Thrombospondin 2 inhibits metastasis of human malignant melanoma through microenvironment-modification in NOD/SCID/γ_C^{null} (NOG) mice

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Abstract. Thrombospondin (TSP) 2 interacts with matrix metalloproteinases (MMPs) and matrix serine proteases such as plasminogen activator (PA). Malignant melanoma is an aggressive human neoplasm showing aggressive metastatic features. We examined the effects of TSP2 gene introduction in the human malignant melanoma cell line A375. We established three clones transfected with human TSP2 (A375/TSP2). The *in vitro* invasiveness was remarkably suppressed (42-61%) in the TSP2-transfectants, while growth properties were preserved. The A375/TSP2 showed significantly decreased liver metastatic potential (liver weight: 3.88±0.30 g in A375/TSP2, 7.07±0.67 g in vector-transfectant (A375/V), $p<0.01$, Mann-Whitney U test) in super immuno-deficient mice (NOD/SCID/γ_C^{null}, NOG). The PA inhibitor-1 (PAI-1) and PAI-2 mRNAs were significantly overexpressed in A375/TSP2. The increased activities of PAI-1 and PAI-2 were confirmed by reverse zymography. The vascularity of metastatic lesions was significantly decreased in A375/TSP2 (vascular density: 0.62±0.15% in A375/TSP2, 4.96±0.61% in A375/V, $p<0.01$, Welch test). These results suggest that TSP2 suppresses hematogenous metastasis through microenvironment-modification including PAI up-regulation and anti-vascularization in human malignant melanoma.

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Introduction

Thrombospondin (TSP) is a family of glycoproteins with at least five subtypes encoded by independent genes, among which TSP1 and TSP2 contain three properdin-like type-1 repeats, unlike the other TSPs (1-3). TSP2 has recently attracted attention as an endogenous negative regulator of angiogenesis in tumorigenesis (4,5). Since TSP2 shows only low sequence homology to TSP1 in the procollagen region but good matches in the properdin-like type-1 repeats region, it has been suggested that the anti-angiogenic activity of TSP2 maps to the properdin-like type-1 repeats (6). Human TSP2 mRNA is expressed at high levels in aortic, cardiac, muscle, fetal, endocrine, immune, and nervous tissues (7). However, the detailed functions and properties of TSP2 are not well understood, while many studies have revealed those of TSP1 (4,7).

Malignant melanoma is one of the most aggressive human tumors. In recent decades, the incidence of malignant melanoma has steadily increased. A particularly worrying feature of the tumor is its increasing incidence and its capacity for rapid metastatic spread. Because of their resistance to current therapies, such as surgical excision, systemic chemotherapy with dacarbazine, and immunotherapy with vaccines, melanomas remain a significant cause of mortality. To achieve improvement in overall survival, it is important to develop effective new therapies.

We previously reported that TSP2 gene expression is significantly correlated with vascularity in various tumors (non-small cell lung cancer, colon cancer, and glioma) (8-10). In addition, there have been a number of reports concerning the biological activities of TSP2. Murine and bovine TSP2 protein inhibits the migration of bovine adrenal capillary endothelial cells and neovascularization induced in the rat cornea (1). *In vivo* tumor growth and angiogenesis of human

squamous cell carcinoma cell lines were inhibited by transfection with murine TSP2 complementary DNA (cDNA) (2). Our previous reports demonstrated that human colon cancer cell lines transfected with human TSP2 cDNA showed downregulation of matrix metalloproteinase (MMP) 2 and MMP9 mRNA (11,12). The activities of matrix proteases are correlated with the potential for tumor invasion. The proteolytic plasminogen activation system plays an important role in malignant melanoma (13). These facts suggest that TSP2 plays important roles as an inhibitor of tumor growth and angiogenesis. However, the biological characteristics of human TSP2 in human malignant melanoma are not well understood.

For the study of human cancers, including melanoma, tumor xenografts in athymic nude mice are well-established animal models. Several human melanoma cell lines were reported to metastasize in athymic nude mice or severe combined immuno-deficiency (SCID) mice (14,15). We recently reported the distant metastatic potentials of 4 human melanoma cell lines (A2058, A375, G361, and HMY-1) in newly developed NOD/SCID/γ_C^{nul} (NOG) mouse models employing systemic injections (16). It was confirmed that the experimental metastasis model of human melanoma using NOG mice was more sensitive and easier than that using SCID mice, due to its multiple immunological dysfunctions in not only cytokine production capability but also the functional competence of T, B and NK cells (17). In the present study, we isolated and transfected human TSP2 cDNA into A375 cells, which had previously been shown to have low expression of TSP2 mRNA and a high rate of liver metastases in NOG mice (16). Stable transfectants overexpressing TSP2 (A375/TSP2) were established. The *in vitro* properties of these transfectants and their *in vivo* potential for distant metastases in NOG mice were examined. We present the results here and discuss whether TSP2 affects the cell invasion *in vitro* and metastatic potential *in vivo* of human malignant melanoma, and conclude that TSP2 expression is related to the expression of matrix proteases and their inhibitors.

Materials and methods

Cell culture. Human amelanotic melanoma cell line A375 was obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). A375 cells were maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin.

