Antiangiogenic Treatment with Thrombospondin-1 Enhances Primary Tumor Radiation Response and Prevents Growth of Dormant Pulmonary Micrometastases after Curative Radiation Therapy in Human Melanoma Xenografts

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ABSTRACT

Thrombospondin-1 (TSP-1) is a potent antiangiogenic factor that has been shown to inhibit tumor growth by preventing endothelial cells from responding to a wide variety of angiogenic stimulators. We have demonstrated previously that a-12 primary tumors (human melanoma xenografts) suppress the growth of their spontaneous pulmonary micrometastases by secreting TSP-1 into the blood circulation. The same tumor model was used in the present work to study antitumor effects of combined radiation therapy and antiangiogenic treatment with TSP-1. Curative radiation treatment of a-12 primary tumors resulted in rapid growth of previously dormant micrometastases. Growth of dormant micrometastases could be prevented by treating the host mice with exogenous TSP-1 after the radiation treatment. Treatment with exogenous TSP-1 after subcurative radiation treatment reduced the growth rate of recurrent primary tumors in addition to suppressing metastatic growth. TSP-1 suppressed tumor growth at both primary and metastatic sites by inducing apoptosis in tumor-associated microvascular endothelial cells. Treatment with exogenous TSP-1 before radiation treatment enhanced the antitumor effect of the radiation treatment. The radiopotentiation by TSP-1 involved at least two distinctly different mechanisms, i.e., TSP-1 reduced the fraction of radiobiologically hypoxic parenchymal tumor cells and increased the radiation sensitivity of the tumor microvasculature by promoting radiation-induced endothelial cell apoptosis. In conclusion, the present preclinical study showed that TSP-1 has antiangiogenic, antimetastatic, and radiopotentiation properties that merit additional investigation in clinical studies.

INTRODUCTION

Angiogenesis is necessary for primary tumors and their metastases to grow beyond a certain size, given by the diffusion distances of oxygen and other nutrients (1). Tumor parenchymal cells and immune cells infiltrating tumor tissue produce and secrete several proteins that may stimulate or inhibit angiogenesis (2). VEGF, IL-8, PDGF, platelet-derived endothelial cell growth factor, and bFGF are among the most potent proangiogenic factors identified thus far, whereas the most important antiangiogenic factors include angiostatin and endostatin (3). The rate of tumor angiogenesis is determined by the balance between pro- and antiangiogenic factors (4). Cancer treatment may modify this balance and, hence, change the rate of angiogenesis in the primary tumor and at metastatic sites (5–12).

Treatment strategies based on the use of endogenous antiangiogenic factors or agents inhibiting the action of proangiogenic factors are being developed currently (13). Antiangiogenic therapy may inhibit tumor growth significantly when used as a single treatment modality, but the therapeutic benefit may even be greater when used in combination with established treatment modalities such as surgery, chemotherapy, or radiation therapy (14, 15). Preclinical studies of the potential benefit of giving antiangiogenic treatment in combination with ionizing radiation have given particularly promising results (16–25).

Thus, several laboratories have shown that antiangiogenic treatment may enhance the antitumor effect of radiation therapy without increasing the radiation damage to normal tissues (16, 17, 20, 21, 23). Antiangiogenic agents that have been found to enhance the effect of ionizing radiation in studies of experimental tumors include angiostatin (17, 18), endostatin (25), antibodies against VEGF or VEGFR-2 (10, 19, 21), the soluble extracellular component of VEGFR-2 (22), and small molecular kinase inhibitors such as minocycline, TNP-470, SU5416, and SU6668 (16, 23, 24). The mechanisms underlying the enhanced antitumor effects of the combined treatments have not been determined conclusively for any of these agents, but they seem to differ among the agents and may also depend on how the antiangiogenic treatment is administered relative to the radiation treatment (10, 16, 17, 19, 21, 22, 24, 25). However, there is evidence from studies in vitro that antiangiogenic agents may enhance the antitumor effect of radiation therapy by increasing the radiation sensitivity of tumor endothelial cells (10, 17, 22, 24, 25).

