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Induction of Apoptosis by Recombinant Soluble Human TRAIL in Jurkat Cells[1]

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Objective To investigate the therapeutic potential of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, and to analyze TRAIL-induced apoptosis in Jurkat cells. **Methods** Expression of TRAIL receptors (DR4 and DR5) was detected by reverse transcriptase-polymerase chain reaction (RT-PCR). Cytotoxic effects were determined by colony formation assay and a cell counting kit. The effects of recombinant TRAIL on apoptosis of Jurkat cells were determined by DNA fragmentation (DNA ladder) and PI staining. Changes in mitochondrial membrane potential were detected with JC-1 fluorescence. **Results** TRAIL inhibited the proliferation and induced internucleosomal DNA fragmentation (characteristic of apoptosis) and loss of mitochondrial membrane potential. **Conclusion** Recombinant soluble TRAIL can be used as a therapy for cancer.

Key words: TRAIL; Apoptosis; Jurkat cells

INTRODUCTION

Tumor necrosis factor (TNF) is a prototypic member of the cytokine family functioning in immune regulation and inflammatory response. Members of this family interact with a corresponding set of receptors. Signals induced by these interactions have diverse functions such as cell differentiation, proliferation and activation or induction of apoptosis[1-2]. TNF-related apoptosis inducing ligand (TRAIL), also known as Apo2L, belongs to TNF superfamily. In 1995, TRAIL was initially identified and cloned based on the sequence homology to Fas/Apo 1 (FasL) and TNF[3-5]. Studies showed that members of TNF receptor (TNFR) could actively instruct individual cells to die. Instructive apoptosis plays a physiological role in deletion of unnecessarily activated lymphocytes and in elimination of virus-infected cells and oncogenically-transformed cells[6-7].

Dysregulation of normal apoptotic mechanisms provides a growth advantage to cancer cells. Studies suggested that activation of specific apoptotic mechanisms in cancer cells has effectiveness in treating cancer[8]. The death receptors of TNF receptor family induce apoptosis upon binding to their specific ligands *via* activation of caspases. These death receptor pathways offer a promising method to induce apoptosis in cancer and thereby serve as a therapy for cancer[9-11]. Despite the ability of TNF and FasL to induce apoptosis in cancer cells, severe toxic effects hamper the use of both TNF family ligands in anticancer therapy. TNF could cause a leathal inflammatory response that resembles septic shock. This effect is mediated primarily by TNF activation of the proinflammatory transcription factor NF-κB in many cells. Infusion of anti-Fas antibody causes leathal liver damage caused by induction of Fas-dependent apoptosis in hepatocytes, which express high levels of Fas[6,12-14]. In contrast to TNF and FasL, TRAIL can selectively induce apoptosis in a wide variety of tumor cells, but not in normal cells[15-19].

The ability of TRAIL to induce apoptosis in a large number of tumors cells has stimulated interest in TRAIL as a tumor therapeutic agent. Therefore, recombinant soluble human TRAIL is a candidate for cancer therapy. Studies showed that different

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recombinant versions of TRAIL vary widely in biochemical properties and potential for cancer treatment. Lawrence developed a version of human TRAIL that lacks exogenous sequence tags (residues 114-281) and demonstrated that this version of recombinant TRAIL could induce apoptosis in many cancer cell lines[20]. In the present study, we obtained a highly purified recombinant human TRAIL with similar version to Lawrence from *Escherichia coli.* The apoptotic cell death and related mechanism in Jurkat cells were determined with treatment of this recombinant TRAIL. The study might shade light into the treatment of this kind of disease with TRAIL.

MATERIALS AND METHODS

Materials

Trizol regent was purchased from BioBasic Inc (Scarborough Ontario, Canada). Taq polymerase enzyme, M-MLV reverse transcriptase and rRNasin (ribonuclease inhibitor) were from Promega (Madison, WI, USA). Cell counting kit was from Dojin Laboratories (Kumamoto, Japan). PI and RNAse A were obtained from Sigma (St Louis, MO, USA). JC-1 mitochondrial membrane potential detection assay kit was from Biotium Inc (Hayward, CA, USA). All other commercially available reagents were of the highest grade.

