Novel antiangiogenic pathway of thrombospondin-1 mediated by suppression of the cell cycle

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We have recently reported that keratin 14-promoter-driven vascular endothelial growth factor (VEGF)-E_{NZ-7} transgenic mice have a significant number of capillary vessels in subcutaneous tissue. However, these vessels are generated in a layer some distance from the epithelial basal cells that express VEGF-E_{NZ-7}, suggesting that one or more antiangiogenenic molecules may exist very near the basal cell layer. By screening keratinocyte-conditioned medium, we found that thrombospondin-1 (TSP-1) is produced from keratinocytes and suppresses human umbilical vein endothelial cells (HUVEC) growth as well as tubular formation in a HUVEC–fibroblast coculture system. Different to the known mechanism of CD36-dependent endothelial cell apoptosis, the HUVEC we used did not express CD36 at detectable levels, indicating a new mechanism for TSP-1-induced antiangiogenesis. We found that TSP-1 induces little apoptosis of endothelial cells but causes cell-cycle arrest, increasing the amounts of $p21^{CIP/WAF-1}$ and unphosphorylated retinoblastoma (Rb) in HUVEC. CD36-binding peptide in TSP-1 and CD36-neutralizing antibody did not block the TSP-1-induced cell-cycle arrest. Our results strongly suggest that TSP-1 utilizes a novel pathway for its antiangiogenic effect independent of CD36, and suppresses the cell cycle. (*Cancer Sci* 2007; 98: 1491–1497)

The formation of new blood vessels (angiogenesis) is critical to both vascular development in embryogenesis and the progression of various diseases in adulthood. Under pathological conditions such as tumorigenesis and chronic inflammation, it is well established that the degree of angiogenesis correlates with the degree of malignancy. Based on its importance, angiogenesis has been considered an attractive target for the development of new therapeutic molecules.[1–3]

Vascular endothelial growth factor (VEGF) is a key regulator essential for many angiogenic processes in normal and abnormal states, and has been well characterized. The major biological roles of VEGF are in the proliferation, migration and tubular formation of vascular endothelial cells as well as the induction of microvascular permeability.

Currently, the VEGF family includes VEGF-A, placenta growth factor, VEGF-B, VEGF-C, VEGF-D, VEGF-E and snake venom (sv)VEGF. We have demonstrated that VEGF-E_{NZ-7}, a variant of VEGF-E, specifically binds and activates VEGFR-2. We also showed that keratin 14 (K14)-promoter-driven VEGF-E_{NZ-7} transgenic mice have a significant vascular network in the dermis with very few abnormal effects such as edema and hemorrhage.[4] Interestingly, however, these vascular networks were formed at a distance from the basal epidermal layer that expresses VEGF-E_{NZ-7}, suggesting an antiangiogenic molecule near the layer.

Several endogenous antiangiogenic factors have been described, including thrombospondin-1 (TSP-1), bone morphogenetic protein-4, soluble VEGFR-1 and angiostatin.[5–7] Thus, we hypothesized that keratinocytes, a major component of the basal epidermal layer, secrete a molecule with antiangiogenic properties.

In the present study, we found that keratinocytes secrete TSP-1 as an angiogenic inhibitor, using an *in vitro* angiogenesis assay and proliferation assay with human umbilical vein endothelial cells (HUVEC). Although the CD36-mediated apoptosis of endothelial cells is considered to be the major pathway for the antiangiogenic effect of TSP-1, our results indicate that TSP-1 has another important mechanism for antiangiogenesis whereby it induces endothelial growth arrest via upregulation of $p21^{CIP/WAF-1}$ expression, dephosphorylation of retinoblastoma (Rb), and phosphorylation of p53, in a CD36-independent manner.

Materials and Methods

Materials. Recombinant human $VEGF_{165}$ and hepatic growth factor (HGF) were purchased from R & D Systems (Minneapolis, MN, USA). Basic fibroblast growth factor (bFGF) was obtained from Oncogene Research Products (Cambridge, MA, USA). Angiopoietin-1 (Ang-1) was kindly provided by Dr D. G. Yancopoulos (Regeneration Pharmaceuticals, Tarrytown, NY, USA). Purified human TSP-1 was purchased from Calbiochem (Darmstadt, Germany).

Cell culture. HUVEC and human aortic endothelial cells (HAEC) were purchased from Kurabo (Osaka, Japan) and grown in HuMedia-EG2 medium (Kurabo). Human microvascular endothelial cell (HMVEC) were purchased from Kurabo and grown in HuMedia-MvG medium (Kurabo). Human epidermal keratinocytes derived from neonatal foreskin (HK cells) were purchased from Kurabo and grown in HuMedia-KG2 medium (Kurabo). Mouse keratinocytes (MK cells) were isolated from BALB/c nude mouse (SLC, Shizuoka, Japan) skin. Briefly, the skin samples were cut into pieces and incubated with a 0.25% trypsin solution (Invitrogen, Carlsbad, CA, USA) for 1–3 h at 37°C. After the epidermis was separated from the dermis, the epidermal sheets were dispersed into individual cells. The cells were collected by centrifugation and were cultured further in HuMedia-KG2 medium. NIH3T3-KDR[8,9] and NIH3T3-Flt-1 cells[10] were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan), supplemented with 10% calf serum (CS), 2 mM L-glutamine (Invitrogen), 40 μg/mL kanamycin (Wako, Tokyo, Japan), and 200 μg/mL G418 (Wako). Cells were cultured at 37°C with 5% CO_2.

