Stem Cells, Tissue Engineering and Hematopoietic Elements

Evidence for a Role of TNF-Related Apoptosis-Inducing Ligand (TRAIL) in the Anemia of Myelodysplastic Syndromes

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Myelodysplastic syndromes (MDS) are characterized by impaired erythropoiesis, possibly caused by proapoptotic cytokines. We focused our study on the cytokine TRAIL (TNF-related apoptosis-inducing ligand), which has been shown to exhibit an anti-differentiation activity on erythroid maturation. Immunocytochemical analysis of bone marrow mononuclear cells (BMMC) showed an increased expression of TRAIL in MDS patients with respect to acute myeloid leukemia (AML) patients and normal BM donors. TRAIL expression was increased predominantly in myeloid precursors of granulocytic lineage and in a subset of monocytes and pro-erythroblasts. Significant levels of soluble TRAIL were released in 21 of 68 BMMC culture supernatants from MDS patients. On the other hand, TRAIL was detected less frequently in the culture supernatants of AML (4 of 33) and normal BMMC (0 of 22). Analysis of peripheral blood parameters revealed significantly lower levels of peripheral red blood cells and hemoglobin in the subset of patients whose BMMC released TRAIL in culture supernatants compared to the subgroup of patients who did not release TRAIL. Moreover, TRAIL-positive BMMC culture supernatants inhibited the differentiation of normal glycophorin A⁺ erythroblasts generated in serum-free liquid phase. Thus, increased expression and release of TRAIL at the bone marrow level is likely to impair erythropoiesis and to contribute to the degree of anemia, the major clinical feature of MDS. (*Am J Pathol* 2005, 166:557–563)

The myelodysplastic syndromes (MDS) comprise a heterogeneous group of clonal disorders, usually characterized by a normal or hypercellular marrow with dysplastic features leading to peripheral blood cytopenias and a variable incidence of transformation into acute myeloid leukemia (AML).¹ MDS generally occurs in elderly patients, the median age at diagnosis being 65 to 70 years.² One mechanism invoked to explain the apparent discrepancy between cellular marrow and peripheral blood cytopenias in patients with MDS is apoptosis, which occurs with increased frequency in MDS marrow.^{3–8} The decrease of erythrocytes, the major clinical feature of MDS, has been attributed to an enhanced sensitivity to inhibitory cytokines.^{9–14} In fact, it has been shown that several cytokines, known to have proapoptotic properties, such as IL-1 β , TNF- α , FasL, and TGF- β 1, are up-regulated in many patients with MDS. However, it is clear from those studies that the regulation of hemopoiesis in MDS is complex and it is likely that multiple factors are involved. Moreover, conflicting data are available on the functional integrity of the BM microenvironment in MDS, which plays a key role for the long-term survival and maintenance of the progenitor cells.^{15,16}

TNF-related apoptosis-inducing ligand (TRAIL)/Apo-2L exists as either a type II membrane protein or as a soluble form.^{17–19} TRAIL interacts with four high-affinity membrane receptors belonging to the apoptosis-inducing TNF-receptor (R) family. TRAIL-R1 (DR4) and TRAIL-R2 (DR5) are able to transduce apoptotic signals on binding of TRAIL, while TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) are homologous to DR4 and DR5 in their cysteine-rich extracellular domain, but lack apoptosis-inducing capability.²⁰ TRAIL shows the unique property to induce apoptosis in a variety of neoplastic cells,

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Table 1. Characteristics of the Study Groups

Patient group	Number of cases	Gender (male/female)	Age (median and range)
Controls	22	12/10	48 (38–64)
- normal BM	9		
- NHL*	13		
MDS (FAB subtypes)	68	37/31	70 (37–87)
- RA	34		
- RARS	8		
- RAEB	15		
- RAEB-T	4		
- CMMoL	7		
AML FAB (subtypes)	33	15/18	67 (32–84)
- M0	4		
- M 1	3		
- M 2	4		
- M 3	4		
- M 4	9		
- M 5	8		
- M 7	1		

