Differential regulation of thrombospondin-1 expression and antiangiogenesis of ECV304 cells by trichostatin A and helixor A

Susie Hong, Seo-Yoon Chang, Dong-Hoon Yeom, Jung-Hoon Kang and Kyong-Ja Hong

Trichostatin A and helixor A increased thrombospondin-1 expression by ECV304 cells at both mRNA and protein levels by transcriptional activation through the enhancement of *tsp-1* promoter activity. The induction of thrombospondin-1 by these agents potently reduced ECV 304 cell migration and capillary-like tube formation on Matrigel; these findings were confirmed by the neutralization of thrombospondin-1 using a specific antibody. In the presence of exogenous vascular endothelial growth factor, however, these agents had a different effect on the vascular endothelial growth factor-induced tube formation; trichostatin A remarkably inhibited tube formation regardless of the presence of exogenous vascular endothelial growth factor, whereas helixor A reduced it to 70–80% of the control level. Interestingly, when the helixor A-generated conditioned media were concentrated three-fold and the endogenous vascular endothelial growth factor was removed, tube formation was remarkably inhibited compared with the effect of three-fold concentrated conditioned media that had endogenous vascular endothelial growth factor. Additionally, in media with endogenous vascular endothelial growth factor that were concentrated five-fold, tube formation was markedly blocked regardless of the presence of exogenous or endogenous vascular endothelial growth factor. Thus, our results indicate that trichostatin A-induced or helixor A-induced antiangiogenesis is mediated by both agents; increased, absolute and relative levels of thrombospondin-1 to the vascular endothelial growth factor level are critical in angiogenesis.
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Introduction

An increase in neovascularization not only increases the supply of nutrition and oxygen for rapid tumor growth, but also provides a means for the tumor to migrate to distant sites through the circulation. Thus, it has been recognized that solid-tumor proliferation and metastases are dependent on angiogenesis. Angiogenic processes are tightly regulated by the local balance between inhibitory and stimulatory factors. Thrombospondin-1 (TSP-1) is recognized as an inhibitory factor; its inhibitory pattern of expression is inverse to the increased expression pattern of stimulatory factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [1–3]. Recently, it has been reported that TSP-1 plays a biphasic role in angiogenesis; it acts both as a proangiogenic factor at lower concentration levels (1–10 μg/ml) and as an antiangiogenic factor at higher concentration levels (> 15 μg/ml) [4–7]. Thus, TSP-1 expression must be constantly maintained at higher concentration levels to suppress growth and spread of tumors. Currently, it is known that the TSP-1 level is cell-specifically regulated by extracellular stimuli and pathological state [8–15]. In addition to the cell-type regulation and stimulus-specific regulation, the expression of TSP-1 is dependent on the epigenetic modification; persistent maintenance in histone acetylation upregulates TSP-1 expression, but methylation on the *tsp-1* promoter downregulates TSP-1 expression [16–18].

A specific histone deacetylase inhibitor, trichostatin A (TSA), negatively regulates the VEGF level, VEGF-mediated signaling and matrix metalloproteinase-2 activation; these result in the inhibition of tumor growth and neovacularization [19–21]. No reports, however, dealing with the effects of TSA on the expression of negative regulators such as TSP-1 in angiogenesis have been published.

Helixor A (HA) is a complex therapeutic agent that contains lectins, viscotoxins and alkaloids. It is an

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aqueous cold extract derived from fresh European mistletoe (*Viscum album abietis*) that is grown on the host tree tannenbaum; its production has been standardized and is produced by Helixor Heilmittel (Rosenfeld, Germany) [22,23]. HAs are given in combination with traditional chemotherapeutic drugs to improve the tolerances and toxicities of the regimens, as palliative therapy to improve quality of life, and as an adjuvant therapy for preventing relapse [22,23]. Interestingly, recent reports have shown that mistletoe reduced tumor growth and metastasis through apoptosis and antiangiogenesis [24–27]. Its precise mechanism of action, however, has not been clarified; other than enhancing natural killer (NK) cell activity, it is not known which mediators are involved in HA-induced apoptosis and antiangiogenesis [26,28–31].

In this study, the effects of both therapeutic agents, TSA and HA, on TSP-1 expression, its levels, and the resulting modulation of cell migration and angiogenesis were assessed. Both agents elevated TSP-1 expression at both the mRNA and protein levels by transcriptional activation. The presence of TSP-1 inhibited cell migration, and the capillary-like tube formation of ECV304 cells was assessed and clearly confirmed by neutralization of TSP-1 by a specific antibody. Additionally, in the presence of exogenous VEGF, HA reduced VEGF-induced tube formation to a lesser extent, compared with the complete inhibition seen with TSA. Compared with the three-fold concentrated conditioned media ($3 \times$ CM) in which endogenous VEGF remained, the removal of endogenous resulted in a greater reduction of tube formation. In contrast, when the absolute TSP-1 level was changed without changing its level relative to the VEGF level and HA was introduced to CM that was five-fold concentrated, but from which VEGF had not been removed, there was complete inhibition of tube formation, regardless of whether endogenous or exogenous VEGF was present. Our data showed that TSA elevated TSP-1 level sufficiently to block VEGF-induced angiogenesis, whereas HA increased the TSP-1 level to just threshold levels and thus could not tip the balance towards antiangiogenesis because of the resulting small change in the VEGF level.

