Application of flow cytometry to molecular medicine: Detection of tumor necrosis factor-related apoptosis-inducing ligand receptors in acute myeloid leukaemia blasts

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Received July 8, 2005; Accepted August 18, 2005

Abstract. TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), a cytokine belonging to the TNF (tumor necrosis factor) family, is currently regarded as a potential anti-cancer agent. Nevertheless, several types of cancer cells display a low sensitivity to TRAIL or are completely resistant to this pro-apoptotic cytokine. TRAIL signalling is dependent on four receptors. Two of them, death receptors 4 and 5 (DR4 and DR5), induce apoptosis, whereas decoy receptors 1 and 2 (DcR1 and DcR2) are unable to evoke cell death upon TRAIL binding. TRAIL resistance may be related to the expression of TRAIL decoy receptors. TRAIL has been proposed as a novel therapeutic agent for the treatment of haematological disorders, including acute myeloid leukaemia (AML). Surprisingly, however, very limited information is available concerning the expression of TRAIL receptors in AML blasts. Here, we have evaluated, using flow cytometry, TRAIL receptor surface expression and sensitivity to TRAIL-dependent apoptosis of AML blasts from 30 patients. We observed frequent expression of TRAIL DcR1 and DcR2, while expression of DR4 and DR5 was less frequent. Nevertheless, the expression of DR4 or DR5 in leukaemic cells was always matched by a similar expression of one of the decoy receptors. Leukaemic blasts were invariably resistant, even to a high concentration (1000 ng/ml) of TRAIL. We suggest that AML blasts are resistant to TRAIL apoptosis *in vitro*. Therefore, it is unlikely that TRAIL alone might be used in the future as an innovative pharmacological agent for the treatment of AML.

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Key words: tumor necrosis factor-related apoptosis-inducing ligand, acute myeloid leukaemia, receptors, surface expression, apoptosis

Introduction

TRAIL is an important member of a family of death ligands which also includes TNF (1,2). TRAIL is a type II membrane protein and its C-terminus can be processed proteolytically to form a soluble ligand. TRAIL binds to four high-affinity transmembrane receptors which belong to the apoptosis-inducing TNF-receptor family. TRAIL-R1 (DR4) and TRAIL-R2 (DR5) (also referred to as 'death receptors') transduce apoptotic signals upon interacting with TRAIL, whereas TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) (also referred to as 'decoy receptors') are homologue to DR4 and DR5 in their cysteine-rich extracellular domain but lack the intracellular death domain and apoptosis-inducing capability (3). Therefore, the decoy receptors can inhibit cell killing by sequestration of the death ligand. TRAIL has the unique capability of inducing apoptosis in a large variety of neoplastic cells, including several haematological malignancies (4), while displaying minimal or absent toxicity on most normal cells (5). For this reason, TRAIL is considered as a potential tumour-specific therapeutic agent.

AML is a disease characterized by a severe prognosis and the end-limit for chemotherapeutic treatment has probably already been reached. Therefore, innovative therapeutic strategies are required to improve the outcome of this disorder. It has been shown that TRAIL suppresses the growth of AML progenitors (6). Therefore, TRAIL is currently regarded as a potential agent for the treatment of AML, also in combination with chemotherapeutic drugs (4). The applicability of TRAIL to the therapy of AML would require detailed knowledge about the expression of TRAIL receptors in AML blasts.

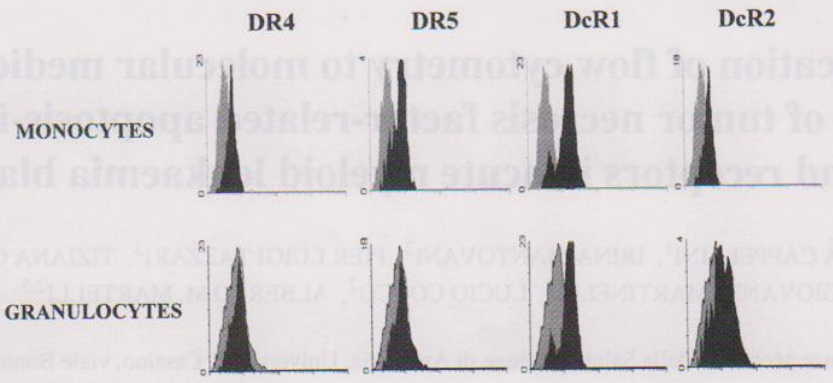

Figure 1. Flow cytometric detection of TRAIL receptors in granulocytes and monocytes from healthy donors. Grey histograms represent control samples (samples incubated with an unrelated isotype-matched, PE-conjugated antibody).

