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Research Article

TRAIL promotes the survival, migration and proliferation of vascular smooth muscle cells

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Abstract. Human and rat primary sub-cultured vascular smooth muscle cells (VSMCs) showed clear expression of the death receptors TRAIL-R1 and TRAIL-R2; however, recombinant soluble TRAIL did not induce cell death when added to these cells. TRAIL tended to protect rat VSMCs from apoptosis induced either by inflammatory cytokines tumor necrosis factor-α + interleukin-1β + interferon-γ or by prolonged serum withdrawal, and promoted a significant increase in VSMC proliferation and migration. Of note, all the biological effects induced by TRAIL were significantly inhibited by pharmacological inhibitors of the ERK pathway. Western blot analysis consistently showed that TRAIL induced a significant activation of ERK1/2, and a much weaker phosphorylation of Akt, while it did not affect the p38/MAPK pathway. Taken together, these data strengthen the notion that the TRAIL/TRAIL-R system likely plays a role in the biology of the vascular system by affecting the survival, migration and proliferation of VSMCs.

Key words. VSMCs; TRAIL; signal transduction; apoptosis; migration; proliferation.

The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)/Apo-2L is a member of the TNF family of cytokines, which play important roles in regulating cell death and inflammation [1]. TRAIL exists either as a type II membrane or as a soluble protein, and interacts with four high-affinity membrane receptors, belonging to the apoptosis-inducing TNF-receptor (R) family. TRAIL-R1 and TRAIL-R2 transduce apoptotic signals upon binding of TRAIL, while TRAIL-R3 and TRAIL-R4 lack the intracellular death domain and apoptosis-inducing capability and have been proposed to function as decoy receptors, protecting certain normal cells, including endothelial cells, from apoptosis [1, 2]. Despite its potential as an anti-cancer therapeutic agent both in vitro and in vivo [3–5], the wide expression of TRAIL and TRAIL-Rs in many normal tissues [1] suggests that the TRAIL/TRAIL-R system likely has uncharacterized biological functions in many tissues. Various groups of investigators, including our own, have shown that endothelial cells express all four transmembrane TRAIL-Rs on their surface [6, 7]. On the other hand, although a study has described TRAIL expression in the tunica media of big vessels in the mouse [8], the role of the TRAIL/TRAIL-R system in the biology of vascular smooth muscle cells (VSMCs) has not been elucidated. Of interest therefore, was to investigate the effects of

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TRAIL on VSMC biology. In particular, we have analyzed the ability of soluble recombinant TRAIL to modulate the survival, proliferation and migration of VSMCs. Moreover, we correlated these biological end-points with the ability of TRAIL to activate intracellular signal transduction pathways, which play a key role in VSMC biology [9–18].

Materials and methods

Materials

Recombinant human histidine 6-tagged $TRAIL_{(114–281)}$ was produced in bacteria, as previously described [7], and tested for cytotoxicity by using HL-60 cells (American Tissue Culture Collection, Rockville, Md.). The absence of endotoxin contamination in the recombinant TRAIL preparations (< 0.1 endotoxin units/ml) was assessed by the *Limulus* amebocyte lysate (LAL) assay (BioWhittaker; Walkersville, Md.). Since recombinant human TRAIL has been previously shown to act on rat cells also [19, 20], it was used for treatments of both human and rat cell cultures, as described below. The optimal TRAIL concentration (10 ng/ml), used in most experiments, was determined in ancillary preliminary assays in which scalar TRAIL doses (ranging from 1 to 1000 ng/ml) were tested. In selected experiments, we also used a recombinant soluble TRAIL (CC-mutant; Alexis Biochemicals, Lausen, Switzerland), which is characterized by an inserted mutation that stabilizes the multimeric conformation. Recombinant rat interferon-γ (IFN-γ; 40 ng/ml) and platelet-derived growth factor (PDGF, 10 ng/ml) were from Peprotec (London, UK); TNF-α (20 ng/ml) and insulin (1 µM) were from Sigma (St Louis, Mo.), while interleukin-1β (IL-1β; 2 ng/ml) was from Roche Diagnostics (Mannheim, Germany). In neutralization experiments, TRAIL was pre-incubated with TRAIL-R1-Fc and/or TRAIL-R2-Fc chimeras, according to the supplier's instructions (R&D Systems, Minneapolis, Minn.). PD98059 was purchased from Calbiochem (La Jolla, Calif.). In preliminary experiments, several doses of PD98059 (from 1 to 100 µM) were tested for their ability to inhibit ERK phosphorylation. A complete and specific abrogation of ERK phosphorylation was observed at concentrations ranging between 25 and 75 µM. Therefore, in most of the following experiments, PD98059 was used at the concentration of 50 μM.

For Western blot analyses, the following antibodies (Abs) were used: anti-human TRAIL (R&D Systems), anti-rat TRAIL (ABR Affinity Bioreagents, Golden, Colo.), anti-TRAIL-R1 (DR4, clone H-130; Santa Cruz Biotechnology; Santa Cruz, Calif.) and anti-TRAIL-R2 (DR5, Chemicon; Temecula, Calif.). The anti-TRAIL-R1 and anti-TRAIL-R2 are reactive against both human and rat antigens. We also employed anti-caspase-3 (sc-7148; Santa Cruz Biotechnology), anti-caspase-6, -7, -8 and -9 (Cell Signaling Technology, Beverly, Mass.), anti-caspase-10 (Medical & Biological Laboratories, Naka-ku Nagoya, Japan), anti-Akt (Transduction Labororatories, Lexington, Ky.), anti-phospho-Akt, anti-ERK1/2, anti-phospho-ERK1/2, anti-p38, anti-phospho-p38 (all from New England Biolabs, Beverly, Mass.).

