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TEM8 expression stimulates endothelial cell adhesion and migration by regulating cell–matrix interactions on collagen

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Abstract

The TEM8 gene is selectively expressed in tumor versus normal blood vessels, though its function in endothelial cell biology is not known. Towards the goal of clarifying this function, we tested whether TEM8 overexpression, or blocking TEM8's function with a dominant negative protein, would modulate endothelial cell activities. We found that TEM8-expressing endothelial cells migrated at a rate 3-fold greater than control cells in a monolayer denudation assay. Also, the addition of recombinant TEM8 extracellular domain (TEM8-ED) specifically inhibited both chemokinetic and chemotactic migration on collagen in the denudation and Boyden chamber assays, respectively. The TEM8-ED binds preferentially to collagen, and TEM8 expression enhanced endothelial adhesion to collagen 3-fold; the latter response was antagonized by the TEM8-ED. Consistent with the TEM8-ED acting as a dominant negative inhibitor of endogenously expressed protein were data showing that the TEM8-ED had no effect on the activation of β1 integrin. TEM8 protein is present in human umbilical vein in situ and is expressed in low passage HUVEC in vitro. TEM8 protein expression in HUVEC was increased 5-fold by the initiation of tube formation, correlating expression of TEM8 with the angiogenic response. Taken together, these results indicate that TEM8 plays a positive role in endothelial cell activities related to angiogenesis.

Keywords: TEM8; Angiogenesis; Endothelial cells; Cell migration; Cell adhesion

Introduction

Angiogenesis, the growth of new blood capillaries from existing vessels, is essential for embryonic development, tissue regeneration and remodeling [1,2], and contributes to many pathologic conditions including tumor growth and metastasis [3,4]. Angiogenesis is a complex multistep process involving degradation of the extracellular matrix (ECM), endothelial cell (EC) migration, proliferation, and re-differentiation into patent vessels [1,2]. While these processes are common to neovascularization in all tissues, the tumor vasculature is phenotypically and structurally abnormal. Tumor vessels are highly disorganized, tortuous, and dilated, with excessive branching and shunts [5]. The endothelial cells (ECs) associated with these vessels have numerous fenestrae, abnormally large inter-endothelial cell junctions, and do not always exist as a monolayer. Moreover, the vessels often have a discontinuous basement membrane, and peri-vascular support cells, such as pericytes and smooth muscle cells, are often absent. These aberrant characteristics result in an abnormally leaky network of vessels in the tumor.

These phenotypic changes, triggered by the local tumor microenvironment, are accompanied by genetic

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changes that up-regulate molecular markers on angiogenic ECs that do not exist on resting ECs or other cells. A variety of experimental strategies have been used to identify differentially expressed genes in angiogenic/tumor-derived versus quiescent ECs [6,7]. The nine Tumor Endothelial Markers (TEMs) are one such group of genes discovered by SAGE analysis of human colon cancer [6]. TEM8 contains a single membrane spanning domain, and a 220-amino acid intracellular domain that does not contain consensus sequences for any known structural or functional polypeptides. The TEM8 extracellular domain (ED) contains a region that is highly related to von Willebrand factor type A (vWA) domains. This domain is a well-characterized protein interaction site in α-integrins and many ECM components [8]. The TEM8 gene is expressed as three alternatively spliced transcripts [9] that share a common ED. The original cDNA transcript was designated splice variant 1 (SV1). Splice variant 2 contains the membrane spanning region and only a short cytosolic tail, while SV3 diverges just before the transmembrane region such that it does not contain a recognizable membrane anchoring sequence. SV2 was identified by an expression cloning strategy to be a receptor for the PA subunit of anthrax toxin and has been designated as anthrax toxin receptor or ATR [9].

