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ARTICLE

Expression of the TRAIL Receptors in Blood Mononuclear Cells in Leukemia

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TRAIL receptors are differentially expressed on restricted subpopulations of normal blood cells. In the present study, we investigated the utility of individual TRAIL receptors in evaluating the presence of circulating tumor cells in blood. Patients with chronic myeloid leukemia (CML) carrying the t(9;22) translocation were compared with patients in whom no translocation was detected, with patients with multiple myeloma and with a group of healthy individuals. TRAIL receptor expression was analyzed by RT-PCR in blood mononuclear cells. Blood mononuclear cells of healthy subjects expressed the TRAIL-R1 and TRAIL-R2 death receptors and the TRAIL-R4 decoy receptor while the other decoy receptor TRAIL-R3 was not detectable. This normal expression pattern was also observed in all cases with multiple myeloma and in almost all patients without translocation (42/43; 97.7%). However, in 24/56 (42.9%) of the translocation-positive patients, the expression pattern was completely different. In this group the TRAIL-R4 receptor alone or in combination with TRAIL-R1 disappeared from blood mononuclear cells, while the TRAIL-R2 was expressed at normal level, indicating that the loss of expression is specific for the TRAIL-R4 and TRAIL-R1. This expression pattern was also confirmed by real-time PCR. The differences between the translocation-positive and –negative groups for the TRAIL-R4 and TRAIL-R1 expression were highly significant (p=0.0001 and p=0.0004, respectively). However, the differential expression pattern did not correlate with the number of leukemic cells. Our results suggest a correlation between the presence of leukemic cells in circulation and the differential expression pattern of TRAIL receptors in blood mononuclear cells. (Pathology Oncology Research Vol 13, No 4, 290–294)

Key words: TRAIL receptors; expression; circulating tumor cells; translocation

Introduction

The initial observation that the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in tumor but not in normal cells has invoked interest in it as a potential anti-cancer molecule.[1] The true physiological role of TRAIL is still not clear and its anti-tumorigenic properties are limited by potential toxicity to the liver.[2] However, although the preconceived mechanisms of action may be misleading, TRAIL is still a promising anti-tumor agent since resistant tumor cells can be sensitized by HDAC inhibitors. There are five known TRAIL receptors, two of which are death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2), leading to apoptosis via cytoplasmic death domains. The other two serve as "decoy" receptors (DcR) incapable of transmitting an apoptotic signal. DcR1 (TRAIL-R3) lacks a death domain while DcR2 (TRAIL-R4) possesses a truncated, nonfunctional death domain. Finally, a fifth receptor for TRAIL, osteoprotegerin (OPG), exists in a dimeric, soluble form and acts in a concentration-dependent manner promoting cell survival. Initially, widespread expression of the DR4 and DR5 on both malignant and normal cells at similar levels could not explain the observed TRAIL selectivity.[3] Exclusive expression of decoy receptors in normal cells[2] was considered to be the answer for the selective TRAIL activity.[3,4] It has been hypothesized that decoy receptors on normal cells could sequester TRAIL diverting it away from the death receptors, whereas their absence on cancerous cells leaves them susceptible to TRAIL-induced death. However, recent studies suggest that the TRAIL

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decoy/death receptor system is more complex as cells expressing one or both DcRs are not necessarily resistant to TRAIL, and cells lacking these receptors can still be protected from TRAIL-induced apoptosis.[5]

Some distinct expression profiles for TRAIL receptors have been reported. TRAIL-R1 has been mainly observed on B lymphocytes, TRAIL-R2 on monocytes, TRAIL-R3 on neutrophils, and TRAIL-R4 on CD8+ and NK cells.[6] However, the results on the expression of TRAIL receptors at cell surface protein level on blood cells are not consistent.[7-9] The primary reason for the conflicting results appears to be the use of different antibodies against the receptors. Assuming that normal and tumor cells might display different expression profiles of TRAIL receptors, our aim in the present study was to investigate whether the mRNA expression profile of TRAIL receptors in the blood mononuclear cell fraction (BMC) would reflect the presence of tumor cells in blood circulation. Two death and two decoy receptors were investigated in relation to tumor cell presence in the circulation of patients in whom leukemic cells carrying t(9;22) translocation were detected. Patients with chronic myeloid leukemia (CML) carrying the t(9;22) translocation were compared to patients in whom no translocation was detected and to healthy subjects. Patients with multiple myeloma (MM), a disease of plasma cells of B-cell origin that reside in the bone marrow while the number of circulating tumor cells is low,[10-13] served as a further control group.

