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Evidence for a Proangiogenic Activity of TNF-Related Apoptosis-Inducing Ligand[1]

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Abstract

Starting from the observation that tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)/Apo-2L protein is expressed in both malignant and inflammatory cells in some highly vascularized soft tissue sarcomas, the angiogenic potential of TRAIL was investigated in a series of *in vitro* assays. Recombinant soluble TRAIL induced endothelial cell migration and vessel tube formation to a degree comparable to vascular endothelial growth factor (VEGF), one of the best-characterized angiogenic factors. However, the proangiogenic activity of TRAIL was not mediated by endogenous expression of VEGF. Although TRAIL potentiated VEGF-induced extracellular signal–regulated kinase (ERK) phosphorylation and endothelial cell proliferation, the combination of TRAIL + VEGF did not show additive effects with respect to VEGF alone in inducing vessel tube formation. Thus, although TRAIL has gained attention as a potential anticancer therapeutic for its ability to induce apoptosis in a variety of cancer cells, our present data suggest that TRAIL might also play an unexpected role in promoting angiogenesis, which might have therapeutic implications.

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Introduction

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)/Apo-2L is a member of the TNF family of cytokines, which is broadly expressed at the mRNA level in many normal tissues and tumor cell lines [1]. TRAIL is a type II membrane protein, which can be proteolytically cleaved by cysteine proteases to a soluble form [2] as previously shown also for TNF-α and CD95 (Apo-1/Fas) ligand. The unique feature of TRAIL, compared to other members of the TNF family, is its ability to induce apoptosis in a variety of malignant cells both *in vitro* and *in vivo*, displaying minimal or absent toxicity on normal cells and tissues [3,4].

TRAIL interacts with four high-affinity transmembrane receptors belonging to the apoptosis-inducing TNF-R family. TRAIL-R1 (DR4) and TRAIL-R2 (DR5) transduce apoptotic signals on binding of TRAIL, whereas TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) are homologous to DR4 and DR5 in their cysteine-rich extracellular domain, but lack intracellular death domain and apoptosis-inducing capability and have been proposed to function as decoy receptors, protecting normal cells, including endothelial cells, from apoptosis [5,6]. Although little is known about possible nonapoptotic effects induced by TRAIL, it has been shown that endothelial cells express all TRAIL-Rs [6–8], whereas TRAIL protein is expressed in the medial smooth cell layer of the aorta and pulmonary artery [9]. Whereas cleavage of Fas ligand from the cell surface requires the action of zinc-dependent metalloproteases, generation of soluble TRAIL involves the action of cysteine proteases [2]. Notably, the vessel wall is a rich source of cysteine proteases [10], which suggests that the TRAIL/TRAIL-R system likely plays a physiological role in vascular biology. In this respect, we have recently demonstrated that addition of TRAIL to human umbilical vein endothelial cells induces the rapid phosphorylation and activation of extracellular signal–regulated kinase (ERK) and Akt [7,8]. Because these intracellular pathways are known to be involved in endothelial cell survival, proliferation, and migration [11,12], in this study, we have investigated whether TRAIL induced angiogenesis by using various *in vitro* assays. Taking into consideration that several recent studies have confirmed the hypothesis that tumor growth, in general, is dependent on angiogenesis [13,14], we have also analyzed the expression of TRAIL protein in soft tissue sarcomas because these tumors are often highly vascularized [15].

Materials and Methods

Reagents and Cells

Recombinant histidine6-tagged TRAIL was produced in bacteria and purified by chromatography on Ni^{2+} affinity resin,

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as described [7]. The concentration of TRAIL used in most assays (10 ng/ml) was determined in preliminary dose–response (0.1–1000 ng/ml) experiments. For neutralization experiments, TRAIL was preincubated with TRAIL-R1-Fc and/or TRAIL-R2-Fc chimeras, according to the supplier's instructions (R&D, Minneapolis, MI). Vascular endothelial growth factor (VEGF; Peprotec, London, UK), was used at the final concentration of 10 ng/ml. Polymyxin B (Calbiochem, La Jolla, CA), was used at the final concentration of 10 μg/ml. A pharmacological inhibitor of the ERK pathway (PD98059; final concentration: 10 μM) was from Calbiochem.

Primary human umbilical vein endothelial cells (HUVECs) were obtained as described previously [8] and were used between the third and sixth passages *in vitro*. Cells were grown on 0.2% gelatin-coated tissue culture plates in M199 endothelial growth medium (BioWhittaker, Walkersville, MD) supplemented with 20% fetal bovine serum (FBS), 10 μg/ml heparin, and 50 μg/ml Endothelial Cell Growth Factor (ECGF) (Sigma, St. Louis, MO).

Neoplastic Samples and Immunohistochemistry

Formalin-fixed and paraffin-embedded tissues of human sarcomas were obtained from 10 surgically treated patients. The tumors consisted of five liposarcomas, two leiomyosarcomas, one rhabdomyosarcoma, one angiosarcoma, and one Kaposi's sarcoma. Immunohistochemical study was performed on 4 μm sections using a streptavidin–biotin complex immunoperoxidase technique with a polyclonal anti–TRAIL antibody (Ab; clone H-257; Santa Cruz Biotechnology, Santa Cruz, CA). Isotype-matched irrelevant antibodies, used as negative control, gave absence of background. For the evaluation of tumor vascularization, sections were stained with Ab anti-CD31 (clone JC/70A; BioGenex, San Ramon, CA) and angiogenesis was quantified by assessment of microvessel density using previously described techniques [16,17]. Briefly, the number of vessel was counted throughout the entire core specimen in serial sections. Consecutive per ×20 high-power fields were examined, a median count per field was calculated, and a simple high-density/low-density score was used. Microvessel density was analyzed blinded toward the result of the TRAIL staining.

Cell Migration and Cell Invasion Assays

Cell migration was analyzed using a modified Boyden chamber assay, as described previously [18], by using 24-well plates with inserts containing 8 μm pore size gelatinized polycarbonate membranes separating the two chambers of each well (Transwell; Costar, New York, NY).