Conditioned medium. Conditioned medium (CM) was collected from proliferating primary human keratinocytes (HK) or primary mouse keratinocytes (MK cells) after a 72-h culture in HuMedia-KG2 medium. The collected medium was centrifuged to remove any residual cells, filtered through a 0.22-μm pore size filter (Millipore Corp., Bedford, MA, USA) and frozen at –80°C.

Assay of angiogenesis *in vitro*. Angiogenesis *in vitro* was assessed as the formation of capillary-like structures in HUVEC cocultured with human fibroblasts. The experimental procedure followed the instructions provided with the angiogenesis kit (Kurabo). Briefly, cells were cultured with medium containing test substances at day 1 and the medium was replaced at days 4, 7 and 9. At day 11, the cells were fixed with paraformaldehyde and stained with the antihuman CD31/PECAM antibody or antihuman von

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Willebrand Factor (vWF) antibody. Tubule-like structures were analyzed under a bright-field microscope and the total length in a fixed area of five randomly chosen fields per well was determined by using Angiogenesis Image Analyzer Ver. 1.0.0 (Kurabo).

Mass spectrometry. Protein bands plus blank regions were excised from gels visualized with silver staining, and destained using a SilverQuest Silver Staining Kit (Invitrogen) according to the manufacturer's instructions. After destaining, samples were subjected to in-gel digestion with a trypsin solution overnight at 37°C. Supernatants were then collected; washed with 0.1% trifluoroacetic acid (TFA) (Wako); eluted with a 50% acetonitrile solution containing 0.1% TFA, and analyzed via liquid chromatography–matrix-assisted laser desorption ionization–time of flight–mass spectrometry (LC-MALDI-TOF-TOF-MS). Analysis of the resulting data was carried out using the Mascot search engine (http://www.matrixscience.com).

Gel chromatography. Concentrated CM was applied to a Superdex 200 HR 10/30 column (Amersham Biosciences Co., Piscataway, NJ, USA) equilibrated previously with 50 mM Tris (pH 7.5) and 10% glycerol, and eluted by using a fast protein liquid chromatography (FPLC) system (Amersham Biosciences Co.).

Western blotting. Cells were lysed in lysis buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM ethylene glycol bis(βaminoethyl ether)-N,N,N′,N′-tetra-acetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 U/mL aprotinin and 1 mM Na_3VO_4) or alternatively in sodium dodecylsulfate (SDS) sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol (DTT), 4% SDS, 0.2% bromophenol blue (BPB) and 20% glycerol). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore Corp.). The membrane was blotted with specific antibody and visualized with a chemiluminescence substrate (Amersham Pharmacia or PerkinElmer Life Science, Buckinghamshire, UK, and Boston, MA, USA, respectively).

Antibodies. The antibodies used were as follows: antihuman TSP-1/2 (H-300) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); antihuman $p21^{CIP/WAF-1}$ (Cip1) (Transduction Laboratories, San Jose, CA, USA); and antihuman $p27^{Kip}$ (p27 kip) (Upstate Biotechnology, Lake Placid, NY, USA). Anti-human p15, antihuman p16 ($p16^{Ink4}$), anti-cyclin D1 (M-20), anti-cyclin E (M-20), anti-CDK2 (M2-G) and anti-CDK4 (C-22) were from Santa Cruz, antiphospho-Rb (Ser780, Ser807/811) and antiphospho-p53 (Ser15) were from Cell Signaling Technology (Beverly, MA, USA), anti-actin and anti-p53 were from Chemicon International (Temecula, CA, USA), horseradish peroxidase (HRP)-conjugated antimouse/rat/rabbit immunoglobulin G (IgG) (Amersham Biosciences), HRP-conjugated antigoat IgG (Santa Cruz) and antiphosphotyrosine (PY20) (BD Biosciences, San Diego, CA, USA) were also used.

Anti-human VEGFR-2 (KDR), antiphospho-KDR (PY1175) and antihuman VEGFR-1 (Flt-1) were prepared previously.[10,11] Anti-phospholipase C (PLC)-γ was purchased from Santa Cruz. Anti-phospho-PLC-γ1 (Thr783), antiphospho-p44/42 mitogen-activated protein kinase (MAPK) (Thr202/Tyr204) and antip44/42 MAPK were purchased from Cell Signaling Technology. Anti-TSP-1 neutralizing antibody (Ab-1) was acquired from Laboratory Vision (Fremont, CA, USA). Anti-CD36 neutralizing antibody (FA6-152) was obtained from Immunotech (Marseille Cedex, France). Anti-transforming growth factor (TGF)-β neutralizing antibody was purchased from Abcam (Cambridge, UK).

Cell growth assay. Cells were seeded on 96-well collagen-coated plates. approximately 6 h later, the medium was replaced with growth medium containing materials to be tested, and the cells were incubated at 37°C for 72 h. Cell numbers were determined by using a Cell Titer 96 (Promega, Madison, WI, USA) according to the manufacturer's directions. When the fractions separated by FPLC were used, each fraction was concentrated 10-fold, and used at a 5% concentration in the HuMedia-EG2 medium. To examine the effect of a caspase inhibitor, Z-VAD-FMK (Calbiochem), HUVEC were seeded on 96-well collagen-coated plates and incubated at 37°C for 6 h. The medium was then replaced with HuMedia-EG2 containing 50 μM Z-VAD-FMK or dimethylsulfoxide (DMSO; final concentration 5 mM). After HUVEC were treated with Z-VAD-FMK at 37°C for 2 h, the medium was replaced with HuMedia-EG2 containing 25 nM TSP-1, and incubated at 37°C for 72 h.

Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling assay. Apoptotic cells were detected using a terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) kit (Roche Diagnostics, Mannheim, Germany). To assess the nuclear morphology of the cells, the samples were stained with Hoechst 33258 (Wako). The samples were then observed under a fluorescence microscope and photographed.

Immunohistochemistry. To examine the ratio of Ki67-positive cells, HUVEC were seeded on eight-well collagen-coated culture slides. After 6 h the medium was replaced with HuMedia-EG2 containing TSP-1, and the cells were incubated at 37°C for 72 h. The cells were then fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.1% (v/v) Triton X-100, and treated with 5% bovine serum albumin in PBS. They were incubated with antihuman Ki-67 antibody (Ab) (DAKO A/S, Glostrup, Denmark) overnight at 4°C, and further with an antirabbit rhodamine-conjugated goat IgG fraction (ICN Biochemicals, Costa Mesa, CA, USA). To assess the nuclear morphology of the cells, the samples were stained with Hoechst 33258. The samples were then observed under a fluorescence microscope and photographed.

Flow cytometry. To analyze the cell cycle, HUVEC were seeded on collagen-coated culture dishes and incubated at 37°C for 6 h. The medium was replaced with HuMedia-EG2 containing 25 nM TSP-1, and the cells were incubated at 37°C for 72 h. The cells were trypsinized, collected and fixed with 70% ethanol for 2 h on ice. After fixation, the cells were treated with 0.25 mg/mL RNase (Wako) at 37°C for 15 min and the nuclei were stained by incubation in 50 μg/mL propidium iodide (PI) (Wako) at 4°C for 30 min. They were analyzed using FACS Aria (Becton Dickinson, Franklin Lakes, NJ, USA). Results were analyzed using FLOWJO software (Tree Star, Ashland, OR, USA).

To assess the expression level of CD36 on endothelial cell (EC), cells were trypsinized, collected and treated with IgG-purified immunoglobulin (Sigma-Aldrich, St Louis, MO, USA) at 4°C for 10 min. The cells were incubated with antihuman fluoroscein-5-isothiocyanate (FITC)-conjugated CD36 antibody (BD Biosciences) or FITC-conjugated mouse IgMκ (BD Biosciences) as an isotype control at 4°C for 30 min, washed with PBS, and further fixed with a 0.5% formaldehyde solution. The nuclei were stained with PI and each sample was applied to FACS Aria. Results were analyzed using FLOWJO software.

Results

TSP-1 is a major antiangiogenic factor secreted from keratinocytes. To examine the possible antiangiogenic factors near the basal epidermal layer in the skin of K14-VEGF-E_{NZ-7} transgenic mice, we tested whether the CM of keratinocytes had antiangiogenic activity or not. An antiangiogenic effect was clearly detected in both HK CM and MK CM (Fig. 1A, and Supplementary Fig. 1A) by using a tubular formation assay in cocultures of HUVEC and human fibroblasts that mimic angiogenesis *in vivo*.[12] To reveal which factor has antiangiogenic activity, we tested the angiogenesis-inhibitory activity of each fraction of HK CM separated by gel chromatography. As shown in Fig. 1B, fractions 9 and 10 inhibited both the tubular formation and growth of HUVEC. Next, we attempted to identify the antiangiogenic factors in these fractions. The specific protein