Immunohistochemistry

Tissue sections of human internal thoracic artery were obtained in accordance with institutional guidelines and processed using standard procedures. Briefly, endogenous peroxidase was blocked with 0.3% H_2O_2 in PBS for 30 min, followed by incubation with avidin and biotin blocking solutions (Vector Laboratories; Burlingame, Calif.), before incubation with the primary anti-human TRAIL mAb (R&D Systems; 1:50 in PBS/1% BSA) for 1 h at room temperature. Slides were subsequently incubated with a secondary biotinylated polyclonal rabbit IgG before exposure to chromogenic substrates in accordance with instructions for the Vectastain Elite ABC Kit (Vector Laboratories). Control tissue sections were developed after incubation with only the secondary Abs.

Cell cultures

Primary human aortic VSMCs were obtained from BioWittaker (Walkersville, Md.) and cultured in SmGM3 medium (BioWittaker) according to the manufacturer's instructions. Primary rat aortic VSMCs were isolated as previously described [21], and routinely maintained in modified Eagle's medium (MEM) containing 10% fetal bovine serum (FBS). Prior to selected experiments, cells were made quiescent by using serum-reduced medium (MEM + 0.1% FBS), without affecting cell viability. VSMCs were used between passages 4 and 8. Induction of apoptosis was carried out either by treatment for 48 h with pro-inflammatory cytokines (IFN-γ + TNF-α + IL-1β), followed by cell washing and culture in serum-free medium for an additional 18 h, or by prolonged (96 h) culture in serum-free medium. Analysis of VSMC viability was performed by light microscopy after Trypan blue dye staining and by fluorescence microscopy after propidium iodide (PI) staining of cells cultured in chamber-slides. Moreover, substrate-attached VSMCs were harvested, pooled with floating cells and apoptotic cells were evaluated by PI/annexin V-FITC staining followed by flow cytometric analysis (see below).

Flow cytometric analyses

Surface expression of TRAIL receptors was evaluated in human VSMC, by indirect staining followed by flow cytometric analyses. Surface cell staining was performed at 4 °C for 40 min by incubating 3×10^5 cells in 200 µl of PBS (containing 1% BSA and 5% human plasma) with the primary mAbs anti-human TRAIL-R1, TRAIL-R2,

TRAIL-R3 and TRAIL-R4 (all from Alexis Biochemicals), followed by PE-conjugated anti-mouse secondary Abs (Immunotech, Marseille, France). Non-specific fluorescence was assessed using normal isotype-matched mouse IgG (Immunotech) followed by secondary antibodies.

For apoptosis evaluation, substrate-attached rat VSMCs were harvested, pooled with floating cells and apoptotic cells were identified by PI/annexin V-FITC double staining (Trevigen, Gaithersburg, Md.) followed by flow cytometric analysis, according to the manufacturer's instruction, and as previously detailed [7]. Flow cytometric analyses were performed by FACScan (Becton Dickinson. San José, Calif.).

Protein preparation and immunoblotting analysis

Aortas were removed from adult Sprague-Dawley rats, kept according to the ethical principles and guidelines for scientific experiments on animals, and homogenized with a glass Teflon homogenizer in freshly made ice-cold lysis buffer (50 mM Tris-HCL, pH 7.5, 0.1 mM EDTA, 0.1 EGTA, 0.1% SDS, 0.1% sodium deoxycholate, 1% NP40, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF). For the studies of intracellular signal transduction pathways, rat VSMCs were plated in 10-cm dishes at a cell density of 20,000 cells/cm^2, and grown at sub-confluence before treatments. To minimize activation by serum, VSMCs were subject to partial FBS reduction (to 0.1%) for 48 h prior to the addition of cytokines. Equal amounts of protein (50 µg) for each sample were migrated in acrylamide gels and blotted onto nitrocellulose filters. Specific reactions were then revealed with the enhanced chemiluminescence reagent (ECL) detection system (DuPont-NEN, Boston, Mass.). Densitometric values were expressed in arbitrary units and estimated by the ImageQuant software (Molecular Dynamics, Piscataway, N. J.). Multiple film exposures were used to verify the linearity of the samples analyzed and to avoid saturation of the film.

[^{3}H] thymidine incorporation

Rat VSMCs were seeded onto 96-well plates and the next day the medium was changed to basal medium containing 0.1% FBS (starvation medium) for 36–48 h. Cells were then incubated with cytokines for 30 h and [^{3}H]thymidine (1 µCi, Perkin Elmer Life Sciences, Boston, Mass.) was added to each well during the last 6 h of incubation. When indicated, cells were pre-treated with PD98059 for 1 h before treatment with cytokines. [^{3}H]thymidine-labeled DNA was then measured by liquid scintillation counting. Assays were done in triplicate.

Cell migration assay

Cell migration was analyzed in a modified Boyden chamber assay [22], using 24-well plates with inserts containing 8-µm pore size collagen-treated polycarbonate membranes separating the two chambers of each well (Transwell, Costar, New York, N. Y.). Briefly, exponentially growing cells were starved (0.1% FBS) for 18–20 h, then resuspended in migration buffer (MEM + 0.5% bovine serum albumin) and placed in the upper compartment of the chambers. Cell viability was always >95%. When indicated, cells were pre-treated with PD98059 for 1 h before seeding. TRAIL, insulin or PDGF were added in the lower chambers. After 5 h 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 triplicate.

Statistical analysis

The medians and ranges, as well as the means ± SD were obtained for each group of experiments. The results were evaluated using analysis of variance with subsequent comparisons by Student's t test and with the non-parametric Mann-Whitney rank-sum test. Statistical significance, evaluated by both statistical tests, was defined as $p < 0.05$.

Results

TRAIL is expressed in the artery wall

In the first group of experiments, we investigated whether TRAIL was expressed in human and rat arteries. As shown in figure 1A, immunohistochemistry demonstrated that TRAIL was clearly expressed in the wall of human internal thoracic artery, with expression prevalent in the smooth muscular layers of the tunica media. Since no reliable antibodies are available for performing TRAIL immunohistochemistry in rat tissues, TRAIL expression was analyzed, and detected, in rat arteries by Western blot performed on aortic ring lysates. An ~45-kDa band, corresponding to the glycosylated full form of TRAIL [23], was clearly detected in rat aortic lysate (fig. 1B).

