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Figure Legends

Fig. 1 Expression of DGK□ in human melanoma cells and melanocytes. (A) 70W, AKI, G361, MMAc, SK-mel-23 and SK-mel-118 human melanoma cells were harvested and the cell lysates (15 □g of protein) were analyzed by Western blotting using anti-DGK□ antibody. Lysis buffer alone (–) was used as a control. Size marker is indicated on the left in kDa. (B) AKI human melanoma cells and NHEM were harvested and the cell lysates (15 □g of protein) were analyzed by Western blotting using anti-DGK□ antibody (upper panel) or anti-actin antibody (lower panel). Size markers are indicated on the left in kDa. (C) Total RNAs of AKI human melanoma cells and NHEM were isolated and the aliquots (500 ng) were subjected to RT-PCR. Upper and lower panels in each set show the amplified 282- and 983-bp cDNA fragments of DGK□ (30 cycles) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 25cycles), respectively, in agarose gel electrophoresis.

Fig. 2 Effects of overexpression of DGKs □, □ and □ on apoptosis in AKI melanoma cells. AKI melanoma cells were transfected with expression plasmids encoding GFP alone, GFP-DGK□-WT, GFP-DGK□-KD, GFP-DGK□-WT or GFP-DGK□-WT as indicated. After 24 h, cells were further incubated with or without 50 ng/ml of TNF-□ for 24 h. (A and B) The cells were fixed with 3.7 % formaldehyde and permeabilized with 0.1 % Triton X-100. Cell apoptosis was detected by TUNEL assay. Cells were examined using an inverted confocal laser scanning microscope (Zeiss LSM 510). A representative of three repeated experiments is shown (A). Bar = 100 □m. (B) Quantification of apoptosis of AKI cells. Random fields (ten fields per experiment) including (A) were analyzed, and TUNEL-positive cells were counted. For each experiment, >1000 cells were counted. The results are presented as the percentage of the TUNEL-positive cell and represent the means ± S. D. of the values obtained in three separate experiments. Statistical significance was determined using the Student's *t* test. (C) After incubation with TNF-□, the cells were harvested, and cell lysates (10 □g of protein) were analyzed by Western blotting with anti-GFP antibody. Size markers are indicated on the left in kDa.

Fig. 3 Effects of DGK□-knockdown on apoptosis in AKI melanoma cells. AKI melanoma cells were transfected with or without siRNA targeting either DGK□ or GFP (control) as indicated. After 24 h, cells were further incubated with or without 50 ng/ml of TNF-□. (A) After incubation with TNF-□ for 24 h, the cells were harvested, and cell lysates (10 □g of protein) were analyzed by Western blotting with anti-DGK□ antibody (upper panel) or anti-actin antibody (lower panel). The relative intensity of each band is shown below upper panel. A representative of three repeated experiments is shown. (B, C) After incubation with TNF-□ for 24 h, the cells were fixed with 3.7 % formaldehyde and permeabilized with 0.1 % Triton X-100. Cell apoptosis was detected by TUNEL assay. Cells were examined using an inverted confocal laser scanning microscope (Zeiss LSM 510). A representative of three repeated experiments is shown (B). Bar = 100 □m. (C) Quantification of apoptosis of AKI cells. Random fields (ten fields per experiment) including (B) were analyzed, and TUNEL-positive cells were counted. For each experiment, >1000 cells were counted. The results are presented as the percentage of the TUNEL-positive cell and represent the means ± S. D. of the values obtained in three separate experiments. Statistical significance was determined using the Student's *t* test. (D)

After incubation with TNF-□ for 48 h, the cells were treated with RNase A, stained with propidium iodide (PI) and analyzed by FACS. Apoptotic cells fall in the sub-G1 gate. A representative of three repeated experiments is shown.

Fig. 4. Effects of DGK□-knockdown on apoptosis in SK-mel-118 melanoma cells. SK-mel-118 melanoma cells were transfected with siRNA targeting either DGK□ or GFP (control) as indicated. After 24 h, cells were further incubated with or without 50 ng/ml of TNF-□ for 24 h. (A) The cells were harvested, and cell lysates (10 □g of protein) were analyzed by Western blotting with anti-DGK□ antibody (upper panel) or anti-actin antibody (lower panel). The relative intensity of each band is shown below upper panel. A representative of three repeated experiments is shown. (B and C) After incubation with TNF-□, the cells were fixed with 3.7 % formaldehyde and permeabilized with 0.1 % Triton X-100. Cell apoptosis was detected by TUNEL assay. Cells were examined using an inverted confocal laser scanning microscope (Zeiss LSM 510). A representative of three repeated experiments is shown (B). Bar = 100 □m. (C) Quantification of apoptosis of SK-mel-118 cells. Random fields (ten fields per experiment) including (B) were analyzed, and TUNEL-positive cells were counted. For each experiment, >500 cells were counted. The results are presented as the percentage of the TUNEL-positive cell and represent the means ± S. D. of the values obtained in three separate experiments. Statistical significance was determined using the Student's *t* test.

Fig. 5. Effects of overexpression and knockdown of DGK□ on Akt, ERK and NF-□B activities in AKI melanoma cells. (A and B) AKI melanoma cells were transfected with siRNA targeting either DGK□ or GFP (control) as indicated. After 24 h, cells were further incubated with or without 50 ng/ml of TNF-□. After incubation with TNF-□ for 24 h, the cells were harvested, and cell lysates (10 □g of protein) were analyzed by Western blotting with anti-phospho-Akt antibody (p-Akt), anti-Akt antibody (Akt), anti-phospho-ERK antibody (p-ERK) or anti-ERK antibody (ERK) as indicated. (C) AKI melanoma cells were co-transfected with pNF-□B-Luc Vector, pSV-□-Galactosidase Control Vector, and with either pEGFP, pEGFP-DGK□-WT or pEGFP-DGK□-KD as indicated. After 24 h, cells were further incubated with or without 50 ng/ml of TNF-□ for 12 h. Luciferase activity was measured and was normalized to □-galactosidase activity. The results are presented as the percentage of the value of GFP alone (TNF-□ (–)) and represent the means ± S. D. of the values obtained in three separate experiments. Statistical significance was determined using the Student's *t* test. (D) AKI melanoma cells were co-transfected with pNF-□B-Luc Vector, pSV-□-Galactosidase Control Vector, and with siRNA targeting either DGK□ or GFP (control) as indicated. After 24 h, cells were further incubated with or without 50 ng/ml of TNF-□ for 12 h. Luciferase activity was measured and was normalized to □-galactosidase activity. The results are presented as the percentage of the value of control siRNA (TNF-□ (–)) and represent the means ± S. D. of the values obtained in three separate experiments. Statistical significance was determined using the Student's *t* test.

