



# Potential mechanisms of resistance to TRAIL/Apo2L-induced apoptosis in human promyelocytic leukemia HL-60 cells during granulocytic differentiation

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Received 10.1.00; revised 24.4.00; accepted 25.5.00  
Edited by JC Reed

## Abstract

Human promyelocytic leukemia HL-60 cells are well known to differentiate into granulocytes or monocytes in the presence of some agents such as DMSO or PMA, respectively. Differentiated HL-60 cells become resistant to some apoptotic stimuli including anticancer drugs or irradiation though undifferentiated cells significantly respond to these stimuli. TRAIL (TNF-related apoptosis-inducing ligand) which is also known as Apo2 ligand (Apo2L), a new member of TNF family, can induce apoptosis in some tumor cells but not in many normal cells. We show here that apoptosis is well induced in HL-60 cells by TRAIL, but susceptibility to TRAIL is reduced during granulocytic differentiation by DMSO. We also suggest some possible mechanisms by which granulocytic differentiated cells become resistant to TRAIL-induced apoptosis. First, in granulocytic differentiated cells, expression of antagonistic decoy receptors for TRAIL (TRAIL-R3/TRID/DcR1/LIT and TRAIL-R4/TRUNDD/DcR2) were enhanced. In addition, expression of Toso, a cell surface apoptosis regulator, seemed to block activation of caspase-8 by TRAIL via enhanced expression of FLIP<sub>L</sub> in granulocytic differentiated cells. These findings suggest that differentiated cells are resistant using plural mechanisms against various apoptosis-inducing stimuli rather than undifferentiated cells. *Cell Death and Differentiation* (2000) 7, 939–946.

**Keywords:** HL-60; differentiation; TRAIL/Apo2L; TRAIL receptor; apoptosis; FLIP

**Abbreviations:** TNF, tumor necrosis factor; FLIP, FLICE-inhibitory protein

## Introduction

Human promyelocytic leukemia HL-60 cells are known to become resistant to various apoptotic stimuli such as anticancer drugs, irradiation which involve mitochondrial pathways, after differentiation is induced by some reagents.<sup>1–4</sup> Recently, it was reported that expression of Mcl-1 which is one of the members of Bcl family was increased when HL-60 cells were induced to monocytic differentiation by 1,25-dihydroxyvitamin D<sub>3</sub>.<sup>5</sup> This increase of Mcl-1 inhibited apoptosis induced by VP-16, calcium ionophore by reducing release of cytochrome c from mitochondria.

On the other hand, TRAIL/Apo2L, a new cytotoxic ligand which is the member of TNF family,<sup>6–8</sup> and its specific receptors<sup>9–18</sup> have been identified. Expression of TRAIL is detected in various normal cells. Similarly to other TNF family molecules, TRAIL can induce apoptosis by ligation to its specific cell surface receptors (TRAIL-Rs). Interestingly, although TRAIL can induce apoptosis in tumor cells, little or no effect on normal cells has been reported.<sup>6,7</sup> We found that apoptosis was induced in HL-60 cells by TRAIL, but HL-60 cells became resistant to TRAIL-induced apoptosis when they were induced to granulocytic differentiation. TRAIL transduces its apoptotic signal via cell surface receptor, like Fas or TNF $\alpha$ . Recently, it was reported that in receptor-mediated apoptosis, Bid, a member of Bcl family, was cleaved by activated caspase-8, and cytochrome c was released from mitochondria by cleaved Bid.<sup>19–21</sup> The cleavage of Bid by caspase-8 was also found in TRAIL-induced apoptosis in BJAB cells.<sup>22</sup> However, the inhibition of receptor-mediated apoptosis cannot be explained by only inhibition of cytochrome c release from mitochondria. Therefore, the mechanisms of resistance to TRAIL-induced apoptosis during granulocytic differentiation may not be sufficiently explained by inhibition of cytochrome c release from mitochondria by Bcl family proteins such as Bcl-2, Bcl-x<sub>L</sub>, Bax, Bfl-1 and Mcl-1. So it is expected that there are other changes in the TRAIL-induced apoptotic pathway from the ligation of TRAIL with receptor to the final process of apoptosis during granulocytic differentiation.

When TRAIL binds to its specific receptors, TRAIL-R1 (DR4) or -R2 (DR5, TRICK2, KILLER), which have intracellular death domain, caspase-8 is cleaved and activated followed by caspase-3 activation.<sup>9,10</sup> Another two receptors, TRAIL-R3 (TRID, DcR1, LIT) which has no cytoplasmic death domain, and TRAIL-R4 (TRUNDD, DcR2) which has incomplete death domain act as inhibitor of TRAIL-induced apoptosis by competition with TRAIL-R1, -R2.<sup>10,11,17,18</sup> Besides the changes of expression of TRAIL receptors, another possible mechanism that contributes to resistance to TRAIL is expression of intracellular proteins that inhibit activation of caspases. It has been reported that FLIP<sub>L</sub>

and IAP family proteins (XIAP, c-IAP1 and c-IAP2) directly inhibit activation of caspase-8 and caspase-3, -7, respectively.<sup>23–31</sup> Recently, it was shown that in T cells, inhibition of caspase-8 activation by FLIP<sub>L</sub> was related with resistance to Fas-induced apoptosis caused by TCR activation.<sup>27</sup> Moreover, a cell surface apoptosis regulator, named Toso, which regulated induction of FLIP<sub>L</sub> expression was identified.<sup>32</sup> Toso can inhibit apoptosis induced by Fas, TNF $\alpha$  or FADD, but not by staurosporine or ceramide. It has been demonstrated that the consistently weak expression of mRNA for Toso is detected in some cell lines including HL-60 cells.