Cell invasion was investigated by using the Chemicon Cell Invasion Assay kit (Chemicon International, Temecula, CA) according to the manufacturer's instruction. This assay is performed in an invasion chamber, a 24-well tissue culture plate with cell culture inserts containing an 8-μm pore size polycarbonate membrane, over which a thin layer of extracellular matrix (ECM) is dried.

For the assays, exponentially growing cells were harvested with trypsin, centrifuged, resuspended at 0.5 $\times 10^6$ cells/ml in migration buffer [M199 medium, 10 mM HEPES, pH 7.4, and 0.5% bovine serum albumin (BSA)], and placed in the upper compartment of the chambers. TRAIL or VEGF, used alone or in combination, was added in the lower chambers. After 4 hours (for the migration assay) or 48 hours (for the invasion assay) of incubation at 37°C, cells on the upper face of the membrane were scraped using a cotton swab and cells on the lower face were fixed and stained with Mayer's hematoxylin solution. The number of migrated cells on the lower face of the filters was counted in five fields under ×100 magnification. Assays were done in triplicates.

Tube Formation Assays

In vitro formation of tubular structures was studied on BioCoat Matrigel tissue culture plates (BD Biosciences, Bedford, MA). Briefly, HUVECs were plated at 3.5 $\times 10^5$ cells/well in 24-well plates precoated with a solution of Matrigel basement membrane matrix, and left untreated or exposed to TRAIL or VEGF. After 48 hours of incubation at 37°C, the cell 3D organization was examined under an inverted photomicroscope and photographed (×40). Each treatment was performed in triplicate.

In vitro angiogenesis was assessed as formation of capillary-like structures of HUVECs cocultured with matrix-producing cells that had been UV-irradiated before plating of primary HUVECs (TCS Biologicals, Buckingham, UK) [19]. Briefly, cultures were left untreated or stimulated with TRAIL or VEGF, used alone or in combination, at day 3. When indicated, PD98059 or the vehicle (0.25% DMSO), previously diluted in medium, was added to the cultures 45 minutes before exposure to TRAIL or VEGF. Medium and treatments were replaced every 2 to 3 days. At day 12, the cells were fixed and HUVECs were stained using an anti–CD31 Ab (TCS Biologicals, Buckingham, UK), according to the instructions provided with the kit. Images were captured and analyzed. In particular, to measure the formation of the capillary network, the number of connections between three or more capillary-like structures and the total length of tubes were quantified by image analysis at ×40 magnification. Four-six different fields were analyzed per well.

Western Blot Analysis

For Western blots, HUVECs were plated in 10-cm dishes and grown at subconfluence before treatments. In order to minimize activation by serum, HUVECs were subject to partial fetal calf serum (FCS) reduction (to 0.5%) and complete growth factor withdrawal for 18 hours prior to the addition of TRAIL or VEGF, used alone or in combination. Cells were harvested in lysis buffer containing 1% Triton X-100, Pefablock (1 mM), aprotinin (10 μg/ml), pepstatin (1 μg/ml), leupeptin (10μg/ml), NaF (10 mM), and Na_3VO_4 (1 mM). Protein determination was performed by Bradford assay (Bio-Rad, Richmond, CA). Equal amounts of protein (50 μg) for each sample were migrated in acrylamide gels and blotted onto nitrocellulose filters. Blotted filters were probed with antibodies for the phosphorylated ERK1/2 and p38/MAPK (all from New England Biolaboratories, Beverly, MA). After incubation with peroxidase-conjugated

anti–rabbit or anti–mouse IgG (Sigma), specific reactions were revealed with the Enhanced Chemiluminescence (ECL) Western blotting detection reagent. Membranes were stripped by incubation in Re-Blot 1 × Ab stripping solution (Chemicon International) and reprobed for the respective total protein kinase content or β-actin (New England Biolaboratories) for verifying loading evenness.

Densitometric values, expressed in arbitrary units, were estimated by the ImageQuant software (Molecular Dynamics, Piscataway, NJ). Multiple film exposures were used to verify the linearity of the samples analyzed and avoid saturation of the film.

[^{3}H]Thymidine Incorporation

HUVECs were plated onto 96-well plates at a density of 5 $\times 10^3$ cell/well. On the next day, the medium was changed to endothelial cell basal medium containing 0.5% FBS and 0.1% BSA (starvation medium). The cells were then pretreated with PD98059 for 1 hour and incubated with TRAIL or VEGF, used alone or in combination, for 30 hours. [^{3}H]thymidine (1 μCi) was added to each well during the last 6 hours of incubation. [^{3}H]thymidine-labeled DNA was then measured using a Beckman (Fullerton, CA) model LS6000IC liquid scintillation counter.

Statistical Analysis

Data were analyzed using the two-tailed, two-sample *t*-test (statistical analysis software; Minitab, State College, PA). Values of $P < .05$ were considered significant.

Results

TRAIL Is Expressed in Highly Vascularized Soft Tissue Sarcomas

Having previously demonstrated that TRAIL activates intracellular signal transduction pathways [7,8], which have been involved in promoting angiogenesis [13,14], in the first group of experiments, we have investigated the expression of TRAIL in some cases of malignant mesenchymal tumors because these tumors are often highly vascularized [15,20]. As shown in Figure 1*A*, the cells of a low-grade gastric leiomyosarcoma stained negative for TRAIL and were poorly vascularized. However, the malignant neoplastic cells of a high-grade leiomyosarcoma showed a strong expression of TRAIL and were characterized by prominent neovascularity. To ascertain that TRAIL expression was not confined to leiomyosarcomas, we have also analyzed TRAIL expression in some cases of angiogenetic liposarcomas (Figure 1*B*). In these sarcomas, a clear-cut positivity for TRAIL was noticed in both malignant cells as well as in tumor-infiltrating lymphocytes and plasma cells (Figure 1*B*). Finally, a high expression of TRAIL was documented also in tumors characterized by tumultuous angiogenesis, such as malignant vascular sarcomas (Kaposi's sarcoma; Figure 1*C*).

