TRAIL counteracts the pro-adhesive activity of inflammatory cytokines in endothelial cells by down-modulating CCL8 and CXCL10 chemokine expression and release

Short title: Anti-adhesive activity of TRAIL

Paola Secchiero¹, Federica Corallini¹, Maria Grazia di Iasio¹, Arianna Gonelli¹, Elisa Barbarotto¹, Giorgio Zauli²

¹Department of Morphology and Embryology, Human Anatomy Section, University of Ferrara, Via Fossato di Mortara 66, 44100 Ferrara Italy;
²Department of Normal Human Morphology, University of Trieste, Via Manzoni 16, 34138 Trieste, Italy.

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Corresponding author: Paola Secchiero, Ph.D., Department of Morphology and Embryology, Human Anatomy Section, University of Ferrara, Via Fossato di Mortara 66, 44100 Ferrara, Italy; Fax: 39-0532-207351; Tel: 39-0532-291574; E-mail: secchier@mail.umbi.umd.edu.

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Abstract

Exposure of endothelial cells to recombinant TNF-related apoptosis inducing ligand (TRAIL) induced a modest (two-fold) increase of HL-60 cell adhesion, as compared to TNF-α (forty-fold) or IL-1β (twenty-fold). However, pretreatment of endothelial cultures with TRAIL determined a significant reduction of the pro-adhesive activity induced by both TNF-α and IL-1β. Unexpectedly, the anti-adhesive activity of TRAIL was not due to interference with the NF-kB-mediated upregulation of surface ICAM-1, VCAM-1 and E-selectin adhesion molecules in response to inflammatory cytokines. In searching for the molecular mechanism underlying this biological activity of TRAIL, a cDNA microarray analysis was performed. TRAIL pre-treatment variably down-modulated the mRNA steady-state levels of several TNF-α-induced chemokines, and, in particular, it abrogated the TNF-α-mediated up-regulation of CCL8 and CXCL10. Of note, the addition of optimal concentrations of recombinant CCL8 plus CXCL10 to endothelial cultures completely restored the pro-adhesive activity of TNF-α. Moreover, experiments performed with agonistic anti-TRAIL-receptor antibodies demonstrated that both TRAIL-R1 and TRAIL-R2 contributed, although at different levels, to TRAIL-induced chemokine modulation. Taken together, our data suggest that TRAIL might play an important role in modulating leukocyte/endothelial cell adhesion, by selectively down-regulating CCL8 and CXCL10 chemokines.
Introduction

Vascular endothelium is a dynamic tissue that possesses important secretory and metabolic functions and has a central role in controlling leukocyte migration into different tissues in adult life.\textsuperscript{1,2} The migration of leukocytes into extravascular tissues involves a cascade of molecular events, including the elaboration of chemotactic factors and chemokines, the response to these factors, the interaction of leukocytes with endothelial cells, and leukocyte transmigration through the blood vessel wall.\textsuperscript{2-5}

The first step in the canonical pathway of leukocyte migration involves transient selectin-mediated interactions between rolling leukocytes and the endothelium. Next, integrins on leukocytes are activated by chemokines that have been produced locally; they are presented on glycosaminoglycans, resulting in firm adhesion between leukocytes and endothelial cells. Finally, leukocytes extravasate through the vascular wall and into the surrounding tissue.\textsuperscript{2-5} Together with adhesion molecules, chemokines regulate the appropriate “addressing and delivery” of each leukocyte subtype to healthy or diseased body compartments.\textsuperscript{5} It is also noteworthy that an abnormal increase of leukocyte adhesion to endothelial cells is considered an early step in endothelial cell dysfunction.\textsuperscript{6-8}