We therefore investigated changes of expression of these anti-apoptotic proteins in addition to four distinct receptors for TRAIL during granulocytic differentiation of HL-60 cells. In this study, we show that HL-60 cells become resistant to TRAIL-induced apoptosis during granulocytic differentiation. And the results suggest that the upregulation of TRAIL-R3, -R4, and enhanced expression of FLIP<sub>L</sub> induced by Toso participate in resistance to TRAIL-induced apoptosis in granulocytic differentiation of HL-60 cells.

## Results

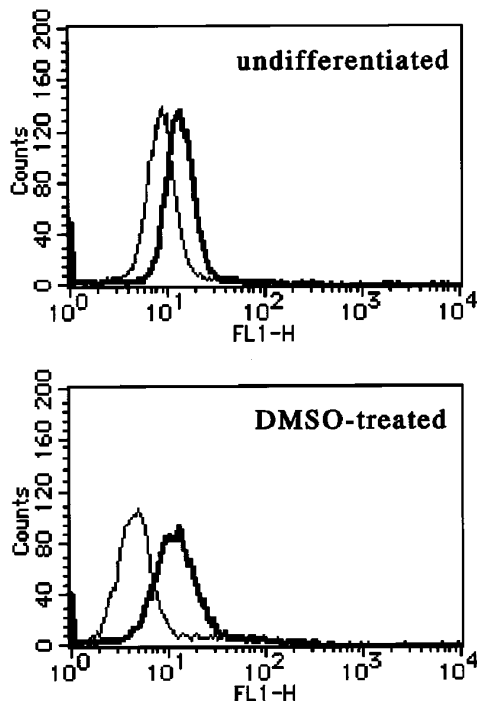
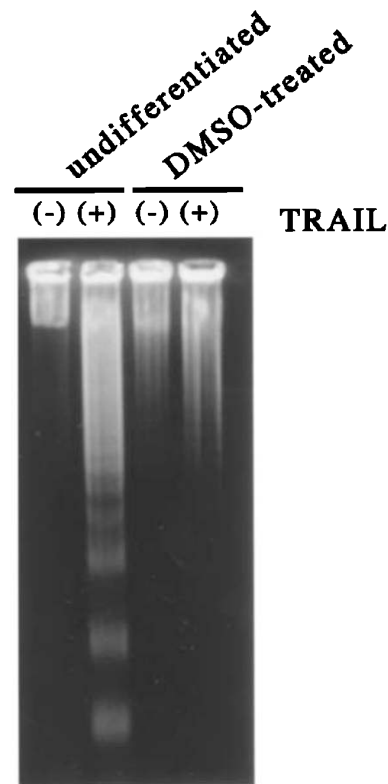
### Apoptosis is induced by TRAIL in HL-60 cells but not granulocytic differentiated cells

Granulocytic differentiation of HL-60 cells was confirmed by expression of cell surface CD11b as shown in Figure 1A. We

studied the susceptibility of HL-60 cells to human recombinant TRAIL. Undifferentiated HL-60 cells underwent apoptotic cell death by incubation with 1  $\mu$ g/ml of TRAIL for 4 h. Figure 1B shows TRAIL-induced DNA fragmentation confirmed by agarose gel electrophoresis. In undifferentiated cells, DNA fragmentation was observed after incubation with TRAIL, whereas in granulocytic differentiated cells, decreased DNA fragmentation was observed. To quantify apoptotic cell death induced by TRAIL, flowcytometric analysis was performed using propidium iodide. Figure 1C shows reduction of fragmented DNA in subdiploid area during granulocytic differentiation. These findings suggest that granulocytic differentiated HL-60 cells become increasingly resistant to the induction of apoptosis by TRAIL.