Materials and methods

Expression of TRAIL receptors was analyzed in three groups of subjects. The first group consisted of patients (n =99; 61 men, 38 women; mean age 46.4±18.1 years) who were admitted to the Institute for a translocation analysis to confirm a CML diagnosis or to evaluate the minimal residual disease during follow-up. Cases with positive translocation were considered as those with tumor cells present in circulation. The second group consisted of patients diagnosed with multiple myeloma (n=44; 25 men, 19 women; mean age 60.7±10.5 years). A group of 20 healthy individuals (7 men, 13 women; mean age 39.8±11.9 years) was used as the control group. All study participants were informed on the study.

Blood was taken into EDTA-containing tubes and mononuclear cells were immediately separated by Ficoll-gradient centrifugation. The cells were collected, washed twice and stored at -80 °C until use. RNA isolation was performed as previously reported.[14] First-strand (cDNA) synthesis was carried out using the ImProm-II cDNA synthesis kit (Promega, Madison, USA) according to the manufacturer's instructions. Integrity check of cDNA molecules and quantitation for subsequent analysis was performed by amplifying GAPDH as the housekeeping gene.

PCR reactions were performed in a final volume of 25 µl containing 1x reaction buffer with 25 µM $MgCl_2$, 200 µM dNTP mix, 300 pg of each primer, 1 Unit Taq polymerase (Takara, Shiga, Japan), and 1.5 µl cDNA, using the Progene thermocycler (Techne, Burlington, USA). PCR conditions were the same for all receptor genes and consisted of 30 cycles of denaturation at 94 °C for 30 sec, annealing at 64 °C for 30 sec and extension at 72 °C for 30 sec. Primer sequences and the sizes of the PCR products are given in *Table 1*. The products of conventional PCR were resolved on a 1.5% agarose gel and evaluated using a video gel documentation system (Vilber-Lourmat, Marne-La-Vallée, France). Expression status of TRAIL receptors was confirmed by real-time PCR on the LightCycler platform (Roche Diagnostics, Mannheim, Germany) using the double-strand binding dye SYBR Green I, using the same primers. The TRAIL receptor expression was normalized using the GAPDH gene as reference. LightCycler reaction was performed using 2 µl of 10x FastStart SYBR Green mix (Roche Diagnostics), 3 mM $MgCl_2$, 0.2 µM of each primer and 1.5 µl cDNA. The protocol consisted of a 2-min initial denaturation, followed by 45 cycles of denaturation at 95 °C for 10 sec, annealing at 64 °C for 10 sec, and extension for 10 sec at 72 °C. Temperature transition rate was set at 20 °C /sec.

Analysis of the t(9;22) translocation was performed by conventional PCR according to van Dongen et al[18] and the products were analyzed as described above. Quantitation of circulating t(9;22)-positive leukemic cells by quantita-

Table 1. **Primer sequences used**

Gene	*Sequence*	*Product size (bp)*	*Reference*
TRAIL-R1	5′-CTGAGCAACGCAGACTCGCTGTCCAC-3′ (F) 5′-TCCAAGGACACGGCAGAGCCTGTGCCAT-3′ (R)	505	15
TRAIL-R2	5′-CCTCATGGACAATGAGATAAAGGTGGCT-3′ (F) 5′-CCAAATCTCAAAGTACGCACAAACGG-3′ (R)	501	15
TRAIL-R3	5′-GAAGAATTTGGTGCCAATGCCACT-3′ (F) 5′-CTCTTGGACTTGGCTGGGAGATGT-3′ (R)	611	16
TRAIL-R4	5′- CTTTTCCGGCGGCGTTCATGTCCT-3′ (F) 5′- GTTTCTTCCAGGCTGCTTCCCTTTTA-3′ (R)	460	16
GAPDH	5′-CCACCCATGGCAAATTCCATGGCA-3′ (F) 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (R)	573	17

tive real-time PCR was performed using a commercially available kit (Roche Diagnostics) according to the instructions of the manufacturer. This provides a relative value as the ratio of the t(9;22)-positive cells to cells expressing the housekeeping gene GAPDH. Using samples with a known concentration, a standard curve was generated for GAPDH. Then, for each sample, the t(9;22) and GAPDH reactions were run in parallel, and the t(9;22)/GAPDH ratio was calculated from the standard curve.

