Thrombospondin-1-Mediated Metastasis Suppression by the Primary Tumor in Human Melanoma Xenografts

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Some cancer patients show accelerated growth of pre-existing metastases after removal of the primary tumor. The purpose of this study was to investigate whether primary tumor-induced metastasis suppression can be mediated by thrombospondin-1 in melanoma. Human melanoma xenografts (D-12, R-18, and U-25) were used as models of melanoma in humans. Melanoma angiogenesis, lung colonization, and spontaneous pulmonary metastasis were inhibited in mice bearing D-12, U-25, or thrombospondin-1 overexpressing R-18 tumors, which showed high thrombospondin-1 expression and secreted large quantities of thrombospondin-1 into the blood, but not in mice bearing wild-type R-18 tumors, which were negative for thrombospondin-1. D-12 tumors suppressed the growth of their own spontaneous metastases. The anti-angiogenic and anti-metastatic effects of D-12 and U-25 tumors were blocked in mice treated with thrombospondin-1 neutralizing antibody. Dormant avascular microcolonies having an elevated apoptotic activity were seen in the lungs of mice bearing D-12 or U-25 tumors, whereas only neovascularized lung macrocolonies were seen in control and antibody-treated mice. This study suggests that some melanoma patients may benefit from combined local treatment and long-term anti-angiogenic therapy involving thrombospondin-1. ***Key words: angiogenesis inhibition/melanoma/metastasis suppression/thrombospondin-1. J Invest Dermatol 117:1042–1049, 2001***

Growth of metastases in regional and distant sites is the major cause of death of cancer patients (Fidler and Ellis, 1994). The probability that cancer patients develop metastatic disease is reduced after surgical removal of the primary tumor (Sugarbaker *et al*, 1977); however, in some patients surgical removal of the primary tumor is followed by accelerated growth of pre-existing metastases in secondary organ sites (Sugarbaker *et al*, 1977). Folkman (1995) has proposed an interesting hypothesis to explain this phenomenon. The hypothesis states that a primary tumor, although being capable of promoting neovascularization in its own vascular network by secreting angiogenesis stimulators, can inhibit neovascularization of a metastasis by generating angiogenesis inhibitors, and is based on the assumptions that the angiogenesis stimulators are in excess of the angiogenesis inhibitors at the primary site and that the angiogenesis inhibitors are in excess of the angiogenesis stimulators at the metastatic site, owing to the primary tumor having a larger mass than the metastasis and the angiogenesis inhibitors having a longer half-life in the blood circulation than the angiogenesis stimulators. Folkman's hypothesis is supported by theoretical considerations (Ramanujan *et al*, 2000) and experimental data on murine tumors (Holmgren *et al*, 1995). Thus, the neovascularization and growth of Lewis lung carcinoma and T241 fibrosarcoma spontaneous pulmonary metastases have been shown to be inhibited by the primary tumor (Holmgren *et al*, 1995). The inhibitory effect was found to be caused by angiostatin (O'Reilly *et al*, 1994), a 38 kDa internal fragment of plasminogen, which in the case of the Lewis lung carcinoma most likely was produced by metalloelastase secreted by tumor-infiltrating macrophages (Dong *et al*, 1997).

Thrombospondin-1 (TSP-1), a 450 kDa trimeric glycoprotein secreted by a wide variety of tumor types, is another potent angiogenesis inhibitor that has been suggested to mediate primary tumor-induced suppression of metastatic growth (Volpert *et al*, 1998). This protein inhibits tumor angiogenesis by inducing apoptosis in endothelial cells by binding to the transmembrane receptor CD36 (Jiménez *et al*, 2000), a mechanism that explains its ability to prevent endothelial cells from responding to a wide variety of angiogenesis stimulators, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Campbell *et al*, 1998). Studies of bladder carcinoma (Grossfeld *et al*, 1997) and melanoma (Grant *et al*, 1998) have suggested that 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 tumors with mutant p53. This suggestion is consistent with the observations that the p53 protein is a transcriptional activator of the TSP-1 gene and that increased expression of wild-type p53 results in increased expression of TSP-1 in fibroblasts (Dameron *et al*, 1994). TSP-1 expression has been shown to be an important prognostic factor independent of tumor stage, lymph node status, and histologic grade in the transitional cell carcinoma of the urinary bladder (Grossfeld *et al*, 1997). Studies of experimental tumors suggesting that primary tumors can suppress the growth of metastases by secreting TSP-1 have been performed. Thus,

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Abbreviations: TSP-1, thrombospondin-1; VEGF, vascular endothelial growth factor.

HT1080 human fibrosarcomas growing subcutaneously in athymic mice were shown to produce large quantities of TSP-1 and to prevent induction of angiogenesis by bFGF-containing pellets implanted in the mouse cornea and to inhibit lung colonization of B16 murine melanoma cells inoculated in the mouse tail vein (Volpert *et al*, 1998); however, experimental studies demonstrating that primary tumors can secrete sufficient quantities of TSP-1 to inhibit the growth of their own spontaneous metastases have not been reported so far. Such data have not been published for any other angiogenesis inhibitor produced directly by parenchymal tumor cells either.

Several human melanoma xenograft lines having retained essential biologic features of the donor patients' tumors, including organ-specific metastatic pattern and the capacity to induce angiogenesis, have been established previously in our laboratory (Rofstad, 1994). These tumor lines were used in this study to demonstrate that the development of spontaneous pulmonary metastases in human melanoma in mice can be suppressed by TSP-1 secreted by the primary tumor.

MATERIALS AND METHODS

Cell lines Human melanoma cell lines (D-12, R-18, and U-25) with wild-type p53 were used. They were established from large regional subcutaneous metastases of patients admitted to the Norwegian Radium Hospital as described elsewhere (Rofstad, 1994). D-12 and U-25 cells show high expression of TSP-1, whereas the expression of TSP-1 in R-18 cells is insignificant. The R-18 line has been subcloned, and a subclone showing high TSP-1 expression has been established. Most cells of both the parent, wild-type line, and the TSP-1 overexpressing subclone have near-triploid chromosome numbers; the number of chromosomes per cell has been found to range from 49 to 67 (median: 57) in the wild type and from 52 to 68 (median: 61) in the subclone. The main difference between the karyotypes is that chromosome 15 is trisomic in the majority of the cells of the subclone, whereas chromosome 15 is absent in most cells of the wild type. All cell lines were maintained in monolayer culture in RPMI 1640 (25 mM HEPES and L-glutamine) supplemented with 13% bovine calf serum, 250 mg penicillin per l, and 50 mg streptomycin per l. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air, and subcultured twice a week. The cell lines were verified to be free from *Mycoplasma* contamination.

