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ORIGINAL ARTICLE *Early pregnancy*

Soluble TRAIL is elevated in recurrent miscarriage and inhibits the *in vitro* adhesion and migration of HTR8 trophoblastic cells

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STUDY QUESTION: What is the potential physiopathological role of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) in recurrent miscarriage (RM), characterized by at least three consecutive pregnancy losses.

SUMMARY ANSWER: The levels of serum TRAIL immediately after miscarriage in RM patients are significantly elevated with respect to that in first-trimester normal pregnant women, and recombinant TRAIL inhibits the adhesion and migration of HTR8 trophoblastic cells *in vitro*.

WHAT IS KNOWN ALREADY: Both TRAIL and its trans-membrane receptors (TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4) have been documented in the placenta, but their physiopathological role is incompletely understood.

STUDY DESIGN, SIZE, DURATION: The study populations consisted of RM patients ($n = 80$) and first-trimester normal pregnant women ($n = 80$). Blood samples were obtained within 24 h after abortion (RM) or at gestational 12-week (normal pregnant women). As additional controls, third-trimester normal pregnant women ($n = 28$) were examined before (within 72 h) and after (within 24 h) partum.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The concentrations of TRAIL were analysed in serum samples by ELISA. In parallel, the effect of soluble recombinant TRAIL (0.1–1000 ng/ml) was analysed on the survival of primary extravillus trophoblasts (EVTs) and on the survival, proliferation, adhesion and migration of trophoblastic HTR8 cells.

MAIN RESULTS AND THE ROLE OF CHANCE: The circulating levels of TRAIL in RM women (median: 52.5 pg/ml; mean and SD: 55.5 ± 24.4 pg/ml) were significantly higher with respect to first-trimester normal pregnant women (median: 44.9 pg/ml; mean and SD: 47 ± 15.1 pg/ml) and third-trimester normal pregnant women, as assessed before (median: 45.1 pg/ml; mean and SD: 46 ± 12.4 pg/ml) and after partum (median: 35.4 pg/ml; mean and SD: 38 + 17.5 pg/ml). Both primary EVT and HTR8 cells expressed detectable levels of TRAIL death receptors, but exposure to soluble recombinant TRAIL did not induce cell death of trophoblastic cells. On the other hand, TRAIL dose-dependently inhibited the adhesion of HTR8 cells to decidual endothelial cells (DEC) as well as the migration of HTR8 in transwell assays using either fibronectin or DEC.

LIMITATIONS, REASONS FOR CAUTION: Although this study suggests that TRAIL might have a pathogenic role in RM by inhibiting both the adhesion and migration capabilities of first trimester trophoblastic cells, there is a possibility that the elevated serum levels of TRAIL in RM are not cause but rather the result of RM.

WIDER IMPLICATIONS OF THE FINDINGS: Our current findings together with data of other authors suggest that circulating TRAIL should be further analysed as a potential important biomarker in different physiopathological settings.

[†]These authors contributed equally to this work.

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Key words: TRAIL / trophoblasts / cell adhesion / cell migration / recurrent miscarriage

Introduction

Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a TNF superfamily member present in detectable amounts as a soluble cytokine in the serum of normal individuals (Secchiero and Zauli, 2008). TRAIL is as a homotrimer which interacts with four transmembrane TNF-related apoptosis-inducing ligand receptors (TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1 and TRAIL-R4/DcR2) and with the soluble receptor osteoprotegerin (OPG) (Zauli *et al.*, 2009). TRAIL-R1 and TRAIL-R2 play an essential role in mediating the apoptotic response upon the binding of TRAIL. However, other experimental data indicate that TRAIL-R1 and TRAIL-R2 also mediate non-apoptotic intracellular signal transduction pathways (Di Pietro and Zauli, 2004). TRAIL-R3, TRAIL-R4 and OPG are decoy receptors for TRAIL, but their ability to neutralize the biological activity of TRAIL is still under debate. It should also be noticed that TRAIL and its receptors are widely expressed, suggesting that the TRAIL/TRAIL receptor system likely plays unrecognized physiological roles in different human tissues (Di Pietro and Zauli, 2004). In particular, among different tissues, TRAIL and its receptors are expressed in the placenta (Phillips *et al.*, 1999; Phillips *et al.*, 2001; Lonergan *et al.*, 2003; Chen *et al.*, 2004; Keogh *et al.*, 2007; Klugman *et al.*, 2008; Bai *et al.*, 2009; Fluhr *et al.*, 2009; Sokolov *et al.*, 2009; Groebner *et al.*, 2010). Although TRAIL has been proposed to contribute to the establishment of immune privilege during pregnancy (Phillips *et al.*, 1999; Phillips *et al.*, 2001), the potential physiopathological role of TRAIL in the placenta has not been deeply investigated.

