Participation of Thrombospondin-1 in the Activation
of Latent Transforming Growth Factor-β
in Malignant Glioma Cells

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Abstract

Malignant glioma cells secrete transforming growth factor-β (TGF-β) and can activate latent TGF-β. However, the mechanism of the latent TGF-β activation has not yet been determined. This study examined whether thrombospondin-1 (TSP-1) secreted by malignant glioma cell lines participates in the activation of latent TGF-β secreted by the glioma cells. Western blot analysis revealed that TSP-1 was present in both the cell lysates and the culture supernatants of all three malignant glioma cell lines (T98G, A172, and U251). A bioassay for TGF-β activity revealed that all malignant glioma cell lines used in this study could activate latent TGF-β by themselves. Latent TGF-β activation, evaluated by enzyme-linked immunosorbent assay, was inhibited by more than 50% by the addition of neutralizing anti-TSP-1 monoclonal antibody or anti-TSP-1 polyclonal antibody. These results indicate that TSP-1 has a predominant role in the activation of latent TGF-β in malignant glioma cells.

Key words: latent transforming growth factor-β activation, thrombospondin-1, malignant glioma cells

Introduction

Most cells secrete transforming growth factor-β (TGF-β) in the latent form, and the biological functions of TGF-β are only manifested after conversion to the active form. TGF-β forms a latent complex by association with its propeptide, the latency-associated peptide, through noncovalent interactions, from which TGF-β must be released to manifest its biological activity. This process is called latent TGF-β activation or TGF-β formation. Latent TGF-β activation is involved in tumor progression, since active TGF-β induces immunosuppression, and promotes extracellular matrix formation and angiogenesis.

Thrombospondin-1 (TSP-1) was earlier identified as a thrombin-sensitive protein released by the activation of human platelets. TSP-1 is a member of a family of structurally related proteins encoded by different genes, which includes four new members designated TSP-2, TSP-3, TSP-4, and TSP-5/cartilage oligomeric matrix protein. TSP-1 and TSP-2 are similar in overall structure but differ from TSP-3, TSP-4, and TSP-5. TSP-1 is a trimeric high molecular weight glycoprotein with a monomeric molecular mass of about 140 kD, and is secreted by platelets and synthesized by many cell types, including endothelial and tumor cells. TSP-1 activates latent TGF-β secreted by cells as well as purified forms of small and large latent TGF-β in a chemically defined system via binding interactions. The activation of latent TGF-β has been localized to the unique sequence arginine-phenylalanine-lysine (RFK) found between the first and second type I repeats of TSP-1 (amino acids 413-415) by the use of synthetic peptides. The peptide with the corresponding sequence in TSP-2, arginine-isoleucine-arginine, is inactive. Recently, TSP-1 was found to be significant in latent TGF-β activation in vivo.

Previous studies have shown that malignant glioma cells secrete TGF-β1 and/or TGF-β2, and have the potential to activate latent TGF-β. Malignant glioma cells also secrete TSP-1 and both TGF-β and TSP-1 are present in malignant glioma tissues. It is important to investigate the mechanism of activation of latent TGF-β in malignant glioma cells, because the presence of active TGF-β in tumor tissues may contribute to the progression of the tumor. However, the mechanism...
of latent TGF-β activation in gliomas has not yet been determined. This study examined whether TSP-1 secreted by malignant glioma cells participates in the activation of latent TGF-β secreted by malignant glioma cells.