TRAIL, also known as Apo-2 ligand (L), is a 40 kDa protein that is structurally related to the TNF family of cytokines.\textsuperscript{9,10} TRAIL is expressed as a type-II transmembrane protein, however, its extracellular domain can be proteolytically cleaved from the cell surface and act as a soluble cytokine.\textsuperscript{11} TRAIL interacts with four high-affinity transmembrane and one soluble receptors belonging to the TNF-receptor (R) family. TRAIL-R1 (DR4) and TRAIL-R2 (DR5) contain cytoplasmic “death domains” and mediate pro-apoptotic signals by activating the apical caspases 8
and 10 via the adaptor protein FADD. However, increasing experimental data indicate that TRAIL-R1 and TRAIL-R2 can also mediate cell type-dependent pro-survival and proliferation signals mainly by activating the ERK/MAPK and NF-κB pathways.\textsuperscript{11} The other three receptors appear to act as “decoys”, although their function is much less characterized. TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) have homology to the extracellular domains of TRAIL-R1 and TRAIL-R2. TRAIL-R4 has a truncated cytoplasmic death domain, while TRAIL-R3 lacks a cytosolic region and is anchored to the plasma membrane through a glycoprophospholipid moiety. Both receptors are therefore incapable of transmitting an apoptosis signal, while the potential role of these receptors in transducing other intracellular signals is unclear.\textsuperscript{11} The soluble “decoy” receptor osteoprotegerin (OPG) was initially discovered to bind the TNF superfamily member RANKL. Later, OPG was found to bind also TRAIL, although, similarly to the transmembrane decoy receptors TRAIL-R3 and TRAIL-R4, its role in modulating the biological activity of TRAIL is not firmly established.\textsuperscript{11}

While TNF-α, the prototype member of the TNF family of cytokines, strongly induces leukocyte adhesion to endothelial cells\textsuperscript{1}, the role of TRAIL on leukocyte adhesion has not been characterized so far, in spite of the fact that endothelial cells express all four transmembrane TRAIL receptors.\textsuperscript{12,13} In this context, we have previously demonstrated that recombinant TRAIL promotes the survival/proliferation of endothelial cells through activation of the Akt and ERK/MAPK pathways\textsuperscript{13,14} and Li et al\textsuperscript{15} have provided preliminary evidence that TRAIL might have pro-adhesive activity in endothelial cells. Therefore, the aim of this study was to address the role of TRAIL on endothelial/leukocyte interactions. For this purpose, a series of \textit{in vitro} studies were performed in order to investigate the biological activity of TRAIL used
alone and in combination with canonical pro-inflammatory cytokines, such as TNF-α and IL-1β.
Methods

Materials

Recombinant histidine 6-tagged TRAIL was produced as described. Recombinant TNF-α and IL-1β were purchased from Sigma (Saint Louis, MO), CCL8, CXCL10, CCL20 and CXCL1, were purchased from R&D Systems, IL-8, VEGF and bFGF were purchased from Peprotech (London, UK). The optimal concentrations of TRAIL, IL-1β, TNF-α, VEGF and bFGF (10 ng/ml each) were determined in preliminary dose-response experiments. The selective activation of single TRAIL-receptors was performed by using agonistic goat anti-human TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4 polyclonal Abs, and neutralization of IL-8 was performed by using the Ab (clone 6217) anti-IL-8 (all from R&D System, Minneapolis, MN).

For Western blot analyses, the following antibodies were used: anti-IκBα and IκBε Abs (both from Santa Cruz Biotechnology, Santa Cruz, CA), anti-tubulin Ab (Sigma). Enhanced chemiluminescence reagent (ECL) detection system was from DuPont-NEN (Boston, MA).

Flow cytometric analyses were performed by FACScan (Becton Dickinson; San José, CA), using anti-human TNF receptor (TNF-R)1 and anti-human TNF-R2 Abs (both from Alexis Biochemical; Lausen, Switzerland); PE-conjugated anti-mouse secondary Ab (Immunotech; Marseille, France); FITC-conjugated anti-E-selectin, anti-ICAM-1 (Bender Medical System; Wien, Austria), and anti-VCAM-1 (Cymbus Biotechnology; Chandlers Ford, UK) Abs. Non-specific fluorescence was assessed using normal mouse IgG followed by second layer (as above) or by incubation with irrelevant isotype-matched conjugated Abs.
Measurement of chemokines in endothelial cell culture supernatants was performed with chemokine-specific ELISAs (Search Light Human Chemokine Arrays; Pierce, Rockford, IL), following the manufacturer’s instructions.

**Cell cultures**

Primary human umbilical vein endothelial cells (HUVEC), obtained as described. Approval was obtained from the University of Ferrara institutional review board and informed consent was provided according to the Declaration of Helsinki. Cells grown on 0.2% gelatin-coated tissue culture plates in M199 endothelial growth medium (BioWhittaker; Walkersville, MD) supplemented with 20% FBS, 10 μg/ml heparin, and 50 μg/ml ECGF. Human aortic endothelial cells (HAEC) were purchased from BioWhittaker and cultured in EGM basal medium supplemented with 2% FBS, 12 μg/ml BBE, 1 μg/ml hydrocortisone and 10 ng/ml ECGF (all from BioWhittaker). In all experiments cells were used between the 3rd and 5th passage in vitro.