At subconfluence, cells were washed twice with phosphate-buffered saline (PBS) to remove residual FBS and incubated for 24 h in serum-free DMEM medium. Harvested conditioned media were centrifuged at 500 x g for 10 min at 4˚C to remove cellular debris and then concentrated by ultrafiltration (Centricon YM-10, Millipore, Billerica, MA, USA). Total cellular RNA was extracted from wild-type (WT) A375 cells and from each transfectant by the acid guanidinium thiocyanate-phenol-chloroform extraction method.

Transfection of human TSP2 cDNA. The human TSP2 cDNA was constructed previously in our laboratory (11). A375 cells were transfected with the human TSP2 expression plasmid and vector alone, by using Lipofectin® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Stable transfectants were selected with 600 μg/ml G418 for 4-8 weeks, and resistant clones were isolated and used for experiments.

Northern blot analysis. Total cellular RNA was extracted as described above. Aliquots of 20 μg of total RNA were electrophoresed through 1% agarose gels containing 6% formaldehyde, and the RNA was transferred onto Gene Screen Plus nylon membranes (Du Pont NEN, Boston, MA, USA). Immobilized RNA was hybridized using a BcaBest labeling kit (Takara Biomedicals, Tokyo, Japan), [^{32}P]-dCTP (Du Pont NEN) and cDNA probe, which was designed to combine with human TSP2 (8-10). The membrane was washed and exposed to JB film (Eastman Kodak, Rochester, NY, USA). To control for loading and blotting variation, the gel was stained with ethidium bromide and photographed.

Quantitive evaluation of gene expression. Complementary DNA was synthesized from 1 μg of total RNA according to our previous reports (18,19). Quantitative reverse transcription PCR (RT-PCR) for TSP1 and 2, vascular endothelial growth factor (VEGF)-A, plasminogen activator inhibitor (PAI) 1 and 2, and ß-actin mRNA was performed according to the manufacturer's recommendations. The primers used for TSP2 and VEGF-A were as follows and were described previously (16,20): TSP2, 5'-primer: GCTGGTTCAGACAGCCAACTC; 3'-primer: TAACCAAAGACGAAGCCAGCAT; probe: FAM-TGCCACTGAAGTCCACAGACCCAAACT-TAMRA. The primers for TSP1 (Hs00170236), PAI-1 (Hs00167155), and PAI-2 (Hs00234032) were purchased from TaqMan® Gene Expression Assays (PE Applied Biosystems, Foster City, CA, USA). We used TaqMan® Universal PCR Master Mix (PE Applied Biosystems) for the real-time PCR. For the internal controls, ß-actin-probe-primer mixture was used (human ACTB, 4310881E, PE Applied Biosystems). The positive control standards were as follows: A2058 for TSP2 and PAI-2, A172 (human glioma cell line) for TSP1, HCT116 (human colorectal carcinoma) for VEGF-A, SW480 (human colorectal adenocarcinoma) for PAI-1. Real-time RT-PCR assays were run on an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems) as described previously (16). All the samples were assayed in quadruplicate and values were normalized by the respective amounts of ß-actin expression.

Growth analyses of transfectants in vitro and in vivo. The cells were seeded at $1.0x10^3$ cells/well in 2-cm dishes. The cell number was determined by trypan-blue exclusion with a hemacytometer at 5 time-points during 126 h. Quadruplicate cultures of each cell line were prepared at all time-points.

NOG mice (9-12 weeks of age, male) were maintained in the specific pathogen free facilities of the Central Institute for Experimental Animals (CIEA, Kanagawa, Japan). The cells ($1.0x10^5$/mouse) were subcutaneously inoculated into the flank of NOG mice. The smallest (a) and largest (b) tumor diameters were measured with calipers during 4 weeks, and tumor volumes were calculated using the following formula:

Volume = 1/2 x a^2 x b (11). All experiments involving laboratory animals were performed in accordance with the care and use guidelines of the CIEA.

Western blot analysis. Western blot analysis was performed to examine TSP2 protein production as described previously (21). Briefly, samples of conditioned media were boiled in denaturing sample buffer and then electrophoresed on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred onto a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK). After the non-specific binding was blocked (5% dry milk and 0.3% Tween-20 in PBS, for 1 h), the blots were incubated with TSP2 antibody (1:500, sc-7655, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and horseradish peroxidase-labeled secondary antibody (Amersham). The protein was visualized using an ECL nucleic acid labeling and detection system (Amersham).

Zymography analyses. Gelatin zymography and reverse zymography were performed as follows: the expression of MMPs in conditioned media was analyzed by gelatin zymography (22). The conditioned medium of each cell type were mixed with sample buffer (4.5% SDS, 0.125 M Tris-HCl, 22% glycerol and 0.01% bromophenol blue). Electrophoresis was carried out on a 0.1% SDS 10% polyacrylamide gel containing 1 mg/ml of gelatin. Following electrophoresis, the gel was rinsed twice with renaturing buffer (2.5% Triton X-100/50 mM Tris-HCl, pH 7.4) and incubated at 37˚C for 16 h in developing buffer (150 mM NaCl/10 mM $CaCl_2$/ 0.05% NaN_3/50 mM Tris-HCl, pH 7.4). The expression of protease inhibitors was analyzed by reverse zymography. For the analysis of tissue inhibitor of metalloproteinase (TIMP), the type of gel described above except that it also contained 20 ng/ml proMMP-2 was used and the rest of the procedures were the same as above (23). The gel was stained with 0.05% Coomassie blue in 10% ethanol and 10% acetic acid, and destained with 10% ethanol and 10% acetic acid. White lysis zones in gelatin zympgraphy indicated degrading activity and blue zones against a clear background in reverse zymography indicated inhibitory activity.