Local treatment of primary tumors may modify the balance between pro- and antiangiogenic factors at metastatic sites (6, 12, 26). Some primary tumors inhibit or even prevent neovascularization of their metastases by secreting antiangiogenic factors into the blood circulation (27). Surgical resection of the primary tumor has then been shown to result in accelerated growth of distant metastatic deposits (5, 28, 29). The effect of treating the primary tumor with curative radiation therapy rather than surgery, is unclear (12, 26). Radiation therapy of the LLC-LM Lewis lung carcinoma, which is known to generate angiostatin, resulted in angiogenesis and growth of dormant pulmonary metastases (26). In contrast, radiation therapy of the PC-3 prostate carcinoma and the FSA-II fibrosarcoma, unlike surgical resection, caused reduced neovascularization at distant sites, presumably because the radiation therapy resulted in enhanced blood levels of endostatin (12). It is also unclear whether any accelerated metastatic growth induced by primary tumor radiation therapy may be prevented by combining the radiation therapy with antiangiogenic treatment.

TSP-1 is a potent antiangiogenic factor that is produced and secreted by many tumor types (30). The expression of TSP-1 in tumors is associated with the mutational status of the p53 tumor suppressor gene, i.e., tumors with wild-type p53 are more likely to show high TSP-1 expression than are tumors with mutant p53 (31, 32). TSP-1 inhibits tumor angiogenesis by binding to the transmembrane receptor CD36 and inducing apoptosis in endothelial cells (33), a mechanism that explains its ability to prevent endothelial cells from responding to
a wide variety of proangiogenic factors, including VEGF and bFGF (34). Clinical studies have indicated that high TSP-1 expression is an independent favorable prognostic factor in several tumor types (31, 35, 36). Studies of experimental tumors have shown that treatment with exogenous TSP-1 may inhibit angiogenesis at both primary and metastatic sites (29, 37, 38). However, studies of antitumor effects of combined radiation therapy and antiangiogenic treatment with TSP-1 have not been reported thus far.

Human melanoma xenograft models showing angiogenic activities and organ-specific metastatic patterns similar to those of the donor patient tumors have been established in our laboratory (39). Primary tumors of one of the models, the D-12 melanoma, have been shown to suppress metastatic growth in distant organ sites by secreting large quantities of TSP-1 into the blood circulation of the host mice (29). Thus, surgical resection of D-12 primary tumors was followed by neovascularization and accelerated growth of pulmonary micrometastases. In the work reported here, we show also that curative radiation therapy of D-12 primary tumors resulted in increased metastatic growth, whereas curative radiation therapy followed by antiangiogenic treatment with TSP-1 reduced the incidence of metastases. Moreover, we show that TSP-1 treatment increased the antitumor effect of radiation therapy in D-12 primary tumors, partly by inhibiting angiogenesis and, hence, tumor regrowth and partly by enhancing the effect of the radiation treatment. The radiopotentiation effect of TSP-1 involved at least two different mechanisms, i.e., TSP-1 treatment reduced the fraction of radiation-resistant hypoxic tumor cells and promoted radiation-induced apoptotic death in tumor endothelial cells.

MATERIALS AND METHODS

Mice and Tumors. Adult (8–10 weeks of age) female BALB/c-nu/nu mice were used as host animals for xenografted tumors. The experiments were performed with intradermal tumors of the D-12 human melanoma line (39). Intradermal D-12 tumors grow to a volume of $\approx 1000$ mm$^3$ in 5 weeks and develop pulmonary metastases in BALB/c-nu/nu mice (40). Aliquots of $3.5 \times 10^5$ cells suspended in 10 $\mu$L of Ca$^{2+}$- and Mg$^{2+}$-free HBSS were inoculated intradermally in the left mouse flank (39). Tumor-bearing mice were allocated to treatment groups at day 13 after tumor initiation. Only tumors with volumes within the range of 60–90 mm$^3$ were included in experiments. Two orthogonal tumor diameters were measured with calipers, and tumor volume ($V$) was calculated as $V = \pi a \times b^2$, where $a$ is the longer and $b$ is the shorter of the two diameters. The animal experiments were approved by the Institutional Committee on Research Animal Care and were performed according to the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing, and Education (New York Academy of Sciences, New York, NY).

Radiation Treatment. Tumors were irradiated at a dose rate of 5.1 Gy/min by using a Siemens Stabilipan X-ray unit, operated at 220 kV, 19–20 mA, and with 0.5-mm Cu filtration (41). The irradiation was performed 14 days after the tumors were initiated. Hypoxic tumors were obtained by occluding the tumor blood supply with a clamp 5 min before irradiation (42).

