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Advances in Brief

Thrombospondin-1 Associated with Tumor Microenvironment Contributes to Low-Dose Cyclophosphamide-Mediated Endothelial Cell Apoptosis and Tumor Growth Suppression

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Abstract

Low-dose cyclophosphamide (LDC) induces selective apoptosis of endothelial cells within the vascular bed of tumors. Here, we investigated a hypothesis that the effect of LDC is mediated by the pro-apoptotic action of endogenous inhibitors of angiogenesis. Tumors treated with LDC demonstrate similar expression of matrix metalloproteinases and also basement membrane-derived angiogenesis inhibitors when compared with wild-type tumors, whereas the expression of thrombospondin-1 (TSP-1) is significantly elevated in LDC-treated tumors. We used mice with an absence of type XVIII collagen (endostatin) or type IV collagen α3 chain (tumstatin) or TSP-1 to assess the contribution of these endogenous inhibitors of angiogenesis on LDC-mediated tumor suppression. Lack of TSP-1 in the host in addition to tumor cells leads to diminished capacity of LDC to suppress tumor growth, whereas the absence of endostatin and tumstatin did not alter the effect of LDC. LDC treatment predominantly induces selective expression of TSP-1 in tumor cells and peri-vascular cells and facilitates apoptosis of proliferating endothelial cells, with minimal direct effect on tumor cells and peri-vascular cells. These studies indicate that TSP-1 contributes to tumor growth suppression induced by LDC and suggest that tumors that express high basal level of TSP-1 may be more susceptible to tumor suppression by such a regimen. This study also makes a strong case for TSP-1 expression levels as a potential predictive marker for the successful use of LDC in cancer patients.

Introduction

Antiangiogenic (metronomic or low-dose) chemotherapy is a strategy for optimizing the effects of chemotherapeutics by administrating traditional cytotoxic drugs (*i.e.*, cyclophosphamide, vinblastine, etc.) at lower concentration without rest periods (1–4). The target is the genetically stable endothelial cells within the vascular bed of a tumor, rather than tumor cells with a high rate of mutations. Therefore, antiangiogenic scheduling holds the promise of not inducing acquired drug resistance, which is often seen with traditional chemotherapy scheduling (1, 3, 5). Antiangiogenic chemotherapy also demonstrates significantly lower side effects compared with the maximum tolerated dose regimen in mice, presumably due to a high degree of selectivity and sensitivity to endothelial cells (1, 2, 6). However, it remains unclear how low-dose chemotherapy induces selective apoptosis of endothelial cells.

Endogenous proteins and fragments of proteins such as thrombospondin (TSP)-1 and -2, angiostatin, endostatin, arresten, canstatin, and tumstatin have been shown to possess antiangiogenic activity (7–13). Recent genetic experiments have shown that tumstatin (type IV collagen α3 chain NC1 domain)-deficient mice and TSP-1-deficient mice exhibit increased growth of implanted or spontaneous tumors when compared with their wild-type counterparts, suggesting that both tumstatin and TSP-1 serve as key endogenous angiogenesis regulators in the body (7, 14, 15). Endostatin (type XVIII collagen)-deficient mice exhibit altered retinal vascular development (16).

In this study, we investigated the hypothesis that the effect of low-dose cyclophosphamide (LDC) is mediated via up-regulation of endogenous angiogenesis inhibitors in the tumor microenvironment. Our cell biological and genetic studies suggest that TSP-1 in the tumor microenvironment is a key mediator of antiangiogenic effects observed with low-dose antiangiogenic scheduling of cyclophosphamide.