Casein/plasminogen zymography and one-phase reverse zymography were performed as follows: the expression of urokinase-type plasminogen activator (uPA) was analyzed by casein/plasminogen zymography using 0.1% SDS 15% polyacrylamide gels containing 1 mg/ml α-casein and 10 μg/ml plasminogen (23). After electrophoresis, gels were incubated for 6 h at 37˚C in 50 mM Tris-HCl, pH 7.5. For PAIs, the gel was incubated for proteolysis with 0.5 IU/ml human uPA (24). The gels were stained and analyzed as described above.

Enzyme-linked immunosorbent assay (ELISA). PAI-1 ELISA quantikine kits were from R&D Systems (DSE100, Minneapolis, MN, USA). This kit detects the active and latent forms of PAI-1 (minimum detectable concentration 0.014 ng/ml) but does not detect PA-complexed PAI-1. Samples of conditioned media were assayed according to the manufacturer's recommendations in duplicate. Readings were made at 450 nm using Molecular Devices SpectraMax 250 (GMI Inc., Ramsey, MN, USA).

In vitro invasion/migration assays. Invasion was assayed in BD BioCoat™ Matrigel™ Invasion Chambers (24-well, 8-μm pore, Becton Dickinson Labware, Bedford, MA, USA). Control insert chambers were used for migration assays. DMEM supplemented with 5% FBS was used as a chemoattractant. Cells ($2.5x10^3$) were suspended in serum-free DMEM and seeded onto invasion chambers and control chambers. After 24 h of incubation, cells were fixed with methanol and stained with crystal violet for 15 min. Cells remaining on the upper face of the membranes were scraped and those on the lower face were counted using an inverted microscope. All assays were performed in quadruplicate. The results were calculated by using the following formula: % Invasion = (Mean count of invading cells)/(Mean count of migrating cells) x 100.

In vivo liver metastasis assays. Experimental liver metastases using NOG mice (9-12 weeks of age, male) were generated by intrasplenic injection of cancer cells ($1.0x10^5$/mouse) and splenectomy (25). The mice were autopsied after 4 weeks of injection to evaluate the metastatic foci of the melanoma cells in liver. The weights of the livers, fixed in 40% formaldehyde for 3 days, were measured as an index for metastatic hepatomegaly. The metastatic lesions were also histologically confirmed.

Vascularization. Estimation of vascularity was performed as described previously (26). The tissue sections were deparaffinized and dehydrated through a xylene and alcohol series. After antigen retrieval (autoclaving at 121˚C for 10 min in 0.01 M citrate buffer) and blocking of endogenous peroxidase activity (0.3% H_2O_2 in methanol for 30 min), sections were incubated with rabbit anti-factor VIII polyclonal antibody (DakoCytomation, Glostrup, Denmark) overnight at 4˚C. The sections were then incubated with peroxidase-labelled polymer conjugated goat anti-rabbit antibody (Histofine Simplestain Mouse Max-PO; Nichirei, Tokyo, Japan) for 30 min at room temperature. The amplified products were visualized by a 3,3'-diaminobenzidine tetrahydrochloride (DAB) reaction. The vessel counts and densities were evaluated at magnification x200 (x20 objective and x10 ocular, 0.739 mm^2 per field) using a computerized image analysis system (Interactive Build Analysis System, Zeiss, Germany). Each specimen was measured from nine different view-points.

Statistical analysis. Statistical comparisons of data sets to the control (A375/V) were performed by a 2-sample t-test for inequality distribution (Welch test) or nonparametric analysis using the Mann-Whitney U test. Population and nodular doubling times were analyzed by one-way factorial ANOVA. Data are shown as means ± standard error of mean (SEM). These analyses were performed using JMP version 6 software (SAS Institute Inc., Cary, NC, USA). P-values <0.05 were considered statistically significant.

Results

Establishment of TSP2-transfectants. We transfected human TSP2 cDNA into the human melanoma cell line A375, and selected TSP2-overexpressing clones. The gene expression of TSP2 was confirmed by Northern blotting (data not shown) and