Treatment with TSP-1. TSP-1 treatment was given by using purified TSP-1 derived from activated human platelets. TSP-1 was diluted in HBSS and administered by i.p. injection, usually in doses of 50 $\mu$L. Radiopotentiation by TSP-1 was studied by giving two doses of TSP-1. The first dose was administered 24 h before and the second dose 1 h before radiation treatment, i.e., at days 13 and 14 after tumor initiation. Antiangiogenic effects of TSP-1 were studied by giving three doses of TSP-1 per week (Monday, Wednesday, and Friday). The first dose was administered at day 15 after tumor initiation, i.e., 24 h after irradiation, and the treatment continued during the whole observation period. The two treatment schedules were used jointly to study the antitumor effects of TSP-1-induced radiopotentiation and TSP-1-induced angiogenesis inhibition in combination.

Treatment with Anti-TSP-1 Antibody. Anti-TSP-1 treatment was given by using an antihuman TSP-1 mouse monoclonal antibody (TSP Ab-1; clone A4.1; NeoMarkers, Union City, CA). The antibody was diluted in PBS and administered by i.p. injection in doses of 100 $\mu$L. The effect of anti-TSP-1 treatment on the development of metastases was studied by treating tumor-bearing mice with daily doses of antibody for 3 weeks. The first dose was administered at day 14 after tumor initiation.

Tumor Growth Delay Assay. Tumor volume was measured weekly after irradiation as described above. Tumor growth delay was defined as the time after irradiation at which a treated tumor reached twice its volume at irradiation minus the median time required by unirradiated control tumors to double their volumes.

Tumor Disaggregation. Single cell suspensions were prepared from tumors by using a standardized mechanical and enzymatic procedure (43). The tumors were minced in cold HBSS with a scalpel before enzymatic treatment at 37°C for 2 h. The enzyme solution consisted of 0.2% collagenase, 0.05% Pronase, and 0.02% DNase in HBSS.

Tumor Cell Survival Assay. The cell survival of tumors treated in vivo was measured in vitro by using a plastic surface colony assay (41, 42). Tumor cell suspensions were prepared as described above, and aliquots of the suspensions were plated in 25-cm$^2$ tissue culture flasks. Plated tumor cells were incubated at 37°C for 14 days in a humidified atmosphere of 5% CO$_2$ in air. The cell-surviving fraction of a treated tumor was calculated from the plating efficiency of the cells of the tumor and the mean plating efficiency of the cells of 6 untreated control tumors. The plating efficiency of the control tumors was within the range of 30–50%.

Determination of Hypoxic Fraction. The fractions of radiobiologically hypoxic cells in untreated tumors and tumors pretreated with TSP-1 were determined by using the paired survival curve method. Tumors were irradiated with 10 Gy under unclamped or clamped conditions. Single cell suspensions were prepared from the tumors immediately after the radiation exposure, and cell survival levels were measured in vitro, using procedures described above. Hypoxic fractions were calculated as SF$_{10}$ (unclamped)/SF$_{10}$ (clamped). The determination of the fraction of radiobiologically hypoxic cells in D-12 tumors from measurements of SF$_{10}$ has been justified previously (41).

Metastasis Assay. The mice were killed 35 days after the primary tumors were initiated, irrespective of the primary tumor treatment. The lungs were removed, rinsed in HBSS, fixed in Bouin’s solution for 24 h, and examined for macroscopic metastases by stereomicroscopy. The presence of pulmonary metastases was always confirmed by histological examinations.

ELISA. A commercial ELISA kit (Chemicon International, Temecula, CA) was used according to the instructions of the manufacturer to measure TSP-1 concentrations in blood plasma. Blood was collected from control and tumor-bearing mice by heart puncture. Plasma samples were prepared and analyzed promptly, because samples stored frozen were found to show lower TSP-1 levels than fresh samples.

Immunohistochemical Detection of Apoptotic Endothelial Cells. Primary tumors and lungs with metastatic deposits were snap-frozen in liquid nitrogen. Histological sections were prepared and stained for either endothelial cells or apoptotic cells. Apoptotic cells were visualized by using an in situ apoptosis detection kit (Apotag; Oncor, Gaithersburg, MD) as described by the manufacturer. To avoid erroneous identification of apoptotic cells because of light staining of necrotic cells, only brown-stained nuclei with morphological characteristics associated with apoptosis were scored as apoptotic. Endothelial cells were visualized by using an avidin-biotin immunoperoxidase method. A1 anti–CD31 rat monoclonal antibody (MEC 13.3; Research Diagnostics, Flanders, NJ) was used as primary antibody. Controls included omission of the primary antibody and incubation with blocking peptide before staining. Immunostained sections were counterstained with hematoxylin. Apoptotic frequencies in tumor-associated endothelial cells were determined by examining adjacent sections, one stained for endothelial cells and the other stained for apoptotic cells.