Production of Recombinant TRAIL

The extracellular portion of TRAIL molecular (residues 114-281) was cloned into pET28a vector (Novagen, Madison, WI) and expressed in *Escherichia coli.* The rHis-TRAIL fusion production was purified by metal chelate column chromatography using Ni-NTA rasin, according to the manufacturer's recommendations (Qiagen, Hilden, Germeny).

Determination of DR4 and DR5 by Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from Jurkat cells by the Trizol method. RNA concentrations were quantified with a spectrophotometer at 260 nm. Two μg of total RNA was reverse-transcribed by reverse transcription-polymerase chain reaction (Perkin-Elmer Cetus DNA Thermal Cycler, Perkin-Elmer, CT, USA). Then the first strand complementary was gained in the presence of M-MLV reverse transcriptase and oligo-dT primers. After RT reaction, 2 μL of the incubation mixture was used as the template for the following PCR. The following components were added to the mixture: 5 μL 10×PCR buffer, 1 μL (10 pmol) of both sense and antisense primers (DR4 sense primer: 5'-CTGAGCAACGCAGACTCGCTGTCCAC-3', DR4 antisense primer: 5' -TCAAAGGACACGGCAGAGCCTGTGCCA-3', DR5 sense primer: 5'-ATGGAA -CAACGGGGACAGAAC-3', antisense primer: 5'-TTAGGACATGGCAGAGTCTGCATTAC-3'), 1 μL 10 mmol/L mixture of all four deoxynucleotide triphosphates, 1 μL Taq DNA polymerase, and 12 μL nuclease-free water added to adjust the final volume to 25 μL. After an initial incubation at 94℃ for 1 min, temperature cycling was initiated with each cycle as following: denaturation at 94℃ for 30 s, annealing at 65℃ for 30 s and elongation at 72℃ for 1 min. Then the reaction was followed by a second round of 30 cycles of denaturation at 94℃ for 30 s, annealing at 65℃ for 30 s, and elongation at 72℃ for 1 min. The reactions were terminated by incubation at 72℃ for 5 min. The PCR products were then separated on 1.5% agarose gel containing 0.5 μg/mL ethidium bromide. The gel was put on an UV-transilluminator and photographed.

Jurkat Cell Colony Assay

Jurkat cell colony formation was detected as previously described[21]. Briefly, 1×10^5 nonadherent Jurkat cells were plated in 6-well round-bottomed microtiter plates and incubated at 37℃ in a humidified atmosphere containing 5% CO_2. Recombinant human TRAIL was added at the initiation of culture at concentrations of 30 ng/mL and 300 ng/mL. Cell colony was evaluated under a microscope at 12 h and 24 h, respectively.

Cell Cytotoxicity

Cell viability was assessed with a cell counting kit (Dojin Laboratories, Kumamoto, Japan) as previously described[22] with minor modifications. Briefly, Jurkat cells were plated on a 96-well (100 μL /well) microplate at a density of 1×10^6 cells/mL. TRAIL at various concentrations was added and the plate was incubated at 37℃ in a humidified atmosphere containing 5% CO_2 for 12, 24, and 48 h. Ten μL of a solution containing WST-8 (4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl) -2H-5-tetraz--olio]-1, 3-benzene disulfonate sodium salt) was added to each well and incubated for an additional 2 h. The absorbance was measured at 450 nm on an automated microplate reader (Bio-Rad Model 550).

DNA Laddering Detection

To examine apoptosis by electrophoresis of nucleosomal fragments, a standard procedure for

precipitating cytosolic nucleic acid was used[23]. Briefly, 1×10^6 TRAIL–treated cells were pelleted (200×g, 5 min) and lysed at 4℃ for 15 min (250 μL, 0.4% Triton-X, 20 mmol/L Tris, and 0.4 mmol/L EDTA). Nuclei were then pelleted (13 000×g, 5 min, 4℃) and the supernatants were transferred to a clean microfuge tube. Nucleosomal fragments were precipitated overnight with an equal volume of isopropanol after adjustment to 0.5 mol/L NaCl. The pellet was washed twice in 70% ethanol, dried briefly, and resuspended in 40 μL TE (10 mmol/L Tris-HCl, 0.1 mmol/L EDTA, pH 8.8). The extracted DNA was separated on a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide and visualized under an UV-transilluminator and photographed. Fragmented DNA, shown as DNA ladder in the gel, indicates apoptotic cell death.

