Experimental and Molecular Pathology 87 (2009) 184–188

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Thrombospondin-1 (TSP-1) up-regulates tissue inhibitor of metalloproteinase-1 (TIMP-1) production in human tumor cells: Exploring the functional significance in tumor cell invasion

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ARTICLE INFO

Article history:
Received 20 August 2009
Available online 9 September 2009

Keywords:
Thrombospondin-1
TIMP-1
Tumor Cell Metastasis
Tumor Cell Invasion

ABSTRACT

Thrombospondin-1 (TSP-1), a matrix-bound adhesive glycoprotein, has been shown to modulate tumor progression. We previously demonstrated that TSP-1 up-regulates matrix metalloproteinases MMP-2 and MMP-9. Our studies suggested that the balance between MMPs and tissue inhibitors of metalloproteinases (TIMPs) is a key determinant in tumor cell invasion. We now report that TSP-1 up-regulates TIMP-1 expression in both human breast and prostate cancer cell lines. The effect of TSP-1 on TIMP-1 expression was examined in human breast adenocarcinoma cell lines (MDA-MB-231) and human prostate cancer cell lines (PC3-NI and PC3-ML) treated with exogenous TSP-1. TIMP-1 expression was also examined in TSP-1 stably transfected breast cancer cell line (MDA-MB-435). Northern and western blot analysis revealed TIMP-1 mRNA and TIMP-1 protein expression increased with increasing concentrations of TSP-1. This effect was inhibited by antibodies against the type I repeat domain of TSP-1 further suggesting that TSP-1 mediates TIMP-1 secretion. Inhibition of TSP-1 induced TIMP-1 levels increased tumor cell invasion. We conclude that TSP-1 is involved in influencing the critical balance between MMPs and their inhibitors, maintaining the controlled degradation of the extracellular matrix needed to support metastasis and our results may provide an explanation for the divergent activities reported for TSP-1 in tumor progression.

Introduction

The process of metastasis involves the survival of the primary tumor through a series of steps including: tumor cell invasion, extravasation from the blood or lymphatic circulation, and finally tumor cell colonization and angiogenesis to form the metastatic lesion (Stetler-Stevenson et al., 1993). Once a tumor cell is formed, it relies on the body's extracellular matrix (ECM) to survive and grow. ECM proteins such as laminin, collagen, and thrombospondin-1 (TSP-1) have been shown to stimulate tumor cells to secrete proteolytic enzymes needed for tumor cell metastasis (Haas et al., 1998; Song et al., 1997; Tuszynski et al., 1987).

TSP-1 is a 450 kDa trimeric glycoprotein involved in a variety of processes including cell adhesion, cell migration, and angiogenesis (Qian and Tuszynski, 1996). Each monomer of TSP-1 is composed of repeating homologous amino acid sequences, with specific receptors responsible for the numerous cellular processes involving TSP-1 (Lawler, 1986; Lawler et al., 1978). The complexity of the TSP-1 molecule and the many receptors makes the study of TSP-1 difficult, often leading to conflicting results especially in the fields of angiogenesis and tumor progression.

One of the mechanisms by which TSP-1 influences tumor cell invasion and metastasis is through the regulation of several proteolytic enzyme families, including the metalloproteinases (MMPs). MMPs are used by tumor cells to invade the basement membrane and its underlying connective tissue, providing a mechanism for tumor cells to enter the small blood vessels and lymphatics (Parsons et al., 1997). Our laboratory was also the first to identify that TSP-1 is capable of stimulating MMP-9 production in bovine aortic endothelial cells and in pancreatic tumor cells, stimulating TSP-1 mediated tumor progression and endothelial tube formation (Qian et al., 1997).

Critical to the function of MMPs is the balance of the enzyme to its physiologic inhibitor, tissue inhibitor of metalloproteinase (TIMPs). The TIMPs are comprised of a family of four members, TIMP-1, TIMP-2, TIMP-3, and TIMP-4. A balance of inhibitor to proteolytic enzyme is needed to achieve tumor cell invasion as unregulated enzyme action has been shown to actually inhibit tumor cell invasion *in vivo* (Qian et al., 1997).

In this study, we report the finding that TSP-1 stimulates the expression of TIMP-1 in both breast and prostate carcinoma cell lines.

Abbreviations: ECM, extracellular matrix; MMP, metalloproteinase; SDS-PAGE, sodium dodecyl sulfate-polyacylamide gel electrophoresis; TIMP, tissue inhibitor of metalloproteinase; TSP-1, thrombospondin-1; SDS-PAGE, sodium dodecyl sulfate-polyacylamide gel electrophoresis.

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doi:10.1016/j.yexmp.2009.09.002

We hypothesize that the control of net proteolysis of the ECM by TSP-1 is through both up-regulation of MMP-9 and its inhibitor TIMP-1 leading to a controlled proteolytic system.

Materials and methods

Materials

All reagents, unless specified otherwise, were reagent grade and purchased from Sigma Chemical Co. (St. Louis, MO). Tissue culture supplies were purchased from Fisher Scientific (Malvern, PA). Reagents for sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad Laboratories (Richmond, CA). Laminin, type IV collagen and fibronectin were purchased from Collaborative Research (Bedford, MA). Rabbit anti-human TIMP-1 and mouse anti-human TIMP-1 were purchased from Triple Point (Forest Grove, OR) and Oncogene Science (Cambridge, MA), respectively. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG were purchased from Boehringer Mannheim (Indianapolis, IN). Goat polyclonal anti-human TSP-1 IgG and rabbit polyclonal CSVTCG antibody were raised in our laboratory. Type I repeat peptides and irrelevant peptides were purchased from Peptidogenic (Livermore, CA).