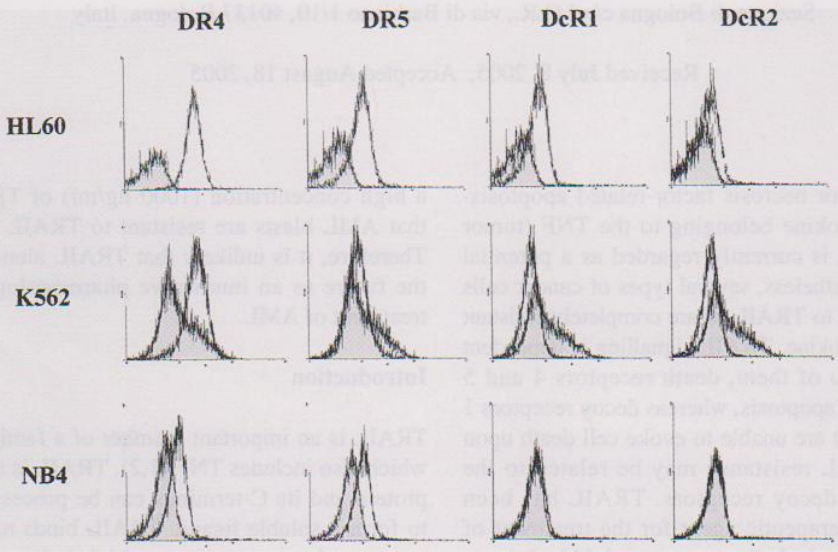

Figure 2. Flow cytometric analysis of TRAIL receptor expression on human myeloid leukaemia cell lines. Grey histograms represent control samples (see Figure 1 legend). A representative of three separate experiments is shown.

Surprisingly, however, very limited information is available concerning the expression of TRAIL surface receptors in AML blasts. In a recent report, the expression of DR4 and DR5 in AML blasts was investigated by immunocytochemistry (7). However, in this study, cells were fixed by acetone so that positive staining could reflect the presence of intracellular TRAIL receptors being transported to the plasma membrane (8), given that acetone also acts as a permeabilizing agent (9,10). For example, in TRAIL-resistant colon cancer cells, a defective transport system for DR4 to the plasma membrane has been described in which DR4 accumulated in the cytosol (11). Moreover, neither the expression of decoy receptors nor the actual TRAIL sensitivity of AML blasts were analyzed. In other studies performed on AML blasts, TRAIL receptor expression was evaluated only at the mRNA level and no correlation was made between the TRAIL sensitivity of leukaemic cells and TRAIL receptor expression (12,13).

With the above in mind, we decided to analyze the expression of surface TRAIL receptors (both death and decoy) in AML blasts using flow cytometry and we also correlated the receptor expression with apoptosis induced *in vitro* by TRAIL. We have found that AML blasts frequently express decoy receptors whereas surface expression of DR4 and DR5 was slightly less frequent. Moreover, leukaemic blasts were resistant to TRAIL-mediated apoptosis.

Materials and methods

Chemicals and antibodies. RPMI-1640, fetal bovine serum (FBS), bovine serum albumin (BSA, Fraction V), and L-glutamine were from Sigma, St. Louis, MO, USA. Ficoll-Paque Plus (1.077 g/ml) was from Amersham Biosciences, Milan, Italy. Phycoerythrin (PE)-conjugated monoclonal antibodies to human TRAIL receptors (DR4, DR5, DcR1, DcR2) were from eBiosciences, San Diego, CA, USA. Human recombinant TRAIL was from Calbiochem, La Jolla, CA, USA. Fluorescein isothiocyanate (FITC)-conjugated anti-CD33 monoclonal antibody was from BD Biosciences, Erembodegem, Belgium.