Taking into account of the previous studies, our current study aimed to analyse the serum levels of TRAIL in women with recurrent miscarriage (RM, with at least three consecutive pregnancy losses) with respect to normal pregnant women, assessed during the first trimester and before and after delivery. Furthermore, *in vitro* studies were designed to investigate the potential biological activity of soluble TRAIL on the apoptosis induction, proliferation rate, adhesion to different substrated and migration in a human first-trimester-derived throphoblastic cell line (HTR8).

Materials and Methods

Study population and TRAIL measurements

The study population consisted of RM patients ($n = 80$) and first-trimester normal pregnant women ($n = 80$). Blood samples were obtained within 24 h after abortion (RM) or at gestational week 12 (normal pregnant women). As additional controls, third-trimester normal pregnant women ($n = 28$) were examined before (within 72 h) and after (within 24 h) partum. The procedures followed were in accordance with the Declaration of Helsinki and approved by the institutional review board (Institute for Maternal and Child Health, IRCCS Burlo Garofolo of Trieste). All participant subjects gave written informed consent. The samples were immediately centrifuged, aliquoted and the sera were frozen at −80°C until biochemical measurements. The levels of circulating TRAIL were analysed by ELISA (R&D Systems, Minneapolis, MN, USA) in duplicate serum samples, as previously described (Campioni *et al.*, 2005). Sensitivity of the assay was 2.86 pg/ml and the upper limit of detection was 1000 pg/ml. Selected samples were run in each ELISA plates as internal controls to confirm the reproducibility of the determinations over time.

Cell isolation and cultures

Trophoblast cells were purified from first trimester placental specimens (range: 8–12 weeks of gestation), as previously described (Agostinis *et al.*, 2012). Briefly, after incubation with HBSS plus 0.25% trypsin plus 0.2 mg/ml DNase (Roche, Milan, Italy) for 20 min at 37°C, cells were fractionated through Percoll gradient. Leucocytes were eliminated by immunomagnetic beads coated with mAb to CD45 (Dynal, Invitrogen, Milan, Italy). The remaining cells were seeded in 25-cm^2 flask coated with 5 $\mu g/cm^2$ fibronectin (FN, Roche) in RPMI 1640 (Gibco, Invitrogen) plus 10% fetal calf serum (FCS). After overnight culture, cells were detached by using trypsin–EDTA and characterized for the expression of cytokeratin 7 (95% positivity) and of HLA-G (>70% positivity), a marker of extravillous trophoblasts (EVTs).

The human EVT cell line, HTR8/SVneo (Graham *et al.*, 1993), was provided by Peeyush K. Lala (Department of Anatomy and Cell Biology, University of Western Ontario, Canada) and grown in RPMI 1640 medium plus 10% FBS. Decidual endothelial cells (DEC) were isolated from decidual biopsy specimens as previously described (Agostinis *et al.*, 2010). At flow cytometry, cells showed a positivity of 95% for von Willebrand factor (Dako-Cytomation). DEC were plated in 12.5-cm^2 flask precoated with 5 $\mu g/cm^2$ of fibronectin (FN, Roche Diagnostics, Mannheim, Germany) and cultured in endothelial serum-free basal medium (Gibco, Invitrogen) plus 20 ng/ml of basic fibroblast growth factor and 10 ng/ml of epidermal growth factor (EGF) (Gibco, Invitrogen). In some experiments, DEC cells were cultured for 24 h in RPMI supplemented with 10% FCS and the decidual cell-conditioned medium (DCCM) was collected, stored at −80°C and used as the source of chemotactic stimuli in the migration assays.