TSP-1 expression *in vitro* The expression of TSP-1 in exponentially growing monolayer cell cultures was studied by western blotting. The cells were washed in phosphate-buffered saline and boiled in Laemmli lysis buffer for 5 min. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. Membranes were incubated with anti-human TSP-1 mouse monoclonal antibody [TSP Ab-4 (clone A6.1); NeoMarkers, Union City, CA] for 60 min. Bound antibody was detected by a biotin–streptavidin alkaline phosphatase staining procedure. TSP-1 (Calbiochem, San Diego, CA) purified from human platelets was used as positive control. The specificity of the antibody–antigen interaction was confirmed by the incubation of membranes in solutions without primary antibody. Protein molecular weights were estimated by using prestained standards according to the manufacturer's instructions (sodium dodecyl sulfate–polyacrylamide gel electrophoresis standards; Bio-Rad Laboratories, Hercules, CA).

Treatment with TSP-1 and TSP-1 neutralizing antibody *in vitro* Possible anti-proliferative, apoptotic, or cytotoxic effects of TSP-1 (Calbiochem) and TSP-1 neutralizing antibody [TSP Ab-1 (clone A4.1); NeoMarkers] were investigated *in vitro*. D-12 cells were cultured in RPMI 1640 (25 mM HEPES and L-glutamine) supplemented with 13% bovine calf serum, 250 mg penicillin per l, and 50 mg streptomycin per l in the absence or presence of 5 μg TSP-1 per ml or 5 μg TSP-1 neutralizing antibody per ml for up to 8 d. The number of cells in the cultures was determined 2, 4, 6, or 8 d after the cultures were initiated by counting cells in a hemocytometer. Apoptotic cells were detected by immunofluorescence using an apoptosis detection kit (Apotag; Oncor, Gaithersburg, MD) based on the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling method as instructed by the manufacturer. The colony forming efficiency of the cells was measured at the same time points by plating 200 cells in 25 cm^2 culture flasks, incubating the cells at 37°C in a humidified atmosphere of 5% CO_2 in air for 10 d, and then counting colonies containing more than 50 cells by using a stereomicroscope.

Mice Adult (8–12 wk of age) female BALB/C *nu/nu* mice, bred at the Institute for Cancer Research (The Norwegian Radium Hospital, Oslo, Norway), were used as host animals for xenografted tumors. The mice were maintained under specific pathogen-free conditions at constant temperature (24–26°C) and humidity (30–50%). Sterilized food and tap water were given *ad libitum*. Animal care and experiments were performed according to the guidelines of the National Animal Research Authority and were approved by the Institutional Committee on Research Animal Care.

Intradermal flank tumors Approximately 3.5×10^5 melanoma cells in 10 μl of Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS) were inoculated intradermally in the flanks of mice by using a 100 μl Hamilton syringe. The tumors were deposited above the subcutaneous muscle tissue in the deeper part of the dermis. The dermis and the subcutaneous muscle were infiltrated and gradually replaced by malignant tissue during tumor growth. Tumor volume (V) was calculated as $V = \pi/6 \times ab^2$, where a is the longer and b is the shorter of two perpendicular diameters, measured with calipers. The histologic appearance of the tumors has been described in detail previously (Rofstad, 1994). The tumors of all lines express bFGF and show VEGF-dependent angiogenesis (Rofstad and Halsør, 2000). Moreover, D-12 tumors form spontaneous pulmonary metastases, whereas R-18 and U-25 tumors do not (Rofstad, 1994; Rofstad and Halsør, 2000).

TSP-1 expression *in vivo* The expression of TSP-1 in tumors was studied by immunohistochemistry, using an avidin–biotin immuno-peroxidase method. Tumors were fixed in phosphate-buffered 4% paraformaldehyde. Anti-human TSP-1 rabbit polyclonal antibody (TSP Ab-8; NeoMarkers) was used as the primary antibody. Controls included omission of the primary antibody, incubation with normal rabbit immunoglobulin or normal rabbit serum, and incubation with blocking peptide before staining. The sections were counterstained with hematoxylin.

The concentration of TSP-1 in mouse plasma was determined by western blotting using purified human platelet TSP-1 (Calbiochem) as a standard. Samples of 35 μg of total plasma protein were resuspended in loading buffer containing 7.5% β-mercaptoethanol and boiled for 5 min. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted to a polyvinylidene membrane. Membranes were incubated with TSP Ab-4 (clone A6.1), and bound antibody was detected by a biotin–streptavidin alkaline phosphatase staining procedure.

Angiogenesis assay The capacity of D-12 cells to induce angiogenesis was assessed by an intradermal angiogenesis assay (Rofstad, 1994). A 100 μl Hamilton syringe was used to inoculate aliquots of 1.0×10^6 cells suspended in 10 μl of HBSS intradermally in the abdominal region of mice (day 0). The mice were killed on day 5 after the cell inoculation. Small vascularized tumors had developed in the inoculation sites at that time. The skin around the inoculation sites was removed, and the tumors were located with a dissecting microscope. The capillaries in the dermis oriented toward the tumors were counted, and the diameters of the tumors were measured, using an ocular with a scale. The number of capillaries was corrected for the background, determined after the injection of 10 μl of HBSS. The number of tumor-oriented capillaries per tumor and the number of tumor-oriented capillaries per millimeter of tumor circumference were used as parameters for D-12-induced angiogenesis.

Experiments were performed in control mice, in mice bearing D-12, R-18, or U-25 intradermal flank tumors, and in mice treated with TSP-1 or TSP-1 neutralizing antibody. Four doses of 50 μg of purified human platelet TSP-1 (Calbiochem) or four doses of 100 μg of anti-human TSP-1 mouse monoclonal antibody (TSP Ab-1 (clone A4.1); NeoMarkers) were given in intervals of 24 h. The first dose was given 24 h after the inoculation of D-12 cells (day 1). The TSP-1 and TSP-1 antibody solutions were diluted in HBSS and administered to the mice in volumes of 0.25 ml by i.p. injection.

Lung colonization assay The lung colonization efficiency of D-12 cells was assessed by inoculating aliquots of 1.0×10^6 cells suspended in 0.2 ml of HBSS into the lateral tail vein of mice. The mice were killed and autopsied at predetermined times after the cell inoculation. The lungs were removed, rinsed in HBSS, and fixed in Bouin's solution for 24 h to facilitate the scoring of colonies. The number of macroscopic surface colonies was determined by using a stereomicroscope. Experiments were performed in control mice and in mice bearing D-12, R-18, or U-25 intradermal flank tumors.

Spontaneous metastasis assay The capacity of D-12 tumors to form spontaneous pulmonary metastases was assessed as described previously (Rofstad and Halsør, 2000). Briefly, primary tumors were initiated by inoculating aliquots of 3.5×10^5 cells suspended in 10 μl of HBSS intradermally into the left flanks of mice. The tumors were removed surgically at predetermined times, and the wounds were closed with surgical clips. The mice were killed and autopsied at predetermined times or when moribund. The lungs were examined for the presence of macroscopic surface metastases as described above. Experiments were performed in control mice, in mice bearing R-18 or U-25 intradermal tumors in the right flanks, and in mice treated with TSP-1 neutralizing antibody. TSP Ab-1 (clone A4.1) was administered to the mice in daily doses of 100 μg as described above.

