

Interferon- α -induced apoptosis via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-dependent and -independent manner

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Abstract. IFN- α regulates tumor cell growth at least through induction of apoptosis. We have recently demonstrated that IFN- α causes apoptosis through upregulation of TNF-related apoptosis-inducing ligand (TRAIL) in Daudi B lymphoma and U266 myeloma cells. However, other cell lines such as Ramos and RPMI 8226 underwent apoptosis without any apparent involvement of TRAIL following IFN- α stimulation. In this study, we examined whether the IFN- α -induced upregulation of TRAIL is essential for the induction of apoptosis. IFN- α -induced early phase (48 h) of loss of $\Delta\Psi_m$ was substantially prevented in Daudi B lymphoma cells overexpressing the dominant-negative form of Fas-associated death domain (dnFADD) compared with vector control, whereas a late phase (72 h) of $\Delta\Psi_m$ was comparable to the control. The IFN- α -induced early phase of apoptosis was also reduced in the dnFADD-expressing cells, while the late phase of apoptosis was unaffected. IFN- α -induced upregulation of TRAIL protein in the dnFADD-expressing Daudi or U266 cells was comparable to their control cells, suggesting that FADD is not involved in the IFN- α -induced upregulation of TRAIL. Moreover, the early phase of mitochondrial depolarization was severely prevented by the presence of fusion protein of TRAIL receptor 1 and Fc portion of immunoglobulin (TRAIL-R1:Fc) and TRAIL-R2:Fc. Together, IFN- α induces apoptosis in a TRAIL-dependent or -independent manner, depending on the course of the apoptotic process.

Introduction

Interferon- α (IFN- α) has multiple functions in a variety of cell types including modulation of immune responses, cell growth arrest and cell death (1). IFN- α has been employed for the treatment of some tumors including melanoma, renal cell carcinoma and chronic lymphatic leukemia (2-4), although the detailed molecular mechanisms by which IFN- α prevents tumor cells remains unresolved. We and others have previously demonstrated that IFN- α induces apoptotic cell death in several cell types (5-8), at least through upregulation of the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (9-12), a member of the TNF family of proteins. TNF family proteins containing the death domain are composed of at least 18 members including Fas (CD95) and TRAIL (13). Interaction of TRAIL with its receptors results in recruitment of adaptor protein Fas-associated death domain (FADD) and pro-caspase-8, making up death-inducing signaling complex (DISC), leading to the activation of effector caspases such as caspase-3 (extrinsic pathway) (14). The caspase 8 activation in most cell types is amplified in mitochondria, the function of which is regulated at least by Bcl-2 family members. The Bcl-2 family proteins are composed of pro-apoptotic (Bax- α and Bak) and anti-apoptotic (Bcl-2 and Bcl-xL) components and the balance of both members plays a crucial role in determining the cell fate, survival or cell death (intrinsic pathway) (15-18). Mitochondrial dysfunction associated with cell death in response to multiple stimuli appears to involve the activation of pro-apoptotic members, Bax- α and Bak, which constitute gates to the intrinsic apoptotic pathway. Thus, cells deficient of both Bax- α and Bak, but not those lacking one of these components, fail to undergo apoptosis in response to diverse apoptotic stimuli including chemotherapeutic agents and radiation (19). The anti-apoptotic Bcl-2 family members prevent activation of Bax- α and Bak through sequestration of sequestering BH3-only proteins (20). The mitochondrial dysfunction induced by some apoptotic stimuli including IFN- α is accompanied by the loss of mitochondrial membrane potential ($\Delta\Psi_m$), cytochrome C release and caspase activation, a hallmark of apoptosis (15,16,18,21). The loss of $\Delta\Psi_m$ by multiple apoptotic stimuli is accompanied by modulation of Bcl-2 family members, including down-

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Abbreviations: IFN- α , interferon- α ; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; $\Delta\Psi_m$, mitochondrial membrane potential; FADD, Fas-associated death domain; dnFADD, dominant-negative form of FADD

Key words: interferon- α , human, apoptosis, tumor cells, TNF-related apoptosis-inducing ligand

regulation of Bcl-2/Bcl-xL or upregulation of Bax- α or Bak (15,18,22,23). Moreover, we and others have recently demonstrated that the truncated form of Bax- α , p18 Bax- α , is generated by some stimuli such as IFN- α and chemotherapeutic agents (7,23-27).