Materials and Methods

Cell Lines and Knock Out Mice. Lewis lung carcinoma (LLC) and B16F10 melanoma (B16F10) cells were grown at 37°C in 5% CO_2 in DMEM with 10% heat-inactivated fetal bovine serum and 5 ng/ml plasmocin. LLC and B16F10 cells are gifts from Dr. Judah Folkman from Children's Hospital (Boston; Ref. 10). Primary mouse lung endothelial cells were isolated as shown previously (14). 10T1/2 cells are a gift from Dr. Patricia A. D'Amore from Schepens Eye Research Institute (Boston; Ref. 17). NIH-3T3 cells were purchased from American Type Culture Collection (Manassas, VA). The tumstatin (type IV collagen α3 chain)-deficient mice were described in a previous publication (14). The endostatin (type XVIII collagen)-deficient mice were originally described by Fukai *et al.* (16) and provided as a gift by Dr. Bjorn R. Olsen from Harvard Medical School (Boston). The TSP-1-deficient mice were originally described by Lawler *et al.* (18) and provided as a gift by Dr. Jack Lawler from Beth Israel Deaconess Medical Center (Boston). For *in vitro* assay, 0.35, 3.5, and 35 μM 4-hydroperoxycyclophosphamide (4-HC) was added into the culture medium and incubated with cells for 24 h. 4-HC is a gift from Dr. Susan M. Ludeman from Duke University (Durham, NC), and the treatment was performed as recommended by Flowers *et al.* (19) to avoid the toxic effects of volatile metabolites.

***In Vivo* Tumor Studies.** Age- and sex-matched mice on a C57BL/6 background that are deficient in tumstatin, endostatin, or TSP-1 or normal wild-type mice were used for these studies. All mouse studies were reviewed and approved by the animal care and use committee of Beth Israel Deaconess Medical Center. LLC or B16F10 cells were injected s.c. on the backs of mice (1 or 0.5×10^6 cells/mouse). Each experimental group had five mice, and the experiments were repeated twice. When tumor volumes reached 100 mm^3, 170 mg/kg cyclophosphamide (Sigma, St. Louis, MO) were injected into peritoneal cavity every 6 days according to the protocol reported previously (1). The tumors were measured as described previously (13). These mice were sacrificed, and the tumors were collected at the end of each *in vivo* experiment.

Immunostaining. Immunohistochemical staining was performed as described previously (14). Four-μm frozen sections were incubated with various primary antibodies, *i.e.*, rat anti-CD31 (BD PharMingen, San Diego, CA), rabbit anti-epidermal growth factor receptor (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-α-smooth muscle actin (Sigma), rabbit antifibroblast specific protein-[FSP-1; a gift from Dr. Eric G. Neilson, Vanderbilt University

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School of Medicine, Nashville, TN; Ref. 20], goat anti-TSP-1 (Santa Cruz Biotechnology), rabbit anti-metalloproteinase (MMP)-9, rabbit anti-MMP-2, rabbit anti-MMP-3, and goat anti-MMP13 (Chemicon, Temecula, CA) antibodies. *In situ* cell death detection kit (Roche, Indianapolis, IN) was used to detect apoptotic endothelial cells of blood vessels. In each group, the numbers of CD31-positive apoptotic endothelial cells of blood vessels were counted at ×200 magnification in a blinded fashion for 10 separate fields and averaged.

Western Blotting. Serum-free medium conditioned by cells after 24 h of culture was collected and concentrated with Amicon membranes with 10,000 M_r cutoff (Millipore, Billerica, MA), and samples containing 15 μg of protein were subjected to electrophoresis on polyacrylamide gels. Western blotting was performed as described previously (14). Mouse anti-TSP and TSP-1 (NeoMarkers, Fremont, CA) antibodies were used as primary antibodies in this study.

Survival/Proliferation Assay. Survival/proliferation assay was performed using methylene blue staining method as described previously (12). All experimental groups represent quadruplicate samples.

Statistical Analysis. Statistical differences between two groups were calculated using Student's *t* test or Welch's *t* test. ANOVA was used to determine statistical differences between three or more groups. As needed, additional analysis was carried out using *t* test with Bonferroni correction to identify significant differences. $P < 0.05$ was considered statistically significant.

Results and Discussion

***In Vitro* Cell Survival/Proliferation.** In all our *in vivo* studies, we used a dosage of 170 mg/kg cyclophosphamide as reported by Browder *et al.* (1). This working concentration translated to 7.5 mM cyclophosphamide. Cyclophosphamide, however, is considered inactive in all *in vitro* experiments, because it requires liver hepatocyte p-450 system to generate active cytotoxic metabolites (21). Therefore, in all our *in vitro* experiments, we used 4-HC, which spontaneously converts to 4-hydroxycyclophosphamide in aqueous solution. In these experiments, 0.35 and 3.5 μM 4-HC did not exhibit significant effect on the proliferation of primary endothelial cells, LLC cells, B16F10 cells, 10T1/2 fibroblastic cells, and NIH-3T3 fibroblasts, whereas 35 μM 4-HC was enough to inhibit proliferation of these cells (Fig. 1*A*).