Histologic examinations of lung colonies and metastases Lungs were snap-frozen in liquid nitrogen or fixed in phosphate-buffered 4% paraformaldehyde. The mitotic frequency of the parenchymal melanoma cells in lung colonies and spontaneous pulmonary metastases was scored in sections stained with hematoxylin and eosin. Apoptotic cells were detected by immunohistochemistry, using an *in situ* apoptosis detection kit (Apotag; Oncor, Gaithersburg, MD) based on the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling method. Briefly, a solution of 30% terminal deoxynucleotidyl transferase was applied at 37°C for 30 min to link deoxyuridine triphosphate-digoxigenin to the 3′-hydroxy ends of fragmented DNA. Anti-digoxigenin peroxidase conjugate was applied for 30 min to detect labeled nucleotides. Diaminobenzidine was used as chromogen. Negative controls received no terminal deoxynucleotidyl transferase. To avoid erroneous identification of apoptotic cells because of light staining of necrotic cells, only brown-stained nuclei with morphologic characteristics associated with apoptosis were scored as apoptotic. Parallel sections were used for immunohistochemical detection of endothelial cells, using an avidin–biotin immunoperoxidase method. Anti-mouse 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 of parenchymal melanoma cells and tumor-associated endothelial cells were determined by examining histologic sections at a magnification of × 400.

Statistical analysis Data are presented as arithmetic mean ± SEM. Statistical comparisons of data were performed by one-way ANOVA under conditions of normality and equal variance. Under other conditions, comparisons were performed by nonparametric analysis using the Kruskal–Wallis one-way ANOVA on ranks. When significant differences were found, the Bonferroni method (parametric tests) or the Dunnett method (nonparametric tests) was used to identify data sets that differed from control data. $p < 0.05$ was considered significant. All p-values were determined from two-sided tests. The statistical analysis was performed by using SigmaStat statistical software (Jandel Scientific GmbH, Erkrath, Germany).

RESULTS

Experiments with D-12, U-25, and wild-type R-18 cells and tumors D-12 and U-25 cells showed significant expression of TSP-1, whereas R-18 cells did not, as revealed by western blot analysis of cells derived from exponentially growing monolayer cultures (**Fig 1*A***). The expression of TSP-1 was higher in U-25 cells than in D-12 cells in five of five independent experiments. Immunohistochemical preparations showed that D-12 and U-25 tumors stained positive for TSP-1, whereas R-18 tumors were negative (**Fig 1*B***). The TSP-1 staining was relatively homogeneous within a tumor and appeared to be localized in the cytoplasm of the cells. Although a few clusters of negative cells were observed in some U-25 tumors, U-25 tumors generally showed stronger staining than D-12 tumors. Moreover, D-12 and U-25 tumors showed significant secretion of TSP-1, whereas R-18 tumors did not. The TSP-1 concentrations in the plasma of mice bearing 800–1000 mm^3 tumors were 4 ± 1 μg per ml (D-12; n = 8) and 6 ± 1 μg per ml (U-25; n = 7). TSP-1 could not be detected in the plasma of control mice and mice bearing 800–1000 mm^3 R-18 tumors.

Treatment with TSP-1 or TSP-1 neutralizing antibody had no anti-proliferative, apoptotic, or cytotoxic effects on D-12 cells; the total number of cells and their apoptotic frequency were not significantly different in treated and control cultures ($p > 0.05$) and the colony forming efficiency of treated cells was not significantly different from that of control cells ($p > 0.05$), irrespective of treatment (data not shown).

Experiments were designed to investigate whether D-12, R-18, or U-25 intradermal flank tumors secreted sufficient quantities of TSP-1 to inhibit D-12-induced angiogenesis, lung colonization of D-12 cells, or spontaneous pulmonary metastasis of D-12 tumors.

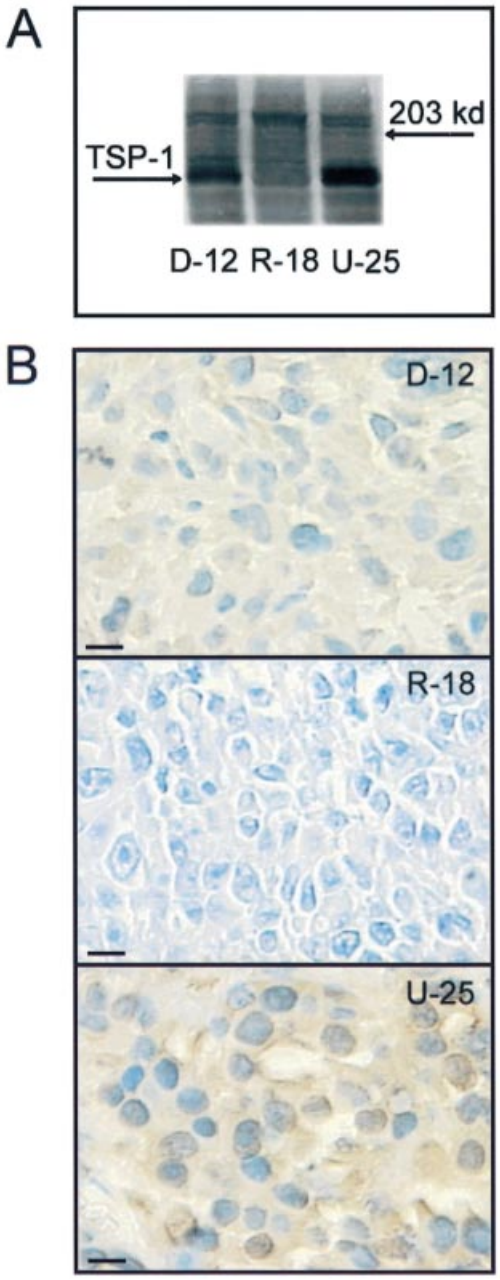

Figure 1. D-12 and U-25 show TSP-1 expression *in vitro* and *in vivo*, whereas R-18 does not. (*A*) Western blot showing the expression of TSP-1 in exponentially growing D-12, R-18, and U-25 monolayer cell cultures. Proteins from 5.0×10^5 cells were loaded into each lane. The *arrow* to the left indicates the TSP-1 specific band. The *arrow* to the right indicates the position of a standard with a known molecular weight of 203 kDa. (*B*) Immunohistochemical preparations of D-12, R-18, and U-25 tumors stained with anti-TSP-1 antibody by an avidin–biotin immunoperoxidase method. The sections were counterstained with hematoxylin. *Scale bars:* 10 μm.