**Materials and Methods**

I. **Cell lines and cell culture**

Human malignant glioma cell lines (T98G, U251, and A172) and mink lung epithelial cell (Mv1Lu) were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 μg/ml kanamycin. Previous studies indicated that these malignant glioma cell lines can activate latent TGF-β.25,29 and that all cell lines expressed TSP-1 messenger ribonucleic acid.16

II. **Generation of anti-TSP-1 polyclonal antibody**

A rabbit polyclonal antibody specific for TSP-1 was manufactured by Sawady Technology Inc. (Tokyo) using a synthetic peptide including a sequence RFK that can activate latent TGF-β.30 The RFK sequence, which is unique to TSP-1 and not found in TSP-2, is located between the first and the second type I repeats of TSP-1 (amino acids 413–415). Briefly, rabbits were immunized with the synthetic peptide NH2-TCHIQECDKRFK-COOH (amino acids 404–415). After the third immunization, samples of total blood were obtained from rabbits, and the antibody isolated by affinity purification. The specificity of the anti-TSP-1 antibody was determined by Western blot analysis using TSP-1 purified from platelets (Athens Research & Technology, Inc., Athens, Ga., U.S.A.)

III. **Western blot analysis**

The subconfluent human malignant glioma cells (T98G, U251, and A172) were cultured in 2.5 ml of serum-free medium in 6 cm dishes for 18 hours and the supernatants were collected and filtered (0.22 μm). Cellular protein was extracted by lysing 1 × 10⁶ cells with 50 μl of lysis buffer (40 mM Heps-NaOH, pH 7.0, 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 5 μg/ml aprotinin). The cell lysates or the culture supernatants were mixed with equal amounts of 2 × gel-loading buffer (125 mM Tris-HCl, pH 7.0, 4.6% SDS, 20% glycerol, and 10% 2-mercaptoethanol) and boiled for 5 minutes, then 40 μg of cellular protein or 25 μl of 2-fold diluted culture supernatants were separated in a 3–10% gradient SDS polyacrylamide gel (Page; Atto Co., Tokyo) and electroblotted on polyvinylidene difluoride membrane (Clear blot membrane-P; Atto Co.). After blocking with 10% nonfat dry milk in Tris-buffered saline, the membranes were incubated at room temperature for 1 hour with the anti-TSP-1 mouse monoclonal antibody (Genzyme, Cambridge, Mass., U.S.A.) at a dilution of 1:500. The blot was subsequently probed by a polymer reagent labeled with the second antibodies and peroxidase (Envision polymer reagent; DAKO Japan Co., Kyoto), and developed with diaminobenzidine chromogen. TSP-1 purified from platelets (Athens Research & Technology, Inc.) was dissolved in 1 × gel-loading buffer (62.5 mM Tris-HCl, pH 7.0, 2.3% SDS, 10% glycerol, and 5% 2-mercaptoethanol) and boiled for 5 minutes, and 250 ng of dissolved TSP-1 was used as a control.

IV. **Bioassay for TGF-β activity**

To determine the capacity for latent TGF-β activation in malignant glioma cell lines used in this study, the TGF-β activity in the culture supernatants were examined by the quantitative bioassay using Mv1Lu mink lung epithelial cells described elsewhere.25 Briefly, T98G, U251, and A172 cells were cultured in 6-well plates. Subconfluent cells were washed with phosphate-buffered saline and 2 ml of serum-free RPMI1640 medium with 1 ng/ml bovine serum albumin added to each well. After incubation for 24 hours, supernatants were collected, filtered (0.22 μm), and stored at −80°C. Aliquots (25 μl) of the supernatants from T98G, U251, and A172 cells were placed in 96-well plates in triplicate and 25 μl of RPMI1640 medium was added to each well. To determine whether the growth inhibition of Mv1Lu cells is due to TGF-β, the supernatants (25 μl) were pretreated with neutralizing anti-TGF-β1/β2/β3 antibody solution (25 μl) (final concentration 30 μg/ml; Genzyme) for 1 hour at 37°C. To convert all latent TGF-β to the active form, the supernatants were acid-activated (1.5 μl 6N HCl/200 μl supernatant) for 15 minutes at room temperature and then neutralized with 1.5N NaOH to a final pH of 7–7.4. Aliquots (50 μl) of Mv1Lu cells (2000 cells) were added to each well. Finally, all samples were diluted by 4-fold. After incubation for 72 hours at 37°C in 5% CO₂, the supernatants were decanted and the cells stained with 0.5% crystal violet in 20% methanol for 5 minutes. After washing 10 times with tap water, the dye was eluted and the absorbance at 570 nm was determined with a spectrophotometer (Titeretek Multiskan; Flow Laboratories Inc., Helsinki, Finland). Human recombinant TGF-β2 (Austral Biologicals, San Ramon, Calif., U.S.A.) was used as an internal standard. The values of TGF-β in each
V. Blocking of latent TGF-β activation by anti-TSP-1 antibodies