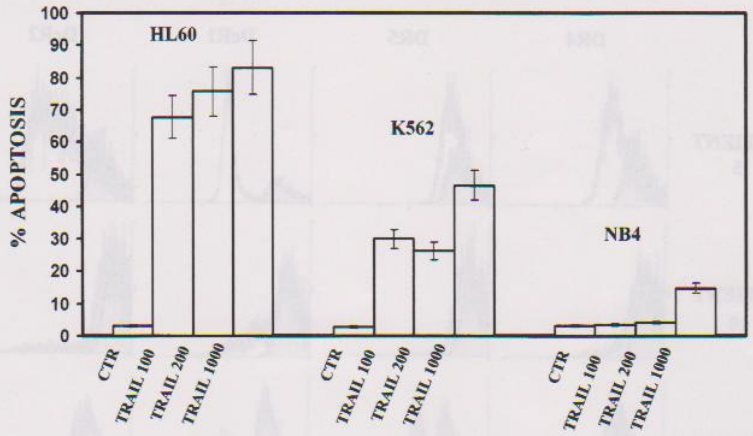

Figure 3. Analysis of TRAIL-dependent apoptosis in human leukaemia cell lines. Cells were incubated for 24 h with the indicated TRAIL concentrations (ng/ml), then analyzed by flow cytometry after being stained with PI. CTR, control (untreated cells). Results are the mean ± SD of three separate experiments.

Cell culture. NB4, HL60, and K562 cell lines were cultured in a humidified atmosphere at 37˚C, with 5% (v/v) CO_2, in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, and 2 mM L-glutamine at an optimal density of 3 to $8x10^5$ cells/ml. The medium was changed every 3 days.

Patients. Samples were obtained at diagnosis after informed consent in accordance with institutional guidelines. Peripheral blood mononuclear cells (PBL) and bone marrow cells (BM) were isolated by density gradient centrifugation (Ficoll-Paque Plus, 1.077 g/ml) and were frozen at a concentration of 30 to $60x10^6$ cells/ml in 10% dimethylsulfoxide, 45% RPMI-1640 and 45% FBS. Cryopreservation was performed using a programmable freezer according to an electronically monitored program that allows a cooling rate of -1˚C/min to -120˚C. Samples were maintained in the vapour phase of liquid N_2. The AML cases were defined according to the classification of the French-American-British (FAB) committee. The percentage of blasts in the samples ranged from 75 to 91% and was checked by flow cytometry staining, depending on the phenotype of leukaemia (usually CD13, CD33, CD34, alone or in combination).

Flow cytometric analysis of surface TRAIL receptors. Cell surface immunostaining was performed at room temperature for 1 h by incubating $3x10^5$ cells/sample in 100 μl of phosphate-buffered saline (PBS, containing 1% BSA) with the indicated monoclonal antibodies. Dilution was 1:10 for all of the antibodies, corresponding to 0.2 μg antibody/sample. Non-specific fluorescence was assessed by incubation with irrelevant isotype-matched PE- or FITC-conjugated monoclonal antibodies. To confirm that the blasts were leukaemic in PBL or BM samples, before staining with anti-TRAIL receptor antibodies, cells were incubated for 1.5 h at 4˚C with an FITC-conjugated antibody to CD33 (Beckman-Coulter, Miami, FL, USA). In some cases, cells were fixed with Reagent 1 of the Intraprep kit, according to the Manufacturer's instructions (Beckman-Coulter). Cells were then permeabilized with saponin-based Reagent 2 and incubated at 4˚C for 2 h, then immunostained overnight with anti-TRAIL receptor

Table I. Biological characteristics of AML patients.

Patients	Age	Sample	FAB classification	Karyotype
1	38	PBL	M1	normal
2	68	PBL	M2	Inv9, dup1p
3	74	BM	M1	normal
4	64	PBL	M5	NA
5	57	PBL	M5	normal
6	48	PBL	M1	normal
7	39	PBL	M0	normal
8	85	PBL	M4	46xy, inv16
9	21	BM	M2	normal
10	65	PBL	M0	NA
11	84	BM	M2	normal
12	61	BM	M1	NA
13	57	BM	M1	NA
14	45	BM	M3	t(15;17)
15	39	PBL	M3	t(15;17)
16	45	PBL	M1	complex
17	66	PBL	M4	46xy, inv(3) (q21q26)
18	58	PBL	M4	46xy, inv(3) (q21q26)
19	58	BM	M1	46xx, inv(3) (q21q26)
20	63	PBL	M5	46xx, t(4,11) (q21q23)
21	47	PBL	M1	46xx, inv(3) (q21q26)
22	31	PBL	M5	complex
23	45	BM	M5	normal
24	86	PBL	M0	+8
25	41	PBL	M4	46xy, inv16
26	35	BM	M2	t(8;21)
27	61	PBL	M2	t(8;21)
28	55	BM	M2	NA
29	47	BM	M5	NA
30	56	PBL	M2	NA

NA, not available.