Statistical Analysis. Experimental data are presented as arithmetic mean ± SE unless otherwise stated. Statistical comparisons of data sets were performed by using the Student’s $t$ test (single comparisons) or by one-way ANOVA (multiple comparisons) when the data sets complied with the conditions of normality and equal variance. Under other conditions, comparisons were performed by nonparametric analysis using the Mann-Whitney rank-sum test (single comparisons) or the Kruskal-Wallis one-way ANOVA on ranks (multiple comparisons). The Bonferroni’s method (parametric tests) or the Dunn’s method (nonparametric tests) was used to identify the data sets that
Results

Pulmonary Metastases. The effect of radiation therapy of the primary tumor on the development of pulmonary metastases was investigated by comparing the metastatic frequency in untreated mice and mice treated with anti-TSP-1 antibody, surgery, subcurative radiation therapy, or curative radiation therapy (Fig. 1). The tumor control dose, i.e., the radiation dose that results in local control in 50% of the tumors, has been determined to be 30 Gy for D-12 tumors (42), and, therefore, subcurative radiation therapy was given as a single dose of 15 Gy and curative radiation therapy as a single dose of 30 Gy. The percentage of mice that developed macroscopic surface metastases was used as a parameter for metastatic frequency. The metastatic frequency in mice given 30 Gy was similar to that in mice treated with anti-TSP-1 antibody or surgery and significantly higher than that in untreated mice ($P = 0.0000044$). In contrast, the metastatic frequency in mice given 15 Gy did not differ from that in untreated mice.

The significance of TSP-1 as an inhibitor of the development of metastases was investigated by measuring the TSP-1 concentration in the blood plasma of untreated mice and mice treated with surgery, 15 Gy, or 30 Gy (Fig. 2). An inverse correlation was found between TSP-1 concentration and metastatic frequency. Thus, the TSP-1 concentration was significantly lower in mice treated with surgery or 30 Gy than in untreated mice and mice given 15 Gy ($P < 0.0000010$). Moreover, the TSP-1 concentration in mice given 15 Gy did not differ from that in untreated mice, and the TSP-1 concentration in mice given 30 Gy did not differ from that in mice treated with surgery.

The effect of exogenous TSP-1 on the development of pulmonary metastases was investigated by comparing the metastatic frequency in untreated mice and mice treated with 50-$\mu$g-doses of TSP-1 alone, 30 Gy alone, or 30 Gy in combination with 25-$\mu$g-doses or 50-$\mu$g-doses of TSP-1 (Fig. 3). The metastatic frequency was significantly higher in mice treated with 30 Gy alone than in untreated mice ($P = 0.0000028$), consistent with the data in Fig. 1. Untreated mice showed a significantly higher metastatic frequency than mice treated with 50-$\mu$g-doses of TSP-1 alone ($P = 0.00021$), and mice given 30 Gy alone showed a significantly higher metastatic frequency than mice treated with 30 Gy in combination with 25-$\mu$g doses ($P = 0.0000010$) or 50-$\mu$g doses ($P < 0.0000010$) of TSP-1. The metastatic frequency in mice treated with 30 Gy in combination with 25-$\mu$g-doses of TSP-1 was similar to that in untreated mice, and the metastatic frequency in mice treated with 30 Gy in combination with 50-$\mu$g doses of TSP-1 was similar to that in mice treated with 50-$\mu$g doses of TSP-1 alone.

The lungs of mice used in the metastasis experiments described above were subjected to histological examinations in an attempt to identify mechanisms underlying the antimetastatic effect of TSP-1. Micrometastases were detected in the lungs of untreated mice and mice treated with 15 Gy alone, 50-$\mu$g doses of TSP-1 alone, or 30 Gy in combination with 25-$\mu$g doses or 50-$\mu$g doses of TSP-1. They were seen either as perivascular cuffs of 5–10 cell layers or as pleural surface colonies of 2–4 cell layers, and did not differ in size or shape between the treatment groups. Sections stained with antibody against CD31 showed no evidence of neovascularization within the micro-
metastases. In contrast, the lungs of mice treated with anti-TSP-1 antibody, surgery, or 30 Gy showed only highly vascularized macroscopic metastases. Angiogenic activity was seen adjacent to nonvascularized micrometastases as well as vascularized macrometastases. Twenty pairs of lungs with micrometastases and 20 pairs of lungs with macrometastases only were subjected to quantitative studies of apoptotic activity. The apoptotic frequency in endothelial cells adjacent to micrometastases (21.5 ± 3.1%) was significantly higher than that in endothelial cells adjacent to macrometastases (8.2 ± 2.0%; P = 0.00089). There was no significant difference in endothelial cell apoptotic frequency between micrometastases in mice with untreated primary tumors and micrometastases in mice treated with 30 Gy in combination with 25-μg doses or 50-μg doses of TSP-1.