Fig. 6. Effects of overexpression and knockdown of DGK□ on nuclear localization of NF-□B in AKI melanoma cells. (A and B) AKI melanoma cells were transfected with expression plasmids encoding GFP alone, GFP-DGK□-WT or GFP-DGK□-KD as indicated. After 24 h, cells were further incubated with or without 50 ng/ml of

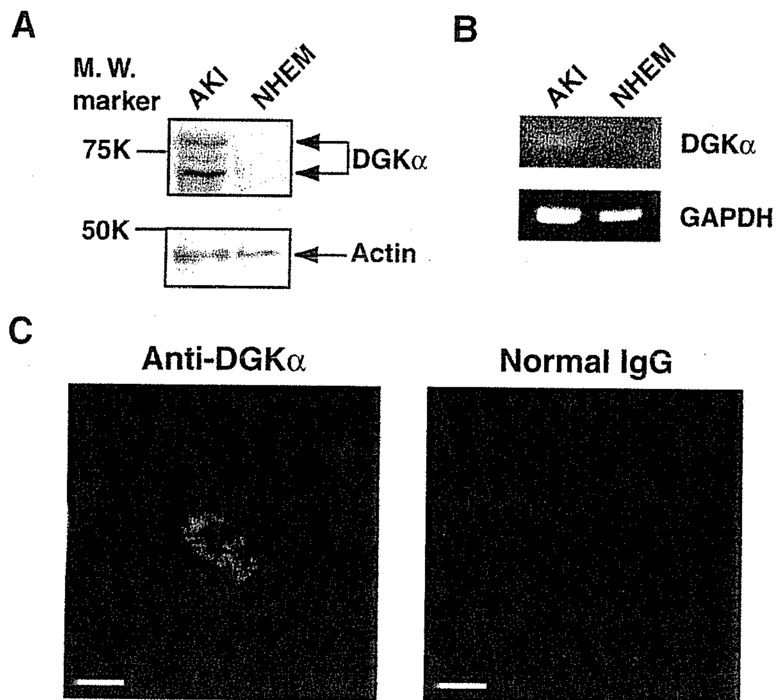
TNF-□ for 12 h. A, The cells were fixed with 3.7 % formaldehyde, permeabilized with 0.1 % Triton X-100. NF-□B was labeled with anti-NF-□B p65 antibody and Alexa Fluor 594-conjugated secondary antibody. Cells were examined using an inverted confocal laser scanning microscope (Zeiss LSM 510). A representative of three repeated experiments is shown (A). Bar = 10 □m. (B) The nucleus/cytoplasm (N/C) ratio of NF-□B (fluorescence intensity) was quantified using Image J software. For each experiment, >20 cells were analyzed. The results represent the means ± S. D. of the values obtained in three separate experiments. Statistical significance was determined using the Student's *t* test. (C and D) AKI melanoma cells were transfected with siRNA targeting DGK□ or GFP (control) as indicated. After 24 h, cells were further incubated with or without 50 ng/ml of TNF-□ for 12 h. The cells were fixed with 3.7 % formaldehyde, permeabilized with 0.1% Triton X-100. NF-□B was labeled with anti-NF-□B p65 antibody and Alexa Fluor 594-conjugated secondary antibody. Cells were examined using an inverted confocal laser scanning microscope (Zeiss LSM 510). A representative of three repeated experiments is shown (C). Bar = 10 □m. (D) The N/C ratio of NF-□B (fluorescence intensity) was quantified using Image J software. For each experiment, >20 cells were analyzed. The results represent the means ± S. D. of the values obtained in three separate experiments. Statistical significance was determined using the Student's *t* test. (E) AKI melanoma cells were transfected with siRNA targeting DGK□ or GFP (control) as indicated. After 24 h, cells were further incubated with 50 ng/ml of TNF-□ for 0.5, 2 and 12 h as indicated. Cytosol (*C*), membrane/organelle (*M*), nuclear (*N*) and cytoskeletal (*CS*) fractions were isolated using the ProteoExtract Subcellular Proteome Extraction Kit. Each fraction (10 □l) was analyzed by Western blotting with anti-NF-□B p65 antibody. The blots are quantified and relative intensities of the bands are shown below each panel. A representative result of two repeated experiments is shown.

Fig. 7. Effects of NF-□B inhibitor on apoptosis and NF-□B activity enhanced by DGK□ overexpression in AKI melanoma cells. (A) AKI melanoma cells were co-transfected with pNF-□B-Luc Vector, pSV-□-Galactosidase Control Vector, and either pEGFP or pEGFP-DGK□-WT as indicated. After 23 h, cells were further incubated with or without 5 □M of MG-132 for 1 h. Then, cells were incubated with 50 ng/ml of TNF-□ for 12 h. Luciferase activity was measured and was normalized to □-galactosidase activity. The results are presented as the percentage of the value of GFP alone (MG-132 (–)) and represent the means ± S. D. of the values obtained in three separate experiments. (B and C) AKI melanoma cells were transfected with expression plasmids encoding GFP alone or GFP-DGK□-WT as indicated. After 23 h, cells were further incubated with or without 5 □M of MG-132 for 1 h. Then, cells were incubated with 50 ng/ml of TNF-□ for 24 h. The cells were fixed with 3.7 % formaldehyde and permeabilized with 0.1 % Triton X-100. Cell apoptosis was detected by TUNEL assay. Cells were examined using an inverted confocal laser scanning microscope (Zeiss LSM 510). A representative of three repeated experiments is shown (B). Bar = 100 □m. (C) Random fields (ten fields per experiment) including (B) were analyzed, and TUNEL-positive cells were counted. For each experiment, >500 cells were counted. The results are presented as the percentage of the TUNEL-positive cell and represent the means ± S. D. of the values obtained in three separate experiments.

Table 1
Expression of Type I DGKs in human melanoma cells

Melanoma cell line	WB			RT-PCR		
	□	□	□	□	□	□
70W	+	N.D.	N.D.	+	N.D.	N.D.
AKI	+	N.D.	N.D.	+	N.D.	N.D.
G361	+	N.D.	N.D.	+	N.D.	N.D.
MMAc	+	N.D.	N.D.	+	N.D.	N.D.
SK-mel-23	+	N.D.	N.D.	+	N.D.	N.D.
SK-mel-118	+	N.D.	N.D.	+	N.D.	N.D.