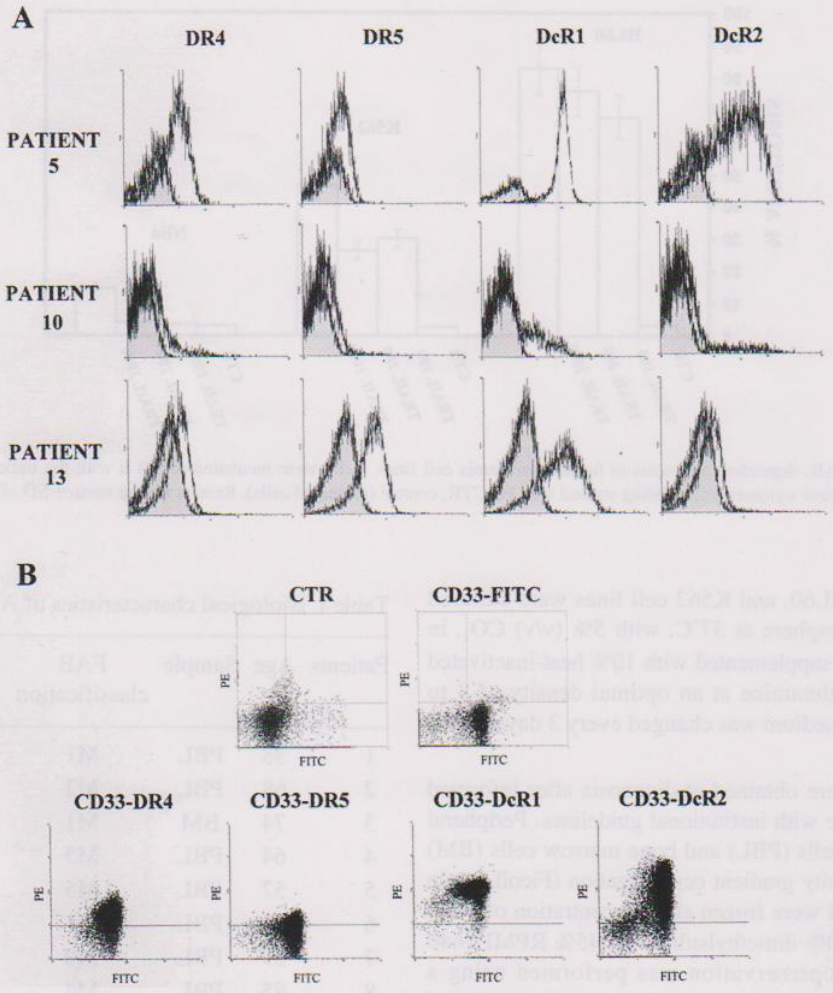

Figure 4. Expression of TRAIL receptors on AML blasts detected by flow cytometry. A, single staining (grey histograms represent controls). B, dot plots of double staining with PE-conjugated anti-TRAIL receptor antibodies and FITC-conjugated anti-CD33 antibody. CTR, control, stained with unrelated isotype-matched antibody.

antibodies, as reported elsewhere (14). Flow cytometric analysis was performed by a Coulter Epics XL-MCL flow cytometer (Beckman-Coulter) using SystemII dedicated software.

Induction and detection of apoptosis. Apoptosis was induced by treatment with human recombinant TRAIL. Cells ($1x10^6$/ml) were seeded in 6-well culture plates containing 3 ml of RPMI-1640 plus 10% FBS and incubated at 37°C for 24 h in the presence of TRAIL (100, 200 or 1000 ng/ml). Apoptotic cells were detected by staining with propidium iodide (PI) and analyzed by flow cytometry. For PI staining, cells were fixed in 70% cold ethanol for at least 20 h at -20°C. They were then washed in PBS and incubated for 30 min at room temperature with 20 μg/ml PI. Apoptotic cells were detected as a hypodiploid peak.