TRAIL Promotes Endothelial Cell Migration and Invasion

Because angiogenesis is a tightly regulated process, which involves the coordinated migration, differentiation, and morphogenetic organization of endothelial cells into new capillary structures [18], these aspects of angiogenesis were next examined in a series of *in vitro* assays.

To test whether recombinant soluble TRAIL could affect endothelial cell motility, HUVECs were incubated in a modified Boyden chamber with recombinant soluble TRAIL (10 ng/ml). For comparison, cells were treated also with VEGF (10 ng/ml). TRAIL significantly ($P < .01$) increased HUVEC migration (Figure 2*A*). The increase in the number of migrated cells detected with TRAIL (approximately two-fold) was similar to that observed in the presence of VEGF (2.5-fold). Interestingly, however, the simultaneous addition of optimal concentrations of TRAIL and VEGF did not show additive effects on endothelial cell migration (data not shown).

To form new blood vessels, endothelial cells have to migrate and cross basement membranes. This invasive capacity of HUVECs in response to TRAIL was investigated by measuring the invasion of an ECM layer. Addition of recombinant TRAIL significantly ($P < .01$) increased HUVEC invasion through ECM (Figure 2*B*). Again, maximal stimulation, corresponding to a 1.5-fold increase in the number of migrated cells in response to TRAIL, was similar to that observed in response to VEGF. The specificity of these biological effects was confirmed by preincubation of TRAIL with TRAIL-R1-Fc (Figure 2, *A* and *B*) or TRAIL-R2-Fc (data not shown) chimeric proteins, which completely ($P < .01$) abrogated the ability of TRAIL to promote cell migration and invasion (Figure 2, *A* and *B*), without exhibiting, by themselves, any effect on endothelial cell migration or invasion (data not shown). These data demonstrate, for the first time, that TRAIL induces endothelial cell migration and invasion through the basement membrane. Importantly, the TRAIL concentration used in these assays was in the range reported to be present in the plasma of patients affected by hematological malignancies (1–10 ng/ml) [21].

TRAIL Induces Morphological Endothelial Differentiation

To examine whether TRAIL induces morphogenetic changes resembling capillary-like structure tube formation, HUVECs were plated on 3D Matrigel plates. After 48 hours, untreated endothelial cultures showed both cells with small round shape (that remained isolated) and elongated shape, forming connections but an incomplete network of tubes (Figure 3*A*). However, cultures exposed to TRAIL (10 ng/ml) exhibited a distinct phenotype by assuming a more elongated shape, forming thin cords of inteconnecting cells (Figure 3*A*). Similar effects were also observed with VEGF (10 ng/ml) (Figure 3*A*). Morphometric quantitation of the vessel-like structures in the 3D cultures revealed that Fc-TRAIL-R1 completely abrogated this response to TRAIL (Figure 3*B*). These data demonstrate that TRAIL, like VEGF, is able to mediate morphogenetic effects leading to differentiation into vascular structures, which represent an obligatory step for the sprouting of endothelial cells and tube formation.

Because tubules formed in standard Matrigel assays are short and relatively homogeneous, the ability of TRAIL to induce angiogenesis was further investigated by using the