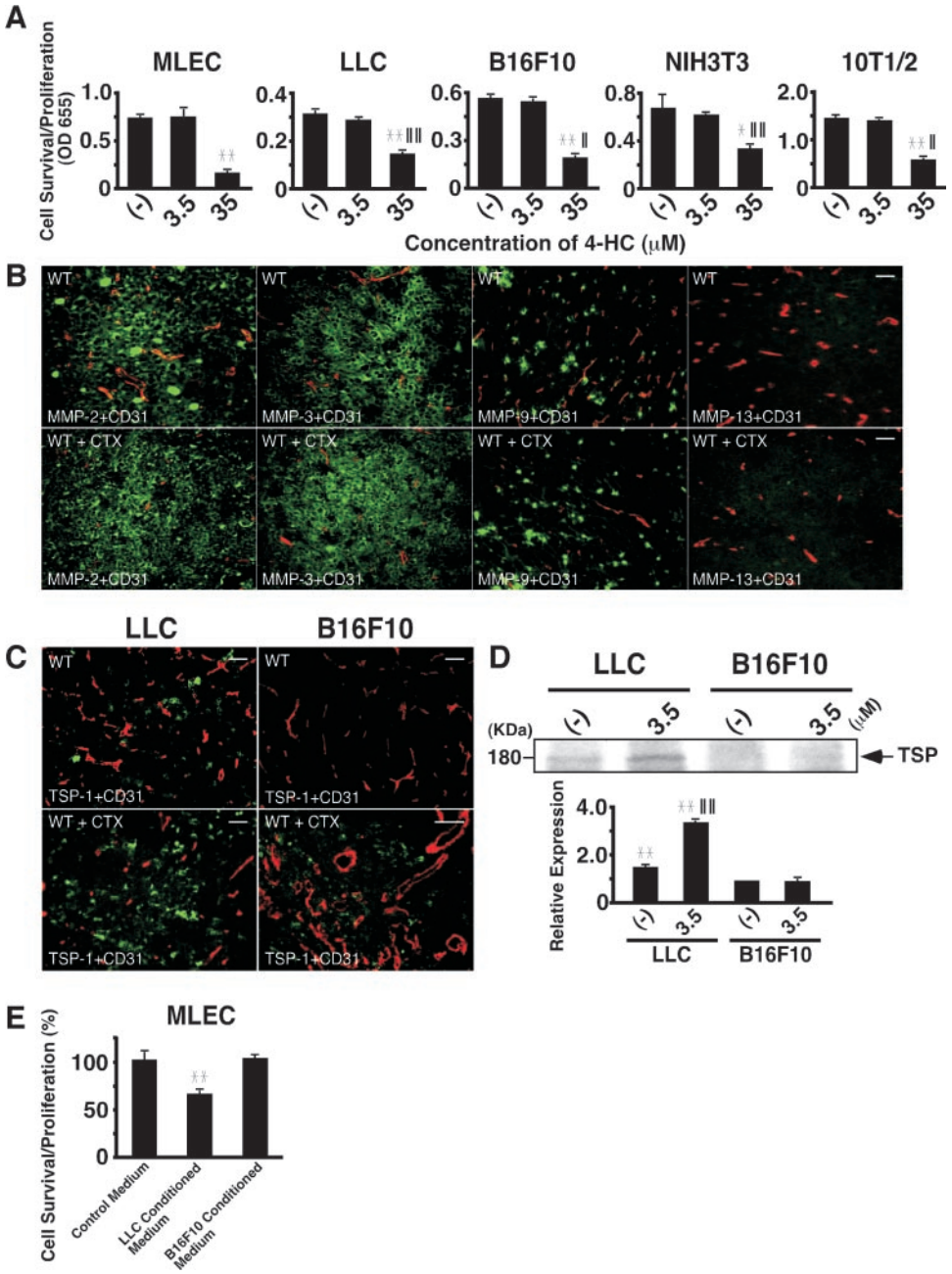

Fig. 1. *In vitro* cell survival/proliferation and the expression of MMPs and TSP-1 in control tumors and LDC-treated tumors. *A*, low and high concentration of *4-HC* was added into culture medium supporting primary mouse lung endothelial cells (*MLEC*), LLC cells, B16F10 melanoma cells, NIH 3T3 cells, and 10T1/2 fibroblasts for 24 h. Cell survival/proliferation was estimated using methylene blue staining. The results are shown as the mean ± SE. ** and * indicate $P < 0.01$ and 0.05, respectively, compared with nontreatment groups. ‖ ‖ and ‖ indicate $P < 0.01$ and 0.05, respectively, compared with MLEC treated with 35 μM 4-HC. *B*, frozen sections from LLC tumor in the wild-type mice with or without LDC treatment (*WT* and *WT+CTX*, respectively) were stained with anti-MMP-2, -3, -9, and -13 antibodies (*green*). Anti-CD31 antibody was used as a marker for endothelial cells (*red*). *Scale bar* = 50 μm. *C*, frozen sections from LLC and B16F10 tumors in the wild-type mice with or without LDC treatment (*WT* and *WT+CTX*, respectively) were stained with anti-TSP-1 antibody (*green*). Anti-CD31 antibody was used as a marker for endothelial cells (*red*). *Scale bar* = 50 μm. *D*, TSP expression in LLC cells was compared with B16F10 cells using Western blotting. Relative expression of TSP as determined by band intensity is shown by a bar graph. ** indicates $P < 0.01$ compared with B16F10 cells with nontreatment. ‖ ‖ indicates $P < 0.01$ compared with LLC cells with nontreatment. *E*, serum-free media conditioned by LLC and B16F10 cells were incubated with MLEC for 24 h, and cell survival/proliferation was estimated using methylene blue staining. DMEM medium was added as a control. The results are shown as the mean ± SE. ** indicates $P < 0.01$ compared with B16F10 groups.

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These experiments reveal statistical difference in the survival capacity of these cells by 35 μM 4-HC, demonstrating that endothelial cells are more susceptible to the effects of cyclophosphamide (4-HC) than other cells (Fig. 1*A*).

