TRAIL regulates normal erythroid maturation through an ERK-dependent pathway

Paola Secchiero, Elisabetta Melloni, Markku Heikinheimo, Susanna Mannisto, Roberta Di Pietro, Antonio Iacone, and Giorgio Zauli

In order to investigate the biologic activity of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) on human erythropoiesis, glycoporphin A (GPA)− erythroid cells were generated in serum-free liquid phase from human cord blood (CB) CD34+ progenitor cells. The surface expression of TRAIL-R1 was weakly detectable in the early-intermediate phase of erythroid differentiation (days 4-6; dim-intermediate GPA expression), whereas a clear-cut expression of TRAIL-R2 was observed throughout the entire course of erythroid differentiation (up to days 12-14; bright GPA expression). On the other hand, surface TRAIL-R3 and -R4 were not detected at any culture time. Besides inducing a rapid but small increase of apoptotic cell death, which was abrogated by the pan-caspase inhibitor z-VAD-fmk, the addition of recombinant TRAIL at day 6 of culture inhibited the generation of morphologically mature erythroblasts. Among the intracellular pathways investigated, TRAIL significantly stimulated the extracellular signal-regulated kinase 1/2 (ERK1/2) but not the p38/mitogen-activated protein kinase (MAPK) or the c-Jun NH2-terminal kinase (JNK) pathway. Consistently with a key role of ERK1/2 in mediating the negative effects of TRAIL on erythroid maturation, PD98059, a pharmacologic inhibitor of the ERK pathway, but not z-VAD-fmk or SB203580, a pharmacologic inhibitor of p38/MAPK, reversed the antidiagnostic effect of TRAIL on CB-derived erythroblasts. (Blood. 2004; 103:517-522)

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Materials and methods

Purification of the cells

Cord blood (CB) specimens were collected according to institutional guidelines. CB mononuclear cells were isolated by density gradient centrifugation (Ficoll/Histopaque 1077 g/mL) and left to adhere to plastic for at least 2 hours at 37°C. After removal of adherent cells, CD34+ cells were isolated using a magnetic cell-sorting program Mini-MACS and the CD34 isolation kit (Miltenyi Biotech, Auburn, CA) in accordance with the manufacturer’s instructions. The purity of CD34+ selected cells was determined by FACScan (Lysys II program; Becton Dickinson, San José, CA) using a fluorescein isothiocyanate (FITC)–conjugated monoclonal antibody (MoAb), which recognizes a separate epitope of the CD34 molecule (HPCA-2; Becton Dickinson). The purity of CD34+ preparations ranged between 93% to 98%.

Supported by Fondo per l’Incentivazione della Ricerca di Base (FIRB) (P.S. and G.Z.), Associazione Italiana per la Ricerca sul Cancro (AIRC) (G.Z.), and “Jusselius Foundation” (M.H.) grants.

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In vitro generation of erythroid cells and culture treatments

CB CD34+ cells were cultured in Ex-vivo-20 (BioWhittaker, Walkersville, MD) serum-free medium supplemented with nucleosides (10 μg/mL each), 0.5% bovine serum albumin (BSA, Chon fraction V), 10−6 M BSA-adsorbed cholesterol, 10 μg/mL insulin, 200 μg/mL iron-saturated transferrin, and 5 × 10−5 M 2-β-mercaptoethanol (all purchased from Sigma Chemical, St. Louis, MO). Cells were adjusted to an optimal cell density of 5 × 106/mL and seeded in culture in the presence of stem cell factor (SCF), 50 ng/mL + interleukin-3 (IL-3), 10 ng/mL + erythropoietin (EPO, 4U/mL) to induce erythroid differentiation. All cytokines were purchased from Genzyme (Cambridge, MA). Fresh cytokines were added every 2 to 3 days and the cell density was readjusted to 4 × 107/mL.

Erythroid differentiation was monitored by analysis of surface glycoporphin A (GPA) and by cell morphology examination. Surface expression of GPA was evaluated by flow cytometry as detailed in "Flow cytometric analyses." For cell morphology examination, cells were spun on coverslips, fixed, stained with May-Grünwald-Giemsa, and observed at light microscopy with an Axiophot Zeiss microscope, equipped with a CoolScan video camera (both from Lambda Photometrics, Ratford Mill, United Kingdom).