During the extensive study on IFN- α -induced apoptosis in multiple cell lines, we found some cell lines undergo apoptosis via upregulation of a death ligand such as TRAIL, while others are independent of TRAIL. In the present study, we examined whether TRAIL-induced extrinsic pathway is essential for IFN- α -induced apoptosis in human B cell lines. The early phase of IFN- α -induced apoptosis was dependent on TRAIL, whereas the late phase of IFN- α -induced apoptosis functioned via mitochondrial intrinsic pathway, independent of TRAIL. These findings would be useful for understanding IFN- α -induced apoptosis and hopefully for the design of new treatment modalities for certain tumors.

Materials and methods

Cell cultures. Two lymphoma cell lines (Daudi and Ramos) and two myeloma cell lines (U266 and RPMI 8226) (American Type Culture Collection; Manassas, VA, USA) were maintained in RPMI-1640 medium supplemented with 10% v/v fetal bovine serum (FBS), 2 mM glutamine, 50 μ M mercaptoethanol and 100 μ g/ml kanamycin in humidified air with 5% CO₂.

Flow cytometric analysis of membrane TRAIL expression, $\Delta\Psi_m$ and apoptosis. The cells (5x10⁵/ml) were stimulated with or without recombinant IFN- α 2a (Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) for various times and assayed for expression of TRAIL, $\Delta\Psi_m$ and apoptosis, as previously described (11). Briefly, the cells were incubated with mouse anti-TRAIL monoclonal antibody (mAb), followed by staining with fluorescein-conjugated goat anti-mouse IgG. Samples were analysed on a flow cytometer (FACSCalibur, Nippon Becton Dickinson Company Ltd., Tokyo, Japan) using CELL Quest software (Becton Dickinson Immunocytometry System, San Jose, CA). The fluorescence distribution histograms were plotted as the number of cells versus fluorescence intensity on a logarithmic scale. For determination of $\Delta\Psi_m$ or apoptosis, cells were loaded with 20 nM DiOC₆ or were stained with annexin V-Cy5, using Annexin V-Cy5 Apoptosis Detection kit (BioVision, Mountain View, CA, USA), followed by analysis on a flow cytometer. In some experiments, cells pretreated with both 2 μ g/ml TRAIL-R1:Fc and 2 μ g/ml TRAIL-R2:Fc (Alexis Biochemicals, San Diego, CA, USA) for 15 min were stimulated with 25 U/ml IFN- α for 36 h, followed by assay for $\Delta\Psi_m$.

Construction of expression vector and transfection into lymphoid cell lines. The cDNA for the dominant-negative form of FADD (dnFADD) (28) was generated by reverse transcription-polymerase chain reaction (RT-PCR) using primers (forward, 5'-CGGGAATTCATGGACTTCGAGCGGGGGCG-3'; reverse, 5'-GCAGCGGCCGCTCAGGACGCTTCGGAGGTAG-3') specific to FADD and subcloned at EcoRI and NotI sites into expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA). The dnFADD construct was