The myeloid HL-60 leukemic cell line (American Tissue Culture Collection, Rockville, MD) was routinely grown in RPMI supplemented with 10% FBS.

**Adhesion assay**

Confluent HUVEC or HAEC, seeded in 24-well plates, were treated with TRAIL and/or inflammatory cytokines for 18 hours. In other experiments, HUVEC were pretreated for 1, 2, 3 or 4 days with TRAIL before exposure to inflammatory cytokines for additional 18 hours. When indicated, endothelial cells were pre-treated with agonist anti-TRAIL-receptors Abs for 3 days before stimulation with inflammatory cytokines. After treatments, the culture medium was removed and...
endothelial cultures were washed twice before adding HL-60 (350×10^3/well) in order to avoid any cytotoxic effect of recombinant TRAIL on HL-60. In selected experiments, recombinant chemokines were added to the endothelial cultures together with HL-60. After 1 hour of coculture of endothelial cells and HL-60, unbound HL-60 cells were removed by gently washing with medium. Endothelial-leukocyte cocultures were photographed under a light microscope (10X magnification), and adhered leukocytes were counted/scored in at least 6 random fields for each treatment.

The viability of both endothelial cells and adherent HL-60 was routinely monitored at light microscopy by Trypan blue dye exclusion. In some experiments, parallel set of endothelial cells (treated and untreated) were assessed using MTT and neutral red staining, performed as described, while the viability of unbound HL-60 was analyzed by propidium iodide (PI) staining and flow cytometric analysis of apoptosis, performed as described.

**Western blot and assay for NF-kB DNA binding**

For Western blot analysis, HUVEC were grown at sub-confluence prior to the addition of TRAIL or TNF-α. Cells were harvested in lysis buffer containing 1% Triton X-100, Pefablock (1 mM), aprotinin (10 µg/ml), pepstatin (1 µg/ml), leupeptin (10 µg/ml), NaF (10 mM), and Na3VO4 (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 primary Abs for IκBα and IκBε and for tubulin to verify loading eveness.

NF-kB induction was measured using the Trans-AM NF-kB p65 and p50 kit (Active Motif; Rixensart, Belgium), which measures the level of active form of NF-
kB contained in cell extracts, able to specifically bind to an oligonucleotide containing the NF-kB consensus site (5′-GGGACTTTCC-3′), attached to a 96-well plate. Assays were performed in triplicates, according to the manufacturer’s instructions. NF-kB DNA binding activity was determined as absorbance values measured by using a microplate reader (Multiskan Ascent; Dasit, Milan Italy). Increase in fluorescence was linear over extract concentration.

**cDNA microarray analysis**

RNA was isolated with a Qiagen RNeasy kit (Hilden, Germany) from HUVEC, either left untreated or stimulated with TNF-α, in the absence or presence of a pretreatment for 3 days with TRAIL. Labeled cDNA was hybridized with a customized cDNA microarray containing an array of 367 inflammation associated genes together with housekeeping genes at the SuperArray core facility (the list of the genes is available elsewhere). Alterations imposed by the different treatments on the basal gene expression were determined as ratio of relative gene expression compared to unstimulated cells. The fold change in expression between unstimulated and treated cultures was considered significant when greater than 2. However, only those genes that were differentially expressed >3-fold were selected to reliably detect differential expression in the RT-PCR confirmation assays (by SingleGene PCR kit, purchased from Bioscience Corporation, GEArray).
Statistical analysis

For each set of experiments, values are reported as means ± SD. The results were evaluated by using analysis of variance with subsequent comparisons by Student’s t-test. Statistical significance was defined as p<0.05.
Results

Recombinant TRAIL alone induces a modest HL-60 cell adhesion to endothelial cells, as compared to pro-inflammatory cytokines

The first group of experiments was designed to investigate whether TRAIL modulates the adhesion properties of endothelial cells. As shown in Figure 1A, in 12 separated experiments, TRAIL (10 ng/ml) induced an approximately two-fold increase of HL-60 adhesion to HUVEC. However, the TRAIL-induced HL-60 cell adhesion was extremely modest, as compared to canonical pro-inflammatory cytokines, such as TNF-α and IL-1β (both used at 10 ng/ml), which induced approximately 40-fold (p<0.01) and 20-fold (p<0.01) increase, respectively, over the background levels. Moreover, at variance to TNF-α or IL-1β, TRAIL did not modulate the surface expression of adhesion molecules ICAM-1, VCAM-1 and E-selectin (Figure 1B) and it did not activate the NF-kB pathway (Figure 1C) in endothelial cells.