70W, AKI, G361, MMAc, SK-mel-23 and SK-mel-118 human melanoma cells were harvested, and the cell lysates (15 □g of protein) were analyzed by Western blotting using anti-DGKs antibodies. Moreover, total RNAs were isolated and the aliquots (500 ng) were subjected to RT-PCR. +: specific bands were detected; N.D.: not detected.



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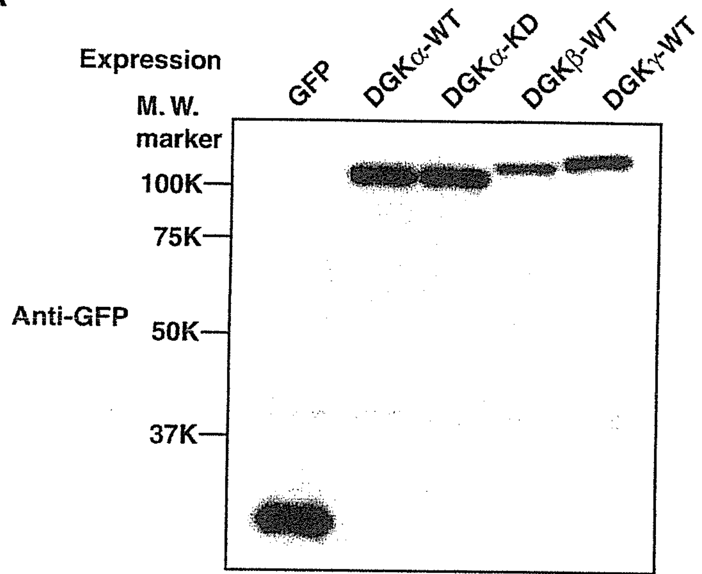

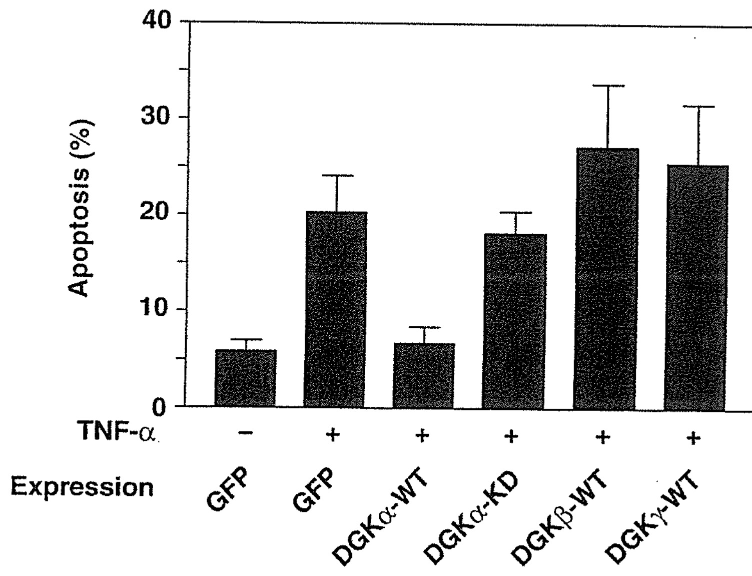

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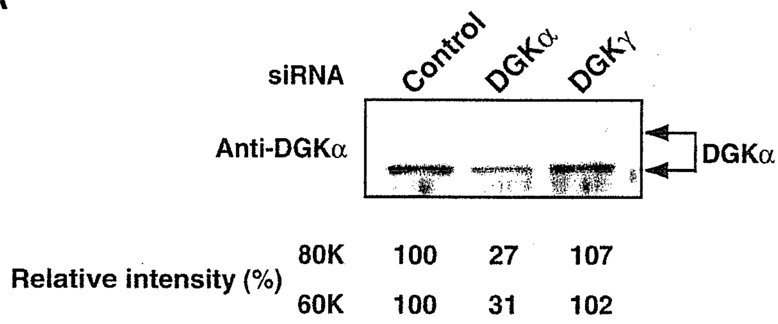

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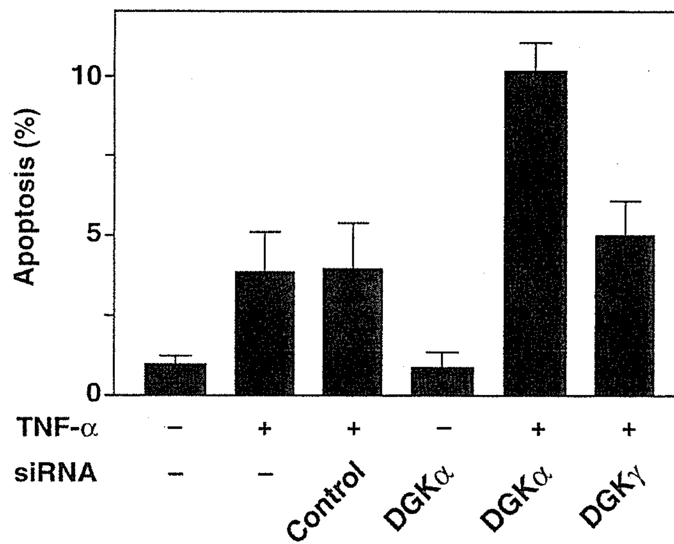