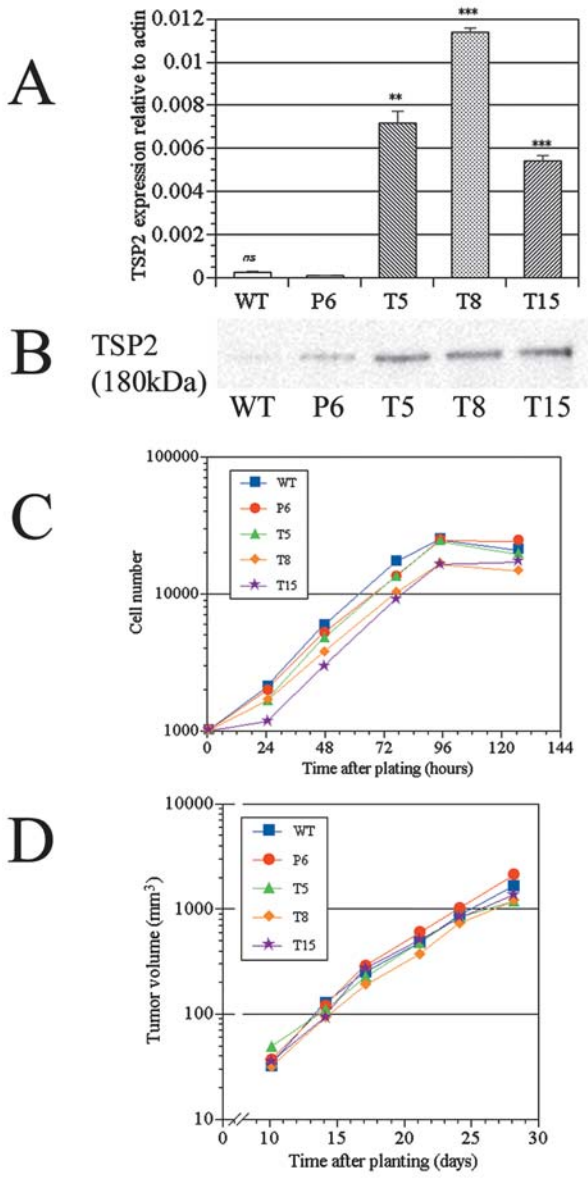

Figure 1. Establishment of TSP2-transfectants. (A) Real-time PCR of TSP2. TSP2-transfectants (T5, T8 and T15) showed >50-fold increased expression of TSP2 mRNA in comparison to vector-transfectant (P6), while there was no significant difference between wild-type A375 (WT) and P6 (n=4, $^{**}p<0.01$, $^{***}p<0.001$, Welch test). Columns, mean; bars, SEM. (B) Western blot analysis of TSP2 in condition media. TSP2-transfectants secrete TSP2 protein more than WT and P6. (C) Growth curves of WT and each transfectant *in vitro*. Cells were seeded at $1.0x10^3$ cell/well in 6-well plates. There was no significant difference among their doubling times; WT, 17:16±0:20; P6, 19:12±0:57; T5, 17:38±1:10; T8, 20:03±0:11; T15, 17:48±0:48; values are hour:minute, (n=4, ns, ANOVA). (D) Growth curves of WT and each transfectant *in vivo*. Cells were subcutaneously inoculated at $1.0x10^5$ cell/NOG mouse. There was no significant difference among their doubling times; WT, 4.26±0.53 days; P6, 3.87±0.21; T5, 3.81±0.21; T8 3.58±0.24; T15, 4.48±0.31; (n=6, ns, ANOVA).

real-time RT-PCR (Fig. 1A). TSP2-transfectants, A375/TSP2 (T5, T8 and T15), expressed TSP2 mRNA more strongly than WT and a vector-transfectant (A375/V, P6). Western blot analysis revealed increased protein levels of TSP2 in TSP2-transfectants (Fig. 1B).

Growth characteristics of TSP2-transfectants. We examined the growth characteristics of A375/TSP2 in cell culture

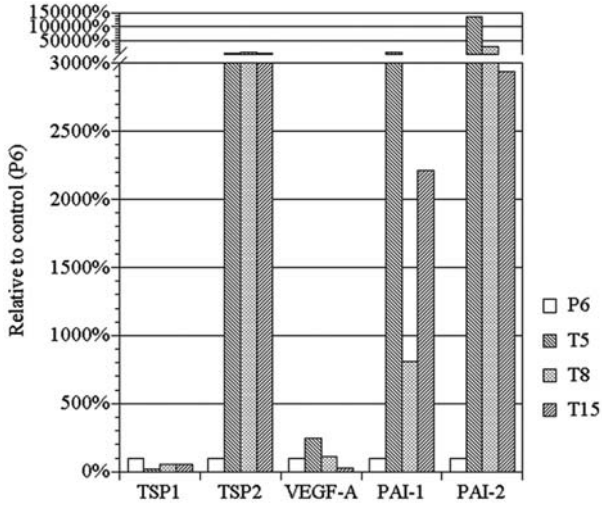

Figure 2. Gene expression levels measured by real-time RT-PCR. The values for each gene were standardized with the vector-transfectant as the control.

conditions (Fig. 1C). The doubling times of the cells were as follows: WT, 17:16±0:20; P6, 19:12±0:57; T5, 17:38±1:10; T8, 20:03±0:11; T15, 17:48±0:48; Values are hours:minutes. TSP2-transfectants, vector-transfectants and WT showed similar growth curves in cell culture without any significant differences in the doubling times (ANOVA, p=0.11). There were no apparent morphological differences in the transfectants. We also xenotransplanted the cell lines subcutaneously into NOG mice (Fig. 1D). Their doubling times were as follows: WT, 4.26±0.53 days; P6, 3.87±0.21; T5, 3.81±0.21; T8 3.58±0.24; T15, 4.48±0.31. There was no significant difference of doubing time among these cell lines (ANOVA, p=0.31). Therefore we used T5, T8 and T15 for the following experiments, and P6 as a control.

