ABSTRACT Concomitant tumor resistance refers to the ability of some large primary tumors to hold smaller tumors in check, preventing their progressive growth. Here, we demonstrate this phenomenon with a human tumor growing in a nude mouse and show that it is caused by secretion by the tumor of the inhibitor of angiogenesis, thrombospondin-1. When growing subcutaneously, the human fibrosarcoma line HT1080 induced concomitant tumor resistance, preventing the growth of experimental B16/F10 melanoma metastases in the lung. Resistance was due to the production by the tumor cells themselves of high levels of thrombospondin-1, which was present at inhibitory levels in the plasma of tumor-bearing animals who become unable to mount an angiogenic response in their corneas. Animals carrying tumors formed by antisense-derived subclones of HT1080 that secreted low or no thrombospondin had weak or no ability to control the growth of lung metastases. Although purified human platelet thrombospondin-1 had no effect on the growth of melanoma cells in vitro, when injected into mice it was able to halt the growth of their experimental metastases, providing clear evidence of the efficacy of thrombospondin-1 as an anti-tumor agent.

Concomitant tumor resistance is an unusual phenomenon wherein animals harboring large primary tumors are resistant to the growth of distant smaller tumors (reviewed in refs. 1 and 2). This resistance depends on circulating factors and disappears upon surgical resection of the primary tumor. In stunning advances over the past several years, O'Reilly and Folkman (3–7) have shown that, in syngeneic rodent systems, the circulating factors responsible for such resistance are inhibitors of angiogenesis. Two of these have been identified: angiostatin and endostatin. In these model systems, primary tumors secrete inducers of angiogenesis and also generate inhibitors of angiogenesis. In the microenvironment of the large primary tumor, the inducers are apparently sufficient to overcome the effects of the inhibitors because the new vessels essential for progressive tumor growth are present. However, when inducers and inhibitors are shed from the tumor bed into the circulation, levels of the more labile inducers fall off rapidly whereas levels of the more stable inhibitors rise, creating a systemic antiangiogenic environment that prevents small nests of metastatic cells from generating neovascularization. As a result, these incipient tumors remain small and dormant. On removal of the primary tumor, inhibitor levels fall and the previously dormant metastases expand vigorously.

Concomitant tumor resistance likely also occurs in human cancer patients. There are a variety of anecdotal clinical reports in the literature describing the fast and disastrous development of what must have been preexisting metastases following surgical removal of a primary tumor, (for example, see ref. 8 and citations in refs. 1 and 2). One such case is that of the patient from whom the widely used human fibrosarcoma line HT1080 was derived and who died from widespread metastases within 3 mo of removal of the primary tumor (9). In addition, an occasional human tumor cell line or primary explant is able to drive nude mice in which it is growing into an antiangiogenic state in which the animals become unable to mount a corneal angiogenic response that is induced in tumor-free controls (5, 6). The molecules generated by these human tumors that are responsible for this effect have not been identified.

The HT1080 line is unusual both in that it was derived from a patient whose history is compatible with concomitant tumor resistance and in that, unlike most sarcomas that have inactivated the p53 tumor suppressor gene (10–12), it retains wild-type p53 activity (13). In fibroblasts, wild-type p53 supports the production of thrombospondin-1 (TSP-1) (14, 15), a potent inhibitor of angiogenesis (16, 17). As might be predicted for a fibroblast-derived tumor line that retains wild-type p53, HT1080 cells secrete relatively high levels of TSP-1. Here, we show that this tumor-derived TSP-1 is active and can drive tumor-bearing animals into an antiangiogenic state that blocks the growth of experimental metastases. Exogenous TSP-1 also blocked the growth of metastases. This work identifies a molecule produced directly by a human tumor that is responsible for concomitant tumor resistance and provides direct evidence for the anti-tumor efficacy of the antiangiogenic agent TSP-1.

MATERIALS AND METHODS

Cell Culture. Human fibrosarcoma cell line HT1080 (American Type Culture Collection) and pigmented mouse melanoma line B16/F10P (a kind gift from P. Polverini, University of Michigan) were maintained in DMEM with 10% fetal calf serum. A second highly metastatic but unpigmented strain of the B16/F10 cell line (18, 19) was kindly provided by I. J. Fidler (University of Texas M. D. Anderson Cancer Center, Houston) and maintained in MEM supplemented with glutamine, nonessential amino acids, and 10% fetal calf serum. Bovine adrenal capillary endothelial cells, BP10T8, a kind gift of J. Folkman (Children’s Hospital, Harvard Medical School, Boston) were maintained in MEM supplemented with glutamine, and 100 μg/ml endothelial cell mitogen (R & D Systems) and used at passage 14 or 15. All of the cells were incubated at 37°C at 7% CO2.