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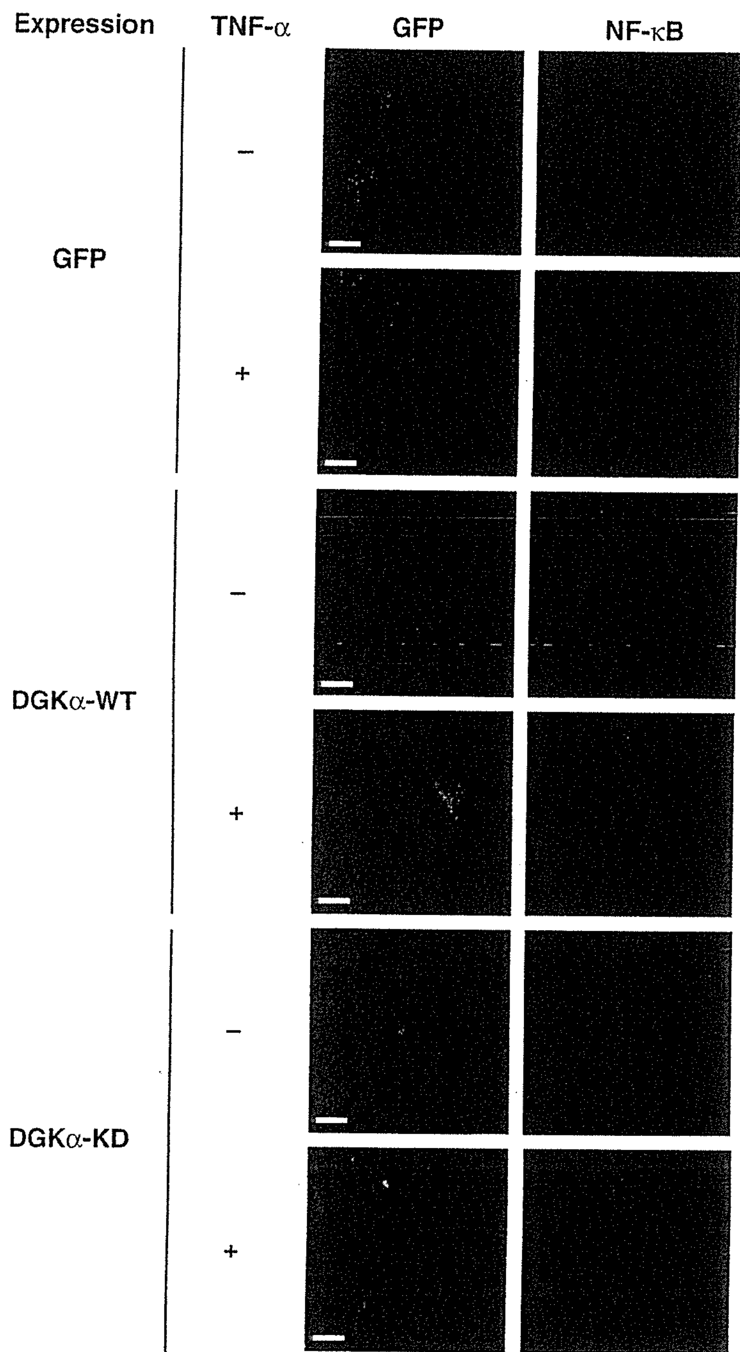