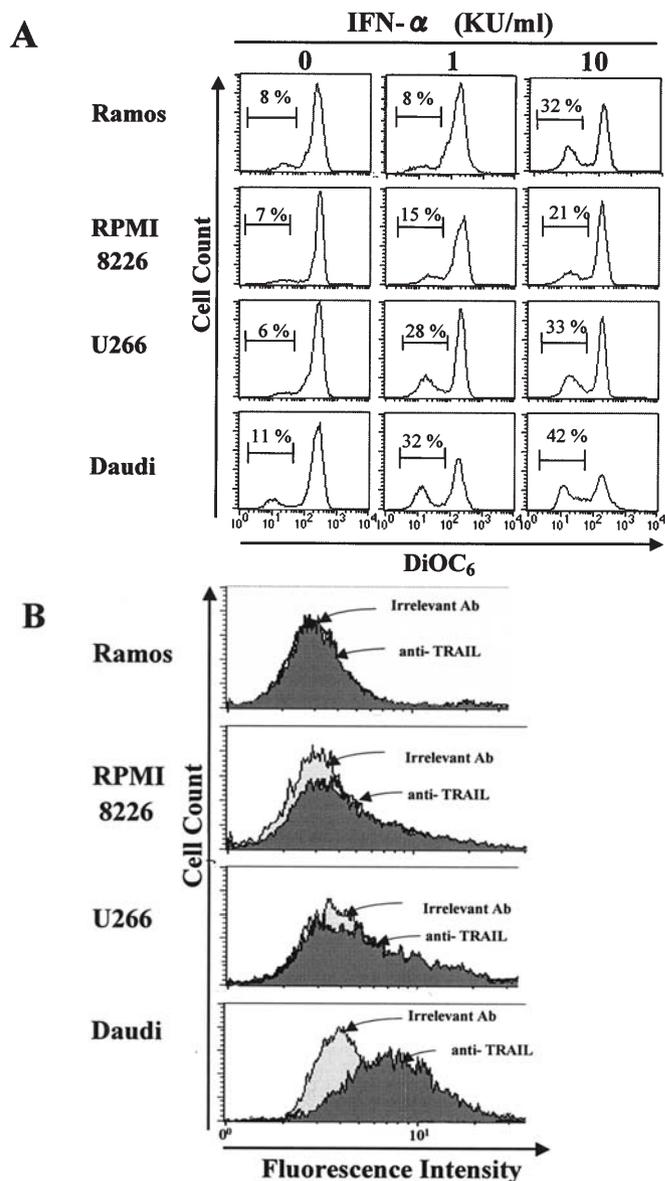


Figure 1. IFN- α -induced loss of mitochondrial membrane potential does not correlate with the IFN- α -induced upregulation of TRAIL in B lineage cell lines. Two lymphoma cell lines (Daudi and Ramos) and myeloma cell lines (U266 and RPMI 8226) were cultured with IFN- α for 48 h and assayed for mitochondrial membrane potential (A) and expression of TRAIL (B). The experiments were done three times, with essentially similar results.

confirmed to be accurate by sequence analysis. Daudi or U266 cells were transfected with the dnFADD construct or control vector alone, as previously described (11). After incubation with selection media containing G418, several transformants were generated, followed by a limiting dilution to obtain individual clones.

Detection for dnFADD expression by RT-PCR. RT-PCR was carried out, as previously described (11). Briefly, total RNA was reverse transcribed using RNA PCR kit (Takara, Tokyo, Japan) for PCR. PCR reactions were done Taq polymerase (Takara) using the following primers: human dnFADD (forward, 5'-GGAGCTGACACGGAAGAT-3'; reverse, 5'-GCAGCGGCCGCTCAGGACGCTTCGGAGGTAG-3'); GAPDH (forward, 5'-GTGGAAGGAAGTCAATGACCAC-3';