TRAIL counteracts the TNF-α- and IL-1β-induced leukocyte adhesion to endothelial cells

To examine the effect of TRAIL on the biological response induced by pro-inflammatory cytokines, HUVEC were cultured with TRAIL, added in combination with TNF-α or IL-1β. When TRAIL was added at the same time with TNF-α or IL-1β, it modestly affected the pro-adhesive activity of both inflammatory cytokines (Figure 2A). On the other hand, a 2-4 day pre-exposure to TRAIL before addition to inflammatory cytokines induced a clear-cut (p<0.05) decrease in the number of adherent HL-60 cells to HUVEC (Figure 2A). This effect was dose-dependent and
showed a plateau at TRAIL concentration of 10 ng/ml (Figure 2B). Of note, the anti-adhesive activity of TRAIL in TNF-α-treated cultures was not due to a down-regulation of surface TNF-R1 or TNF-R2 in HUVEC, as shown by flow cytometric analysis performed 3 days after TRAIL treatment (Figure 2C). The possibility that the observed effects were due to cellular toxicity induced by TRAIL or TRAIL+TNF-α, was ruled out by assessing endothelial cell viability following treatments. As expected on the basis of previous studies of our and other groups of investigators, incubation of endothelial cells for 2-4 days with TRAIL, irrespective of TNF-α addition, did not induce any significantly cytotoxicity as determined by MTT staining (Figure 2D). Moreover, the decreased adhesiveness of HL-60 cells also to human aortic endothelial cells in response to TNF-α (data not shown), allowed us to exclude the possibility that the anti-inflammatory activity of TRAIL was restricted to HUVEC.

Next experiments were designed to elucidate whether TRAIL interfered with the ability of TNF-α to activate the NF-kB pathway, which plays a key role in the regulation of ICAM-1, VCAM-1 and E-selectin surface adhesion molecules. As shown in Figure 3A, TRAIL did not modulate the NF-kB pathway and, consistently, did not affect the surface expression of ICAM-1, VCAM-1 and E-selectin induced by TNF-α (Figure 3B).

The anti-inflammatory activity of TRAIL is mediated by a down-regulation of the mRNA steady-state levels of CCL8 and CXCL10 chemokines

Since chemokines play a major role in promoting the adhesiveness of leukocytes to endothelial cells, the expression profile of a set of chemokines was next analyzed by cDNA microarray in: i) unstimulated HUVEC, ii) HUVEC...
treated with TNF-α for 18 hours and iii) HUVEC treated with TNF-α for 18 hours after a pre-exposure for 3 days to TRAIL. As shown in Figure 4A, TNF-α induced an increase (comprised from two- to fourteen-fold) of the mRNA steady-state levels of several chemokines with respect to unstimulated cultures. The TNF-α-induced chemokines were divided in two groups on the basis of their low or high basal steady-state mRNA levels in unstimulated endothelial cells. Among the low basal steady-state mRNA group of chemokines, TRAIL partially decreased the expression of CCL20/MIP-3α and CXCL5/ENA-78, while it completely abrogated the TNF-α-mediated upregulation of CXCL10/IP-10 and CCL8/MPC-2 (Figure 4A). Among the high basal steady-state mRNA group of chemokines, TRAIL modestly affected the expression of CXCL1/GROα, CXCL2/GROβ, CXCL3/GROγ and CCL2/MPC-1, while it increased the expression CXCL8/IL-8 (Figure 4A).

Validation of the microarray results was performed by semi-quantitative RT-PCR and it was accompanied by ELISA measurement of release in culture media for selected chemokines. The most striking effect associated to TRAIL-pretreatment both at mRNA and protein levels (Figure 4A-C) was the almost complete abrogation of the TNF-α-induced steady-state mRNA levels and protein release of CCL8/MCP-2 and CXCL10/IP-10. On the other hand, the release in culture supernatant of other chemokines, belonging to the low (CCL20/MIP-3α) and high (CXCL1/GROα) basal steady-state mRNA groups and whose mRNA level was variably decreased by TRAIL, was unaffected by TRAIL pretreatment (Figure 4C). In addition, and in agreement with the microarray results (Figure 4A), TRAIL-pretreatment (p<0.05) further increased the release in culture of CXCL8/IL-8 protein in response to TNF-α (Figure 4C).
To investigate whether the TRAIL-mediated down-regulation of CCL8/MCP-2 and CXCL10/IP-10, and up-regulation of CXCL8/IL-8 were mediated by a specific TRAIL receptor, HUVEC were challenged with agonistic polyclonal Abs anti-TRAIL receptors which mimic the interaction between TRAIL and each TRAIL receptor, before exposure to TNF-α. Pretreatment for 3 days with either anti-TRAIL-R1 or anti-TRAIL-R2 antibodies significantly (p<0.05) decreased, although at different levels, the pro-adhesive activity of TNF-α, while anti-TRAIL-R3 did not show any significant biological activity (Figure 5A). Moreover, both anti-TRAIL-R1 and anti-TRAIL-R2, but not anti-TRAIL-R3, decreased the release in culture of CCL8/MCP-2 and CXCL10/IP-10 and promoted the release of CXCL8/IL-8 (Figure 5B).