*, Non-Hodgkin's lymphoma with no bone marrow involvement.

including MDS blasts,^{21,22} displaying minimal or absent toxicity on most normal cells. Despite its potential as an anti-cancer therapeutic agent both *in vitro* and *in vivo*,^{23,24} the wide expression of TRAIL and TRAIL-Rs in many normal tissues^{17,18} suggests that the physiological role of TRAIL is more complex than induction of apoptosis in cancer cells. In this respect, although TRAIL is not cytotoxic on normal clonogenic hematopoietic progenitors,^{21,22,25} it has been shown that TRAIL selectively affects erythroid development by specifically targeting immature erythroblasts.^{25–28} In particular, we have recently demonstrated that TRAIL displays an anti-differentiative activity on erythroid maturation.²⁷ On this basis, the present study was designed to investigate the expression and the potential role of TRAIL in the induction of anemia in MDS patients.

Materials and Methods

Patients

Sixty-eight patients with MDS were investigated (Table 1). The patients were subdivided according to the French-American-British (FAB) classification: refractory anemia (RA, 34 patients); refractory anemia with ringed sideroblasts (RARS, 8 patients); refractory anemia with excess of blasts (RAEB, 15 patients); refractory anemia with excess of blasts in transformation (RAEB-t, 4 patients); and chronic myelomonocytic leukemia (CMMoL, 5 patients). Patients were also classified according to World Health Organization criteria. Moreover, we have studied 33 patients with AML (including FAB subtypes M0–M7) at diagnosis; 9 healthy volunteer donors and 13 patients with non-Hodgkin lymphoma (NHL) without BM involvement, which were included in this study for comparison with MDS patients (Table 1). All individuals considered in this study had given informed consent in accordance with

institutional guidelines. Samples were routinely processed for phenotyping and cytogenetic analyses.

Preparation of BM-Conditioned Media for ELISA

BM specimens of patients and controls were obtained by aspiration with Jamshidi needle; 1 ml from each sample was gently run through a 22-gauge needle to homogenize the marrow particles. Cell suspensions were fractionated on Lymphocyte-H (Cedarlane Laboratories, Hornby, Ontario, Canada) by centrifugation at 2000 rpm, for 20 minutes. Cells were washed twice with PBS 1X (Life Technologies, Renfrewshire, UK). After gradient, BMMC samples were suspended in 1 ml serum-free RPMI medium at a cell density of 1.5×10^6 /ml, supplemented with 1% penicillin/streptomycin and 2% glutamine (Hyclone Europe, Cramlington, UK). The suspension was incubated for 24 hours at 37°C in 5% CO₂. After overnight culture, the conditioned medium was collected, filtered on 0.22-μm Millipore sterile filters (Millipore Corporation, Bedford, MA), and stored at –20°C. The level of soluble TRAIL, released in the culture medium by BMMC, was determined by a commercial ELISA kit (R&D Systems, Minneapolis, MN), following the manufacturer's instructions.

TRAIL Immunostaining

Immunocytochemical detection of TRAIL was performed using alkaline phosphatase anti-alkaline phosphatase (APAAP) technique in BM smears and/or cytopsin preparations of BMMC obtained from patients and controls. Cytopsin were stained with May-Grunwald-Giemsa and used for immunostaining with anti-TRAIL mAb (1:20 dilution, clone 75402.11, R&D Systems). For this purpose, cytopsin were incubated with the specific mAb for 30 minutes at room temperature, then washed in TBS; rabbit anti-mouse Ig and APAAP complex (DAKO, Copenhagen, Denmark) were added and incubated for further 30 minutes. After addition of the substrate, the samples were washed in TBS, mounted in a suitable aqueous medium, and examined under light microscopy with an Axyophot Zeiss microscope equipped with a CoolScan video camera (Photometrics, Livingston, UK). The specificity of the staining was ensured by using an isotype-matched irrelevant antibody, as a substitute for primary antibody, and by pre-incubating the TRAIL mAb in the presence or absence of a 10-fold excess of recombinant TRAIL (used as blocking peptides). Slides were analyzed blinded by two independent investigators.