### TRAIL induces apoptosis in undifferentiated HL-60 cells via activation of caspase-8 and -3, but not in granulocytic differentiated cells

Next, we investigated whether caspase-8 and caspase-3 were activated by TRAIL. To confirm the activation of both caspase-8 and -3, cleavages of them were determined by Western blot analysis. Activation of caspase-8 yields 12 kDa and 20 kDa active subunits from the 43 kDa inactive form, while 17–20 kDa active subunits are cleaved from a 32 kDa inactive form during caspase-3 activation. Figure 2 shows reduced expression of inactive form of caspase-8 in granulocytic differentiated cells. In addition, cleaved products

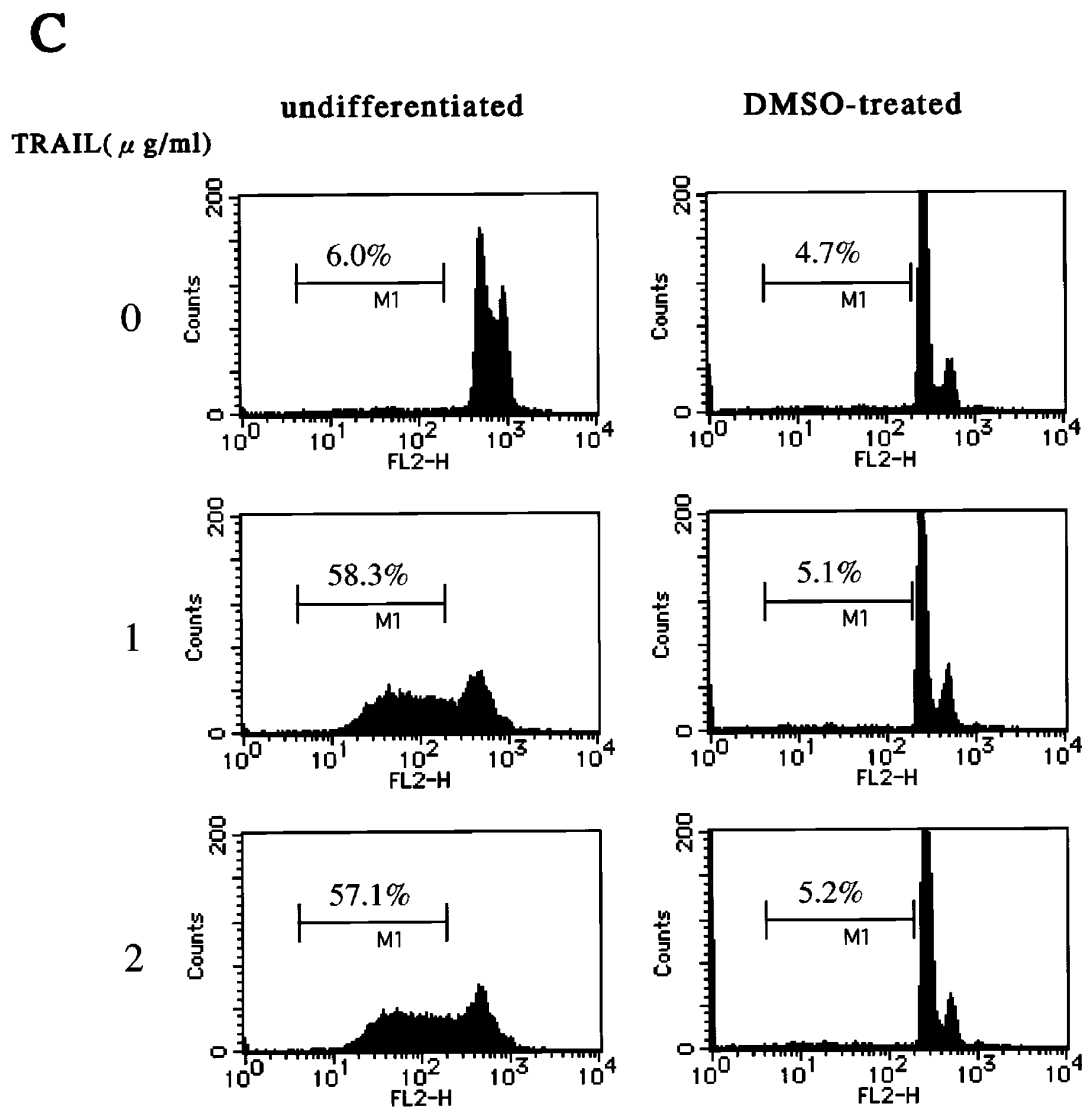
**A**

**B**


from both caspase-8 and -3 were detected in undifferentiated cells, whereas they were little detected in granulocytic differentiated cells after incubation with 1  $\mu$ g/ml of TRAIL, suggesting that resistance of granulocytic differentiated cells to TRAIL-induced apoptosis is related with inhibition of activation of caspase-8. Additional experiment using caspase inhibitors was performed. As shown in Figure 3, when preincubated with Ac-IETD-CHO which is known as a caspase-8 inhibitor, TRAIL-induced apoptosis was decreased in undifferentiated HL-60 cells. Similar result was obtained by using caspase-3 inhibitor, Ac-DEVD-CHO, but ability to inhibit TRAIL-induced apoptosis was weaker than that of caspase-8 inhibitor. Whereas, Z-VAD-FMK, which is a pan-caspase inhibitor, almost completely blocked TRAIL-

induced apoptosis. These findings confirmed the sequential activation of caspases during TRAIL-induced apoptosis in undifferentiated HL-60 cells.