Boyden chamber invasion assay

Breast tumor cell invasion was measured using the modified Boyden chamber. Polycarbonate filters, 8 μm pore size (Millicell, Millipore Corporation, Bedford, MA), were coated with 100 μg Type IV collagen (1 mg/ml 60% EtOH) and dried overnight at 25 °C. Blind-well Boyden chambers were filled with 700 μl of serum-free media containing 0.1% BSA in the lower compartment, and the coated filters were mounted in the chamber. Approximately 50,000 cells (tested to be greater that 95% viable) suspended in 300 μl of the same media were placed in the upper chamber of the apparatus and allowed to settle onto the collagen-coated membrane. Neutralizing antibodies as well as peptides were placed in the upper chamber. After an incubation period of 3–6 h at 37 °C, the cells on the upper surface of the filter were removed with a cotton swab. The filters were fixed in 3% glutaraldehyde solution and stained with 0.5% crystal violet solution. Invasive cells adhering to the under-surface of the filter were counted using a phase contrast microscope (400×). The data were expressed as the summation of the number of invasive tumor cells in five representative fields.

Cell culture and treatment

The human breast adenocarcinoma cell line MDA-MB-231 was purchased from the American Type Culture Collection (CRL 10317, Rockville, MD). The human prostate cancer cell lines, PC3-NI and PC3-ML, were kindly provided by Dr. Mark Sterns, Drexel School of Medicine, Philadelphia, PA. The TSP-1 transfected breast adenocarcinoma cell line, MDA-MB-435, was kindly provide by Dr. David Roberts, National Cancer Institute, Bethesda, MD. The origin of the MDA-MB-235 cell line has been in question with some studies suggesting that the line was identical to a M14 melanoma line, however recent published data are consistent with both M14 and MDA-MB-235 cell lines being of breast cancer origin (Chambers, 2009). The lines obtained from Dr. Roberts include three lines: a vector control (TH5), a high TSP-1 producer (TH26), and a COOH-terminally truncated TSP-1 producer (TH50). These cells were transfected with the pCMVBamNeo vector. All cells were grown at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/ml of penicillin, 50 μg/ml of streptomycin, and 50 μg/ml of gentamicin sulfate (Sigma Chemical Co). The TSP-1 transfected cells were cultured with media supplemented with 50 μg/ml G418 antibiotic to maintain the transformed phenotype. Cells were cultured in 6-well plates for TIMP-1 analysis or T75 flasks for RNA isolation. Cells were grown to 85% confluence and were washed and incubated in serum-free medium containing 0.1% BSA. Different concentrations of TSP-1 (20–60 μg/ml) and/or 10 μg/ml of antibody IgG, conrol IgG or peptides were added. After 48–72 h of culture, the conditioned medium was collected, clarified by centrifugation, and assayed by enzyme-linked immunoadsorbant assay (ELISA) and Western blot analysis for TIMP-1. The amount of conditioned media analyzed was corrected for the total number of cells in each well so that TIMP-1 secretion was compared from the same amount of cells from each treatment group. Cell viability after treatments was assessed using trypan blue exclusion.

ELISA analysis

Conditioned media from cells were analyzed for TIMP-1 levels using commercial TIMP-1 ELISA kits (Oncogene Science, Cambridge MA). Statistical analysis was performed using GraphPad Prizm software, San Diego CA.

Northern blot analysis

Total RNA was isolated from tumor cells by Rneasy Total RNA Kits (QIAGEN Inc. Chatsworth, CA) following the manufacturer's direction. 15 μg of total RNA was electrophoresed on 1% agarose/formaldehyde gels and blotted onto nitrocellulose paper. The paper was hybridized with the appropriate ^{32}P-labeled cDNA probes and exposed for autoradiography at −80 °C with an intensifying screen. After recording the results, the same paper was re-probed with a beta-actin cDNA probe and exposed at −80 °C. The cDNAs were radiolabeled with ^{32}P by the random primer labeling method using the Stratagene labeling kit (Stratagene, La Jolla, CA).

Thrombospondin-1 purification

TSP-1 was purified from Ca^{+2} ionophore A23187-activated platelets in our laboratory as previously described (Tuszynski et al., 1985). Purity was assessed by SDS-PAGE using Coomassie blue or silver staining. All TSP-1 used was further purified to remove all bound TGF-β1 according to the procedure of Murphy-Ullrich et al. (1992). TGF-β1 levels was monitored by human TGF-β1 ELISA kits (Quantikine, R&D Systems, Minneapolis, MN).

Western blotting

Concentrated conditioned media was fractionated on 8–12% SDS-PAGE and then transferred to a nitrocellulose membrane using a Pharmacia Phast gel electrophoresis system. Nonspecific sites of the membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h. The immunoblots were incubated with primary antibodies diluted in TBS-T for 1 h at a concentration of 1 μg/ml. After washing, the immunoblots were incubated in TBS-T with horseradish peroxidase-conjugated secondary antibodies for 1 h at a concentration of 0.1 μg/ml. The immunoreactivity was detected using enhanced chemoluminescence (ECL) system (Amersham, Arlington Heights, IL).

Results

TSP-1 treatment stimulates TIMP-1 production in a dose-dependent manner

MDA-MB-231, a ductal breast adenocarcinoma cell line, and PC3-NI and PC3-ML, a non-invasive and a highly metastatic prostate cancer