T98G cells were cultured in a 24-well plate. Anti-TSP-1 monoclonal antibody (Genzyme) or the rabbit polyclonal antibody which neutralizes latent TGF-β activation was added in different concentrations (1, 10, and 30 μg/ml) to fresh serum-free RPMI1640 medium with 1 mg/ml of bovine serum albumin in T98G cell culture. Normal mouse or normal rabbit immunoglobulin was added to each well as controls. After incubating for 24 hours at 37°C in 5% CO₂, the supernatants were collected, filtered (0.22 μm), and stored until use at −80°C. The amounts of active TGF-β1 in the T98G culture supernatants with or without neutralizing anti-TSP-1 antibodies were determined using the TGF-β1 enzyme-linked immunosorbent assay kit (Genzyme) which detects only the active form of TGF-β1. The assay followed the manufacturer's instructions.

Results

I. TSP-1 protein expression in cell lysates and culture supernatants

TSP-1 was detected in both the cell lysates and the culture supernatants of all cell lines examined by Western blot analysis (Fig. 1). T98G cells produced and secreted a high amount of TSP-1, whereas A172 cells secreted only a low amount of TSP-1.

II. TGF-β activity in the culture supernatants of malignant glioma cells

The culture supernatants from T98G, U251, and A172 cells showed significant TGF-β activity, indicating that tumor cells can activate latent TGF-β (Fig. 2). The concentrations of active TGF-β in the supernatants from T98G, U251, and A172 were 1.76 ± 0.33, 1.44 ± 0.04, and 0.52 ± 0.03 ng/ml (mean ± SD, n = 3), respectively, and the total TGF-β concentrations were 24.8 ± 2.3, 22.4 ± 6.1, and 8.8 ± 1.1 ng/ml, respectively. The percentages of active TGF-β in total TGF-β were 7.1%, 6.4%, and 5.9%, respectively. This suggests that a relatively constant amount of latent TGF-β was activated in the supernatants.
III. Blocking of TGF-β1 activation by anti-TSP-1 antibodies

The concentration of active TGF-β1 in the culture supernatant of T98G cells was decreased by treatment with neutralizing anti-TSP-1 antibodies in a dose-related manner (Fig. 3). Activation of latent TGF-β1 was almost completely inhibited by the addition of 30 μg/ml of anti-TSP-1 monoclonal antibody, and inhibited by nearly 50% by the addition of 30 μg/ml of anti-TSP-1 polyclonal antibody. This difference in the rate of inhibition might be caused by a difference of the affinity of the antibodies.

Discussion

The present study demonstrated that malignant glioma cells produced and secreted TSP-1 protein, and that latent TGF-β activation by the tumor cells was inhibited by neutralizing anti-TSP-1 antibodies. These results indicate that TSP-1 secreted by malignant glioma cells participates in the activation of latent TGF-β. Latent TGF-β is activated by several mechanisms, including glycosidases (endoglycosidase-F, sialidase, neuraminidase, N-glycanase),22) serine proteinases, plasmin and cathepsin D,19,20) cocultures of vascular endothelial cells and pericytes,1) and TSP-1.10,21–33) Among these, TSP-1 seems to be predominant in the activation of latent TGF-β in malignant glioma cells, since the present study found that anti-TSP-1 antibodies inhibited the activation by more than 50%.