Recombinant histidine 6–tagged TRAIL (114–281) was produced in bacteria, purified by chromatography as previously described. The absence of endotoxin contamination in the recombinant TRAIL preparation (< 0.1 endotoxin units/mL) was assessed by limulus amebocyte lysate (LAL) assay (BioWhittaker). The optimal TRAIL concentration (100 ng/mL), used in most experiments, was determined based on preliminary assays in which scalar TRAIL doses (ranging from 0.01 to 1 μg/mL) were tested. Pharmacologic inhibitors of the caspase (z-VAD-fmk), extracellular signal-regulated kinase 1/2 (ERK1/2, PD98059), p38/mitogen-activated protein kinase (MAPK, SB203580) pathways, and the inhibitor of lipopolysaccharide (polymyxin B) (all from Calbiochem, La Jolla, CA) were used at the concentrations indicated in "Results.”

Flow cytometric analyses

For flow cytometric analyses, surface cell staining was performed at 4°C for 40 minutes by incubating 3 × 106 cells in 200 μL phosphate-buffered saline (PBS, containing 1% BSA and 5% human plasma) with the indicated antibodies. CD34 and GPA expression were detected using FITC-conjugated anti-CD34 and phycoerythrin (PE)-conjugated anti-GPA MoAbs (BD Pharmingen, San Diego, CA), respectively. Nonspecific fluorescence was assessed by incubation with irrelevant isotype-matched conjugated MoAbs. GPA expression is reported in the text as percentage of positive cells and/or as mean fluorescence intensity (MFI). Surface expression of TRAIL receptors was evaluated by indirect staining with primary MoAbs against transmembrane TRAIL receptors (TRAIL-R1, R2, R3, and R4) on freshly purified CB CD34+ hematopoietic progenitor cells (day 0), as well as at early-intermediate (day 6) and late (days 12-14) culture times (Figure 1). As shown in Figure 1A, highly purified populations of CB CD34+ cells did not express any TRAIL receptors. On the other hand, after 6 days of serum-free liquid culture in the presence of SCF + IL-3 + EPO, when most of the cells showed a dim-intermediate expression of GPA, an intermediate surface expression of TRAIL-R2 and a dim surface expression of TRAIL-R1 became apparent (Figure 1B-C). At later culture times (days 12-14), when most erythroblasts showed a bright expression of GPA, surface TRAIL-R2 expression was increased with respect to earlier culture times, while the dim expression of surface TRAIL-R1, observed at earlier time points, declined, becoming barely detectable in most experiments (Figure 1B-C). On the other hand, TRAIL-R3 and TRAIL-R4 were never expressed on the surface of GPA+ erythroblasts (Figure 1B). The first conclusion of this group of analyses is that CB CD34+ cells are not affected by TRAIL, as previously shown by us and by others in adult CD34+ cells, due to the lack of expression of surface TRAIL receptors.

Western blot analyses

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 nM), 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. The following antibodies were used: rabbit Abs anti-ERK1/2, anti-phospho-ERK1/2, anti-p38, anti-phospho-p38, anti-c-Jun NH2-terminal kinase (JNK1), anti-phospho-JNK1 (all from New England Biolabs, Beverly, MA), and MoAb anti-tubulin (Sigma). Blotted filters were first probed with antibodies for the phosphorylated forms of ERK1/2, p38, and JNK1. After incubation with peroxidase-conjugated anti-rabbit or anti-mouse IgG (Sigma), specific reactions were revealed with the enhanced chemiluminescence (ECL) Western blotting detection reagent. Membranes were stripped by incubation in Re-Blot 1X Ab stripping solution (Chemicon International, Temecula, CA) and reprobed for the respective total protein kinase content or tubulin for verifying loading evenness. Densitometry values were estimated by the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Multiple film exposures were used to verify the linearity of the samples analyzed and to avoid saturation of the film.

