

Thrombospondin-1 Is Downregulated by Anoxia and Suppresses Tumorigenicity of Human Glioblastoma Cells

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Abstract

Angiogenesis, the sprouting of new capillaries from preexisting blood vessels, results from a disruption of the balance between stimulatory and inhibitory factors. Here, we show that anoxia reduces expression of thrombospondin-1 (TSP-1), a natural inhibitor of angiogenesis, in glioblastoma cells. This suggests that reduced oxygen tension can promote angiogenesis not only by stimulating the production of inducers, such as vascular endothelial growth factor, but also by reducing the production of inhibitors. This downregulation may significantly contribute to glioblastoma development, since we show that an increase in TSP-1 expression is sufficient to strongly suppress glioblastoma cell tumorigenicity *in vivo*.

Key words: tumor • angiogenesis • glioma • p53 • GD-AIF

Introduction

Glioblastoma is the most frequent human primary brain tumor and represents the ultimate and most malignant stage of astrocytoma progression. This progression is accompanied by a sequential series of genetic alterations including mutation or loss of the *TP53* gene on chromosome 17p (1) and the *PTEN* gene on chromosome 10q (2, 3).

Malignant astrocytomas are among the most vascularized human tumors, suggesting that angiogenesis is a crucial event during their genesis. Despite this prominent vascularization, the supply of oxygen and nutrients seems to be insufficient to support such rapidly expanding tumors, and necrosis appears (4). The switch to the angiogenic phenotype of a tumor is thought to result from a shift in the balance between the secretion of angiogenesis inducers and inhibitors. Glioblastoma cells secrete many angiogenic factors, including acidic and basic fibroblast growth factors

(aFGF and bFGF)¹ (5), IL-8 (6), and especially vascular endothelial growth factor (VEGF), which is a specific endothelial cell mitogen (7, 8).

Both the physiological changes that gradually develop during malignant progression of astrocytoma and the genetic alterations arising during this evolution are able to affect the neovascularization of this tumor type. Physiological regulation of angiogenesis in astrocytoma is mediated through stimulation by angiogenic factors. VEGF and IL-8 expression is induced in cells lining necrotic tumor areas where hypoxia upregulates their mRNA levels (7–10). Genetic alterations can affect both angiogenic stimulators and inhibitors in glioblastoma. Wild-type (wt) p53 has been demonstrated to repress the *bFGF* gene, while mutant forms of the protein can activate it *in vitro* (11). Loss of p53 function may also cause an increase in VEGF levels, as

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¹Abbreviations used in this paper: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; GD-AIF, glioma-derived angiogenesis inhibitor factor; TIMP-1, tissue inhibitor of metalloproteinase 1; TSP-1, thrombospondin-1; VEGF, vascular endothelial growth factor; wt, wild-type.

p53 has been suggested to negatively regulate VEGF expression in glioma cells (12). Furthermore, p53-null glioblastoma cells are able to release an inhibitor of angiogenesis, called glioma-derived angiogenesis inhibitory factor (GD-AIF), upon restoration of wt p53 function (13). p53 was also shown to positively regulate the expression of thrombospondin-1 (TSP-1), a physiological inhibitor of angiogenesis, in fibroblasts of Li-Fraumeni patients (14). It is unclear whether p53 also controls TSP-1 expression in glioblastoma and whether TSP-1 and GD-AIF are identical. TSP-1 expression is also upregulated by a potential tumor suppressor gene(s) on chromosome 10 that is lost during the final progression to glioblastoma (15).

TSP-1 is a 450-kD homotrimeric extracellular matrix glycoprotein. It has a complex structure and modulates cellular behaviors like motility, adhesion, and proliferation that are important for tumor growth and metastasis (16, 17). Furthermore, TSP-1 has been shown to inhibit angiogenesis both in vitro by inhibiting endothelial cell proliferation, migration, and cord formation (18–21) and in vivo, in the rat cornea (18). In addition, peptides from TSP-1 type 1 properdin repeats can compete with bFGF for binding to endothelial cells and prevent their bFGF-induced proliferation and migration (22). Both intact TSP-1 and derived peptides have been shown to induce apoptosis in endothelial cells (23). The in vitro antiangiogenic activity of TSP-1 has been demonstrated to be mediated by the CD36 receptor expressed on endothelial cells (24).

Here, we wished to examine whether TSP-1 is regulated by p53 in glioblastoma, whether a decrease in oxygen tension as occur in tumors could alter its expression, and whether increase in TSP-1 levels would affect glioblastoma tumorigenesis.

Materials and Methods

Cell Culture and Anoxic and Cobalt Chloride Treatments. Glioblastoma cells were grown in DME supplemented with 5% FCS and subjected to anoxia as described (9). Cobalt chloride treatment was performed by incubating LN-229 cells with cobalt chloride at different concentrations (100, 200, and 400 μ M) for 24 h. Fresh culture medium was added at the beginning of incubation. At time zero, RNA extraction was performed immediately after medium change.