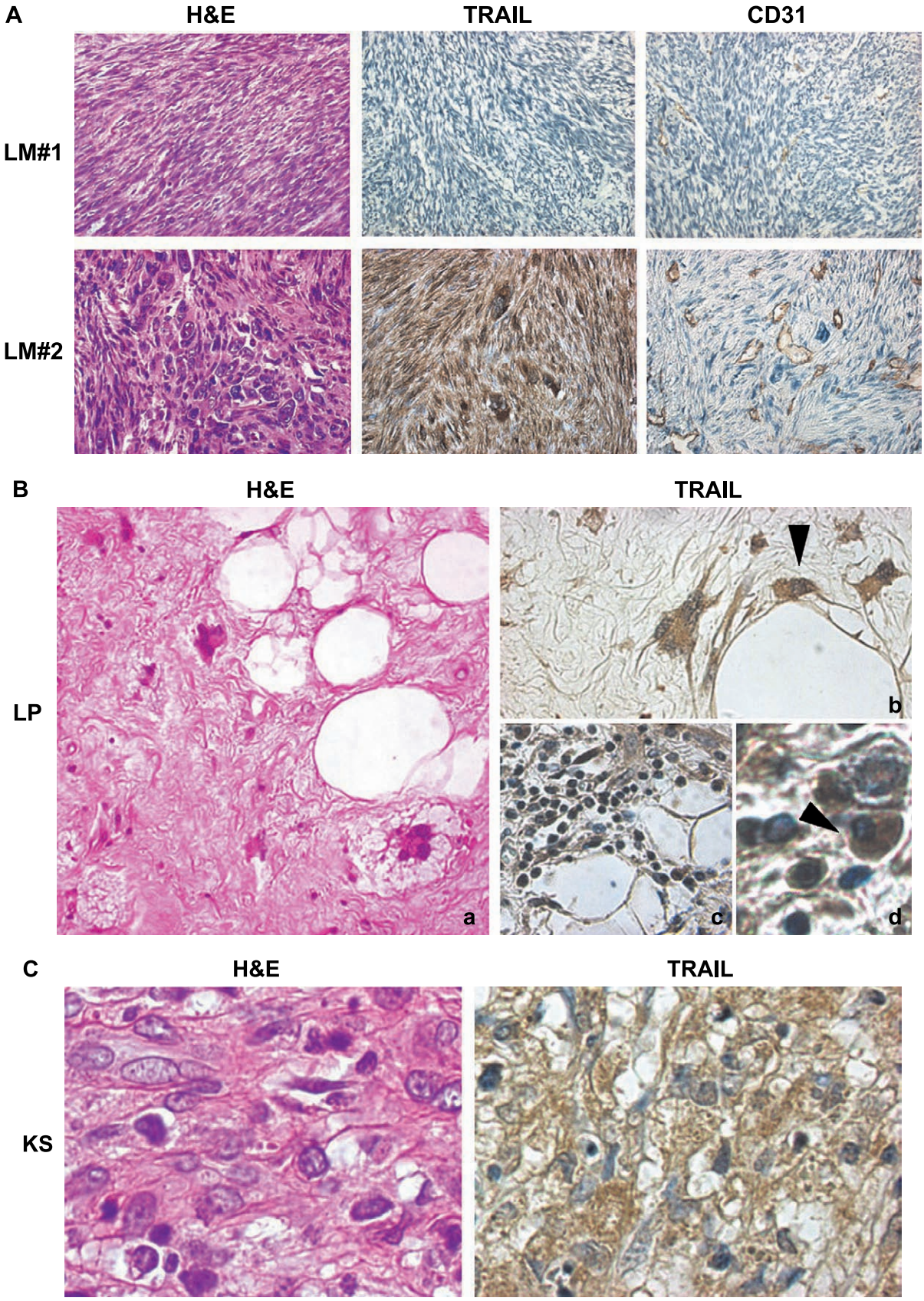

Figure 1. *Expression of TRAIL in human sarcomas. Sections of sarcomas were examined immunohistochemically by using an anti–TRAIL Ab. (A) Representative sections of leiomyosarcomas at low (LM 1) and high vascularity (LM 2) are shown. Stainings with anti–CD31 Ab to detect endothelial cells and vascular structures, and hematoxylin and eosin (H&E) are also shown. Original magnification, ×20. (B) Liposarcoma (panel a, H&E staining, ×40) showing TRAIL expression in malignant neoplastic cells (arrowhead in panel b; ×40), tumor-infiltrating lymphocytes (panel c; ×40), and plasma cells (arrowhead in panel c; ×100). (C) Kaposi's sarcoma showing diffuse expression of TRAIL. Original magnification, ×40.*

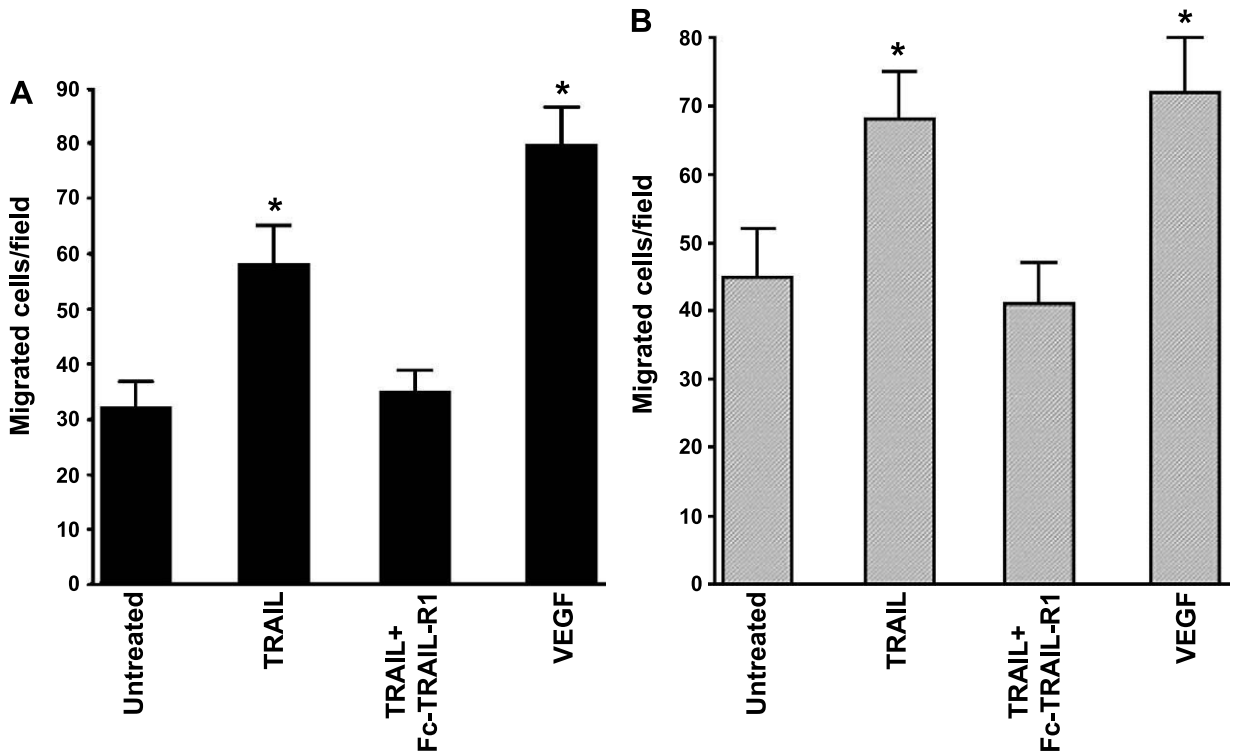

Figure 2. *Effect of TRAIL on HUVEC migration and invasion. Cell migration (A) and cell invasion (B) assays were performed in 24-well Transwell plates, as described in Materials and Methods section. Endothelial cells were seeded in the upper compartments, whereas TRAIL or VEGF was added in the lower compartments. For neutralization experiments, TRAIL was preincubated with TRAIL-R-Fc chimera. Cells migrated through the gelatinized membranes and ECM-coated membranes were counted after 4 hours (A) and 48 (B) hours, respectively. Data are expressed as the number of migrated cells in 10 high-power fields and are mean ± SD of results from four experiments each performed in triplicate. *P < .01 compared to untreated cells.*

angiogenesis coculture assay, which appeared significantly heterogeneous, consisting of both short and long interconnecting tubules that more closely resembled capillaries (Figure 4). In this assay, vessels develop where they are well protected (e.g., between layers of fibroblasts) and morphogenetic processes of tubule formation occur in HUVECs completely surrounded by stromal cells, as *in vivo*. The ability of fibroblasts to support tubule formation has been attributed to their capacity of producing considerable quantities of collagen, fibronectin, and other matrix molecules [19].

To rule out the possibility that the angiogenic activity of TRAIL might be mediated by upregulation of endogenous VEGF, the production of VEGF released in the culture medium was analyzed by enzyme-linked immunosorbent assay (ELISA) in both untreated and TRAIL-treated cultures. In HUVECs, VEGF was not detected in the culture supernatants of both untreated and TRAIL-treated cultures analyzed up to 72 hours (data not shown). In angiogenesis coculture assay, VEGF was endogenously produced, but the levels were similar in untreated (256 ± 36 pg/ml) and TRAIL-treated (240 ± 45 pg/ml) cultures.

