Low-Density Lipoprotein Receptor–Related Protein Contributes to the Antiangiogenic Activity of Thrombospondin-2 in a Murine Glioma Model

Constance Y. Fears,1,2 J. Robert Grammer,1 Jerry E. Stewart, Jr.,1 Douglas S. Annis,3 Deane F. Mosher,1 Paul Bornstein,4 and Candece L. Gladson1,2

Abstract

Host antiangiogenesis factors defend against tumor growth. The matricellular protein, thrombospondin-2 (TSP-2), has been shown to act as an antiangiogenesis factor in a carcinogen-induced model of skin cancer. Here, using an in vivo malignant glioma model in which the characteristics of the tumors formed after intracerebral implantation of GL261 mouse glioma cells are assessed, we found that tumor growth and microvessel density were significantly enhanced in tumors propagated in TSP-2−/− mice. Mechanistically, matrix metalloproteinase (MMP)-2 has been associated with neoangiogenesis and has been proposed that the levels of available MMP-2 may be down-regulated by formation of a complex with TSP-2 that is internalized by low-density lipoprotein receptor–related protein 1 (LRP1). We found elevated expression of MMP-2 and MMP-9 in tumors propagated in TSP-2−/− mice, with a preferential localization in the microvasculature. In wild-type mice, MMP-2 was coexpressed with TSP-2 in the tumor microvasculature. In vitro, addition of recombinant (rec) TSP-2 to mouse brain microvessel endothelial cells reduced MMP-2 levels and invasion through mechanisms that could be inhibited by a competitive inhibitor of ligand binding to LRP1 or by siLRP1. Thus, the antiangiogenic activity of TSP-2 is capable of inhibiting the growth of gliomas in part by reducing the levels of MMP-2 in the tumor microvasculature. This mechanism is mediated by LRP1. (Cancer Res 2005; 65(20): 9338-46)

Introduction

The thrombospondins (TSP) are a family of large, multimeric, matricellular glycoproteins (TSP-1, TSP-2, TSP-3, TSP-4, and TSP-5/COMP) that modulate cell adhesion, proliferation, and motility in certain cell types (reviewed in ref. 1). TSP-1 and TSP-2, which represent a subgroup of this family, share a similar domain structure and significant sequence identity. TSP-1 is a potent inhibitor of angiogenesis in several malignant and nonmalignant processes; however, less is known regarding the role of TSP-2 in neovascularization (2). TSP-2 is expressed in a variety of human fetal and adult tissues, including the heart, kidney, and brain (3, 4), and has been localized to the vasculature and connective tissue of the developing mouse (5). Stromal TSP-2 expressed in the dermis has been shown to inhibit angiogenesis in response to a foreign body implant (6), and stromal TSP-2 has been shown to reduce neovascularization in the developing mouse iris and in sutured mouse cornea (7). TSP-2 can also inhibit angiogenesis in tumors derived from the skin epithelium. In a chemical carcinogen-induced model of skin papilloma, tumors that developed in the carcinogen-treated TSP-2−/− mouse exhibited increased angiogenesis, accelerated conversion to malignant tumors, and reduced tumor cell apoptosis (8). When similar tumor cells (squamous carcinoma cells) were modified to express TSP-2 by stable transfection and then propagated in the s.c. tissue of the BALB/c nude mouse, decreased angiogenesis, smaller tumors, and increased tumor necrosis were found (9). Furthermore, in a s.c. xenograft model of squamous cell carcinoma, i.p. injection of a recombinant (rec) TSP-2 protein containing the type 1 repeat domains resulted in decreased tumor angiogenesis and tumor volume (10). This rec-TSP-2 protein also inhibited human dermal microvessel endothelial cell (MvEC) tube formation on Matrigel (10). These reports suggest that TSP-2 expressed by stromal cells, by tumor cells after stable transfection, or when given systemically can inhibit tumor angiogenesis.