VSMCs express TRAIL-R1 and TRAIL-R2, but are resistant to TRAIL-mediated cytotoxicity

In parallel experiments, cultured human and rat VSMCs were analyzed for the expression of TRAIL receptors. In flow cytometric analysis, human aortic VSMCs showed significant surface levels of the 'death receptors' TRAIL-R1 and TRAIL-R2, but not of 'decoy receptors' TRAIL-R3 and TRAIL-R4 [1] (fig. 2A). Again, commercial antibodies cross-reacting with rat TRAIL-Rs are available for Western blot but not for flow cytometric analysis. Therefore the expression of the 'death receptors' TRAIL-R1 and TRAIL-R2 in cultured rat aortic VSMCs was ex-

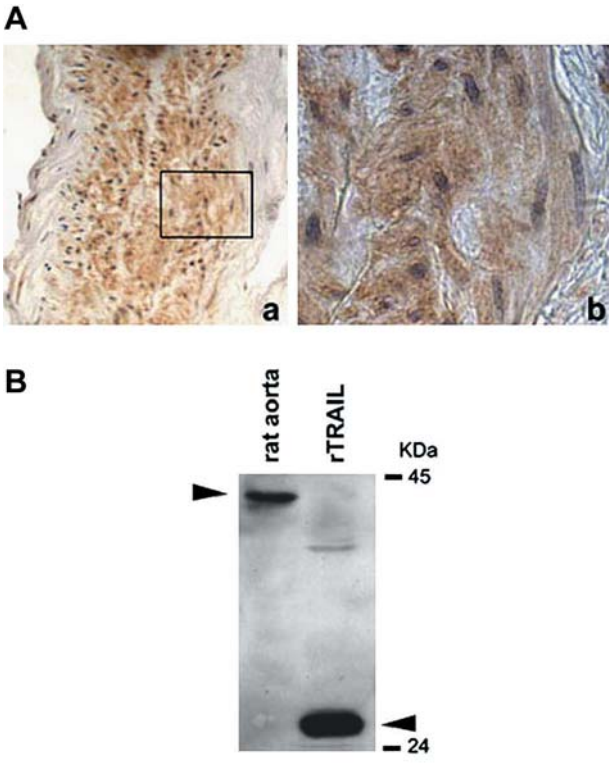

Figure 1. Expression of TRAIL in human and rat arteries. (*A*) Sections of a human internal thoracic artery were examined immunohistochemically using an anti-TRAIL mAb (a, original magnification, ×20). TRAIL expression is predominantly localized in the smooth muscular layers of tunica media (b shows a magnification of the framed region of a, original magnification, ×80). (*B*) Cell lysate of rat aorta analyzed by Western blot with the anti-TRAIL mAb. Molecular-size markers are indicated on the right. The antibody recognizes a protein band of approximately 45 kDa (glycosylated full form of TRAIL). Recombinant soluble $TRAIL_{(114–281)}$ (rTRAIL; approximately 24 kDa) was used as a positive control. A representative of three separate experiments is shown.

amined by Western blot on membrane-cell lysates. As shown in figure 2B, a clear-cut expression of both TRAIL-R1 and TRAIL-R2 was also documented in rat VSMCs. However, despite the expression of both TRAIL-R1 and TRAIL-R2, treatment for 24–48 h with recombinant soluble TRAIL did not induce apoptosis (constantly <5% over background levels) in either human (data not shown) or rat VSMCs, even when used at high concentrations (2 µg/ml), while it significantly induced cell death in HL-60 cells (used at only 100 ng/ml for 24 h) (fig. 2C). Lack of cytotoxicity was noticed even when a polymeric form of TRAIL (CC-mutant) was used to enhance the potency of TRAIL (fig. 2C). This lack of apoptosis induction was not due to a defective expression of procaspases in these cells (fig. 2D).

TRAIL protects rat VSMCs from apoptosis

Taking into consideration the previous demonstration that Fas/CD95 ligand (L), a TNF family member closely