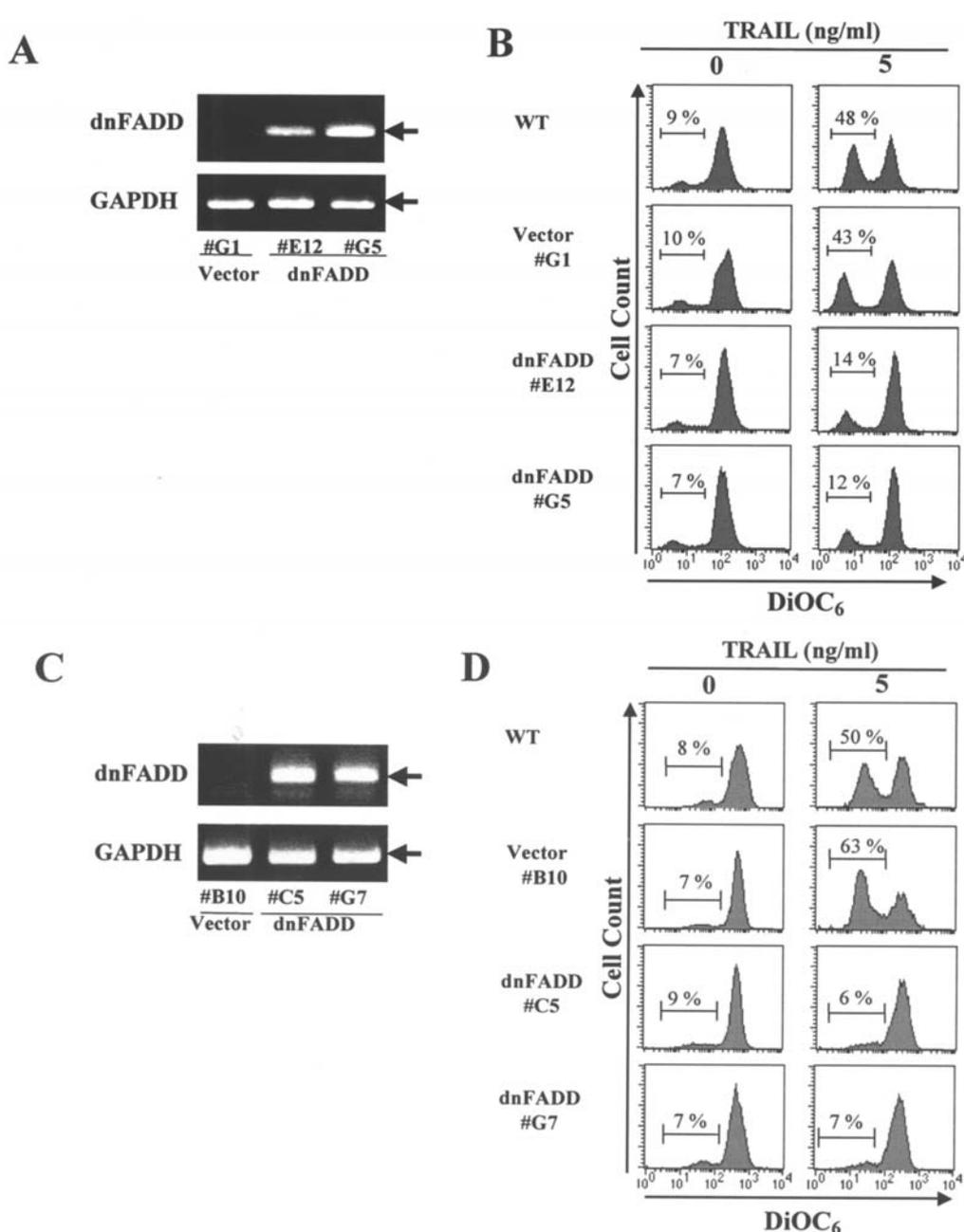


Figure 2. Establishment of Daudi and U266 cell lines overexpressing dnFADD. Daudi and U266 cells were transfected with dnFADD construct or control vector alone and cultured with a medium containing G418 to obtain transformants. The clones derived from Daudi (A,B) and U266 (C,D) were confirmed to express dnFADD by RT-PCR (A,C) and assessed by mitochondrial membrane potential after TRAIL stimulation (B,D). The experiments were done three times, obtaining essentially similar results.

reverse, 5'-TCGTTGTCATACCAGGAAATG-3'). PCR products were resolved on 0.8 % agarose gels and visualized with ethidium bromide.

Subcellular fractionation. Cells were subfractionated by using mitochondrial/cytosol fractionation kit (BioVision). Briefly, cells were lysed in cytosol extraction buffer containing 1 mM DTT and protease inhibitors and incubated for 10 min on ice. After homogenization, samples were centrifuged at a low speed to remove nuclei. The resultant supernatant was centrifuged at 10,000 g for 30 min at 4°C to obtain the mitochondrial fraction (pellet) and this supernatant was used as cytosolic fraction.

Western blot analysis. Western blot analysis was done, as previously described (11). Briefly, cells were solubilized in lysis buffer (10 mM Tris-HCl, pH 7.4, 1% Triton X100, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin). Samples were resolved on 12.5% SDS-PAGE, and transferred to membranes. Blots were incubated with primary Abs: anti-Bax-α, anti-Bcl-xL and anti-XIAP). After several washes, the blots were developed using a horseradish peroxidase (HRP)-labeled second Ab and enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham Biosciences, Buckinghamshire, UK). The intensity of each band was scanned and calculated using NIH image.