We next performed a quantitative analysis by calculating both the total length of tubes and the number of capillary connections per field in cocultures left untreated or exposed to VEGF, TRAIL, and combination of the two cytokines (10 ng/ml each). As shown in Figure 5, the basal formation of capillary-like structures was significantly ($P < .01$) increased after stimulation with either TRAIL or VEGF, and TRAIL-R1-Fc chimeric protein significantly ($P < .01$) inhibited TRAIL-induced total tube length and capillary connections. Moreover, preincubation of TRAIL with 5 μg/ml polymyxin B, which complexes and inactivates endotoxin, did not abrogate the angiogenetic activity of TRAIL, further indicating that these responses to TRAIL are specific. However, the simultaneous addition of TRAIL + VEGF did not show any additive or synergistic effect, and it was not statistically different from VEGF alone (Figure 5).

TRAIL Potentiates VEGF-Induced ERK1/2 But Not p38/MAPK Phosphorylation

The findings illustrated above suggest that TRAIL and VEGF might compete for the same intracellular signal transduction pathways. Therefore, in the next group of experiments, we have investigated the effect of TRAIL and VEGF, used alone or in combination, on mitogen-activated protein (MAP) kinase family members, which have been involved in different aspects of angiogenesis. Although ERK1 and ERK2 are strongly activated on stimulation of cells with mitogens [11], p38/MAPK plays a complex role in angiogenesis by promoting cell migration and inhibiting endothelial cell survival [21–24]. After exposure to either TRAIL or VEGF, induction of phospho-ERK1/2 was observed starting at 5 minutes of treatment (Figure 6*A*). Remarkably, the

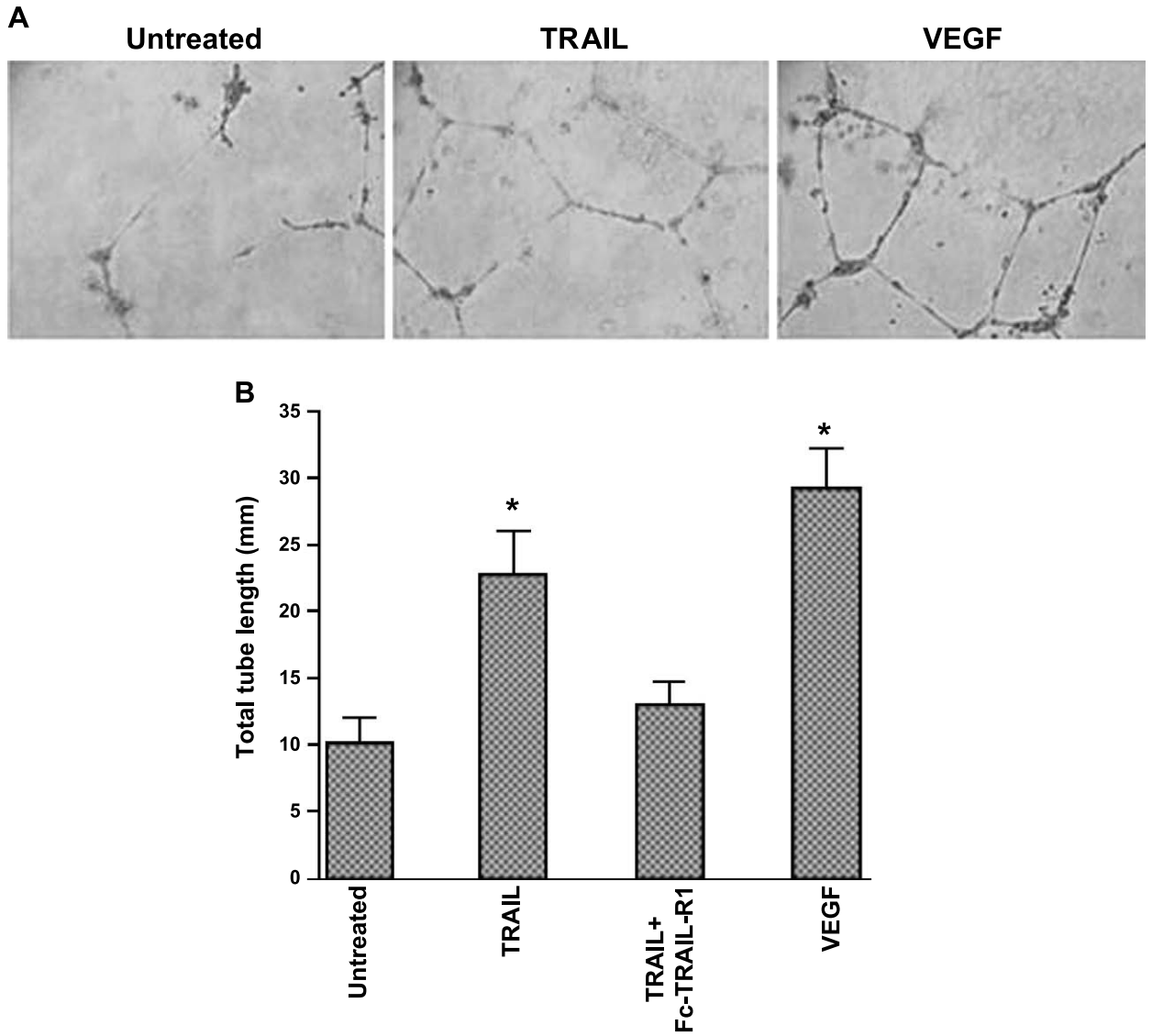

Figure 3. *Effect of TRAIL on in vitro tube formation. HUVECs were seeded into 24 wells containing 3D Matrigel in the absence and presence of TRAIL ± TRAIL-R-Fc or VEGF. (A) Photographs (×40) were taken at 48 hours of cultures. Representative images of at least five experiments with similar results are shown. (B) Five to seven random fields were photographed and recorded, and tube length was quantified by measuring the total cell projection length and individual tubular structure. Tubular length per field is reported as mean ± SD. *P < .01 compared to untreated cells.*