Short-Term Hematopoietic Colony Assay

BMMC from MDS patients and controls were isolated as previously described, washed in PBS and plated in triplicate at 1×10^5 cell-density in 35-mm Petri dishes in 1.1 ml of a methylcellulose semisolid medium (Methocult H4434, Stem Cell Technologies, Vancouver, Canada). Petri dishes were incubated at 37°C in a fully humidified atmosphere with 5% CO₂ and scored at day 14 under inverted microscope (Wilovert-Will, Wetzlar, Germany)

for the presence of hematopoietic progenitor colonies: CFU-GM and burst-forming-unit erythroid (BFU-E) as previously described.²⁹

In Vitro Generation of Erythroid Cultures and Treatments

Cord blood (CB) specimens were collected according to institutional guidelines. CB CD34⁺ cells were isolated using a magnetic cell-sorting program Mini-MACS and the CD34 isolation kit (Miltenyi Biotech Auburn, CA) as previously described.²⁶ CD34⁺ cells were cultured in Ex-vivo-20 serum-free medium supplemented with nucleosides (10 µg/ml each), 0.5% BSA (Chon fraction V), 10⁻⁴ M BSA-adsorbed cholesterol, 10 µg/ml insulin, 200 µg/ml iron-saturated transferrin, 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 × 10⁴/ml and seeded in culture in the presence of stem cell factor (SCF, 50 ng/ml) + 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, the cell density was re-adjusted to 4 × 10⁵/ml and erythroid differentiation was monitored by analysis of surface glycoprotein A (GPA), as previously described.^{26,27} At day 6, fresh medium was replaced together with 50% vol:vol of MDS BMMC culture supernatants, in which the presence and the levels of TRAIL were predetermined by ELISA. For TRAIL-neutralization, culture supernatants were pre-incubated with recombinant Fc-TRAIL-R2 chimera (R&D Systems). Erythroid differentiation was monitored after additional 6 to 8 days of culture by flow cytometric analysis of GPA.

Statistical Analysis

Results were evaluated by using the χ^2 analysis to compare the control and study groups for the frequency of TRAIL detection. Box plots were used to show the median, minimum, and maximum values and 25th to 75th percentiles for each group. When appropriated on the basis of the distribution of the values, a parametric (Student's *t*-test) or a non-parametric (Mann-Whitney rank-sum test) analysis was performed. Statistical significance was defined as *P* < 0.05.

Results

TRAIL Expression Is Increased in BM Cells in MDS Patients with Respect to Either AML Patients or Normal Controls

In the first group of experiments, we have performed immunocytochemistry analysis in BMMC samples obtained from patients affected by MDS, AML, or normal controls. As shown in Figure 1, A and B, TRAIL was expressed in most metamyelocytes and granulocytes in normal BMMC. Moreover, a small percentage of

Figure 1. TRAIL expression in normal and pathological BMMC. TRAIL expression was examined by immunohistochemistry by APAAP, using anti-TRAIL mAb. In **A**, BMMC slides, obtained from 6 to 10 different individuals per study group were examined. Data are expressed as mean $\pm$ SD of percentage of TRAIL-positive cells calculated by scoring at least six random fields for each slide. Representative fields of BMMC from normal donors (**B**), from MDS (**C**), and AML (**D**) patients, examined immunohistochemically for TRAIL are shown. Stainings with May-Gruenwald-Giemsa of the same samples are also shown. In **B**, some TRAIL-positive metamyelocytes and granulocytes (white asterisks, left panels) and megakaryocytes (white arrow, right panel) are indicated, while erythroblasts (black arrowhead, left panel) are negative for TRAIL expression. In **C**, several myeloid cells at different level of differentiation exhibit a bright positivity for TRAIL. While erythroblasts are negative (asterisks in left and right panels), a subset of proerythroblasts (arrowhead, right panel) are positive. In **D**, monocytoid blast cells show either negativity (bottom panel) or a dim positivity (top panel) for TRAIL. Original magnification, $\times 20$.