Northern Blot Analysis. Northern blot analysis was done as previously described (9) with 10 μ g of total RNA. 18S rRNA was stained by immersing the membrane in 0.02% methylene blue, 0.3 M sodium acetate, pH 5.5, for 45 s. The membrane was then destained in water for 3–4 min, photocopied, and completely destained in 0.2 \times SSC, 1% SDS for 15 min. The probes used were a 1.4-Kb BamHI fragment of the human TSP-1 cDNA from plasmid pcDNATS1 (25), a 1.8-Kb BamHI fragment of human p53 cDNA from plasmid pc53SN3, a 1-Kb NotI-EcoRI fragment of human CDKN1 cDNA from plasmid pCEP-WAF1-S (26), a 0.5-Kb EcoRI-BamHI fragment of human VEGF cDNA from plasmid pBspT-KS-VEGF165 (10), and a 0.6-Kb EcoRI-KpnI fragment of human tissue inhibitor of metalloproteinase (TIMP)-1 cDNA from plasmid pBSTIMP1 (provided by W.G. Stetler-Stevenson, National Cancer Institute, Be-

thesda, MD). The probe for fibronectin was synthesized by reverse transcriptase PCR on total RNA from LN-229 cells using primers 5'-GGCGACAGGACGGACATCTTTGGT-3' (forward) and 5'-ATGCTGATGAGCTGGCCCTCGTATAC-3' (reverse). After 3 min of initial denaturation, 35 cycles of amplification were performed at 95°C for 60 s (denaturation) and 70°C for 90 s (annealing and elongation).

Immunoprecipitation. Immunoprecipitations were performed as previously described on cellular extracts (27). The mAbs used were G59-12 (PharMingen) for human p53 and clones A6.1 and A4.1 (GIBCO BRL) for human TSP-1.

Western Blot Analysis. Serum-free conditioned media from 1.25×10^5 (LN-229), 8×10^5 (U87MG), 4×10^4 (D247MG), and 2.6×10^5 (LN-Z308) cells grown under normoxia or anoxia were electrophoresed on a reducing 5% SDS-polyacrylamide gel and then transferred onto Hybond C membranes (Amersham Pharmacia Biotech). The blots were first incubated with a mouse mAb against human TSP-1 (Ab-4, clone A6.1; Neomarkers) and then with a horseradish peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich). The blots were developed with the BM chemiluminescence Blotting Substrate POD kit (Boehringer Mannheim). Western blot analysis was performed on serum-free conditioned media from 10^5 cells of the clones stably transfected with TSP-1 cDNA (C8, C9, E7) or the cloning vector (A7, A8, A9).

Silver Staining. Serum-free conditioned media, corresponding to 10^5 (U87MG, D247MG), 2.5×10^5 (LN-Z308), and 37.5×10^5 (LN-229) cells grown under normoxia or anoxia were precipitated with TCA. Proteins were electrophoresed on a reducing 8% SDS-PAGE using ProSieve 50 acrylamide (FMC Bioproducts). Silver staining was performed using a commercial kit (Amersham Pharmacia Biotech).

Nuclear Run-on Assays. Nuclear run-on assays were previously described (10) using nuclear extracts from LN-229 cells exposed to normoxia for 24 h or to anoxia for 12 and 24 h. The plasmid DNAs deposited on the membrane contained cDNAs for human TSP-1, human VEGF₁₆₅, and mouse lymphocyte β -actin (10). Empty plasmid vectors, pcDNA1neo for TSP-1 and pBS for VEGF₁₆₅, were used as controls for nonspecific hybridization.

Transfection. Cells were transfected with 10 μ g of TSP-1 expression plasmid pcDNATS1 (25) or the pcDNA1neo control vector (Invitrogen) using the calcium phosphate procedure. After 24 h, G418 (600 μ g/ml) was added, and single clones were isolated after 2–3 wk of selection.

ELISA. Human TSP-1 purified from thrombin-activated platelets (28) was used to generate polyclonal antibodies in rabbits. Specificity of this antiserum was examined by Western blot analysis on purified human TSP-1 and bovine TSP-2. No cross-reactivity with TSP-2 was found (not shown). 96-well microplates were coated overnight at 4°C with 0.2 ml per well of rabbit polyclonal anti-TSP-1 antiserum diluted 1:40 in 20 mM Tris-HCl buffer, pH 9. Saturation of the nonspecific binding sites was then performed by a 40-min incubation at 37°C in the presence of 2% BSA in coating buffer. After two PBS washes, samples were added to the wells and incubated for 2 h at 37°C under gentle shaking in the presence of 2% BSA, 0.05% Tween 20, and 0.25 μ g/ml of mouse anti-human TSP-1 mAb (Boehringer-Mannheim). Purified human TSP-1 (0–10 ng) was used as a standard for the calibration curve. The plates were then rinsed three times with PBS-Tween 0.05%, and 0.2 ml per well of alkaline phosphatase-coupled goat anti-mouse IgG (Bio-Rad), diluted 1:500 in PBS-Tween 0.05%–3% BSA, was added for 40 min at 37°C. After seven PBS-Tween washes, the reaction was revealed using *p*-nitrophenyl phosphate as a substrate, and the OD at 405

nm was measured on a microplate densitometer. The detection limit of the assay is 0.3 ng TSP-1. The standard deviation between multiple determinations using this assay was <12%. ELISAs for bFGF and VEGF were performed with Quantikine Immunoassays (R & D Systems, Inc.).

Immunostaining. Immunostaining for microvessels was performed as previously described (29) using a biotin anti-mouse CD31 Ab (clone MEC 13.3; PharMingen). Microvessels were counted in a 100× power field with a 5 × 5 grid. Only vessels crossing the intersection points of the grid were counted. For each tumor, five random fields were counted in a blinded manner by three independent investigators. (M. Tenan, M.-F. Hamou, and J.-F. Brunet [University of Lausanne, Lausanne, Switzerland]).