Quantitative analysis of the gene expression in real-time PCR. The gene expression levels of TSP1 and 2, VEGF-A, and PAI-1 and 2 in the A375/TSP2 clones and vector-transfectant were evaluated using real-time RT-PCR. While the gene-expression level of TSP2 in P6 was as low as that in the WT (P6, $1.10±0.14x10^{-4}$; WT, $2.39±0.53x10^{-4}$), the A375/TSP2 cells expressed the TSP2 gene at a significantly higher level than P6 cells (T5, $7.15±0.59x10^{-3}$, $p<0.01$; T8, $1.14±0.02x10^{-2}$, $p<0.001$; T15, $5.42±0.22x10^{-3}$, $p<0.001$; Welch test). In TSP1 and VEGF-A, A375/TSP2 clones showed no increased gene expression than A375/V (TSP1, 52.42±2.60 in P6 to 12.90±4.57 - 31.84±10.77 in A375/TSP2; VEGF-A, $7.78±0.15x10^{-2}$ to $2.67±0.31x10^{-2}$ - $19.34±0.95x10^{-2}$. The expression levels of PAI-1 and 2 mRNA were significantly increased in all transfectants as compared with P6 (PAI-1, $8.59±3.81x10^{-4}$ in P6, $9.74±0.75x10^{-2}$ in T5 ($p<0.001$), $6.95±0.75x10^{-3}$ in T8 ($p<0.01$), $1.90±0.15x10^{-2}$ in T15 ($p<0.001$); PAI-2, $2.34±0.83x10^{-1}$ in P6, $3.18±0.20x10^{2}$ in T5 ($p<0.001$), 7.09±0.62x10 in T8 ($p<0.01$), 6.89±1.04 in T15 ($p<0.01$); Welch test). These values are shown relative to that for A375/V in Fig. 2.

Secretion and activity of matrix proteases and inhibitors. Gelatin-zymography analysis of MMPs in the conditioned

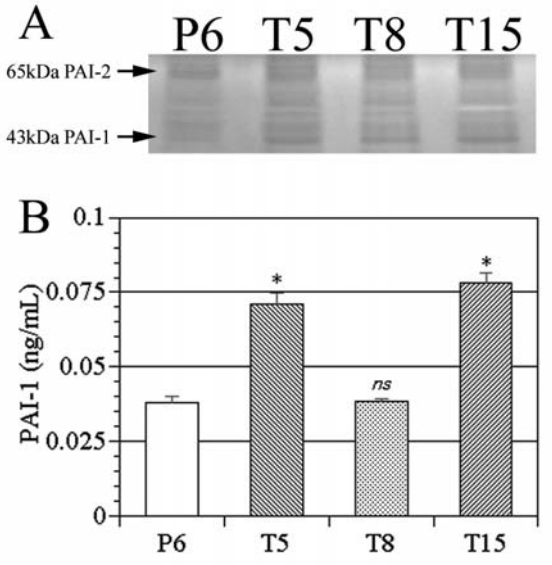

Figure 3. Matrix proteases and inhibitors. (A) One-phase reverse zymography, (B) PAI-1 ELISA. Reverse zymography showed that both PAI-1 and 2, inhibitors of PAs, were increased in A375/TSP2 more than in P6. ELISA showed that the total amounts of active and latent PAI-1 were significantly increased in T5 and T15. Columns, mean; bars, SEM (n=2, *p<0.05, Welch test).

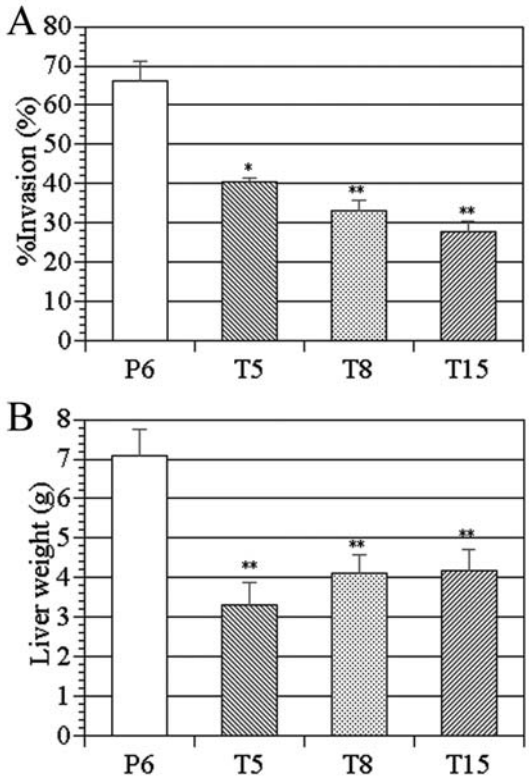

Figure 4. Invasiveness *in vitro* and *in vivo*. (A) *In vitro* invasion/migration assays. TSP2-transfectants invaded significantly less than the vector-transfectant (n=4, *p<0.05, **p<0.01, Welch test). (B) *In vivo* liver metastasis assays. The liver weights of NOG mice were measured as an index for metastatic hepatomegaly 4 weeks after inoculation. The livers of TSP2-transfectants showed significantly milder hepatomegaly than the controls (n=8, P6; n=9, others; **p<0.01, Mann-Whitney U test). Columns, mean; bars, SEM.