Next experiments were performed to evaluate the biological significance of TRAIL interference on TNF-α-induced CCL8/MCP-2, CXCL10/IP-10 and CXCL8/IL-8 steady-state mRNA levels and protein release in mediating the anti-adhesive activity of TRAIL. For this purpose, increasing concentrations of recombinant CCL8/MCP-2 plus CXCL10/IP-10 were added to HUVEC pre-exposed to TRAIL and then treated with TNF-α. As shown in Figure 6A, the addition in culture of CXCL10/IP-10 + CCL8/MCP-2, dose-dependently counteracted the anti-adhesive activity of TRAIL in TNF-α-stimulated cultures. Thus, optimal concentrations (10 ng/ml each) of the two chemokines added together, restored the TNF-α-induced cell adhesion, completely abrogating the anti-adhesive effect of TRAIL (Figure 6A-B). In parallel, the addition of CCL8/MCP-2 and CXCL10/IP-10 to untreated or TRAIL-treated endothelial cells showed that these chemokines were unable to increase the adhesive properties of endothelial cells in the absence of the pro-inflammatory stimulus (data not shown). On the contrary, the addition of other recombinant chemokines (CXCL1/GROα + CCL20/MIP-3α), whose release in
culture was not significantly affected by TRAIL pretreatment, did not revert the anti-adhesive activity of TRAIL (Figure 6B). Similarly, the addition in culture of recombinant CXCL8/IL-8, which has been shown to promote leukocyte adhesion to endothelial cells and it is abundantly produced in endothelial cultures treated with TRAIL+TNF-α, did not revert the TRAIL-mediated inhibition (Figure 6C). Moreover, the possibility that the increased release of CXCL8/IL-8 in response to TRAIL+TNF-α (Figure 4C) might be paradoxically involved in mediating the anti-adhesive activity of TRAIL was excluded (Figure 6C). In fact, the addition in culture of anti-CXCL8/IL-8 neutralizing Abs did not modulate the adhesive activity of TRAIL+TNF-α-treated cultures (Figure 6C).

In additional experiments the critical role of CCL8/MCP-2 and CXCL10/IP-10 in mediating the pro-adhesive activity of TNF-α was ascertained by analyzing combinations of TNF-α with other anti-adhesive cytokines. For this purpose, HUVEC were pretreated for three days with the pro-angiogenic cytokines, bFGF and VEGF, which have been shown to down-regulate the TNF-α-mediated pro-adhesive activity. As shown in Figure 7, the addition of recombinant CCL8/MCP-2 plus CXCL10/IP-10 reverted the anti-adhesive activity of both bFGF and VEGF in TNF-α-treated cultures, therefore suggesting that the control of their release in culture represents a common target for several cytokines able to down-modulate the pro-adhesive activity of inflammatory cytokines.
Discussion

Our data demonstrated for the first time that TRAIL significantly counteracts the pro-adhesive activity of canonical inflammatory cytokines, such as TNF-α or IL-1β. However, TRAIL did not significantly modulate the surface level of ICAM-1, VCAM-1 and E-selectin adhesion molecules in HUVEC, either when used alone or in association with TNF-α or IL-1β. Consistently, TRAIL did not affect the ability of TNF-α to potently activate the NF-kB pathway, a pre-requisite for the transcriptional upregulation of ICAM-1, VCAM-1 and E-selectin. Nevertheless, TRAIL induced a modest (two-fold) increase of leukocyte adhesion to HUVEC, a finding in agreement with the data of Li et al, who described a similar pro-adhesive activity of TRAIL. However, at variance to Li et al., and consistently with our previous findings, we did not observe any TRAIL cytotoxic effect or any TRAIL-induced activation of the NF-kB pathway. Although we do not have a ready explanation for this discrepancy, it might be ascribed to the different time frame examined (5-7 hours of TRAIL treatment in the Li’s study versus 18-90 hours in our experiments) and endothelial culture conditions. In this respect, it has recently been shown that the susceptibility of endothelial cells to TRAIL cytotoxicity critically depends on the nature of the substrate on which endothelial cells are cultured. The fact that TRAIL did not modify the TNF-α-mediated up-regulation of ICAM-1, VCAM-1 and E-selectin likely accounts for the incomplete suppression (approximately 60% inhibition) of the TNF-α-induced pro-adhesive activity in endothelial cells pre-exposed to TRAIL.