**Primary Tumors.** Primary tumors were treated with 10 Gy alone or 10 Gy in combination with 50-μg doses of TSP-1 to investigate whether the antitumor effect of radiation was enhanced by TSP-1. Radiopotentiation by TSP-1 was studied by giving two doses of TSP-1 before the radiation treatment. Antiangiogenic effect of TSP-1 was studied by giving three doses of TSP-1 per week after the radiation treatment. TSP-1 was found to have substantial radiopotentiating effect (Fig. 4). Thus, the growth delay was significantly longer for tumors given combined radiation and radiopotentiating TSP-1 treatment (Fig. 4, closed triangles) than for tumors given radiation treatment alone (Fig. 4, closed circles; P < 0.00001), whereas the growth curve for tumors given radiopotentiating TSP-1 treatment alone (data not shown) was indistinguishable from that for untreated control tumors (Fig. 4, closed squares). During the regrowth period, the growth rates of the tumors given radiation treatment alone or combined radiation and radiopotentiating TSP-1 treatment were similar to those of unirradiated control tumors. Moreover, TSP-1 was found to have substantial antiangiogenic effect on both unirradiated and irradiated primary tumors (Fig. 4). Thus, tumors given antiangiogenic TSP-1 treatment alone (Fig. 4, open squares) grew more slowly than untreated control tumors (Fig. 4, closed squares), tumors given combined radiation and antiangiogenic TSP-1 treatment (Fig. 4, open circles) grew more slowly during the regrowth period than tumors given radiation treatment alone (Fig. 4, closed circles), and tumors given radiation treatment in combination with both radiopotentiating and antiangiogenic TSP-1 treatment (Fig. 4, open triangles) grew more slowly during the regrowth period than tumors given combined radiation and radiopotentiating TSP-1 treatment only (Fig. 4, closed triangles).

The mechanisms underlying the radiopotentiating effect of TSP-1 were studied by irradiating tumors under clamped or unclamped conditions. The tumors were treated with 10 Gy alone or 10 Gy in combination with two doses of TSP-1 as above, and the effects of the treatments were measured by using the tumor cell survival assay (Fig. 5A) or the tumor growth delay assay (Fig. 5B). When the radiation treatment was given under clamped conditions, the cell-surviving fractions were not different for tumors given radiation treatment alone, and tumors given combined radiation and radiopotentiating TSP-1 treatment (Fig. 5A). In contrast, when the radiation treatment was given under unclamped conditions, the cell-surviving fractions were significantly lower for tumors given combined radiation and radiopotentiating TSP-1 treatment than for tumors given radiation treatment alone (P = 0.0031; Fig. 5A). Thus, the fraction of radiobiologically hypoxic cells was significantly lower in tumors pretreated with TSP-1 (9% ± 3%) than in tumors given no TSP-1 treatment (30% ± 8%; P = 0.024). These observations showed that TSP-1 caused radiopotentiation, not by increasing the radiation sensitivity of the melanoma cells, but by reducing the fraction of hypoxic melanoma cells. However, this mechanism was probably not the only mechanism by which TSP-1 caused radiopotentiation, because not only tumors irradiated under unclamped conditions, but also tumors irradiated under clamped conditions showed significantly increased growth delays when the radiation treatment was combined with radiopotentiating TSP-1 treatment (P = 0.0088; Fig. 5B).

In an attempt to additionally characterize the radiopotentiating effect of TSP-1, primary tumors were subjected to quantitative studies of the frequency of apoptosis in microvascular endothelial cells. Tumors were treated with TSP-1 alone, 10 Gy alone, or 10 Gy in combination with TSP-1 as above, and the tumors were fixed in liquid nitrogen 16 h after the radiation exposure. Radiopotentiating TSP-1 treatment was found to promote apoptosis (Fig. 6A). Thus, the endothelial cell apoptotic frequency was significantly higher in tumors
given combined radiation and TSP-1 treatment than in tumors given TSP-1 treatment alone ($P = 0.038$) or radiation treatment alone ($P = 0.0032$). Moreover, tumors treated with TSP-1 alone or radiation alone showed significantly higher endothelial cell apoptotic frequencies than untreated control tumors ($P = 0.0023$ and 0.036, respectively).