Figure 2. Release of TRAIL in the supernatants of normal and pathological BMMC. Culture supernatants derived from BMMC of control individuals (ie, BM not involved by any pathology) and of patients affected by AML or MDS were analyzed for soluble TRAIL by ELISA. The absolute number of TRAIL-positive samples in each study group are reported. Each dot was determined in duplicate. *, Statistically significant differences in the frequency of TRAIL detection ($P < 0.05$).

megakaryocytes and monocytes also showed positivity for TRAIL. In BMMC of MDS patients, most metamyelocytes and granulocytes examined were also positive for TRAIL expression (Figure 1A), but usually showed a greater intensity of staining with respect to normal BM (Figure 1C). Moreover, in MDS patients, a significant ($P < 0.05$) increase of TRAIL positivity was noticed in BM monocytes as well as in a subset of pro-erythroblasts (sometimes including megaloblasts) (Figure 1, A and C). In AML samples, blast cells were mostly negative or in a few cases dimly positive for TRAIL and only the residual metamyelocytes, granulocytes, and monocytes expressed TRAIL, at levels comparable to those of non-pathological BM (Figure 1D). Of note, in all normal and MDS samples examined, polychromatophil and orthochromatic erythroblasts were constantly negative for TRAIL expression (Figure 1A).

TRAIL Is Released in the Culture Supernatant of BMMC in a Subset of MDS Patients

In the next group of experiments, we have investigated whether TRAIL was released by BMMC obtained from 22 individuals without BM involvement, 33 patients affected by AML and 68 patients affected by MDS (Table 1). None of the 22 conditioned media from BMMC of control individuals released detectable amounts of TRAIL (Figure 2). Among the conditioned media derived from AML BMMC, only 4 of 33 were positive for TRAIL protein (mean value $\pm$ SD: 29 ± 23 pg/ml) (Figure 2). The positive cases belonged to the M0, M2, M3, and M7 FAB subtypes. On the other hand, MDS BMMC showed a significant ($P < 0.05$) increase in the frequency of TRAIL detection (in 21 of 68 patients; mean value $\pm$ SD: 56 ± 95 pg/ml) in their culture supernatants with respect to either normal and AML BMMC (Figure 2). No significant differences were noticed among the RA, CMMoL, and RARS subgroups of MDS, with a percentage of positive cases ranging from

Figure 3. Peripheral blood (PB) and BM hematological parameters. PB hematological parameters (**A**) and BM hematopoietic progenitors (**B**) were measured in the control group as well in the MDS patients. Comparisons between the patients in which soluble TRAIL was (TRAIL-positive) or was not (TRAIL-negative) detected in the supernatants of MDS BMMC are indicated. Horizontal bars are median, upper and lower edges of box are 75th and 25th percentiles, lines extending from box are 10th and 90th percentiles. Parameters are expressed as follow: RBC $\times 10^3/\mu\text{l}$; Hb g/dl; Platelets (Plts) $\times 10^3/\mu\text{l}$; WBC $\times 10^3/\mu\text{l}$; BFU-E/ 10^5 BMMC; CFU-GM/ 10^5 BMMC. *, Statistically significant differences (<0.05).

~35 to 45% of patients/subgroup, while none of the four RAEB-t examined released TRAIL protein in the conditioned medium.

Release of Soluble TRAIL by MDS-BMMC Correlates with Relevant Clinical Parameters

MDS patients were subdivided in two groups on the basis of the ELISA TRAIL-positivity/negativity to evaluate whether the release of TRAIL at the BM level might be associated to baseline hematological clinical parameters. As far as the cytogenetic characteristics, the frequency of clonal chromosome abnormalities was equally distributed among TRAIL positive (36%) and TRAIL-negative (37.5%) MDS patients. However, analysis of peripheral blood parameters (Figure 3A) revealed significantly ($P < 0.05$) lower levels of peripheral red blood cells and of hemoglobin in the subset of patients whose BMMC released TRAIL in culture supernatants (mean value $\pm$ SD of peripheral red blood cells, $3104 \pm 780 \times 10^3/\mu\text{l}$; mean value $\pm$ SD of hemoglobin, 8.8 ± 2.2 g/dl) with respect to the subgroup of patients who did not release TRAIL (mean value $\pm$ SD of peripheral red blood cells:

Figure 4. Effect of conditioned media of MDS BMMC on erythroid maturation. CB-derived CD34⁺ cells were cultured in the presence of EPO + IL-3 + SCF for 6 days. At this time point, cultures were supplemented with conditioned media of TRAIL-positive MDS BMMC, in the presence or absence of TRAIL-R2-Fc chimeric protein. At 12 days of cultures, cells were analyzed for GPA expression by flow cytometry. **Shadowed histograms** represent cells stained with mAb anti-GPA, while **unshadowed histograms** represent the background fluorescence obtained from the staining of the same cultures with isotype-matched control mAb. Mean fluorescence intensity (MFI) values are indicated. Similar results were observed in three independent experiments.

3560 ± 930 × 10³/μl; mean value ± SD of hemoglobin: 10.3 ± 1.9 g/dl). On the other hand, no significant differences could be established by evaluating the number of the platelets and leukocytes, even if they were lower in TRAIL-positive patients (Figure 3A). Moreover, when we have analyzed BM hematopoietic progenitor cells, the number of BFU-E and CFU-GM was significantly ($P < 0.01$) lower in MDS patients (mean value ± SD of BFU-E, 8.5 ± 11; mean value ± SD of CFU-GM, 21 ± 18) than in normal controls (mean value ± SD of BFU-E, 35 ± 13; mean value ± SD of CFU-GM, 42 ± 8), as expected based on previous findings of our group.²⁹ Of note, among MDS patients, the number of BFU-E and CFU-GM showed a tendency to increase in the TRAIL-positive subgroup (mean value ± SD of BFU-E, 12 ± 14; mean value ± SD of CFU-GM, 26 ± 19) with respect to the TRAIL-negative subgroup (mean value ± SD of BFU-E, 7 ± 10; mean value ± SD of CFU-GM, 18 ± 16), although not to a significant extent (Figure 3B).

TRAIL Released in the Culture Supernatant of MDS-BMMC Impairs the Maturation of Normal Erythroid Progenitors

To further investigate whether TRAIL endogenously released by BMMC of MDS patients might be involved in the impairment of erythroid maturation, which characterizes the natural history of MDS, pools of BM-conditioned media from three MDS patients showing significant release of soluble TRAIL were added to normal CD34-derived erythroblasts cultured in serum-free liquid phase. The addition of the MDS-BM-derived culture supernatants induced a significant ($P < 0.01$) impairment of erythroid maturation, as assessed by the levels of GPA examined by flow cytometry at 12 to 14 days of culture (mean value ± SD of GPA MFI, 240 ± 60 in TRAIL-treated cultures versus 780 ± 120 in control cultures) (Figure 4). Of note, this inhibitory effect was counteracted by pre-incubation of the culture supernatant with Fc-TRAIL-R2 chimera (mean value ± SD of GPA MFI, 640 ± 80) (Figure 4), demonstrating the crucial role of released

soluble TRAIL in impairing the maturation of erythroid precursors.

Discussion

Ineffective erythropoiesis is a common feature of MDS. Increased cellular proliferation, along with exaggerated apoptosis, is one paradigm that has been used to explain the presence of a cellular or even hypercellular marrow concurrently with peripheral blood cytopenias in patients with MDS.^{1,2} Variable cytopenias in the face of normocellular to hypercellular BM in patients with MDS have been attributed to excessive intramedullary apoptosis. Studies have distinguished in myelodysplastic BM between the ability of immature progenitors to proliferate and the propensity of more mature precursor cells to undergo apoptosis.^{3–14} Moreover, it has been shown that the apoptosis of differentiated MDS erythroid cells during culture mimics the apoptosis of erythroid mature cells in MDS bone marrow biopsy specimens and smears. Apoptosis could be mediated by either a lack of positive (survival) signals or an overproduction of negative (death) signals.^{3–14} Such a pattern could arise with an excess of bifunctional cytokines showing a positive impact on early precursors and mediating negative signals for cells at later stages of differentiation.