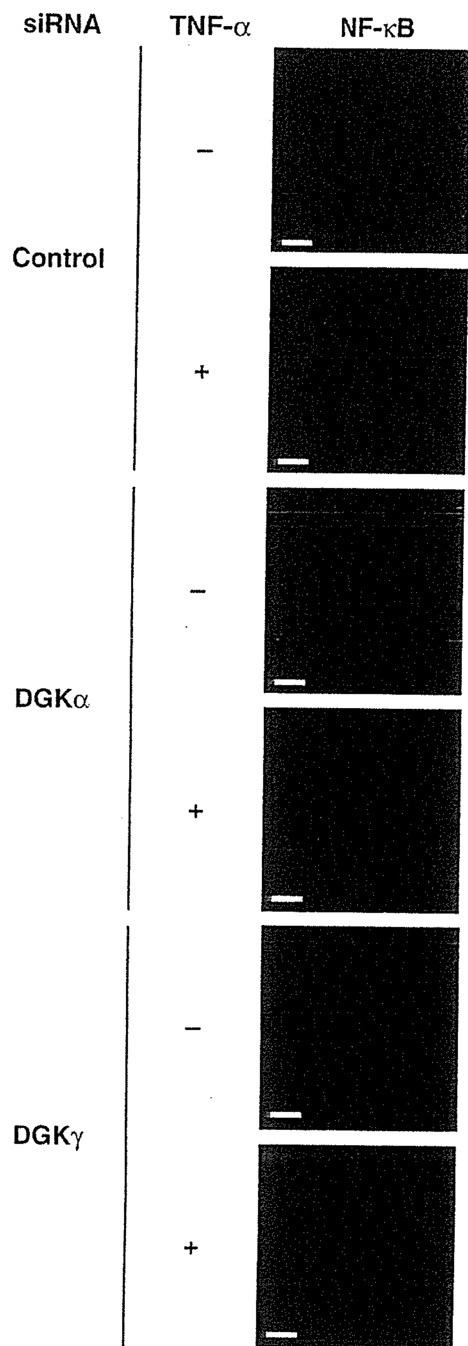

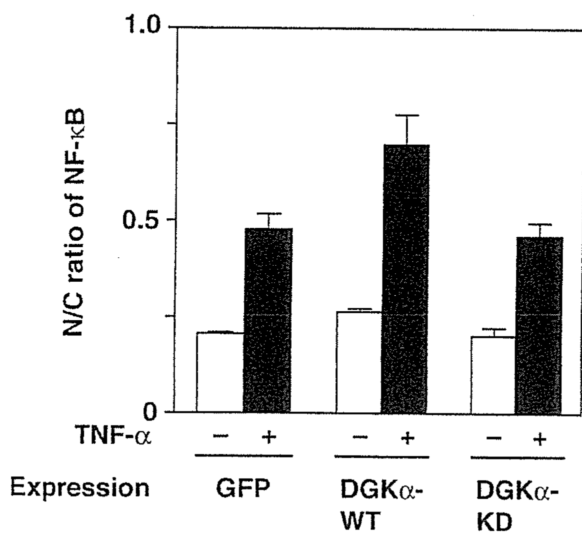

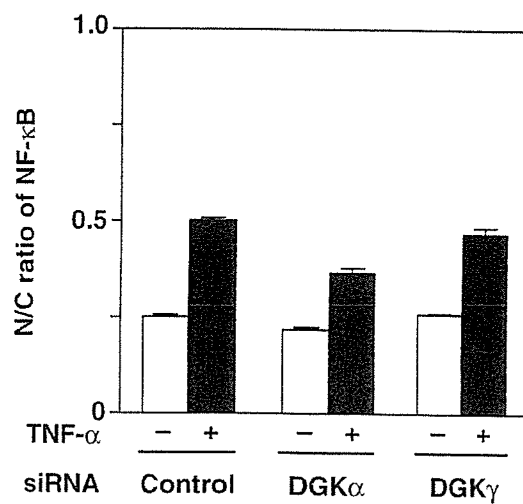

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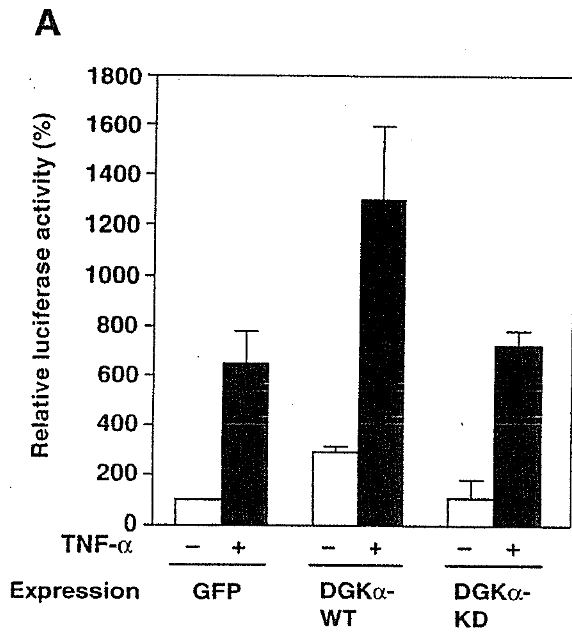

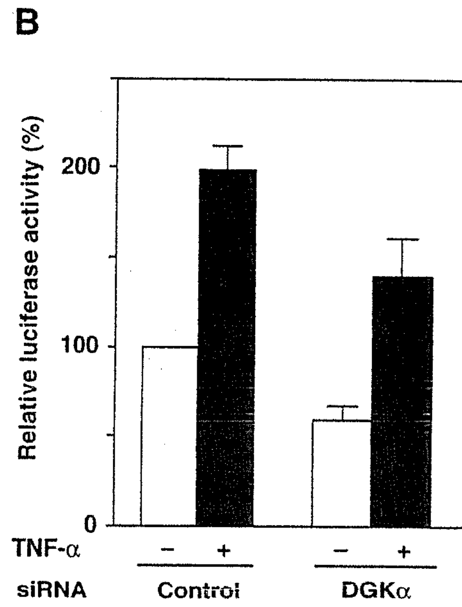

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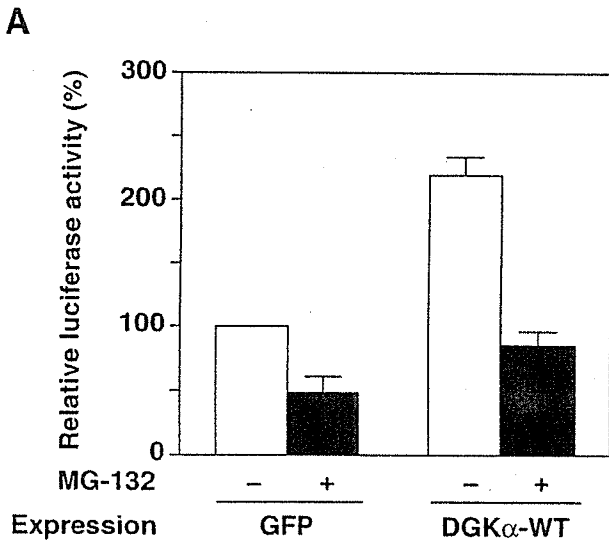

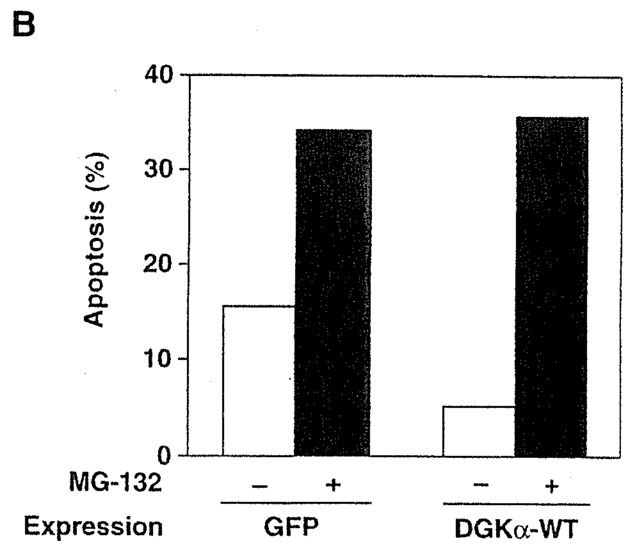

図６　柳澤

The increased caspase-2 activity can be a new novel marker associated with IFN-β induced apoptosis in malignant melanoma

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SHORT TITLE

Caspase-2 is essential for IFN-β induced apoptosis in melanoma

Abbreviations: IFN, interferon; TRAIL, tumor necrosis factor related apoptosis-inducing ligand; TNF α, tumor necrosis factor alpha

ABSTRACT

The management of malignant melanoma is a difficult challenge. Among various therapeutic approaches, interferon (IFN) is believed to be one of the most effective anti-melanoma agents. Specifically IFN-β has the ability to induce apoptosis in melanoma cells. In order to characterize the signaling pathway involved in the IFN-β-mediated apoptosis, we analyzed biological effect of IFN-β on the cell growth and death of melanoma cells and activation of caspases. Among four human melanoma cell lines tested, MM418, SK-mel-23 and SK-mel-118 showed growth inhibition by IFN-β, but G361 was not affected by it. TUNEL assay detected the increased apoptotic cell population in IFN-sensitive cell lines. Caspase fluorometric assay also showed the up-regulation of caspase-3 activity in IFN-sensitive cell lines. Furthermore, caspase-2 activity, in addition to caspase-3, was up-regulated in the IFN-β-sensitive cell lines, but not in G361. The involvement of caspase-3, 8 and 9 is well known in the process of apoptosis, however, the role of caspase-2 has not yet been elucidated. Our results suggest that activation of caspase-2 is commonly associated with the IFN-β-mediated apoptosis of melanoma cells.

INTRODUCTION

The management of malignant melanoma has been a difficult challenge for physicians and scientists. Among various therapeutic approaches interferon (IFN) is believed to be one of effective anti-melanoma agents.

Although the mechanisms of IFN-mediated cell death have not been fully elucidated, IFN is known as an effective anti-tumor agent (Fisher *et al*, 1985; Stark *et al*, 1998; Pfeffer *et al*, 1998). IFN induces many biological responses by

regulating IFN-stimulated genes (ISGs) (Chawla-Sarkar *et al*, 2003). Several ISGs, such as double-stranded RNA-activated protein kinase (PKR), myxovirus resistance protein A (MxA), melanoma differentiation associated gene-5 (mda-5) and tumor necrosis factor related apoptosis-inducing ligand (TRAIL) are related anti-tumor effects (Leaman *et al*, 2003; Kang *et al*, 2004).