Regulation of the Expression of Endogenous Angiogenesis Inhibitors and MMPs by LDC. To assess the contribution of endogenous inhibitors of angiogenesis in the tumor microenvironment induced by LDC, we evaluated the expression levels of matrix MMPs and TSP-1. Basement membrane (BM)-derived fragments with antiangiogenic activities require MMPs for their generation, thus MMP expression levels likely must go up to generate angiogenesis inhibitors tumstatin, canstatin, and arresten (14, 22). The most prominent BM-degrading enzymes are considered to be MMP-2, MMP-3, MMP-9, and MMP-13 (22, 23). However, the expression of these MMPs was unaltered in LLC tumors treated with LDC (Fig. 1*B*). Similar results were also observed when B16F10 tumors treated with LDC were analyzed (data not shown). Concomitantly, the expression of different isoforms of type IV collagen (the precursor source of arresten, canstatin, and tumstatin) was also not altered in the LDC-treated LLC and B16F10 tumors (data not shown). In contrast, the levels of TSP-1 were significantly elevated in the LLC and B16F10 tumor tissues treated with LDC, and the enhanced expression was associated predominantly with nonendothelial cells (Fig. 1*C*). We also performed the immunohistochemical studies using liver tissue that normally lacks TSP-1 expression and also the kidney, lung, and spleen tissues that express basal levels of TSP-1 from LDC-treated and untreated control mice. However, there was no difference in TSP-1 expression pattern in these organs between untreated and LDC-treated wild-type mice (data not shown). Additionally, using Western blotting technique, we demonstrated that LLC cells expressed significant levels of TSP-1 while B16F10 cells revealed minimal expression of TSP-1, and a dosage of 3.5 μM 4-HC, a concentration that did not effect the survival of cells directly (Fig. 1*A*), could induce expression of TSP-1 in LLC cells, 10T1/2 fibroblastic cells, and NIH-3T3 fibroblasts (Fig. 1*D*; data not shown). In fact, serum-free conditioned media from LLC cells inhibited the proliferation of endothelial cells (Fig. 1*E*). Such enhancement in TSP-1 expression was not seen in B16F10 tumor cells, suggesting that the upregulation of TSP-1 in the B16F10 tumor tissue is potentially contributed by nonendothelial cells and noncancer cells (Fig. 1, *C–E*).