Table 2. **Expression pattern of TRAIL receptors in the patients and controls**

	Positivity of expression			
	TRAIL-R1	*TRAIL-R2*	*TRAIL-R3*	*TRAIL-R4*
Controls	20/20 (100%)	20/20 (100%)	0	20/20 (100%)
Multiple myeloma	44/44 (100%)	44/44 (100%)	0	44/44 (100%)
CML	81/99 (81.8%)	99/99 (100%)	0	74/99 (74.7%)
Translocation (-)	42/43 (97.6%)	43/43 (100%)	0	42/43 (97.6%)
Translocation (+)	39/56 (69.6%)	56/56 (100%)	0	32/56 (57.1%)

Fisher's exact test was used to evaluate the significances of correlations between the leukemic translocation and the TRAIL receptor expression in BMCs. The unpaired t-test was used to compare the quantitative values, and differences with $p \leq 0.05$ were considered significant.

Results

To investigate the relationship between the presence of tumor cells in the circulation and the expression pattern of the TRAIL receptors in mononuclear cells, we first analyzed the expression pattern in healthy subjects. This analysis revealed a differential expression pattern for the individual TRAIL receptor genes. Except for TRAIL-R3, all three genes were expressed in mononuclear cells (*Table 2*). TRAIL-R2 expression was relatively high, while TRAIL-R1 and TRAIL-R4 were detectable at much lower levels. In general, the expression of TRAIL-R1 was slightly higher than that of TRAIL-R4 (*Fig. 1a*). This pattern was considered as the 'normal expression pattern' for subsequent analysis. Next we analyzed TRAIL receptor expression in patients with multiple myeloma. In all cases with multiple myeloma, the same expression pattern was observed as in the normal subjects (*Table 2*).

Then, we analyzed the TRAIL receptor expression pattern in relation to the presence of leukemic cells in the circulation of which 56.6% (56/99) were positive for the t(9;22) translocation, while no translocation was found in 43 cases. In 42 of 43 samples without translocation (97.7%), the normal expression pattern found in the control subjects was observed (*Table 2*). However, in 24 of 56 the translocation-positive patients (42.9%), the expression pattern was completely different. In this group the TRAIL-R4 receptor alone or in combination with TRAIL-R1 disappeared from BMCs. TRAIL-R2 was expressed at normal levels, indicating that the loss of expression is specific for the TRAIL-R4 and TRAIL-R1 receptor genes (*Table 2*). A representative gel is shown in *Fig. 1b*. The difference between the translocation-positive and -negative groups with regard to the expression of TRAIL-R4 or TRAIL-R1 was highly significant ($p=0.0001$ and $p=0.0004$, respectively), indicating a correlation between the presence of leukemic cells in circulation and the absence of TRAIL-R1 and TRAIL-R4 expression in BMCs.

The differential expression pattern was also confirmed by real-time PCR. *Fig. 2* shows the results of TRAIL-R2 and TRAIL-R4 receptor genes in two patients. Peaks seen in the figure were generated by gradually increasing the temperature when amplification was complete and represents the sum of fluorescence measured during amplification. Thus, they correspond to positive expression. In both samples, TRAIL-R2 is positive as it was the case in all samples, while in one of the patients TRAIL-R4 was not expressed as represented by the lack of the melting peak.