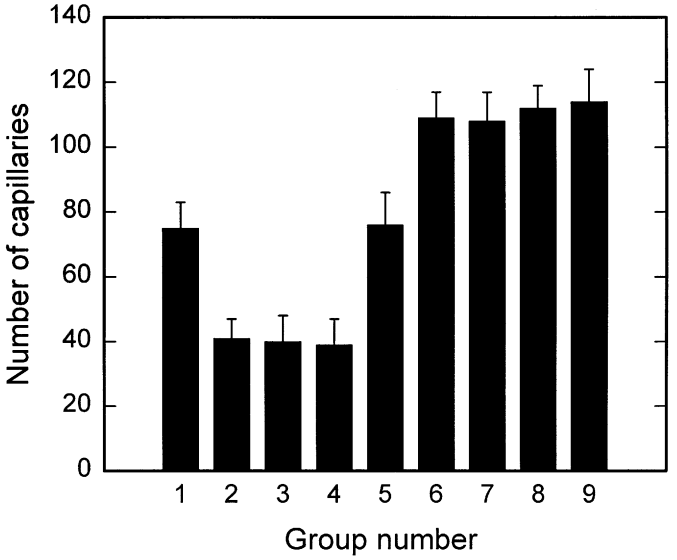

Figure 2. D-12-induced angiogenesis is inhibited by D-12 and U-25 flank tumors, but not by R-18 flank tumors. Aliquots of 1.0×10^6 D-12 cells were inoculated intradermally in the abdominal region of female BALB/c-*nu/nu* mice for evocation of angiogenesis and tumor formation (day 0). The mice were bearing 800–1000 mm^3 D-12, R-18, or U-25 intradermal tumors in the right flanks or were without flank tumors. Mice treated with TSP-1 were given four doses of 50 μg (days 1–4). Mice treated with TSP-1 neutralizing antibody were given four doses of 100 μg (days 1–4). D-12-induced angiogenesis was quantified by counting the capillaries in the dermis oriented toward the tumors formed in the abdominal region (day 5). The plot shows the number of tumor-oriented capillaries per tumor in the nine groups of mice included in the experiments. Columns and bars represent mean values and standard errors of 20 tumors. Group 1: Control mice. Group 2: Mice treated with TSP-1. Group 3: Mice bearing D-12 tumors. Group 4: Mice bearing U-25 tumors. Group 5: Mice bearing R-18 tumors. Group 6: Mice treated with TSP-1 neutralizing antibody. Group 7: Mice bearing D-12 tumors treated with TSP-1 neutralizing antibody. Group 8: Mice bearing U-25 tumors treated with TSP-1 neutralizing antibody. Group 9: Mice bearing R-18 tumors treated with TSP-1 neutralizing antibody.

The results of the angiogenesis experiments are presented in **Fig 2**, which shows D-12-induced angiogenesis expressed as the number of tumor-oriented capillaries per tumor for the nine groups of mice included in the experiments. The number of D-12-induced capillaries was higher in control mice, i.e., mice without D-12, R-18, or U-25 flank tumors (group 1), than in mice treated with TSP-1 (group 2; $p = 0.035$), mice bearing D-12 tumors (group 3; $p = 0.027$), and mice bearing U-25 tumors (group 4; $p = 0.020$). Mice bearing R-18 tumors (group 5) and control mice showed numbers of D-12-induced capillaries that were not different ($p > 0.05$). The number of D-12-induced capillaries was lower in control mice than in mice treated with TSP-1 neutralizing antibody, irrespective of whether the mice were without flank tumors (group 6; $p = 0.035$) or were bearing D-12 tumors (group 7; $p = 0.045$), U-25 tumors (group 8; $p = 0.016$), or R-18 tumors (group 9; $p = 0.0089$). Similar results and identical conclusions were achieved by expressing D-12-induced angiogenesis as the number of tumor-oriented capillaries per millimeter of tumor circumference (data not shown).

Intradermal flank tumors also influenced the lung colonization efficiency of D-12 cells (**Fig 3**). Eight groups of mice were included in the experiments (**Fig 3*A***), and the number of macroscopic surface lung colonies per mouse was used as a parameter for lung colonization efficiency (**Fig 3*B***). The lung colonization efficiencies in control mice, i.e., mice without D-12, R-18, or U-25 flank tumors, examined 3 wk (group 1) or 6 wk (group 2) after the inoculation of D-12 cells were not different ($p > 0.05$). Control mice showed a higher lung colonization efficiency than mice bearing D-12 tumors during the whole experimental period of 3 wk (group 3; $p = 0.0019$), mice bearing U-25 tumors during the whole experimental period of 3 wk (group 4; $p = 0.0031$), and mice bearing U-25 tumors during the whole experimental period of 6 wk (group 5; $p = 0.000062$). Mice that had their D-12 tumors removed 3 wk after the inoculation of D-12 cells and were examined for lung colonies 3 wk later (group 6) showed a lung colonization efficiency that was higher than that of the mice in group 3 ($p = 0.00071$) and not different from those of the mice in the two control groups ($p > 0.05$). Similarly, mice that had their U-25 tumors removed 3 wk after the inoculation of D-12 cells and were examined for lung colonies 3 wk later (group 7) showed a lung colonization efficiency that was higher than those of the mice in group 4 ($p = 0.00055$) and group 5 ($p = 0.00014$) and not different from those of the mice in the two control groups ($p > 0.05$). The lung colonization efficiencies in control mice and mice bearing R-18 tumors during the whole experimental period of 3 wk (group 8) were not different ($p > 0.05$).

The development of spontaneous D-12 pulmonary metastases was also influenced differently by R-18 and U-25 intradermal flank tumors (**Fig 4**). Seven groups of mice were included in the experiments (**Fig 4*A***), and the percentage of mice that developed macroscopic surface metastases was used as a parameter for metastatic frequency (**Fig 4*B***). The D-12 primary tumors were removed 4 wk after they were initiated in all experimental groups. Control mice, i.e., mice without R-18 or U-25 flank tumors, examined for metastatic status 4 wk (group 1) or 8 wk (group 2) after the removal of the primary tumors showed metastatic frequencies that were not different ($p > 0.05$). The metastatic frequency was higher in control mice than in mice bearing U-25 tumors during the whole experimental period until they were examined for metastatic status 4 wk (group 3; $p = 0.0084$) or 8 wk (group 4; $p = 0.0017$) after the removal of the D-12 primary tumors. Mice that had their U-25 tumors removed 4 wk after the removal of the D-12 primary tumors and were examined for metastatic status 4 wk later (group 5) showed a metastatic frequency that was higher than those of the mice in group 3 ($p = 0.0038$) and group 4 ($p = 0.0017$) and not different from those of the mice in the two control groups ($p > 0.05$). Similarly, mice that were bearing U-25 tumors during the whole experimental period until they were examined for metastatic status 8 wk after the removal of the D-12 primary tumors and were treated daily with TSP-1 neutralizing antibody the last 4 wk before they were examined for metastatic status (group 6) showed a metastatic frequency that was higher than those of the mice in group 3 ($p = 0.0084$) and group 4 ($p = 0.0038$) and not different from those of the mice in the two control groups ($p > 0.05$). The metastatic frequency of mice bearing R-18 tumors during the whole experimental period until they were examined for metastatic status 4 wk after the removal of the D-12 primary tumors (group 7) was not different from that of control mice ($p > 0.05$). The volumetric growth rate of the D-12 primary tumors was not influenced significantly by the R-18 or U-25 tumors (data not shown). It was confirmed experimentally that R-18 and U-25 flank tumors did not give rise to spontaneous pulmonary metastases.