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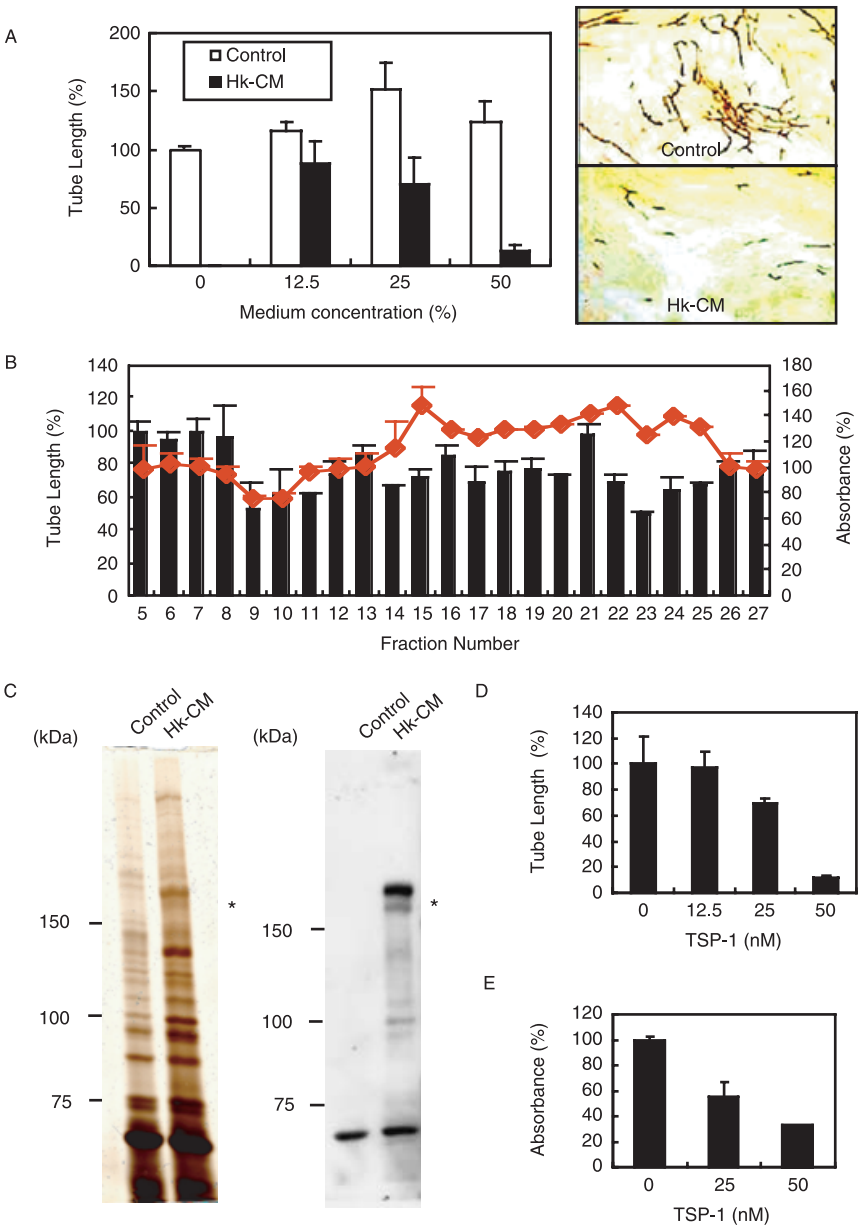

Fig. 1. A major antiangiogenic factor secreted from keratinocytes is thrombospondin-1 (TSP-1). (A) Human keratinocyte (HK) conditioned medium (CM) inhibits tubular formation of human umbilical vein endothelial cells (HUVEC) in a HUVEC–fibroblast coculture system. The results in the left panel indicate tube length with or without HK CM. The right panel shows representative tubular structures with (50%) or without HK CM. (B) Fractions 9 and 10 from the gel chromatography inhibit the proliferation and tubular formation of HUVEC. (C) Silver-stained sodium dodecylsulfate–polyacrylamide gel electrophoresis for concentrated HK CM and control medium. The TSP-1-specific band indicated with an asterisk at 180 kDa was detected by western blotting using anti-TSP-1 antibody (right panel). (D,E) TSP-1 inhibits the tubular formation (D) and proliferation of HUVEC (E) in a dose-dependent manner. Results represent the mean ± SD from several independent experiments.

band at ~180 kDa in HK CM (Fig. 1C left panel) was analyzed by LC-MALDI-TOF-TOF-MS and found to consist of TSP-1. We confirmed the presence of TSP-1 at the 180-kDa position in HK CM by western blotting using anti-TSP-1 Ab (Fig. 1C right panel). As expected, TSP-1 bands were clearly detected in fractions 9 and 10 (Supplementary Fig. 1B). Furthermore, TSP-1 inhibited the tubular formation and proliferation of HUVEC (Fig. 1D,E) *in vitro* in a dose-dependent manner. However, TSP-1 did not interfere with the proliferation of human fibroblasts (data not shown). These results indicate that keratinocytes secrete TSP-1 as a major antiangiogenic factor.

TSP-1 inhibits angiogenesis in a CD36-independent manner. TSP-1 is an antiangiogenic molecule mainly acting through CD36, one of the TSP-1 receptors, on endothelial cells.[13–18] However, several reports have described that HUVEC do not express a high level of CD36.[15,19] Thus, we examined whether the HUVEC used in the present study expressed CD36 or not by flow cytometry. As shown in Fig. 2A, CD36 was undetectable on these HUVEC.

Next, we tested whether a TSP-1-derived peptide corresponding to the CD36-binding site, Val-Thr-Cys-Gly (VTCG),[16,20] could inhibit angiogenesis *in vitro* using HUVEC. Although a synthetic peptide (peptide A: SPWSSCSVTCGDGVITRIR), which included the VTCG sequence, inhibited the growth of HMVEC expressing CD36 (data not shown), this peptide did not inhibit either the tubular formation or growth of HUVEC (Fig. 2B,C). Furthermore, anti-TSP-1 neutralizing antibody (Ab-1), which blocks the binding of TSP-1 to CD36, and anti-CD36 neutralizing antibody did not suppress the inhibitory effect of TSP-1 on either proliferation or tubular formation (Fig. 2B,C). In addition, phosphorylation of p38 and JNK, which are mediated by activation of a CD36 pathway, are not induced by TSP-1 in HUVEC (Fig. 2D). These results strongly suggest that the antiangiogenic effects of TSP-1 in these assays occurred independently of CD36.