Recently, it has been unraveled that IFN has apoptotic effect in several tumors. It was reported that IFN-β induces apoptosis in the melanoma cells more significantly than IFN-α and γ do (Chawla-Sarkar *et al*, 2001; Leaman *et al*, 2003). TRAIL known as Apo2 ligand, is also a member of the TNF family of transmembrane protein, which leads tumor cells to apoptosis by stimulating death receptors (Griffith *et al*, 1998; Kimberley and Screaton, 2004; Zhang and Fang, 2005). It was suggested that the involvement of TRAIL is essential for the apoptotic cascade induced by IFNs in certain melanoma cell lines, as well as other tumors cell lines (Chawla-Sarkar *et al*, 2001; Chen *et al*, 2001; Morrison *et al*, 2005).

Apoptosis is divided into two signal pathways. One is through apoptosis inducing ligands such as Fas ligand, TRAIL and TNF-α stimulating death receptors. The other is through cytochrome c release from mitochondria. In these pathways it is evident that functions of caspase-3, 8, and 9 are essential. On the other hand, caspase-2, which is considered to be an initiator caspase and involved in apoptotic pathways, is located diversely in the cytoplasm and nuclei. However, the biological role of caspase-2 has yet been controversial compared with that of other caspases (Zhivotovsky *et al*, 1999; van Loo *et al*, 2002; Zhivotovsky and Orrenius, 2005). The correlation between TRAIL and caspase-2 has been indicated recently in TRAIL mediated apoptotic pathways, in which caspase-2 processes procaspase-8 or cleaves Bid, a proapoptotic Bcl-2 family member (Wagner *et al*, 2004; Shin *et al*, 2005). Although the interaction among apoptosis-inducing ligands, IFNs and caspase-2 has recently been clarified to some extent, the role of caspase-2 for IFN-induced apoptosis still remains to be elucidated.

In this study we are interested in clarifying the biological role of caspases, especially caspase-2 in IFN-β induced apoptotic pathway, and examined two classes of human melanoma cell lines that are either sensitive or resistant to apoptosis by IFN-β treatment.

RESULTS

IFN-β inhibited growth and viability of melanoma cell lines (SK-mel-118, SK-mel-23 and MM418 but not G361).

To assess the inhibitory effect of IFN on the cell growth, each cell line treated with IFN-α2 or IFN-β and the cell number was measured by gentian violet dye-binding assay (Fig 1). IFN-α2 or IFN-β inhibited the growth of SK-mel-118, SK-mel-23, and MM418 cell lines in all cell lines. The growth-inhibition effects of IFN-β were significantly higher than those of IFN-α2. However, the cell growth of G361 was inhibited by neither IFN-α2 nor IFN-β. Furthermore, to assess the viability of these IFN-treated cell lines, MTT cell viability assay was performed (Fig 2). Cell viability of SK-mel-118, SK-mel-23 and MM418 were reduced with IFNs treatment. The reduction of cell viability by IFN-β was significantly higher than those by IFN-α2. The cell viability of G361 was inhibited by neither IFN-α2 nor IFN-β. These results indicated that these four melanoma cell lines were

divided into two groups, one is the group which is inhibited their growth and viability by IFNs, the other is not inhibited.

Induction of ISGs after treatment of IFNs

To assess whether tested melanoma cell lines can transduce intracellular signaling by IFN treatment, we investigated mRNA expression of ISGs, such as PKR, MxA, mda-5 and TRAIL in tested cell lines (Fig 3). The mRNA expressions of PKR, MxA and mda-5 were upregulated by IFN treatment all the cell lines including G361, which was resistance to anti-growth and anti-viability effects of IFN. These results indicated that IFN signaling pathway was not suppressed in all tested cell lines. TRAIL mRNA induction of SK-mel-118 and SK-mel-23 were markedly higher than those of MM418 and G361 by IFN-treatment. Impairment of TRAIL upregulation by IFNs in MM418 and G361 may cause in a TRAIL-specific manner.

Induction of apoptosis by IFN-β treatment in melanoma cells

We investigated the mechanism of cell growth inhibition and cell death induced by IFN-β. We analyzed the alteration of sub-G_0 population before and after the cell lines were treated with IFN-β. After treatment with IFN-β, FACS analysis showed IFN-β induced increase of sub-G_0 population in SK-mel-118, SK-mel-23 and MM418 except G361. The sub-G_0 populations of MM418 and SK-mel-23 treated by IFN-β were remarkably increased compared with untreated ones (Fig 4). IFN-β also reduced G1 phase population significantly in SK-mel-118, SK-mel-23 and MM418. Furthermore, we performed TUNEL assay in these four cell lines if these increase of sub-G_0 population occurred by apoptosis or other types of cell deaths when they were treated with IFN-β. This assay showed that BrdU-FITC-positive cells corresponding to apoptotic population, which cause DNA fragmentation, increased in the cell lines, which increased sub-G_0 populations by treatment with IFN-β. However, apoptotic cells, which are BrdU-positive, in G361 were not increased by treatment with IFN-β (Fig 5). SK-mel-23, SK-mel-118 and MM418 had a greater degree of apoptosis than G361. SK-mel-118 and SK-mel-23 expressed TRAIL-mRNA by IFN-β treatment (Fig 3). The apoptosis induction seems to relate with the TRAIL expression. However, MM418, which showed apoptosis induced by IFN treatment, did not express TRAIL mRNA even after IFN treatment (Fig 3).

Caspase-2 activity was significantly upregulated by IFN-β treatment in melanoma cell lines during apoptosis induction.

Activation of caspase cascade is critical signal for initiation of apoptosis. We performed caspase fluorometric assay to detect upregulation of caspase activity when melanoma cells were induced apoptosis by IFN treatment. Consistent with the result of TUNEL assay (Fig 5), caspase-3 activity treated with IFN-β for 48 h showed high-fold increase in SK-mel-118, SK-mel-23, and MM418, but not G361 (Fig 6). To characterize the related regulation of caspase cascade, we furthermore analyzed activation of caspase-2, 8, 9 and 10 after IFN-β treatment. The results of IFN-sensitive cell lines, SK-mel-118, SK-mel-23, and MM418, showed significantly upregulation of caspase-2 activities compared with other caspase activities after IFN-β treatment. These results agreed with the result of apoptosis induction, but

not TRAIL mRNA expression in case of MM418. IFN-resistant G361 cell had no significant upregulation of any caspase activity and TRAIL expression after treatment of IFN-β, even though the IFN signal transuduction pathway was not impaired in G361 cells.