Analysis of Hypodiploid Cells

Analysis of hypodiploid cells was performed using PI staining[24]. In brief, Jurkat cells (1×10^6) in 1 mL RPMI 1640 were added to each well of 12-well plates and treated at 37℃ with various concentrations of recombinant TRAIL for 12 h and 24 h respectively. The cells were fixed in 75% ethanol for 30 min at room temperature, and stained with propidium iodide staining buffer (Trixon X-100, EDTA, RNAse A, PI) for 10 min in the dark. The fluorescence of PI was monitored with a FACSCalibur flow cytometer (Becton-Dickson, Immunocytometry System, San Jose, CA) at an excitation wavelength of 488 nm and an emission wavelength of 625 nm. Apoptotic cells were determined on a PI histogram as a hypodiploid. For each sample, 20 000 cells were analyzed. The data were analyzed by Cellquest software (Palo Alto, CA, USA).

Analysis of Mitochondrial Membrane Potential by JC-1 Fluorescence

JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (525 nm) to red (590 nm). JC-1 is most widely applied in the detection of mitochondrial depolarization during the early stages of apoptosis[25]. In our study, 1×10^6 Jurkat cells were incubated in a complete medium with 2.5 ug/mL JC-1 in the dark for 10 min at room temperature. Then, cells were washed twice in PBS, resuspended in 400 μL PBS and analyzed by flow cytometry. Prior to the FCM analysis, the cell suspension was suctioned three times through a needle and filtered through a 50 um-nylon mesh to minimize cell aggregation. For assessment of individual samples for JC-1 staining, a total of 10000-gated events were analyzed per sample by flow cytometer. The sample running rates were approximately 100-300 events/sec. A 488 nm filter was used for excitation of JC-1. Emission filters of 535 nm and 595 nm were used to quantify the population of Jurkat cells with green (JC-1 monomer) and orange (JC-1 aggregates) fluorescence, respectively. Frequency plots were prepared for FL-1 (green) and FL-2 (orange) to determine the percentage of the population stained green and orange. In addition, all samples were viewed under fluorescence microscope to confirm JC-1 labeling patterns. Confirmation of apoptotic cells was done by morphological assessment of cytospin preparations of cells.

RESULTS

Expression of DR4 and DR5

Since expression of TRAIL death receptors (DR4 and DR5) could regulate the sensitivity of cells to TRAIL, we assessed the expression of TRAIL receptors in Jurkat cells by RT-PCR. The results indicated that both death receptors were expressed in Jurkat cells. The sizes of PCR products for DR4 and DR5 are shown in Fig. 1.

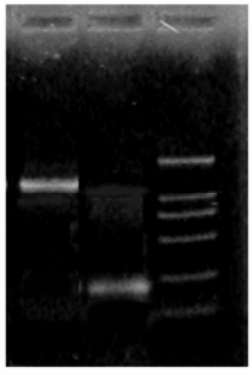

FIG. 1. Expression of TRAIL receptors (DR4 and DR5) in Jurkat cells determined by RT-PCR. M stands for DNA marker, 1 for DR5, and 2 for DR4.

TRAIL Inhibited Colony Formation and Decreased Viability of Jurkat Cells

Colony formation in Jurkat cells was significantly inhibited by recombinant human TRAIL (Fig. 2). As shown in Fig. 2a, there were a great deal of clones and cells in the representative field of control group. However, as shown in Fig. 2b, the number of clones and cells was markedly decreased in the TRAIL-treated group.

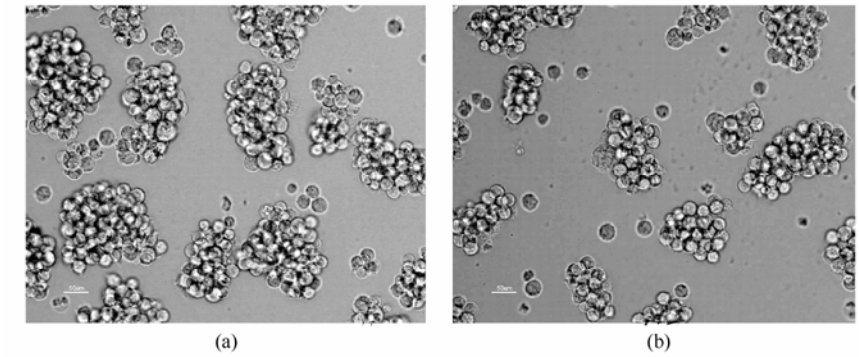

FIG. 2. Colony formation inhibition in Jurkat cells not treated with TRAIL (a) and treated TRAIL (b).