Tumorigenesis Assay. For each clone, ten Swiss *nu/nu* 6–7-wk-old male mice were injected subcutaneously with 6×10^6 cells. Each mouse was injected with cells from a TSP-1-expressing clone (C8, C9, or E7) in the right flank and with a pool of cells from control clones (A7, A8, A9) in the left flank. Tumor sizes were measured weekly with a caliper. When mice were killed, tumors were resected, weighed, and frozen for histological studies. Tumor volume (mm^3) was calculated as $(\text{length} \times \text{width}^2)/2$.

Results

p53 Does Not Regulate TSP-1 in Glioblastoma Cells. To first establish whether TSP-1 might be related to GD-AIF and mediate p53 control on angiogenesis, we examined whether p53 could regulate TSP-1 expression in glioblastoma cells. TSP-1 expression was analyzed in LN-Z308 glioblastoma cells, where p53 can be regulated by tetracycline (13). Cellular TSP-1 levels were measured in two clones conditionally inducible for p53 (wt4, wt11) in the presence (no induction) or absence (p53 induction) of tetracycline and in a control clone not inducible for p53 (wt1). The amounts of immunoprecipitated TSP-1 protein did not differ upon wt p53 induction in both wt4 and wt11 clones (Fig. 1). Moreover, Northern blotting on five glioblastoma cell lines with wt (U87MG, D247MG) or mutant (LN-229, LN-319, LN-Z308) *TP53* alleles (30) did not show any correlation between TSP-1 mRNA expression and *TP53* gene status (data not shown).

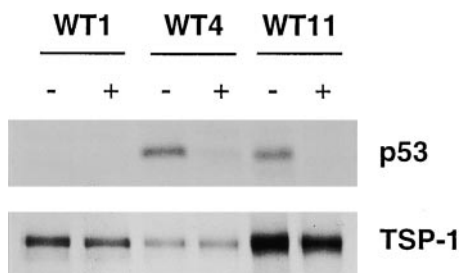


Figure 1. Immunoprecipitation of cellular p53 and TSP-1. Clones wt4 and wt11, derived from parental LN-Z308 cells, contain a wt *TP53* gene under a tetracycline-regulated promoter (+, with tet; –, without tet). In the wt1 clone, p53 is not induced upon tetracycline removal and served as a negative control (13). The antibodies used were A6.1 (GIBCO BRL) for TSP-1 and G59-13 (PharMingen) for p53. Similar results were obtained with anti-TSP-1 antibody A4.1 (GIBCO BRL) (not shown).

To examine whether TSP-1 expression and *TP53* gene status of tumor cells might correlate in vivo, we performed immunostainings for TSP-1 on a series of 10 human WHO grade II/grade IV astrocytoma pairs from patients with recurrence for which we established the status of the *TP53* gene (31). In four cases (no. 4, 7 mutant *TP53* and nos. 15 and 18, wt *TP53*), tumor cells staining for TSP-1 were detected in the low grade but not in the recurrent higher grade astrocytoma. In one case (no. 2, mutant *TP53*), positive cells were observed in the recurrent glioblastoma but not in the initial low-grade tumor. In five cases (nos. 1, 3, and 9, mutant *TP53*, and nos. 16 and 25, wt *TP53*), no staining was observed. These observations did not correlate with *TP53* gene status (data not shown).

TSP-1 Synthesis Is Reduced by Decreased Oxygen Tension. Since variation in oxygen tension is an important factor regulating angiogenesis mediators such as VEGF or IL-8 in glioblastoma, we explored the possibility that decreased oxygen could also influence TSP-1 expression. Four glioblastoma cell lines were placed for 24–48 h in conditions of normoxia and anoxia using anaerobic chambers. By Northern blotting (Fig. 2 a) we found, as expected, an important upregulation of VEGF mRNA expression when cells were exposed to anoxia. On the contrary, TSP-1 mRNA levels were significantly decreased under the same conditions. Densitometric analysis revealed a reduction of up to eightfold (D-247MG) in TSP-1 mRNA levels. The specificity of these observations was further verified by rehybridizing with a probe for fibronectin and TIMP-1, another inhibitor of angiogenesis (32). Furthermore, no evidence for mRNA degradation was observed under anoxia. Secreted TSP-1 was then examined by Western blotting in conditioned media from the same cell lines exposed to normoxia or anoxia (Fig. 2 b). TSP-1 levels were significantly reduced up to sevenfold (D247MG, LN-Z308) under anoxia as compared with normoxia. This reduction in TSP-1 levels was not likely due to protein degradation under anoxia, as no degradation products were observed. To examine whether reduction of protein expression was a general phenomenon under anoxia, we compared levels of secreted proteins under anoxia and normoxia by silver staining. Levels of secreted proteins were not altered for the vast majority of proteins under anoxia. A few large proteins showed reduced secretion (Fig. 2 c). This was not observed on extracts of intracellular proteins (not shown), suggesting impaired secretion of a selected subset of large proteins under anoxia. These data suggest that under anoxic conditions, both a reduction in TSP-1 mRNA levels and a decreased TSP-1 secretion are responsible for the final decrease in released TSP-1. Due to the combination of these mechanisms, glioblastoma cells stressed by low oxygen tension will release high levels of VEGF and reduced levels of TSP-1. This will result in a strong imbalance in favor of angiogenesis stimulation.

