Paradoxical TRAIL Activity in Acute Promyelocytic Leukemia (APL) Cell Line AP-1060

Yiwu Sun, Hongbao Ma

Department of Medicine, Brookdale University Hospital and Medical Center, Brooklyn, NY 11212, USA
yiwu_sun@hotmail.com

Abstract: TRAIL as a member of TNF ligands is involved in the anti-tumor mechanisms by its cytotoxic activity inducing apoptosis, and has been explored as a tumor target reagent in the therapy of different tumors. In acute promyelocytic leukemia (APL), TRAIL was used to be token as a crucial molecule that was up-regulated by retinoic acid and induced the blast cell to differentiation and apoptosis. However, our findings indicated that AP1060 cell, an APL cell line, produced a high level of TRAIL, and its growth and proliferation depended on the auto-secreted TRAIL. Furthermore, the above phenomenon may be related to the TRAIL-mediated NF-kB activation. It suggests that there may be a new therapy approach to the subtype of APL.

Keywords: TRAIL; acute promyelocytic leukemia; retinoic acid; induced differentiation

1. Introduction

TNF-related apoptosis-inducing ligand (TRAIL) belongs to the superfamily of TNF ligands and is composed of 281 amino acids and has characteristics of a type II transmembrane protein. TRAIL has five death receptors including TRAIL-R1 (DR4), TRAIL-R2 (DR5), osteoprotegerin, and two decoy receptors. TRAIL is not only required for natural killer cell-mediated immune-surveillance against the tumor progression and metastasis (Takeda 2002), but also for Fur and dendritic cell-mediated innate and adaptive immunity in suppression of tumor progression (Schmaltz 2002, Taieb 2006). Alpdogan TRAIL induces apoptosis in tumor cells by binding to the death receptors DR4 and DR5 (Sheridan 1997). Once trimeric TRAIL cross-links with the receptor DR4 or DR5, there is an aggregation of the death domains, which leads to recruitment of the adaptor molecule FADD and activation of caspase 8-mediated cascade. Caspase 8 is responsible for initiating activation of caspase 3, ultimately committing tumor cells to undergo apoptosis (Kischkel 2000, Rudner 2005). In some cases, the mitochondrial type II pathway is involved in TRAIL-induced apoptosis (Song 2008). TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), which do not contain a cytoplasmic domain (DcR1) or contain a truncated death domain (DcR2), functions as a TRAIL-neutralizing decoy-receptor to protect normal cells from TRAIL-induced apoptosis in physiological mechanisms (Avi Ashkenazi 1999).

Unlike its other homologous ligands such as TNF-A and FasL, TRAIL exerts a cytotoxic activity against the majority of tumor cell lines, while it spares normal tissues (Kelley and Shankar 2004, Younes 2003). In mouse, the TRAIL deficiency made mice suffer from a greater than 25% increased risk of developing lymphoid malignancies after 500 days of age or enhances lymph node metastasis of squamous cell carcinoma (Zerafa 2005, Grosse-Wilde 2008). During the last few years, the anti-tumor activity of TRAIL has been reported in hematologic malignancies, including multiple myeloma cells and Philadelphia chromosome-positive leukemia in which it was shown that TRAIL was able to induce apoptosis (Chen 2001, Uno 2003). In acute promyelocytic leukemia (APL), Studies have documented that APL blasts are sensitive to TRAIL-mediated apoptosis and that retinoic acid induces TRAIL expression and thereafter killing of the leukemic cells (Altucci 2001). A phenomenon that AML blasts, including APL blasts, are resistant to TRAIL-mediated apoptosis is seemingly related to the expression of TRAIL deco receptor on these cells (Riccioni 2005). However, Soucek reported that a combination treatment with all-trans retinoic acid (ATRA) and TGF-beta1 led to the enhancement of ATRA-induced suppression of cell proliferation, which is accompanied by inhibition of ATRA-induced apoptosis and increasing of anti-apoptotic molecules (c-FLIP(L) and Mcl-1) in human leukemia HL-60 cells (Soucek 2006).