Results

Expression of surface TRAIL receptors on human monocytes and granulocytes. Since a very critical issue for the findings of this paper was represented by the specificity of the antibodies to TRAIL receptors, we decided to test them first on human monocytes and granulocytes from healthy donors and then to compare the results with the data available in the literature. We reasoned that this could be a better approach than testing them on cell lines because it would probably present some variability, depending on the laboratory, due to the existence of different clones. As presented in Fig. 1, monocytes mainly expressed DR5 and DcR1 whereas DR4 and DcR2 were detected to a lower extent. In contrast, granulocytes were positive for DcR1 and DcR2 and negative for DR4 and DR5. Overall, our findings were in agreement with the literature (15-18).

Surface TRAIL receptor expression and TRAIL sensitivity of human leukaemia cell lines. We then analyzed the expression of surface TRAIL receptors in myeloid leukaemia cell lines, HL60, NB4, and K562, using flow cytometry. HL60 cells displayed all four TRAIL receptors. In K562 cells, we detected the presence of TRAIL-DR4 and, to a lower extent, DR5, but not the decoy receptors. Finally, NB4 cells expressed only the two death receptors at low levels and were negative for the decoy receptors (Fig. 2).

Table II. TRAIL receptor expression in AML patients.

Patients	DR4	DR5	DcR1	DcR2
1	+-	+-	+-	+
2	+-	+-	+-	+-
3	+-	+-	+-	+
4	+	-	+-	-
5	++	+	++++	++++
6	+-	+-	+-	+-
7	+-	+-	+-	+-
8	+-	+-	+-	+-
9	+	+-	+	+
10	+-	+-	+-	+-
11	+	+	++	+
12	+-	++	++	++
13	+-	++	++	+-
14	+	+-	+	++
14 perm	+	+	++++	++
15	+-	+-	+-	+-
15 perm	+-	+	+	+-
16	+++	+	+++	++++
17	+	+	++	+
18	+-	+-	+	+-
19	+	+	++	+
20	+	+	+	+
21	+	+	++	+
22	+-	++	+-	++
23	+++	++	++	++
24	+-	+-	+-	+-
25	++	+	+	+-
26	+	+-	+	+
27	+++	++	++++	+++
28	+	+	++	+++
28 perm	+	+	+++	+++
29	+++	+	++	+++
29 perm	+++	+	+++	+++
30	+-	++	+	+-
30 perm	+-	++	+++	+

+-, 1-20% of positive cells; +, 20-40%; ++, 40-60%; +++, 60-80%; ++++, 80-100%. Perm, cells were permeabilized prior to incubation with antibodies to TRAIL receptors.

We then treated all the cell lines with different concentrations of TRAIL to investigate if there was an apoptotic effect as a result of cytokine interaction with the receptors. In spite of the considerable expression of decoy receptors, HL60 cells presented a high sensitivity to TRAIL-induced apoptosis. Indeed, in the presence of 1000 ng/ml of TRAIL, nearly 85% of cells were apoptotic after a 24-h incubation (Fig. 3). By treating K562 cells with TRAIL, we observed a lower percentage of apoptotic cells (approximately 50%) than those in HL60 cells, even at the highest concentration of the cytokine. In NB4 cells, which do not express decoy receptors and have very low levels of DR4 and DR5, the percentage of apoptosis following TRAIL treatment was very scarce (approximately 18% at the highest concentration; Fig. 3). These results confirmed the ability of these TRAIL preparations to kill cells expressing DR4 and DR5.

Surface TRAIL receptor expression and TRAIL sensitivity of human AML blasts. In a subsequent group of experiments, the surface expression of all four TRAIL receptors was investigated using samples taken at diagnosis from 30 patients diagnosed as having AML (Table I). It is worth noting here that, for this investigation, we used frozen samples which were rapidly thawed on ice and then analyzed. The majority of samples were constituted of PBL, whereas the remaining 30% of samples were isolated from BM. To ensure that we considered the expression of TRAIL-receptors only in leukaemic blast cells, we always performed double immunostaining with anti-CD33 antibody (Fig. 4A and B).