TSP-1 is synthesized and secreted by many cultured human tumor cell lines, including those derived from squamous carcinoma, melanoma, glioma, osteosarcoma,8) and breast adenocarcinoma.3,7,42,43) Malignant progression in some cell lines is associated with reduced TSP expression.6,42,43) In contrast, suppression of TSP-1 expression by anti-sense TSP-1 complementary deoxyribonucleic acid suppressed the metastatic activity of the highly active squamous carcinoma cell line.5) However, TSP-1 injected intravenously into mice enhanced lung tumor colony formation two- to threefold.36) These observations suggest that the net effect of TSP-1 on tumor progression varies with tumor type. In our previous study, malignant glioma tissues were found to be rich in both TSP-1 and TGF-β proteins compared to low grade gliomas.10) The present results suggest that TSP-1 and TGF-β secreted by malignant glioma cells may interact to produce active TGF-β. Thus, overexpression of TSP-1 may relate to tumor progression in malignant gliomas.

TGF-β is a potent growth inhibitor of many different cell types and is important in growth control during development and differentiation.21,23) We previously found that malignant glioma cell lines, which have functional TGF-β receptors, are resistant to growth inhibition by active TGF-β.15) Loss of sensitivity to growth inhibition by TGF-β is a frequent event in many cell lines established from human tumors including gliomas.12) Cells with resistance to the growth-inhibiting effect of TGF-β would obtain a growth advantage by activation of latent TGF-β during tumor progression. Therefore, malignant glioma cells may acquire these two important features, resistance to the growth-inhibiting effect of TGF-β and the capacity to activate latent TGF-β by TSP-1, which may be profoundly related to increased malignancy and tumor progression.

The present study is the first to demonstrate the participation of TSP-1 in the activation of latent TGF-β.
TGF-β in malignant glioma cells. TSP-1 is a multifunctional matrix protein implicated in cancer cell adhesion, migration, invasion, and angiogenesis, as well as latent TGF-β activation. Further studies to clarify the biological importance of the expression of TSP-1 in malignant glioma cells or tumor tissues are needed.

References

27) Roberts DD: Regulation of tumor growth and...


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Commentary

This interesting basic research article investigates the autocrine activation of malignant glioma cells in culture. TGF-β is known to be an important factor in the pathogenesis and evolution in malignant gliomas. Ordinarily, this factor is present in a latent form and needs to be activated in order to express its gross stimulatory capability. This research shows that one of the activating factors present in malignant glioma cells in culture is thrombospondin-1. The implication is clear that the presence of thrombospondin may activate, in an autocrine fashion, the activation and expression of TGF-β. This may be an important part of the mechanism of glioma growth and development, and it may ultimately prove to be a target for therapy.

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In this study, the authors demonstrated that active TGF-β is detected in the culture supernatants of malignant glioma cells. They also showed that thrombospondin-1 (TSP-1), a potential activator of latent TGF-β, is expressed in those glioma cell lines. Therefore, they speculate that TSP-1 is critical in the activation of latent TGF-β in malignant glioma cells. This speculation is supported by their finding that addition of neutralizing anti-TSP-1 antibody could inhibit the latent TGF-β activation. Although TGF-β is known to suppress proliferation of many different cell types, the authors previously showed that most malignant glioma cells are insensitive to growth inhibition by TGF-β. Therefore, they hypothesize that glioma cells with resistance to the growth-inhibiting effect of TGF-β
may conversely obtain a growth advantage by TSP-1-activated latent TGF-β. To test this hypothesis, the authors need to examine whether inhibition of TSP-1 by antibody or the synthetic peptide GGWSHW, an inhibitor of the TSP-1 activation mechanism, can suppress the growth of malignant glioma cell. These data may provide important information for developing new therapeutical approaches for malignant gliomas.

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Sasaki et al. carefully studied the role of TSP-1 in the activation of latent TGF-β in malignant glioma cells using enzyme-linked immunosorbent assay. They demonstrated that latent TGF-β was activated by TSP-1 and the activation was inhibited by more than 50% by anti-TSP-1 antibodies. The findings were important and clinically relevant because TGF-β is known to be secreted by glioma cells and play a role in tumor progression.

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