TSP-1 and not Endostatin or Tumstatin Contributes to the Antiangiogenic Effect Observed with LDC. We next used mice deficient in TSP-1, type XVIII collagen α1 chain (endostatin), and type IV collagen α3 chain (tumstatin) to assess the contribution of these endogenous angiogenesis inhibitors to the antiangiogenic effect observed with LDC (14, 16, 18). LLC tumors in the wild-type mice and the three different knock out mice were treated with LDC starting when 100 mm^3, and the study was suspended when the tumors

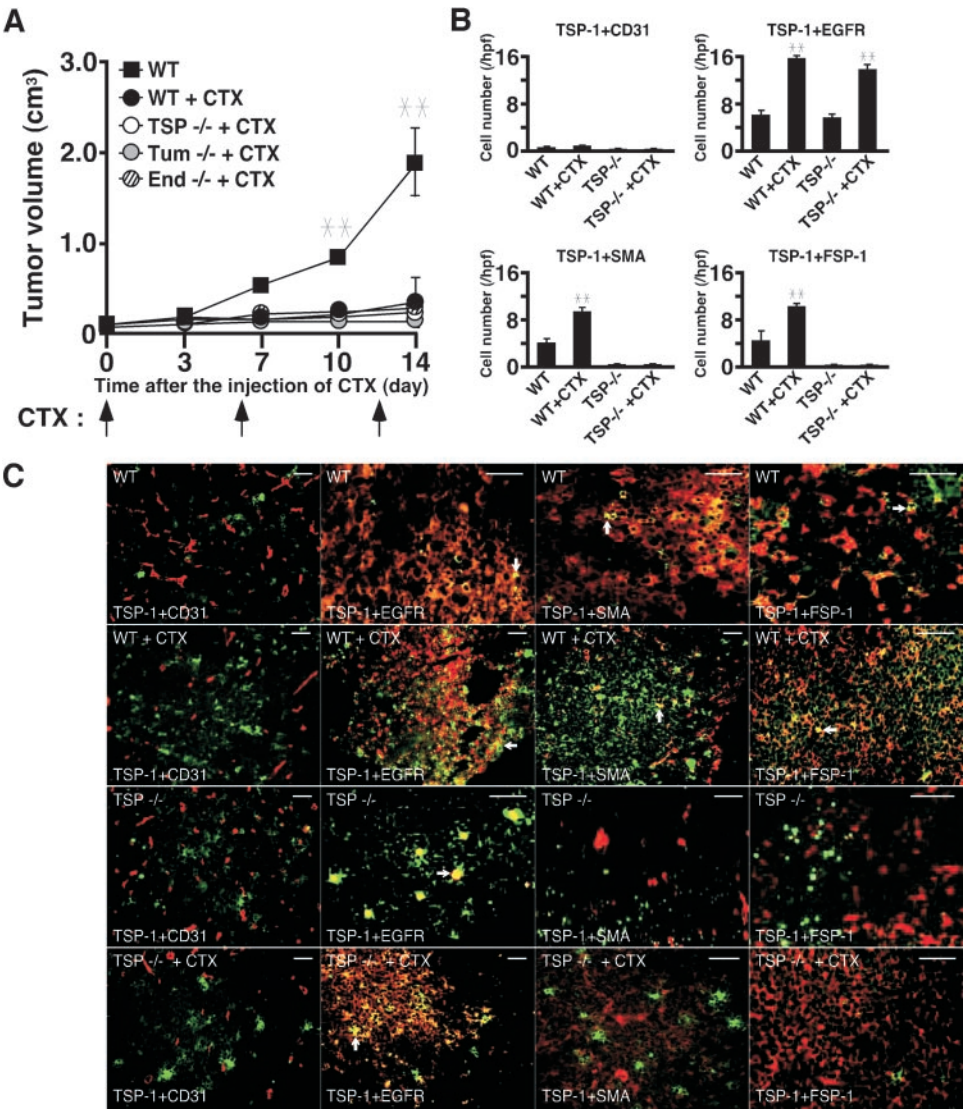

Fig. 2. LDC treatment of LLC tumors in mice. *A*, the LLC tumors in wild-type (WT), tumstatin (type IV collagen α3 chain; *Tum*), TSP-1 (*TSP*), and endostatin (type XVIII collagen; *End*)-deficient mice were treated with administration of 7.5 mM cyclophosphamide into the peritoneal cavity every 6 days, and the tumor sizes were measured. Data are