Materials and methods

Cell stimulation, cell viability and protein concentration assay

ECV304 cells, a human endothelial cell line, were plated and cultured at an initial density from 1×10^5 to 2×10^5 cells/ml on 6-well, 12-well and 48-well culture plates with 2, 1 and 0.2 ml Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, New York, USA) supplemented with 10% heat-inactivated fetal bovine serum and gentamycine (50 μg/ml) at 37°C in 5% CO_2. After 24 h, the cells were washed twice with calcium/magnesium-free phosphate-buffered saline (pH 7.2) and further grown with serum-free DMEM medium in the presence of TSA (10–500 ng/ml; Sigma, St Louis, Missouri, USA) or HA (5–500 ng/ml; donated by Boryong, Seoul, South Korea) at 37°C for the time periods indicated. As TSA and HA were dissolved with dimethylsulfoxide (DMSO), the control group consisted of unstimulated cells that were also cultured in the DMEM-containing DMSO in the absence of TSA or HA. Culture supernatants were sequentially harvested and stored at −70°C until use. When necessary, the harvested culture supernatants were concentrated as described previously by Kim *et al.* [14]. Total protein concentrations in the culture supernatants were routinely estimated using the Coomasie protein assay reagent (Pierce, Rockford, Illinois, USA), with bovine serum albumin as the standard.

Cell viability was determined by the MTT assay, and expressed as the mean ± SD based on duplicate tests.

Northern blot analysis

About 10–15 μg of total cellular RNA extracted by STAT-60 (Tel-Test, Friendwool, Texas, USA) was electrophoresed in 1% agarose formaldehyde gel and transferred in $20 \times$ SSC onto nylon membranes (Schleicher & Shuell, Dassel, Germany). The blotted RNAs were ultraviolet cross-linked by Stratalinker (Stratagene, La Jolla, California, USA) and hybridized with digoxigenin-11-dUTP-labeled human TSP-1 cDNA (Boehringer Manheim, Germany). The hybridized membrane was developed using the digoxigenin chemiluminiscent detection kit (Boehringer Manheim). The cDNA probe of human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also prepared as the internal control.

Western blot analysis

An aliquot (20 μg/lane) obtained from the culture supernatants was electrophoresed and transferred to the nitrocellulose membrane (Schlicher & Schuell) by the routine method. The separated bands were visualized using anti-TSP-1 monoclonal mouse antibody (Neomarkers, Fremont, California, USA) or anti-VEGF monoclonal mouse antibody (Neomarkers) using the enhanced chemiluminiscent kit (Amersham Biosciences, Arlington Height, Illinois, USA). As TSP-1 is a well-known extracellular protein, the TSP-1 identified by Western blot was the secreted form of TSP-1 found in the extracellular media. Hence, the total protein concentration in the culture supernatant was determined using the Coomassie protein assay and equal protein amounts were applied to each lane.

Construction of the reporter plasmid bearing the *tsp-1* promoter

The human *tsp-1* promoter containing 2954 bp from the −2200 to + 754 region was prepared and amplified using a previously described method [15].

Transient transfection and luciferase activity assay

ECV304 cells (2×10^5 cells/ml) in 0.4 ml Opti-MEM (Gibco BRL) were seeded into a 12-well plate and transiently transfected with 1 or 2 μg of the reporter plasmid at 37°C for 4 h. They were incubated with Plus reagent (Invitrogen, Carisbad, California, USA) at room temperature for 15 min; in addition, the cells were mixed with Opti-MEM Lipofectamine (Invitrogen) at room temperature for 30 min. One milliliter of the medium containing fetal bovine serum was added into each well and the cells were cultured overnight at 37°C. These cells were stimulated with TSA (100 ng/ml) or HA (100 ng/ml) for 24 h and lysed with 100 μl of reporter lysis buffer (Promega; Madison, Wisconsin, USA). Luciferase activity was measured using a TD-20/20 Luminometer (Turner Design, Sunnyvale, California, USA). The luciferase activities were expressed as the mean ± SD from three separate experiments. Moreover, to check the transfection efficiency, the luciferase activities were normalized using the protein concentration.

Wounded cell migration assay

Cells wounded by scraping were washed twice with phosphate-buffered saline and incubated in media supplemented with TSA (100 ng/ml) or HA (50–100 ng/ml). To confirm whether the effects induced by these agents were mediated by agent-enhanced TSP-1 and exogenous TSP-1 (15–25 μg/ml), neutralization test was carried out using TSP-1 antiserum (1 : 1000 dilution, Ab-9; NeoMarkers). Anti-IgG mouse antibody (NeoMarkers) was also used to evaluate the specific neutralization caused by anti-TSP-1 antibody. After 24 h of cell migration, the results were photographed at a × 40 magnification.

Capillary-like tube formation

Trypsinized cells (5×10^5 cells/ml) were placed on the Matrigel-coated 48-well plates containing serum-free DMEM and grown for 6–24 h. Although HA-stimulated ECV 304 cell secreted higher levels of TSP-1 than unstimulated cells, the HA-induced TSP-1 level was lower than that induced by TSA, resulting in a lower level of antiangiogenesis with HA. To investigate definitively the tube formation induced by HA, the media harvested at the indicated times were concentrated three-fold (designated as 3 × CM) and then added to cells. 3 × CM – enVEGF was prepared using immunological precipitation by adding a sufficient amount of specific VEGF antibody (Ab-4, 1 : 500 dilution; NeoMarkers). Moreover, to investigate the effect on tube formation of the absolute TSP-1 level in the HA-induced CM containing endogenous VEGF, HA-generated CM was concentrated five-fold without removing endogenous VEGF (designated as 5 × CM). To neutralize the effect of TSP-1 on capillary-like tube formation, specific TSP-1 and anti-IgG antisera were pretreated as described for the cell migration experiment. The resulting morphologic changes were photographed at a × 40 magnification.