We found that AP1060 cell, an acute promyelocytic leukemia cell line (Sun 2004), highly expressed TRAIL during the characterization of this cell line that was developed from a multiple-relapse patient clinically-resistant to both ATRA and arsenic trioxide (ATO). In this tentative study, we observed the effects of auto-secreted TRAIL on AP1060 cell.

2. Material and Methods

Cell Culture: AP1060 cell line was established from the patient who had relapsed for the fourth time after achieving an initial, transient clinical response...
on ATO therapy. The cells were incubated in Iscove’s modification of Dulbecco’s medium (IMDM, Invitrogen, USA) containing 10% FBS and 10ng/ml G-CSF (Amgen, Thousand Oaks, CA, USA). HL60, NB4 and Jurkat cells were maintained in RPMI-1640 Medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Invitrogen, USA). The incubator was set at 37°C and 5% carbon dioxide (CO2).

All-trans Retinoic acid (ATRA, Sigma, USA) dissolved in ethanol to a concentration of 1mM and stored at -20°C. The dose of the induced differentiation was referred to Y Sun et al.19 The percentage of viable and non-viable cells was determined by the exclusion or uptake, respectively, of the vital trypan blue (Life Technologies) from/by a minimum of 200 cells manually counted using a hemocytometer under microscopic observation. Specific inhibition of TRAIL-induced cell death was performed using TRAIL and DcR1 neutralizing monoclonal antibody (R&D, USA), and the respective no-neutralizing antibodies were used as control. The evaluation of cytolytic features by Wright’s stains and the performance of the nitroblue tetrazolium (NBT) test.

RNase Protection Assays: RNA was extracted from the cultured cells using Trizol (Invitrogen, USA). The RNase protection was performed with 5ug RNA with the human apoptosis detection kits (hApo2, hApo3d and hApo5, Parmingen, USA) as specified by the manufacturer. In brief, RNA was hybridized overnight with the vitro-translated 32P-labeled probes. Following hybridization, samples were treated with RNases A and T1 plus proteinase K, phenol chloroform extracted, and ethanol precipitated. The protected fragments were resolved by electrophoresis on a 6% acryl amide-urea gel and exposed on a Phosphor Imager screen (Molecular Dynamics Phosphor Imager). The intensity of each band was determined by using a Molecular Dynamics Phosphor Imager.

Western blot analysis: The total protein was prepared from the control cells and treated cells with RIP buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0 and 1x Roche protease inhibitor cocktail). Equal amounts of protein were electrophoresed in 10% polyacryl amide gels containing 0.1% SDS and then transferred to nitro-cellulose membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were probed with an antibody directed against the TRAIL (1:1000 antibody dilution), and antibody binding was detected with the ECL chemiluminescence system (Amersham Life Science Inc., Arlington Heights, IL).

Evaluation of apoptosis: Sub G1 cells were detected by Propidium iodide (PI). Briefly, 10⁶ cells were washed and fixed in a suspension of 70% ethanol on ice and stored at -20°C overnight. Following fixation, the cells were centrifuged and resuspended in 1 mL of Hank’s balanced salt solution (HBSS). One ml of 0.2 M Na2HP03 - 0.1 M citric acid buffer, pH 7.8, was then added and incubated at room temperature for 10 min. After centrifugation, 1 mL of HBSS containing 20 mg/ml of propidium iodide and 5 Kunitz units of DNase-free RNase was added to the cells, followed by incubation for 30 min at room temperature. The TUNEL (terminal deoxynucleo-tidyl transferase-mediated deoxyuridine triphosphate [dUTP] nick-end labeling) assay was performed by use of the cell death detection kit (Invitrogen, USA). DNA strand breaks generated by cleavage of genomic DNA during apoptosis can be identified in this assay by labeling free 3-OH termini with fluorescein isothiocyanate (FITC) -conjugated dUTP. Within 1 hour, the cells were analyzed at 488nm in a FACSCAN cytometer (Becton Dickinson Labware).