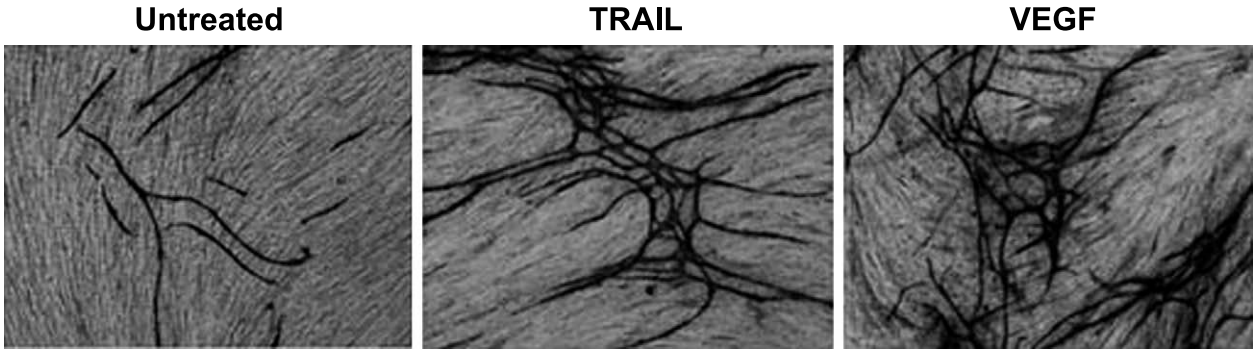

Figure 4. *Effect of TRAIL on the capillary-like network in a coculture angiogenesis assay. Formation of capillary-like structures of HUVECs cultured with matrix-producing cell types was analyzed after 12 days of culture treatment as indicated. Representative example of the morphology endothelial structures was detected after staining with anti–CD31 Ab in the in vitro angiogenesis assay. Rare capillary structures were observed in the angiogenesis assay left untreated, whereas both TRAIL and VEGF induced a diffuse network of capillary structures. Original magnification, ×10.*

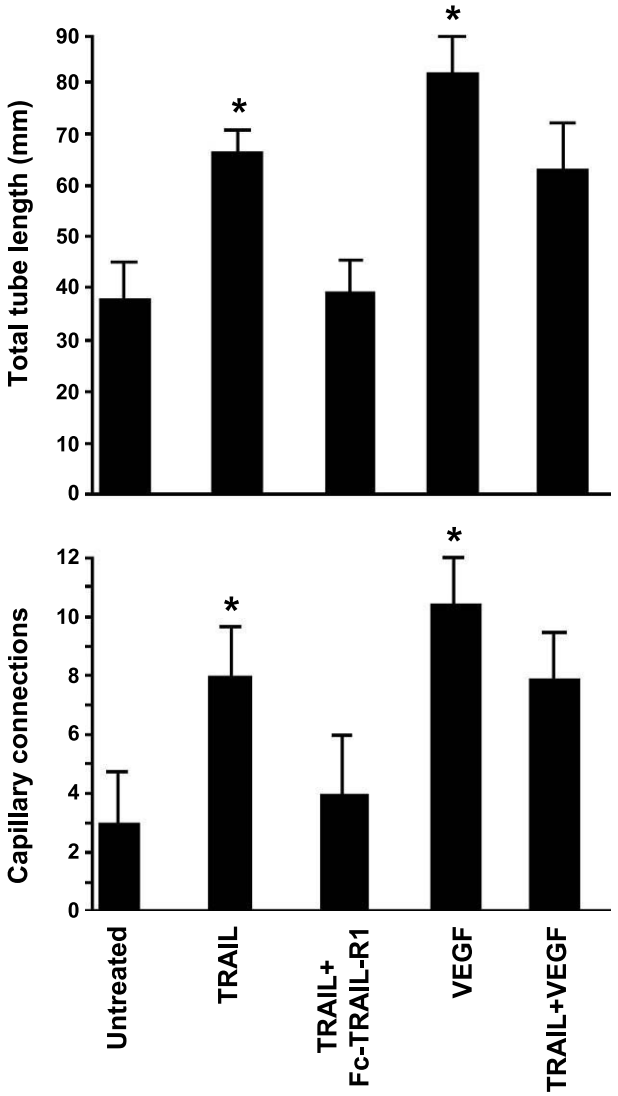

Figure 5. *Lack of additive effect of TRAIL + VEGF on the capillary-like network in coculture angiogenesis assay. Cultures treated as indicated were observed after 12 days and results were recorded for quantitative analysis of the total length of tubes per field and the number of capillary connections per field. Data are expressed as mean ± SD of results from at least five experiments each performed in duplicate. *P < .01 compared to untreated cells.*

simultaneous addition of TRAIL + VEGF resulted in a prolonged activation of ERK phosphorylation with respect to each cytokine used alone (Figure 6*A*). However, although TRAIL was unable to induce p38 phosphorylation, it decreased somewhat the VEGF-induced phosphorylation of p38/MAPK (Figure 6*B*). Consistent with a key role of the ERK pathway in endothelial proliferation, thymidine incorporation assay showed that the combination of VEGF + TRAIL showed an additive effect ($P < .01$) with respect to VEGF or TRAIL used alone (Table 1). Preincubation with the cell-permeable PD098059 compound (20 μM), a commonly used inhibitor of the ERK pathway, completely inhibited thymidine uptake induced by any cytokine combination (Table 1), clearly confirming that the activity of ERK is required for TRAIL- and VEGF-induced mitogenesis.

Moreover, PD098059 treatment strongly suppressed the total tube length and number of tube interconnections in control, TRAIL-treated, and VEGF-treated cocultures (Figure 7), underscoring the key role of ERK pathway in the whole process of tube formation evaluated in the angiogenesis assay.