Statistical analysis

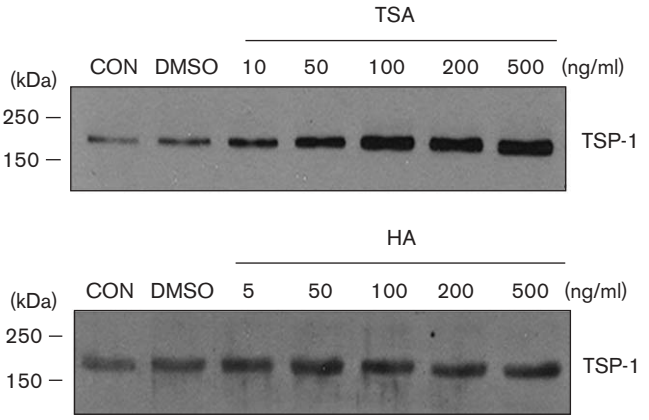
Data are expressed as mean ± SD obtained from duplicate or triplicate experiments. Statistical significance was evaluated using the unpaired Student's *t*-test; $P < 0.05$ was accepted as indicating significance.

Results

The increase in the thrombospondin-1 protein level depends on the trichostatin A or helixor A concentration

To check the effective concentrations of TSA and HA in TSP-1 synthesis, ECV304 cells were exposed to various concentrations of these agents and the TSP-1 protein level was determined by Western blot analysis (Fig. 1). With increasing concentrations, TSA gradually increased the TSP-1 protein level reaching a maximum at 100 ng/ml. TSA at 500 ng/ml maintained the TSP-1 level at the same level as TSA at 5–100 ng/ml. At a concentration of 5 ng/ml, HA increased the TSP-1 protein level; the maximum TSP-1 protein level was attained with an HA concentration of 50–100 ng/ml. The increase in TSP-1 protein level was, however, not large between low-dose (5 ng/ml) and high-dose (50–100 ng/ml) HA; with HA over 200 ng/ml, the TSP-1 protein level decreased slightly and then was consistently maintained up to an HA

Fig. 1



The increase in the TSP-1 protein level depended on the dose of TSA or HA to which the ECV304 cells were exposed. About 2×10^5 cells were cultured in serum-supplemented media for 24 h and then further cultured in the serum-free media containing TSA (10–500 ng/ml) or HA (5–500 ng/ml) for 24 h. Subsequently, culture supernatants were harvested and Western blot analysis was performed with monoclonal TSP-1 antibody diluted at 1 : 1000. The migrated TSP-1 was visualized by an enhanced chemiluminiscent detection kit. CON, control in the absence of DMSO; DMSO, control in the presence of DMSO. As TSP-1 is an extracellular protein, it is difficult to determine the specific protein to be used as the appropriate internal control. The total protein concentration in the culture supernatant was, therefore, quantified and an equal amount of protein was applied to each lane. DMSO, dimethylsulfoxide; HA, helixor A; TSA, trichostatin A; TSP-1, thrombospondin-1.

concentration of 500 ng/ml. Thus, further experiments were conducted using a TSA dose of 100 ng/ml and an HA dose of 50–100 ng/ml. Compared with control, TSA markedly upregulated the TSP-1 protein level (maximum approximately 11-fold), whereas HA only weakly upregulated the TSP-1 protein level (maximum approximately three-fold). DMSO increased the TSP-1 level only a negligible amount. For cell viability, wounded cell migration and tube formation assays, therefore, DMSO was added to the control cells to ensure that they were exposed to the same conditions as the HA-stimulated and TSA-stimulated cells.

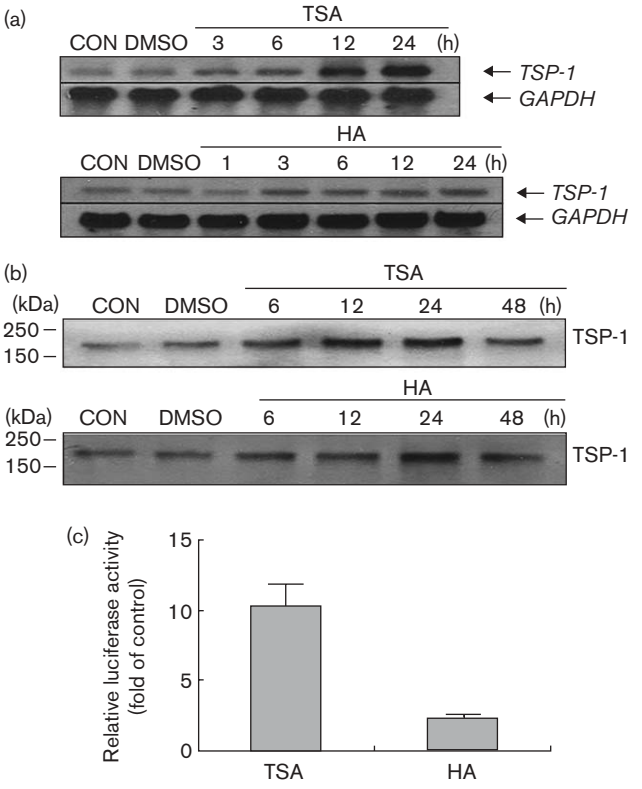
Time-dependent thrombospondin-1 expression at the mRNA level induced by trichostatin A or helixor A