Antisense Clones. The central fragment of human TSP-1 cDNA (nucleotides 883-2572) was amplified by PCR by using primers with HindIII linkers, 5'-ggg agctt CCGCAACTAAC- dIII linkers, 5'-ggg agctt CCGCAACTAAC-3' (left) and 5'-ggg agctt GTACTGGCAGTT-3' (right), and cloned into the polylinker of pRC

This paper was submitted directly (Track II) to the Proceedings office.
Abbreviations: TSP-1, thrombospondin-1; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor.
To whom reprint requests should be addressed. e-mail: n-bouck@nwu.edu.
CMVNeo (Invitrogen) in reverse orientation. HT1080 fibrosarcoma cells were transfected with plasmid expressing antisense TSP-1 were selected in G418, and screened individually for the loss of TSP-1 secretion by Western blotting of serum-free conditioned media. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were measured in serum-free conditioned media by using commercial kits (R & D Systems). TSP-1 was quantitated using Western blots with purified human platelet TSP-1 as a standard.

**Western Analysis.** Seven to 10 μg of total protein from conditioned media, or 40 μg from mouse plasma, was resuspended in loading buffer containing 7.5% β-mercaptoethanol, boiled 3–5 min, run on 6% SDS/PAGE and blotted to Hybond-N membrane (Amersham). The membrane was probed with anti-thrombospondin mAbs A4.1 (16) or MA-1 (24), developed with an enhanced chemiluminescence kit (Amersham) and exposed to x-ray film for 1–20 s.

**Thrombospondin (TSP-1)** was purified from human plasma as described (21, 22) by heparin affinity column with subsequent sizing chromatography on Sephacryl-200 (Pharmacia) and exposed to x-ray film for 1–20 s.

**Assays.**

**In vitro** migration was assayed as described (25) by using BP10T8 bovine capillary endothelial cells in a modified Boyden chamber (Neuroprobe). DMEM with 0.1% BSA served as negative control, and recumbent human FGF (10 ng/ml; R & D Systems) was used instead of antisense to reduce TSP-1, similar control of tumor growth was due to TSP-1 because if antibodies were used instead of antisense to reduce TSP-1, similar control of tumor growth was seen. Animals in which parental HT-1080 cells were strongly angiogenic and the cells formed tumors readily in animals. The TSP-1 secreted by the tumor cells was active because a fraction of high molecular weight secretions containing all of the TSP-1 inhibited endothelial cell migration, and this inhibitory activity was sensitive to antibodies known to neutralize TSP-1 (data not shown). To better understand how these cells became angiogenic in the face of active inhibitor, inducers were examined. Neutralizing antibodies against a variety of inducers demonstrated that VEGF was responsible for the majority of the angiogenic activity present in HT1080-conditioned medium with bFGF accounting for the remainder (data not shown). When quantitated by ELISA, these two inducers were found to be expressed at dramatically high levels, 20 pg/μg for bFGF and 510 pg/μg for VEGF, concentrations that are 10-fold over those seen in tumorigenic fibroblasts that have down-regulated TSP-1 (27).

The thrombospondin secreted by HT1080 cells was active in vivo. HT1080 cells were transfected with an antisense TSP-1 construct and a series of stable subclones isolated that expressed various reduced levels of TSP-1. All of the clones grew at similar rates in vitro; yet, when these clones were tested in vivo, their latent periods were shortened in proportion to the decrease in their TSP-1 levels (Table 1). This acceleration of tumor growth was due to TSP-1 because if antibodies were used instead of antisense to reduce TSP-1, similar control of primary tumor growth was seen. Animals in which parental HT1080 cells had been seeded and grown to a size of 5 mm in diameter were injected daily with 40 mg/kg of neutralizing protein.

