico cDNA walking and 5' RACE was used to derive sequences covering the entire coding region. Four TEMs (TEM1, TEM5, TEM7, and TEM8) had sequence characteristics, indicative of cell surface proteins (Fig. 1 and Table 1). Additionally, the presence of the signal peptides confirmed that we had identified the complete open reading frames of these genes. Each of these four TEMs was unique with respect to each other and to other proteins in the databases.

TEM1 was predicted to encode a type I transmembrane protein of 757 amino acids (Table 1). The majority (685 amino acids) of the sequence was predicted to be extracellular, with only a short COOH-terminal cytoplasmic tail. A homology search revealed that the extracellular region of TEM1 has three EGF-like domains, as well as a C-lectin-like carbohydrate recognition domain with similarity to thrombomodulin (Fig. 1). In addition, amino acids 164–230 bear weak homology to a Sushi/SCR/CCP domain.

TEM5 is predicted to encode a seven-pass transmembrane protein of 1331 amino acids (Table 1). The hydrophobic domains lie within a 300-amino acid region that shares homology with seven-pass transmembrane proteines of the secretin family (class II) of GPCRs (13), suggesting that TEM5 also may be a GPCR. In the NH2-terminal extracellular region, TEM5 contains four simple LRRs, one LRR of the COOH-terminal type, one immunoglobulin-type domain, and a hormone-receptor domain (Fig. 1). The 300-amino acid region containing the LRRs shares homology with the membrane glycoprotein, known as LIG-1 (14), and the secreted SLIT proteins (15). In the extracellular region immediately adjacent to the first transmembrane domain, TEM5 contains a putative GPCR proteolysis site (16) domain typical of class II family members and required for endogenous proteolysis (Ref. 17; Fig. 1). Within its seven-pass transmembrane region, TEM5 is most similar to the cadherin-related Celsr1 proteins (18–20) and members of the calcium-independent a-latrotoxin receptor family (21). Taken together, these data suggest that TEM5 is likely to be a novel member of the GPCR superfamily involved in transmitting signals across the cell membrane, although its function as a G-protein coupled receptor remains to be proven.

Similar to TEM1, TEM7 was found to encode a type I transmembrane protein with a large extracellular domain, a hydrophobic transmembrane domain, and a short cytoplasmic tail (Fig. 1 and Table 1). The extracellular region of TEM7 contains a plexin-like domain and has weak homology to the ECM protein nidogen. The function of these domains, which are usually found in secreted and extracellular matrix molecules, is unknown. Interestingly, during the course of performing 5' RACE to extend the sequence identified previously by the TEM3 SAGE tag, we obtained sequences of the gene we had identified previously as TEM7. Further investigation revealed that TEM3 and TEM7 represent alternative transcripts of the same gene, including signal recognition sequences and transmembrane domains, indicative of cell surface proteins (Fig. 1 and Table 1). Additionally, the presence of the signal peptides confirmed that we had identified the complete open reading frames of these genes. Each of these four TEMs was unique with respect to each other and to other proteins in the databases.

Bioinformatics. Amino acid alignments were performed using the ClustalW program (7). Hydrophobicity plots were created using DAS software (8). Signal peptides were determined using the SignalP program (9). Domain structures were found using SMART software (10) and Pfam software (11). Signaling sites were predicted using ScanSite software (12).