TSP-1 mRNA and protein levels can accumulate with increasing incubation time. Thus, as shown in Fig. 2a and b, the TSP-1 levels obtained from controls with or without DMSO were determined at 24 h to allow a proper comparison with the maximal TSP-1 levels measured in the TSA-stimulated and HA-stimulated samples. TSA and HA always increased the TSP-1 level at 3–24 h. HA-stimulated TSP-1 expression at 1 h was, however, slightly lower than that of DMSO, which may be the result of transient instability of the agent-treated cells during the early stage (within 1 h). As shown in Fig. 2a, TSP-1 expression at the mRNA level was enhanced by the stimulating agent, depending on the time course. With TSA, the TSP-1 expression level began to increase at 3 h; the increase was sustained and reached a maximal level at 24 h. With HA, the TSP-1 expression level started to increased at 3 h and the increased level was consistently sustained with little changes until 24 h. The TSP-1 protein level was continuously upregulated by both agents until 24 h (21.4–25.5 μg/10^6 cells/24 h by TSA and 5.6–7.3 μg/10^6cells/24 h by HA); a slight decrease in the TSP-1 protein level was noted at 48 h with both agents (Fig. 2b). TSP-1 expression in the protein level was time-dependently parallel to the mRNA, whether with TSA or HA. To examine whether the agent-induced TSP-1 expression occurred through transcriptional activation, *tsp-1* promoter activities were determined by measuring *tsp-1* promoter-mediated luciferase activity (Fig. 2c). Luciferase activity relative to the control level was increased 10.4-fold by TSA and 2.3-fold by HA. The lower promoter activity of HA compared to that of TSA was consistent with the increase in the mRNA (Fig. 2a) and protein (Fig. 2b) levels. Hence, the increase in mRNA and protein levels were due to transcriptional activation.

The effects of trichostatin A and helixor A on cell viability

First, to determine whether the dose that was effective for TSP-1 expression was appropriate for the cell-migration and capillary-like tube-formation experiments, agent-induced cytotoxicity was investigated using the MTT assay. As shown in Fig. 3, TSA and HA had no cytotoxic effect on ECV304 cells at any of the doses used in the experiments. Given the data shown in Fig. 3 and in Fig. 1, 100 ng/ml of TSA and 100–200 ng/ml of HA were given for 24 h for the cell-migration and capillary-like tube-formation experiments. No difference in cell viabilities was seen between the controls with DMSO and those without DMSO (data not shown); thus, controls with DMSO were used for the cellular function assays, as shown in Figs 3–7 and 9.

Fig. 2



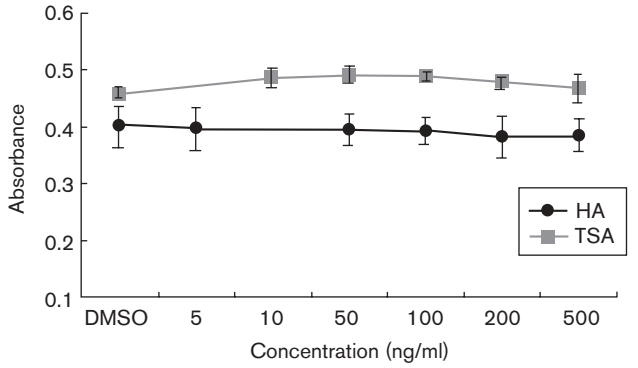
Overexpression of TSP-1 by TSA or HA was time dependent. TSP-1 mRNA and protein levels were determined by Northern blot analysis (a) and Western blot analysis (b) in the presence of a fixed dose of TSA (100 ng/ml) or HA (100 ng/ml). CON, control in the absence of DMSO; DMSO, control in the presence of DMSO. GAPDH mRNA was also determined as an internal control for equal loading. (c) Transcriptional activity of *tsp-1* promoter inserted into the luciferase reporter plasmid and transfected into the TSA- or HA-stimulated ECV304 cells for 18 h. pCMV-β-gal plasmid (0.1 μg) was cotransfected to determine transfection efficiency. Luciferase activities are expressed as the relative activity to that of the control. Luciferase data are represented as the mean ± SD from three separate experiments. DMSO, dimethylsulfoxide; HA, helixor A; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TSA, trichostatin A; TSP-1, thrombospondin-1.

Trichostatin A and helixor A suppression of wounded cell migration

As endothelial cells migrate from the parent vessel and eventually form a lumen, angiogenesis can be inhibited by halting endothelial cell migration. The ECV304 cells

wounded by scraping were allowed to migrate from the position shown by the black arrow in Fig. 4 to the right in the presence of TSA (100 ng/ml) or HA (100 ng/ml) at 37°C for 24 h. Control cells (DMSO) moved markedly to the right, whereas cell migration was blocked by the agents. Cells exposed to HA (lower panel in Fig. 4) migrated densely in crowds, whereas those exposed to TSA spread freely with less dense migration. These differences might reflect differences in cell condition. Overall, the agents reduced cell migration; these inhibitory effects were inhibited by specific TSP-1 antibody. HA at a concentration of 50 ng/ml inhibited cell migration to the same extent as HA at a concentration of 100 ng/ml (data not shown). As TSP-1 is a well-known extracellular protein, the TSP-1 activity could be easily counteracted by neutralizing with TSP-1 antibody. In contrast to TSP-1 antibody, nonspecific mouse IgG antibody (IgGAb) did not reverse the suppressive effects of these agents. These results demonstrate that the reduction in cell migration induced by these agents was mediated by alterations in the TSP-1 level.