Phenotypic analyses, apoptosis and proliferation assays

The expression of TRAIL receptors was analysed by using PE-conjugated mAb anti-human TRAIL-R1 (FAB347P), TRAIL-R2 (FAB6311P), TRAIL-R3 (FAB6302P) and TRAIL-R4 (FAB633P; all from R&D Systems) as previously described (Secchiero *et al.*, 2007; Zauli *et al.*, 2007). Cell survival was evaluated by Trypan blue dye exclusion and MTT assays, as previously described (Milani *et al.*, 1996), while apoptosis was evaluated after treatment with 0.1–100 ng/ml of recombinant TRAIL for 24–96 h, as previously described (Zauli *et al.*, 1996; 2008). In order to analyse the degree of apoptosis in the entire cell population, adherent HTR8 cells were recovered with 0.25% trypsin–EDTA and added to floating cells. After double-staining with PI and FITC-conjugated Annexin V (Alexis Biochemical, Lausen, Switzerland,), cells were analysed by flow cytometry as previously detailed (Campioni *et al.*, 1995; Secchiero *et al.*, 2009). In selected experiments, cells were pre-incubated with recombinant human OPG (R&D Systems) at a 1:1 molar ratio.

Cell proliferation was analysed by using the DP version of the xCELLigence real-time cell analyzer RTCA (Roche Diagnostics, Mannheim, Germany), which records changes in impedance [reported as a cell index (CI)] over a prolonged time course in a non-invasive system. All data were then analysed using the xCELLigence software (version 1.2.1). The background impedance of RTCA DP E-Plates 16 was performed using the standard protocol provided in the software with 100 μl of cell culture medium. Cells were seeded in quadruplicate at three different concentrations before treatment with recombinant TRAIL.

Cell adhesion and migration assays

For cell adhesion assay, confluent DEC were co-cultured in 96-microwell plates (Iwaki, Bibby Scientific Italia, Milan, Italy) for 45 min at 37°C with HTR8 (2×10^5 cells/100 μl) labelled with the Fast DiI fluorescent dye (Molecular Probes, Invitrogen) and previously incubated with TRAIL. Floating HTR8 cells were removed by washing with Dulbecco-PBS containing Ca^{2+} and Mg^{2+} (0.7 mM), and adherent HTR8 cells were scored with Infinite200 (ABS 544 nm, EM 590 nm) (TECAN Italia S.r.l., Milano, Italy), as previously described (Agostinis *et al.*, 2010).

The migration assay through FN was performed in transwell chambers using Fast DiI-labelling (Molecular Probes, Invitrogen). HTR8 (2×10^5 cells) were resuspended in RPMI with 0.1% bovine serum albumin (BSA) and added to the upper chamber. The cells were allowed to migrate through HTS FluoroBlokTM 8-μm pore inserts (Becton Dickinson, Falcon, Milan, Italy) coated on the lower side with FN (20 μg/ml). DCCM diluted 1:4 in RPMI with 0.1% BSA was added to the lower chamber as a chemoattractant. The number of cells transmigrated to the lower side of the insert after overnight incubation at 37°C was evaluated with Infinite200 (ABS 544 nm, EM 590 nm). For the transendothelial migration assay, DEC (2×10^4) were seeded onto 20 μg/ml FN-coated polycarbonate inserts of a 24-well FloroBlock Transwell system (6.5 mm diameter, 8-μm pores; BD Falcon) and these were used 6 days after plating. HTR8 (2×10^5 cells/100 μl) labelled with Fast DiI were added to the upper compartment of the transwell. Cells were allowed to migrate overnight using 1:4 diluted DCCM as a chemoattractant. Cells present in the lower chamber or adherent to the lower surface of the transwell were counted as indicated above, with multiple reads of same well, and the number of migrated cells was expressed as a percentage with reference to a calibration curve established with increasing numbers of labelled HTR8 plated in the lower chamber.

Statistical analysis

For each set of experiments, values are reported as means ± SD. For selected experiments, the results are reported as box plots showing the median, minimum and maximum values and 25–75th percentiles. The results were evaluated by using Student's *t*-test and the Mann–Whitney rank-sum test. Statistical significance was defined as $P < 0.05$.