In Situ Hybridization. DIG-labeled antisense RNA probes were generated by PCR amplification of 500–600-bp products incorporating T7 promoters into the antisense primers. In vitro transcription was performed with DIG RNA labeling reagents and T7 RNA polymerase according to the manufacturer's instructions (Roche, Indianapolis, IN). Tumors and normal tissues were dissected, embedded in OCT, frozen in a dry ice-methanol bath, and cryosectioned at 7 μm. Embryos were prefixed in buffered 4% paraformaldehyde, infused with 20% sucrose overnight, rinsed in PBS, embedded in OCT, and cryosectioned. All sections were immediately fixed with 4% paraformaldehyde, permeabilized with pepsin, blocked with ISH solution (Dako, Carpenteria, CA), and incubated with RNA probes (100 ng/ml) overnight at 55°C. After washing twice in 2× SSC and then once in TNE buffer [10 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 1 mM EDTA], sections were incubated at 37°C with RNase mixture (Ambion, Austin, TX) diluted 1:35 in TNE. Slides were stringently washed twice in 2× SSC/50% deionized formamide (American Bioanalytical, Natick, MA) and then once with 0.1× SSC at 55°C. Before immunodetection, tissues were treated with peroxidase blocking reagent (DAKO) and blocked with 1% blocking reagent (Roche; DIG Nucleic Acid Detection kit) containing purified, nonspecific rabbit immunoglobulins (DAKO). For signal amplification, a horseradish peroxidase-rabbit anti-DIG antibody (DAKO) was used to catalyze the deposition of Biotin-Tyramide (GenPoint kit; DAKO). Further amplification was achieved by adding horseradish peroxidase-rabbit anti-biotin (DAKO), biotin tyramide, and then alkaline phosphatase rabbit anti-biotin (DAKO). Signal was detected with the alkaline phosphatase substrate Fast Red TR/Napthol AS-MX (Sigma Chemical Co., St. Louis, MO). All sections were exposed for 10 min. Cells were counterstained with hematoxylin and mounted with Crystal/Mount (Biomeda, Foster City, CA).

Table 1. Structural characteristics of TEMs

<table>
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<tr>
<th>Name</th>
<th>Accession no.</th>
<th>Signal peptide</th>
<th>TM domains</th>
<th>AA* length</th>
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*a AA, amino acid.

** TEM3 encodes an alternate transcript of TEM7.
with the differences simply attributable to the use of alternative polyadenylation sites. Because the predicted open reading frame of the two transcripts was the identical 500-amino acid protein, we refer to the gene product of both as TEM7.

Finally, completion of the TEM8 sequence also revealed a type I transmembrane protein (Fig. 1 and Table 1), 564 amino acids in length. The 220-amino acid cytoplasmic tail of TEM8 is much larger than that of the other cell surface TEMs. In the extracellular region, TEM8 was found to contain a vWF A domain containing a metal ion-dependent adhesion motif (MIDAS; Refs. 22 and 23). The vWF A domain is also known as an I-domain when present in integrins (24). TEM8 was found to contain a vWF A domain containing a metal ion-dependent adhesion motif (MIDAS; Refs. 22 and 23). The vWF A domain is also known as an I-domain when present in integrins (24). The TEM8 domain is most similar to that of D integrin, which has plexin-like domains lie within the conserved NH2-terminal region. The cytoplasmic tail is also conserved but is unrelated to other known proteins. Importantly, TEM7R and mTEM7R both contain putative signal peptides and transmembrane regions in the same relative positions as TEM7.

**TEMs in Tumor Angiogenesis.** To confirm the mRNA localization of the full-length cell surface TEMs in tumor endothelium and to explore the expression of TEM7R, we modified our nonradioactive *in situ* hybridization technique to achieve increased sensitivity (see “Materials and Methods” for details). Acquisition of the complete sequences for TEM1, TEM5, TEM7, TEM7R, and TEM8 allowed us to compare multiple RNA probes, further improving the sensitivity of the *in situ* hybridization protocol. As controls, various endothelial cell markers, such as *vWF*, *CD31 (PECAM)*, VE-cadherin, P1H12, and VEGFR2, were used. Classic endothelial cell markers such as *vWF*, *CD31* and VE-cadherin were detected predominantly in the larger vessels. Although P1H12 detected many microcapillaries, VEGFR2 was the best pan endothelial marker and appeared to detect most microvessels in addition to the larger vessels. Thus, we chose to use VEGFR2 as our positive control.