TSP-1 induces growth arrest in HUVEC. To investigate the mechanism of CD36-independent antiangiogenesis by TSP-1, we examined whether TSP-1 blocks a certain type of angiogenic

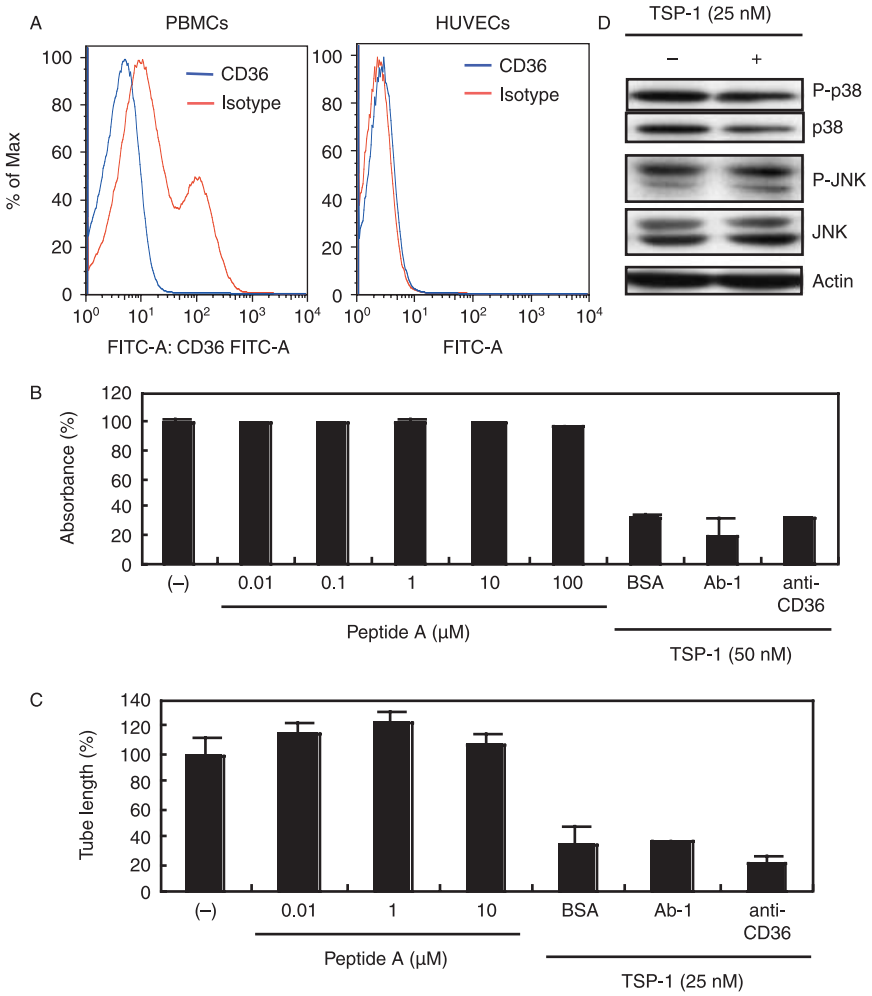

Fig. 2. Thrombospondin-1 (TSP-1) inhibits angiogenesis in a CD36-independent manner. (A) Expression of CD36 is undetectable on human umbilical vein endothelial cells (HUVEC) using flow cytometry. Peripheral blood mononuclear cells (PBMC): a positive control collected from human peripheral blood using Ficoll-Paque plus. (B,C) A synthetic peptide (peptide A), which blocks CD36–TSP-1 binding, does not inhibit the tubular formation and proliferation of HUVEC. A neutralizing antibody against TSP-1 named Ab-1, which binds to the CD36 recognition site on TSP-1, and CD36 neutralizing antibody do not rescue the inhibition of HUVEC proliferation by TSP-1. TSP-1 and Ab-1 (50 μg/mL) were preincubated for 1 h before use. Anti-CD36 antibody (10 μg/mL) was added 1 h before the addition of TSP-1. Results represent the mean ± SD of several independent experiments. (D) Phosphorylation of either p38 or JNK was not detected in HUVEC treated with TSP-1 using western blotting. The HUVEC sample was obtained 30 min after addition of TSP-1.

factor such as VEGF. However, TSP-1 almost equally inhibited angiogenesis induced by VEGF, bFGF, HGF and Ang-1 (Supplementary Fig. 2A). Moreover, no obvious inhibitory effect of TSP-1 was detected on the VEGF–VEGFR signaling pathway (Supplementary Fig. 2B). These results suggest that the antiangiogenic activity of TSP-1 is not caused by inhibition of a specific angiogenic ligand and receptor.

Next, we examined whether this antiangiogenic effect was caused by induction of apoptosis or to other mechanisms. The results showed that apoptosis was not significantly induced by TSP-1 in a TUNEL assay, annexin V staining, and a matrigel assay (Supplementary Fig. 3A,D,E). Also, Z-VAD-FMK, which inhibits all caspases, only weakly suppressed the antiangiogenic effect of TSP-1 (Supplementary Fig. 3C). Furthermore, addition of TSP-1 at earlier time points suppressed angiogenesis more effectively than at middle or late stages without severe apoptosis in the tubular formation assay (Supplementary Fig. 3B and data not shown), suggesting that the major antiangiogenic effect of TSP-1 is not mediated by apoptosis.

Next, we examined whether the inhibitory effect of TSP-1 on HUVEC was caused by cell-cycle arrest or not. As shown in Fig. 3A, the proportion of Ki67-positive proliferating cells was decreased markedly among TSP-1-treated HUVEC. In addition, cell-cycle analysis by flow cytometry revealed the percentage of G_2/M-phase and S-phase cells to have decreased, and that of G_0/G_1-phase cells to have increased in the TSP-1-treated HUVEC population compared with the control cells (Fig. 3B). These results indicate that TSP-1 induces growth arrest in HUVEC.