The current paradigm for the antiangiogenic effect of TSP-1 and TSP-2 invokes a proapoptotic mechanism, based on the reports that TSP-1 promotes apoptosis of dermal MvEC in a CD36 receptor—, Fas—, and caspase-dependent mechanism, and is supported by the fact that the type 1 repeat domains of TSP-1 and TSP-2 are homologous (11–13). Other mechanisms by which TSP-2 could inhibit angiogenesis include the recently identified caspase-independent antiproliferative effect shown in dermal MvEC propagated in vitro (14) and by its interaction with matrix metalloproteinase (MMP)-2 (15, 16). TSP-2 and TSP-1 interact with the zymogens of MMP-2 and MMP-9 and can inhibit their activation (15, 17). Low-density lipoprotein receptor–related protein 1 (LRP1) is known to mediate the cellular internalization and lysosomal degradation of TSP-1 and TSP-2 (16, 18–24), and the level of extracellular MMP-2 was shown to be regulated by LRP1 and TSP-2 in fibroblasts propagated in vitro (16). The involvement of a LRP1-mediated mechanism in the antiangiogenic effects of TSP-2 or TSP-1 has been proposed previously (16, 25), although the expression and localization of LRP1 in vivo were not examined. In addition, a relationship of the in vivo antiangiogenic effect of TSP-1 or TSP-2 to a LRP1-mediated clearance of a TSP-1 or TSP-2/MMP-2 complex as well as to reduced levels of MMP-2 has not been shown in a tumor model.

Malignant gliomas are highly vascularized, proliferative, and infiltrative primary brain tumors that are associated with a poor
prognosis (short survival) for patients (26). In the present study, we tested the antiangiogenic activity of TSP-2 in a malignant glioma that is representative of tumors in which MMP-2 is known to promote angiogenesis (27–29). There are few published data regarding the expression or function of host stromal TSP-2 in malignant gliomas. We found that the absence of TSP-2 in the brain resulted in a significantly greater tumor volume, greater microvessel density, and higher levels of MMP-2 and MMP-9 in the gliomas propagated in the TSP-2−/− mouse brain. In addition, we colocalized MMP-2, TSP-2, and LRPI to the microvasculature in tumors propagated in the wild-type (WT) mouse brain. In vitro, we found that the addition of rec-TSP-2 to mouse brain MvEC reduced the level of MMP-2 in the conditioned medium and that this effect required LRPI. Furthermore, we show that rec-TSP-2 inhibits mouse brain MvEC invasion through a LRPI-dependent mechanism that is Fas independent. These data suggest that LRPI internalization of a TSP-2/MMP-2 complex in the microvasculature is a mechanism whereby TSP-2 could act to inhibit angiogenesis in malignant gliomas.

Materials and Methods

Reagents. The following antibodies were purchased: monoclonal antibody (mAb) anti–glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Cell Signaling Technologies, Inc., Flanders, NJ); mAb anti-MMP-2, which recognizes mouse and human MMP-2 (mAb 3308, Chemicon, Temecula, CA); rabbit anti-mouse MMP-9 IgG (AB190947, Chemicon); rat anti-mouse CD31 IgG (Pharmingen, San Diego, CA); mAb anti-LRP1, which recognizes human and mouse LRPI (mAb 5A6B5; Maine Biotechnology/Biodesign International, Saco, ME); mAb anti-actin (Sigma Chemical Co., St. Louis, MO); mAb anti-TSP-1 IgG, which recognizes human and mouse TSP-1 (mAb A6.1, Neomarkers, Fremont, CA); and goat anti-TSP-2 IgG (sc-12313), which recognizes mouse and human TSP-2 (Santa Cruz Biotechnology, Santa Cruz, CA). mAb anti-human TSP-2, 6B5.36-1, was isolated from a panel of mouse mAbs produced against rec-overlapping pieces of human TSP-2. This mAb cross-reacts by immunoblotting and ELISA with rec-human and mouse TSP-2, and it was subcloned and used for immunohistochemical analyses of TSP-2 in mouse samples. Rec receptor-associated protein (RAP; denoted RAP) was purchased from Maine Biotechnology/Biodesign International and dialyzed to remove sodium azide before use. Human rec TSP-2 (denoted rec-TSP-2) produced in baculovirus-transduced insect cells has been described previously (30).

Animal experiments. Malignant mouse glioma cells, GL261, were harvested with buffered EDTA, washed, and resuspended in PBS, and 1 × 10⁶ cells in 10 µL PBS were injected with stereotactic assistance into the mouse brain as described previously (31, 32). After 12 days, the animals were euthanized, and the brains were harvested and fixed in buffered formalin before embedding in paraffin or snap-frozen. TSP-2−/− mice were generated on a 129/C57Bl/6 genetic background and have been described previously (33). The housing and method of euthanasia were in accordance with the guidelines and regulations established by the Animal Welfare Act (PL99-158) and the Guide for the Care and Use of Laboratory Animals as part of the fully accredited (American Association for the Accreditation of Laboratory Animal Care International) Animal Resources Program and in accordance with University of Alabama at Birmingham animal approval (04050989).