Electrophoretic mobility shift assay (EMSA): Nuclear extracts were prepared from AP1060 cells as previously described (Haas 1998), in which protein concentration 1 to 5mg/mL determined by the bicinchoninic acid protein assay (Pierce Chemical Co). The oligo nucleotide containing the prototypical NF-kB binding site from murine Igk, 5’ -CA-GAGGGACTTTCCGAGA-3’, was radio-labeled with [32P]dCTP (>3000Ci/mmole, Amersham). The specificity of binding was verified using excessively unlabeled consensus oligonucleotide (x10) corresponding to the above sequence as a competitor in the binding reaction. The DNA binding reaction contained 5ug protein and 10 cpm radio-labeled DNA probes was performed as described (Haas 1998). Following incubation, the samples were loaded onto a 5% native polyacrylamide gel (acylamide/bisacrylamide at 40:1 in 0.3x TBE) for electrophoresis resolution. The intensity of each protein/DNA complex was determined by using a Molecular Dynamics Phosphor Imager.

3. Results

Highly expressed TRAIL in AP1060 cell: In order to explore the mechanism of AP1060 cell immortalization, we examined the expression level of the apoptosis molecules in the different myeloid cell lines (NB4, HL60 and AP1060), which belong to human promyelocytic leukemia cells and could be induced to differentiation with Retinoic acid, by apoptosis detection kits. It was found AP1060 cell expressed high level of TRAIL and significant level of its receptors (DcR1, DcR4 and DcR5) comparing
with NB4 and HL60 (Fig. 1). A great amount of TRAIL was also detected in AP1060 cell cultural medium with immune-precipitation, and no mutation was detected in its cDNA (data not shown).

Fig 1. Expression of the apoptosis gene set. Cells were cultured for 72 h in the presence of medium. Total RNA from growing cells was analyzed for distinct mRNA species using multi-probe RNase protection assay system with the Apo2, hAPO3d and Apo5 multi-probe template set. Free probe and other single-strand DNA molecules were digested with RNases. The RNase-protected probes were purified, resolved on denaturing polyacrylamide gels, and imaged by autoradiography. Bands of house-keeping genes (L32 and GAPDH) were included for normalizing signals. The high expression of TRAIL presented in AP1060 comparing with NB4 and HL60 both of which could be induced to differentiation with ATRA.

Down-regulation of TRAIL by Retinoic acid: It was commonly reported that TRAIL was increased in both of transcriptional and protein level in ATRA-induced differentiation. We also observed the mRNA expression of TRAIL by RNase-protected probes in Apo3d set. The cells were treated with 100 nM ATRA and 1 uM ATRA which was able to stimulate the terminal differentiation of virtually 100% of cells into neutrophilic granulocytes which was determined with nitroblue tetrazolium (NBT) test. The expression of TRAIL was dramatically down-regulated by ATRA treatment in AP1060 cell.

Fig 2. Analysis of possible genes involved in apoptosis during the induced differentiation with Retinoic acid. 100nM and 1uM ATRA stimulated cells to differentiate into 100% neutrophilic granulocytes which was determined with nitroblue tetrazolium (NBT) test. The expression of TRAIL was dramatically down-regulated by ATRA treatment in AP1060 cell.

Fig 3. TRAIL protein expression is consistent with mRNA levels. Cells were treated with 1 uM ATRA to be induced into terminal differentiation. 100 ug total protein per lane was loaded for electrophoresis. After 96 hours induction, the trace of TRAIL was seen in AP1060 cell.

Bio-activity of TRAIL: It was known the Jurkat cell, a lymphocytic leukemia cell line, was very sensitive to TRAIL, which could efficiently induce the cell apoptosis, and used for the evaluation of TRAIL activity. 2.5 x 10^5/ml AP1060 cells were incubated for 48 hours, and the supernatant was collected as the condition medium for TRAIL activity
The 2.5 x 10^5/ml cells were plated in the above condition medium. 24 hours later, the viable and dead cells were counted by Trypan blue staining. The percentage of dead cells in the condition medium was more than 15% contrast to 3.2% in control medium.