Results

Circulating levels of TRAIL are significantly increased in RM with respect to control pregnant women

In previous studies, we have demonstrated that TRAIL serum levels are similar in the first and second trimester of pregnancy (Zauli *et al.*, 2011) and that TRAIL serum levels determined in the first trimester do not show significant variations between normal women and women who will develop pre-eclampsia in the third trimester of gestation (Zauli *et al.*, 2012). Since the primary aim of this study was to investigate the physiopathological significance of serum TRAIL during pregnancy, we have analysed the serum levels of TRAIL in a group of RM patients ($n = 80$) in comparison with normal first-trimester pregnant women ($n = 80$) (Fig. 1). The reason for choosing first-trimester normal pregnant woman as a control group was made on the assumption that placental-derived hormones could exhibit effects on TRAIL serum levels in both RM patients and normal pregnant women. Unexpectedly, we found that the values of circulating soluble TRAIL were significantly ($P < 0.01$) higher in RM patients with respect to first-trimester pregnant women (Fig. 1). Since we could analyse the serum levels of TRAIL in RM patients only after the miscarriage (within 24 h), it was necessary to rule out the possibility that the increase in serum TRAIL merely reflects the interruption of pregnancy. Therefore, in additional experiments we analysed the serum levels of TRAIL in paired samples harvested from women ($n = 28$) before and after partum (within 24 h). As shown in Fig. 1, after delivery, TRAIL levels decreased significantly ($P < 0.05$). Consequently, also TRAIL levels in RM patients were still significantly elevated when compared with third-trimester pregnant women both before and after delivery (Fig. 1), allowing us to exclude the possibility that the increased levels of serum TRAIL in RM patients was due to the interruption of pregnancy.

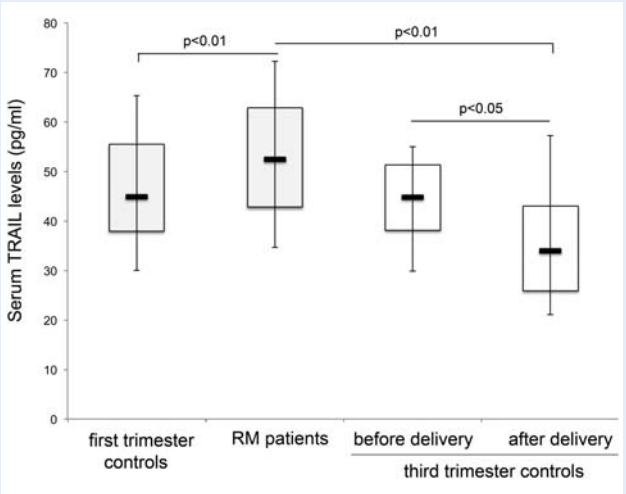

Figure 1 Serum TRAIL is significantly increased in patients affected by recurrent miscarriage (RM). Comparative analysis of soluble TRAIL levels measured in serum samples of RM patients ($n = 80$) and of control pregnant woman (first trimester; $n = 80$; third trimester $n = 28$, assessed both before and after delivery). Levels of TRAIL were determined by ELISA. Horizontal bars are median, upper and lower edges of the box are 75th and 25th percentiles, lines extending from the box are 10th and 90th percentiles.

Human trophoblastic cells express TRAIL receptors, but do not show susceptibility to TRAIL-induced apoptosis

In order to investigate the potential physiopathological role of excessive TRAIL release during gestation, we have used, as an *in vitro* model system, the first trimester-derived HTR8 trophoblastic cell line (Graham *et al.*, 1993). Initially, we evaluated the phenotypic surface