Fig. 3

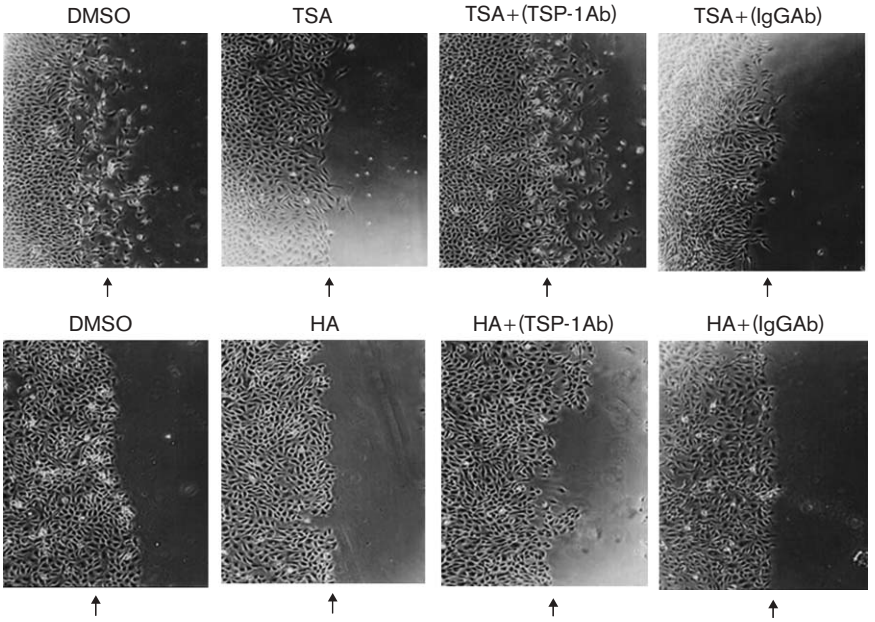


Effects of TSA and HA on cell viability. Cell viability was estimated using the MTT assay at 570 nm. Cells were grown for 24 h in the presence of HA (100 ng/ml; -●-) or TSA (100 ng/ml; -■-) and assayed at the times indicated. DMSO, absorbance of the unstimulated cells in the culture media containing DMSO. All data are expressed as the mean ± SD of two separated experiments. DMSO, dimethylsulfoxide, HA, helixor A; TSA, trichostatin A.

Suppression of angiogenesis by trichostatin A or helixor A

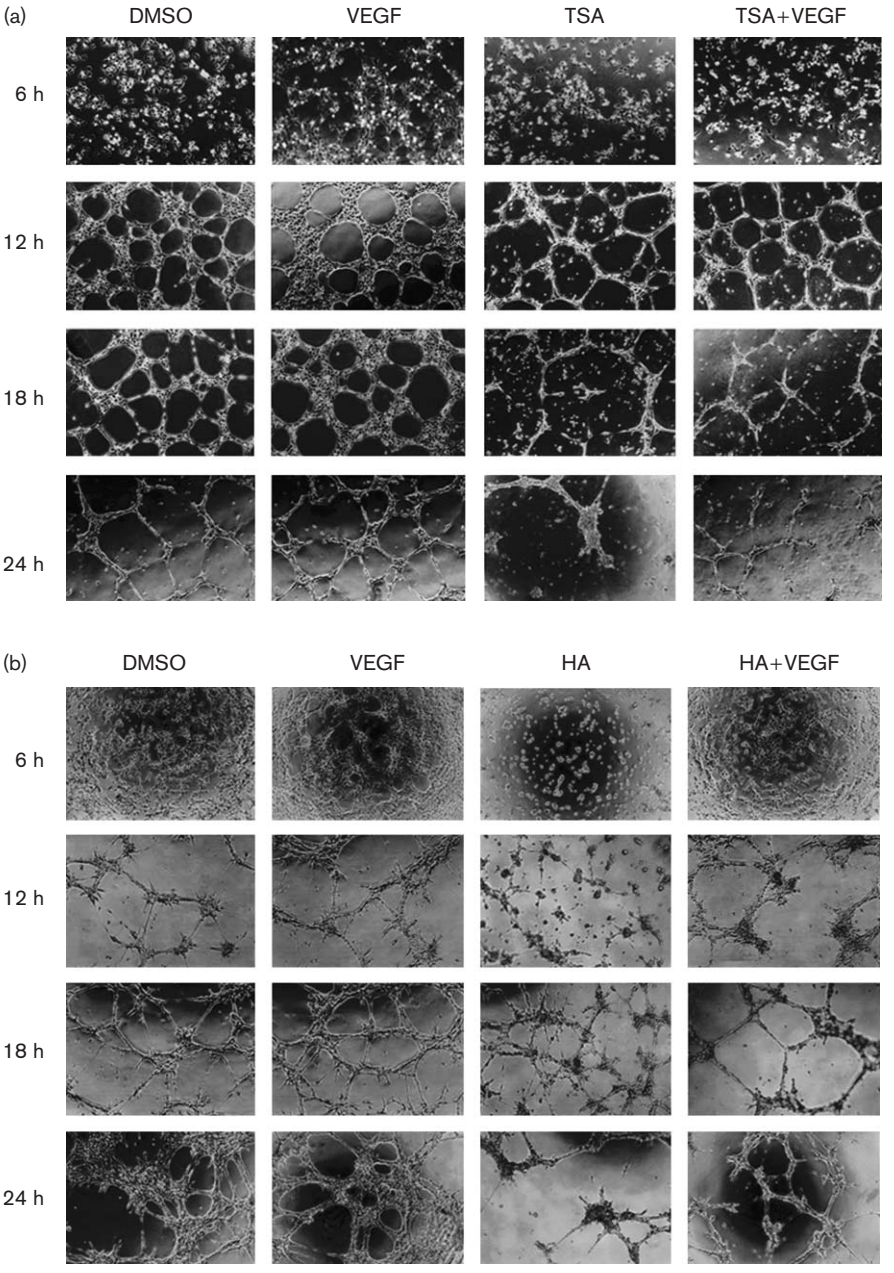
To investigate the effects of the agents on neovascularization, ECV304 cells were cultured on the Matrigel-coated plate with stimulation by TSA (100 ng/ml), HA (100 ng/ml) or VEGF (10 ng/ml) for 3–24 h; angiogenesis was evaluated based on capillary-like tube formation (Fig. 5). In the absence of TSA or HA, ECV304 cells formed tubular structures in a time-dependent manner; tubular forms started to appear at 6 h and developed into microvessel networks from 12 to 24 h. In addition,

Fig. 4



Suppression of wounded cell migration by TSA (100 ng/ml) or HA (100 ng/ml). Cells wounded by scraping were stimulated for 24 h and sequentially photographed under phase microscopy at a ×40 magnification. To check whether TSP-1 was involved in the agent-induced suppression of cell migration, they were pretreated with neutralizing antibody to TSP-1 (TSP-1Ab) at a dose of 10 μg/ml. Mouse IgG antibody (IgGAb) was also used to evaluate the nonspecific effect of antiserum. Black arrows indicate the edge wounded by mechanical scraping. DMSO, dimethylsulfoxide; HA, helixor A; TSA, trichostatin A; TSP-1, thrombospondin-1.