**RESULTS**

**HT1080 Secreted Active Antiangiogenic Thrombospondin-I.** In cells of the fibroblastic lineage, the wild-type p53 tumor suppressor gene supports the secretion of high levels of antiangiogenic thrombospondin-1 that prevents the cells from inducing angiogenesis (14, 15). As such cells progress toward tumorigenicity in vitro, wild-type p53 is inactivated and such inactivation is essential for the development of an angiogenic phenotype (27). The HT1080 human fibrosarcoma cell line is atypical in that it has retained wild-type p53 and, as expected, secretes high levels of thrombospondin. Yet, despite the TSP-1 present, the secretions of the HT1080 cells were strongly angiogenic and the cells formed tumors readily in animals. The TSP-1 secreted by the tumor cells was active because a fraction of high molecular weight secretions containing all of the TSP-1 inhibited endothelial cell migration, and this inhibitory activity was sensitive to antibodies known to neutralize TSP-1 (data not shown). To better understand how these cells became angiogenic in the face of active inhibitor, inducers were examined. Neutralizing antibodies against a variety of inducers demonstrated that VEGF was responsible for the majority of the angiogenic activity present in HT1080-conditioned medium with bFGF accounting for the remainder (data not shown). When quantitated by ELISA, these two inducers were found to be expressed at dramatically high levels, 20 pg/μg for bFGF and 510 pg/μg for VEGF, concentrations that are 10-fold over those seen in tumorigenic fibroblasts that have down-regulated TSP-1 (27).

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**Table 1. Latency of tumors growing from clones transfected with TSP-1 antisense**

<table>
<thead>
<tr>
<th>Clone</th>
<th>TSP-1 level</th>
<th>µg TSP-1 per µg secreted protein</th>
<th>Latency, days</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-1080 (parent) high</td>
<td>0.14</td>
<td>23 ± 2</td>
<td>0.02.</td>
</tr>
<tr>
<td>HT-13</td>
<td>intermediate</td>
<td>0.034</td>
<td>15 ± 3*</td>
</tr>
<tr>
<td>HT-16</td>
<td>intermediate</td>
<td>0.0037</td>
<td>13 ± 4*</td>
</tr>
<tr>
<td>HT-18</td>
<td>low</td>
<td>0.0016</td>
<td>8 ± 2*</td>
</tr>
</tbody>
</table>

*Significantly different from parental HT1080, n of 3 or 4, P < 0.02.
TSP-1 antibody, A4.1, and tumor size was measured after 14 days of treatment. Tumor weights were 0.45 ± 0.15 g in buffer-injected animals but much larger, 2.0 ± 0.6 g, in animals injected with anti-TSP-1 antibodies.

**Tumor-Derived TSP-1 Accumulated in the Circulation Causing a Systemic Antiangiogenic Effect.** To determine whether the TSP-1 secreted at such high levels by HT1080 tumor cells accumulated in the circulation of tumor-bearing animals, plasma samples were collected and tested by Western analysis for human TSP-1 using an antibody, MA-1, which fails to recognize mouse TSPs (Fig. 1A). In all cases, the amount of TSP-1 in the circulation of tumor-bearing mice was directly proportional to the amount of soluble TSP-1 the cells secreted in vitro. Dilution curves run on Western blots using human platelet TSP-1 as a control suggested that the actual circulating levels of human TSP-1 in these animals was 6.2 ± 0.8 µg/ml of plasma for those bearing HT1080 tumors, 0.82 ± 0.18 µg/ml of plasma for those bearing HT-13 tumors, and 0.13 ± 0.036 µg/ml of plasma for those bearing HT-23 tumors.

The presence of TSP-1 in the plasma made it antiangiogenic. Plasma from normal mice, when tested at 20 or 50 µg/ml protein, was not stimulatory or inhibitory in vitro (Fig. 1B, Center) or in vivo (Table 2, line 1). However, plasma derived from animals bearing HT1080 tumors producing high levels of TSP-1 was distinctly different. It inhibited both migration (Fig. 1B, Left) and neovascularization (Table 2) induced by bFGF, and this inhibition was due to TSP-1 because neutralizing antibodies alleviated the effect. Plasma from animals bearing tumors that produced low, negligible levels of TSP-1 was weakly angiogenic in vitro and in vivo (Fig. 1B, Right; Table 2), possibly due to the massive amounts of angiogenic inducers that escape from these tumors.

Moreover, animals bearing HT1080 tumors that therefore had high circulating levels of TSP-1 were systemically antiangiogenic in that they became unable to mount an angiogenic response when pellets of bFGF were implanted in their corneas (Fig. 1C) whereas in control animals bearing tumors that secreted a low level of TSP-1 or having no tumor, vigorous ingrowth of vessels was seen.