Results

CD34-derived erythroblasts show persistent expression of TRAIL-R2

Having observed that TRAIL protein is expressed in human adult bone marrow and in human fetal livers at different ages of gestation (8-22 gestational weeks; data not shown) in the first group of experiments, we have analyzed the phenotypic surface expression of transmembrane TRAIL receptors (TRAIL-R1, R2, R3, and R4) on freshly purified CB CD34+ hematopoietic progenitor cells (day 0), as well as at early-intermediate (day 6) and late (days 12-14) culture times (Figure 1). As shown in Figure 1A, highly purified populations of CB CD34+ cells did not express any TRAIL receptors. On the other hand, after 6 days of serum-free liquid culture in the presence of SCF + IL-3 + EPO, when most of the cells showed a dim-intermediate expression of GPA, an intermediate surface expression of TRAIL-R2 and a dim surface expression of TRAIL-R1 became apparent (Figure 1B-C). At later culture times (days 12-14), when most erythroblasts showed a bright expression of GPA, surface TRAIL-R2 expression was increased with respect to earlier culture times, while the dim expression of surface TRAIL-R1, observed at earlier time points, declined, becoming barely detectable in most experiments (Figure 1B-C). On the other hand, TRAIL-R3 and TRAIL-R4 were never expressed on the surface of GPA+ erythroblasts (Figure 1B). The first conclusion of this group of analyses is that CB CD34+ cells are not affected by TRAIL, as previously shown by us and by others in adult CD34+ cells, due to the lack of expression of surface TRAIL receptors.

[3H]Thymidine incorporation assay

Erythroid cells were plated onto 96-well plates at a density of 5 × 105 cell/well. The cells were then pretreated with inhibitors or vehicle for 1 hour and then incubated with TRAIL for 18 hours in the presence of [3H]Thymidine (1 μCi [0.037 MBq]). [3H]Thymidine-labeled DNA was then assayed by harvesting the cells using Brandel Harvester 96 (Brandel, Gaithersburg, MD). [3H]Thymidine levels were then measured using a Beckman model LS6000IC liquid scintillation counter (Beckman Coulter, Fullerton, CA).

TRAII exhibits antidifferentiative effects when added to immature erythroblasts

In order to characterize the biologic activity of TRAIL on CB erythropoiesis, recombinant TRAIL was added to erythroid cultures at day 6, when GPA+ immature erythroblasts showed an intermediated expression of TRAIL-R2 and a dim expression of...
Figure 1. Surface expression of TRAIL receptors in erythroid cultures. Surface TRAIL receptor (TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4) expression was evaluated by flow cytometry in CD34+ cells freshly purified (A) and in CD34+ cells cultured in the presence of EPO + IL-3 + SCF (B-C). In panels B-C, erythroid differentiation was monitored at 6 and 12 days of liquid culture by analysis of surface GPA expression. In panels A-B, shaded histograms represent cells stained with MoAbs specific for the indicated surface antigens (CD34, glycoporphin A, TRAIL receptors), whereas unshaded histograms represent the background fluorescence obtained from the staining of the same cultures with isotype-matched control MoAbs. In panel C, surface TRAIL-R1 and TRAIL-R2 expression was analyzed in combination with surface glycoporphin A at 6 and 12 days of culture. Horizontal axis indicates the TRAIL-R1 or TRAIL-R2 expression detected by Cy-chrome fluorescence intensity. Vertical axis indicates the TRAIL-R1 or TRAIL-R2 expression detected by indirect PE fluorescence intensity. A representative of 5 (A-B) and 3 (C) separate experiments is shown.

TRAIL-R1 (Figure 1B). The potential interference of the prosurvival signal transduction pathways elicited by the cytokine cocktail used to induce erythroid differentiation21,22 was avoided by adding TRAIL 48 hours after the last addition of SCF + IL-3 + EPO. Every 3 days after TRAIL addition, cultures were monitored for cell viability, surface GPA expression levels, and morphology. TRAIL induced a small decrease in the total number of viable cells (data not shown) as well as of percentage of GPA+ erythroblasts (Figure 2A) evident at day 9, which, however, did not reach statistical significance. On the contrary, TRAIL induced a marked decrease in the mean fluorescence level of surface GPA (Figure 2B and 2C), observed after 3 days from the addition of TRAIL (day 9 of culture, $P < .01$) and persisting at later time points (day 12 of culture, $P < .01$) (Figure 2B). The possibility that TRAIL treatment was shifting erythropoiesis to another lineage was unlikely because the percentage of GPA+ cells generated in culture was not significantly affected by TRAIL. Moreover, the antidifferentiative activity of TRAIL was confirmed by morphologic analysis of cytopsin samples, which showed decreased number of mature orthochromatic erythroblasts with respect to control cultures (Figure 2D), thus ruling out the possibility that TRAIL effect could just be on GPA cell surface expression. Of note, these biologic effects of TRAIL were not due to potential contaminating endotoxin, as polymyxin B did not affect TRAIL activity (data not shown).