However, the most striking result of our study was the ability of TRAIL to significantly down-modulate the potent pro-adhesive activity of inflammatory cytokines through a down-regulation of CCL8/MCP-2 and CXCL10/IP-10 chemokine
steady-state mRNA levels and protein release. Chemokines can be divided broadly into two categories: (i) inflammatory chemokines, which recruit leukocytes in response to physiological stress, and (ii) homeostatic chemokines, which are responsible for basal leukocyte trafficking and the forming and architecture of secondary lymphoid organs. Both CCL8/MCP-2 and CXCL10/IP-10 belong to the group of inflammatory chemokines, which are expressed in inflamed tissues by resident and infiltrated cells after stimulation by pro-inflammatory cytokines or during contact with pathogenic agents. This group of chemokines is specialized for the recruitment of effector cells, including monocytes, granulocytes and effector T cells.

The ability of TRAIL to down-regulate CCL8/MCP-2 and CXCL10/IP-10 expression and release explains how TRAIL counteracts the pro-adhesive activity of TNF-α without interfering with the TNF-α-mediated upregulation of the surface expression of adhesion molecules in endothelial cells. It is noteworthy that although it has been shown that chemokines often show redundant biological activity, the addition in culture of other recombinant chemokines (CXCL1/GROα and CCL20/MIP-3α) was unable to vicariate CCL8/MCP-2 and CXCL10/IP-10. The discrepancy between the levels of endogenous CCL8/MCP-2 and CXCL10/IP-10 detected in culture supernatants of endothelial cells by ELISA and the concentrations of recombinant CCL8/MCP-2 and CXCL10/IP-10 required to fully restore the pro-adhesive activity of TNF-α can be explained at least in part by the fact that chemokines mainly accumulate on the endothelial cell layer through glycosaminoglycan binding, where they induce conformational changes in the adhesion molecules of endothelial cells, increasing their adhesiveness property. The glycosaminoglycan-bound chemokines are not measured by ELISA performed on culture supernatants.
We cannot completely exclude the possibility that the down-regulation of CCL8/MCP-2 and CXCL10/IP-10 might be selectively due to the recruitment of only one of the TRAIL receptors (TRAIL-R1 or TRAIL-R2), able to elicit an intracellular signal transduction pathway. However, this possibility is unlikely since in experiments performed with agonistic anti-TRAIL-R1 and anti-TRAIL-R2 polyclonal Abs, both Abs mimicked the activity of recombinant TRAIL in terms of inhibition of CCL8/MCP-2 and CXCL10/IP-10 and stimulation of CXCL8/IL-8.

Although we have not investigated the molecular mechanisms underlining the TRAIL-mediated down-regulation of CCL8/MCP-2 and CXCL10/IP-10, the involvement of the NF-kB transcription factor family members was excluded. A complex interplay exists between TRAIL and NF-kB in different cell models, but we have clearly demonstrated that TRAIL does not induce NF-kB activity in endothelial cells nor it perturbs the TNF-α-mediated induction of NF-kB. To the best of our knowledge, little is known on the effect of TRAIL at the transcription level, but it has been previously shown in a lymphoid model that TRAIL negatively regulates the transcription of an important member of the AP-1 family, c-Fos, which might contribute to explain the observed CCL8/MCP-2 and CXCL10/IP-10 down-regulation.