TEM8 (SV1) mRNA is highly expressed in colon tumor vasculature but undetectable in ECs from healthy tissue. Expression of the mouse homologue is up-regulated in the vasculature of the developing embryo, indicating that TEM8 may have an important role in neovascularization [10]. Considering the potential for interaction between the vWA domain of TEM8 and the ECM, these data suggest a principal role for TEM8 in modulating angiogenesis. However, the functional roles played by the TEM gene products in EC biology are not currently known. We report that TEM8 protein is expressed on the EC of human umbilical vein in situ and in vitro, and is up-regulated 5-fold in HUVEC undergoing differentiation/tube formation on collagen. In EC engineered to express TEM8 (SV1), migration was increased 3-fold but no effect was observed on proliferation, in vitro differentiation on Matrigel, or the ability of EC to survive an apoptotic challenge. We also observed that the ED of TEM8 preferentially binds collagen 1 and that attachment of EC expressing TEM8 is enhanced 3-fold on collagen-based matrices exclusively. The use of a recombinant TEM8 extracellular domain (TEM8-ED) as a dominant negative inhibitor of TEM8 function confirmed that the endogenous expression of TEM8 in HUVEC does contribute significantly to the angiogenic response. The TEM8-ED ablated adhesion of HUVEC to and motility on collagen-based matrices. Together, these data suggest that TEM8 plays a prominent role in promoting EC activities contributing to neovascularization.

Materials and methods

Isolation of endothelial cells and cell culture

Human venous endothelial cells (HUVEC) were isolated from umbilical cords and maintained in culture as previously described [11,12]. Confluent HUVEC monolayers (passages 1–3) were used in the experiments described below. Microvascular rat epididymal fat pad endothelial cells (RFPEC) and HEK293 were cultured as previously described [11,13].

Cloning and expression of FL-TEM8 and TEM8-ED in stable cell lines

The 1695 nucleotide full-length TEM8 (FL-TEM8) cDNA (Accession NM_032208, nucleotides 144–1838) was isolated by a two-step protocol. In the first step, nucleotides 1–855 (nucleotide 1 starting with the initiating ATG) were PCR amplified from cDNA isolated from an expressed sequenced tag (Accession AL542724), and nucleotides 830–1695 isolated using RT-PCR and HUVEC RNA. In the second step, PCR was performed using oligonucleotides corresponding to the extreme 5′ and 3′ TEM8 DNA sequences and aliquots of the products from the first step of the protocol. The final PCR product was cloned into the pEF6/myc-V5-His TOPO TA expression plasmid (Invitrogen), and transfected into RFPEC using the Effectene reagent (Qiagen) according to the manufacturer's instructions. Stable transfectants were selected for 2 weeks, isolated and expanded in medium containing 2 μg/ml blasticidin.

The TEM8-ED cDNA (nucleotides 1-906 of FL-TEM8) was PCR amplified using FL-TEM8-encoding cDNA and the oligonucleotides 5′-AAAGGATCCA-GGCC**ATG**GCCACGGCGGAGCGGAGA and 5′-AAA-GCGGCCGCAGGCCATCGTTCATGCTGACCTG. The ATG initiation codon is indicated as bold, and restriction sites were included (underlined) to facilitate cloning into the pcDNA4/myc-His expression plasmid (Invitrogen). The expression plasmid was transfected into HEK293 cells using the GenePORTER reagent (Gene Therapy Systems) according to the manufacturer's instructions. Stable transfectants were selected for 2 weeks, isolated, and expanded in medium containing 200 μg/ml zeocin.

Recombinant TEM8-ED from transfected HEK293 conditioned media was used in our studies as a dominant negative inhibitor to antagonize cellular TEM8 function. Conditioned media was prepared by culturing HEK293 or TEM8-ED expressing HEK293 for 5 days, after which the media was collected and centrifuged at 200 × *g* for 5 min to remove cellular debris. In addition, TEM8-ED synthesized by in vitro transcription/translation (IVT) was prepared from plasmid DNA (Fig. 2B) using the TNT T7 Quick Coupled System (Promega).