In early studies, a role for the Fas/FasL as well as for TNF-α/TNF-R1 systems has been proposed to explain the pathogenesis of dyserythropoiesis in MDS.^{4–14} However, a recent study has challenged the hypothesis that blocking TNF-α might be of clinical relevance in MDS, despite the elevation of TNF-α at the BM level.¹³ In fact, inhibition of TNF-α production using the soluble TNF receptor (Enbrel) was well tolerated at the dosages used in MDS, but its efficacy as a single agent was very modest.¹³ All these studies underline the complexity of the pathogenesis of anemia in MDS.

In this study, we have documented by immunocytochemistry that TRAIL protein was constitutively expressed by BM metamyelocytes in both normal BM donors and MDS patients, but the intensity of the staining was significantly stronger in MDS BMMC samples. Of note, the expression of TRAIL in MDS patients was not restricted to the granulocytic lineage but it was extended also to a subset of monocytes, megakaryocytes, and to a small subset of proerythroblasts and megaloblasts. The expression of TRAIL by 15% of pro-erythroblasts and by 30% monocytes in BM MDS is particularly noteworthy, since cells of the erythroid lineage are known to develop in BM niches in close contact between each other and with BM monocytes.²⁵ Thus, considering the crucial role of BM monocytes in the regulation of erythropoiesis, it is plausible that an increased TRAIL expression in BM monocytes importantly contributes to the ineffective erythropoiesis characteristic of MDS. In this respect, we have previously demonstrated in a series of *in vitro* studies that erythropoiesis is most heavily impaired by TRAIL with respect to the other hematopoietic lineages.^{26,27}

TRAIL, a member of the TNF superfamily of cytokines, is expressed as a type II membrane protein or, on cleav-

age from the cell surface, as a soluble protein.¹⁹ Measurement of soluble TRAIL, released in culture media, revealed undetectable levels of TRAIL in all normal BMMC cultures examined, while it was detected by a significant subset of MDS BMMC. Consistent with our present findings, it has been recently demonstrated by using the cDNA microarray technology that several genes including TRAIL are selectively up-regulated in a subset of MDS patients.³⁰ TRAIL release does not necessarily correlate with the level of cellular expression, since several MDS BMMC samples, which displayed a strong positivity for TRAIL at immunocytochemistry, did not release soluble TRAIL in the culture supernatant. Therefore, although the mechanisms involved in TRAIL release are still poorly understood, these data suggest a potential role of BM microenvironment in the control of TRAIL protein release.

It is also particularly noteworthy that endogenous TRAIL was predominantly detected in early disease (RA and RARS MDS), which are entities characterized by lower number of blasts. On the other hand, the RAEB-t group of MDS examined, like the AML transformed from MDS, were negative for TRAIL release by BMMC. These findings are compatible with a potentially protective role of TRAIL against the expansion of the blast cell population, as suggested in a previous study,²¹ although it is also possible that the selective pressure of TRAIL on the blast cell population ultimately leads to the emergence of more malignant clones.

Remarkably, among the MDS patients examined, the ability of BMMC to release TRAIL correlates positively with the degree of anemia, suggesting that TRAIL is an important pathogenetic determinant in the induction of anemia. These findings are particularly noteworthy as no correlations were previously observed between the levels of other death-inducing ligands, such as TNF- α , and any clinical parameter.¹³ The hypothesis that TRAIL has a pathogenetic role in worsening the degree of anemia in MDS patients was strengthened by the observation that endogenous TRAIL, released in the culture supernatants by MDS BMMC, significantly impaired normal erythroid maturation in *in vitro* assays. Thus, an increased expression and release of TRAIL protein at the BM level seems to be involved in the impairment of erythropoiesis, which characterizes MDS and its major clinical feature: the induction of anemia.

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