Immunostaining. Tissue sections (5 µm) cut from paraffin-embedded blocks were deparaffinized, rehydrated, permeabilized with 0.1% pepsin, and the endogenous peroxidases were blocked, before reaction with 10 µg/mL anti-primary antibody (TSP-2, MMP-2, MMP-9, or LRPI) IgG or nonspecific IgG as described (34). Subsequently, the sections were reacted with a secondary biotin-conjugated multikin antibody, washed, reacted with streptavidin-conjugated to horseradish peroxidase (HRP), and developed with 3,3′-diaminobenzidine substrate for 5 minutes using a kit from Biogenex (San Ramon, CA) according to instructions. Sections were counterstained with H&E or methyl green and analyzed by a blinded investigator using a Leica DMR microscope.

Quantification of microvessel density. Two sections containing tumor from each animal were immunostained with anti-CD31 IgG as described above. Six fields of tumor were analyzed at ×20 magnification for microvessel density, which is expressed as the number of capillaries or venules (diameter ≤ 1 mm²) in 4-mm² area of tumor.

Estimation of the quantity of matrix metalloproteinase-2 and matrix metalloproteinase-9 associated with the microvasculature of tumors. The densitometric intensity of substrate on three microvessels per ×20 magnification photomicrograph and from six photomicrographs of tumor per animal brain were measured using Adobe Photoshop. The background densitometric intensity was subtracted.

Quantification of tumor volume. Mouse brains were serially sectioned, and one section per 80 µm was stained with H&E and digitally photographed using a ×12 objective. A pixel number was obtained for the tumor area in each section in each tumor using Adobe Photoshop, and the identification of tumor cells was determined by the neuropathologist. The pixel numbers for each section from each tumor was then summed to recreate tumor volume as described (31, 32).

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay. The ApoTag Plus Peroxidase In situ Apoptosis Detection Kit (Chemicon) was used according to the manufacturer's instructions as described (32).

Real-time reverse transcription-PCR. Total RNA was obtained using the RNasey kit (Qiagen, Valencia, CA) from tumor tissue isolated by laser capture microdissection (LCM). RNA was quantitated using Ribogreen (Molecular Probes, Eugene, OR), and equivalent amounts of RNA were reverse transcribed to cDNA as described previously (32). Quantitative reverse transcription-PCR (RT-PCR) analysis was carried out with the SYBER Green PCR Master Mix (Applied Biosystems, Foster City, CA) using the GeneAmp 5700 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. The quantitative RT-PCR reaction was done using 4 µL of the synthesized cDNA, 12.5 µL SYBR Green PCR Master Mix, 1 µL of primer, and 7.5 µL water in a final 25-µL volume. MMP-2 and MMP-9 cDNA were amplified using sequence-specific primers for mouse MMP-2-forward 5′-CTGTTGACCTCTGAGTCTCT3′ and reverse 5′-CTGTTTCTTACACGCTAGCT3′ or mouse MMP-9-forward 5′-TAAG-GAAAGGCCCTGTAAAT-3′ and reverse 5′-CTACGGCGCTCTCTCTAG-3′ (Invitrogen, Carlsbad, CA). Samples were assayed in duplicate and the values were normalized to the relative amounts of actin.

Mouse brain microvessel endothelial cells. Immortalized mouse brain MvEC, a gift from Dr. Lena Claesson-Welsh (Uppsala, Sweden), were isolated from the brain of a temperature-sensitive SV40 large T antigen transgenic mouse (35) and propagated in Ham's F-12 medium with 10% fetal bovine serum (FBS) (36).

Immunoblot analysis. Mouse brain and MvEC were lysed in 0.01M Tris Base pH 7.4, 0.15M NaCl, 1% Deoxycholate, 1% Triton X-100 and 1.0% SDS, (RIPA) lysis buffer with protease inhibitors as described previously (32, 34). The lysates or conditioned medium were electrophoresed on SDS-PAGE, transferred to an Immobilon P membrane, probed with primary antibody (4°C, overnight), washed, reacted with a HRP-conjugated secondary antibody (Bio-Rad, Hercules, CA), and developed using a chemiluminescent kit (Amersham, Buckinghamshire, United Kingdom). Band intensities were evaluated by densitometry of three different autoradiographs.