Moreover, D-12 primary tumors secreted sufficient quantities of TSP-1 to inhibit the growth of their own spontaneous pulmonary metastases (**Fig 5**). Four groups of mice were included in the experiments (**Fig 5*A***), and as above, the percentage of mice that developed macroscopic surface metastases was used as a parameter for metastatic frequency (**Fig 5*B***). Control mice, i.e., mice bearing D-12 primary tumors during the whole experimental period until they were examined for metastatic status 5 wk after the primary tumors were initiated (group 1), showed a lower metastatic frequency than mice that had their primary tumors removed 2 wk after they were initiated and were examined for metastatic status 3 wk later (group 2; $p = 0.025$), mice bearing the primary tumors for 5 wk that were treated daily with TSP-1 neutralizing antibody the last 3 wk before they were examined for metastatic

status (group 3; $p = 0.043$), and mice that had their primary tumors removed 5 wk after they were initiated and were examined for metastatic status 3 wk later (group 4; $p = 0.015$). The metastatic frequencies of the mice in the latter three groups were not different ($p > 0.05$).

Histologic examinations of lungs of mice used in lung colonization experiments revealed microcolonies of melanoma cells in mice that were bearing D-12 or U-25 flank tumors during the whole experimental period until the lungs were removed, i.e., groups 3–5 in **Fig 3**. The lung microcolonies were seen either as perivascular cuffs of five to 10 cell layers or as pleural surface colonies of two to four cell layers. Immunohistochemical staining with antibody against CD31 showed no evidence of neovascularization within the microcolonies. In contrast, lungs of control mice, i.e., groups 1 and 2 in **Fig 3**, lungs of mice bearing D-12 or U-25 tumors that were removed 3 wk before the lungs were removed, i.e., groups 6 and 7 in **Fig 3**, and lungs of mice bearing R-18 tumors, i.e., group 8 in **Fig 3**, showed only highly neovascularized macrocolonies. Angiogenic activity was detected adjacent to microcolonies as well as macrocolonies. The apoptotic activity differed between macrocolonies and microcolonies, as revealed by the analysis of five pairs of lungs from each of the eight experimental groups. The frequency of apoptotic melanoma cells was higher in the microcolonies of groups 3–5 ($5.2 \pm 1.4\%$) than in the macrocolonies of groups 1 and 2 ($0.8 \pm 0.3\%$; $p = 0.0068$) and groups 6–8 ($0.7 \pm 0.3\%$; $p = 0.0020$). Moreover, the apoptotic frequency of the endothelial cells adjacent to the microcolonies of groups 3–5 ($19.1 \pm 3.4\%$) was higher than those of the endothelial cells adjacent to the macrocolonies of groups 1 and 2 ($7.9 \pm 1.8\%$; $p = 0.019$) and groups 6–8 ($8.5 \pm 2.2\%$; $p = 0.013$). In contrast, the frequency of mitotic melanoma cells in the microcolonies of groups 3–5 ($1.0 \pm 0.2\%$) was not significantly different from those in the macrocolonies of groups 1 and 2 ($0.9 \pm 0.2\%$; $p > 0.05$) and groups 6–8 ($1.1 \pm 0.2\%$; $p > 0.05$). Corresponding results were achieved by subjecting the lungs of mice used in spontaneous pulmonary metastasis experiments to histologic examinations.

Experiments with TSP-1 overexpressing and wild-type R-18 tumors Tumors initiated from the TSP-1 overexpressing R-18 subclone were not able to grow beyond a volume of 300–500 mm^3. The TSP-1 overexpressing R-18 tumors were poorly vascularized, developed extensive necrotic regions, and showed substantially stronger TSP-1 immunostaining than did D-12 and U-25 tumors (**Fig 6*A***). In contrast, wild-type R-18 cells developed well vascularized tumors without necrosis that stained negative for TSP-1 (**Fig 6*B***). The concentration of TSP-1 in the plasma of mice bearing 300–500 mm^3 TSP-1 overexpressing R-18 tumors was 8 ± 2 μg per ml ($n = 5$), which is higher than that in the plasma of mice bearing 800–1000 mm^3 D-12 or U-25 tumors by factors of approximately 2.0 and 1.3, respectively.

Two experiments were performed to investigate whether D-12-induced angiogenesis and D-12 lung colonization also could be inhibited by TSP-1 overexpressing R-18 tumors. In each experiment, aliquots of 1.0×10^6 D-12 cells were inoculated intradermally in the abdominal region or intravenously in the tail vein in 10 mice bearing 300–500 mm^3 TSP-1 overexpressing R-18 tumors and in 10 control mice bearing 300–500 mm^3 wild-type R-18 tumors. TSP-1 overexpressing R-18 tumors prevented D-12-induced angiogenesis and D-12 lung colonization completely; neither angiogenesis nor lung colonies could be detected in the mice bearing TSP-1 overexpressing R-18 tumors. The angiogenesis and the lung colonization in the control mice were consistent with the control data in **Figs 2** and **3**. Moreover,