Cytotoxic Effects of TRAIL on Jurkat Cells

As shown in Fig. 1, both receptors were expressed in Jurkat cells. Therefore, to evaluate the cytotoxicity of recombinant TRAIL, Jurkat cells were treated with 30 ng/mL and 300 ng/mL TRAIL for 12 h and 24 h, respectively. Viability tests were performed using a cell counting kit. As shown in Fig. 3, treatment of Jurkat cells with TRAIL for 12 and 24 h significantly decreased the cell viability. However, Jurkat cells treated with TRAIL for a relatively longer time (48 h) appeared to have no significant difference in cell viability. Therefore, we analyzed the effects of recombinant TRAIL on Juakat cells at 12 and 24 h, respectively.

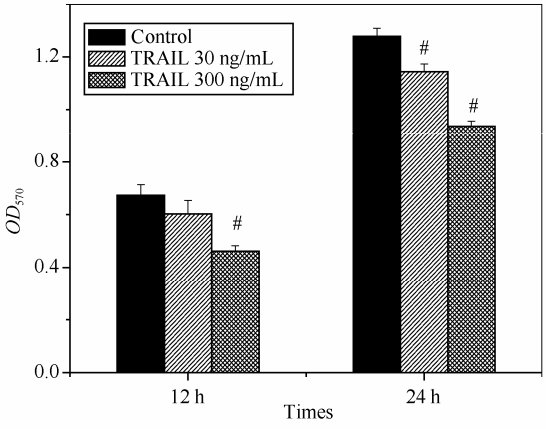

FIG. 3. Decrease in viable cells number of Jurkat cells after treatment with recombinant TRAIL . $^{\#}P<0.05$ *vs* control group.

DNA Fragmentation of Jurkat Cells

To investigate the mechanism by which recombinant TRAIL causes cytotoxicity in cultured Jurkat cells, DNA was examined. According to Hughes[26], DNA laddering is commonly used to establish if a decrease in cell viability due to apoptosis rather than necrosis. As shown in Fig. 4, agarose gel electrophoresis for soluble DNA in TRAIL-treated Jurkat cells revealed DNA fragmentation characteristics of apoptotic cells (DNA ladder). The results indicate that exposure of Jurkat cells to 30 ng/mL and 300 ng/L recombinant TRAIL could lead to DNA fragmentation in 12 h and 24 h, suggesting that TRAIL induced apoptosis. The results are consistent with observations of decreased cellularity of Jurkat cells after TRAIL treatment.

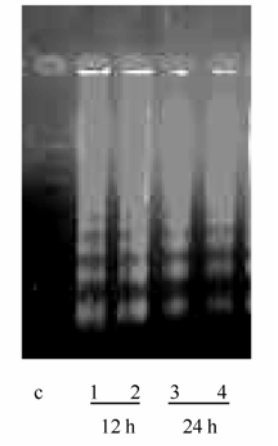

FIG. 4. Agarose gel electrophoresis of DNA fragments in Jurkat cells cultured for 12 h and 24 h.

Apoptosis Determination by Hypodiploid Cells Analysis

To further confirm that TRAIL induced apoptosis, the cells were stained with PI and analyzed for hypodiploid cells by flow cytometry. The fraction of apoptotic cells was identified on a DNA histogram as a sub-G1 hypodiploid population. Jurkat cells in the control group showed less sub-G1 hypodiploid population (Fig. 5a, arrow). Figure 5b (arrow) shows the hypodiploid sub-G1 phase in cells treated with

recombinant TRAIL, confirming its apoptotic effect. The results indicate that Jurkat cells treated with TRAIL for 12 h and 24 h had a sub-diploid peak and the percentage of cells with fragmented DNA increased compared with the control group. Once again, this observation substantiated the conclusion that TRAIL could induce apoptosis of Jurkat cells. Figure 5c shows the percentages of apoptotic cells.