TSP-1 upregulates $p21^{CIP/WAF-1}$ and phosphorylation of p53 in HUVEC. To understand the molecular basis of the TSP-1-induced growth arrest in HUVEC, we examined the expression and phosphorylation levels of several cell-cycle regulators in HUVEC with or without TSP-1. As shown in Fig. 4A, the expression of $p21^{CIP/WAF-1}$, a major cyclin-dependent kinase inhibitor (CDKI), was strongly induced by TSP-1. However, the level of phosphorylated Rb was decreased in TSP-1-treated HUVEC. These results strongly suggest that TSP-1 induces growth arrest of HUVEC by upregulating the expression of $p21^{CIP/WAF-1}$, which inhibits the phosphorylation of Rb.

To understand the molecular basis underlying the TSP-1-induced cell-cycle arrest in HUVEC, we examined the levels of phosphorylated p53 using western blotting. Phosphorylation of p53 was increased approximately four-fold by TSP-1 (Fig. 4B). These results suggest that TSP-1 induces cell-cycle arrest through upregulation of $p21^{CIP/WAF-1}$ expression mediated by p53.

Discussion

In the present study, we found that keratinocytes secrete TSP-1, and the TSP-1 suppresses the proliferation and tubular formation of HUVEC *in vitro*. Very little, if any, CD36-dependent apoptosis was detected under these conditions. Cell-cycle arrest of HUVEC accompanies an upregulation of $p21^{CIP/WAF-1}$ expression, phosphorylation of p53 and dephosphorylation of Rb (Fig. 5).

Using immunostaining, Yano *et al*. found that TSP-1 is expressed in the epidermal layer, including basal cells.[21] Our

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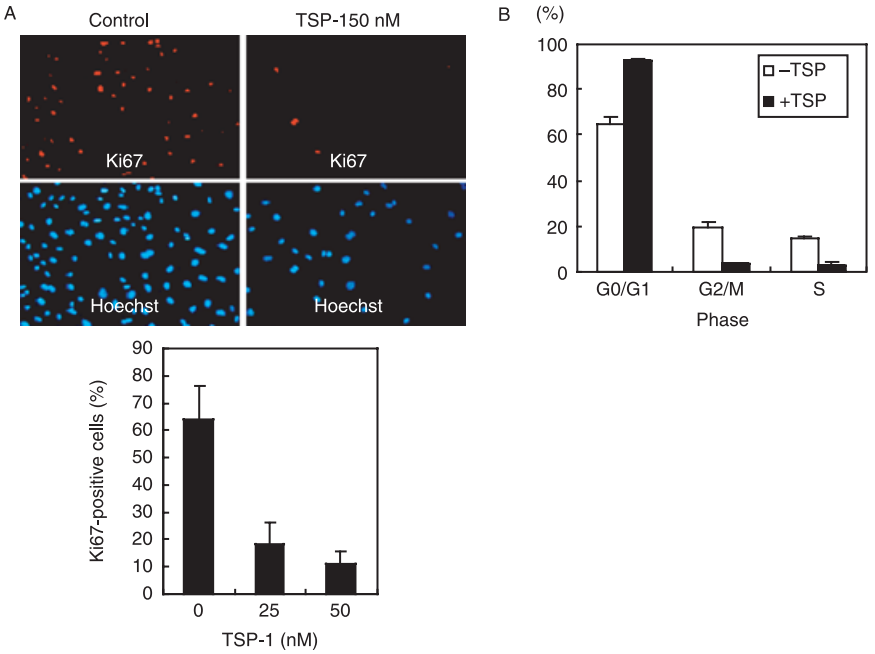

Fig. 3. Thrombospondin-1 (TSP-1) induces growth arrest in human umbilical vein endothelial cells (HUVEC). (A) Ki-67-positive proliferating cells dramatically decreased in number among TSP-1-treated HUVEC. Representative results for the TSP-1-treated HUVEC are shown in the upper panels. Hoechst 33258 was used for nuclear staining. The graph below shows the percentage of Ki-67-positive cells. (B) TSP-1 impairs S-phase progression in HUVEC. Cell-cycle analysis by flow cytometry was carried out using HUVEC treated with TSP-1 (25 nM) for 72 h. The results indicate a quantified ratio of cells at each phase as the mean percentage ± SE from several independent experiments.