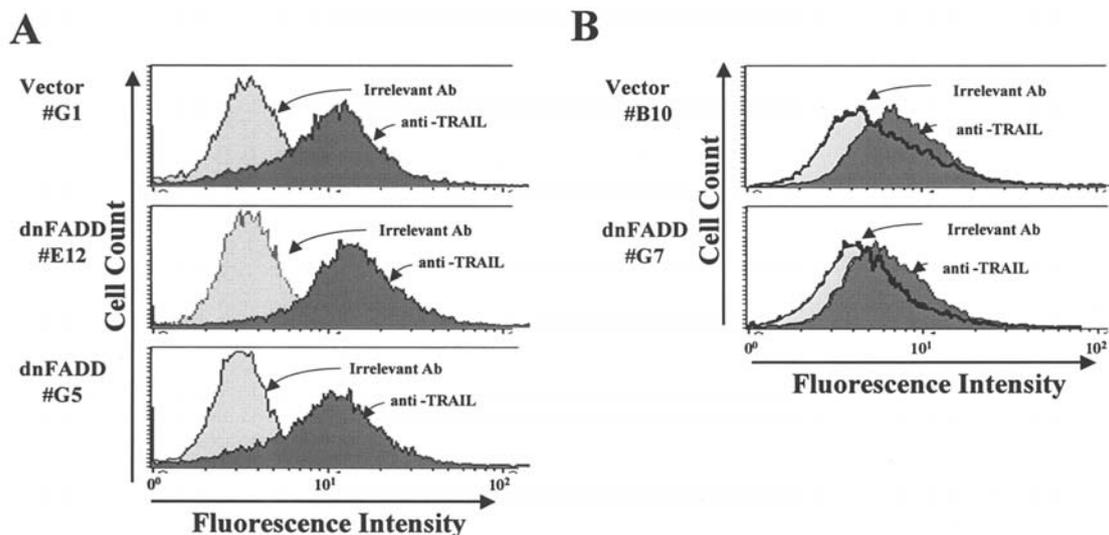


Figure 3. IFN- α induces upregulation of TRAIL in the dnFADD cell lines. The clones from Daudi (A) and U266 (B) were cultured with IFN- α for 48 h and assayed for expression of TRAIL by flow cytometry. Experiments were done three times, with essentially similar results.

Statistical analysis. Data were expressed as the means \pm SD for each group. Statistical significance was determined by Student's t-test and a difference of $p < 0.05$ was considered significant.

Results

IFN- α -induced loss of mitochondrial membrane potential does not correlate with the IFN- α -mediated upregulation of TRAIL in several B cell lines. Two lymphoma cell lines (Daudi and Ramos) and two myeloma cell lines (U266 and RPMI 8226) were cultured with IFN- α (1k and 10k U/ml) or medium alone for 48 h and assayed for $\Delta\Psi_m$ and membrane TRAIL expression. All 4 cell lines underwent substantial levels of apoptosis following stimulation with IFN- α (Fig. 1A). Although the substantial upregulation of TRAIL was achieved in the IFN- α -stimulated U266 and Daudi cells (Fig. 1B) (11), it was almost undetectable in the IFN- α -stimulated Ramos and RPMI 8226 cells. These results suggest that the IFN- α -induced loss of $\Delta\Psi_m$ does not always correlate with IFN- α -mediated TRAIL upregulation.

Establishment of Daudi and U266 cells overexpressing dnFADD. To analyze the participation of TRAIL in the IFN- α -induced loss of $\Delta\Psi_m$, cell lines overexpressing dnFADD, which could block TRAIL-induced apoptosis, were established (Fig. 2A and 2C). Daudi and U266 cells overexpressing dnFADD were confirmed to show reduced responses to TRAIL, compared with control cells expressing vector alone (Fig. 2B and 2D). The IFN- α -induced upregulation of TRAIL was found in the dnFADD-overexpressing cell lines to comparable levels as controls (Fig. 3A and 3B). These results indicate that dnFADD prevents TRAIL-induced loss of $\Delta\Psi_m$, but not upregulation of TRAIL.

IFN- α -induced loss of mitochondrial membrane potential in the early phase is considerably reduced in the dnFADD-

expressing cells, but not in the late phase. To analyze the role of FADD in the IFN- α -induced loss of $\Delta\Psi_m$, the dnFADD-overexpressing cell lines were stimulated with IFN- α for 48 h or 72 h. At 48 h after stimulation, the IFN- α -induced loss of $\Delta\Psi_m$ was substantially reduced in the dnFADD-expressing cells, compared with control cells (Fig. 4A). At 72 h after IFN- α stimulation, however, comparable levels of loss of $\Delta\Psi_m$ were obtained in the dnFADD-expressing and control cells (Fig. 4B). These observations suggest that the IFN- α -induced loss of $\Delta\Psi_m$ in the early phase depends on TRAIL, while in the late phase is independent of TRAIL.

IFN- α -induced apoptosis in the early phase is reduced in the dnFADD-expressing cells, but not in the late phase. To analyze the role of FADD in the IFN- α -induced apoptosis, the IFN- α -stimulated dnFADD-expressing or control cells were assayed for apoptosis using annexin V staining. The IFN- α -induced apoptosis was substantially abrogated in the dnFADD-expressing cells 48 h after stimulation with 250 and 1,000 U/ml (Fig. 5A), whereas almost similar levels of apoptosis were found at 72 h, compared with control cells (Fig. 5B). These results further suggest that TRAIL is involved in IFN- α -induced apoptotic events in the early phase, but not in the late phase.