Overall, flow cytometric analysis revealed a low immunoreactivity of AML blasts for death receptors. According to a previous report (7), we considered a sample to be positive when at least more than 20% of cells displayed surface staining. Variable expression of DR4 and DR5 was detected in approximately 50% of the patients (16/30), whereas 70% of patients were positive for DcR1 (21/30) and 60% were positive for DcR2 (18/30) (Table II). Samples that displayed a higher positivity for DR4 or DR5 (or both), expressed at the same time a sizable amount of at least one of the decoy receptors. For example, patient 5 displayed high levels of DR4, moderate levels of DR5 and elevated amounts of both the decoy receptors, whereas in patient 13 there was a high level of DR5 which matched the DcR1 expression (Fig. 4A). Some patients were negative for all four TRAIL receptors (patient 10, Fig. 4A). In a recent report about TRAIL receptor expression in AML blasts, it has been shown that the expression of DR4 was limited to AML cases with monocytic features (19). Even though we found that 3 of the patients with the highest DR4 expression (cases 5, 23 and 29; Table II) were of FAB M5 subtype (acute monocytic), other M5 subtype patients expressed low (cases 4 and 20) or almost undetectable (case 22) levels of DR4. In addition, we found an M1 subtype (case 16) and a M2 subtype (case 27) with high levels of DR4 (Table II).

Effect of membrane permeabilization on surface TRAIL receptor expression of human AML blasts. In 5 AML samples, we performed cytofluorimetric immunostaining for TRAIL receptors, prior to and after a permeabilization step to detect the difference (if any) between the two techniques. One of these cases is represented by patient 14; after permeabilization, we noticed an increase in the immunoreactivity for DcR1 (Fig. 5). This was also true of the other 4 patients we analyzed with and without permeabilization (Table II).

AML blasts are resistant to TRAIL. TRAIL-dependent apoptosis of AML cells was investigated next, by flow cytometric analysis of PI-stained samples. All the AML samples (even those with a high expression of DR4 and/or DR5) displayed a very low sensitivity to a high TRAIL concentration (1000 ng/ml for 24 h) (Table III).

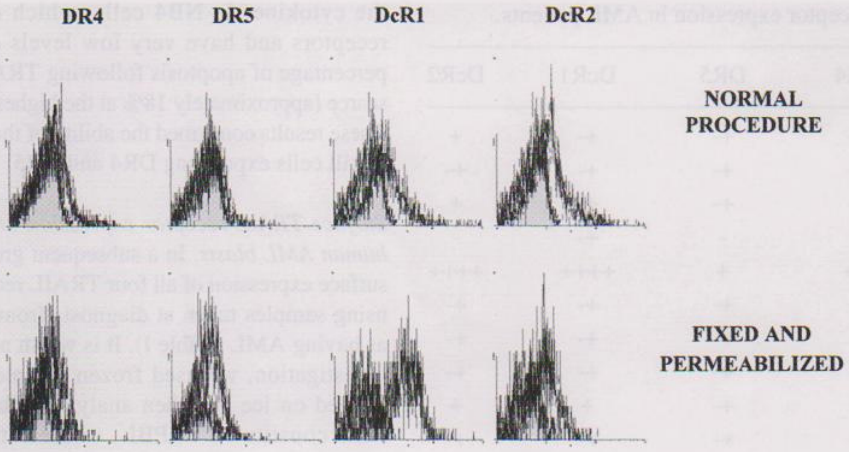

Figure 5. Flow cytometric analysis of the effect of fixation and permeabilization on immunostaining for TRAIL receptors of AML blasts. Grey histograms represent negative controls.

Discussion

Clinical trials have been initiated with either TRAIL or agonistic monoclonal antibodies to TRAIL receptors, DR4 and DR5, in patients with advanced solid tumours or non-Hodgkin's lymphoma. TRAIL has been proposed to be an innovative form of therapy for other haematological malignancies, including AML. To maximize therapeutic benefits, it is essential to ascertain whether or not a primary tumour contains TRAIL receptors, prior to initiating a therapy. Surprisingly, however, our knowledge about surface expression of TRAIL receptors in AML blasts is scarce. Here, we have demonstrated that malignant cells isolated from AML patients are resistant to apoptosis induction by TRAIL. This phenomenon was accompanied by the frequent expression of TRAIL decoy receptors, whereas DR4 and DR5 receptors were less commonly expressed on AML blasts.

In a previous investigation performed by immunocytochemical staining on acetone-permeabilized cells it was arbitrarily defined that, when more than 20% of blasts were stained by anti-DR4 or -DR5 antibody, the case was positive (7). Accordingly, it was found that 20 out of 29 patients (69%) were positive for DR4, whereas positivity for DR5 was 100%. In this investigation, neither were decoy receptors studied nor the actual TRAIL sensitivity of AML blasts analyzed but if we had considered the cases showing more than 20% of the cells stained by the antibodies to be positive, we would have found 16 out of 30 cases (53%) positive for either DR4 or DR5 (or both). This discrepancy between our findings and theirs could not be explained by the fact that they performed immunostaining on permeabilized cells because, in our study, cell permeabilization did not affect the expression of either DR4 or DR5 but did slightly increase the staining with the anti-DcR1 antibody. Also, the difference could not be ascribed to the fact that we investigated samples which had been stored frozen because they also employed cryopreserved samples in their study. It might be that the difference depends on the type of antibody or the technique (immunocytochemistry versus flow cytometry) employed. While our work was in progress, a study of the surface expression of TRAIL receptors

Table III. TRAIL-dependent apoptosis in AML blasts.