media of A375/TSP2 did not show altered activities of MMP-2 or 9. There were no differences between A375/TSP2 and P6 regarding the activities of TIMP-1, TIMP-2, and uPA seen on reverse and casein/plasminogen zymograms. One-phase reverse zymography demonstrated the increased activities of PAI-1 remarkably and PAI-2 mildly in A375/TSP2 (Fig. 3A). We also performed ELISA to examine the total amount of PAI-1 in the conditioned media. The concentrations of PAI-1 were as follows: 1.21±0.87x10^{-2} (ng/ml) in P6, 5.73±1.57x10^{-2} in T5, 2.60±0.18x10^{-2} in T8, 4.86±2.78x10^{-2} in T15 conditioned medium. T5 and T15 showed significantly increased secretion of PAI-1 (p<0.05, Welch test, Fig. 3B).

In vitro invasion/migration assays. To investigate the invasion ability of A375/TSP2, Matrigel™ invasion assays were performed (Fig. 4A). The percent invasions of each cell line were as follows: P6, 66.02±5.14%; T5, 40.50±1.08%; T8, 33.14±2.54%; T15, 27.91±2.54%. These results revealed significant differences in comparison to A375/V (p<0.05 in T5, p<0.01 in T8 and T15, Welch test).

In vivo liver metastasis assays. A375/TSP2 and A375/V cells (1.0x10^{5} cells) were transportally inoculated into NOG mice (n=9). One mouse of P6 was excepted because of a splenic abnormality. Amelanotic metastatic lesions, which consisted of solid white masses, were detected in all of the livers (Fig. 5A). The livers of A375/TSP2 clearly had fewer metastatic foci than the vector-transfectant. The liver weights obtained after inoculation of each of the cell lines were as follows: P6, 7.07±0.67 g; T5, 3.33±0.57 g; T8, 4.11±0.46 g; T15, 4.19±0.53 g. Statistical analysis revealed that there were significant differences among these values (n ≥8, p<0.01, Mann-Whitney U test, Fig. 4B). It was also confirmed that spouting microvessels inside the metastatic lesions were decreased in A375/TSP2 by immunohistochemical staining of factor VIII (Fig. 5B and C). Quantitative analysis revealed that microvessel counts (per view) and densities (%) were significantly decreased in A375/TSP2 (Fig. 6); count, 335.44±31.42/view in P6, 113.89±24.93 in T5 (p<0.001, Welch test), 55.89±12.55 in T8 (p<0.001), 62.78±5.18 in T15 (p<0.001); density, 4.96±0.61% in P6, 0.96±0.42 in T5 (p<0.01), 0.38±0.07 in T8 (p<0.001), 0.50±0.12 in T15 (p<0.001).

Discussion

In this study, we examined the biological role of TSP2 in the human malignant melanoma cell line A375. Real-time PCR revealed that the gene expression levels of TSP1 and VEGF-A were preserved in A375/TSP2 clones. TSP2-overexpressing clones showed increased activities of PAI-1 and 2. The *in vitro* invasiveness and *in vivo* metastasis were significantly suppressed, and microvessel deficiency was seen in the TSP2-transfectants. These results suggest that the effects of TSP2 are mediated by up-regulation of PAI activation mainly in human melanoma and result in the suppression hematogenous metastasis and in enhanced anti-vascularization.

MMPs and matrix serine proteases are major groups of secretory proteinases that play essential roles in various physiological and pathological processes, including tumor invasion and metastasis. The proteolytic plasminogen activation