The mechanisms underlying the antiangiogenic effect of TSP-1 were also studied by quantitating the apoptotic frequency in microvascular endothelial cells of primary tumors. Tumors were treated with TSP-1 alone, 10 Gy alone, or 10 Gy in combination with TSP-1. Three doses of TSP-1 were given per week. The analysis was performed by using tumors from the experiment described in Fig. 4, implying that the tumors were fixed in liquid nitrogen 3 weeks (untreated controls), 4 weeks (TSP-1 alone), 5 weeks (10 Gy alone), or 6 weeks (10 Gy in combination with TSP-1) after the radiation treatment. Also, antiangiogenic TSP-1 treatment was found to induce apoptosis (Fig. 6B). Thus, the apoptotic frequency of the endothelial cells was significantly higher in tumors treated with TSP-1 alone than in untreated control tumors ($P = 0.0018$), and significantly higher in tumors given combined radiation and TSP-1 treatment than in tumors given radiation treatment alone ($P = 0.014$). The endothelial cell apoptotic frequency in tumors given combined radiation and TSP-1 treatment was similar to that in tumors treated with TSP-1 alone, and the endothelial cell apoptotic frequency in tumors treated with radiation alone was similar to that in untreated control tumors.

**DISCUSSION**

**TSP-1 and Radiation-induced Metastasis.** We have demonstrated previously that D-12 primary tumors suppress angiogenesis and growth of their pulmonary micrometastases by secreting large quantities of TSP-1 into the blood circulation of the host mice (29). In the work reported here, we showed that curative radiation therapy of D-12 primary tumors, in conformity with surgical treatment and treatment with anti-TSP-1 antibody but in contrast to subcurative radiation therapy, resulted in neoangiogenesis and increased metastatic growth in the lungs, primarily because of a reduced concentration of TSP-1 in the blood.

Thus, BALB/c-nu/nu mice bearing D-12 tumors model those cancer patients who show accelerated metastatic growth in regional and distant organ sites after surgical resection or curative radiation therapy of the primary tumor. Very few experimental tumor models of this subgroup of patients have been characterized thus far. However, accelerated metastatic growth after curative radiation therapy has also been reported for the murine Lewis lung carcinoma LLC-LM (26). Curative radiation therapy of LLC-LM primary tumors, analogous to surgical resection (5, 28), was followed by explosive growth of previously dormant pulmonary metastases. The metastatic deposits were kept dormant in mice with untreated primary tumors because angiotatin, probably generated by metalloelastase secreted by macrophages in the primary tumors (44), prevented neoangiogenesis of the metastases (26). The metastatic suppression by D-12 primary tumors reported here probably did not involve angiotatin or endostatin. Neither angiotatin nor endostatin could be detected in the blood plasma of mice bearing untreated or irradiated (15–30 Gy) 800–1000 mm$^3$ D-12 primary tumors, using Western blotting and a specific antimouse angiotatin rabbit monoclonal antibody for angiotatin detection and an ELISA kit (Accucyte Assay; Cytoimmune Sciences, College Park, MD) for endostatin detection.

There is no a priori reason to believe that curative radiation therapy and surgical treatment of a primary tumor should modify angiogenesis and metastatic growth at distant sites in similar ways. The rate of angiogenesis in tumor tissue is governed by the balance between pro- and antiangiogenic factors (4). At a metastatic site, this balance is determined by the rate of synthesis of pro- and antiangiogenic factors by the metastasis and by the supply of blood-borne pro- and antiangiogenic factors, produced by the primary tumor or the tumor host (5, 8, 11). Surgical resection of a primary tumor leads to reduced concentrations of both pro- and antiangiogenic factors in the blood circulation. If the primary tumor is responsible for synthesis of angiogenic inhibitors like TSP-1 and angiotatin, surgical resection may result in reduced blood ratios of antiangiogenic:proangiogenic factors and, hence, increased angiogenesis at distant metastatic sites (7, 9, 28), primarily because antiangiogenic factors have longer half-lives in the blood circulation than proangiogenic factors (27). On the other hand, the effect of curative radiation therapy on the balance between pro- and antiangiogenic factors at distant metastatic sites is less predictable. Malignant and normal cells are metabolically active for a considerable period after having been inactivated by ionizing radiation. Radiation therapy may cause increased expression of pro- and antiangiogenic factors in tumor tissues, both because irradiation may activate transcription factors directly and because radiation treatment may deteriorate the tumor microenvironment and, hence, prepare for transcriptional activation by tissue hypoxia and acidity (45). It has been reported that the expression of VEGF can be up-regulated in tumor cells in vitro and experimental tumors in vivo after exposure to ionizing radiation (10), and that the blood level of VEGF can be elevated during radiation therapy in patients with brain tumors (6). Moreover, radiation therapy may cause increased production of proangiogenic cytokines in normal tissues within the radiation field (46, 47). Thus, in contrast to the observations reported here for D-12 tumors, curative radiation therapy of the PC-3 prostate
carcinoma and the FSA-II fibrosarcoma, unlike surgical resection, resulted in reduced bFGF-induced angiogenesis in window chambers at distant sites, presumably because of higher endostatin blood levels in mice given radiation therapy than in mice treated with surgery (12).