**The Growth of Metastases Was Severely Curtailed by Tumors Producing TSP-1 and by Exogenous TSP-1.** Concomitant tumor resistance could be seen in animals bearing tumors that produced high levels of TSP-1 but not in animals bearing tumors that produced low levels. Athymic mice were injected in the flank with HT1080 cells or with subclones of HT1080 carrying an antisense TSP-1 construct and therefore secreting intermediate or low levels of TSP-1. When the tumors reached ~5 mm in diameter, tumor-bearing animals, along with a control group, were challenged with a tail vein injection of B16-F10(P) cells, a pigmented melanoma line. Fourteen days later, animals were killed and pigmented metastases in the lung were measured and counted (Fig. 2). Gross examination of the lungs showed a significant decrease in the number and size of metastases in animals with TSP-1-secreting HT1080 tumors, compared with animals bearing TSP-1-negative tumors (Fig. 2A). Quantitative analysis (Fig. 2B) showed a 15- to 20-fold reduction in the number of metastases and a sixfold reduction in their size when animals with TSP-secreting tumors were compared with animals without tumors or with tumors secreting low levels of TSP-1. Clone HT-13, which secreted intermediate levels of TSP-1, caused a notable fourfold decrease in the size of experimental lung metastases but no decrease in their number (Fig. 2C).

TSP-1 appeared to be solely responsible for the restricted growth of melanoma colonies in the lungs of HT1080 tumor-bearing mice because similar restriction was obtained with injections of purified human TSP-1. For these experiments, a “classic” B16-F10 melanoma line that is not pigmented but is known to complete seeding within 48 hr (28) was used. Mice were injected in the tail vein with melanoma cells on day 1, and from days 2 through 23, mice were injected i.p. twice a day with 10 mg/kg purified TSP-1. In the TSP-1-treated animals, the number of surface metastases were reduced to 6–10% of those present in the PBS-treated controls, and their lung weights were reduced to 30% that of controls (Fig. 3, A–B).
The TSP-1 used for in vivo assays had been pretreated with polymixin B to remove endotoxin, and assays indicated endotoxin levels were below detection limit (<0.05 units/μg TSP-1). To eliminate the possibility that a very low level of endotoxin was influencing metastases, an additional control was performed. Animals were injected with the usual two doses of TSP-1, once a day before the introduction of the melanoma cells, and again 1 day after the tumor cell injection. This regime did not alter the seeding or the growth of metastases (Fig. 3, pulse of TSP). TSP-1 injections also were effective against metastases derived from the melanotic B16-F10 because injections of 5 mg/kg day TSP-1 reduced the number of visible surface metastases from this strain by 90% (data not shown).

TSP-1 was not toxic to the B16 melanoma tumor cells themselves because when TSP-1 treatments were halted, metastases grew out in numbers that were not significantly different from those in untreated controls (Fig. 3, reversal). In addition, the growth of the melanoma cells over 5 days in culture was unaffected by the presence of 5 μg/ml of TSP-1, which was renewed daily, and their cloning efficiency on plastic remained unchanged in concentrations of TSP-1 that ranged from 0.75 to 25 μg/ml (data not shown).

**DISCUSSION**

Data presented above show that the human fibrosarcoma cell line HT1080 is able to inhibit the growth of experimental metastases. These results suggest that this inhibition is due to the antiangiogenic activity of circulating TSP-1 that is released by the tumor cells in vivo, circulates at effective levels, and makes the tumor-bearing animals unable to mount an angiogenic response. The data also demonstrate the in vivo efficacy of soluble TSP-1, showing it can be used as a drug to prevent the growth of experimental metastases.

HT1080 produced concomitant tumor resistance directly by secreting active TSP-1, unlike previously studied rodent tumors that create an antiangiogenic state when the tumor cells, or associated stromal elements (29), secrete enzymes or activators of enzymes (30) that generate inhibitors from other molecules. We saw no significant evidence for in vivo breakdown of tumor-derived TSP-1 to fragments smaller than the 140 kDa monomer, a molecule that is as effective an antiangiogenic agent as the intact TSP-1 (16, 17). Although there was not enough TSP-1 available to titrate the minimal amount needed to control metastases in vivo, both 5 and 20 mg/kg/d

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**Table 2. Angiogenic activity of plasma from tumor-bearing animals was due to TSP-1**

<table>
<thead>
<tr>
<th>Primary tumor</th>
<th>Secreted TSP-1</th>
<th>Tested alone</th>
<th>+ bFGF</th>
<th>anti-TSP</th>
<th>+ bFGF and anti-TSP</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>none</td>
<td>0/3</td>
<td>3/3</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>HT-23</td>
<td>low</td>
<td>2* /3</td>
<td>nd</td>
<td>3* /3</td>
<td>nd</td>
</tr>
<tr>
<td>HT1080</td>
<td>high</td>
<td>0 /3</td>
<td>0/3</td>
<td>3* /3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

Pellets containing plasma derived from tumor-bearing mice mixed with additives where indicated were implanted into avascular rat corneas and vigorous vessel ingrowth at 7 days and scored as a positive response.