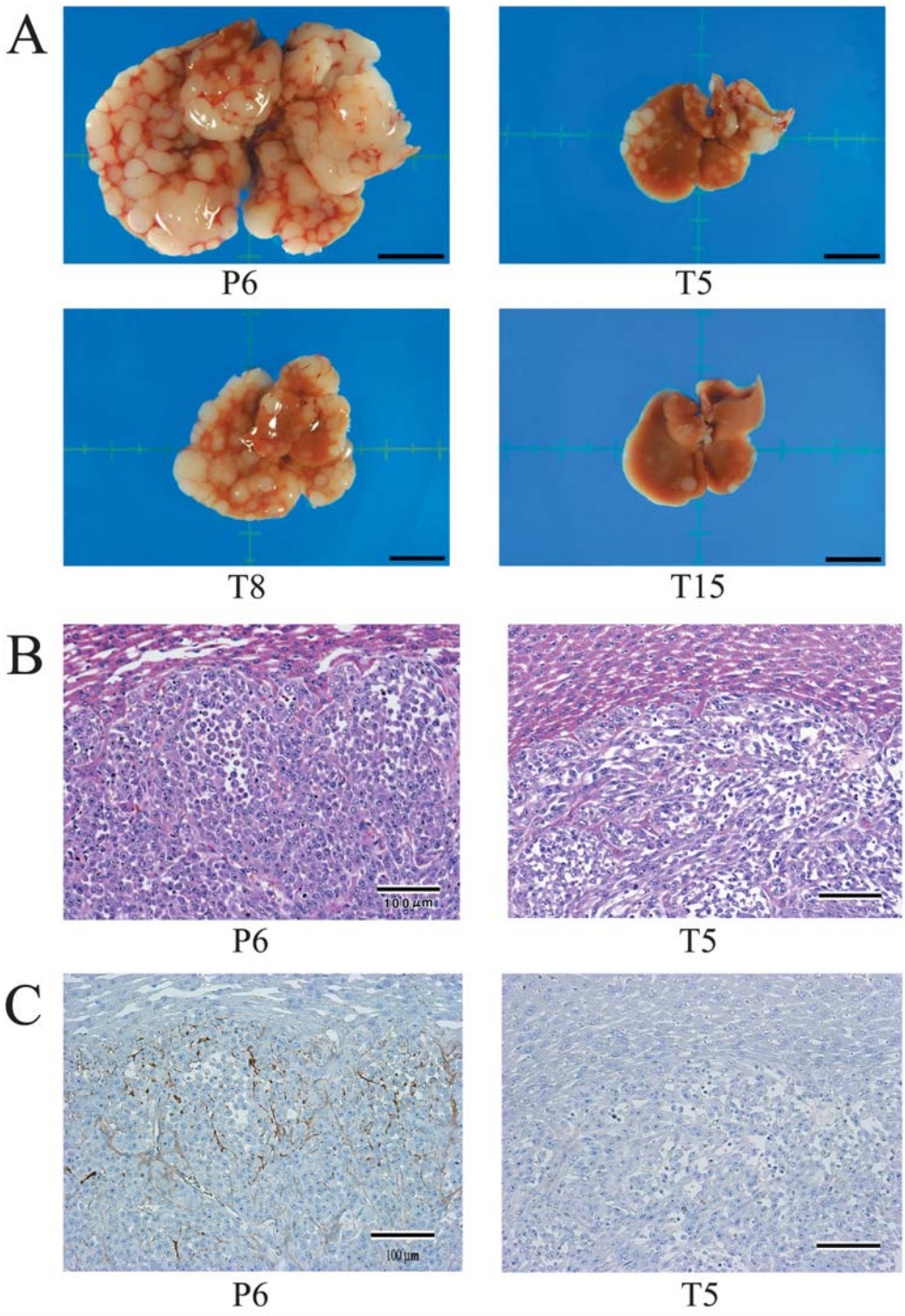

Figure 5. Liver metastasis of amelanotic melanoma in NOG mice. (A) Each type of transfectant cells was transportally inoculated into NOG mice from spleen ($1.0x10^5$/mouse, n=8 or 9). The mice were autopsied after 4 weeks to evaluate the liver metastasis. Amelanotic metastatic lesions, which consisted of solid white masses, were detected in all livers. (Bar, 1 cm). (B) Histological sections of livers from TSP2-transfectant and vector-alone NOG mice (hematoxylin and eosin). (C) Immunohistochemical sections of factor VIII antigens. There were clearly fewer vascular endothelial cells in TSP2-transfectants than in vector-alone; bar, 100 μm.

system plays an especially important role in the invasion and metastatis of malignant melanoma (13). Two functionally and structurally different PAs, uPA and tissue-type PA (tPA), are both capable of not only catalyzing the conversion of the inactive zymogen plasminogen to the active proteinase plasmin but also of degrading extracellular matrix macromolecules. Various melanoma cell lines were reported to produce large amounts of uPA and tPA (13.27). Moreover, plasmin transferred from plasminogen by PAs is potent in activating the latent forms of many MMPs. Generally, the activity of MMPs and PAs is correlated with the potential for tumor invasion and metastasis (28-30). The activation of plasminogen to plasmin is regulated by two specific plasminogen activator inhibitors, PAI-1 and PAI-2 (13). The role of PAIs in the prevention of tumor progression is still under investigation. In human melanoma progression, expression of uPA and

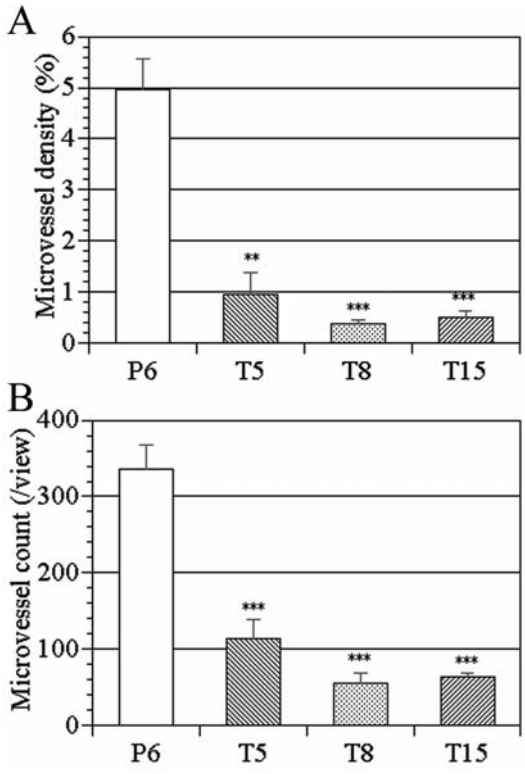

Figure 6. Quantitative analysis of microvessels. (A) Microvessel density. (B) Microvessel count. Immunohistochemical staining of factor VIII for sprouting microvessels inside the metastatic lesions were evaluated using a computerized image analysis system. The microvessel densities (%) and counts (per view) were significantly decreased in TSP2-transfectants (n=9, $^{**}p<0.01$, $^{***}p<0.001$, Mann-Whitney U test).

