Available online at www.sciencedirect.com
SCIENCE DIRECT
Microvascular Research 70 (2005) 189 – 197
MVR Microvascular Research
www.elsevier.com/locate/ymvre

Modulation of tumor endothelial cell marker 7 expression during endothelial cell capillary morphogenesis

Xue-Qing Wang [a,*], Nader Sheibani [b], James C. Watson [a]

[a] Department of Surgical Oncology, Fox Chase Cancer Center, Philadelphia, PA 19111-2497, USA
[b] Department of Ophthalmology and Visual Science, University of Wisconsin, Madison, WI 53792, USA

Received 22 July 2005; accepted 30 August 2005
Available online 3 October 2005

Abstract

Angiogenesis, the process of new blood vessel formation from preexisting capillaries, is a multistep process. The principle cell type in this process is endothelial cells which are required to undergo proliferation, migration, differentiation, and coalescence into cord-like structures with a central lumen. However, the molecular and cellular mechanisms that regulate this process require further investigation. In this study, we have used the fibrin/fibrinogen-based three-dimensional culture to enrich for primary cultured mouse aortic endothelial cells, and to induce endothelial cell capillary morphogenesis. We found among all the cell surface markers examined that only TEM7 expression was up-regulated upon endothelial cells capillary morphogenesis. In addition, inhibition of capillary morphogenesis by serum stimulation completely blocked TEM7 expression. In contrast, stimulation of endothelial cell capillary morphogenesis with PMA enhanced TEM7 expression. Furthermore, incubation of endothelial cells with a recombinant extracellular domain of TEM7 blocked capillary morphogenesis in three-dimensional cultures. These results suggest that TEM7 is a novel protein whose cell surface expression is essential during endothelial cell capillary morphogenesis.

Keywords: TEMs; Angiogenesis; Fibrinogen

Introduction

Angiogenesis, the process of new blood vessel formation from preexisting capillaries, is very tightly regulated by a balanced production of positive and negative factors which affect the vascular cell's proliferation and differentiation (Folkman, 1982; Folkman and Klagsburn, 1987). The process of angiogenesis can be divided into multiple overlapping steps. It begins with loosening of the supporting vascular cells and increased local blood vessel permeability and extravasation of plasma proteins such as fibrin/fibrinogen (Wojtukiewicz et al., 2001), followed by degradation of the basement membrane and extracellular matrix proteins by proteinases, which facilitates the proliferation and migration of endothelial cells towards the source of the angiogenic factor (Davis et al., 2002). Finally, during the maturation stage, the newly formed endothelial cell sprouts ultimately differentiating to form lumens, reestablishing a new basement membrane, and collaterializing into a functional network of microvessels (Davis et al., 2002). Throughout these angiogenic processes, the control of endothelial cell phenotype plays an essential role. In particular, the angiogenic properties of the endothelial cells require coordinated changes in endothelial cell morphology, function, and gene expression. An alteration in the angiogenic balance contributes to the pathogenesis of a variety of vascular diseases, such as cancer, arthritis, and a variety of eye diseases (Folkman, 1995). Although considerable work has been performed in identifying factors that promote or inhibit angiogenic responses, less efforts have focused on gene(s) that control individual and/or groups of endothelial cells that assemble into capillary-like structures during angiogenesis. Identifying new molecular targets involved in endothelial cell capillary morphogenesis may be critical in efforts to inhibit angiogenesis in many human diseases with a neovascular component.

Recently, St. Croix et al. (2000) identified 9 new endothelial cell genes by comparing gene expression patterns of endothe-

* Corresponding author. Department of Medicine, Divisions of Pulmonary Science and Critical Care Medicine, University of Colorado at Denver and Health Science Center, 4200 E. 9th Ave., C272, Denver, Colorado 80262, USA. Fax: +1 303 315 1356.
E-mail address: xue-qing.wang@uchsc.edu (X.-Q. Wang).

0026-2862/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.mvr.2005.08.004

lial cells derived from blood vessels of normal and malignant colorectal tissues by SAGE (serial analysis of gene expression) technique. These were called tumor endothelial cell markers (TEM1 to TEM9), for which only EST sequences, but no other information, were available. They confirmed by RT-PCR and in situ hybridization that these TEMs were prominently expressed in tumor endothelial cells but were barely detectable in normal tissue endothelial cells and endothelial cells maintained in culture (St. Croix et al., 2000). However, whether these cell surface markers are involved in specific endothelial cell functions remains largely unknown.

Differentiating endothelial cells act differently in both cell functions and gene expressions in contrast to the cells grown in monolayer or quiescent. Bussolati et al. (2003) recently compared normal HUVEC cell function and gene expression with endothelial cells from 9 highly vascularized human renal carcinomas. They found that endothelial cells from renal carcinomas exhibit enhanced survival and angiogenic properties, such as proliferation at low serum concentration (0–5%) and enhanced capillary morphogenesis when plated in Matrigel (Bussolati et al., 2003). These cells also continually expressed markers of endothelial cell activation, and growth factor receptors (Bussolati et al., 2003). Given the specific expression of TEMs on endothelial cell isolated from tumors, and the fact that the majority of these genes have not been previously characterized, the exploration of their expression pattern, and their relation to the development of angiogenesis will provide new insight into our understanding of the process of angiogenesis.