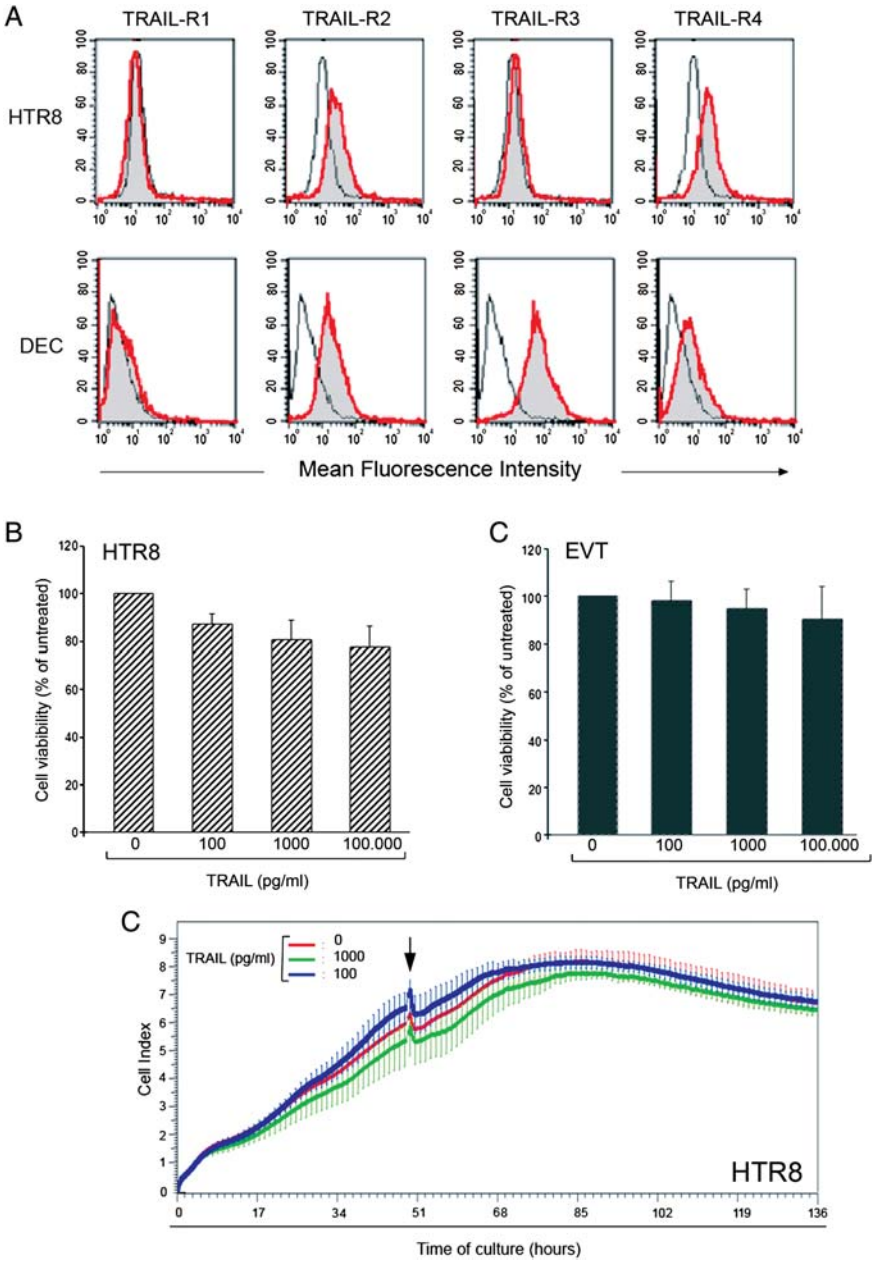

Figure 2 Soluble TRAIL does not affect HTR8 cell viability and proliferation. In (**A**), the surface TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4 expression was evaluated in HTR8 and DEC by flow cytometry analysis. Shaded histograms represent cells stained with mAbs specific for the indicated surface antigens, while unshaded histograms represent the background fluorescence obtained from the staining of the same cultures with isotype-matched control mAbs. Representative phenotypes are shown. In (**B**) and (**C**), HTR8 cells and primary EVT were cultured in the absence or presence of recombinant human TRAIL (used at the indicated concentrations). Cell viability was assessed after 48 h of treatment and the results are expressed as percentages with respect to untreated cultures. In (**D**) cell proliferation, in the absence or presence of recombinant human TRAIL (used at the indicated concentrations), was evaluated using the xCELLigence real-time cell analyzer (RTCA) and was expressed as CI. Arrow, time of TRAIL addition. A representative profile of HTR8 proliferation is shown.

expression of transmembrane TRAIL receptors (TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4). HTR8 showed a clear-cut expression of the death receptor TRAIL-R2 and of the decoy receptor TRAIL-R4 (Fig. 2A). On the other hand, DEC, used as controls, showed the expression of TRAIL-R2 and TRAIL-R4 and a high expression of TRAIL-R3 (Fig. 2A).