Of 56 translocation-positive patients, 32 (57.1%) were newly diagnosed while the remaining cases were follow-up patients evaluated for the MRD. *Table 3* shows the distribution of cases with differential expression with respect to diagnosis status. The difference between these two sub-

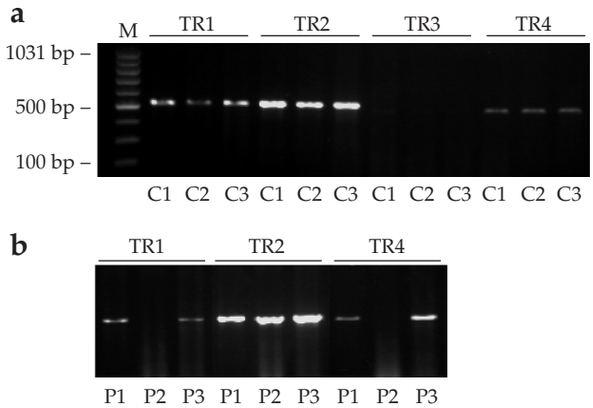

Figure 1. *Expression pattern of TRAIL receptors in blood mononuclear cells from normal subjects* ***(a)*** *and leukemia patients* ***(b).*** *P1 and P2 are patients with a positive t(9;22) translocation, while P3 is translocation-negative. Note that no TRAIL-R1 and -R4 expression is detected in P2. M, molecular size marker; bp, base pairs; C, control; P, patient*

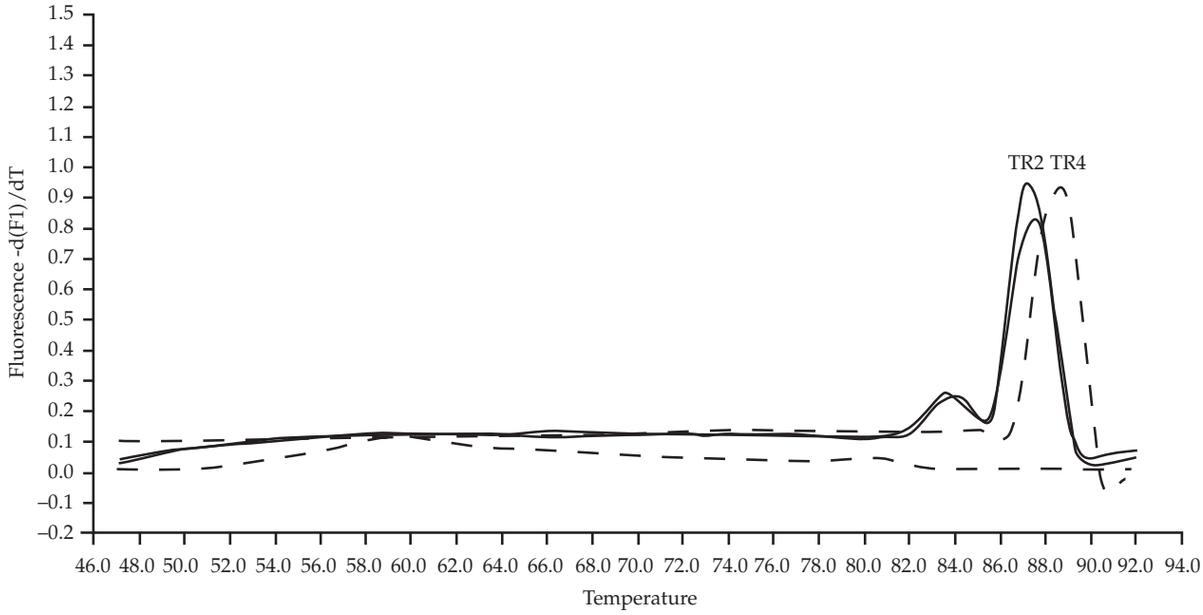

Figure 2. *Analysis of the expression of TRAIL receptors by real-time PCR. After completing the amplification, melting curves were generated by increasing the temperature gradually from 45 °C to 95 °C with continuous measurement of fluorescence. The curves were then converted to melting peaks by plotting the negative derivate of the fluorescence with respect to temperature (-dF/dT) against temperature. Shown are TRAIL-R2 and -R4 from two patients with CML. Note that TRAIL-R4 was not expressed in one of the patients.*

groups was not significant ($p>0.05$). In a further experiment, we investigated possible correlations between the levels of circulating leukemic cells and the expression pattern of TRAIL receptors. Quantitation of t(9;22)-positive leukemic cells was performed and compared in subgroups with a normal or differential expression pattern. A relative value expressed as the ratio of the t(9;22) to GAPDH was determined using a standard curve for each sample. The mean values were 8.9×10^{-4} and 5.5×10^{-4} in the subgroups displaying normal and differential expression, respectively. The difference between the mean levels was not significant ($p>0.05$), indicating that the differential expression pattern was not related to the number of leukemic cells.