Discussion

The growth of microvessels is an integral component of tissue remodeling during a variety of normal and pathological events, such as the female reproductive cycle, fetal development, wound healing, inflammation, diabetic retinopathy, and tumor progression [13,14]. The way in which vessels form is being intensively studied because this complex morphogenetic process is very important in medicine. Although a true understanding of the morphogenetic processes involved in tubule formation is still lacking, all these angiogenic events are orchestrated by a network of extracellular factors, including several classes of cytokines, ECM, and integrins, and by their cognate receptors. Several cytokines have been involved in angiogenesis and, in particular, in tumor-associated angiogenesis [13,14]. Besides its involvement in vascular development, VEGF has been demonstrated to play a key role in both physiologic and tumor angiogenesis in adult mammals [25].

It has also been shown in previous studies that some angiogenic regulators belong to the TNF family [26]. Ligands of this family trigger biological activities by binding and signaling through their corresponding receptors in the TNF receptor family. The majority of the TNF family members mediate host defense, inflammation, and immunological regulation, but some of these ligands also regulate endothelial cell functions [26]. For instance, it has been demonstrated that TNF-α modulates endothelial cell behavior; however, its effects are complex. TNF-α inhibits endothelial cell growth yet induces capillary tube formation *in vitro* [27,28]. It also can be antiangiogenic in the context of solid tumors, or angiogenic in corneal settings *in vivo* [27–29]. In a recent study, we have demonstrated that TRAIL functions as an anti–apoptotic factor for endothelial cells [7], and we have hypothesized that TRAIL may contribute to endothelial cell integrity by acting as a survival factor for newly formed blood vessels.

In this study, we have demonstrated for the first time that TRAIL induces a proangiogenic phenotype in human endothelial cells. This phenotype includes both early (increase in migration, invasion, and proliferation) and late (differentiation into vascular cords) angiogenic events. More importantly, TRAIL is angiogenic in a variety of *in vitro* and *in vivo* assays to a degree comparable to VEGF. However, the interplay between TRAIL and VEGF appears rather complex, with TRAIL unable to potentiate VEGF activity in most assays. In fact, TRAIL increased VEGF-induced ERK phosphorylation and HUVEC proliferation; however, TRAIL did not potentiate VEGF-induced p38/MAPK phosphorylation or capillary formation in the

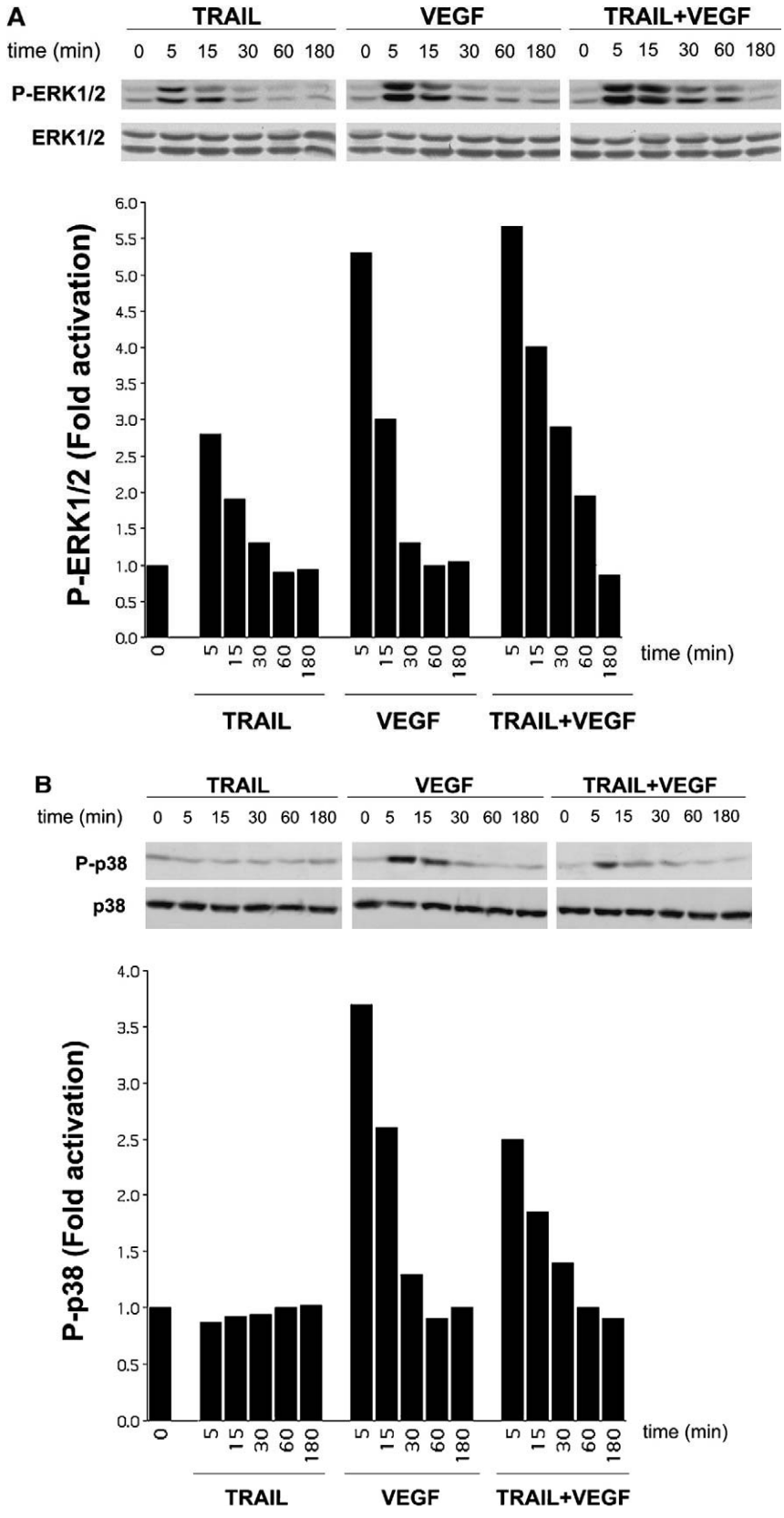

Figure 6. *Phosphorylation of ERK1/2 and p38/MAPK in response to TRAIL and VEGF. Quiescent HUVECs were stimulated with either TRAIL, VEGF, or VEGF + TRAIL for 0 to 180 minutes. Cell lysates were analyzed for ERK1/2 (A) and p38/MAPK (B) activation by Western blot analysis of total and phosphorylated (P) proteins using specific antibodies. Protein bands were quantified by densitometry, and levels of P-ERK1/2 and P-p38 were calculated for each time point, after normalization to ERK1/2 and to p38/MAPK, respectively. Unstimulated basal expression was set as unity. Results are representative of four separate experiments.*