the representative of two such independent experiments. The results are shown as the mean ± SE. ** indicates $P < 0.01$ compared with wild-type mice with treatment. *B* and *C*, frozen sections (4 μm) were cut from tumor tissues in the wild-type and TSP-1-deficient mice with or without LDC treatment (*WT*, *WT+CTX*, *TSP −/−*, and *TSP−/−+CTX*), and they were double-stained with indicated antibodies. *B*, the numbers of double-stained cells in each staining were counted and averaged at ×200 magnifications. ** indicates $P < 0.01$ compared with each nontreatment group. *C*, representative pictures in each staining are also shown. TSP-1-positive cells are shown as *green*, whereas the other antibody-positive cells are shown as *red*. *Arrows* indicate double-stained cells (*yellow*). *Scale bar* = 50 μm.

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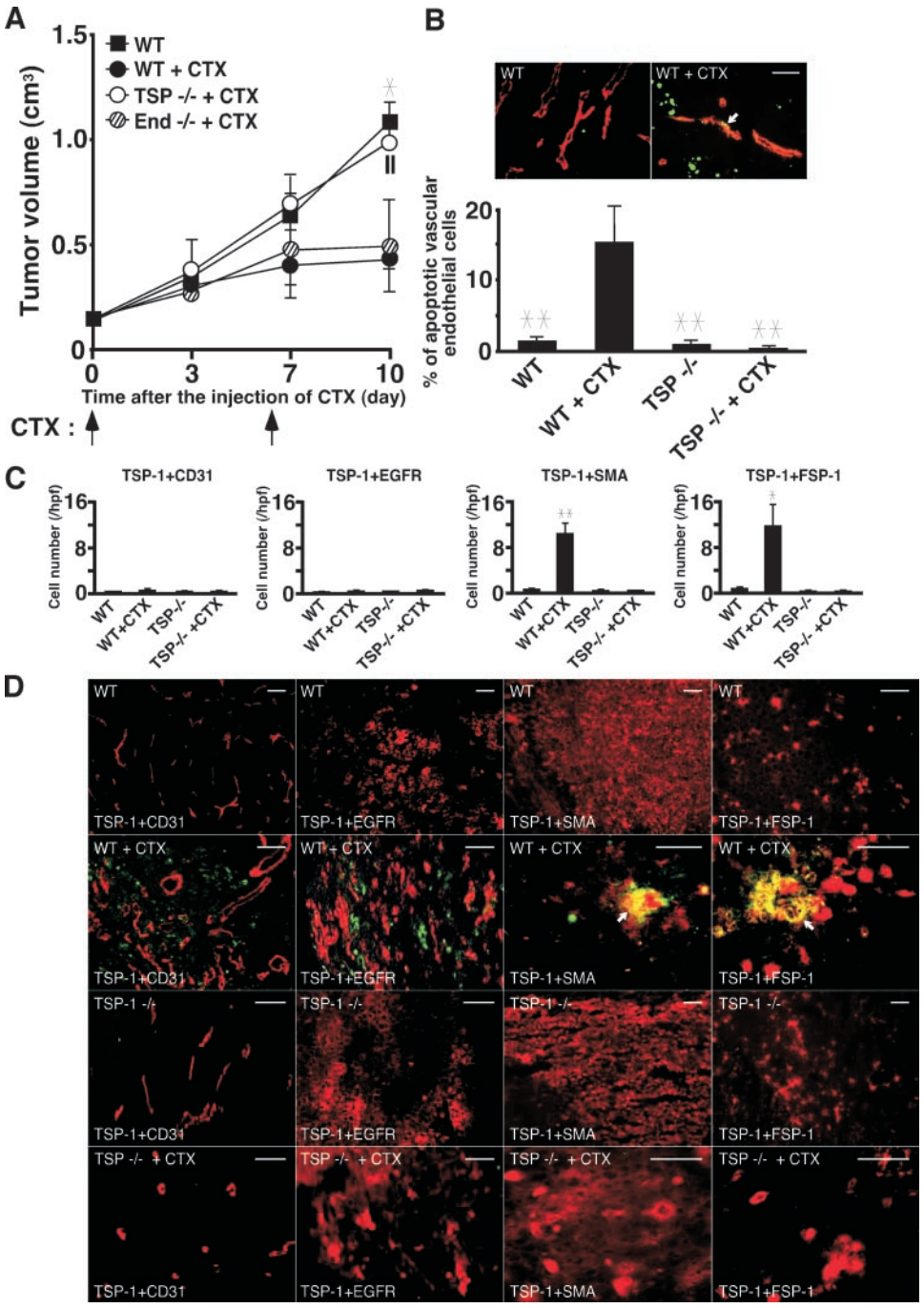