Prolonged exposure to anoxia can induce programmed cell death in mammalian cells (33). Therefore, we examined whether the reduction in TSP-1 mRNA and protein levels we observed could be the consequence of an apop-

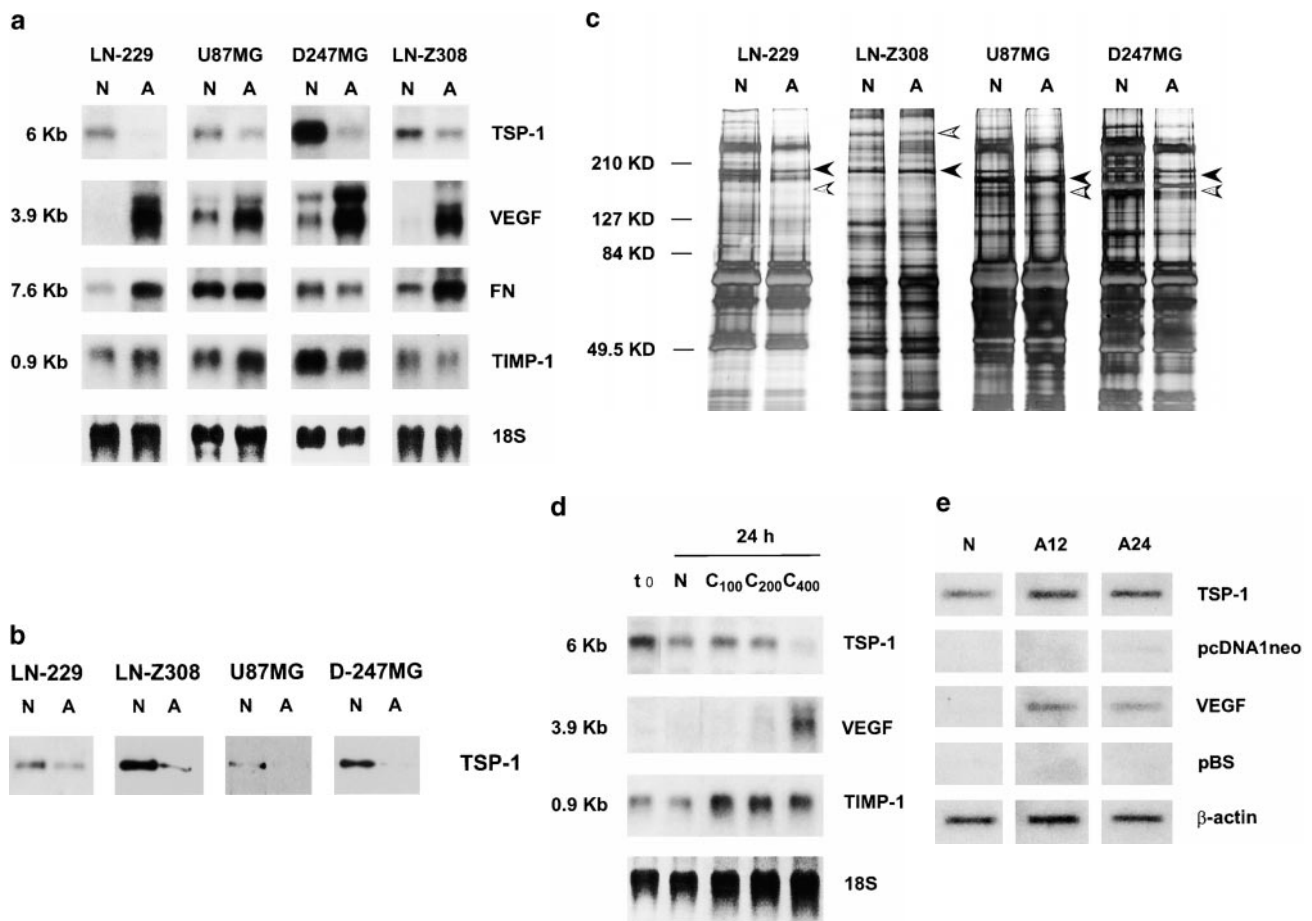


Figure 2. (a) Northern blotting on total RNA of glioblastoma cell lines exposed for 24 or 48 h (D247MG) to normoxia (N) and anoxia (A) (LN-229, $n = 3$; LN-Z308, U87MG, and D247MG, $n = 2$). cDNA probes for TSP-1, VEGF, fibronectin (FN), and TIMP-1 were used. 18S rRNA (18S) was revealed with methylene blue staining. (b) Analysis of TSP-1 expression by Western blotting on 24- or 48-h (D247MG) conditioned media from glioblastoma cell lines exposed to normoxia (N) and anoxia (A) ($n = 3$). Mouse anti-TSP-1 mAb A6.1 (Neomarkers) was used. (c) Silver staining of secreted proteins from glioblastoma cell lines exposed to normoxia (N) and anoxia (A) for 24 and 48 h (D247MG) ($n = 5$). Black arrows provide examples of large proteins for which secretion is not affected by anoxia. Open arrows show proteins for which secretion is decreased under anoxia. Proteins from 10^5 (U87MG, D247MG), 2.5×10^5 (LN-Z308), and 37.5×10^5 (LN-229) cells were loaded. (d) Northern blotting on total RNA of LN-229 glioblastoma cells treated with cobalt chloride at different concentrations (C_{100} , 100 μ M; C_{200} , 200 μ M; and C_{400} , 400 μ M) for 24 h ($n = 2$). Time zero, t_0 (no cobalt treatment); normoxia for 24 h (N). (e) Nuclear run-on assay for TSP-1 mRNA from LN-229 cells. Nuclear extracts were prepared after 24 h normoxia (N), 12 h anoxia (A12), and 24 h anoxia (A24) ($n = 2$). Empty vector DNAs were used as controls (pcDNA1neo for TSP-1; pBSpt-KS for VEGF). The controls of this experiment were previously reported (10).