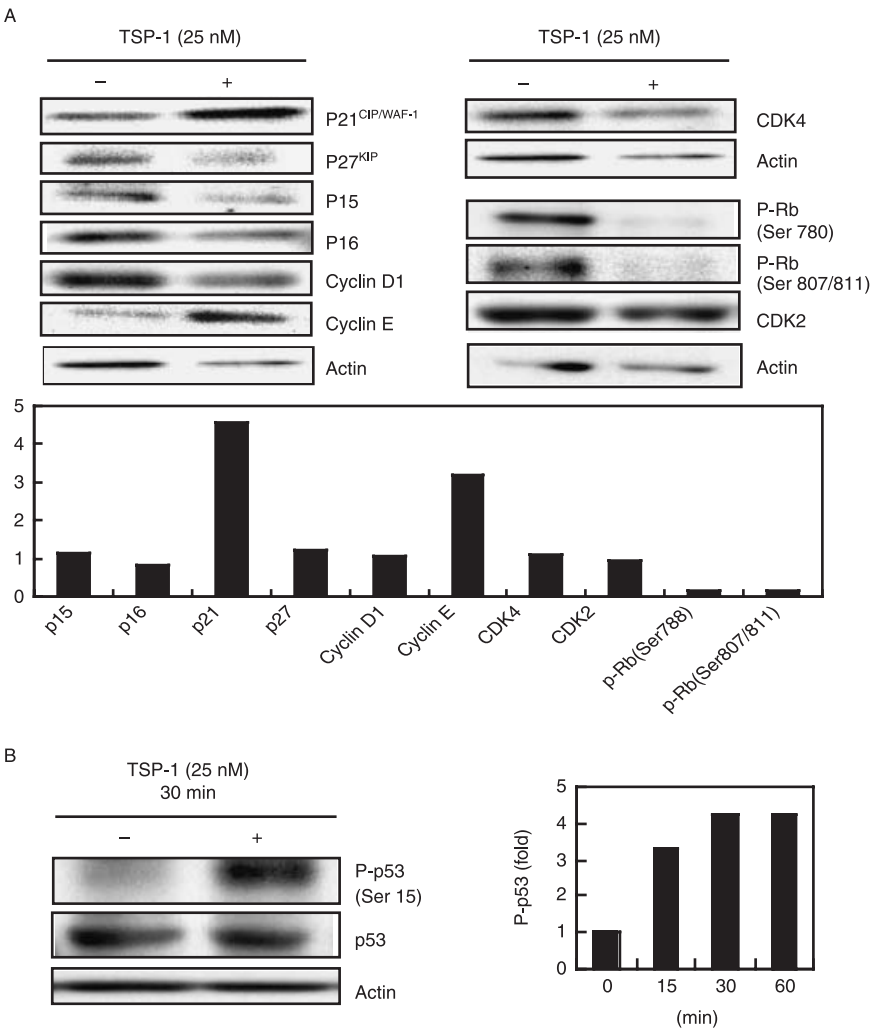

Fig. 4. Upregulation of $p21^{Cip1/WAF-1}$ expression, phosphorylation of p53, and dephosphorylation of retinoblastoma (Rb) in human umbilical vein endothelial cells (HUVEC) treated with thrombospondin-1 (TSP-1). (A) HUVEC were treated with or without TSP-1 (25 nM) at 37°C for 24 h. Cell lysates were used directly for sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by western blotting using the specific antibodies indicated at the right of the figure. The graph below shows quantified results obtained using NIH image 1.6 software. (B) HUVEC were treated with or without TSP-1 (25 nM) at 37°C for 15, 30 and 60 min. Cell lysates were separated by SDS-PAGE, and analyzed by western blotting using the antibodies indicated at the right of the figure. Representative results are shown in the left panels. The graph shows quantified results obtained using NIH image 1.6 software.

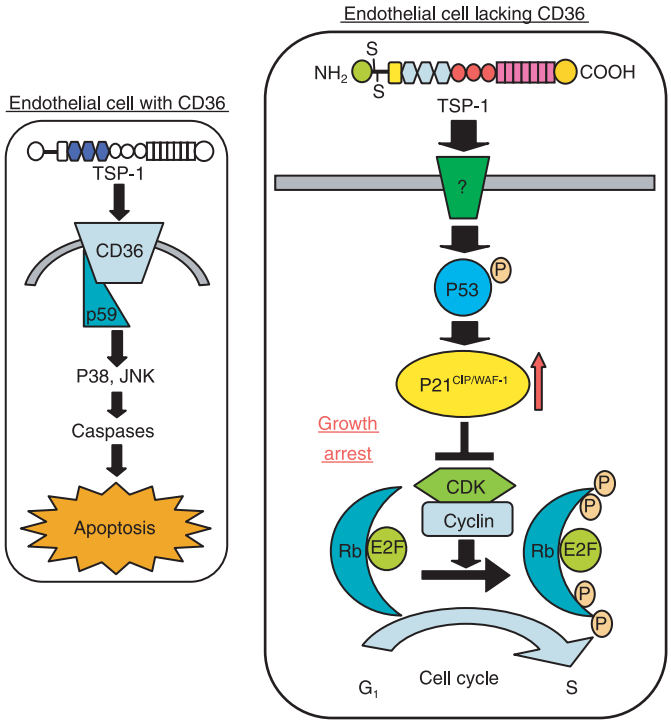

Fig. 5. Schematic model of the effects of thrombospondin-1 (TSP-1) on endothelial cells dependent on or independent of CD36. TSP-1 is reported to bind CD36, activate caspases through p38 and JNK, and induce apoptosis in endothelial cells expressing CD36 (left). However, our results suggest that TSP-1 induces endothelial cell growth arrest mediated by an upregulation of $p21^{CIP/WAF-1}$ expression, phosphorylation of p53, and dephosphorylation of Rb in a CD36-independent manner (right).

results showed a similar staining pattern both in wild-type and VEGF-E_{NZ-7} transgenic mice (data not shown). The structure of the epithelial layer as well as basal cells in K14-promoter-driven VEGF-E_{NZ-7} transgenic mice was well maintained compared with those in the wild-type mice.[4] We therefore suggest that the level of TSP-1 in the keratinocytes of the transgenic mice was not significantly different from that in the wild-type mice.