Discussion

The differential expression of the individual TRAIL receptors on normal blood cells may be used for differential diagnosis of hematological and lymphoid malignancies. Although there are inconsistent results on the expression of TRAIL receptors in subpopulations of blood cells, a recent study has shown that TRAIL-R1 and -R4 are predominantly expressed on subpopulations of lymphocytes, while monocytes and neutrophils express TRAIL-R2 and TRAIL-R3, respectively.[6] The main purpose of the present study was to investigate the expression pattern of TRAIL receptors in the blood mononuclear cell fraction in leukemia. Translocation-positive samples were compared to normal subjects and to those without translocation. Patients with multiple myeloma served as a second control group for the TRAIL receptor expression analysis.

Our results show that normal BMCs express the death receptors TRAIL-R1 and TRAIL-R2, and the decoy receptor TRAIL-R4, while the other decoy receptor TRAIL-R3 is not detectable. This pattern is in accordance with the expression of the decoy receptor TRAIL-R3 on neutrophils.[6] Expression of TRAIL-R2 was very high while TRAIL-R1 and TRAIL-R4 showed comparable, lower expression levels. As might be expected, the expression pattern was the same in patients with multiple myeloma without exception. In CML patients, almost all cases (97.7%) without the t(9;22) translocation also displayed a normal expression pattern. However, in a subgroup of subjects with a positive t(9;22) translocation in BMCs, the TRAIL-R4 and, to a lesser extent, also TRAIL-R1 disappeared from BMCs. Loss of TRAIL-R1 and/or

Table 3. **Differential expression with respect to diagnosis status in translocation-positive cases**

	Normal expression	*Differential expression*
Patients at diagnosis	18/32 (56.3%)	14/32 (43.7%)
Patients during follow-up	14/24 (58.3%)	10/24 (41.7%)

TRAIL-R4 expression from the BMC fraction in the presence of leukemic cells may indicate that cells expressing TRAIL-R1/TRAIL-R4 are absent or substantially lower in number. This assumption is supported by the fact that the ratio of lymphocytes to mature monocytes in chronic leukemia decreases, as well as by our quantitative analysis of leukemic cells in the BMC fraction with respect to the expression pattern of TRAIL receptors. This analysis revealed that the disappearing TRAIL-R4 or -R1 expression from BMCs did not correlate with level of leukemic cells. This also confirms that the death receptor TRAIL-R1 and decoy receptor TRAIL-R4 are not expressed by reactive and leukemic mature monocytes, but probably by subpopulations of lymphocytes as previously reported.[6] The normal expression pattern from healthy individuals and patients with multiple myeloma in whom sufficient number of lymphocytes are present supports this finding. It appears that in presence of leukemic cells subpopulations of lymphocytes expressing the TRAIL-R1 or -R4 are suppressed. Assuming that TRAIL-R4, for which differential expression was most pronounced, is expressed by T cells,[6] our results are in line with leukemic blast-induced T-cell anergy. Leukemic cells that present tumor-specific antigens but do not express costimulatory molecules may be responsible for paralysis of the anti-leukemic immune response.[19] On the other hand, the absence or presence of costimulatory molecules on leukemic blasts may be the reason for the difference that the majority of translocation-positive CML patients display a normal pattern for TRAIL receptors while a subgroup has differential pattern. However, the exact mechanism underlying this difference has still to be elucidated. It needs also to be evaluated whether this distinct expression profile at the mRNA level is also reflected at the protein level on the cell surface.

In conclusion, in a substantial portion of translocation-positive leukemia cases a different expression pattern for TRAIL receptors is observed, although this was not correlated with the number of leukemic cells. To our knowledge, this is the first study to investigate the expression of TRAIL receptors in relation to the t(9;22) translocation and CML. In chronic lymphocytic leukemia and mantle cell lymphoma the apoptotic signal through TRAIL acts via the death receptor TRAIL-R1.[20,21] In our case, TRAIL-R2 may be the predominant receptor since TRAIL-R1 does not appear to be expressed by leukemic cells. Modulation of the TRAIL signaling by targeting regulatory receptors may be a promising strategy in cancer therapeutics.

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