Fig. 5



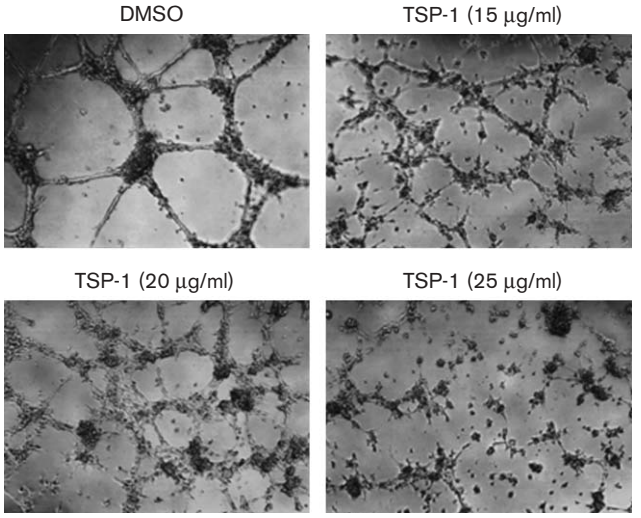
Inhibition of capillary-like tube formation by TSA (100 ng/ml, a) or HA (100 ng/ml, b) at the various time periods from 6 to 24 h. To test whether these agents inhibited the VEGF-induced tube formation, exogenous VEGF (10 ng/ml) was also given with TSA (100 ng/ml), TSA + VEGF (a) or HA (100 ng/ml), HA + VEGF (b). Morphological changes were photographed at a ×40 magnification. DMSO, dimethylsulfoxide; HA, helixor A; TSA, trichostatin A; VEGF, vascular endothelial growth factor.

exogenous VEGF was associated with the formation of capillary-like tubes that were completely organized into networks and were surrounded by a thick cell lining. TSA or HA, however, markedly inhibited the tube formation; partially formed tubes encircled by discontinuous thin cell linings were noted with short-term incubation (6–18 h) and sequentially destroyed networks were found at 24 h. Interestingly, when pretreatment with exogenous VEGF (10 ng/ml) was performed, TSA strongly blocked the tube formation to the same level that was seen in the absence of exogenous VEGF (Fig. 5a). HA, however, weakly blocked tube formation and there was a considerable level of organized networks with VEGF stimulation (Fig. 5b).

Trichostatin A- or helixor A-induced inhibitory activities with respect to capillary-like tube formation mediated by overexpressed thrombospondin-1

The data shown in Figs 1 and 2 indicate that agent-enhanced TSP-1 might be related to ECV304 cell migration and tube formation. Therefore, the effects of exogenous TSP-1 on these cellular functions were examined (Fig. 6). Cell migration and tube formation were reduced in a concentration-dependent pattern when exogenous TSP-1 was given at concentrations from 15 to 25 μg/ml. In particular, capillary-like tube formation was completely inhibited with exogenous TSP-1 given at higher than 20 μg/ml; many lumps of aggregated cells and broken networks were noted. Additionally, to confirm whether agent-induced TSP-1 was involved in the specific inhibition of these cellular functions, a neutralization test was carried out using diluted specific TSP-1 antiserum (1 : 1000) (Fig. 7). The inhibitory activities of the agents with respect to tube formation were reversed by adding neutralizing antibody, whereas nonspecific anti-IgG antiserum (1 : 1000) did not reverse the effects. These results suggest that these agents downregulated angiogenesis by increasing TSP-1. As shown in Fig. 5a and b, as HA had lower activity than TSA, HA partially inhibited these networks and tube formation after 18 h, whereas TSA resulted in complete inhibition. In addition, HA-induced inhibitory activity was completely reversed by neutralization and partially reversed with strong TSA inhibition (Fig. 7). Thus, on the basis of data presented in Figs 5b and 7, subsequent tube-formation experiments were carried out to verify the correlation between the VEGF and TSP-1 levels.

Fig. 6

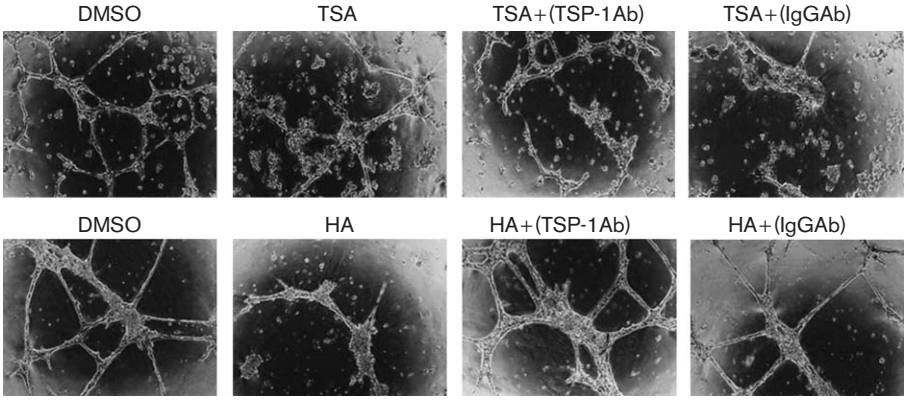


Reduction of capillary-like tube formation by exogenous TSP-1. As TSP-1 negatively affected angiogenesis at high doses (over 15 μg/ml), exogenous TSP-1 was given at a dose from 15 to 25 μg/ml. Morphological features were photographed at a ×40 magnification. DMSO, dimethylsulfoxide; TSP-1, thrombospondin-1.