G361 caused apoptosis by addition of exogenous TRAIL.
To elucidate the mechanism of IFN-resistance in G361, we performed that G361 were treated with apoptosis-inducing ligands. When G361 was treated by TRAIL, TNF-β or anti-Fas (CD95) antibody, only TRAIL significantly reduced its cell viability and induced cell death, although others did not (Fig 7a). Furthermore, cell viability was assessed after that G361 was treated with IFN-β, followed by apoptotic inducing ligands. Interestingly, IFN-α2 pretreatment followed by TRAIL stimulation significantly reduced the cell viability compared with TRAIL stimulation without treatment with IFN-β. IFN-β pretreatment followed by anti-Fas (CD95) antibody also reduced the cell viability slightly. We next analyzed the upregulation of caspase activity when treated with TRAIL, or with IFN-β, followed by TRAIL. The result treated with TRAIL showed remarkable upregulation of caspase-2 and -3 activity as IFN-sensitive cell lines showed, in which IFN-β induced apoptosis (Fig 7b). Furthermore, IFN-β pretreatment synergetically enhanced the upregulation of caspase-2 and -3, as similar to apoptosis induction.

DISCUSSION

In this study we found that the cell growth arrest and cell death were promoted by IFN-β and β treatment in human melanoma cell lines (SK-mel-118, SK-mel-23, and MM418), and that IFN-β preferentially induced these two cytotoxic effects than IFN-β did. We also found that this cell death induced by IFN-β was induced by apoptosis.

It has been reported that IFN-β treatment induces apoptosis which is dependent on TRAIL induction in melanoma cells. It was indicated that the cells, which failed to express TRAIL by IFN-β treatment, were resistant to IFN-induced apoptosis, whereas the cells, which induced TRAIL by IFN-β, were sensitive to IFN-induced apoptosis (Chawla-Sarkar *et al*, 2001). The induction of TRAIL by IFN-β has been shown to initiate the apoptotic cascade in wide variety of tumor cells (Shin *et al*, 2005; Vogler *et al*, 2007). It was shown in melanoma cells that TRAIL had more significant tumoricidal effect than other members of tumor necrosis factor family, such as TNFβ and Fas ligand did (Griffith *et al*, 1998). The induction of TRAIL by IFN-β was shown to be necessary, but still insufficient to induce apoptosis (Chawla-Sarkar *et al*, 2002). In our study two melanoma cell lines, SK-mel-118 and SK-mell-23, revealed such biological reaction in IFN-β induced apoptosis, and upregulated markedly TRAIL mRNA in response to IFN-β treatmentβ In contrast, G361 which was sensitive to IFN-β treatment but unable to induce apoptosis, did not show significant expression of TRAIL mRNA. These results strongly indicated the positive correlation between TRAIL expression and IFN-induced apoptosis in melanoma cells. In addition our study showed a new, unique finding in the study of MM418, which significantly underwent apoptosis by IFN-β treatment but did not reveal significant expression of TRAIL mRNA (Fig 3). This finding indicated that MM418 underwent apoptosis in an alternative pathway from

TRAIL-dependent apoptosis by IFN-β. Importantly, caspase-2 activation of MM418 by IFN-β treatment was much higher than that of SK-mel-118 and SK-mel-23, which underwent TRAIL-dependent apoptosis. Several recent studies demonstrated TRAIL-mediated apoptosis through caspase-2 pathway in other tumor cell lines (Wagner *et al*, 2004; Shin *et al*, 2005). They suggested that caspase-2 is required for upstream of Bid, which leads to caspase-9 activation, in mitochondrial apoptosis pathway (Wagner *et al*, 2004; Bonzon *et al*, 2006). Samraj *et al.* indicated that caspase-2 activation was absent in mutant Jurkat T-cell line with defect of caspase-9 when treated with anticancer drug releasing cytochrome c (Samraj *et al*, 2007). Shin *et al.* also indicated that caspase-2 processed procaspase-8 (Shin *et al*, 2005). Thus caspase-2 appears to have diverse functions in apoptotic processes. Our study may indicate the possible presence of alternative IFN-induced apoptosis pathway, which activates caspase-2 directly without induction of TRAIL. In a previous study, TRAIL was indicated to be a novel useful therapeutic modality for the management of melanomas (Ren *et al*, 2004). We, however, identified TRAIL-independent IFN-β-induced apoptosis in one cell line of MM418.

Another new finding in this study was G361, which showed cell growth arrest by IFN-β but did not show IFN-mediated apoptosis. However, IFN signal transduction seems to be normal in G361. Griffith *et al* previously reported that only TRAIL among apoptotic inducing ligands had significant cytotoxic effect (Griffith *et al*, 1998). In this study TRAIL mRNA was not induced by IFN-β in G361, although other ISGs were expressed significantly. Thus, this loss of TRAIL mRNA induction should be a meaningful biological process for the resistance to IFN-β-induced apoptosis. It has been also suggested that IFN-β pretreatment sensitized TRAIL-mediated apoptosis and XIAP, an inhibitor protein of apoptosis, was cleaved into an inactive form (Chawla-Sarkar *et al*, 2002). In this study, we showed that G361 caused apoptosis by addition of exogenous TRAIL protein (Fig 7a). We also showed that IFN-β pretreatment significantly increased sensitivity against TRAIL-mediated apoptosis in G361, although it didn't increase against TNFβ and anti-Fas antibody mediated stimulation. When G361 was treated with IFN-β followed by additional TRAIL treatment, caspase-2 activity was remarkably upregulated compared with that by TRAIL treatment alone. G361 did not cause apoptosis mediated by IFN-induced TRAIL, because G361 did not produce TRAIL in response to IFN. G361 revealed, however, apoptotic cell death by addition of exogenous TRAIL protein. Thus not only TRAIL but also other factors induced by IFN seem to be important for apoptosis processes, because synergetic activity of TRAIL and IFN-β treatment were observed in this study by treatment with TRAIL and IFN-β. Along with a previous report (Chawla-Sarkar *et al*, 2002), we found in this study that TRAIL/caspase-2 system was important for apoptosis induction in three among four melanoma cell lines tested. On the other hand, MM418 caused IFN-induced caspase-2 and -3 activations and apoptosis without TRAIL expression. Furthermore, neutralizing anti-TRAIL antibody did not inhibit the IFN-induced caspase activation and apoptosis in MM418 by a TRAIL-independent manner (data not shown). Our result further indicated that those melanoma cell lines (SK-mel-118, SK-mel-23, MM418), in which apoptosis was induced by IFN-β, upregulated caspase-2 activity with (SK-mel-118, SK-mel-23) or without (MM418) involvement of TRAIL-related pathway. The finding was a common phenomenon in these cell lines, and the increased apoptotic population

was depended on upregulation of caspase-2 activity, but not on TRAIL induction.