otic process. Morphological analysis of cells under anoxia for up to 48 h did not reveal any signs of apoptosis. Furthermore, using phosphatidyserine membrane translocation and caspase 3 activation assays, we found no significant difference in either apoptotic or necrotic cells under anoxia as compared with normoxia in all four cell lines analyzed (data not shown). This suggests that the drop in TSP-1 mRNA and protein levels is not due to induction of apoptotic cell death under anoxia. Moreover, when reoxygenated after the 24-h anoxic period, the cells resumed cell growth showing cell viability and reversibility of the anoxic stress (not shown).

Next, we investigated whether the mechanism of TSP-1 mRNA decrease by anoxia shared characteristics of the oxygen-sensing and signal transduction pathways reported for hypoxia-inducible factors. For such factors, cell incubation with cobalt imitates the gene induction normally mediated

by oxygen deprivation stress (34, 35). Therefore, two cell lines were treated with cobalt chloride for 24 (LN-229) or 48 h (D247MG). By Northern blot analysis, we established that the minimal concentration of cobalt chloride able to upregulate VEGF was 400 μ M. Precisely at this concentration, TSP-1 mRNA was significantly reduced in D247MG cells (Fig. 2 d) and LN-229 cells (data not shown). This cobalt concentration is not toxic to the cells and does not induce apoptosis, as measured by annexin V assay and morphology (not shown). After densitometric normalization with 18S rRNA for variations in RNA loading, no variation for TIMP-1 mRNA was found. These results suggest that treatment with cobalt can reproduce TSP-1 mRNA reduction caused by anoxia in these cells.

To further explore whether the drop in TSP-1 mRNA under anoxic conditions was the consequence of a direct downregulation of TSP-1 gene transcription, a nuclear

run-on analysis was performed on LN-229 cells. Whereas under anoxia an increase in VEGF transcription (29- and 20-fold at 12 and 24 h of anoxia, respectively) was observed, TSP-1 and β -actin mRNA levels were not affected as compared with normoxia (Fig. 2 e). These results indicate that the decrease in TSP-1 mRNA observed under anoxic conditions is due to posttranscriptional events and that the anoxic treatment applied does not induce a general downregulation of transcription.

Clearly, further work is necessary to investigate the detailed mechanism(s) responsible for TSP-1 downregulation and establish whether, besides regulation by anoxia and cobalt, they share other components with the biochemical mechanisms regulating hypoxia-inducible factors such as VEGF. It is important to stress that regardless of the mechanism responsible for downregulation of TSP-1 and upregulation of VEGF by anoxia, the net result in the tumor will be that anoxia-stressed tumor cells will stay alive and continuously promote angiogenesis.

TSP-1 cDNA Transfection Can Reduce Glioblastoma Growth. To validate the importance of a reduction in TSP-1 levels by anoxia for tumor development, we examined whether increased TSP-1 expression can restrict tumor growth. For this purpose, we selected LN-229 cells, which form tumors in immunocompromised mice, and transfected them with a human TSP-1 expression vector pcDNATS1 or the empty vector pcDNAneo (25). Neomycin-resistant clones were isolated and screened by PCR for TSP-1 cDNA sequence and ELISA for TSP-1 expression. Three clones with increased TSP-1 expression (TSP-1 clones) and three vector control clones were retained. The cells were grown to prepare serum-free conditioned media, and the levels of secreted TSP-1 were measured by Western blot analysis (Fig. 3). Levels of TSP-1, bFGF, and VEGF were also measured by ELISA (not shown). TSP-1 levels were quite uniform among the control clones, whereas the TSP-1 clones showed two- to 28-fold increased levels of expression. Levels of secreted bFGF (7–12 pg/ml) and VEGF (0 ng/ml) were similar in control and transfected clones. The *in vitro* growth rate of the clones was tested, and we did

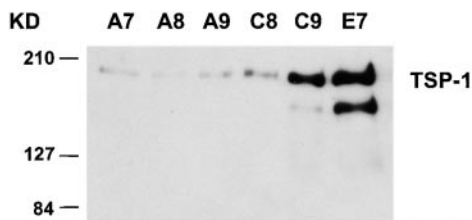


Figure 3. Analysis of TSP-1 expression by Western blotting on serum-free conditioned media from LN-229 clones. Medium from 10^5 cells was loaded for each clone. A7, A8, A9, control clones; C8, C9, E7, TSP-1-expressing clones. The position and size in kilodaltons of molecular mass markers are indicated to the left. In addition to full-length TSP-1, a 160-kD proteolytic fragment was also seen in clones C9 and E7 similar to previous observations (47). Scanning of the 180-kD bands gave the following values: 217 (A7), 82 (A8), 131 (A9), 328 (C8), 2,396 (C9), and 4,100 (E7). The mean of the control clones is 143. Therefore, C8 shows a 2.3-fold, C9 a 16.7-fold, and E7 a 28.6-fold increase in TSP-1 expression.

not observe any consistent differences between control and TSP-1 clones, nor did we see a difference in the capacity to form colonies in soft agar (data not shown).