IFN- α -induced loss of mitochondrial membrane potential in the early phase is abrogated by TRAIL-Rs:Fc. The interaction of ligands with their receptors is abrogated by the fusion protein of receptors and Fc portion of immunoglobulin. To examine whether TRAIL-R:Fc affects the IFN- α -induced loss of $\Delta\Psi_m$, cells were stimulated with IFN- α in the presence or absence of TRAIL-R1:Fc plus TRAIL-R2:Fc, followed by assay for $\Delta\Psi_m$. The addition of TRAIL-Rs:Fc abrogated the IFN- α -induced early phase of loss of $\Delta\Psi_m$ (Fig. 6), observed 48 h after stimulation. These results further indicate that the IFN- α -induced early phase of apoptotic processes is dependent on TRAIL.

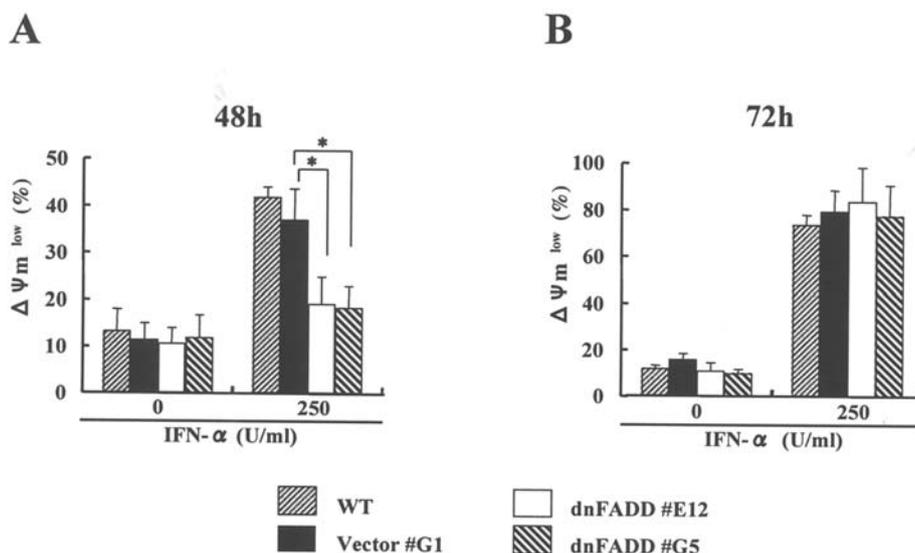


Figure 4. IFN- α -induced early phase, but not late phase, loss of mitochondrial membrane potential is substantially abrogated in the dnFADD-expressing Daudi cells. The dnFADD and control cells were cultured with 250 U/ml IFN- α for 48 h (A) or 72 h (B), followed by assay for mitochondrial membrane potential. Experiments were done three times, obtaining essentially similar results. *Significantly different from dnFADD-expressing cells.

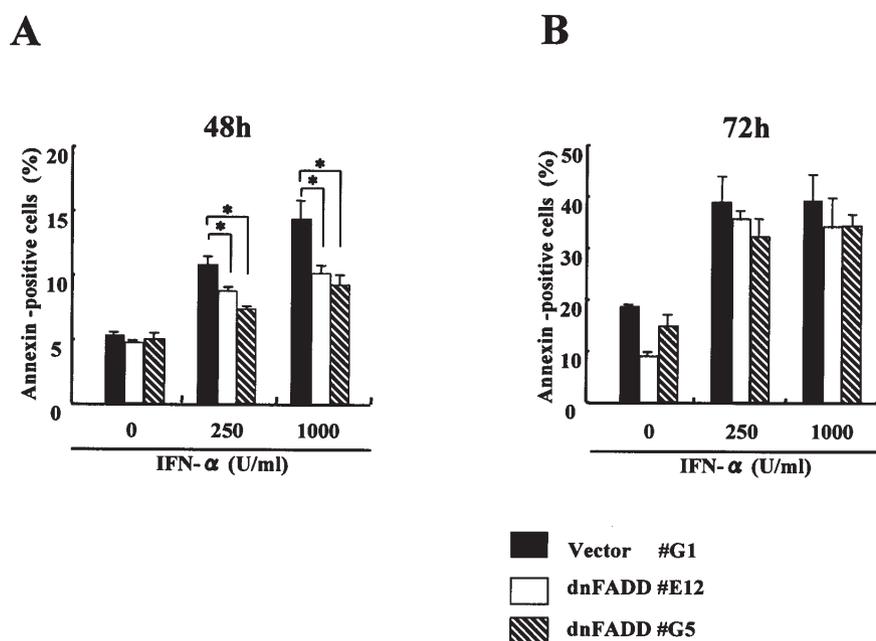


Figure 5. IFN- α -induced early, but not late phase of apoptosis is substantially abrogated in the dnFADD-expressing Daudi cells. The dnFADD and control cells were cultured with IFN- α for 48 h (A) or 72 h (B), followed by assay for annexin V staining. Experiments were done three times, obtaining essentially similar results. *Significantly different from dnFADD-expressing cells.