### Changes of expression of TRAIL receptors during granulocytic differentiation

It is known that there are four distinct receptors for TRAIL. TRAIL-R1 (DR4) and -R2 (DR-5, TRICK2, KILLER) are apoptosis-inducible receptors that contain a death domain. Other two receptors, TRAIL-R3 (TRID, DcR1, LIT) and -R4 (TRUNDD, DcR2) have no ability to induce apoptosis because of the lack of death domain or the incomplete death domain. It has been reported that TRAIL-R1 and -R2 are



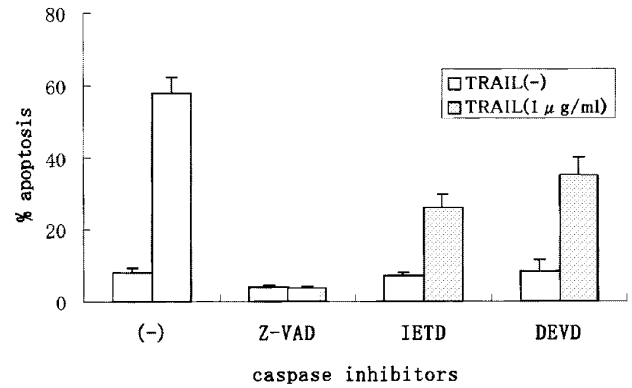
**Figure 1** (A) Granulocytic differentiation of HL-60 cells was confirmed by cell surface CD11b expression. After incubation of  $1 \times 10^6$  cells with or without 1.25% DMSO, anti-CD11b antibody (—) or control IgG (—) was added to each culture, and then flowcytometric analysis was performed using FITC-conjugated secondary antibody. (B) Ladder formation of DNA in HL-60 cells treated with TRAIL. After treatment with or without 1.25% DMSO, total of  $2 \times 10^6$  cells were incubated with or without TRAIL (1  $\mu$ g/ml) for 4 h. Then DNA was extracted, electrophoresed on 2% agarose gel and visualized by ethidium bromide staining. (C) Flowcytometric analysis of apoptosis induced by TRAIL. After incubation with or without TRAIL (1 or 2  $\mu$ g/ml) for 4 h,  $5 \times 10^5$  cells were stained with PI. Percentages of apoptotic cells were determined using a FACSCalibur flow cytometer

widely expressed on both normal and tumor cells, whereas TRAIL-R3 and -R4 are found on normal, but not on tumor cells. Considering that TRAIL selectively induces apoptosis in tumor cells but not in normal cells, sensitivity to TRAIL seems to be negatively regulated by TRAIL-R3 and -R4. So we investigated whether changes of expression of TRAIL receptors could be observed during granulocytic differentiation by RT-PCR using specific primers as described.<sup>33</sup> As shown in Figure 4, mRNA for TRAIL-R1 and -R2 were detected in both undifferentiated and granulocytic differentiated HL-60 cells. But mRNA for TRAIL-R3 was only expressed in differentiated cells, and that for TRAIL-R4 was more strongly expressed in differentiated cells than in undifferentiated cells. These findings suggest one possibility that enhanced expression of decoy receptors for TRAIL contributes to the reduced sensitivity to TRAIL in differentiated cells.

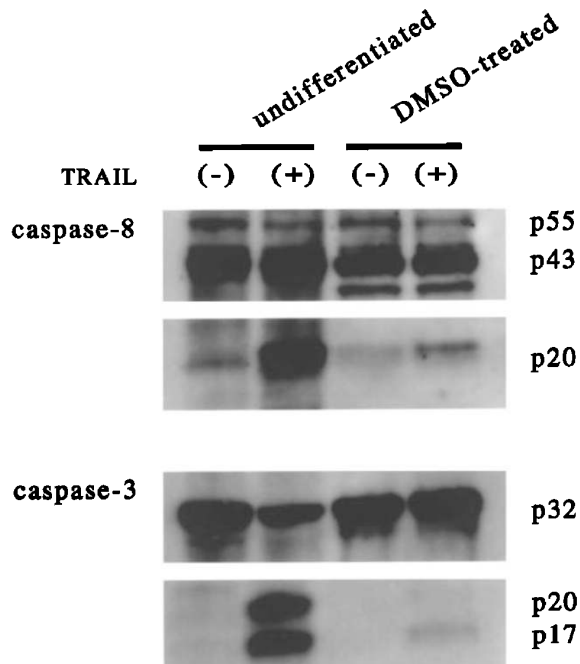
### Enhanced expression of FLIP<sub>L</sub> and induction of Toso during granulocytic differentiation

FLIP<sub>L</sub> inhibits activation of caspase-8 in receptor-mediated apoptosis by FasL or TNF $\alpha$ . The relation between expression of FLIP<sub>L</sub> and resistance to TRAIL-induced apoptosis is unclear, but there are some studies supporting inhibitory effect of FLIP<sub>L</sub> on TRAIL-induced apoptosis.<sup>33</sup> We therefore performed Western blot analysis to detect FLIP<sub>L</sub> expression in HL-60 cells. Expression of FLIP<sub>L</sub> was observed in undifferentiated HL-60 cells, and was enhanced in granulocytic