Inhibition of proliferation by Neutralizing TRAIL: AP1060 cells were cultured in the medium supplemented with TRAIL-neutralizing monoclonal antibody as indicated in following figure 4. The cell growth was obviously inhibited by neutralization of TRAIL with its specific antibody, which corresponded to the increasing dose of the antibody. Respectively, we did not observed significant inhibition effect on AP1060 cell by neutralizing DcR1 with its specific antibody (Fig. 4 B). The AP1060 cells, which were cultured for 5 days in the neutralization treatment and control, were stained with Wright’s, and presented in figure 5.

Fig 4. Neutralizing Tests. A. Inhibition of cell proliferation by Neutralizing TRAIL. B. The cell growth did not be significantly affected by the neutralization of DcR1. 2.5 x10^5 AP1060 cells were seeded in IMDM containing 10% FBS, 20ng/ml G-CSF and the indicated dose of neutralizing monoclonal antibodies. 400ng respective no-neutralizing antibodies were added in control. All cells were cultured with antibody for 5 days. The viable cell was determined by Trypan blue.

Fig 5. AP1060 cells cultured for 5 days. A. treated with TRAIL no-neutralizing antibody (400ng/ml). B. treated with TRAIL neutralizing antibody (400ng/ml).

Modulation of NF-kB activation by neutralizing TRAIL: 2.5 x 10^7 cells were plated and incubated respectively with 400 ng/ml TRAIL neutralizing or no-neutralizing antibody for 96 hours. The translocation of NF-kB was measured by EMSA as described in methods and presented as Figure 7. It was indicated that the activity of NF-kB was markedly inhibited following the treatment of TRAIL neutralizing antibody.

Apoptosis induced by neutralizing TRAIL: Cell cycle was detected by Flow cytometer to analyze the cause of the inhibition of AP1060 proliferation as described in Methods. AP1060 cells were treated with TRAIL neutralizing antibody for 5 days and stained in propidium iodide, and analyzed for cell cycle distribution flowcormetrically using CFlow® software. The population of the cells in sub-G1 phase is increasing with the successively incremental dose of neutralizing TRAIL antibody comparing with the control that was treated with 400 ng/ml no-neutralizing TRAIL antibody (Fig. 5). These results were confirmed by TUNEL assay. The percentage of the cells containing FITC labeled DNA fragments was raising in TRAIL neutralizing antibody treatment, which was consistent with the its sub-G1 cell cycle distribution (Fig. 6). Simultaneously, we did not find any significant effect of neutralizing DcR1 on AP1060 cell (Fig. 5). It indicated that the treatment with TRAIL neutralizing antibody made AP1060 cell undergo apoptosis.
Figure 6. Flow cytometric cell cycle analysis demonstrates sub-G1 accumulation after the treatment with TRAIL neutralizing antibody. The cells were cultured with the indicated neutralizing antibodies (ng/ml). The no-neutralizing antibodies (400ng/ml) were used in controls. A. DNA Histograms of cell cycle (from single experiment). The relative number of cells displaying an apoptotic, sub-G1 DNA content, is given between the marker bars. Cells were treated respectively with 400 ng/ml antibody. B. Dose response. Apoptosis was determined by flow-cytometric detection of nuclear DNA fragmentation. C. TUNEL assay. Bars in B and C represent the mean of cells displaying a sub-G1 DNA or FICT labeled DNA content from three independent experiments.

Figure 7. Inhibition of NF-kB activation by neutralizing TRAIL. Nuclear extracts were prepared from AP1060 cells incubated for 96 hours with TRAIL no-neutralizing antibody (-) and neutralizing antibody (+). The excessively competent reaction was presented as control (c). The presence of NF-kB was determined by EMSA using a radio-labeled probe containing the prototypical NF-kB site.