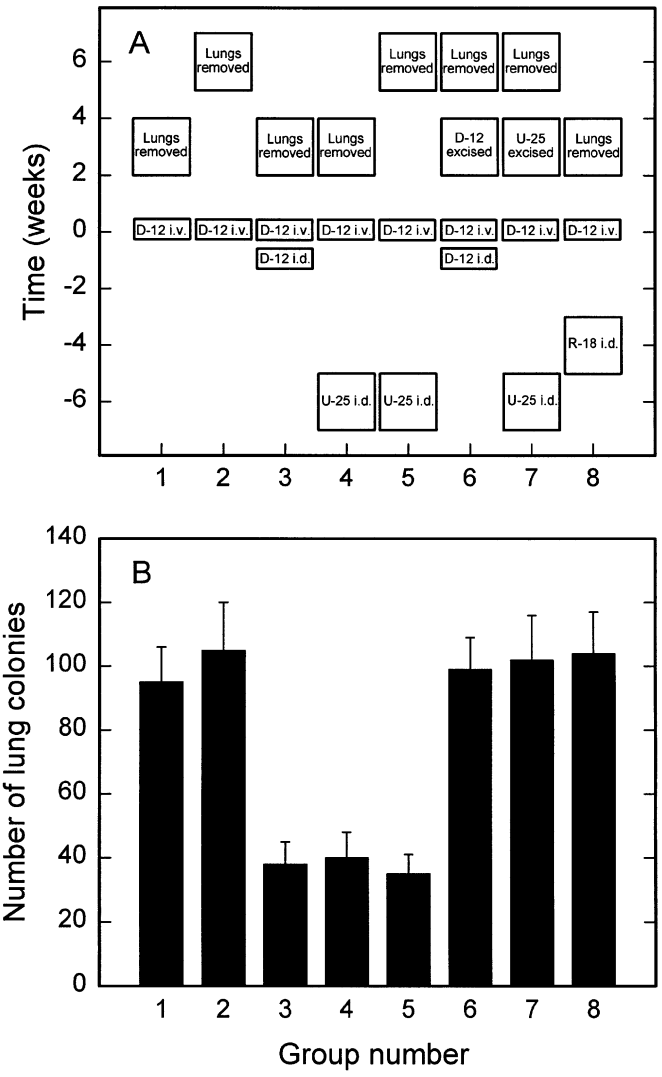

Figure 3. The lung colonization efficiency of D-12 cells is inhibited by D-12 and U-25 flank tumors, but not by R-18 flank tumors. Aliquots of 1.0×10^6 D-12 cells were inoculated intravenously in female BALB/c-*nu/nu* mice for formation of lung colonies. The mice were bearing D-12, R-18, or U-25 intradermal tumors in the right flanks or were without flank tumors. The lungs of the mice were removed at predetermined times and examined for the presence of macroscopic surface colonies. (*A*) Schematic model of the experimental protocol. The flank tumors were initiated 1 wk (D-12), 4 wk (R-18), or 6 wk (U-25) before the intravenous inoculation of D-12 cells (week 0). They had reached volumes of 10–20 mm^3 (D-12), 100–150 mm^3 (R-18), and 30–50 mm^3 (U-25) at week 0, volumes of 800–1000 mm^3 (D-12), 800–1200 mm^3 (R-18), and 150–250 mm^3 (U-25) at week 3, and volumes of 800–1200 mm^3 (U-25) at week 6. (*B*) Number of D-12 lung colonies per mouse in the eight groups of mice included in the experiments. Columns and bars represent mean values and standard errors of 40 mice. Group 1: Control mice, i.e., mice without D-12, R-18, or U-25 tumors; lung colonies were counted 3 wk after the inoculation of D-12 cells. Group 2: Control mice, i.e., mice without D-12, R-18, or U-25 tumors; lung colonies were counted 6 wk after the inoculation of D-12 cells. Group 3: Mice bearing D-12 tumors during the whole experimental period; lung colonies were counted 3 wk after the inoculation of D-12 cells. Group 4: Mice bearing U-25 tumors during the whole experimental period; lung colonies were counted 3 wk after the inoculation of D-12 cells. Group 5: Mice bearing U-25 tumors during the whole experimental period; lung colonies were counted 6 wk after the inoculation of D-12 cells. Group 6: Mice bearing D-12 tumors that were removed 3 wk after the inoculation of D-12 cells; lung colonies were counted 6 wk after the inoculation of D-12 cells. Group 7: Mice bearing U-25 tumors that were removed 3 wk after the inoculation of D-12 cells; lung colonies were counted 6 wk after the inoculation of D-12 cells. Group 8: Mice bearing R-18 tumors during the whole experimental period; lung colonies were counted 3 wk after the inoculation of D-12 cells.

aliquots of 3.5×10^5 and 1.0×10^6 D-12 cells were not able to give rise to macroscopic tumor growth or spontaneous pulmonary metastases when inoculated intradermally in the left flanks of mice bearing 300–500 mm^3 TSP-1 overexpressing R-18 tumors in the right flanks.

DISCUSSION

The experiments reported here show that D-12 lung colonization and spontaneous pulmonary metastasis were suppressed in mice bearing D-12, U-25, or TSP-1 overexpressing R-18 intradermal flank tumors. The observation that D-12 primary tumors inhibited the growth of their own spontaneous metastases is particularly interesting, suggesting that BALB/c-*nu/nu* mice bearing D-12 tumors model patients who show accelerated metastatic growth in regional or distant organ sites following surgical removal of the primary tumor (Sugarbaker *et al*, 1977).

The anti-metastatic effect of D-12, U-25, and TSP-1 overexpressing R-18 flank tumors was most likely mediated by TSP-1, as mice bearing D-12, U-25, or TSP-1 overexpressing R-18 tumors showed high blood plasma concentrations of TSP-1, and the effect was blocked in mice treated with neutralizing antibody against TSP-1. Moreover, D-12 lung colonization and spontaneous metastasis were not suppressed by wild-type R-18 tumors, which stained negative for TSP-1 and did not secrete TSP-1 into the blood circulation. The possibility that other inhibitors also were involved cannot be excluded; however, angiostatin (O'Reilly *et al*, 1994), a potent angiogenesis inhibitor shown to suppress the neovascularization and growth of Lewis lung carcinoma and T241 fibrosarcoma metastases (Holmgren *et al*, 1995), was probably not involved, as angiostatin could not be detected in the serum of mice bearing 800–1000 mm^3 D-12 or U-25 flank tumors by western blotting, using a specific anti-mouse angiostatin rabbit monoclonal antibody (unpublished data).

TSP-1 is a multifunctional protein that regulates attachment, migration, invasion, and differentiation of various cell types, promotes chemotaxis and haptotaxis, and exerts anti-proliferative and anti-angiogenic effects *in vitro* and *in vivo* (Bornstein, 1995). Several observations suggest that the TSP-1-mediated metastasis suppression seen in this study was a result of its anti-angiogenic activity. First, D-12-induced angiogenesis in an intradermal secondary site was inhibited in mice bearing D-12, U-25, or TSP-1 overexpressing R-18 flank tumors and in mice treated with exogenous TSP-1. Second, D-12 lung microcolonies were seen in mice bearing D-12 or U-25 flank tumors during lung colonization and spontaneous metastasis experiments, suggesting that it was primarily the growth of microcolonies and not the seeding of melanoma cells in the lungs that was inhibited. These microcolonies were nonvascularized and surrounded by endothelial cells with an increased apoptotic activity. Moreover, the microcolonies stayed dormant as long as the flank tumors were present, and developed into neovascularized macrocolonies after they were removed. Third, treatment with TSP-1 or TSP-1 neutralizing antibody *in vitro* had no anti-proliferative, apoptotic, or cytotoxic effects on D-12 cells. Our conclusion is consistent with the observation that xenotransplanted tumors of human A431 carcinoma cells transfected with TSP-1 showed lower volumetric growth rate, lower vascular density, and larger necrotic regions than nontransfected control tumors (Streit *et al*, 1999).