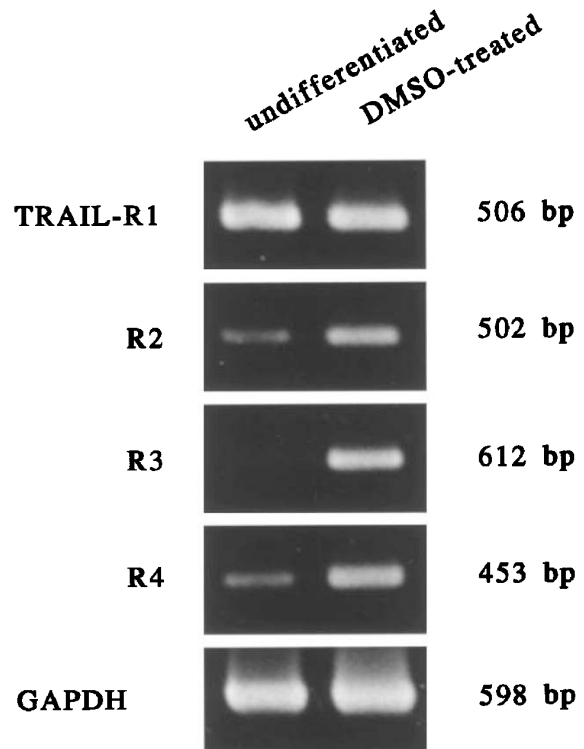
differentiated cells (Figure 5B). There was no significant difference in expression of FADD during differentiation of HL-60 cells. Recently, immunoglobulin domain-containing polypeptide, Toso, was cloned and characterized.<sup>32</sup> This molecule inhibits apoptosis induced by Fas, TNF $\alpha$  or FADD. Toso is



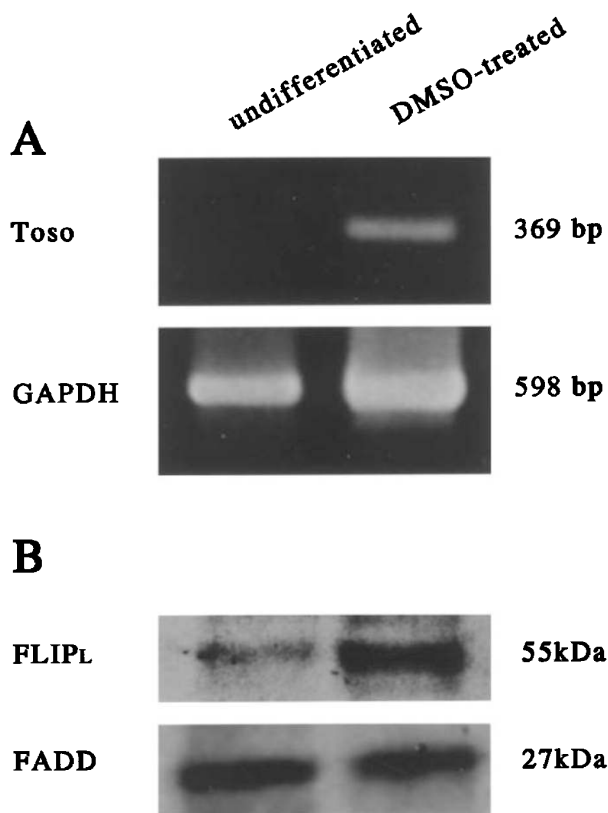
**Figure 3** Effect of caspase inhibitors on TRAIL-induced apoptosis. Each cell was preincubated separately with or without inhibitor of caspase-8 (Ac-IETD-CHO: 100  $\mu$ M), caspase-3 (Ac-DEVD-CHO: 200  $\mu$ M) or pan-caspases (Z-VAD-FMK: 100  $\mu$ M) for 2 h before exposure to TRAIL. After incubation with TRAIL (1  $\mu$ g/ml) (closed) or vehicle (open) for 4 h, percentages of apoptotic cells were determined by flowcytometric analysis as described in Figure 1. The means and standard deviations of three independent experiments are shown



**Figure 2** Activation of caspase-8 and -3 by TRAIL. After treatment with or without 1.25% DMSO, each HL-60 cell was incubated with or without TRAIL (1  $\mu$ g/ml) for 4 h. Then cleavages of caspase-8 and -3 were determined by Western blot using specific antibodies to each caspase. Activation of caspase-8 was confirmed by detection of active form of 20 kDa cleavage, and that of caspase-3 was confirmed by detection of active form of 20–17 kDa products



**Figure 4** Messenger RNA expression of TRAIL receptors. RT-PCR was performed using specific primers to each receptor. Total RNA (5  $\mu$ g) from each HL-60 cell cultured with or without 1.25% DMSO were reverse transcribed and PCR amplified. The length of each PCR product matched that predicted from the sequence data