4. Discussions

The retinoic acid therapy has been established over twenty years and is only successful model in which the patient is cured by inducing the malignant cells to differentiate terminally. A variety of studies about the cellular and molecular characters during the RA-induced differentiation has been carried out to explore its mechanism. It is used to be believed that Retinoic acid-induced apoptosis in acute promyelocytic leukemia cells is mediated by TRAIL that is induced by retinoic acid (Altucci 2001). However, our findings suggest that TRAIL may play a different role in RA-induced differentiation.

The myeloid leukemia is commonly characterized by an arrest of granulocytic differentiation and failure of apoptosis. It was early indicated that the initiation of differentiation required the transcriptional activation of specific genes leading to proliferation arrest and cell cycle exit (Rousselot 1994). The evidence that TRAIL was highly expressed and dramatically down-regulated after retinoic acid treatment was found not only in AP1060 cell line but also in APL specimens (Riccioni 2005). It does not support the hypothesis that TRAIL is related to the ATRA-induced differentiation and apoptosis of APL cells. In addition, our previous data of the genes expression profile of RA-induced differentiation of AP1060...
presented an intricate gene-reprogramming network in which deregulated growth, increased cell survival, processed differentiation were hallmarks (Sun 2011). For example, the inhibition molecules of apoptosis such as BCL related protein 2, were up-regulated while the apoptosis-inducing molecules (TRAIL, SIAH1) were down-regulated. It is consistent with the results of the experiments that were conducted in HL60 cells with the combination treatment of retinoic acid and TGF-β (Soucek 2006). 

Our results indicated that 1060 cell expressed a wild type and bio-effective TRAIL. It is interesting how APL1060 cell could escaped from the TRAIL attack. Many studies have reported that the expression of TRAIL decoy receptors is involved in the mechanisms of resistance of several tumor cell types to TRAIL, including breast cancer, myeloma and osteosarcoma (De Almodovar 2004, Shipman and Bouralexis 2003). Ricconio also ascribed such resistant phenomenon in APL cells to the expression of death decoy receptors (Ricconi 2005). However, in this study the protection of decoy receptors had not been wiped of by neutralizing DcR1 that expressed mainly in AP1060 cells. Furthermore, it was more surprising that AP1060 cells underwent an apoptosis after the treatment with TRAIL neutralizing antibody. It implies that the auto-secreted TRAIL is necessary for the growth and proliferation of AP1060 cells. In fact, it was reported that TRAIL could stimulate the proliferation of leukemia cells and activated NF-κB (Ehrhardt 2003, Degli-Esposti 1997). NF-kB activation is mediated not only by the truncated decoy receptor TRAIL-R4 (DcR2), but also by the death receptors, TRAIL-R1 and -R2 (Degli-Esposti 1997, Harper 2001). Here, the result of EMSA showed that the activation of NF-kb was inhibited by a treatment with TRAIL neutralizing antibody. Therefore the activation of NF-kB leads the transcription of genes that antagonize the death signaling pathway. In other hand, the activation of NF-kB mediated by TRAIL-R1 and TRAIL-R2 depended on the inhibition of apical Caspases (caspase-8 and FADD) (Ehrhardt 2003, Harper 2001). Sensitivity to TRAIL-induced apoptosis was also modulated by inhibition or activation of NF-kB (Harper 2001). We wonder whether the deficiency of Caspase-8 or dysfunction of FADD may harbor in AP1060 cell line and offer it a bio-feature that TRAIL could not kill it but stimulate its proliferation.

In cancer therapy, the apoptosis resistance of tumors is a commonly challenging issue whatever in physiological self-defending system or chemotherapy including APL-retinoid-induced differentiation. Our findings enlighten us to explore the clinical relevant features of APL and the differential strategies of therapy.

Corresponding Author:  
Dr. Yiwu Sun  
Department of Medicine  
Brookdale University Hospital Medical Center  
Brooklyn, NY11212  
E-mail: yiwu_sun@hotmail.com

References


12/22/2011