We then analyzed whether increased TSP-1 expression might affect the ability of these cells to form tumors *in vivo*. Three series of 10 immunodeficient *nu/nu* mice were injected subcutaneously with a pool of cells from control clones on the left side and cells from each TSP-1 clone on the right side. Weekly size measurements showed that control clones gave rise to rapidly growing tumors, whereas TSP-1 clones showed slower or no tumor formation (Fig. 4). After a period of 9 wk, mice had to be killed due to the large size of control tumors and tumor weights were measured (Fig. 5 A). As expected from previous experience with xenografting, we found a high intermouse variability in tumor weights for control cells, demonstrating the importance of injecting both control clones and test clones in the same animals. 25 mice out of 27 showed TSP-1 tumors smaller than controls (Student's *t* test; $P = 0.0002$, $n = 27$).

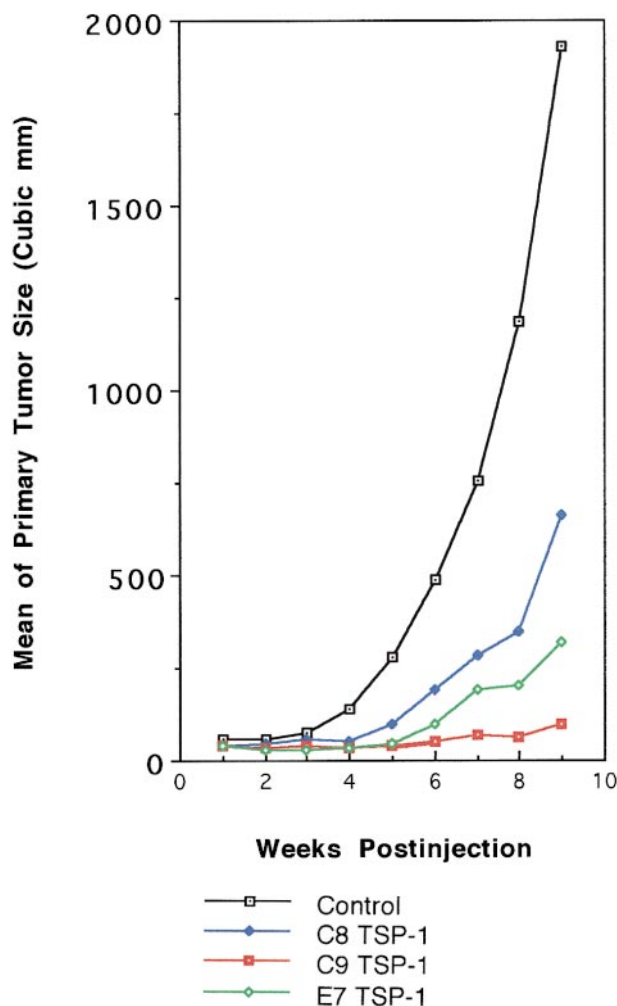


Figure 4. Tumor size measurements in mice injected with a pool of cells from control clones (Control) and cells from individual TSP-1-expressing clones (C8, C9, E7). 5 mice were injected for each clone in two independent experiments (10 mice per clone total).