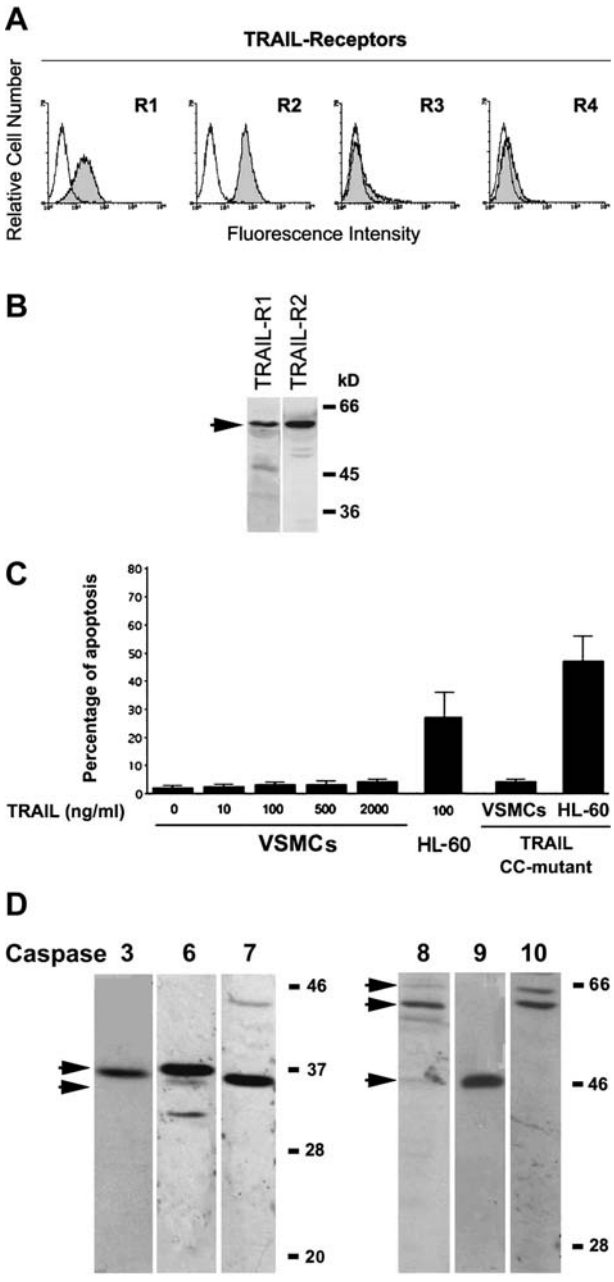

Figure 2. Expression of TRAIL-Rs and lack of TRAIL cytotoxicity on primary VSMCs. (*A*) Surface TRAIL-Rs expression was evaluated in cultured human VSMCs by flow cytometry. Shadowed histograms represent cells stained with Abs specific for the indicated TRAIL-Rs, while unshadowed histograms represent the background fluorescence obtained by staining the same cultures with isotype-matched control Abs. (*B*) TRAIL-R1 and TRAIL-R2 expression was evaluated in rat aortic VSMCs by Western blot analysis of enriched cell membrane lysates. (*C*) Rat VSMCs were treated with the indicated concentrations of soluble recombinant TRAIL or with the multimeric TRAIL (CC-mutant; 100 ng/ml) for 24 h before flow cytometric analysis of apoptotic cells by PI/annexin V staining. HL-60 cells were used as a positive control for their high sensitivity to TRAIL-induced apoptosis. Data represent the means ± SD of five independent experiments. (*D*) Procaspases expressed by VSMCs were analyzed by Western blot using specific Abs. Molecular size markers are indicated on the right (kDa). Results representative of four separate experiments are shown.

related to TRAIL, induces apoptosis of VSMCs after pre-exposure to a mixture of pro-inflammatory cytokines [24], in the next experiments, rat VSMCs were cultured in the presence of IFN-γ (40 ng/ml), plus TNF-α (20 ng/ml) and IL-1β (2 ng/ml) before exposure to TRAIL (10 ng/ml). As expected, pre-treatment with inflammatory cytokines for 48 h, followed by culture in serum-free medium for an additional 18 h, significantly reduced the viability of rat VSMCs. Indeed, at microscopy examination, these cultures showed morphologic alterations typical of apoptosis including cell shrinkage, blebbing, fragmentation into apototic bodies and detachment from the substrate (fig. 3A). Surprisingly, pretreatment of rat VSMCs with inflammatory cytokines for 48 h followed by addition of TRAIL for the additional 18 h of serum-free culture significantly ($p < 0.01$) reduced the apoptosis levels with respect to control cultures. The pro-survival effect of TRAIL was even more pronounced when TRAIL was added simultaneously with the pro-inflammatory cytokines for 48 h before seeding the culture in serum-free medium (fig. 3A). Comparable results were obtained also by flow cytometry analysis after PI/annexin V staining (fig. 3B).

In a different group of experiments, apoptosis was induced in rat VSMCs by serum starvation. At 96 h of serum withdrawal, apoptosis was approximately 30–40% in untreated cells, while again it was significantly ($p < 0.05$) lower in cultures treated with TRAIL (fig. 3C). Of note, the specific cell-permeable compound PD98059, a commonly used inhibitor of the ERK pathway, did not cause any cytotoxic effect, while it significantly ($p < 0.05$) counteracted the protective effect of TRAIL on VSMC apoptosis (fig. 3C).

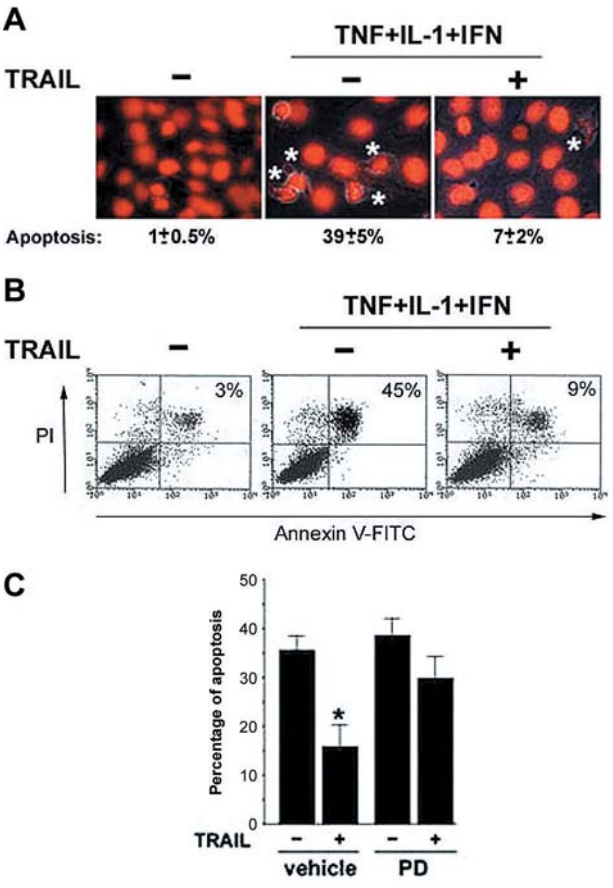

Figure 3. Protection of VSMCs from apoptosis by TRAIL. (*A, B*) Rat VSMCs were either left untreated or treated with pro-inflammatory cytokines (20 ng/ml of TNF-α + 2 ng/ml IL-1β + 40 ng/ml of IFN-γ) in the absence or presence of TRAIL (10 ng/ml), before culture in serum free-medium. Cell viability was analyzed under a fluorescent microscope after nuclear staining with PI. Viable and apoptotic cells were counted (*A*). For each sample, at least 250 cells were counted in different high-power fields, and the percentage of apoptosis is reported. Asterisks indicate representative apoptotic cells (original magnification, ×20). Apoptotic cells were quantitatively evaluated by flow cytometry after PI/annexin-V staining (*B*). The percentage of late-apoptotic cells (upper-right quadrant) is indicated. A representative of three separate experiments, which gave similar results, is shown. (*C*) Rat VSMCs were subjected to serum withdrawal and either left untreated or pre-incubated with PD98059 before exposure to TRAIL. Apoptosis was quantitatively evaluated by flow cytometry after PI/annexin-V staining. Data represent the means ± SD of three to five independent experiments (* $p<0.05$ vs untreated cells).