Increased metastatic growth after curative radiation therapy of D-12 primary tumors could be prevented by treating the host mice with exogenous TSP-1 after the radiation therapy. The metastatic frequency in mice treated with 25-μg doses of TSP-1 after curative radiation therapy was similar to that in mice with untreated primary tumors, and mice treated with 50-μg doses of TSP-1 after curative radiation therapy showed a metastatic frequency that was significantly lower than that in untreated mice. Exogenous TSP-1 suppressed neovascularization and metastatic growth by the same mechanism as endogenous TSP-1 secreted by D-12 primary tumors, i.e., by inducing apoptosis in activated microvascular endothelial cells adjacent to micrometastases. Neither the size of the micrometastases nor the apoptotic frequency in the adjacent endothelial cells differed between mice with untreated primary tumors and mice given combined curative radiation therapy and TSP-1 treatment. Our observations are consistent with that of a recent study having shown that TSP-1 can induce apoptosis in endothelial cells in vitro and in vivo by sequential activation of CD36, p59fyn, caspase-3 like proteases, and p38 mitogen-activated protein kinases (33).

**TSP-1 and Primary Tumor Radiation Response.** Treatment with exogenous TSP-1 not only prevented metastatic growth after curative radiation therapy but also suppressed the growth of unirradiated and irradiated D-12 primary tumors. Unirradiated tumors treated according to the antiangiogenic schedule, i.e., three 50-μg doses of TSP-1 per week, grew more slowly than untreated control tumors, and tumors given combined radiation and antiangiogenic TSP-1 treatment grew more slowly during the regrowth period than tumors given radiation treatment alone. The endothelial cell apoptotic frequency was significantly higher in tumors treated with TSP-1 alone than in untreated control tumors, and significantly higher in tumors given combined radiation and TSP-1 treatment than in tumors given radiation treatment alone, suggesting that exogenous TSP-1 inhibited angiogenesis in well-vascularized, rapidly growing primary tumors by the same mechanism as it prevented neovascularization of dormant micrometastases, i.e., by inducing apoptosis in microvascular endothelial cells. It is noteworthy that the antiangiogenic effect of exogenous TSP-1 was sufficiently strong to suppress angiogenesis also in irradiated primary tumors, knowing that irradiated tissues can show increased expression of several proangiogenic factors, including VEGF, PDGF, and bFGF (10, 46, 47).

Moreover, treatment with exogenous TSP-1 enhanced the radiation response of D-12 primary tumors, i.e., TSP-1 had a radiopotentiating effect. Tumors given combined radiation and TSP-1 treatment according to the radiopotentiating schedule, i.e., two 50-μg doses of TSP-1 before the radiation treatment, showed significantly longer growth delays than tumors given radiation treatment alone, whereas unirradiated tumors given TSP-1 treatment according to the radiopotentiating schedule showed growth curves that were indistinguishable from those of untreated control tumors. The radiopotentiating effect of TSP-1 was distinctly different from the antiangiogenic effect. Thus, unirradiated tumors given TSP-1 treatment according to the antiangiogenic schedule showed decreased growth rates, whereas unirradiated tumors given TSP-1 treatment according to the radiopotentiating schedule did not. Irradiated tumors given radiopotentiating TSP-1 treatment showed increased growth delays without any decrease in growth rate, whereas irradiated tumors given TSP-1 treatment according to the antiangiogenic schedule showed decreased growth rates but no increase in growth delay except for that because of the decreased growth rate. Finally, irradiated tumors given combined radiopotentiating and antiangiogenic TSP-1 treatment showed increased growth delays as well as decreased growth rates during the regrowth period.