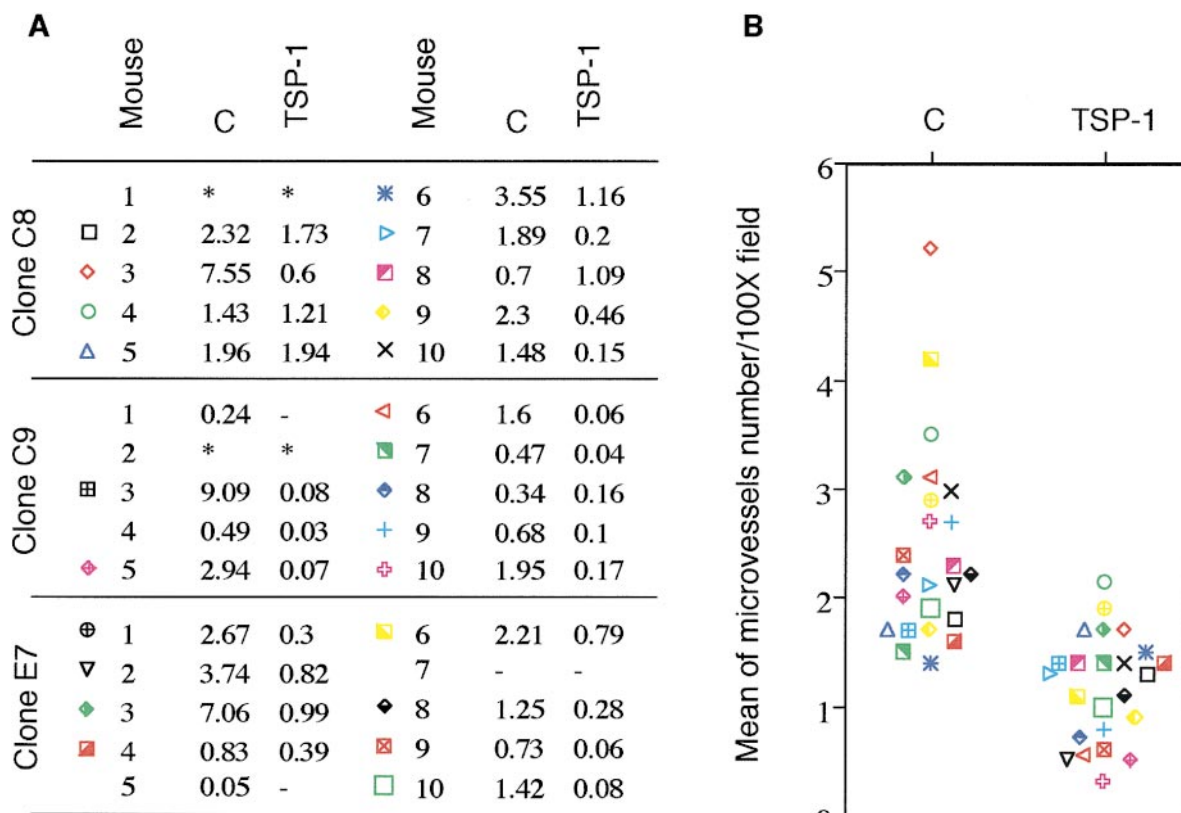


Figure 5. (A) Tumor weights (grams) of mice killed at 9 wk. C, control tumor; TSP-1, TSP-1 tumor. Asterisk (*), mice killed 2 wk after injection due to extensive skin damage after mice fightings. -, no tumor. The difference of mean tumor weight between C and TSP-1 tumors was found to be highly significant (Student's *t* test; $P = 0.0002$, $n = 27$). (B) Measurements of tumor microvessel density. C, control tumor; TSP-1, TSP-1 tumor. Microvessels were counted by three independent investigators. Graphic signs allow one to individually identify microvessel density for tumors of each mouse presented in A. Three cases could not be counted, as no TSP-1 tumor could be detected (mice C9/1 and E7/5), or the tumor was too small to be scored (C9/4). Between C and TSP-1 tumors, the difference in the mean number of microvessels was found to be highly significant (Student's *t* test; $P < 0.0001$, $n = 24$).

These results demonstrated that a two- to 28-fold increase in TSP-1 expression in LN-229 glioblastoma cells suppresses their tumorigenicity *in vivo*.

To verify whether the suppression of tumor growth correlated with a reduction in the extent of angiogenesis, tumors were sectioned and examined for microvessel density. Sections were stained with an antibody against CD31 and microvessels were counted (Fig. 5 B). The mean vessel density in the TSP-1 tumors was 48% of that seen in controls (Student's *t* test; $P < 0.0001$, $n = 24$). Morphological examination of the vessels revealed predominantly capillaries in TSP-1 tumors and large vessels in control tumors (Fig. 6).

Discussion

Glioblastoma is one of the most vascularized human cancers, and the formation of tumor-specific blood vessels occurs early at the onset of tumor growth. The switch to the angiogenic phenotype of a tumor can be related to oncogene activation and tumor suppressor gene loss (36). In fibroblasts of Li-Fraumeni patients, p53 has been shown to regulate the expression of TSP-1 (14). In glioblastoma cells,

p53 inactivation increases bFGF and VEGF expression (11, 12), and p53 overexpression leads to the synthesis of an yet unidentified angiogenesis inhibitor called GD-AIF (13). To establish whether GD-AIF and TSP-1 might be related, we examined whether p53 could control TSP-1 expression in glioblastoma cells. Our data suggest that TSP-1 is not regulated by p53 in glioma and is thus unlikely to be GD-AIF. BAI-1 (brain-specific angiogenesis inhibitor 1), a putative p53-regulated angiogenesis inhibitor, is a new candidate for this activity (37). Loss of TSP-1 expression in the malignant progression of astrocytoma might occur by loss of a positive regulator encoded on chromosome 10 (reference 15) or by downregulation of the TSP-1 gene through methylation of its promoter (38). As LOH (loss of heterozygosity) on chromosome 10 is a hallmark of glioblastoma, this result suggests that downregulation of TSP-1 expression by genetic alteration(s) could be involved in the transition to the last phase of malignant progression. At this stage, the tumor vasculature reaches its ultimate and anarchic expansion and conditions of decreased oxygen supply lead to necrosis.

Hypoxia/anoxia promotes angiogenesis by upregulating the expression of angiogenic molecules. Here, we report