CCL8/MCP-2 and CXCL10/IP-10 were able to restore the pro-adhesive activity of TNF-α also in the presence of the pro-angiogenic cytokines, VEGF and bFGF, implying that the modulation of CCL8/MCP-2 and CXCL10/IP-10 represents a critical step in the control of adhesiveness in response to TNF-α. Moreover, these data suggest that modulation of these two chemokines represent a general mechanism shared by different cytokines to attenuate the pro-adhesive activity of TNF-α. Interestingly, also TGF-β1 has been previously shown to generally reduce the
secretion of several chemokines in TNF-α-activated endothelial cells. Thus, in analogy to TGF-β1, which has a role in the later phases of inflammation when repair and tissue regeneration start to occur, also TRAIL may act as a modulator of inflammation. In this respect, it is also of particular interest our previous demonstration that TRAIL promotes endothelial cell survival and proliferation, which are often observed at the end of an inflammatory process in the transition to repair and regeneration. In keeping with a potential physio-pathological role of TRAIL in counteracting endothelial cell dysfunction, previous studies have shown that the serum level of osteoprotegerin (OPG), which acts as a soluble decoy receptor for TRAIL, is significantly elevated in patients with chronic inflammation states, such as diabetes mellitus and coronary artery disease.

It is noteworthy that the number of chemokines and chemokine receptors is very high and that chemokines have been shown to play redundant biological functions. Consistently, it has been demonstrated that mice with functional deletions in single gene that encode chemokines or their receptors tend to show mild alterations in defensive functions. Thus, although further studies are needed to ascertain the potential role of the TRAIL/TRAIL receptors system in modulating the trafficking of leukocytes in vivo, our data demonstrate for the first time that TRAIL has a potent anti-adhesive effect on in vitro adhesion promoted by inflammatory cytokines and demonstrate that this biological activity is mediated by the selective and simultaneous down-regulation of CCL8 and CXCL10.
References


Figure legends

Figure 1. TRAIL and inflammatory cytokine-mediated leukocyte adhesion. HUVEC cells were either left untreated, or exposed to TRAIL, TNF-α or IL-β. A, HL-60 cell adherence on HUVEC treated (for 18 hours) as indicated. Representative fields of the cultures are shown (10X magnification). Cell adhesion was calculated as described in Methods and is expressed as mean ± SD of twelve experiments, each performed in triplicate. *, p<0.05. B, HUVEC were exposed to cytokines for 18 hours and expression levels of leukocyte adhesion molecules (ICAM-1, VCAM-1 and E-selectin) were evaluated by flow cytometry. The control (unshadowed) histograms represent the background fluorescence obtained from the staining of the same cultures with isotype-matched control antibodies. One of eight experiments with similar results is shown. C, HUVEC were stimulated with TRAIL, TNF-α or IL-β for the indicated time intervals (0-45 min), and cell lysates were analyzed for degradation of IkBα and IkBε by Western blotting. Equal loading of protein in each lane was confirmed by staining with the Ab to tubulin. One of four experiments with similar results is shown.

Figure 2. TRAIL pretreatment alters inflammatory cytokine-mediated leukocyte adhesion. HUVEC cells were either left untreated, or pre-exposed to TRAIL before stimulation with TNF-α or IL-1β (for 18 hours). A, Cells were treated with TRAIL (10 ng/ml) at the time of exposure to inflammatory cytokines (day 0) or 1-4 days before. B, Dose-response effect of 3 days of TRAIL-pretreatment (used at the indicated concentrations) was evaluated on HL-60 cell adhesion to HUVEC stimulated by either TNF-α or IL-1β. In A and B, cell adhesion in the absence of
TRAIL-pretreatment was set as 100%. In A-B, results are expressed as means ± SD of three independent experiments, each performed in triplicate. * p<0.05. C, TRAIL-pretreatment does not affect surface expression of TNF-receptors (TNF-R1 and TNF-R2). The control (unshadowed) histograms represent the background fluorescence obtained from the staining of the same cultures with isotype-matched control antibodies. One of four experiments with similar results is shown. D, Viability of endothelial cultures (treated as indicated) was assessed by MTT assay. Data are shown as average OD ± SD.

**Figure 3. TRAIL pretreatment does not affect TNF-α-induced NF-kB activation.**

HUVEC were either left untreated or pretreated with TRAIL for 3 days before stimulation with TNF-α. A, NF-kB-p65 and NF-kB-p50 DNA binding activity was determined at the indicated time-points as absorbance values and is expressed as percentage of untreated control. B, Analysis of surface leukocyte adhesion molecules (VCAM-1, ICAM-1, E-selectin) was performed by flow cytometry and is reported as mean fluorescence intensity (MFI). Results are expressed as means ± SD of three (for A) or eight (for B) independent experiments.