TRAIL promotes proliferation and migration of VSMCs

Starting from the observation that TRAIL did not induce cytotoxicity and, rather, protected rat VSMCs from apoptosis in the following next experiments we investigated whether TRAIL could affect other relevant biological parameters of VSMCs, such as proliferation and migration. In these experiments, rat VSMCs were also treated with insulin and PDGF, two cytokines known to affect VSMC proliferation and migration [25, 26]. The effect of TRAIL on VSMC proliferation was initially investigated by counting the total number of viable VSMCs 36 h after the addition of the cytokines to subconfluent quiescent cultures. As shown in figure 4A, the total number of VSMCs significantly ($p < 0.05$) increased in TRAIL-treated cultures, as well as in cultures exposed to insulin. This effect was confirmed, and was more evident, by analyzing the thymidine incorporation: TRAIL, insulin and PDGF induced a 2.3-, 3.5- and 2-fold increase, respectively (fig. 4B). In other experiments, the directed migration response of VSMCs toward TRAIL was investigated in Transwell migration chamber assays. We observed a significant increase (>2.0-fold, $p < 0.05$) with respect to control cultures, in directed migration of VSMCs to TRAIL (fig. 4C). As expected [25], insulin induced a weak migration activity, while a significantly ($p < 0.001$) greater migration occurred in response to PDGF, leading to a >6-fold stimulation with respect to control cultures. The specificity of these TRAIL-induced biological effects was confirmed by pre-incubation of TRAIL with TRAIL-R1-Fc or TRAIL-R2-Fc chimeric proteins, which