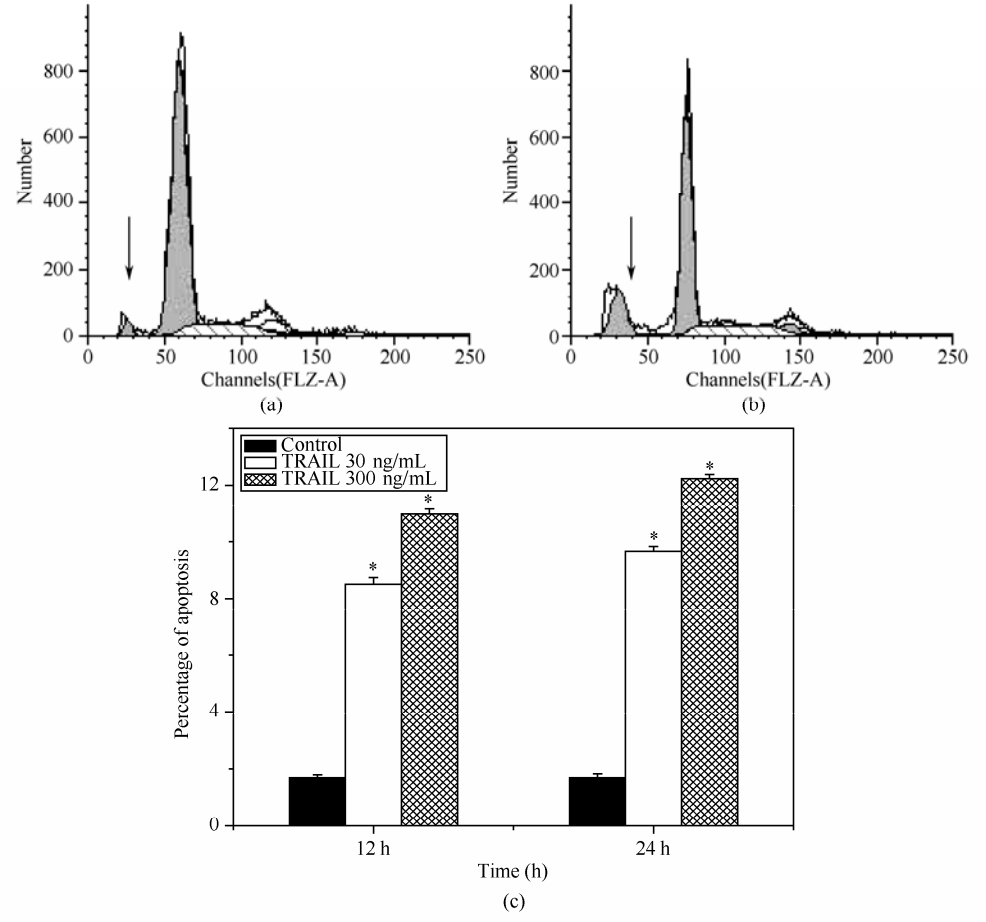

FIG. 5. Hypodiploid cells detected by flow cytometry. (a) representative plot of PI staining of Jurkat cells in control group, (b) representative plot of PI staining of Jurkat cells treated with TRAIL, (c) plotted total percentages of apoptotic Jurkat cells (Sub-G1 hypodiploid population) after 12 h and 24 h of treatment with indicated concentrations of TRAIL. $^{*}P<0.05$ *vs* control group.

Assessment of Changes in Mitochondrial Membrane Potential

Loss of mitochondrial membrane potential is a hallmark for apoptosis. It is an early event coinciding with caspase activation. In non-apoptotic cells, JC-1 exists as a monomer in cytosol (green) and accumulates as aggregates in the mitochondria, which appear red. In apoptotic and necrotic cells, JC-1 exists in a monomeric form and stains the cytosol green. Figure 6a is a representative JC-1 stain of apoptotic and non-apoptotic Jurkat cells. In Fig. 6a, most Jurkat cells, without recombinant TRAIL treatment, showed red and green fluorescence, indicating that they were live cells. However, in Fig. 6b, some Jurkat cells showed green fluorescence, suggesting that TRAIL induced apoptosis of Jurkat cells. Figure 6c and d shows that typical FL-1/FL-2 dot plots for JC-1 stained Jurkat cells with and without apoptosis. Figure 6c shows that less apoptosis of Jurkat cells had red fluorescing J-aggregates. The green fluorescing monomers in the lower left quadrant indicated apoptotic cells (arrow). While the number of green fluorescing monomers increased significantly in Fig. 6d (arrow), indicating that recombinant TRAIL could trigger apoptosis. Figure 6e shows the percentages of apoptotic Jurkat cells analyzed by flow cytometry in different TRAIL-treated groups. The increase in percentages of apoptotic Jurkat cells was observed after treatment with TRAIL for 12 and 24 h. At 12 h, approximately 1.28% of Jurkat cells were apoptotic cells in the