IFN-α2 has been known to upregulate the expression of TRAIL receptor 1 (DR4) and 2 (DR5), to which TRAIL binds and initiates apoptosis in some tumor cells (Meng and El-Deiry, 2001; Merchant *et al*, 2004). It has also been indicated that IFN-β does not upregulate mRNA level of DR4 and DR5, although it is unknown if IFN-β upregulates TRAIL receptor expression (Chawla-Sarkar *et al*, 2001). Caspase-2 induced by IFN-β may upregulate DR4 or DR5 and sensitize melanoma cells more significantly being stimulated by a trace amount of TRAIL. Caspese-2 induced by IFN-β may also inhibit some of apoptosis inhibitors in melanoma cells. Thus our present results clearly indicate that caspase-2 is involved in IFN-β-induced apoptosis. The finding may suggest that measurement of caspase-2 activity in primary culture cells from the excised melanoma tissues can be a novel marker in estimating the extent of cytotoxic effect in IFN-β adjuvant therapy for melanoma. Caspase-2 is indispensable for IFN-induced sensitization of chemotherapeutic drugs to melanoma cells.

Materials and Methods

Cell cultures and reagents

The human melanoma cell lines, SK-mel-118, SK-mel-23, MM418 and G361 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum and antibiotics in 5% CO_2 incubator at 37℃. SK-mel-23 and MM418 are pigmented cell lines, and the others are non- pigmented cell lines. IFN-α2 was purchased from Serotec (Oxford, UK). IFN-β was supplied from Mochida Pharmaceutical (Tokyo, Japan). Cells were treated with 1000 IU/ml of IFNs for different time periods based on the experiment. Recombinant human TRAIL (R&D systems, Minneapolis, MN) was used at the final concentration of 100 ng/ml. Recombinant human TNF-β (R&D systems,) was used at the final concentration of 50 ng/ml. Anti-Fas (CD95) antibody (MBL, Nagoya, Japan) was used at the final concentration of 500 ng/ml.

Cell number and cell viability assay

Cells were plated in 100 μl of medium in 96 well plates with triplicates of 1000 cells/well. After 24 h, cells were treated with IFN-α2 and IFN-β (72 h), or with IFN-β (24 h) followed by PBS washing and addition of TRAIL, TNF-β or anti-Fas (CD95) antibody (48 h). After 48 hr from plating, cells were also treated with TRAIL, TNF-β or anti-Fas (CD95) antibody (48 h) to be compared with IFN-α2 pretreated cells. Cell numbers were determined with a gentian violet dye-binding assay as previously described (Kubota *et al*, 2001). The viability of treated cell was determined with MTT assay, which is a formazan-formation assay, using the Cell Counting Kit-8 (DOJINDO, Kumamoto, Japan) according to manufactures' protocol.

Semiquantitative reverse transcriptase- polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells by using an RNeasy mini kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized using Super Script™-III Rnase H (Invitorogen, Carlsbad, CA) from total extracted RNA (50 ng). The quantitative of the PCR was validated by the linearity of the determination curve at various

concentrations of cDNA. The specific primers (Sigma-Genosys, Ishikari, Japan) were constructed as follows: 5'- TTGGCTCAGGTGGATTTGG-3' and 5'-GGCTTTTCTTCCACACAGTC-3' for PKR, 5'- GCATCCCACCCTCTATTACT-3' and 5'-TGTCTTCAGTTCCTTTGTCC-3' for MxA, 5'-GGAAGTACAATGAGGCCCTACAAA-3', 5'-TCCTCAGTCCTAGTATATTGCTCC-3' for mda-5, 5'-GGCTATGATGGAGGTCCAGG-3' and 5'-GGTCCATGTCTATCAAGTGCTC-3' for TRAIL, 5'-TCCACCACCCTGTTGCTGTA-3' and ACCACAGTCCATGCCATCAC-3' for GAPDH PCR were performed using Taq DNA polymerase (Promega, Wisconsin, WI) as follows: denaturation, 94℃ for 30 s; annealing, 55℃ for 30 s and extension, 72℃ for 1 min. The number of cycles was 35.

Fluorescence activated cell-sorting (FACS) analysis

Analysis of sub-G_0 cell population and TUNEL assay of apoptotic cells were measured by FACS. Cells were plated in 8 ml of medium in 10 cm dishes with $2x10^4$ cells/well. After 72 h, cells were harvested by scraping. For TUNEL assay cells were assayed using the Apo-BrdU In situ DNA fragmentation Assay Kit (MBL, Nagoya, Japan) according to manufactures' protocol. For Sub-G_0 population, harvested cells were stained with propidium iodide using Cycle Test™ PLUS DNA reagent kit (BD Bioscience, San Jose, CA). These stained cells were an analyzed with a FACS Caliber and CellQusest software (Becton Dickinson, San Jose, CA).

Caspase activity assay

Cells were plated in 8 ml of medium in 10 cm dishes with 2×10^4 cells/well. After 24 h, cells were treated with IFN-β and IFN-β (48 h), or with IFN-β (24 h) followed by PBS washing and addition of TRAIL, TNF-β and anti-Fas (CD95) antibody (24 h). After 48 h from plating, cells were also treated with TRAIL, TNF-β and anti-Fas (CD95) antibody (24 h) to be compared with IFN-α2 pretreated cells. Cells were harvested by scraping, and were assayed by commercially available caspase-2, -3, -8, -9 and -10 fluorometric assay kit (MBL, Nagoya, Japan) as procedure was described. Caspase activity was measured by spectrofluorometer Fluoroskan Ascent FL (Labsystems, Helsinki, Finland). Excitation wavelength was at 390 nm and emission wavelength was at 510 nm.

Statistical analysis

Statistical comparisons were made using Student's *t*-test

CONFLICT OF INTEREST

The authors state no conflict of interest.

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