Since the best-characterized activity of TRAIL is to induce apoptosis of different cell types (Secchiero and Zauli, 2008), we next investigated

whether HTR8 cells were susceptible to TRAIL-mediated apoptosis. At both physiological (0.1–1 ng/ml) and pharmacological (100 ng/ml) concentrations, TRAIL induced no apoptosis or only a low level of apoptosis (Fig. 2B). The observations generated in the experiments on HTR8 cells were validated on primary EVTs, obtained as previously described (Agostinis *et al.*, 2012). Indeed, the primary EVTs also showed a constant expression of TRAIL-R2 and of the decoy receptors, with some variability based on the different cell preparations (data not shown). Exposure to soluble recombinant TRAIL did not affect EVT viability, at any TRAIL concentration used (Fig. 2C). In keeping with the lack of apoptosis induction, the overall effect of TRAIL on HTR8 cell proliferation was negligible (Fig. 2D).

TRAIL reduces HTR8 adhesion to DEC

Adhesion of trophoblast cells to the endothelial cells was next assessed using DEC-binding assays to determine whether TRAIL might affect HTR8 trophoblastic adhesion to endothelial cells, a step considered essential in normal placental development (Stoikos *et al.*, 2010). Initial experiments identified that untreated HTR8 cells under control conditions bound efficiently to DEC. There was a significant decrease in binding to the endothelial cells ($P < 0.05$) following HTR8 treatment with 1 ng/ml of recombinant TRAIL (Fig. 3). When TRAIL was pre-incubated with its decoy receptor recombinant OPG, the DEC binding of HTR8 cells showed a small but significant increase with respect to cells treated with TRAIL alone (data not shown).

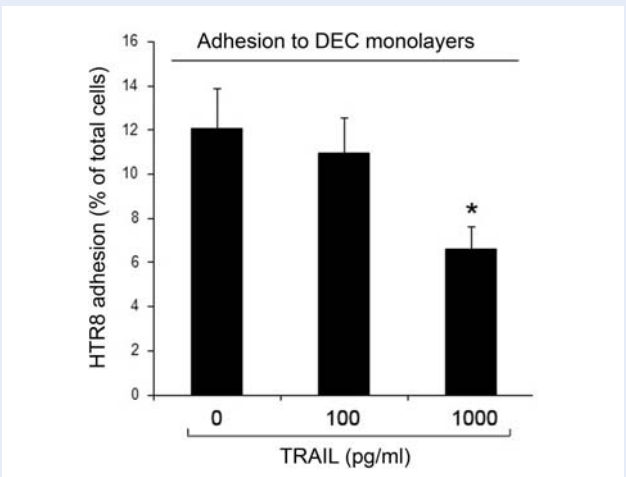

Figure 3 Anti-adhesive property of soluble TRAIL on HTR8 cells. HTR8 cells were labelled with a fluorescent dye and pretreated with recombinant human TRAIL (used at the indicated concentrations) before seeding on a confluent DEC monolayer. HTR8 adhesion was measured after 45 min of culture at 37°C and the results are expressed as percent of adhesion with reference to a standard curve established with an increasing number of labelled cells. Data are means ± SD of results from four experiments each performed in triplicate. Asterisks, $P < 0.05$ with respect to the untreated cultures (TRAIL = 0).

TRAIL counteracts HTR8 trophoblastic cell migration through both FN and DEC

Previous studies have clearly demonstrated that HTR8 cells are able to migrate, mimicking the activity of cytotrophoblast cells (Graham *et al.*, 1993). Cell migration through the endothelial cell matrix component