Patients	% Apoptosis	
	CTR 24h	TRAIL 1000 ng/ml 24h
1	12	14
2	7	11
3	8	14
4	16	20
5	4	9
6	13	15
7	10	11
8	9	12
9	8	9
10	14	14
11	11	15
12	8	13
13	3	4
14	2	6
15	4	11
16	14	15
17	8	13
18	19	21
19	7	15
20	6	10
21	6	8
22	13	14
23	15	18
24	12	16
25	2	9
26	5	9
27	11	14
28	10	12
29	3	7
30	8	9

CTR, untreated cells.

in 79 AML patients was published (19) in which only fresh (not cryopreserved) samples were analyzed by flow cytometry. The percentage of cases positive for DR4 was remarkably similar to ours (50% versus 53%), and this was also true for DcR1 (63% versus 70%) and DcR2 (60% in both studies). The only notable exception regards DR5 expression, which we found expressed in 53% of cases while they observed it only in a minority (10%) of the patients. It remains to be established whether or not the enhanced detection of DR5 that we detected in our samples was due to the fact that we employed cryopreserved samples.

Riccioni *et al* (19) also evaluated TRAIL-dependent apoptosis in AML samples. They found that a 300 ng/ml TRAIL concentration, in up to seven days of incubation, was unable to increase apoptosis in a significant manner. Our results are in total agreement with theirs because we found that an even higher (1000 ng/ml) TRAIL concentration did not increase the percentage of apoptotic AML blasts. The resistance of AML blasts to a lower TRAIL concentration (100 ng/ml) has also been reported in a recent study where a more limited number of cases (n=10) was analyzed (20).

Riccioni *et al* (19) suggested that TRAIL resistance of AML cells could be related to the expression of decoy receptors. However, this conclusion was reached on the basis of a series of experiments which were mainly performed on cell lines or acute promyelocytic blasts induced to differentiate by retinoic acid. We feel that a note of caution should be introduced here because our results obtained with HL60 cells demonstrate that a substantial expression of both the decoy receptors did not render these cells resistant to TRAIL. Moreover, K562 and NB4, which did not display decoy receptors, were much less sensitive to TRAIL than HL60 cells. In some cell types, TRAIL resistance has been demonstrated to be dependent on decoy receptor expression (21-23); however, in other cases, overexpression of proteins such as cFLIP and XIAP, which antagonize caspase-8 activation, has been also shown to negatively influence TRAIL sensitivity in cells of haematopoietic lineage, including HL60 and K562 cells (24-28). Regarding cell differentiation, this could also result in downregulation of cFLIP (26). cFLIP and XIAP proteins were found to be up-regulated in myeloid leukemia cell lines and AML blasts and, in some cases, they have been shown to be involved in TRAIL resistance (29-32). Therefore, the only way to demonstrate that decoy receptors are responsible for the TRAIL resistance of AML blasts would be by down-regulation of these proteins, for example by siRNA.

Whatever the case, our findings and those of other laboratories strongly suggest that AML blasts will probably also be insensitive to TRAIL *in vivo*. Therefore, to reach a therapeutic effect in AML, TRAIL administration should conceivably be supplemented with other treatments known for enhancing TRAIL sensitivity, such as histone deacetylase inhibitors (2,33) and chemotherapeutic agents (13). Alternatively, since cFLIP and XIAP protein expression is under the control of the phosphoinositide 3-kinase/Akt which is frequently up-regulated in AML blasts (reviewed in ref. 34), pharmacological inhibitors of this survival pathway might be usefully employed in the future to enhance the *in vivo* TRAIL sensitivity of AML blasts.

Acknowledgements

This work was supported by grants from: AIRC, Italian MIUR Cofin 2003 and FIRB 2001, 'Hairshow A.I.L.', Fondazione del Monte di Bologna e Ravenna.

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