In the next experiments, we investigated whether the adverse effect of TRAIL on CB erythroid maturation could be secondary to modulation of erythroid cell survival/proliferation or if it was due to a direct antidifferentiative effect of TRAIL on normal erythropoiesis. As shown in Figure 3A, exposure to TRAIL resulted in an increase of apoptosis, which peaked at 24 hours from TRAIL addition ($P < .05$) and was accompanied by a modest but significant ($P < .05$) decrease of thymidine uptake (Figure 3B). As expected on the basis of previous findings on adult erythropoiesis,18,19 the pan-caspase inhibitor z-V AD-fmk completely abrogated the proapoptotic activity of TRAIL (Figure 3A-B). Importantly, however, z-VAD-fmk did not revert the adverse effect of TRAIL on morphologic maturation of erythroblasts, evaluated 3 to 4 days (days 9-10 of culture) from the addition of TRAIL ± z-VAD-fmk in culture (Figure 3C). These data are consistent with a previous study showing that z-VAD-fmk rather arrests the maturation of erythroid progenitors at early stages of differentiation.23

Figure 2. Effect of TRAIL on erythroid maturation. CD34+ cells were cultured in the presence of EPO + IL-3 + SCF for 6 days and then cells were either left untreated or treated with TRAIL. At the indicated times, cultures were analyzed for glycoporphin A expression and cell morphology. In panels A-B, surface glycoporphin A expression, reported either as percentage of positive cells (A) or as mean fluorescence intensity (MFI) (B), was measured by flow cytometry. Data represent the means ± SDs of 4 independent experiments performed in duplicate; *$P < .01$. In panels C-D, representative cell phenotype and cell morphology, examined on day 12 of culture by flow cytometry and by light microscopy after May-Grunwald-Giemsa staining, respectively, are shown. In panel D, some mature (arrowheads) and immature (arrows) erythroblasts are indicated in the untreated (i) and TRAIL-treated (ii) cultures. Original magnification, ×40. Similar results were observed in 3 independent experiments performed in duplicate.
The antiproliferative effect of TRAIL on erythroblasts is mediated by activation of the ERK/MAPK pathway

To date, at least 3 subgroups of mitogen-activated protein kinase (MAPK) family members have been involved in a wide range of cellular responses. The first subgroup includes 2 isoforms of the extracellular signal-regulated kinases, ERK1 and ERK2. The other subgroups are SAPK1/JNK1 (stress-activated protein kinase-1/c-Jun NH2-terminal kinase) and SAPK2/p38. Of note, different family members of the MAPK family have been involved in the control of erythroid maturation. Therefore, we have investigated whether the MAPK pathways are engaged by the interaction between TRAIL and TRAIL receptors in primary erythroid cells, obtained after 6 days of culture. For this purpose, Western blot analyses were performed using antibodies specific for the residues that are phosphorylated in each kinase upon activation (Figure 4). After exposure to TRAIL, a rapid induction of phospho-ERK1/2 was observed at 1 to 10 minutes of treatment (Figure 4A). On the other hand, TRAIL did not activate either p38/MAPK or JNK1 at any time point examined (Figure 4B). Of note, TRAIL addition to 12-day erythroblasts still resulted in the rapid activation of ERK pathway (data not shown).

Since MAPK pathways have been suggested to play regulatory roles in erythroid differentiation, we have assessed the effect of specific cell-permeable inhibitors, such as PD98059 and SB203580, commonly used inhibitors of the ERK and p38/MAPK pathways, respectively, on 6-day erythroblasts, cultured with or without TRAIL. Treatment with PD98059 and SB203580, used alone, showed opposite effects on GAPA expression evaluated after 3 additional days of culture, the former significantly (P < 0.05) increasing and the latter inhibiting (P < 0.05) the mean fluorescence intensity of surface GPA in erythroid cells (Figure 5A). The inhibitory effect of SB203580 on GPA surface expression was comparable with that of z-VAD-fmk (Figure 5A). Remarkably, preincubation with PD98059, used at concentrations that abrogated phospho-ERK activation (Figure 5B), totally abrogated the antiproliferative effect of TRAIL (Figure 5A). On the contrary, preincubation with SB203580 or z-VAD-fmk did not show significant effects on TRAIL-mediated inhibition of GPA surface expression (Figure 5A) and ERK phosphorylation (Figure 5B). U0126, an unrelated inhibitor of the ERK pathway, showed an effect similar to that of PD98059 (data not shown).