coculture assay. Of note, we have also demonstrated that a clear-cut expression of TRAIL was observed in highly vascularized soft tissue sarcomas. At the moment, we have analyzed a small number of sarcomas; experiments aimed to analyze a high number of tumors and a wide variety of tumors, with the aim to quantitatively evaluate potential correlations between microvessel density and the levels of TRAIL expression, are ongoing.

Besides malignant sarcoma cells, TRAIL was expressed also by tumor-infiltrating lymphocytes and plasma cells. Similar findings were obtained by previous authors describing that TRAIL was expressed by tumor-infiltrating lymphocytes as well as by metastatic gastric cancer cells [30]. Although the most frequent hypothesis to explain the expression of TRAIL by cancer cells is a strategy of immune evasion by which TRAIL-positive malignant cells counterattack against activated T lymphocytes [30,31], our data suggest the alternative—not mutually exclusive—hypothesis that TRAIL expression by cancer cells, and, perhaps also by infiltrating lymphocytes, may play a key role in tumor angiogenesis. Consistent with this hypothesis, it has been shown that mouse BALB/c mammary adenocarcinoma cells engineered to express human TRAIL on their membrane grow faster than the parental cell line in both syngeneic and allogeneic mice [32]. In this respect, it has been clearly established that any significant increase in tumor mass must be preceded by an increase in the vascular supply to deliver nutrients and oxygen to the tumor. The ability of a tumor to induce angiogenesis represents an essential step for tumor growth beyond 2 to 3 mm [13]. Consistent with a potentially important role of TRAIL in tumor angiogenesis not confined to soft tissue sarcomas, it has been demonstrated that all primary astrocytic brain tumors analyzed by immunohistochemistry, including astrocytomas and glioblastomas, express TRAIL protein *in vivo* [33,34]. Similarly, TRAIL is expressed by a subset of lymphomas [35], malignant plasmocytoma cells [36], and ovarian carcinomas [37], in which TRAIL expression has been correlated to the degree of malignancy. Therefore, although recombinant TRAIL protein offers great promise as a cancer therapeutic [38], our current demonstration that TRAIL exerts a potent proangiogenic effect adds a cautionary note to the prolonged treatment of cancer patients with pharmacological concentrations of recombinant TRAIL protein or TRAIL-expressing vectors.

Table 1. Thymidine Incorporation Assay in HUVEC Cultures.

	Vehicle	PD098059
Untreated	2700 ± 350	570 ± 80
TRAIL	3850 ± 600	588 ± 98
VEGF	4200 ± 470	550 ± 90
VEGF + TRAIL	5400 ± 550	600 ± 105

Values are expressed as counts per minute (cpm) per well and are mean±SD of three separate experiments.

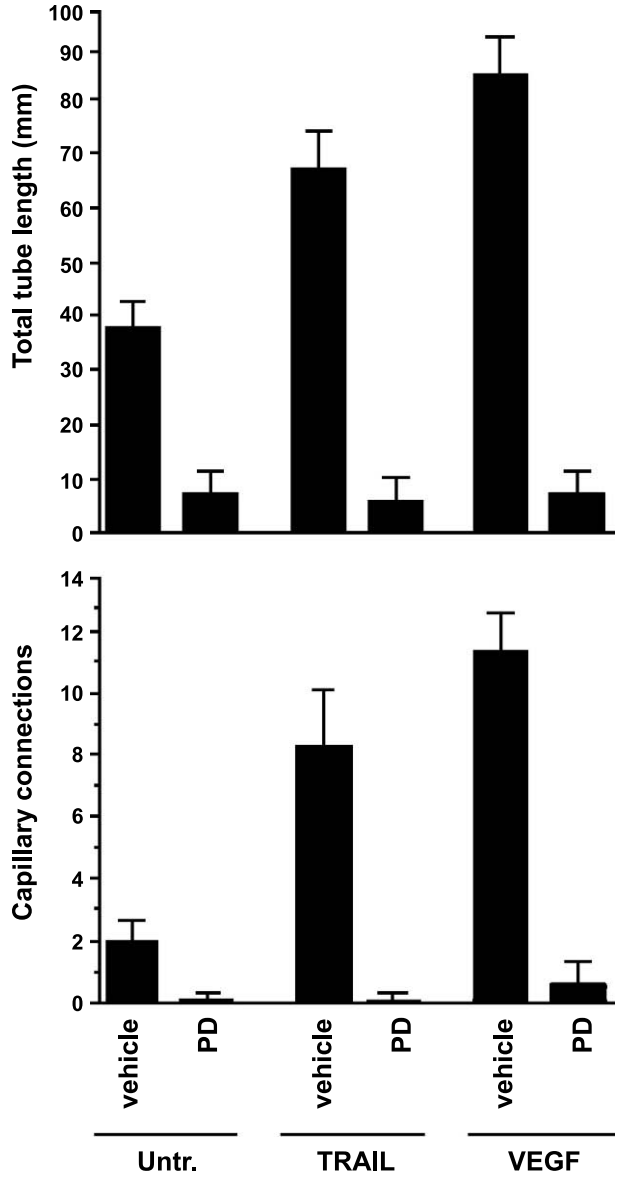

Figure 7. *Role of ERK pathway in capillary-like network formation in the coculture angiogenesis assay. Cultures treated as indicated were observed after 12 days and results were recorded for quantitative analysis of the total length of tubes per field and the number of capillary connections per field. PD, PD98059. Data are expressed as mean ± SD of results from at least five experiments each performed in duplicate.*

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