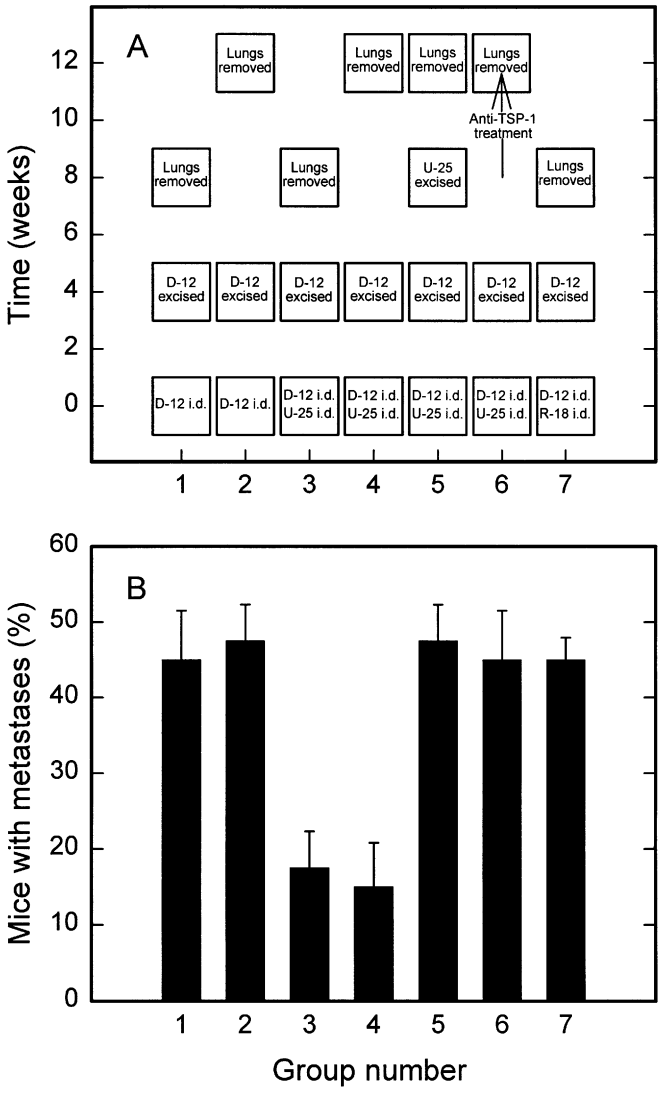

Figure 4. The development of spontaneous D-12 metastases is inhibited by U-25 flank tumors, but not by R-18 flank tumors. D-12 primary tumors giving rise to spontaneous pulmonary metastases were initiated in the left flanks of female BALB/c-*nu/nu* mice. The mice were bearing R-18 or U-25 intradermal tumors or were without tumors in the right flanks. The lungs of the mice were removed at predetermined times and examined for the presence of macroscopic surface metastases. (*A*) Schematic model of the experimental protocol. The D-12 primary tumors were initiated at week 0 and had reached volumes of 800–1000 mm^3 at week 4. The R-18 and U-25 flank tumors were initiated at the same time as the D-12 primary tumors and had reached volumes of 100–150 mm^3 (R-18) and 10–20 mm^3 (U-25) at week 4, volumes of 1000–1500 mm^3 (R-18) and 100–200 mm^3 (U-25) at week 8, and volumes of 800–1200 mm^3 (U-25) at week 12. (*B*) Percentage of mice that developed spontaneous D-12 metastases in the seven groups of mice included in the experiments. Columns and bars represent mean values and standard errors of four independent experiments involving 10 mice each. Group 1: Control mice, i.e., mice without R-18 or U-25 tumors; metastatic status was assessed 4 wk after the D-12 primary tumors were removed. Group 2: Control mice, i.e., mice without R-18 or U-25 tumors; metastatic status was assessed 8 wk after the D-12 primary tumors were removed. Group 3: Mice bearing U-25 tumors during the whole experimental period of 8 wk; metastatic status was assessed 4 wk after the D-12 primary tumors were removed. Group 4: Mice bearing U-25 tumors during the whole experimental period of 12 wk; metastatic status was assessed 8 wk after the D-12 primary tumors were removed. Group 5: Mice bearing U-25 tumors that were removed 4 wk after the D-12 primary tumors were removed; metastatic status was assessed 8 wk after the D-12 primary tumors were removed. Group 6: Mice bearing U-25 tumors during the whole experimental period of 12 wk; metastatic status was assessed 8 wk after the D-12 primary tumors were removed. The mice were treated with daily doses of 100 μg of TSP-1 neutralizing antibody in weeks 8–12. Group 7: Mice bearing R-18 tumors during the whole experimental period of 8 wk; metastatic status was assessed 4 wk after the D-12 primary tumors were removed.

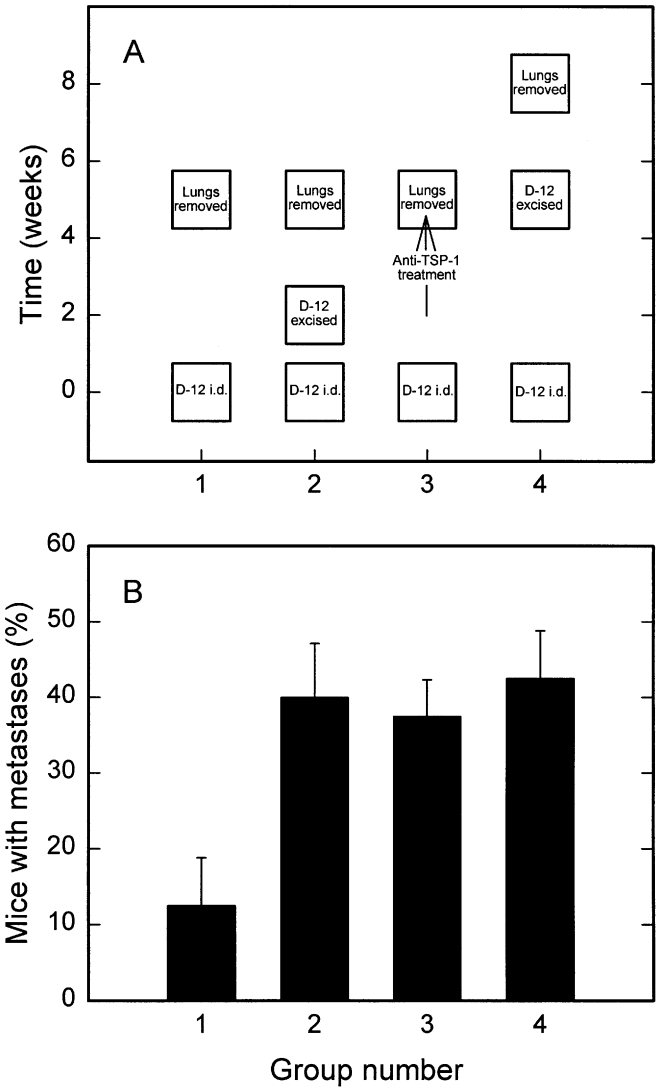

Figure 5. The development of spontaneous D-12 metastases is inhibited by the D-12 primary tumor. D-12 primary tumors giving rise to spontaneous pulmonary metastases were initiated in the left flanks of female BALB/c-*nu/nu* mice. The lungs of the mice were removed at predetermined times and examined for the presence of macroscopic surface metastases. (*A*) Schematic model of the experimental protocol. The D-12 primary tumors were initiated at week 0 and had reached volumes of 50–100 mm^3 at week 2 and volumes of 1000–2000 mm^3 at week 5. (*B*) Percentage of mice that developed spontaneous D-12 metastases in the four groups of mice included in the experiments. Columns and bars represent mean values and standard errors of four independent experiments involving 10 mice each. Group 1: Mice bearing D-12 primary tumors during the whole experimental period of 5 wk before metastatic status was assessed. Group 2: Mice bearing D-12 primary tumors that were removed after 2 wk; metastatic status was assessed 3 wk after the primary tumors were removed. Group 3: Mice bearing D-12 primary tumors during the whole experimental period of 5 wk before metastatic status was assessed. The mice were treated with daily doses of 100 μg of TSP-1 neutralizing antibody in weeks 3–5. Group 4: Mice bearing D-12 primary tumors that were removed after 5 wk; metastatic status was assessed 3 wk after the primary tumors were removed.