The effect of trichostatin A or helixor A on vascular endothelial growth factor levels and on vascular endothelial growth factor-induced angiogenesis

Angiogenesis is regulated by the balance between inhibitory and stimulatory factors, VEGF is a major stimulatory factor that is critical for triggering the balance towards angiogenesis. To determine the differential effects that TSA and HA have in the presence of exogenous VEGF, VEGF protein levels were estimated using Western blot analysis in the presence of TSA (100 ng/ml) or HA (100 ng/ml) (Fig. 8). TSA reduced VEGF levels in a time-dependent manner, so that by 48 h, VEGF levels were minimal. On the other hand,

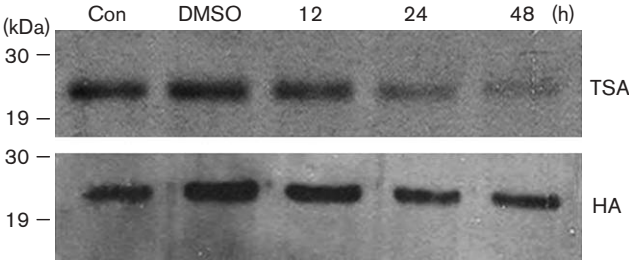
Fig. 7



Reversal of agent-induced inhibition of tube formation by neutralizing TSP-1 antibody. The neutralization test was performed as described in Fig. 4. Morphological changes were photographed at a ×40 magnification. DMSO, dimethylsulfoxide; HA, helixor A; IgGAb, IgG antibody; TSA, trichostatin A; TSP-1Ab, thrombospondin-1 antibody.

DMSO-treated control and HA slightly increased VEGF levels at 12 h and from 12 to 48 h, HA maintained elevated VEGF levels. In the presence of exogenous VEGF, the weak inhibitory activity of HA may be caused by a shift in the balance to angiogenesis, which resulted from the increased net VEGF level. Hence, the effects of HA-generated CM on VEGF-induced capillary-like tube formation were assessed by altering the absolute TSP-1 level without changing its relative level (5 × CM) and altering the relative TSP-1 level by 3 × CM – enVEGF (Fig. 9). Although 3 × CM considerably repressed tube formation in the absence of exogenous VEGF (Fig. 9c), it could not repress tube formation in the presence of exogenous VEGF (Fig. 9d). Notably, 3 × CM – enVEGF reduced tube formation (Fig. 9e) more than 3 × CM containing endogenous VEGF (Fig. 9c). On the other hand, 5 × CM had a strong blocking effect regardless of the presence of exogenous VEGF (Fig. 9f and g). These results demonstrate that absolute and relative TSP-1 levels play an important mediating role in operating the angiogenic switch.

Fig. 8

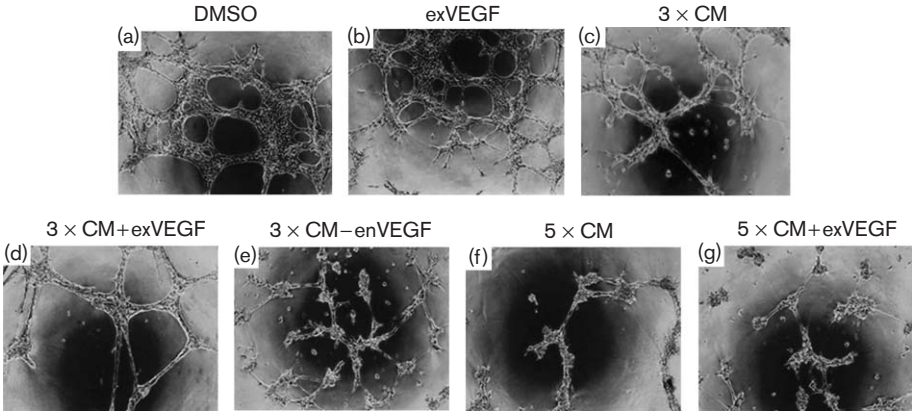


The effects of TSA (100 ng/ml) or HA (100 ng/ml) on the VEGF protein level. VEGF levels were determined by Western blot analysis after the treating agent was applied for the desired times and then visualized by specific VEGF antiserum using the enhanced chemiluminiscent kit. Con, control in the absence of DMSO; DMSO, control in the presence of DMSO. DMSO, dimethylsulfoxide; HA, helixor A; TSA, trichostatin A; VEGF, vascular endothelial growth factor.

Discussion

Normal endothelial cells are quiescent, but tumor vasculature proliferates 30–40 times more quickly than normal endothelial cells. Thus, for most localized tumors, their growth and metastasis could be suppressed by halting angiogenesis. It has been well recognized that tumor vasculature is initiated in a coordinated manner by an angiogenic switch that depends on the balance between proangiogenic and antiangiogenic factors that are present in the environment. Among the many regulatory factors, TSP-1 has been extensively studied as an antiangiogenic factor that is expressed in an inverse manner to the increased expression of proangiogenic factors such as VEGF and FGF-2 [1–3]. In addition, in several preclinical studies, TSP-1 levels were found to have promising anticancer effects: taxol resistance was reduced in the NCI-60 cancer cell lines and metastases were reduced by combining with carboplatin; neovascularization was reduced when three TSP-1 type-1 repeats were added; and survival time of stage III ovarian carcinoma patients was prolonged [32,33]. A recent study demonstrated that TSP-1 exerted a biphasic effect on angiogenesis; at a TSP-1 concentration of 15 μg/ml,