**Figure 5** (A) RT-PCR analysis for mRNA expression of Toso in each HL-60 cell. Before and after differentiation, total RNA from each HL-60 cell were reverse transcribed and PCR amplified with specific primers to Toso and GAPDH. (B) Western blot analysis for FLIPL and FADD in each HL-60 cell. After treatment with or without 1.25% DMSO, cell lysates were extracted and immunoblotted with specific antibodies

expressed on T cell during its activation. The inhibitory effect of Toso on apoptosis is attributable to the induction of FLIPL. So RT-PCR was performed to determine whether mRNA for Toso was expressed or not in both undifferentiated and differentiated HL-60 cells. As shown in Figure 5A, mRNA expression of Toso was observed only in granulocytic differentiation HL-60 cells, suggesting that expression of FLIPL is induced by Toso and it regulates TRAIL-induced apoptosis.

## Discussion

Granulocytic differentiation of HL-60 cells results in spontaneous apoptosis.<sup>34</sup> On the other hand, differentiated cells are resistant against multiple apoptotic stimuli, such as anti-cancer drugs,<sup>1</sup> irradiation or hyperthermia.<sup>2,35</sup> However, it has been demonstrated that expression of Bcl-2 which blocks some kind of apoptosis by inhibiting release of cytochrome c from mitochondria is decreased, and that Bax which reduces anti-apoptotic effect of Bcl-2 by dimerizing Bcl-2, is also decreased in HL-60 cells during granulocytic differentiation.<sup>36</sup> Moreover, induction of granulocytic differentiation by retinoic acid reduces expression of Bcl-x in HL-60 cells.<sup>37</sup> Another experiment

demonstrated the relation of differences in the susceptibility to drug-induced apoptosis with the change of cell cycle during differentiation in HL-60 cells.<sup>38</sup> However, the details of alteration of susceptibility to apoptotic stimuli during differentiation are still unclear. Recently, Mcl-1 protein was found to inhibit apoptosis induced by some anticancer agents and calcium ionophore by inhibiting cytochrome c release from mitochondria in monocytic differentiating HL-60 cells.<sup>5</sup> This protein has homology with Bcl-2 protein and is mainly expressed in differentiating cells. On the other hand, A1, hematopoietic-specific early-response gene product which has homology with bcl-2, has been characterized.<sup>39</sup> This anti-apoptotic protein A1 is constitutively expressed in neutrophils.<sup>40</sup> Moreover, in the myeloid precursor cell line, 32Dcl3, A1 gene expression is stably induced during G-CSF-stimulated granulocytic differentiation. Expression of these anti-apoptotic proteins during granulocytic differentiation effectively inhibits release of cytochrome c from mitochondria and regulates apoptosis.<sup>41</sup> However, in our observation, expression of mRNA of A1 was detected similarly in both undifferentiated and granulocytic differentiated HL-60 cells (data not shown).

On the contrary, in receptor-mediated apoptotic signal pathway, when ligand, such as FasL or TNF $\alpha$ , binds to each specific receptor and multimerization of these receptors is formed, apical caspases, such as caspase-8 and -10 are activated via FADD, subsequently caspase-3 is activated. Human monocytic leukemia U937 cells, which are sensitive to TNF $\alpha$  and FasL, become resistant not only to anticancer agents but also to these ligand-induced apoptosis when they are differentiated to macrophages.<sup>42,43</sup> It has been suggested that the receptor-mediated apoptotic pathway is blocked upstream of the cytochrome c release from mitochondria in differentiated U937 cells. Bid, which is a member of the Bcl-2 family, found to be cleaved by activated caspase-8, and promote release of cytochrome c from mitochondria in receptor-mediated apoptosis.<sup>19</sup> On the other hand, it has been demonstrated that the Fas-mediated apoptotic pathway is independent on p38 mitogen-activated protein kinase (MAPK), whereas stress-induced apoptosis was p38 MAPK-dependent in neutrophils.<sup>44</sup> Furthermore, it has been shown that granulocytic differentiation of HL-60 cells results in a loss in c-Jun NH2-terminal kinase activation with concomitant acquisition of stress-induced p38 MAPK activity.

Recently, TRAIL (TNF-related apoptosis inducing ligand), a member of the TNF family, is identified. TRAIL also induces apoptosis via its specific receptor.<sup>7</sup> Constitutive expression of TRAIL is detected in a wide range of normal human tissues and TRAIL induces apoptosis on tumor cells but not on normal cells.<sup>6,7</sup> In the present study, we investigated whether TRAIL could induce apoptosis on undifferentiated and granulocytic differentiated HL-60 cells. As demonstrated, TRAIL can induce apoptosis on undifferentiated cells within 4 h, but not on granulocytic differentiated cells. This phenomenon seems to be compatible with limited cytotoxicity of TRAIL to tumor cells. When TRAIL binds to its specific receptors, TRAIL-