Previously, Streit *et al.*[22] reported that K14-promoter-driven overexpression of TSP-1 in keratinocytes suppresses wound healing and granulation tissue formation. Thus, exogenous TSP-1 in the skin functions as an angiogenic inhibitor in such a pathological condition. Consistent with their results, our results suggest that endogenous TSP-1 could also suppress angiogenesis and make a thin avascular area below the epidermal basal cell layer.

A major question is whether the cell-cycle arrest of HUVEC with TSP-1 was totally unrelated to CD36. In the blood coagulation system, TSP-1 interacts with CD36, which is expressed in monocytes, macrophages and other types of cells and regulates coagulation, thrombosis and inflammation. We confirmed that CD36 is well expressed in peripheral blood mononuclear cells. However, using the same procedure, we could not detect the expression of CD36 in HUVEC or HAEC, which showed cell-cycle arrest similar to HUVEC in the presence of TSP-1 (Fig. 2A and Supplementary Fig. 5). In addition, neither anti-TSP-1 neutralizing Ab (Ab-1), which blocks the binding of TSP-1 to CD36, nor anti-CD36 neutralizing Ab suppressed the antiangiogenic effects of TSP-1 on HUVEC. Moreover, phosphorylation of either p38 or JNK was not induced by TSP-1, different from CD36-expressing endothelial cells. It might be that *in vivo*, capillary endothelial cells upregulate CD36 expression and this CD36 facilitates the antiangiogenic effect of TSP-1. Although this possibility cannot be completely ruled out at the moment, we did not observe a strong apoptotic response in the skin of K14-driven VEGF-E_{NZ-7} transgenic mice. $CD36^{-/-}$ mice were generated by Febbraio *et al.* but did not show a clear increase in capillary density in subcutaneous tissues,[16,23] suggesting that the CD36-dependent pathway is not a major regulator of the vascular network in the skin. The expression of CD36 in vascular endothelial cells of tumor tissues has been reported by several research groups.[24–26] Because the levels of CD36 in vascular endothelial cells in the pathological angiogenesis is not even (i.e. some endothelial cells appear positive but others are very low or undetectable), we suggest that both mechanisms, CD36-dependent apoptosis and CD36-independent cell-cycle arrest, may exist in the tumor tissues treated with TSP-1.

Tumor cells derived from Li Fraumeni syndrome in humans have been reported to have decreased TSP-1 levels.[27] Although this correlation is not seen consistently in all human tumors, wild-type p53 appears to increase TSP-1 expression in some tumor types, contributing to the suppression of tumor growth. Our results indicate that another type of relationship between p53 and TSP-1 may exist in vascular endothelial cells, where TSP-1 upregulates the p53–p21 pathway. Thus, we suggest that both directions between p53 and TSP-1 are present *in vivo*, depending on the cell type.

Also of interest are the characteristics of the putative TSP-1 receptor other than CD36 on HUVEC. We showed that TSP-1 did not block a specific angiogenic ligand such as VEGF, bFGF, HGF or Ang-1. Therefore, this putative receptor may not be associated with a specific receptor such as VEGFR-2 (Supplementary Fig. 2). Approximately 50% inhibition of HUVEC growth and tubular formation was achieved with 20–50 nM TSP-1. We therefore suggest that the interaction between TSP-1 and the putative receptor on endothelial cells is rather weak, not like that of the growth factor–receptor interaction for which the 50% inhibitory concentration is in the order of 10–100 pM.

It is of interest to know which region of TSP-1 plays an important role in inducing cell-cycle arrest of HUVEC. The type-I repeat domain of TSP-1 was reported to be important for antiangiogenic activity.[28,29] This domain includes the CD36-binding region. However, as we have shown in Fig. 2B,C, a CD36-binding peptide (peptide A) did not inhibit the growth or tubular formation of HUVEC. Another peptide within the type-I repeat, GGWSHWSPWSS, was also reported to inhibit angiogenesis.[20] Studies by Crawford *et al.*[14] and Murphy-Ullrich and Poczatek[30] reported that the KRFK sequence activates TGF-β1. However, the proliferation and tubular formation of HUVEC was not inhibited by either a synthetic peptide (peptide B: KRFKQGGWSHWSPWSSC), which includes the above two sequences, or TGF-β1-neutralizing Ab (Supplementary Fig. 4A,B). Furthermore, a mixture of peptides A and B did not inhibit the tubular formation or proliferation either (Supplementary Fig. 4C and data not shown). No obvious competitive or additive effect was detected using this mixture of peptides in the presence of TSP-1 (Supplementary Fig. 4D,E). Also, our preliminary results suggest that the interaction between CD47 and TSP-1 and that between integrin β1 and TSP-1 do not contribute to the suppressive effect as no obvious effect was observed in the presence of anti-CD47 or anti-integrin β1-blocking antibody (data not shown). These results suggest that TSP-1 inhibits angiogenesis caused by TSP-1 in the HUVEC system, independent of known sequences.

Taken together, our results indicate that TSP-1 induces a unique response in certain types of vascular endothelial cells, blocks the cell cycle, and suppresses angiogenesis *in vivo*.

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