Small interfering RNA experiments. Purified duplex small interfering RNA (siRNA) directed toward mouse LRPI was synthesized by DHarmaco, Inc. (Boulder, CO). The target sequence for the siLRPI was AGAACUCUCA-GUAACAUCA, and the sense sequence for the siRNA was GCAUCUCA-GUAACAUCAATTT and the antisense sequence was UGAUAACUCAU-GAGAUGCdTdT. Purified duplex siRNA, directed against mouse Fas, was purchased from Santa Cruz Biotechnology (SC-29312). Cells plated in Ham's
F-12 medium with 10% FBS were transfected with this siRNA using LipofectAMINE (Invitrogen), and 48 hours later, the cells were lysed in RIPA with protease inhibitors and immunoblotted to evaluate changes in LRP1 or Fas expression.

**Invasion assay.** Mouse brain MvEC were harvested with buffered EDTA, resuspended in serum-free Ham's F-12 medium with 1% bovine serum albumin, and plated (15,000 cells per well) onto 8-µm pore filters coated with growth factor–reduced Matrigel. Ham's F-12 medium with 5% FBS was placed in the bottom chamber. Cells were allowed to invade for 24 hours (33°C, 5% CO2), at which point the cells on the top surface of the filter were removed, and the cells on the bottom surface fixed, stained, and counted. In the absence of added inhibitor, typically 10% of cells invaded.

**Statistics.** A two-sample t test was used for data analysis after determining that the data were normally distributed. \( P < 0.05 \) was required for statistical significance.

**Results**

**Greater microvessel density in malignant gliomas propagated in the TSP-2 \(^{-/-} \) mouse brain.** Host stromal TSP-2 has been reported to act as an inhibitor of angiogenesis in chemically induced squamous cell carcinoma of mouse skin (8). As TSP-2 is expressed in the normal brain (3, 4), we investigated whether host stromal TSP-2 plays a role in regulating angiogenesis in malignant gliomas. The mouse malignant glioma cell line, GL261, was injected into the brains of TSP-2\(^{-/-}\) or WT mice of identical genetic background. Immunostaining of serial sections of the tumors with anti-mouse CD31 IgG showed a 2.5-fold higher microvessel density in tumors propagated in TSP-2\(^{-/-}\) mouse brain (mean \pm\ SE, 2.5 \pm 0.4 microvessels per 4 mm\(^2\) area of tumor) than in tumors propagated in WT mouse brain (1.0 \pm 0.1 microvessels per 4 mm\(^2\) area of tumor; \( P = 0.001; \) Fig. 1A, B, and D). CD31 immunostaining to quantify microvessel density in the TSP-2\(^{-/-}\) mouse has been used previously (6, 7, 37). No morphologic difference in the microvessels of tumors propagated in the TSP-2\(^{-/-}\) versus the WT mouse was detected by histopathologic analysis. Nonspecific mouse IgG did not react with the endothelial cells (Fig. 1C). These findings suggest that brain stromal TSP-2 can inhibit angiogenesis in malignant gliomas.

**Increased tumor volume in malignant gliomas propagated in the TSP-2 \(^{-/-} \) mouse brain.** Angiogenesis has been implicated as the rate-limiting step in tumor growth and progression in a variety of cancers, including malignant gliomas (26). Digital analysis of tumor volume (31, 32) showed that malignant gliomas propagated in TSP-2\(^{-/-}\) mouse brain exhibited a 2-fold greater tumor volume (mean \pm\ SE, 550,000 \pm 90,000 pixels) than tumors propagated in WT mouse brain (280,000 \pm 40,000 pixels; \( P = 0.01; \) Fig. 2A-C). The data shown are combined from two different experiments: one in which seven WT mice and six TSP-2\(^{-/-}\) mice were used and the brains were formalin fixed on harvesting (Fig. 1A-D) and another in which four WT mice and four TSP-2\(^{-/-}\) mice were used and the brains were snap frozen. These data support a role for TSP-2 in inhibiting tumor growth in malignant gliomas most likely through an inhibition of angiogenesis.