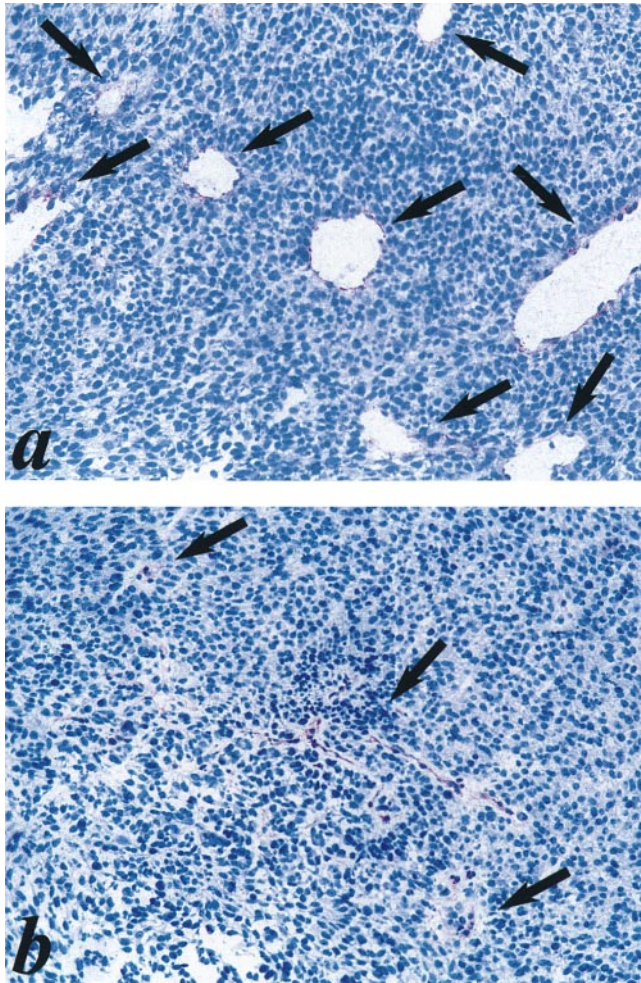


Figure 6. Immunostaining for vessels in tumors from mouse C8/9 using anti-CD31 antibody. (a) Control tumor. (b) TSP-1 tumor. Arrows indicate vessels and capillaries.

the decrease in the production levels of an angiogenesis inhibitor caused by low oxygen tension. We demonstrate that anoxia can dramatically reduce the levels of TSP-1 production and that this occurs at the posttranscriptional level. Posttranscriptional regulation is also one of the major mechanisms accounting for hypoxia/anoxia-induced upregulation of VEGF (39). Recently, in human umbilical vein endothelial cells, TSP-1 mRNA was shown to be stabilized under hypoxia (40). This result might reflect different functions of TSP-1 in different cell types. Upregulation of TSP-1 in endothelial cells might function as a negative feedback mechanism to prevent excessive angiogenesis in physiological conditions of hypoxia. In contrast, downregulation of TSP-1 in tumor cells due to hypoxia might represent an early step to tilt the angiogenic balance in favor of angiogenesis. Later in disease progression, more stable changes due to genetic alterations such as LOH on chromosome 10 might stabilize the angiogenic switch. These results indicate that anoxia should be considered an important process for tumor development for its direct action both on the increase of angiogenic factors and the decrease

in inhibitor(s) of angiogenesis. These mechanisms should prove to be important not only in tumor development but also in other physiological and pathological conditions linked to oxygen depletion.

We show that increased expression of TSP-1 in human glioblastoma cells was able to markedly suppress subcutaneous tumor growth in athymic mice. Since TSP-1 is a potent inhibitor of angiogenesis, we measured vessel density and found an overall reduction of 52%. In tumors from TSP-1 gene-transfected cells, we mainly observed little capillaries, whereas in control tumors the presence of large abnormal vessels was predominant. This is consistent with the primary role of TSP-1 in determining endothelial cell phenotype and differentiation (25). However, since tumors arising from TSP-1 clones were, in the majority of the cases, extremely small, we cannot exclude that reduced vessel numbers are a consequence rather than the cause of smaller size tumors. Furthermore, it will be important to repeat these experiments intracerebrally to evaluate whether TSP-1 can also suppress glioma growth orthotopically.

Because we did not find any direct growth suppressing effects of TSP-1 transfection in culture, the observed *in vivo* suppression of tumor growth is most likely due to other TSP-1 activities. Besides its effect on angiogenesis, TSP-1 can modulate tumor cell adhesion and inhibit proteases (17). TSP-1 expression was previously shown to inversely correlate with malignant progression in human lung and breast carcinoma cell lines (41). Inhibition of angiogenesis and tumor growth has been observed in human breast and skin carcinoma cell lines transfected with TSP-1 cDNA (42, 43). Furthermore, TSP-1 overexpression has been shown to suppress the tumorigenicity of transformed endothelial cells (25). Opposite results were found in a human squamous carcinoma, where reduction of TSP-1 expression reversed the malignant phenotype (44), and in mouse sarcoma and melanoma cells, where TSP-1 promoted tumor cell metastasis (45, 46). The reasons for these apparently discordant results are unclear at this stage. They may reflect the presence of various TSP-1 receptors in diverse cell types and/or different cell responses to different amounts of TSP-1. Understanding the multiple functions of this large protein and the molecular mechanisms regulating its expression in various tissues should help resolve this issue.

In conclusion, we demonstrate that in glioma cells, unlike in fibroblasts, p53 does not regulate TSP-1 expression. We show for the first time that anoxia can reduce the expression of the angiogenesis inhibitor TSP-1. Thus, decreased oxygen tension associated with pathological conditions such as cancer can promote angiogenesis by affecting the regulation of both angiogenesis stimulators and inhibitors. Finally, demonstrating that increased expression of TSP-1 by two- to 28-fold suppresses tumorigenicity of glioblastoma cells in an animal model indicates that even a modest reduction in TSP-1 production might be relevant to human tumor progression. These results suggest that the ability of TSP-1 to restrain tumor growth might be exploited therapeutically, especially in glioblastoma where the possibilities for surgical intervention are often limited.

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