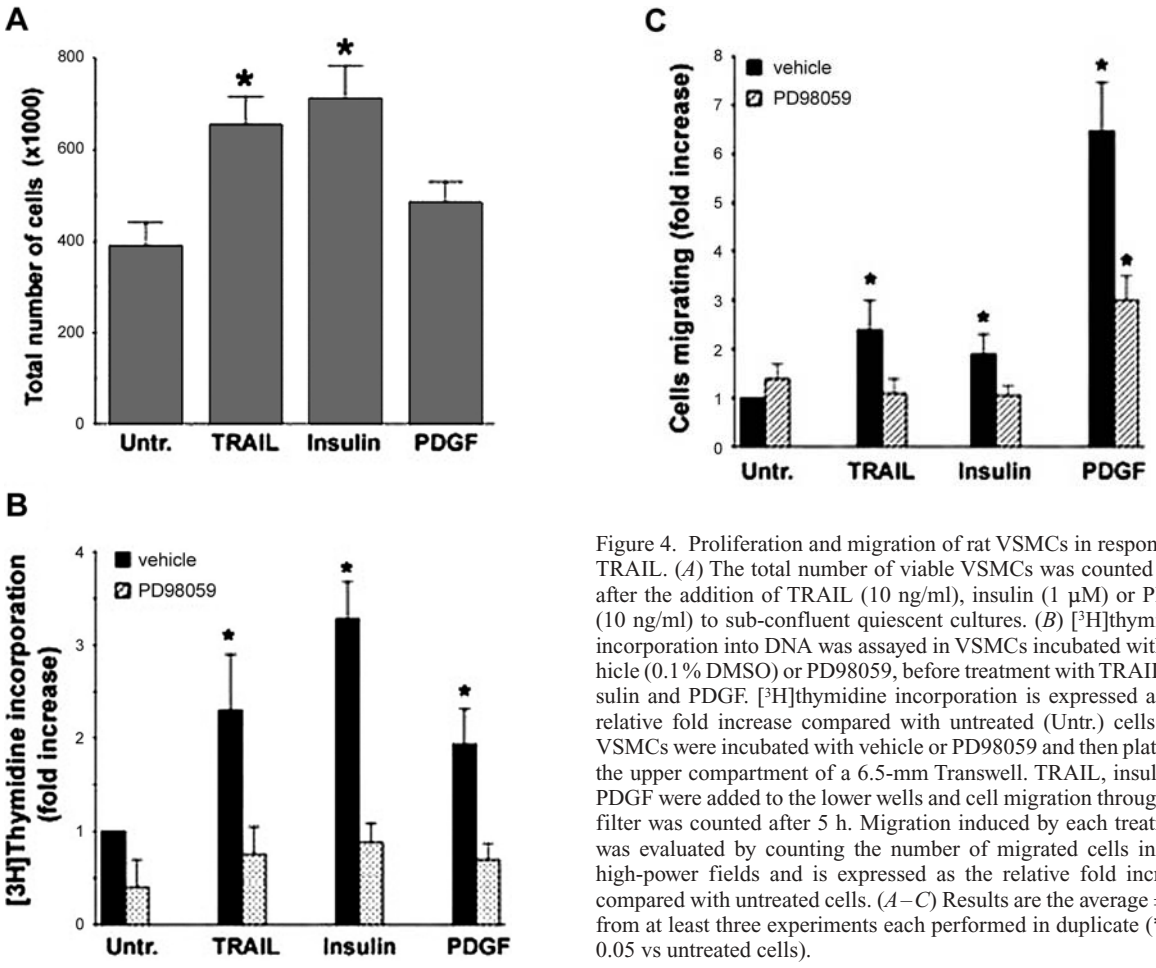

Figure 4. Proliferation and migration of rat VSMCs in response to TRAIL. (*A*) The total number of viable VSMCs was counted 36 h after the addition of TRAIL (10 ng/ml), insulin (1 µM) or PDGF (10 ng/ml) to sub-confluent quiescent cultures. (*B*) [^{3}H]thymidine incorporation into DNA was assayed in VSMCs incubated with vehicle (0.1% DMSO) or PD98059, before treatment with TRAIL, insulin and PDGF. [^{3}H]thymidine incorporation is expressed as the relative fold increase compared with untreated (Untr.) cells. (*C*) VSMCs were incubated with vehicle or PD98059 and then plated in the upper compartment of a 6.5-mm Transwell. TRAIL, insulin or PDGF were added to the lower wells and cell migration through the filter was counted after 5 h. Migration induced by each treatment was evaluated by counting the number of migrated cells in five high-power fields and is expressed as the relative fold increase compared with untreated cells. (*A*–*C*) Results are the average ± SD from at least three experiments each performed in duplicate (* $p < 0.05$ vs untreated cells).

completely ($p < 0.01$) abrogated the ability of TRAIL to promote cell proliferation and migration, without exhibiting by themselves any effect on VSMCs (data not shown). Of note, ERK activation is known to be an important step in both insulin- and PDGF-directed [25, 26] proliferation and migration of VSMCs. Therefore, PD98059 was used to investigate a possible involvement of the ERK pathway in TRAIL-induced proliferation and migration. PD98059 decreased basal cell proliferation as well as that induced by TRAIL, insulin and PDGF ($p < 0.05$) (fig. 4B). Moreover, pre-treatment of VSMCs with PD98059 also significantly ($p < 0.05$) inhibited their migration response toward TRAIL, insulin and PDGF (fig. 4C).

Activation of ERK by TRAIL

To corroborate the above findings obtained with PD98059, suggesting a key role of the ERK pathway in mediating the biological activity of TRAIL on VSMCs, we next examined the effect of TRAIL on ERK/MAPK phosphorylation in VSMCs, by immunoblotting with a phospho-specific ERK1/2-MAPK antibody that recognizes the dually phosphorylated Thr/Glu region derived from the active form of MAP kinase enzymes. The amount of total ERK1/2 was determined in the same cell extracts using an antibody that recognizes ERK1/2 independent of their phosphorylation state. Exposure to TRAIL of quiescent cells induced ERK1/2 phosphorylation from 1 min of treatment onwards (fig. 5A). The peak of activation was observed at 15 min, resulting in a >3-fold increase in phosphorylated, activated ERK/MAPK compared with untreated control ($p < 0.01$). The activation of ERK1/2 by TRAIL was lower, but comparable to that induced by insulin and PDGF, used as positive controls (fig. 5A). Pre-incubation with PD98059 blocked the ability of both TRAIL (fig. 5B), and insulin and PDGF (data not shown) to activate ERK1/2, confirming that the concentration of PD98059 (50 µM), used in the survival, migration and proliferation assays, was effective in suppressing the ERK1/2 pathway. The ability of TRAIL to activate the ERK1/2 pathway was not paralleled by phosphorylation of p38/MAPK (fig. 6A). On the other hand, this pathway was significantly activated by both insulin and PDGF (fig. 6A).

Since the Akt pathway has also been shown to play a significant role in VSMC survival, migration and prolifera-