Fig. 3. LDC treatment of B16F10 melanoma tumors in mice. *A*, the B16F10 tumors in wild-type (*WT*), TSP-1 (*TSP*), and endostatin (type XVIII collagen; *End*)-deficient mice were treated with administration of 7.5 mM cyclophosphamide (*CTX*) into the peritoneal cavity every 6 days. The results are shown as the mean ± SE. ∗ indicates $P < 0.05$ compared with wild-type mice with treatment. ‖ indicates $P < 0.05$ compared with wild-type mice with treatment. *B*, apoptotic vascular endothelial cells were detected using TUNEL technique and CD31 staining in tumor tissues from the wild-type and TSP-1-deficient mice, with or without LDC treatment (*WT*, *WT+CTX*, *TSP−/−*, and *TSP−/−+CTX*). One hundred different high-power fields were counted in a blind fashion. Representative stainings are shown in the figure and *arrow* indicates apoptotic cells in the blood vessels. *Scale bar* = 50 μm. *C* and *D*, frozen sections (4 μm) were cut from tumor tissues in WT and WT+CTX, TSP−/−, and TSP−/−+CTX, and they were double-stained with indicated antibodies. *C*, the numbers of double-stained cells in each staining were counted and averaged at ×200 magnifications. ∗∗ and ∗ indicate $P < 0.01$ and 0.05, respectively, compared with WT nontreatment group. *D*, representative pictures in each staining are also shown. TSP-1-positive cells are shown as *green*, whereas the other antibody-positive cells are shown as *red*. *Arrows* indicate double-stained cells (*yellow*). *Scale bar* = 50 μm.

reached 2.0 cm^3 (Fig. 2*A*). Mice were treated with LDC according to the protocol of Browder *et al.* (1): 170 mg/kg (7.5 mM) every six days. Whereas the untreated wild-type tumors grew very large, the tumors in the LDC-treated wild type and three different knock out mice revealed impressive and similar suppression of tumor growth (Fig. 2*A*). The suppression of tumor growth was associated with significant decrease in CD31-positive vasculature, increase in apoptotic blood vessel-associated endothelial cells, and lack of significant body weight loss (Fig. 2*C;* data not shown). Immunofluorescence experiments showed that in the wild-type mice with LLC tumors, TSP-1 was localized to LLC cells and peri-vascular cells but not endothelial cells, as determined by double staining and colocalization with epidermal growth factor receptor [EGFR; a marker for tumor cells], α-smooth muscle actin (SMA) and FSP-1 (markers for fibroblasts and peri-vascular cells), and CD31 (a marker for endothelial cells), respectively (Fig. 2, *B* and *C*). In the TSP-1-deficient mice with LLC tumors, TSP-1 expression was seen only in the LLC cells and some cancer cells that also expressed stromal markers, but as expected, the host cells were deficient for TSP-1 expression (Fig. 2, *B* and *C*). LDC treatment of LLC tumors in the wild-type mice led to up-regulation of TSP-1 in the LLC cells and peri-vascular cells that also expressed stromal markers, whereas endothelial cells continued to exhibit a lack of TSP-1 expression (Fig. 2, *B* and *C*). LDC-treated LLC tumors in TSP-1-deficient mice revealed up-regulation of TSP-1 expression in the LLC cells, but the host-derived cells remained absent for TSP-1 expression (Fig. 2, *B* and *C*).

