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Research report

Cloning, characterization and neuronal expression profiles of tumor endothelial marker 7 in the rat brain

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Abstract

Tumor endothelial marker7 (TEM7) is a putative transmembrane protein that is highly expressed in the tumor endothelium. In the present study, the expression profile of TEM7 was investigated in TEM7-transfected human embryonic kidney (HEK) 293 cells and the rat brain. The extracellular secretion of the recombinant N-terminal ectodomain of TEM7, not full-length TEM7, was observed in the transiently transfected HEK 293 cells. The full-length TEM7 was found inside and membrane part of cells as demonstrated by confocal microscopy. In situ hybridization study revealed that *TEM7* mRNA expressions were localized to specific neuronal areas, such as cerebellar Purkinje cells, the layer IV and V of cerebral cortex, hippocampal pyramidal cells and hypothalamic magnocellular nuclei. Immunohistochemical investigation of TEM expression with specific antibodies against TEM7 further supported the spatial expression patterns of *TEM7* mRNA. The temporal expression of *TEM7* mRNA in the cerebellar Purkinje cells demonstrated a postnatal developmental regulation of TEM7 expression. Our results indicate that TEM7 plays a role as a transmembrane receptor in some neuronal populations of the vertebrate brains.

Theme: Cellular and molecular biology
Topic: Staining, tracing, and imaging technique

Keywords: TEM7; In situ hybridization; Purkinje cells; Immunocytochemistry

1. Introduction

Tumor endothelial markers (TEMs) are a group of cell surface proteins that are highly expressed in the endothelium of various cancers [3,11,20]. These several novel genes (TEM1-8) have sequence characteristics of cell surface proteins such as signal peptides and the transmembrane domain(s). For example, TEM1, TEM7 and TEM8 have single transmembrane domain, whereas TEM5 appears to be a seven-transmembrane receptor [3]. The putative localization of TEMs in the cell surface would be important for future therapeutic intervention of tumor angiogenesis. Even though the limited expression profile of TEMs in the tumor endothelium represents their restricted functions in the tumor angiogenesis, several recent papers showed evidence implicating a role of TEMs in normal tissues. For example, TEM1 is the lectin-like cell surface protein, endosialin, which is found in several normal tissues [3,4], and TEM8 is recently identified as the receptor for anthrax toxin [2]. It is also known that the mouse homologues of TEM1, TEM5 and TEM8 were abundantly expressed in the endothelium of embryonic liver [3]. Therefore, it seems that TEMs participate in a normal physiological process as well as tumor angiogenesis.

TEM7 is particularly of interest in that it has plexin-semaphorin-integrin (PSI) domain in its presumptive extracellular region [3]. The domain consists of ~50 residues of

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amino acids (aa) and usually contains eight cysteine residues [1]. The cysteine residues are expected to be disulfide bonded. The PSI domain is frequently found in cell surface proteins of the nervous system, such as semaphorin and its receptor, plexin. Semaphorin family is a major repellent that regulates the axon guidance and neuronal migration in the developing nervous system [16,17]. Interestingly, some of these neuronal guidance molecules play an important role outside the nervous system, especially at the vascular system. It was recently reported that semaphorin-3F inhibits angiogenesis [9], and that neuropilin, the semaphorin receptor, functions as a receptor for vascular endothelial growth factor (VEGF) in the endothelial cells [7,13,19]. Reversely, certain angiogenic factors appear to have an important function in the nervous system. For example, VEGF is known to regulate the neuronal cell migration, neurite maturation and neurogenesis [6,10,23].

Based on the PSI containing structure of TEM7 with an emerging role of angiogenic factors in the nervous system, we have speculated the specific function of TEM7 in the nervous system. In the present study, we have cloned and characterized the expression of recombinant TEM7 proteins in human embryonic kidney (HEK) 293 cells. In addition, the expression profiles of *TEM7* mRNA and protein in the rat brain and developing cerebellum were demonstrated using in situ hybridization and immunohistochemical analyses.