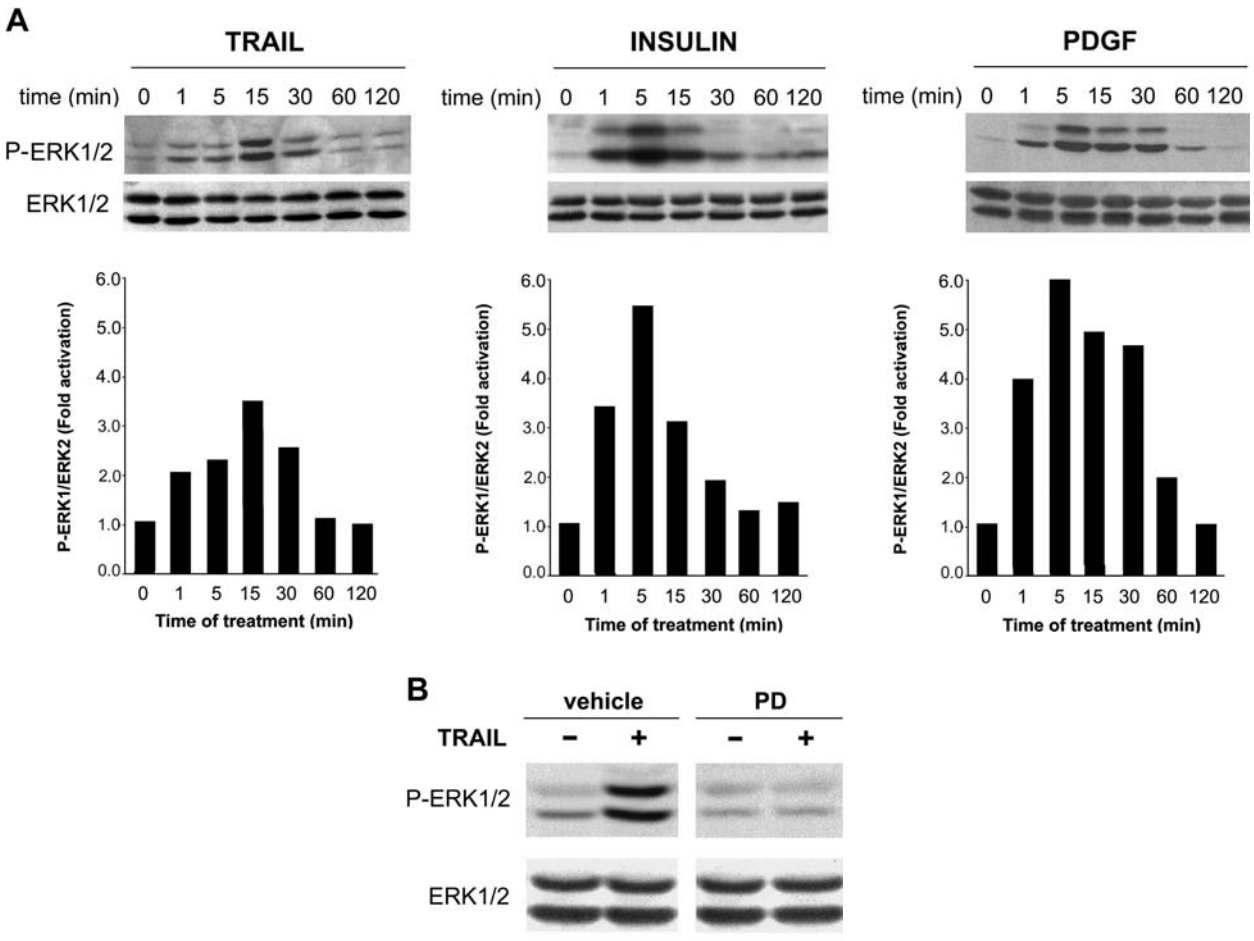

Figure 5. Time-course analyses of ERK1/2 phosphorylation in VSMCs in response to TRAIL. (*A*) rat VSMCs were stimulated with TRAIL (10 ng/ml) for 0–120 min and cell lysates were analyzed for ERK1/2 phosphorylation by Western blot analysis. For comparison, analyses of cells stimulated with insulin (1 µM) or PDGF (10 ng/ ml) are also shown. Protein bands were quantified by densitometry and levels of P-ERK1/2 were calculated for each time point, after normalization to ERK1/2 in the same sample. Unstimulated basal expression was set as unity. (*B*) Cultures were pre-incubated with vehicle (0.1% DMSO) or PD98059, before treatment with TRAIL. Phosphorylation of ERK1/2 was analyzed in cell lysates at 15 min of TRAIL treatment. Equal loading was confirmed by total ERK1/2 staining. Data are representative of three independent experiments that gave similar results.

tion [27], we also evaluated the phosphorylation levels of Akt in response to TRAIL. As shown in figure 6B, only modest Akt phosphorylation was observed in VSMCs upon exposure to TRAIL, in contrast to the potent Akt activation induced by both insulin and PDGF.

Discussion

Expression of TRAIL and TRAIL-Rs mRNAs has been clearly documented in several cells and tissues [**1**], while much less is known for the protein level. In this study, we demonstrated for the first time that TRAIL protein is expressed in both human and rat arteries. In particular, immunohistochemistry revealed that TRAIL is predominantly expressed in the tunica media, as previously suggested for the mouse [8]. Moreover, we demonstrated for the first time that both human and rat VSMCs express the 'death receptors' TRAIL-R1 and TRAIL-R2. However, despite the expression of both death receptors, VSMCs did not undergo apoptosis when treated with recombinant soluble TRAIL, even when these cells were primed with pro-inflammatory cytokines. This implies the existence of intracellular mechanisms which confer resistance to the pro-apoptotic potential of TRAIL. One such mechanism is certainly the activation of the ERK/MAPK pathway. In fact, the ERK1/2 pathway has previously been demonstrated to be involved in the inactivation of the pro-apoptotic protein Bad by phosphorylation and plays a positive role in VSMC survival following oxidant injury [28–30]. Of note, TRAIL activated the ERK/MAPK pathway in VSMCs, similarly to insulin and PDGF. On the other hand, unlike to insulin and PDGF, TRAIL induced only a weak or no activation of Akt and p38 pathways, respectively.