**Matrix metalloproteinase-2 and matrix metalloproteinase-9 localize to the microvasculature in malignant gliomas propagated in the TSP-2 \(^{-/-} \) mouse brain.** The mechanism by which TSP-2 inhibits microvessel density in tumors is unknown. As fibroblasts isolated from TSP-2\(^{-/-}\) mice express elevated levels of MMP-2 protein in the conditioned medium (38), it has been suggested that TSP-2 inhibits angiogenesis by reducing the levels of MMP-2. Both MMP-2 and MMP-9 bind TSP-2 (15, 17) and the expression of MMP-2 and MMP-9 is elevated in the endothelial
cells and associated basement membrane of human malignant glioma tumor biopsies compared with normal brain (39, 40). On densitometric analysis of immunostaining, we found a significantly higher amount (2.5-fold) of MMP-2 localized in the tumor microvasculature of malignant gliomas propagated in the TSP-2−/− mouse brain (mean ± SE, 146 ± 17) than in those propagated in WT mouse brain (57 ± 24; P = 0.01; Fig. 3A-C). Similarly, significantly higher amounts of MMP-9 (mean intensity ± SE, 121 ± 12) were detected in the tumor vasculature propagated in the TSP-2−/− mouse brain than in those propagated in WT mouse brain (mean intensity ± SE, 55 ± 5.0; P = 0.03; Fig. 3E-G). Lower levels of MMP-2 and MMP-9 were detected in association with the glioma cells in the tumors propagated in the TSP-2−/− or WT mouse brain. Specificity was confirmed by the lack of reactivity of non specific mouse IgG with endothelial cells or other cells (data not shown). As the tumors propagated in the TSP-2−/− mouse exhibited a significant elevation in microvessel density, compared with the tumors propagated in the WT mouse (Fig. 1), and the microvessel density data were always tightly clustered in tumors propagated in the WT mice, the immunostaining for MMP-2 and MMP-9 was done on only four of the tumors propagated in the WT mouse. Thus, TSP-2 may modulate microvessel density by regulating MMP levels.

Matrix metalloproteinase-2 and matrix metalloproteinase-9 mRNA levels in malignant gliomas propagated in the wild-type and the TSP-2−/− mouse brain. To determine whether absence of MMP-2 in the TSP-2−/− mouse brain results in enhanced transcription of MMP-2 and MMP-9 or stabilization of mRNA, we isolated total RNA, and did quantitative RT-PCR analysis of the MMP-2 and MMP-9 mRNAs. We found no increase in the relative levels of MMP-2 and MMP-9 mRNAs in three tumors propagated in the TSP-2−/− mouse brain compared with three tumors propagated in the WT mouse brain after normalization of the mRNA levels to the level of β-actin mRNA (Fig. 3D and H, respectively). We cannot entirely rule out the possibility that the tumor cell signals for MMP-2 and MMP-9 mRNAs obscured the signals of the corresponding vascular mRNAs; however, this is thought to be unlikely as protein levels of MMP-2 and MMP-9 were significantly higher in the vasculature than in tumor cells. Thus, enhanced transcription of MMP-2 or MMP-9 was observed only in the tumors propagated in the TSP-2−/− mouse brain.

Expression and localization of thrombospondin-2 in the wild-type mouse brain: brain microvessel endothelial cell express thrombospondin-2 protein in vitro. The immunostaining of TSP-2 protein in malignant gliomas propagated in WT mouse brain indicated a characteristic pattern with localization to the microvasculature, a population of host stromal cells consistent with microgliia, and focally to the neurophil (Fig. 4A). Only a few tumor cells (<8%) synthesized TSP-2 and those that did formed distinct foci (data not shown). The TSP-2 synthesized in the malignant gliomas propagated in WT mouse brains seemed to be predominantly derived from host stromal cells, because immunostaining of tumors propagated in the brains of the TSP-2−/− mice with a mAb to TSP-2 revealed only minimal focal or undetectable levels of TSP-2 protein (Fig. 4B). The absence of TSP-2 in TSP-2−/− mouse brain and its expression in normal WT mouse brain was confirmed by immunoblot analysis of whole mouse brain homogenates (Fig. 4C and D, lanes 1 and 2, respectively).

As MMP-2 and MMP-9 are capable of interacting with TSP-2 through its type 1 repeat domain (15, 17), we determined whether these MMPs could interact with TSP-2 in the gliomas propagated in WT mice using the in vitro mouse brain MvEC model. Immunoblotting of conditioned medium obtained from MvEC cells propagated in serum-free conditions with mAb anti-TSP-2 confirmed that these cells synthesize and secrete TSP-2. Proteins of apparent molecular weights of 170 and 160 kDa were detected, which are consistent with the migration of TSP-2 and a product of proteolysis, respectively. The intensity of the protein bands was proportional to the volume of conditioned medium blotted (Fig. 4E, lanes 1-3). As endothelial cells are known to express TSP-1, we stripped the membrane and reprobed with anti-TSP-1 IgG and found a doublet band with apparent molecular weights of 180 and 160 kDa, consistent with the migration of TSP-1 and a product of proteolysis, respectively. The intensity of these bands was also proportional to the volume of conditioned medium blotted (Fig. 4F, lanes 1-3).
Similar apoptotic indices of malignant gliomas propagated in wild-type or TSP-2−/− mouse brain. The ability of a rec-TSP-2 protein that contains type 1 repeats to induce apoptosis of human dermal MvEC propagated in vitro (10) suggests a mechanism whereby TSP-2 could inhibit angiogenesis. However, a terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay that determines the apoptotic or cell death index of malignant gliomas (tumor and stromal cells) propagated in five TSP-2−/− and five WT mouse brains revealed no significant difference in the tumors propagated in the TSP-2−/− or WT mouse brains (mean ± SE, WT mice 5.6 ± 0.7% and TSP-2−/− mice 7.3 ± 0.7%; P = 0.13; Fig. 4G). In addition, no significant difference in microvascular cell TUNEL positivity was detected in tumors propagated in TSP-2−/− or WT mice (WT mice 13 ± 4% and TSP-2−/− mice 13 ± 3%; P = 0.94).