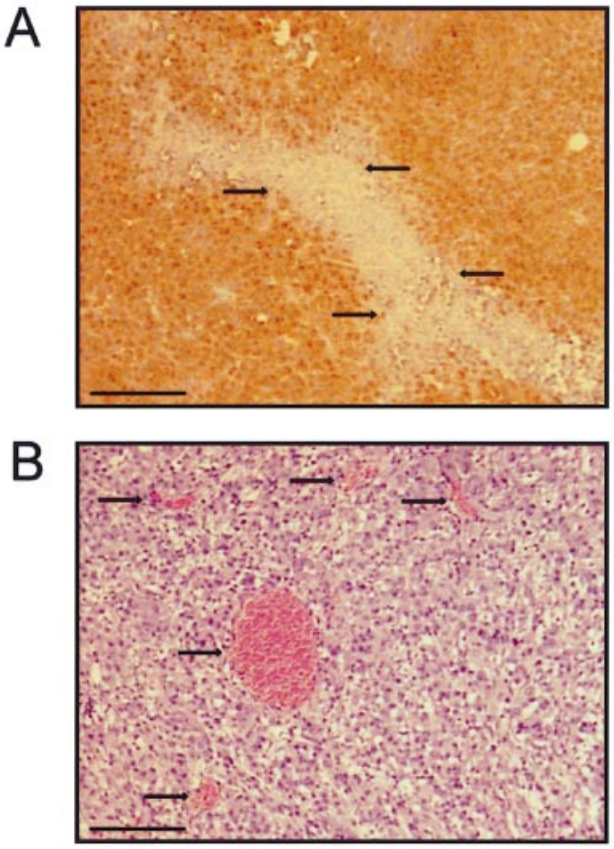

Figure 6. Immunohistochemical preparations illustrating differences between TSP-1 overexpressing and wild-type R-18 tumors. The sections were stained with anti-TSP-1 antibody by an avidin–biotin immunoperoxidase method and counterstained with hematoxylin. *Scale bars:* 150 μm. (*A*) TSP-1 overexpressing R-18 tumor. The tumor showed strong TSP-1 staining and extensive necrotic regions. The *arrows* point at a necrotic region. (*B*) Wild-type R-18 tumor. The tumor showed no TSP-1 staining, no necrosis, and a high vessel density. The *arrows* point at large vessels.

Folkman (1995) has hypothesized that large primary tumors can suppress the growth of their metastases by secreting angiogenesis inhibitors that, owing to a long half-life in the blood circulation, will be in excess of angiogenesis stimulators at the metastatic site. The data reported here are in full agreement with this hypothesis, and suggest that primary tumor-induced metastasis suppression can be mediated by TSP-1 in malignant melanoma. The angiogenesis of D-12 and U-25 tumors is stimulated primarily by VEGF (Rofstad and Halsør, 2000), which has a half-life in blood of approximately 3 min (Folkman, 1995; Ramanujan *et al*, 2000). The half-life of TSP-1, on the other hand, is several hours (Volpert *et al*, 1998; Ramanujan *et al*, 2000). The high rate of TSP-1 secretion of D-12 and U-25 tumors is possibly associated with their p53 mutational status, as melanomas with wild-type p53 generally show higher expression of TSP-1 than melanomas with mutant p53 (Grant *et al*, 1998).

Our suggestion that metastasis suppression by the primary tumor can be mediated by TSP-1 in melanoma is strengthened by a comprehensive study by Volpert *et al* (1998). They showed that the lung colonization of B16 murine melanoma cells inoculated into the tail vein of athymic mice was inhibited by subcutaneous HT1080 human fibrosarcomas secreting TSP-1. Moreover, our suggestion is consistent with a recent clinical observation by Crawford *et al* (1998), who reported rapid growth of cutaneous metastases shortly after surgical resection of a TSP-1-secreting small blue round cell primary tumor, possibly a primitive neuroectodermal tumor, of a 2 mo old girl. Metastasis suppression by the primary tumor may also be mediated by angiogenesis inhibitors other than TSP-1, as angiogenesis in a secondary site has been shown to be inhibited by tumors secreting angiostatin (O'Reilly *et*

al, 1994; Sckell *et al*, 1998) and transforming growth factor-β1 (Gohongi *et al*, 1999). It has indeed been shown that the growth of spontaneous Lewis lung carcinoma metastases can be inhibited by angiostatin produced by metalloelastase secreted by macrophages having infiltrated the primary tumor (O'Reilly *et al*, 1994; Holmgren *et al*, 1995; Dong *et al*, 1997). An experimental study demonstrating that the parenchymal cells of a primary tumor can secrete sufficient quantities of an angiogenesis inhibitor to suppress the growth of spontaneous metastases from that tumor, however, has not been reported until now.

Histologic examinations of lung colonies and spontaneous metastases provided insight into the mechanism of the TSP-1-mediated angiogenesis inhibition and metastasis suppression. The endothelial cells adjacent to dormant microcolonies showed increased apoptotic frequency, suggesting that TSP-1 inhibited neovascularization of lung colonies by induction of apoptosis in activated microvascular endothelial cells. This suggestion is consistent with the observation that TSP-1 can induce apoptosis in endothelial cells *in vitro* and *in vivo* by sequential activation of CD36, $p59^{fyn}$, caspase-3 like proteases, and p38 mitogen-activated protein kinases (Jiménez *et al*, 2000). The mitotic frequency of the D-12 melanoma cells was similar in dormant microcolonies and neovascularized macrocolonies, suggesting that TSP-1 had no significant anti-proliferative effect on D-12 cells *in vivo*, consistent with the *in vitro* data. In contrast, the D-12 apoptotic frequency was higher in microcolonies than in macrocolonies. TSP-1 did not induce apoptosis in D-12 cells *in vitro*, suggesting that the elevated apoptotic activity in microcolonies was not a direct effect of interaction between TSP-1 and D-12 cells. The high frequency of apoptotic D-12 cells in microcolonies was more likely a result of limited access to circulating apoptosis suppressive factors, such as insulin-like growth factor-1 and platelet-derived growth factor (Harrington *et al*, 1994), owing to the lack of adequate perfusion.

The dormancy of Lewis lung carcinoma and T241 fibrosarcoma microcolonies in mouse lungs under angiogenesis suppression by angiostatin has been studied in great detail by Holmgren *et al* (1995), who found that tumor cell proliferation, measured by bromodeoxyuridine incorporation and immunohistochemical staining of proliferating cell nuclear antigen, was not significantly different in dormant and growing colonies, whereas the tumor cells of dormant colonies exhibited more than a 3-fold higher incidence of apoptosis. They concluded that tumor cell proliferation is balanced by an equivalent rate of cell death in dormant metastases, and suggested that angiostatin controls metastatic growth by indirectly inducing apoptosis in tumor cells. The data in this study are in full agreement with the conclusion of Holmgren *et al* (1995) and suggest that the mechanism by which TSP-1 controls metastatic dormancy is similar to that of angiostatin.

This study prompted us to speculate that micrometastases of melanoma patients with large, wild-type p53 primary tumors may escape dormancy after removal of the primary tumor, owing to the disappearance of TSP-1 from the blood circulation. Therefore, some melanoma patients may benefit from combined local treatment and long-term anti-angiogenic therapy involving TSP-1.

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