Multiple mechanisms were responsible for the radiopotentiating effect of exogenous TSP-1 in D-12 primary tumors. First, the fraction of radiobiologically hypoxic melanoma cells was significantly lower in tumors given radiopotentiating TSP-1 treatment than in untreated control tumors. The decrease in hypoxic fraction was associated with an increase in endothelial cell apoptotic frequency, i.e., radiopotentiating TSP-1 treatment induced apoptosis in the tumor microvasculature. TSP-1-induced endothelial cell apoptosis may have resulted in reduced oxygen consumption by the tumor parenchymal cells and, hence, a decrease in the hypoxic fraction. Previous studies of effects of antiangiogenic treatment on tumor oxygenation have given conflicting results, possibly because the effects may differ among antiangiogenic agents and treatment strategies, and also may depend on the tumor model subjected to investigation. Antiangiogenic treatment with TNP-470 resulted in decreased oxygenation of a murine mammary carcinoma (48). Blocking of VEGFR-2 with the DC101 antibody had no significant effect on the oxygenation of small cell lung carcinoma S4A xenografts and glioblastoma multiforme U87 xenografts (21). Treatment with the anti-VEGF antibody A4.6.1 did not modify the oxygenation of colon adenocarcinoma LS174T xenografts (19). However, this treatment caused a significant increase in median pO2 and a significant decrease in the frequency of pO2 readings below 5 mm Hg in U87 tumors (19), consistent with the observation reported here that treatment with TSP-1 reduced the fraction of radiobiologically hypoxic cells in D-12 tumors. Also, antiangiogenic treatment with squalamine or TNP-470 in combination with minocycline has been shown to result in increased tumor oxygenation and response to radiation therapy, both in rat and murine experimental tumors (16, 48, 49).

The radiopotentiating effect of exogenous TSP-1 in D-12 primary tumors was not only a result of a TSP-1-induced decrease in the fraction of radiobiologically hypoxic cells. TSP-1 also increased the radiation sensitivity of the endothelial cells of the tumor microvasculature. Thus, the endothelial cell apoptotic frequency was significantly higher in tumors given combined radiation and radiopotentiating TSP-1 treatment than in tumors given radiation treatment alone or tumors given radiopotentiating TSP-1 treatment alone. A high endothelial cell apoptotic frequency may lead to microvesSEL disintegration and shut down of the blood flow. Tumor cells supplied by microvessels losing their function will gradually be inactivated because of lack of oxygen and nutritional deprivation (50).

TSP-1 is not the only antiangiogenic agent that has the potential to enhance the radiation response of tumors by increasing the radiation sensitivity of tumor endothelial cells. It has been demonstrated that tumors can show enhanced radiation response because of increased endothelial cell radiation sensitivity also after treatment with angiotatin (17, 18), endostatin (25), antibodies and other agents blocking VEGFR-2 signaling (10, 19, 21, 22), the isocoumarin derivative NM-3 (20), and SU6668, an inhibitor of the receptor-tyrosine kinase activity of VEGF, bFGF, and PDGF (23, 24). However, the mechanism by which antiangiogenic agents increase endothelial cell radiation sensitivity may differ among agents. Studies of established endothelial cell lines in vitro suggested that the angiotatin- and NM-3-induced increases in endothelial cell radiation sensitivity were mediated by mechanisms other than apoptosis (17, 20), whereas those induced by endostatin, SU5416, and the soluble extracellular component of VEGFR-2 most likely were apoptosis-mediated (22, 25). In the work reported here, endothelial cell apoptosis was investigated by examining immunohistochemical preparations of the microvasculature of D-12 tumors. Our in vivo study demonstrated clearly that the increased endothelial cell radiation sensitivity induced by TSP-1 was mediated by apoptosis.

The present observations were all based on the use of single large doses of radiation, whereas clinical radiation therapy is given in multiple small dose fractions. It cannot be excluded that the effects of TSP-1 seen here may overestimate those occurring with fractionated
irradiation. Therefore, studies similar to the present ones using clinically relevant fractionation schedules are highly warranted.

In conclusion, preclinical studies with D-12 human melanoma xenografts have suggested that TSP-1 is a potentially useful therapeutic agent in clinical radiation therapy. TSP-1 given before irradiation may enhance the antitumor effect of the radiation treatment by increasing the radiation sensitivity of the tumor microvascular endothelial cells and by reducing the fraction of radiobiologically hypoxic parenchymal tumor cells. TSP-1 given after radiation therapy may prevent angiogenesis and growth of dormant micrometastases at distant sites and inhibit the regrowth of primary tumors failing radiation therapy.

REFERENCES


Antiangiogenic Treatment with Thrombospondin-1 Enhances Primary Tumor Radiation Response and Prevents Growth of Dormant Pulmonary Micrometastases after Curative Radiation Therapy in Human Melanoma Xenografts

Einar K. Rofstad, Kristin Henriksen, Kanthi Galappathi, et al.