control group. The rate of apoptotic Jurkat cells increased to 4.02% after treatment with 30 ng/mL TRAIL. When the concentration of TRAIL increased to 300 ng/mL, the percentage of apoptotic Jurkat cells raised to 6.47%. Analysis of apoptotic cells at 24 h displayed the similar tendency, and the percentage was 1.37%, 5.76%, 9.21% after treatment with 0, 30, and 300 ng/mL of TRAIL, respectively.

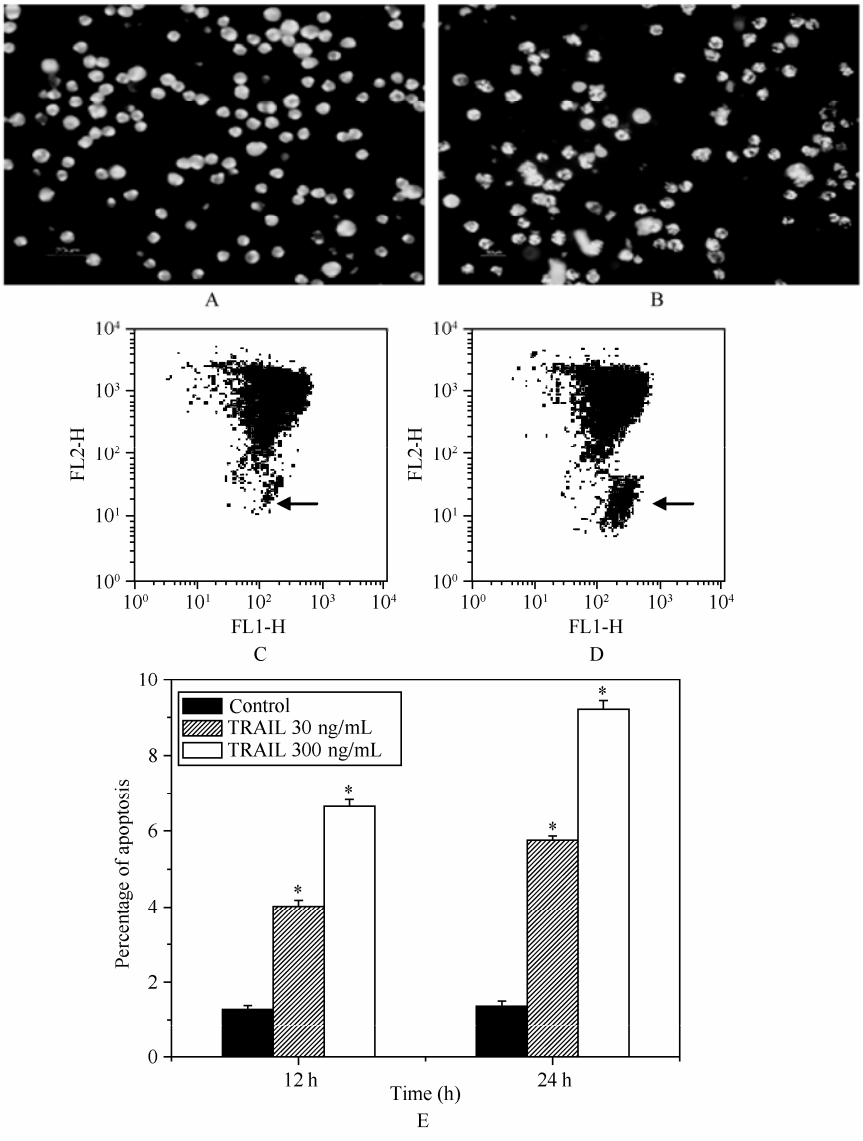

FIG. 6. Representative photos, dot plots and percentages of histogram analysis of Jurkat cells by JC-1 staining. A and B: Polarized mitochondria marked by punctuate orange-red fluorescence staining. A represents the control without apoptosis, B stands for TRAIL-treated sample with apoptotic Jurkat cells. C and D: Representative dot plots of JC-1 stained Jurkat cells. C represents control, D stands for TRAIL treatment. E: Percentages of cells containing polarized or depolarized mitochondria determined by histogram analysis of the ratio of the two fluorescence intensities. The numbers indicate the Jurkat cells expressing green monomer fluorescence only. Data represent means of three independent experiments.