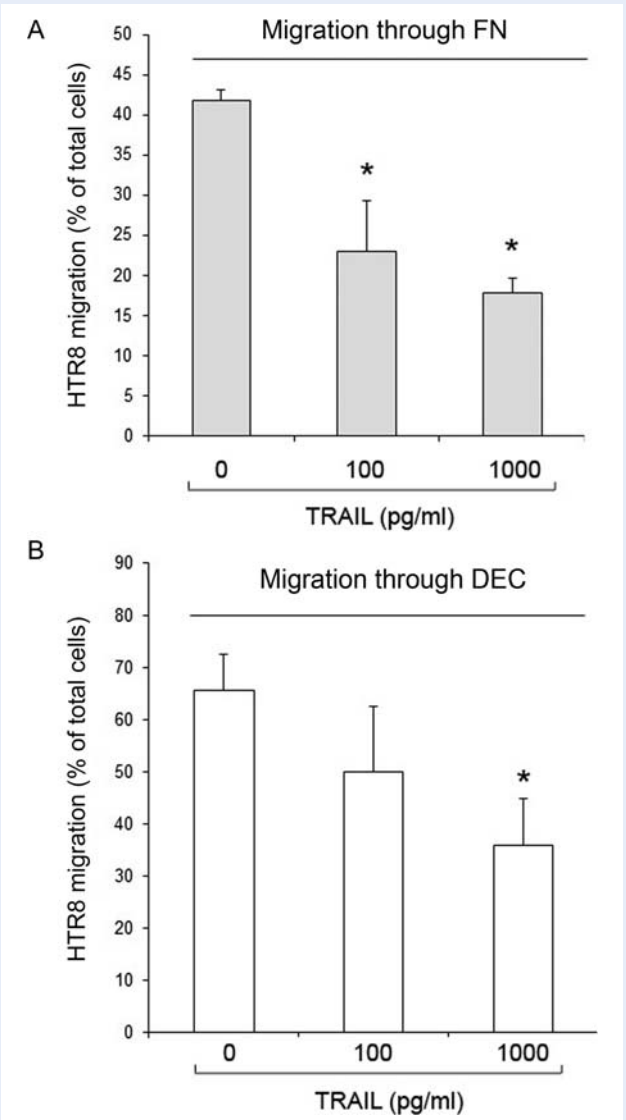

Figure 4 Anti-migratory property of soluble TRAIL on HTR8 cells. In (**A**) and (**B**), HTR8 cells were labelled with a fluorescent dye and treated with recombinant human TRAIL at the indicated concentrations. In (**A**) the migration of HTR8 cells was evaluated after adding the cells to the upper chamber of a transwell and allowing them to migrate overnight through inserts coated with FN in the presence of 1:4 diluted DCCM in the lower chamber as a chemotactic stimulus. In (**B**) transendothelial migration ability of HTR8 cells was evaluated after allowing the cells to migrate overnight through a confluent DEC monolayer. In (A) and (B), the results are expressed as percent of migration with reference to a standard curve established with an increasing number of labelled cells. Data are reported as means ± SD of results from at least four experiments, each performed in triplicate. Asterisks, $P < 0.05$ with respect to the untreated cultures (TRAIL = 0).

FN was assessed using matrix-binding assays to determine whether TRAIL regulates trophoblast migration to this ligand. The directed migration of HTR8 toward DCCM was investigated by measuring the transfilter migration. TRAIL (0.1–1 ng/ml) significantly inhibited DCCM-mediated migration in a dose-dependent manner (Fig. 4A). In parallel experiments, recombinant TRAIL also efficiently counteracted the transendothelial migration of HTR8 cells (Fig. 4B).

Discussion

It has been hypothesized that TRAIL expression at the placenta level represents a key element in the establishment of placental immune privilege by protecting placenta from immune cell attack of maternal cytotoxic lymphocytes and NK cells during normal pregnancy (Phillips *et al.*, 1999; Phillips *et al.*, 2001; Sokolov *et al.*, 2009). In addition, similarly to our data obtained in the HTR8 cell line, Bai *et al.* (2009) demonstrated that recombinant TRAIL failed to induce apoptosis in primary trophoblast cells and this was due to the nuclear localization of the death receptor TRAIL-R2/DR5. Interestingly, only treatment with the pro-inflammatory cytokine TNF-α caused the redistribution of intracellular TRAIL-R2 to the cell surface, potentiating apoptotic susceptibly to exogenously administered recombinant TRAIL (Bai *et al.*, 2009). In keeping with a protective role of placental TRAIL during physiological pregnancy, it has been recently demonstrated (Pantham *et al.*, 2012) that placental TRAIL expression is significantly decreased upon *in vitro* treatment of placental explants with antiphospholipid antibodies, which are involved in the pathogenesis of pre-eclampsia (Katano *et al.*, 1996) as well as of RM (Honig *et al.*, 2010). Thus, in consideration of all previous studies documenting that TRAIL is expressed in the placenta during all trimesters of gestation, and likely plays a physiological role in normal pregnancy, it should also be taken into account that our current data demonstrating elevated serum levels of TRAIL in RM may not be the cause, but rather the result of RM.