The effect of pharmacologic inhibitors on GPA expression in TRAIL-treated cultures could not be ascribed to modulation of cell survival/proliferation since both PD098059 and SB203580 strongly suppressed [3H]thymidine incorporation in cultures without TRAIL (untreated culture set at 100%; + PD098059: 54.2% ± 7%; + SB203580: 50.7% ± 6%; means of 4 independent experiments ± SD) as well as in TRAIL-treated cultures (+ TRAIL alone: 75% ± 7%; + PD098059 + TRAIL: 37.7% ± 4%; + SB203580 + TRAIL: 40.6% ± 5%; means of 4 independent experiments ± SD). These data confirm that both ERK and p38 pathways are involved in mediating erythroid proliferation, and, more importantly, they demonstrate that the differential effect of PD098059 and SB203580 inhibitors on GPA surface expression cannot be ascribed to a differential activity of cell proliferation.
cytometry. Data represent the means by tubulin staining. This experiment is representative of 3 independent experiments analyzed in cell lysates at 5 minutes of TRAIL treatment. Equal loading was compared with untreated culture. (B) The level of phosphorylated ERK1/2 was SB203580 (SB, 10 μM) overnight in cultures were preincubated with vehicle (0.1% DMSO), PD98059 (PD, 10 μM), SB203580 (SB, 10 μM), or z-VAD-fmk (z-VAD, 20 μM) before treatment with TRAIL.

Figure 5. Effect of pharmacologic inhibitors on erythroid differentiation. CD34+ cells were cultured for 6 days in the presence of EPO + IL-3 + SCF. At this time point, cultures were preincubated with vehicle (0.1% DMSO), PD98059 (PD, 10 μM), SB203580 (SB, 10 μM), or z-VAD-fmk (z-VAD, 20 μM) before treatment with TRAIL. (A) Surface glycophorin A expression, reported as MFI, was measured by flow cytometry. Data represent the means ± SDs of 4 different experiments; *P < .05, compared with untreated culture. (B) The level of phosphorylated ERK1/2 was analyzed in cell lysates at 5 minutes of TRAIL treatment. Equal loading was confirmed by tubulin staining. This experiment is representative of 3 independent experiments that gave similar results.

Discussion

In this study, we found that a dim surface expression TRAIL-R1 was detectable only at early stages of erythroid development, while TRAIL-R2 surface expression was detected at early phases of erythropoiesis and showed a progressive increase as erythroid maturation proceeded. These observations suggest that TRAIL-R2, rather than TRAIL-R1, likely plays a mechanistic role in erythroid development.

Among the members of the MAPK family investigated, TRAIL was effective in activating ERK1/2, but not JNK and p38 in erythroid cultures. Moreover, ERK1/2 activation by TRAIL was observed at both early (day 6) and late (days 12-14) culture times strongly implicating TRAIL-R2, the only receptor expressed at both time points, in triggering ERK1/2 phosphorylation. Although we cannot exclude the possibility that TRAIL/TRAIL-receptor interaction stimulates the ERK pathway indirectly, for instance by modulating the conformation of an integral transmembrane protein, this possibility is unlikely in the light of the ability of caspase-8, a major downstream effector of TRAIL-R1/R2, to activate the ERK pathway in lymphoid cells.

MAPKs form a large family of serine-threonine protein kinases conserved through evolution. In mammalian cells, 3 major MAP kinase cascades have been identified: extracellular signal regulated kinases (ERK), c-Jun amino-terminal kinases (JNK) or stress-activated protein kinases (SAPK), and p38 MAP kinase (p38). These kinases, which represent the end of pathways involving multiple serine-threonine kinases activated in a cascade, have become prototypes for the study of structurally related but functionally distinct pathways on cell development.

The potential physiologic significance of our present findings is underscored by the fact that TRAIL is expressed in fetal liver (data not shown) and adult human bone marrow, and that TRAIL protein offers great promise as a cancer therapeutic, its potent antidifferentiative effect on normal erythropoiesis adds a cautionary note to the prolonged treatment of cancer patients with pharmacologic concentrations of recombinant TRAIL protein.

References


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