2. Materials and methods

2.1. Molecular cloning

The ectodomain of human TEM7 (24aa–412aa) was generated using PCR with a human TEM7 cDNA template (a gift from Dr. Vogelstein). The sense PCR primers contained *Sfi*I site whereas antisense primers had *Hin*dIII site. After PCR amplification and restriction enzyme digestion, the PCR fragments were inserted into the AP5 vector (a gift from Dr. Z. He), which contains alkaline phosphatase (AP), Myc and His as tags [21]. A nidogen-like domain (24aa–271aa) or a domain containing PSI repeat (268aa–412aa) of human TEM7 were cloned into the AP5 vector using a PCR cloning strategy. A diagrammatic illustration of the constructs was revealed in Fig. 1A.

To clone the full-length cDNA of mouse TEM7, PCR amplification was performed by using mouse cerebellar cDNA as a template with the sense primer (5′-aagcttctc-agtcccgcaacaccc′) and the antisense primer (5′-ctcgaggtggc-cactgatgtaaat). The specific primers contained *Hin*dIII and *Xho*I sites in its 5′ end of the sense and antisense primers, respectively. The PCR fragments were subcloned into AP5 vectors after the enzyme digestion of *Hin*dIII/*Xho*I, which generated a full-length TEM7 with Myc/His tag at the C-terminus of TEM7 without AP. The nucleotide sequences of cloned materials were verified by the sequencing analysis in an ABI 3700 automated sequencer.

2.2. Generation of polyclonal antiserum against TEM7

We have generated a rabbit polyclonal antiserum raised against a peptide mapping at the 332–344 amino acid of mouse TEM7. The immunoglobulin fractions were purified from the antiserum using a protein-A affinity column, and the sensitivity and specificity were tested with ELISA and Western blot.

2.3. Culture, transfection and Western blot analysis

HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin). The cells were transfected with the expression vectors by calcium phosphate methods. Briefly, the cells were plated on to 100-mm tissue culture dishes at 50–60% confluence. Cells were then transfected with the transfection mixture containing 10 μg DNA, 125 mM $CaCl_2$ and 1 × HBS (140 mM NaCl, 25 mM HEPES, 0.75 mM sodium phosphate, pH 7.05) for 16 h. The transfection mixture was removed and replaced with the growing medium. The conditioned medium and transfected cells were harvested 2 days after transfection, solubilized with 2× sodium dodecyl sulfate (SDS) loading dye at 95 °C for 5 min.

The samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham). The blotted membranes were blocked with 5% non-fat milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST), then were incubated with anti-Myc antibody (Santa Cruz, 1:3000) overnight at 4 °C. Next, the membranes were washed three times with PBST, and incubated with horseradish peroxidase conjugated anti-mouse IgG (Amersham, 1:3000) for 1 h at room temperature. The enhanced chemiluminescence reaction (Amersham) was done to visualize the reaction, and the blot was analyzed using a luminescent image analyzer (LAS-3000, Science Lab).

2.4. Immunofluorescence and immunohistochemistry

The transiently transfected HEK 293 cells grown on uncoated coverslips were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature, washed with PBS and blocked in 10% goat serum in PBS containing 0.3% Triton X-100. The cells were incubated with primary antibodies for 16 h at 4 °C in PBS containing Triton X-100 and 2% bovine serum albumin (BSA). The primary antibodies used were: mouse anti-Myc (Santa Cruz, 1:3000), rabbit anti-His (Santa Cruz, 1:2000), mouse anti-Tem7 (Imgenex, 1:1000). After three washes in PBS, Alexa 488 (Molecular Probe, 1:500) or Cy3 (Amersham, 1:500)-conjugated anti-rabbit and anti-mouse IgG were used as secondary antibodies. In order to stain actin filaments, cultured cells were stained with allexa-phalloidin (1:800,