In our current study, we have analysed the effect of recombinant TRAIL on the survival and proliferation as well as on the adhesive and migratory properties of HTR8, a transformed cell line derived from first trimester placenta culture explants (Graham *et al.*, 1993). We are aware that our *in vitro* data obtained on HTR8 might not reflect the biological activity of TRAIL on primary trophoblasts, neither in terms of apoptosis induction, as HTR8 cells are immortalized, nor in terms of proliferation and migration properties, which are usually mutually exclusive in normal trophoblasts. However, we were able to demonstrate that primary EVTs, although expressing TRAIL receptors, were also resistant to TRAIL cytotoxicity (Soloveva and Linzer, 2012). In spite of the limitations of the HTR8 cell model, it is noteworthy that TRAIL induced a modest cytotoxicity on HTR8 only when used at high concentrations of TRAIL. On the other hand, no significant effects on cell proliferation were observed even at 100–1000 ng/ml of recombinant TRAIL. However, interesting biological effects were noted on the adhesive and migratory properties of HTR8 cultured in the presence of TRAIL. In fact, recombinant TRAIL significantly inhibited HTR8 adhesion to DEC at concentrations of 0.1–1 ng/ml, which are similar to those found in normal human serum/plasma (Zauli *et al.*, 2011). Perhaps more interestingly, recombinant TRAIL also counteracted the migratory activity of HTR8 in two relevant experimental settings: in transwells coated with FN and in transwells coated with DEC. Also in this case, the biological effects of TRAIL were observed at low concentrations (0.1–1 ng/ml), which are likely to be reached in physiological or physiopathological conditions.

With respect to our key finding that the serum levels of TRAIL in a group of RM patients were significantly higher than those found in normal pregnant women (both before and after delivery), it is noteworthy that a recent study (Rull *et al.*, 2012) has independently demonstrated that the circulating levels of TRAIL are elevated in RM patients as compared with first-trimester pregnant women, and has also shown that TRAIL mRNA is significantly elevated at the placenta level in RM patients. While in their study, Rull *et al.* (2012) proposed the use of soluble TRAIL as a biomarker for complicated pregnancies, in our study we offer a potential mechanism for explaining how excessive levels of TRAIL may adversely affect pregnancy. Indeed, a potential pathogenic role of elevated levels of TRAIL in RM might be explained by previous findings of Keogh *et al.* (2007), who proposed a role of TRAIL in remodelling of the uterine spiral arteries during pregnancy, an essential event which transforms them from high to low resistance vessels that lack vasoconstrictive properties. Specifically, trophoblasts isolated from first trimester placenta were shown to express membrane-associated TRAIL and to induce apoptosis of human vascular smooth muscle cells (VSMC), and thus may contribute to VSMC loss during spiral artery remodelling in pregnancy (Keogh *et al.*, 2007). It is possible that high levels of TRAIL induce excessive apoptosis in VSMC. In addition, our current *in vitro* data suggest that, in RM, TRAIL might have an excessive inhibitory effect on key activities of the cytotrophoblast, by inhibiting its adhesive and migratory properties, thus altering the delicate balance required for healthy pregnancy. Clearly, the physiopathological significance of our findings requires additional investigation.

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Authors' roles

C.A. and R.B. carried out experiments, collection and assembly of data, data analysis and interpretation. V.T. carried out experiments, data analysis and interpretation; F.DS. and S.A. contributed to provision of study material. P.S. involved in conception and design, financial support, assembly of data, data analysis and interpretation, manuscript writing; G.Z. involved in conception and design, financial support, manuscript writing, final approval of manuscript.

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Conflict of interest

None declared.

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