*p* weakly angiogenic compared to bFGF-induced responses. nd, not determined.

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**Fig. 2. Experimental metastases in tumor-bearing animals.** Groups of four animals with no tumor (none) or bearing 5-mm diameter tumors derived from parental HT1080 that produced high levels of TSP-1 (1), antisense clone HT-13 that produced intermediate TSP-1 levels (2), or antisense clone HT-23 that produced low levels of TSP-1 (3) and received tail vein injections of 10⁶ melanotic B16-F10(P) cells, and 14 days later, lungs were harvested and photographed (A), metastases counted (B), and their average size measured (C). Errors are SEM. * Indicates significant difference from nontumor-bearing animals (none) *P < 0.01.

**Fig. 3. Experimental metastases in animals treated with exogenous TSP-1.** Animals received an injection of amelanotic B16F10 cells on day 1 and thereafter were divided into groups of four which were treated from day 2 to day 23 twice a day with i.p. injections of 10 mg/kg of purified TSP-1 (daily TSP) or an equal volume of PBS vehicle (PBS). One group was treated with TSP-1 only on the day before and the day after the B16F10 injection (Pulse of TSP). A second group was treated with TSP-1 until day 23 and then remained untreated for 14 days (Reversal). Lungs were harvested and metastases counted (A) and lung weight determined (B). Errors show SEM. * Indicates significant difference from PBS control, *P < 0.05.
of the 450 kDa protein were effective. These amounts are comparable on a molar basis to the 1.5 mg/kg/d of purified 45 kDa angiotatin recently shown to be needed to maintain similar control of Lewis lung metastases in vivo (31). In the tumor-bearing animals, a circulating level of 6.2 μg of TSP-1 per milliliter of plasma halted the growth of metastases whereas 0.82 mg/ml merely slowed their growth and 0.13 μg/ml was ineffective. 

Although TSP-1 is a multifunctional protein, its ability to halt the growth of melanoma metastases seemed to be due to its antiangiogenic activity. TSP-1 did not directly affect the melanoma cells in two in vitro assays. Nor did it kill them in vivo because when TSP-1 treatment was withdrawn after two weeks, metastases grew out in numbers that were not significantly different from those seen in untreated controls. The animals bearing TSP-1-secreting tumors had plasma levels of TSP-1 in the inhibitory range, as defined using in vitro experiments (16, 17), and they were clearly in an in vivo anti-angiogenic state. It enhances metastases in numbers that were not 

The animals bearing TSP-1-secreting tumors had circulating levels of 6.2 mg/ml of plasma halted the growth of metastases in tumor-bearing animals, possibly because the TSP-1 concentration was comparable low in the tumor bed, circulating levels of TSP-1 also could be playing a role in this system. Thrombospondin also has been shown to act in an opposite way. It enhances metastases in mice when injected at high levels, if the injection occurs within 5 min of the injection of the tumor cells (33). This effect requires platelet aggregation (34) and can be explained by the ability of TSP-1 to increase the size of emboli and thereby enhance the frequency with which the injected cells arrest in the lung. We saw no such enhancement in our experiments with tumor-bearing animals, possibly because the TSP-1 concentration was comparatively low in the tumor-bearing mice. In experiments in which TSP-1 was injected, the seeding of melanoma cells in the lung was complete before injections were begun. 

It is not possible at this point to determine whether the patient from whose fibrosarcoma the HT1080 cell line was derived in 1974 had high levels of circulating TSP-1. His patient from whose fibrosarcoma the HT1080 cell line was derived in 1974 had high levels of circulating TSP-1. His 

be demonstrated and for whom TSP-1 replacement therapy may someday be helpful. 

We are grateful to Dr. I. J. Fidler, University of Texas, Houston, TX, for melanoma cells and advice on their use, to Sara S. Tolsma, Northwestern College, Orange City, IA, for help with experimental design, and to the National Cancer Institute and National Heart, Lung and Blood Institute for their support of this work through Grants CA52750 and CA64239 (to N.P.B.) and HL28749 (to J.L.).