Thrombospondin-2 reduces the level of matrix metalloproteinase-2 in the conditioned medium of mouse brain microvessel endothelial cells through a low-density lipoprotein receptor–related protein 1–dependent mechanism. Yang et al. (16) have shown that MMP-2 binds TSP-2, creating a complex that is internalized by LRP1 and targeted for lysosomal degradation. The physiologic relevance of this clearance process is supported by studies using fibroblasts isolated from TSP-2−/− mice in which conditioned medium has been shown to contain elevated levels...
of MMP-2 protein (38). To determine whether LRP1 could internalize a complex of TSP-2 and MMP-2 proteins in mouse brain MvEC and thereby reduce the level of MMP-2 in the microvasculature of tumors propagated in the WT mouse brain, we examined the role of LRP1 in modulating the levels of MMP-2 protein in the conditioned medium of mouse brain MvEC propagated in vitro. Initially, we examined the levels of LRP1 in MvEC by immunoblotting the whole-cell lysate with mAb anti-LRP1 as well as by immunoprecipitating the lysate of the cells with mAb anti-LRP1 followed by immunoblotting with mAb anti-LRP1. We detected a band at 85-kDa consistent with LRP1 (data not shown). We then determined the effect of the addition of rec-TSP-2 (1 or 5 nmol/L) to the serum-free medium of mouse brain MvEC and found that it resulted in a significantly lower level of MMP-2 in the conditioned medium at 24 hours (Fig. 5A, lanes 1-3; lanes 2 and 3 showed a 70% and 90% reduction, respectively, based on densitometry). As the levels of MMP-9 in the serum-free conditioned medium of mouse brain MvEC were very low, we did not examine MMP-9 levels on addition of rec-TSP-2.

The addition of RAP alone also resulted in a slightly higher level of MMP-2 in the conditioned medium of mouse brain MvEC at 24 hours (Fig. 5A, lanes 6 and 8). This finding is consistent with the inhibition of the internalization by LRP1, or a LRP family member, of MMP-2 bound to endogenous TSP-2 or TSP-1 synthesized by mouse brain MvEC. It also is potentially consistent with an inhibition of internalization of a MMP-2/tissue inhibitor of metalloproteinase-2 (TIMP-2) complex by LRP1 based on the recent report by Emonard et al. (42), indicating that LRP1 can internalize MMP-2 bound to TIMP-2 in a TSP-2-independent mechanism.

We then reduced LRP1 with siRNA and examined the effect of rec-TSP-2. At 48 hours after transfection with 200 nmol/L siLRP1, LRP1 was reduced by >95% based on densitometric analysis (Fig. 5B and C); cell morphology and viability were unaffected (data not shown). As a control, we reduced Fas with siRNA. At 48 hours after transfection with 200 nmol/L siFas, Fas was reduced by >80% based on densitometric analysis (Fig. 5D and E); again, cell morphology and viability were unaffected (data not shown). The addition of rec-TSP-2 (1 or 5 nmol/L) to cells transfected with siLRP1 followed 24 hours later by harvesting of the conditioned medium and immunoblotting for MMP-2 showed no significant change in MMP-2 levels (Fig. 5A, lanes 10-12). These findings suggest that LRP1, and not another LRP family member, internalizes the TSP-2/MMP-2 complex in mouse brain MvEC. A similar
Invasion (Fig. 6A). TSP-2 significantly inhibited invasion, suggesting a dose-dependent effect. Addition of RAP (0.1 nmol/L) reduced the level of MMP-2 protein detected in the conditioned medium of brain MvEC at 24 hours (Fig. 6A; \( P < 0.004 \)). A lower level of inhibition of invasion (\( \approx 40\% \)) was seen with 1 or 5 nmol/L rec-TSP-2, suggesting a dose-dependent effect. Addition of RAP (0.1 nmol/L) significantly reversed the inhibition of invasion found with rec-TSP-2 (Fig. 6A; \( P = 0.001 \)), although RAP (0.1 nmol/L) alone did not significantly inhibit invasion. Consistent with our hypothesis that rec-TSP-2 inhibits invasion by promoting the clearance of MMP-2, the MMP-2/MMP-9 inhibitor \((2\text{F})\)-[4-biphenylsulfonylamin]-N-hydroxy-3-phenylpropionamide at 30 nmol/L (the \( IC_{50} \) ref. 45) was also found to inhibit invasion (Fig. 6A). As other investigators have reported that the CD36 receptor mediates the inhibitory effect of TSP-1 on the migration of dermal MvEC toward basic fibroblast growth factor (46), we examined the effect of neutralizing anti-CD36 IgG on the inhibition of invasion observed with rec-TSP-2, but found no reversal of the inhibitory effect of rec-TSP-2 on invasion (Fig. 6A).