Truncated form of Bax- α is generated in the dnFADD-expressing cells only in the late phase of apoptotic events. We have previously demonstrated that IFN- α induces generation of truncated form of p21 Bax- α , p18 Bax- α , which is mainly located in the mitochondrial fraction (21). At 24 h after stimulation with IFN- α , p18 Bax- α was produced in the mitochondrial fraction from control Daudi cells, whereas it was found only after 72 h in the mitochondrial fraction of the dnFADD-expressing cells (Fig. 7). The fidelity of mitochondrial and cytosolic fractions was confirmed by the expression of Bcl-xL and XIAP, respectively (Fig. 7).

Together, the TRAIL-dependent apoptotic events induced by IFN- α occur earlier than the TRAIL-independent processes.

Discussion

IFN- α has been employed for the treatment of cancers such as myeloma, hairy cell leukemia and chronic myelogenous leukemia (2-4). However, the IFN- α -mediated therapy is not satisfactory and we need to know more about the molecular mechanisms underlying IFN- α -induced growth inhibition, which remain largely unresolved (1). We and others have

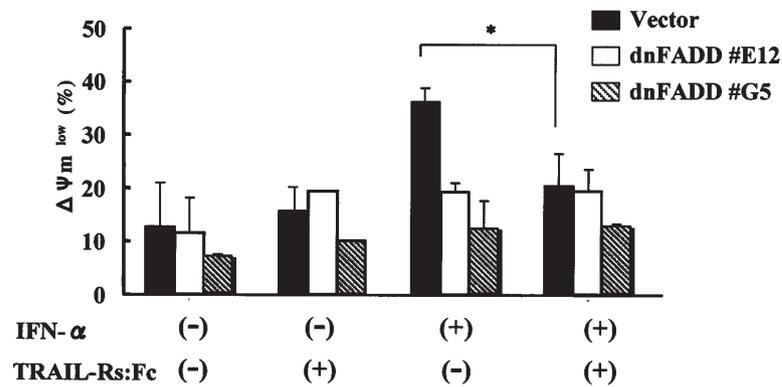


Figure 6. TRAIL-Rs:Fc fusion protein abrogates early phase of loss of mitochondrial membrane potential following stimulation with IFN- α . The early phase of mitochondrial depolarization is substantially blocked by TRAIL-Rs:Fc. Experiments were done twice, obtaining essentially similar results. *Significantly different from group containing TRAIL-R1/R2:Fc.