DISCUSSIONS

It was reported that different recombinant versions of TRAIL vary widely in biochemical properties and potential of cellular and whole animal toxicity[20]. In the present study, we developed a version of human TRAIL that lacks exogenous sequence tags (residues 114-281) similar to those by Jo *et al.*[27].

Studies have shown that sensitivity to

TRAIL-induced apoptosis is regulated by the presence or absence of two receptors (DR4 and DR5) with death-inducing ability[14,27-28]. In the present study, Jurkat cells could express DR4 and DR5 receptors. The presence of DR4 and DR5 in Jurkat cells indicates that TRAIL-induced apoptosis is not an artifact of culture and can be expected.

The results of the current study indicate that recombinant TRAIL could induce apoptosis of Jurkat cells (Figs. 4 and 5). Using a number of techniques, we demonstrated DNA fragmentation (DNA ladder) by gel electrophoresis and DNA cleavage (sub-diploid peak) by PI staining, suggesting that recombinant TRAIL induces apoptosis, which is consistent with observations of decreased cellularity in Jurkat cells. It was reported that expression of TRAIL could influence the biological properties of Jurkat cells[5]. Our results suggest that the soluble form of TRAIL could directly induce apoptosis of Jurkat cells, which express DR4 and DR5 receptors.

Moreover, in this study, we investigated the effects of TRAIL on the mitochondrial membrane potential. It has been shown that mitochondrial permeability transition is an important step in the induction of cellular apoptosis, during which collapse of the electrochemical gradient would occur through the formation of pores in the mitochondria by dimerized Bax or activated Bid, Bak, or Bad proteins. Activation of these pro-apoptotic proteins is accompanied with the release of cytochrome C into the cytoplasm[29]. Bradbury reported that green fluorescence is indicative of compromised mitochondria with a low transmembrane potential by JC-1 staining[30]. In the present study, the percentage of Jurkat cells with green fluorescence increased after TRAIL treatment. After 24 h, the number of Jurkat cells with green fluorescence after treatment with 30 ng/mL TRAIL increased to 5.76% *vs* 1.37% in control group. When the concentration of TRAIL was 300 ng/mL, the percentage of Jurkat cells with green fluorescence increased to 9.21%.

In fact, recombinant TRAIL can induce apoptosis of other types of cells, such as human colon carcinoma cells, human ovarian carcinoma cells, breast cancer cells, hepatocellular carcinoma cells[6,8,14,17,31-33]. However, different recombinant versions of TRAIL vary widely in their toxicity. Ashkenazi developed a recombinant soluble form of human TRAIL that is devoid of foreign sequence (amine acids 114-281)[9], suggesting that recombinant TRAIL can be used in treatment of cancer.

Studies showed that development of a malignant cell clone is due to dysregulation of the balance between cell proliferation and apoptosis[34-36]. Many types of antitumor therapy including radiotherapy exert their effect by activating apoptosis[37]. However, current treatment of tumor has side effects, such as giving rise to host toxicities. Hence, there is a clear need for novel approaches to cancer treatment. Recombinant soluble human TRAIL is a candidate for cancer therapy because it induces apoptosis in a broad spectrum of human cancer cell lines but not in normal cells, and exhibits potent anti-tumor activity without toxicity to normal tissues[20,38-40]. Our results demonstrate that recombinant human TRAIL that lacks exogenous sequence tags (residues 114-281.) could induce apoptosis of Jurkat cells, suggesting that this version of recombinant TRAIL can be used in treatment of this kind of cancer.

In conclusion, recombinant TRAIL is biologically active in decreasing the viability and inducing apoptosis of Jurkat cells. There is increasing evidence that apoptosis relates to a variety of signal transudation events stimulating calcium flux, cAMP production, PLC activation, inositol phosphate generation.

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