R1, -2,<sup>9,12,45</sup> which have complete cytoplasmic death domain, apoptotic signal is transmitted and subsequently caspase-8 is activated.<sup>15</sup> An experiment using caspase-inhibitors supports importance of caspase-8 activation in TRAIL-induced apoptosis. Western blot analysis showed activation of caspase-8 and -3 in undifferentiated, but not in granulocytic differentiated HL-60 cells, suggesting that inhibiting factors of apoptotic signal by TRAIL exist upstream of caspase-8. Reduced expression of the inactive form of caspase-8 in granulocytic differentiated cells was observed. However, it seems not to be the main mechanism of resistance to TRAIL-induced apoptosis, because not enough amount of caspase-8 was detected by Western blot even after granulocytic differentiation. So we examined expression of mRNA for TRAIL receptors, R1, R2, R3 and R4. TRAIL-R1 and -R2, which are apoptosis-inducible receptors, are expressed in a wide range of cells, but TRAIL-R3 and -R4, which are called decoy receptors, are expressed in restricted normal cells.<sup>10,11,15</sup> These decoy receptors cannot mediate the apoptotic signal because R3 has no cytoplasmic domain<sup>15</sup> and R4 has incomplete death domain and induces activation of NF- $\kappa$ B.<sup>17</sup> Therefore, expression of TRAIL-R3 and -R4 is considered to be related with resistance to TRAIL in normal cells.<sup>10,11,18</sup> RT-PCR using specific primers to these receptors<sup>33</sup> revealed constitutive mRNA expression of TRAIL-R1 and -R2 in both undifferentiated and granulocytic differentiated cells. On the other hand, upregulation of TRAIL-R3 and -R4 was observed during granulocytic differentiation, suggesting the possible mechanism of resistance to TRAIL-induced apoptosis in granulocytic differentiated HL-60 cells.

Recently, in T cells, cell surface polypeptide named Toso was found to regulate FLIP<sub>L</sub> expression.<sup>32</sup> Toso is expressed on activated T cells and inhibits receptor-mediated apoptosis through expression of FLIP. Yasumichi H *et al*, screened expression of Toso in several human tissues and cell lines and it was expressed in the spleen, lymph nodes, thymus and peripheral blood leukocytes. Among cell lines, Toso was observed mainly in lymphoid cell lines. In HL-60 cells, weak expression of Toso was reported. So we investigated whether mRNA expression of Toso was observed in HL-60 cells by RT-PCR. The result demonstrated that expression of Toso in mRNA level was observed in granulocytic differentiated, but not in undifferentiated HL-60 cells, suggesting that expression of FLIP may be enhanced by Toso during not only T cell activation but also granulocytic differentiation. FLIP interferes with receptor-mediated apoptotic signaling pathways by inhibiting caspase-8 activation. There are some reports which demonstrate inhibiting role of FLIP in TRAIL-induced apoptosis. Griffith *et al*.<sup>33</sup> showed correlation of FLIP expression and resistance to TRAIL-induced apoptosis in human melanoma cells. In their report FLIP levels were highest in the TRAIL-resistant cells and were low or absent in the sensitive cells. On the other hand, there is a report in which the presence and levels of FLIP expression did not correlate with resistance to TRAIL-induced apoptosis in melanoma cells.<sup>46</sup> This study indicates the possibility of existence of other intracellular proteins that regulate TRAIL-

induced apoptosis in melanoma cells. Though there are opposite possibility whether FLIP inhibits TRAIL-induced apoptosis or not, FLIP seems to be able to block it partially because caspase-8 activation is involved in TRAIL-induced apoptotic pathway. In our study, expression of FLIP<sub>L</sub> was slightly increased in granulocytic differentiation of HL-60 cells, suggesting possible participation of FLIP induced by Toso in resistance to TRAIL-induced apoptosis. These changes of susceptibility to TRAIL-induced apoptosis seem to relate with a role of mature leukocyte in the immune system.

## Materials and Methods

### Reagents

Recombinant human TRAIL was purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA, USA). Antibody to human CD11b was purchased from Chemicon International Inc. (Temecula, CA, USA). Antibodies to caspase-8 and FLIP<sub>L</sub> were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibody to caspase-3 was purchased from PharMingen (San Diego, CA, USA). Antibody to FADD was purchased from StressGen Biotechnologies Corp. (Victoria, BC, Canada). Caspase inhibitors, Z-VAD-FMK, Ac-IETD-CHO and Ac-DEVD-CHO from Peptide Institute, Inc. (Osaka, Japan), were dissolved in dimethyl sulfoxide (DMSO) as stock solution.

### Cell culture and induction of differentiation

The human promyelocytic leukemia cell line HL-60 was maintained in RPMI 1640 (Gibco, BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (EQUITECH-BIO, INC. Ingram, TX, USA), 2 mM L-glutamine and  $1 \times 10^{-5}$  M  $\beta$ -mercaptoethanol in a 5% CO<sub>2</sub> atmosphere at 37°C. Cells were induced to granulocytic differentiation by treatment with 1.25% (v/v) DMSO for 3 days.