Fig. 9



Inhibitory effects of increased TSP-1 levels assessed by changing the absolute or relative TSP-1 level on angiogenesis. CMs were generated by HA (100 ng/ml)-stimulated ECV304 cells. CMs were concentrated using a method described previously [14]. DMSO, control in the presence of DMSO; exVEGF, exogenous VEGF; 3 × CM, three-fold concentrated CM containing endogenous VEGF; 3 × CM + exVEGF, exogenous VEGF (10 ng/ml)-added 3 × CM; 3 × CM–enVEGF, endogenous VEGF-deprived 3 × CM; 5 × CM, five-fold concentrated CM containing endogenous VEGF; 5 × CM + exVEGF, exogenous VEGF (10 ng/ml)-added 5 × CM. To remove endogenous VEGF, 3 × CM was gently shaken after mixing with excess specific VEGF antibody for 6 h at 4°C and placed at room temperature overnight. The precipitated antigen–antibody complex was removed by centrifugation at 12 000*g* for 20 s. DMSO, dimethylsulfoxide; HA, helixor A; TSP-1, thrombospondin-1; VEGF, vascular endothelial growth factor.

capillary-like tube formation was inhibited, whereas at a concentration of 5 μg/ml or less, stabilization was promoted [4–7]. Hence, it is important that the angiogenic balance in the local site be turned towards antiangiogenesis by constantly maintaining a higher level of TSP-1. It has been reported previously that many neoplastically transformed carcinoma cells could not synthesize as much TSP-1 as normal cells. For example, SV-40 transformed fibroblast and fibrosarcoma cells secreted at rates of 8.2 and 4.9 μg/10^6 cells/24 h, respectively, whereas normal fibroblasts secreted TSP-1 at a rate of 32 μg/10^6 cells/24 h or four times as much as transformed fibroblasts [34]. Of note, the overexpression of TSP-1 is essential for reducing tumor proliferation and metastasis.

TSA, a tumor therapeutic agent, blocks tumor growth and neovascularization by decreasing VEGF levels, altering the VEGF-mediated signal pathway and blocking matrix metalloproteinase-2 activation [19–21]. No evidence exists on the effect of TSA on the expression of angiostatic factors.

HA is an antitumor drug that has been used as an alternative medicine and as an adjuvant cancer therapy. Growing evidence is available that HA stimulates immunocompetent cells with the subsequent release of cytokines [29,31,35–38]. In a clinical trial, HA was found to reduce beneficially the side effects of chemotherapy in cancer patients and thus improve quality of life [29,39]. Little, however, is known about the regulatory effects of TSA and HA on TSP-1 expression. In the current study, TSA-regulated or HA-regulated TSP-1 expression, and the effects of the altered TSP-1 levels by these agents on cellular functions such as cell migration and microvessel-like tube formation were investigated. Although TSA and HA elevated ECV304 cell TSP-1 expression at both the mRNA and protein levels through transcriptional activation, they had different effects with respect to TSP-1 elevation. As shown in Figs 1 and 2, compared with control levels, the maximum TSP-1 levels induced by TSA were markedly higher than those induced by HA. As the TSA-elevated TSP-1 level, higher than HA, strongly inhibited tube formation in a neutralization test, TSA-induced inhibition in the presence of higher TSP-1 level was partially reversed by adding TSP-1 antiserum at the same concentration as that in the neutralization of HA activity. Furthermore, it is not excluded that the remaining inhibitory activity was maintained by other TSA-modulated genes, besides the TSP-1 involved in angiogenesis. In particular, when pretreated with exogenous VEGF, TSA strongly and persistently blocked tube formation, whereas HA could only reduce tube formation to about 70–80% of control levels. Given these results, it is conceivable that HA elevated the amount of TSP-1 to a level that barely exceeded the threshold level required to switch off angiogenesis; thus, this effect of HA could be readily affected by a small change in the VEGF level. Indeed, HA in the 3 × CM resulted in a TSP-1 protein level that was just the marginal level required for tube formation (average 16.2 μg/ml; 5.6–7.3 μg/10^6 cells/24 h). To determine whether a small amount of exogenous VEGF (10 ng/ml) disturbed the marginal balance in the presence of high level of endogenous VEGF, changes in the HA-induced VEGF protein level were assessed over time and capillary-like tube formation was observed after removal of endogenous VEGF (3 × CM – enVEGF) or using a more concentration medium (5 × CM) in the presence of exogenous VEGF. It was found that at 48 h, TSA gradually decreased endogenous VEGF to nearly undetectable levels. With HA, however, the endogenous VEGF levels were the same as control levels. Moreover, with 3 × CM-enVEGF (Fig. 9e), tubular networks were greatly suppressed compared with 3 × CM with endogenous VEGF. With medium of higher concentration (5 × CM), HA blocked tube formation regardless of the presence of exogenous VEGF (Figs 9f and g). Our results, shown in Figs 5b, 6, 8 and 9, emphasize that the absolute and relative TSP-1 levels with respect to angiogenic factors are critical for inhibiting angiogenesis. Furthermore, the fact that TSA and HA have different effects indicates that TSA is a strong angiostatic drug through additive effects that occur due to the increase in TSP-1 expression and the decrease in the VEGF level; HA has a weak angiostatic effect and does not decrease the VEGF level, and its activity is easily shifted by a change in the environment VEGF level. As HA is a complex compound containing lectines, viscotoxines, and alkaloids [22,23], its weak activity could be enhanced by using the single component of HA that is responsible for the effect.

As TSP-1 is an extracellular protein, its activity can be neutralized by specific anti-TSP-1 antibody. The neutralization test is the proper means for clearly confirming the agent-induced inhibition of angiogenesis that results from the specific increase of TSP-1 expression. Angiogenesis can be assayed by corneal neovascularization, chick chorioalantoic membrane, aortic ring sprouting and capillary-like tube formation on Martrigel [40,41]. As polymerized Matrigel is composed of mouse extracellular basement membrane (to mimic the physiological environment), the Matrigel system used is an easy and reliable method for evaluating angiogenesis *in vitro*.

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