The *in situ* hybridization analysis of human colorectal cancer demonstrated that all four cell surface TEMs and TEM7R were expressed clearly in the endothelial cells of the tumor stroma but not in the endothelial cells of normal colonic tissue (Fig. 2). Interestingly, all TEMs demonstrated local regions of intense staining throughout the stromal compartment. The microcapillaries were likely to account for much of the staining, because vascular casting techniques have demonstrated the presence of a virtually continuous layer of anastomizing vessels throughout the lamina propria in advanced colorectal cancers (27). However, we cannot rule out the possibility that other stromal cells (*e.g.*, fibroblasts) also expressed these TEMs, although staining was not observed in normal colonic mucosa (Fig. 2).

To analyze expression of mTEMs in murine tumors, B16 mouse melanoma or HCT116 human colon carcinoma cells were implanted s.c. into mice and used for *in situ* hybridization studies. As shown in Fig. 3 and Table 2, mTEM1, mTEM5, and mTEM8 were abundantly expressed in vessels infiltrating both B16 and HCT116 tumors. Unexpectedly, we were unable to detect significant levels of mTEM7 in vessels of either tumor type, despite the fact that its human counterpart, TEM7, was abundantly expressed in human tumor endothelial cells when assessed with the same *in situ* hybridization methods (Fig. 2 and data not shown). Importantly, the lack of mTEM7 signal in tumor vessels was unlikely to be attributable to technical problems, because other cell types were clearly positive (see below). In contrast
to mTEM7, expression of mTEM7R was readily detectable in tumor endothelium (data not shown).

**TEMs in Normal Adult Tissue.** We next assayed expression of mTEMs in various normal adult tissues by in situ hybridization. VEGFR2 was used as a positive control for endothelial cells. Expression of all five TEMs (red stain) was highly specific to tumor endothelial cells and was not detected in the endothelial cells of the normal colonic mucosa. The faint red extracellular staining around the crypts represents nonspecific binding of the in situ hybridization reagents to the mucous. Sections were counterstained with hematoxylin (blue). Bars, 100 μm.

![Fig. 2. TEMs in normal human colon and colorectal carcinoma tissues. Expression of TEM1, TEM5, TEM7, TEM7R, and TEM8 was assessed by in situ hybridization. VEGFR2 was used as a positive control for endothelial cells. Expression of all five TEMs (red stain) was highly specific to tumor endothelial cells and was not detected in the endothelial cells of the normal colonic mucosa.](image)

Discussion

The extent of overlap between tumor angiogenesis in rodents and humans is unclear. Although some cell surface proteins have been identified as important regulators of angiogenesis in both in mice and men...
Fig. 3. mTEMs in mouse tumors. A, expression of mTEM1 in B16 melanoma tumor endothelial cells and HCT116 colon tumor endothelial cells was assessed by in situ hybridization. mTEM1 staining is specific to the endothelial cells of the vessels and microcapillaries, as shown in a low-power field (left) and a high-power field (right). B, expression of mTEM5 and mTEM8 in HCT116 tumor endothelial cells. Note that staining is localized to the endothelial cells. Bars, 100 μm.

(e.g., VEGFR1, VEGFR2, Tie-1, and Tie-2), others may be more species specific. For example, in humans Thy-1 appears to be predominantly expressed in angiogenic vessels, whereas in mice, its expression is most obvious in hematopoietic cells, especially T-lymphocytes (6, 28, 29). The pronounced effects of angiogenesis inhibitors observed in rodent models have, to date, been much less impressive in humans (30). This could be related to the degree of angiogenesis in humans versus mice, species-specific differences in drug sensitivity and tolerance, or species-specific expression of genes regulating tumor angiogenesis.

Here we report the complete coding sequences of four abundant and
differentially expressed TEMs predicted to contain hydrophobic transmembrane domains. The mouse TEM counterparts we identified show a high level of identity with their respective human TEMs, ranging from 77 to 96%. These similarities and conserved expression patterns suggest that we have cloned the mouse orthologues of human TEM1, TEM5, TEM7, TEM7R, and TEM8. Currently, each of the cell surface TEMs has unique features that make it attractive for further investigation.