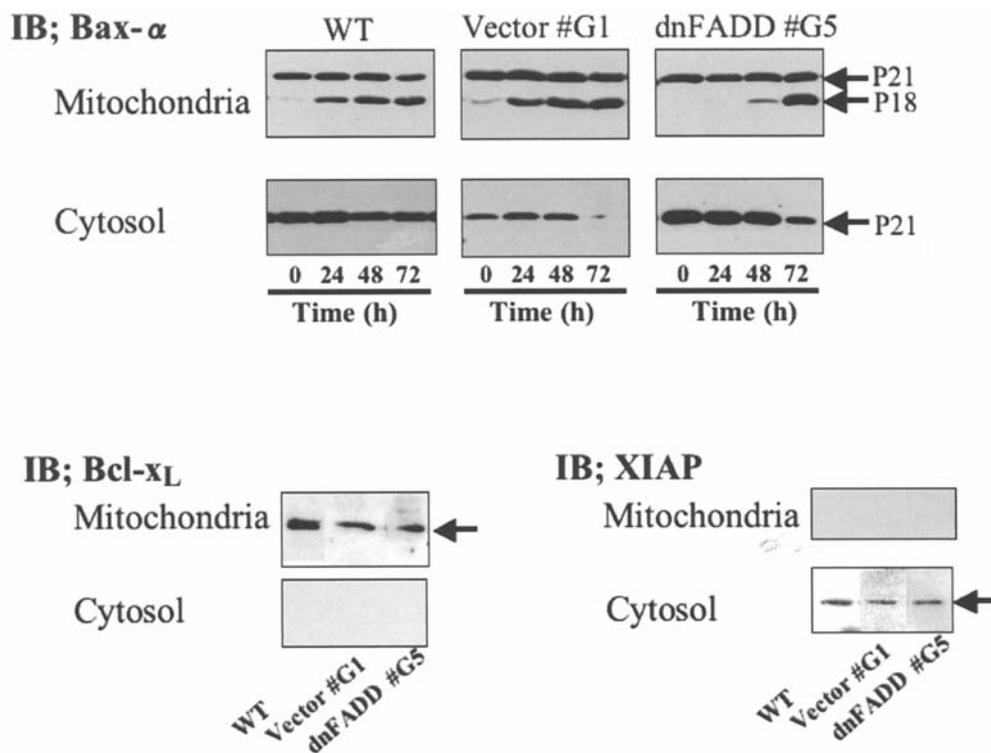


Figure 7. IFN- α generates truncated form of Bax- α in the dnFADD-expressing cells, only in the late phase. The dnFADD-expressing, control, or WT Daudi cells were cultured with 250 U/ml IFN- α at various times and cell lysates were separated into cytosolic and mitochondrial fractions, followed by assay for expression of Bax- α , Bcl-xL, and XIAP, respectively. IB, immunoblot. The experiments were done three times, obtaining essentially similar results.

recently demonstrated that IFN- α induces apoptotic cell death (5-7), at least through upregulation of TRAIL (9-12). The interaction of TRAIL with TRAIL-Rs results in apoptosis, at least through the FADD-mediated caspase-8 activation pathway (14). In the present study, we examined the role of TRAIL in the IFN- α -induced apoptotic events by using dnFADD-expressing cells.

The IFN- α -induced loss of $\Delta\Psi_m$ did not always correlate with upregulation of TRAIL (Fig. 1A and 1B). Gomez-Benito *et al* (29) have reported that no correlation was found between apoptosis and TRAIL upregulation in myeloma cell lines after IFN- α stimulation. The early phase of loss of $\Delta\Psi_m$

in Daudi cells, detected 48 h after stimulation, was substantially blocked by dnFADD or TRAIL-Rs:Fc, whereas the late phase of $\Delta\Psi_m$ was unaffected by dnFADD. These results suggest that the IFN- α -induced apoptosis occurs in a TRAIL-dependent as well as a TRAIL-independent manner.

TRAIL, a member of the TNF family of proteins, is upregulated through JNK activation following stimulation with IFN- α (9,10,12). TRAIL, containing death domains, initiates caspase-8 activation through the formation of complexes comprising of TRAIL-Rs, FADD and pro-caspase-8. The IFN- α -induced caspase-8 activation was amplified through mitochondria, where the truncated form of Bax- α , p18 Bax- α ,



ated 24 h after stimulation (Fig. 7) (7,21). This early phase of the p18 Bax- α was absent in the dnFADD-expressing cells upon stimulation with IFN- α . Moreover, the IFN- α -induced early phase of apoptosis was prevented by TRAIL-Rs:Fc. These results further indicate that death receptor-mediated caspase-8 activation by IFN- α is amplified in the mitochondria in the early phase of the IFN- α -induced apoptotic events.

In addition to the death receptor-mediated apoptotic pathway, some stimuli induce mitochondrial dysfunction, probably determined by the balance between proapoptotic (Bax- α , Bak) and anti-apoptotic (Bcl-2, Bcl-xL) molecules (16,18,30). The TRAIL-independent mitochondrial apoptotic pathway induced by IFN- α was activated 72 h after stimulation in the dnFADD-expressing cells, where p18 Bax- α was produced. Although molecular mechanisms underlying IFN- α -induced modulation of Bcl-2 family proteins remain unresolved in the present study, it is possible that IFN- α -induced sustained activation of JNK somehow affects mitochondrial function involving Bax- α protein, as was indeed demonstrated in some cells including the murine B lymphoma cell line WEHI-231 (31,32).

In the present study, we showed that IFN- α induces apoptotic cell death in both a TRAIL-dependent and -independent manner. In both cases, the IFN- α -induced apoptosis was accompanied by generation of p18 Bax- α , more potent in induction of apoptosis than wild-type Bax- α (33,34). Thus, it would be interesting to apply a combined treatment of IFN- α and p18 Bax- α in the eradication of some tumors. Whatever the mechanism may be, our findings have implications for the IFN- α treatment modalities of patients with tumors.

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