In this study, we used a fibrin/fibrinogen-based three-dimensional culture system to enrich for primary mouse aortic endothelial cells, and to induce capillary morphogenesis of both primary mouse aortic endothelial cells and mouse SVEC4–10 cells. We compared the expression of TEMs, as well as other cell specific markers in endothelial cells during capillary morphogenesis and in regular culture. We found that among all the markers examined only TEM7 expression was up-regulated during capillary morphogenesis. Inhibition of capillary morphogenesis by serum stimulation completely blocked TEM7 expression. In contrast, stimulation of capillary morphogenesis with PMA enhanced TEM7 expression. In addition, incubation of endothelial cells with recombinant extracellular domain of TEM7 blocked endothelial cell capillary morphogenesis in both primary and established endothelial cells. These results suggest that TEM7 is a novel protein on endothelial cell surface whose expression is essential during capillary morphogenesis.

Materials and methods

Reagents

All primers used in this study were synthesized by Nucleic Acid facility of Fox Chase Cancer Center. SuperScript™ II RNase H$^-$ Reverse Transcriptase was from Invitrogen (Carlsbad, CA). Herculase polymerase was from Stratagene (La Jolla, CA). α[^{32}P]-dCTP was from Amersham Life Science (Arlington Heights, IL). Fibrinogen, thrombin, PMA, and actinomycin D were from Sigma (St. Louis, MO).

Cell culture and capillary morphogenesis assays

C57B/L6 mice (from Fox Chase animal facility) aortic disks culture in 0.3% fibrin/fibrinogen clotting medium was as described previously (Berger et al., 2004). At day 6, aortic disks, their associated vessel outgrowths, and parts of the surrounding fibrin/fibrinogen gel were removed and collected. Cells were recovered by incubation with 0.25% trypsin/EDTA, mixed with clotting medium, and plated at the density of 100,000 cells/ml. Capillary morphogenesis was photographed at day 6. At the same time, cells were dissociated with trypsin/EDTA, and subjected to total RNA preparation.

SVEC4–10 mouse endothelial cells were purchased from ATCC (Rockville, MD), and maintained in DMEM with 10% fetal bovine serum. For tube formation assay, 300,000 cells/ml were mixed with 0.3% fibrin/fibrinogen prepared in DMEM medium. Tube-like networks were viewed after 4 h.

RT-PCR analysis

Total RNA was isolated using RNeasy Protect Mini Kit from QIAGEN (Chatsworth, CA). The purified RNA was resuspended in DNase/RNase-free water, and incubated with DNase I (Ambion, Austin, TX) to remove any contaminating genomic DNA. RT-PCR was performed with oligo-(dT)-primed cDNA. 2 μg of RNA was converted into cDNA by suing SuperScript™ II RNase H$^-$ Reverse Transcriptase (Invitrogen, Carlsbad, CA). Gene-specific primers were designed using Primer 3 software (MIT, Boston, MA). All the primers used in this study are listed in Table 1. The reaction mixture contained 10× Herculase polymerase reaction buffer, 250 μM dNTP, 200 ng cDNA, 0.2 μM primers, and 5 units Herculase Enhanced DNA polymerase. After a 3-min initial denaturation at 94°C, amplification conditions were as follows: 94°C 1 min, 56°C 40 s, 65°C 1 min for 35 cycles. PCR products were analyzed by electrophoresis, stained with ethidium bromide, and photographed. Results were normalized to 29S expression, and each experiment was performed at least three times.

Northern blot analysis

The TEM7 and 29S cDNA probes were derived from the RT-PCR products using primers in Table 1. Probes were purified by agarose gel electrophoresis followed by cDNA extraction with the QIAEX gel-extraction kit (QIAGEN, Chatsworth, CA). Each cDNA probe was radiolabeled with α[^{32}P]-dCTP using the Rediprime labeling system (Amersham Life Science, Arlington Heights, IL). 10 μg RNA from each sample was fractionated on 2% denaturing formaldehyde-agarose gel, and transferred to a Hybond-N+ positively charged

Table 1
Primers used in RT-PCR

Genes	Upstream primer (5′–3′)	Downstream primer (5′–3′)
TEM1	GTG TTC TCT GAG CAC CAG AT	GTT GAT GGG CTT TAG AAG TG
TEM5	AGT AGT GCT GGA GAC CTC TG	ACA GAC AGT GCG AGT AGT CC
TEM7	CAT AAC CAC CCC AAC CAC TC	GAG CAG GAG GAA CAC AGG AG
TEM7R	GTG TCC TCG CAG ATT GGT TT	AGA AGA TGC TGG CTG CTG AT
TEM8	ACT TAC ATG CAC GAA GGA TT	AAC ACG TGG TCC TTA CTG TC
CD31	GTC ATG GCC ATG GTC GAG TA	CTC CTC GGC ATC TTG CTG AA
CD144	CCC CGT CTT TAC TCA ATC CA	TTT CCC TGC TTG GTT ATT CG
CD146	CCC TCA ACC CCA CTT CAC TA	ACC CAC ACC TTC CTC TCC TT
FLK-1	TCT TTC GGT GTG TTG CTC TG	CGG CTC TTT CGC TTA CTG TT
29S	GGA GTC ACC CAC GGA AGT T	TTT CCC TGC TTG GTT ATT CG