PAIs is not detectable in early stages but it often appears in later stages, which suggests that plasminogen activation appears to be a late event associated with advanced primary tumors and metastases (31). PAI-1 was reported to inhibit invasion and proliferation of hepatocellular and prostate carcinoma cells (32,33). On the other hand, increased expression of PAI-1 is associated with poor prognosis in many cancers (34). However, the growth of murine melanoma tumors was unaffected by the level of PAI-1 expression in either PAI-1-deficient mice or PAI-1-overexpressing mice (35), while overexpression of PAI-1 in cancer cells inhibited both tumor growth and angiogenesis (32,36). In human malignant melanoma cell lines, it was reported that PAI-1 inhibited tumor growth and angiogenesis and that PAI-2 inhibited tumor cells from degrading the extracellular matrix (ECM) and from metastasizing to distant sites (23,37,38).

The possible correlations between TSP2 and these proteinases have attracted considerable attentions, but are still unproven. In our previous study, TSP2-transfectants of colon cancer cells showed decreased expression of MMP-2 and MMP-9 mRNA (12). In pancreatic cancer cell lines, transfection of TSP2 inhibited *in vitro* cell invasion through the downregulation of MMP-9 and uPA activities (39). TSP protein was suggested to be involved in the regulation of metalloproteinase activity by inhibiting zymogen activation (18). However, it was also reported that TSP2 bound both pro-MMP-2 and MMP-2 directly, but that TSP2 did not function as a direct binding inhibitor of the active protease nor did it prevent the activation of pro-MMP-2 (40). However, active MMP-2 and active MMP-9 were not altered in the present study. Regarding their inhibitors, we previously reported that TSP2-transfected SW480 cells preserved the gene expression levels of TIMP-1 to 3 (12). Albo *et al* reported that TSP1 prevented invasion and metastasis of pancreatic cancer through upregulation of PAI-1 (41). The increased secretion and activities of PAIs can explain the decreased invasion of A375/TSP2 cells through Matrigel-coated membranes in spite of the preservation of MMPs and PAs. PAIs upregulated by TSP2 inhibit PA from transferring plasminogen to plasmin. Moreover, deficiency of plasmin depresses the degradation of fibrin and ECM directly and indirectly, because plasmin is a major activator of pro-MMPs. Therefore, the decrease of plasmin and active MMPs would result in fewer invasions and metastasis of melanoma cells *in vivo*. We conclude that activation of PAIs by TSP2 is essential for the suppressive effects on invasion and metastasis of human malignant melanoma *in vitro* and *in vivo*.

There is increasing evidence that TSP2 plays a more important role in the regulation of primary angiogenesis than TSP1 (1,3,8,9). TSP2 is considered to promote the apoptosis and inhibit the proliferation of microvessel endothelial cells (42,43). Murine and bovine TSP2 protein inhibited the migration of bovine adrenal capillary endothelial cells and neovascularization induced in the rat cornea (1). TSP2-overexpressing squamous cell carcinoma cells showed an inhibitory effect on dermal microvascular endothelial cell migration *in vitro* (2). Colon cancer cell lines transfected with human TSP2 also caused poorer proliferation of microvascular endothelial cells *in vitro* (11). TSP2 mRNA expression was significantly inversely correlated with vascularity in surgical specimens of various cancers (non-small cell lung cancer, colon cancer, and glioma) (8-10). However, these reports disclosed that TSP2 was not correlated with VEGF in squamous cell carcinoma, non-small cell lung cancer, or glioma. In the skin, non-neoplasic angiogenesis is thought to be regulated by the balance between the angiogenic inducer VEGF and the angiogenic inhibitor TSP2 (44). The mediation by TSP2 of the angioinhibitory effect might be performed not only by VEGF but by some matrix proteases (45,46). MMPs play active roles during matrix remodeling and other tissue regeneration processes, including modulation of angiogenesis. In human malignant melanoma, TSP2 suppressed stromal angiogenesis and hematogenous metastasis by activations of PAIs, while VEGF-A expression was preserved.

Tumor cell invasion is a multi-step process that involves cell attachment, proteolysis of matrix components and migration of cells through the disrupted matrix. Hematogenous metastasis occurs as a consequence of a well-characterized set of sequential events. These types of metastasis models address only those events that occur after the entrance of cells into the blood vessels. Our liver metastasis model using intrasplenic injection of small numbers of cancer cells into NOG mice is reliable and quantitative, and more closely mimics the *in vivo* conditions (25). As a consequence, the invasive and metastatic abilities of A375 cell were significantly inhibited by TSP2-transfections *in vitro* and *in vivo*. We are planning further experiments to investigate the mechanism

between TSP2 and PAI-1 or 2 by neutralizing antibody to PAIs or using PAI-knockout mice.

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