We then examined the effect of down-regulation of LRP1 with siRNA on the rec-TSP-2–mediated invasion of brain MvEC through growth factor–reduced Matrigel. The down-regulation of LRP1 blocked the ability of rec-TSP-2 (1 or 50 nmol/L) to inhibit invasion, but the down-regulation of Fas with siRNA had no effect (Fig. 6D). These data strongly suggest that LRP1 is necessary, at least in part, for the inhibitory effect of rec-TSP-2 on invasion. As the addition of rec-TSP-2 reduced the level of MMP-2 protein detected in the conditioned medium of mouse brain MvEC, we suggest that rec-TSP-2 inhibits invasion, at least in part, through the LRP1-mediated clearance of a TSP-2/MMP-2 complex.

**Discussion**

This study examined the mechanism that underlies the ability of TSP-2 to inhibit angiogenesis in a tumor in which MMP-2 is known to promote angiogenesis and tumor growth. The greater microvessel density and tumor volume in tumors propagated in TSP-2/– mice...
TSP-2 Inhibition of Angiogenesis in Murine Gliomas

Figure 6. TSP-2 inhibits invasion of mouse brain MvEC in a LRP1-dependent mechanism. A, mouse brain MvEC were harvested with buffered EDTA, resuspended in serum-free Ham's F-12 medium, and plated (15,000 cells per well) on growth factor-reduced Matrigel-coated filters with or without the addition of rec-TSP-2, RAP, neutralizing anti-CD36 IgG, rabbit IgG, or MMP-2/9 inhibitor at the concentrations indicated. Ham's F-12 medium containing 5% FBS was added to the bottom chamber. Cells were allowed to invade for 24 hours (33 °C, 5% CO₂) followed by removal of the cells on the top surface, fixation, staining, and counting of the cells on the bottom surface of the filter. Conditions were assayed in replicas of three and the results from three experiments were combined. B, mouse brain MvEC were administered 200 nmol/L siLRP1 or siFas, harvested 48 hours later, and plated on growth factor-reduced Matrigel-coated filters with or without rec-TSP-2 at the concentration indicated and allowed to invade as described above. The experiment was repeated and the results were combined. Columns, mean; bars, SE.

brains confirm that TSP-2 exerts a clinically significant antiangiogenic effect in these tumors. Furthermore, the increased levels of MMP-2 and MMP-9 in the microvasculature of tumors propagated in TSP-2−/− mice are consistent with a role for these proteins in this effect. Both the in vitro rec-TSP-2–mediated reduction in the levels of MMP-2 in the conditioned medium of mouse brain MvEC and the rec-TSP-2 inhibition of invasion of mouse brain MvEC occurred in a LRP1-dependent manner. These data suggest that the LRP1-mediated internalization of a TSP-2/MMP-2 complex is a means whereby TSP-2 inhibits angiogenesis in vivo.

The greater microvessel density that was observed in the gliomas propagated in the TSP-2−/− mouse brain is consistent with previous reports of increased microvessel density in models of skin injury, skin tumors, and corneal wound healing in TSP-2-null mice (6–8).

The finding that TSP-2 was expressed at very low levels in the tumors propagated in the TSP-2−/− mouse brain, although the mouse glioma cell line has the potential to produce TSP-2 in vitro, suggests that in vivo the synthesis of TSP-2 primarily reflects a host response to the tumor. This conclusion is consistent with a report that the level of TSP-2 mRNA detected in the stroma of early-stage carcinogen-induced skin papillomas is higher than that in nonneoplastic dermis. This finding also suggests that TSP-2 is expressed as an antiangiogenic response of the host to the tumor (8).