**Figure 4. The anti-adhesive activity of TRAIL is mediated by down-regulation of chemokines.** Differential chemokine gene expression was assessed by cDNA microarray analysis in HUVEC, either left untreated or pretreated with TRAIL for 3 days, before stimulation with TNF-α. A, Ratios represent TNF-α or TRAIL (3 d) + TNF-α values divided by untreated values. The cut-off of 2-fold of induction is shown. Arrows indicate the genes most significantly downmodulated by the TRAIL-pretreatment. B, Microarray results were validated by semi-quantitative RT-PCR (#1-
4: serial scalar dilutions of the RNA templates). C, Chemokine release by HUVEC either left untreated or pretreated with TRAIL for 3 days, before stimulation with TNF-α, was measured by ELISA. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. *, p<0.05.

Figure 5. Role of specific TRAIL receptor triggering in TRAIL-mediated anti-adhesive activity and modulation of chemokine release. HUVEC were either left untreated, or pre-treated for 3 days with the indicated agonistic polyclonal Abs anti-TRAIL receptors before addition of TNF-α. A, TNF-α-induced HL-60 cell adhesion to HUVEC was set as 100%. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. B, Chemokine release was measured by ELISA. Results are expressed as means ± SD of three independent experiments, each performed in triplicate. *, p<0.05.

Figure 6. The anti-adhesive activity of TRAIL is reverted by addition of CXCL10 and CCL8. HUVEC were either left untreated, or pre-treated for 3 days with TRAIL before addition of TNF-α and adhesion assay. When indicated recombinant CXCL10 and CCL-8 (A), CXCL1 and CCL20 (B), IL-8 and neutralizing Ab anti-IL-8 (C) were added to the cultures either alone or in combination. TNF-α-induced HL-60 cell adhesion to HUVEC was set as 100%. Results are expressed as means ± SD of three-four independent experiments, each performed in triplicate.

Figure 7. CXCL10+CCL8 revert the anti-adhesive activity also of VEGF and bFGF. HUVEC were either left untreated, or pre-treated for 3 days with VEGF or bFGF before addition of TNF-α and adhesion assay. At the time of the HL-60 cell
adhesion assay, the indicated recombinant chemokines (10 ng/ml each) were added to the cultures. TNF-α-induced HL-60 cell adhesion to HUVEC was set as 100%. Results are expressed as means ± SD of four independent experiments, each performed in triplicate.
FIGURE 1A
FIGURE 1B

![Graph showing relative cell number and fluorescence intensity for ICAM-1, VCAM-1, and E-Selectin under different treatments: Untreated, TRAIL, TNF-alpha, IL-1beta.]

FIGURE 1C

<table>
<thead>
<tr>
<th></th>
<th>TRAIL</th>
<th>TNF-alpha</th>
<th>IL-1beta</th>
</tr>
</thead>
<tbody>
<tr>
<td>minutes</td>
<td>0 5 15 30 45</td>
<td>0 5 15 30 45</td>
<td>0 5 15 30 45</td>
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<tr>
<td>IKBalpha</td>
<td><img src="#" alt="Image" /></td>
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<td>IKBbeta</td>
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<tr>
<td>Tubulin</td>
<td><img src="#" alt="Image" /></td>
<td><img src="#" alt="Image" /></td>
<td><img src="#" alt="Image" /></td>
</tr>
</tbody>
</table>
FIGURE 2C

FIGURE 2D
FIGURE 4A

Basal level (Untreated)

low

CXCL10/IP-10
CCL8/MCP-2
CXCL6/GCP-2
CX3CL1/Fractalkine
CCL20/MIP-3-alpha
CXCL5/ENA-78
CXCL3/GROgamma
CXCL2/GRObeta
CXCL1/GROalpha
CXCL8/IL-8
CCL2/MCP-1

mRNA steady-state level (ratio)

TNF-alpha
TRAIl (3d) + TNF-alpha

high
FIGURE 4B

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FIGURE 5A

[Graph showing percentage of adhered cells under different conditions.

FIGURE 5B

[Graphs showing CXCL10, CCL8, and CXCL8 levels under different conditions.

[Graph showing percentage of adhered cells under different conditions.]}
FIGURE 6A

![Graph showing the effect of different chemokines on the percentage of adhered cells with TNF-alpha as a variable.](image)

FIGURE 6B

![Graph showing the effect of different chemokines on the percentage of adhered cells with TNF-alpha as a variable.](image)
FIGURE 6C

![Graph showing percentage of adhered cells under different conditions.](image)
FIGURE 7
TRAIL counteracts the pro-adhesive activity of inflammatory cytokines in endothelial cells by down-modulating CCL8 and CXCL10 chemokine expression and release

Paola Secchiero, Federica Corallini, Maria G di Iasio, Arianna Gonelli, Elisa Barbarotto and Giorgio Zauli