TEM1 was the most differentially expressed TEM identified previously in our original SAGE analysis (6). The recent purification of endosialin, a glycoprotein recognized by the FB5 antibody, revealed an amino acid sequence identical to TEM1 (31). Immunostaining with FB5 was specific for tumor microvessels, because a panel of normal tissues was negative for TEM1/endosialin immunoreactivity (32). Thus, TEM1/endosialin appears to be differentially expressed at both the mRNA and protein levels. Interestingly, Rettig et al. (32) reported that radiolabeled FB5 was rapidly internalized into TEM1/endosialin expressing endothelial cells. If this is the case, it may be possible to deliver compounds to TEM1/endosialin-expressing cells for selective uptake.

TEM5 appears to be a member of the class II GPCR family.GPCRs have a history of being excellent drug targets because their natural ligands can often be mimicked for agonistic or antagonistic purposes (33). Other class II GPCRs bind peptide ligands, such as secretin, calcitonin, and vasoactive intestinal peptide, and activate adenyl cyclase and inositol phosphate signaling cascades (34, 35). These findings tentatively suggest that TEM5 may also transmit signals into the cell, but the signaling partners of TEM5 remain to be determined.

In the current study, we found that the human TEM7 gene encodes two transcripts that differed only in the length of their 3′ untranslated region. The murine and human TEM7 proteins are over 80% conserved, but despite these similarities, transcripts of mTEM7 were not detectable by in situ hybridization in mouse tumors (Table 2 and data not shown). There are several possible explanations for this. mTEM7, through the course of evolution, could have acquired different functions than human TEM7. Support for this possibility comes from the specific expression of mTEM7 in Purkinje cells of the mouse brain. Alternatively, there may exist an as yet unidentified mouse counterpart with greater homology than mTEM7 or mTEM7R. Although we cannot formally rule out this possibility, it seems unlikely because we were unable to identify more highly related homologues in EST or mouse genomic databases. The lack of expression of mTEM7 in mouse tumor vessels highlights potentially important differences between mouse and human tumor angiogenesis.

TEM8 is the most highly conserved cell surface TEM, with 96% amino acid identity between the human and mouse proteins. The large cytoplasmic tail of both the human and mouse TEM8 proteins share at least seven potential phosphorylation sites, supporting the hypothesis that TEM8 is involved in transmitting signals into the cell. The expression pattern of TEM8 was especially intriguing in that it is the only human TEM characterized thus far that shows no detectable mRNA expression in either the corpus luteum or healing wounds, suggesting that this gene may be highly specific to tumor angiogenesis and not required for “normal” adult angiogenesis (6). From a clinical point of view, this could be important in designing gene-targeting strategies with the fewest cross-reactivities.

The fact that mTEM1, mTEM5, and mTEM8 were present in the vasculature of developing embryos as well as in the vessels of transplanted syngeneic and human tumors is consistent with the idea that they are markers of neoangiogenesis and not limited to tumor angiogenesis. The data further support the hypothesis that tumor endothelium exploits many of the same genes used by normal developing endothelium.

Our detailed comparison of TEM expression in human and rodent angiogenesis is provocative in that it demonstrates that three of the four cell surface TEMs identified in humans are also expressed in
mouse tumors. The uniqueness of these TEMs was emphasized by comparison with VEGF2, believed to be one of the most specific markers for tumor angiogenesis available. We found that VEGF2 transcripts were expressed in the endothelium of all normal adult tissues studied, whereas expression of the cell surface TEMs was largely restricted to the vasculature of tumors in adult animals. The high degree of sequence conservation of these TEMs, in combination with their restricted expression patterns, suggests that they may be critical regulators of angiogenesis. Continued investigation of all four cell surface TEMs should foster better understanding of the mechanisms of tumor angiogenesis and encourage rational design of interventional strategies.

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References

Cell Surface Tumor Endothelial Markers Are Conserved in Mice and Humans


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