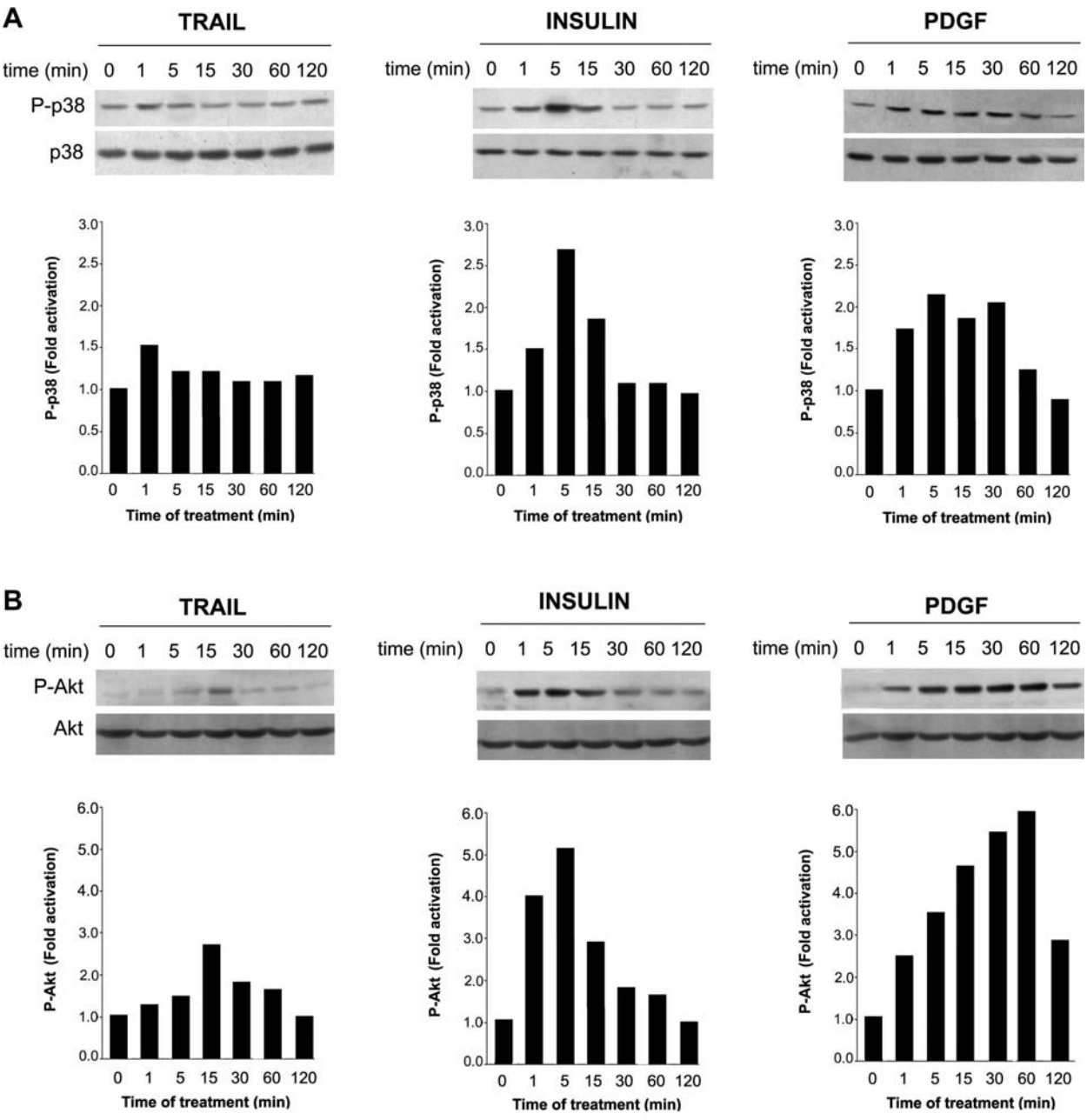

Figure 6. Time-course analyses of p38 and Akt phosphorylation in VSMCs in response to TRAIL. Rat VSMCs were stimulated with TRAIL for 0–120 min and cell lysates were analyzed for p38 (*A*) and Akt (*B*) activation by Western blot analysis of phosphorylated p38 and Akt. For comparison, analyses of cells stimulated with insulin or PDGF are also shown. Protein bands were quantified by densitometry and levels of P-p38 (*A*) and P-Akt (*B*) were calculated for each time point, after normalization to p38 and Akt in the same sample. Unstimulated basal expression was set as unity. A representative of three separate experiments is shown.

Our present findings are noteworthy also in light of our previous data demonstrating that TRAIL also promotes the survival/proliferation of human umbelical vein endothelial cells (HUVECs) [7], strengthening the notion that TRAIL plays an important role in vascular biology. However, several important differences distinguish the effect of TRAIL on endothelial cells versus VSMCs: (i) both HUVEC and human aortic endothelial cells express all TRAIL receptors on their surface [7] while human VSMCs only express TRAIL-R1 and TRAIL-R2; (ii) TRAIL potently activates the Akt pathway in HUVECs [7] while it only modestly activates this pathway in VSMCs; (iii) inhibition of the ERK pathway by PD98059 does not affect the pro-survival activity of TRAIL in endothelial cells [7], while it counteracts the pro-survival activity of TRAIL on VSMCs.

Interestingly, other authors have previously shown that ERK1/2 activity is dramatically induced during VSMC

proliferation in injured arteries of the rat [18] and it is rapidly induced by several cytokines, including insulin and PDGF [25, 26]. Consistent with the previous data that the ERK1/2 pathway is a central element in transducing mitogenic and chemotactic signals in VSMCs [16], we demonstrated here that TRAIL is able to increase VSMC proliferation and migration, and that such effects are abrogated by PD98059. TRAIL acted as a chemoattractant for primary VSMCs at a level comparable to insulin, but significantly lower than that induced by PDGF, one of the most potent chemoattractants for VSMCs [16]. Since the p38/MAPK pathway has been demonstrated contribute significantly to the induction of VSMC migration by PDGF [15–17], the lower activity of TRAIL on the migration of VSMCs when compard to PDGF is likely due to its inability to significantly activate the p38/MAPK pathway in these cells.

TRAIL promoted VSMC migration and proliferation at concentrations (10 ng/ml) in the range of those found in the serum of patients affected by hematological malignancies or HIV-1 disease [31]. Migration and proliferation of VSMCs are crucial events in the formation of intimal thickening in animals and humans with atherosclerosis, which is characterized by additional events, including endothelial cell dysfunction and increased extracellular matrix and lipid deposition. The role of single cytokines or hormones in the pathophysiology of atherosclerosis is very complex [32]. For example, insulin stimulates VSMC migration and proliferation, as also shown in this study, but inhibits migration induced by PDGF [25, 33, 34]. Moreover, during the prolonged process of atherogenesis, complex interactions between the vascular cells and infiltrating immune cells yield local production of a great variety of biologically active substances, such as inflammatory cytokines and growth factors. These bioactive substances may in turn regulate cell functions (such as apoptosis, migration and replication) in a paracrine or autocrine fashion and consequently influence cellularity and morphogenesis of the vessel wall. In particular, pro-inflammatory cytokines, such as INF-γ, a product of activated T lymphocytes, and TNF-α and IL-1β, two cytokines elaborated by activated macrophages, found in atherosclerotic plaques, can profoundly alter the functions and viability of VSMCs [35–37]. We confirmed that the simultaneous exposure to these three cytokines promotes apoptosis of cultured VSMCs, in line with the hypothesis that in atheromatous plaques, activated immune cells may trigger apoptosis of VSMCs by producing these cytokines [32]. Unexpectedly, unlike Fas/CD95L, which plays an important role in the induction of apoptosis in atherosclerotic plaques [38, 39], we demonstrated in this study that TRAIL protected VSMCs from apoptosis triggered by pro-inflammatory cytokines. Therefore, although the overall impact of TRAIL on vascular biology needs to be further addressed in more intact paradigms and in pathological conditions, our findings strongly suggest that TRAIL, which is expressed in the tunica media, likely plays an important role in vascular biology, due to its ability to increase VSMC survival, migration and proliferation.

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