### Detection of DNA fragmentation by agarose gel electrophoresis

After incubation with or without 1  $\mu$ g/ml of rTRAIL for 4 h,  $2 \times 10^6$  cells were washed in PBS(-), lysed in 30  $\mu$ l of 10 mM Tris-HCl, 100 mM EDTA-Na (pH 8.0), containing 0.5% (w/v) sodium-N-lauroylsarcosinate (SDS) and 0.5 mg/ml RNase A (Sigma, St. Louis, MO, USA) and incubated at 50°C for 30 min and then treated with 0.5 mg/ml of proteinase K (Sigma, St. Louis, MO, USA), and incubated at 50°C for 30 min. Electrophoresis was performed on 2% agarose gel in TAE buffer. After electrophoresis, DNA was visualized by ethidium bromide staining.

### Flowcytometric analysis for induction of differentiation and apoptosis

Cell surface CD11b expression was used as a marker of granulocytic differentiation.  $1 \times 10^6$  cells treated with or without 1.25% DMSO were incubated with anti-CD11b antibody or control IgG for 30 min at 4°C. After washing in PBS(-), they were incubated with FITC-conjugated second antibody. For measurement of apoptosis,  $5 \times 10^5$  cells were treated with or without 1  $\mu$ g/ml of rTRAIL for 4 h, and resuspended in a hypotonic buffer containing 0.1% sodium citrate, 0.1% Triton X-100 and 50  $\mu$ g/ml propidium iodide (Sigma, St. Louis, MO, USA) at 4°C in the dark overnight. Flow cytometric analyses were all performed by a FACSCalibur (Becton Dickinson, Mountain View, CA, USA) using CellQuest analysis software.

## Treatment with caspase inhibitors

$5 \times 10^5$  cells were preincubated for 2 h in the presence of caspase inhibitors, Z-VAD-FMK (100  $\mu$ M), Ac-IETD-CHO (100  $\mu$ M) and Ac-DEVD-CHO (200  $\mu$ M) or same volume of DMSO and then rTRAIL or vehicle was added. The percentage of apoptotic cells was determined by flow cytometry described as above.

## SDS-PAGE and Western blot analysis

$5 \times 10^6$  cells were resolved in 100  $\mu$ l RIPA lysis buffer (PBS(-) containing 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS) containing 100  $\mu$ g/ml PMSF, and lysates were centrifuged at 10 000 g for 1 min. The supernatant was added to equal volume of sample buffer (200 mM Tris-HCl (pH 6.7) containing 5% SDS, 20% glycerol, 0.2 mg/ml BPB and 10%  $\beta$ -mercaptoethanol) and then boiled for 3 min. Equal amount of the cell lysates were separated by SDS-PAGE using 10 or 15% polyacrylamide gels and subsequently transferred to a PVDF membrane (ATTO Co., Tokyo, Japan). Blocking was performed in PBS(-) containing 0.1% Tween-20, 1% BSA at 4°C overnight. After several washings in PBS(-) containing 0.1% Tween-20, membranes were incubated with specific antibodies for 1 h at room temperature. After washing, membranes were incubated with HRP-conjugated second antibody for 1 h at room temperature. After extreme washing, specific bands were detected using ECL according to the manufacturer's protocols.

## RT-PCR

Total RNA was isolated from undifferentiated and granulocytic differentiated HL-60 cells using guanidium isothiocyanate method. Five  $\mu$ g of total RNA was reverse transcribed with MuLV reverse transcriptase. Reverse transcription was performed using a thermal program of 42°C for 60 min, 90°C for 5 min. PCR reactions were performed using the following primers: GAPDH, 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3' and 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'; TRAIL-R1, 5'-CTG AGC AAC GCA GAC TCG CTG TCC AC-3' and 5'-TCC AAG GAC ACG GCA GAG CCT GTG CCAT-3'; TRAIL-R2, 5'-GCC TCA TGG ACA ATG AGA TAA AGG TGG CT-3' and 5'-CCA AAT CTC AAA GTA CGC ACA AAC GG-3'; TRAIL-R3, 5'-GAA GAA TTT GGT GCC AAT GCC ACT G-3' and 5'-CTC TTG GAC TTG GCT GGG AGA TGT G-3'; TRAIL-R4, 5'-CTT TTC CGG CGG CTG TCA TGT CCT TC-3' and 5'-GTT TCT TCC AGG CTG CTT CCC TTT GTA G-3'; Toso, 5'-GCC GAG TTA CTC TGA AGC AAT-3' and 5'-CTA CTG AAG ATG CTC TGG ACA-3'. 1 cycle at 95°C for 5 min, 72°C for 2 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min; and 3 min at 72°C. PCR-amplified products were resolved on 2% agarose gel and visualized with ethidium bromide.

## Acknowledgements

We are grateful to K Seki, Z Rahman and J Liu for technical assistance and T Tsuchida for helpful comments.

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