So, why do wild-type and TSP-1-deficient mice show similar suppression of LLC tumors treated with LDC? We propose that TSP-1 expression and its

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up-regulation by LDC in the LLC cells are probably sufficient to overcome the lack of TSP-1 expression by the host cells in the TSP-1-deficient mice. Thus, these experiments suggest that TSP-1 expression levels in the LDC-treated LLC tumors in TSP-1-deficient mice are sufficient to cause endothelial cell apoptosis and death. Therefore, to test this hypothesis, we decided to perform similar experiments with B16F10 cells, because they do not express sufficient levels of TSP-1 (Fig. 1*D*).

B16F10 tumors in the wild-type, TSP-1-deficient, and endostatin-deficient mice were treated with LDC, exactly as described above for LLC tumors. In these experiments, LDC treatment led to significant tumor suppression of B16F10 tumors in wild-type and endostatin-deficient mice but not the TSP-1-deficient mice (Fig. 3*A*). The suppression of tumor growth was associated with significant endothelial cell apoptosis, which was not observed in TSP-1-deficient mice with B16F10 tumors treated with LDC, similar to untreated wild-type and TSP-1-deficient mice (Fig. 3*B*). Evaluation of tumor tissue suggested that LDC-treated wild-type tumors derived most of the TSP-1 from nontumor/nonendothelial cells, such as stromal and peri-vascular cells (Fig. 3, *C* and *D*). Therefore, collectively, our studies demonstrate that cancer cells or perivascular stromal cells can provide adequate protein levels of TSP-1 to mediate the antiangiogenic effect of LDC. It also suggests that LDC can stimulate the expression of TSP-1 in stromal cells and tumor cells without directly killing them, and such up-regulation of TSP-1 has a negative effect on endothelial cell survival (Fig. 1; Fig. 2; Fig. 3).

Collectively, our results also suggest that the antiangiogenic effect of LDC is mediated by TSP-1 and not two other endogenous inhibitors of angiogenesis, namely tumstatin and endostatin. Such evidence is derived from cell biological and genetic experiments using specific individual gene-deleted knock out mice. How LDC induces the expression of TSP-1 in the tumor cells and also peri-vascular stromal cells is yet undetermined. It has been demonstrated that expression of TSP-1 is modulated by hypoxia, oncogenes (*ras*, *myc*, v-*src*, and c-*jun*), tumor suppressor proteins (p53 and PTEN), and transcription factors such as Id1 (24–29). In the future, it would be interesting to assess the impact of LDC on these pathways to facilitate potential up-regulation of TSP-1 protein in the tumor microenvironment. Nevertheless, it is clear from these studies that the contribution of TSP-1 from the endothelial cells is not critical for LDC-mediated antiangiogenic effect in the context of both LLC and B16F10 tumors used in our study. The expression levels of TSP-1 by tumor cells or perivascular stromal cells appear sufficient for inducing specific apoptosis of endothelial cells, and in this regard, it has been shown previously that CD36 expression on microvascular endothelial cells can mediate apoptosis of endothelial cells induced by TSP-1 (30).

Our results also suggest that in the clinic, it might be important to evaluate the protein levels of TSP-1 in the tumor cells using biopsy samples. In such cases in which tumor cells produce TSP-1, LDC would be expected to be more effective. This notion is important to consider in the clinical trials that are in progress at many centers in the world and for also those in the planning at the moment. Lastly, although we present evidence for TSP-1 in the mediation of antiangiogenic effect by LDC, it is likely that other endogenous factors might also be important that were not addressed here. It is quite possible that other chemotherapeutic agents at lower doses could work via distinct endogenous angiogenesis inhibitors.

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Thrombospondin-1 Associated with Tumor Microenvironment Contributes to Low-Dose Cyclophosphamide-Mediated Endothelial Cell Apoptosis and Tumor Growth Suppression

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