The significant elevation in the levels of MMP-2 and MMP-9 localized to the microvasculature of the malignant gliomas propagated in the TSP-2−/− mouse brain is similar to the elevated levels of MMP-2 described in other studies of TSP-2−/− mice, including those of excisional dermal wounds, sponges implanted s.c., and conditioned medium of cultured fibroblasts (6, 37, 38). The findings are also consistent with the high levels of MMP-2 and MMP-9 in microvascular endothelial cells of human malignant gliomas (39, 40). MMP-2 is thought to promote angiogenesis by proteolysis of the endothelial cell basement membrane, thus facilitating endothelial cell sprouting and invasion (44), and it has also been shown that a reduction in MMP-2 levels in malignant gliomas, in an intracerebral animal model, inhibits angiogenesis and tumor growth (29). Therefore, elevated levels of MMP-2 in the microvasculature of malignant gliomas, propagated in the TSP-2−/− mouse brain, likely promote angiogenesis.

The colocalization of LRP1 with TSP-2 and MMP-2 in the microvasculature of tumors propagated in the WT mouse brain led us to test the hypothesis that internalization of a TSP-2/MMP-2 complex by LRP1 could be one mechanism whereby TSP-2 negatively regulates angiogenesis in malignant gliomas. Intense LRP1 expression has been reported in endothelial cells and focally on tumor cells in biopsies of human malignant gliomas (43). Using mouse brain MvEC, we found that addition of rec-TSP-2 reduced the level of MMP-2 in the conditioned medium in a LRP1-dependent manner. Moreover, rec-TSP-2 significantly inhibited the invasion of mouse brain MvEC. The partial reversal by RAP of this inhibition and the ability of siLRP1 to block the inhibitory effect of rec-TSP-2 support the hypothesis that the formation of a TSP-2/MMP-2 complex, followed by LRP1-mediated clearance, inhibits invasion. Although other investigators have shown that TSP-1 inhibits the migration of dermal MvEC in a CD36 receptor–dependent mechanism (46), our finding that neither addition of neutralizing anti-CD36 IgG nor reduction of Fas with siFas reversed the inhibition of invasion seen with rec-TSP-2, indicates that TSP-2–mediated inhibition of MvEC invasion through growth factor–reduced Matrigel is not dependent on the CD36 receptor or Fas.

A variety of molecules can be endocytosed and targeted for lysosomal degradation. These include TSP-2, TSP-1, TSP-1 or TSP-2 complexed with MMP-9, MMP-2 complexed with TIMP-2, the urokinase-type plasminogen activator (uPA) bound to the uPA inhibitor plasminogen activator inhibitor-1 (24), and, possibly, TSP-1 or TSP-2 complexed with MMP-9. As the LRP1−/− mouse is an embryonic lethal, such clearance is likely to be of physiologic relevance (24), although LRP1 is involved in promigratory signaling events in a manner that suggests dependence on the cellular context. For example, other investigators have shown that the amino-terminal Hep I peptide of TSP-1 promotes random and directed migration of bovine aortic endothelial cells through an interaction with calreticulin and that the available pool of calreticulin is complexed with LRP1 (47, 48).
In summary, our data suggest that the antiangiogenic effect of TSP-2 could occur through the LRPI-mediated internalization of a TSP-2/MMP-2 complex. These data also suggest that a change in the current paradigm for the antiangiogenic effects of TSP-2 and TSP-1 is likely to be necessary and that a new model should include a nonapoptotic LRPI-mediated clearance mechanism.

Acknowledgments

Received 5/5/2005; revised 7/14/2005; accepted 8/4/2005.

Grant support: Institutional National Institutes of Health T32HL07119 (C.Y. Fears) and NIH, National Cancer Institute grants CA97110, CA109748 and P50 CA97247, project 5 (C.L. Gladson).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Mrs. Jo Self for assistance in preparing this article.

References

Low-Density Lipoprotein Receptor–Related Protein Contributes to the Antiangiogenic Activity of Thrombospondin-2 in a Murine Glioma Model

Constance Y. Fears, J. Robert Grammer, Jerry E. Stewart, Jr., et al.


**Updated version**
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/20/9338

**Cited articles**
This article cites 46 articles, 26 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/20/9338.full#ref-list-1

**Citing articles**
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/20/9338.full#related-urls

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.