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Research Article

Etoposide sensitizes neuroblastoma cells expressing caspase 8 to TRAIL

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Abstract

TRAIL [TNF (tumour necrosis factor)-related apoptosis-inducing ligand] is a promising agent for clinical use since it kills a wide range of tumour cells without affecting normal cells. We provide evidence that pretreatment with etoposide significantly enhanced TRAIL-mediated apoptosis via up-regulation of DR5 (death receptor 5 or TRAIL-R2) expression in the caspase 8 expressing neuroblastoma cell line, SK-N-MC. In addition, sequential treatment with etoposide and TRAIL increased caspases 8, 9 and 3 activation, Mcl-1 cleavage and Bid truncation, which suggests that the ability of etoposide and TRAIL to induce apoptosis is mediated through activation of an intrinsic signalling pathway. Although TRAIL-R2 expression increased in IMR-32 cells in response to etoposide treatment, cell death was not increased by concurrent treatment with TRAIL compared with etoposide alone, because the cells lacked caspase 8 expression. Restoration of caspase 8 expression by exposure to IFNγ (interferon γ) sensitizes IMR-32 cells to TRAIL. Moreover, pretreatment with etoposide increased TRAIL-induced apoptosis in caspase 8 restored IMR-32 cells through activation of a caspase cascade that included caspases 8, 9 and 3. These results indicate that the etoposide-mediated sensitization of neuroblastoma cells to TRAIL is associated with an increase in TRAIL-R2 expression and requires caspase 8 expression. These observations support the potential use of a combination of etoposide and TRAIL in future clinical trials.

Keywords: caspase 8; death receptor; etoposide; inferferon γ; mitochondrial cascade; TRAIL

1. Introduction

The TRAIL [TNF (tumour necrosis factor)-related apoptosis-inducing ligand], also known as the Apo-2L (Apo-2 ligand), is a member of the TNF family and selectively induces apoptosis in tumour cells (Wu et al., 1997; Wang, 2008). TRAIL interacts with 2 types of receptors, the apoptosis-inducing DR4 (death receptor 4) (TRAIL-R1) and DR5 (TRAIL-R2) and the non-apoptosis-inducing DcR1 [decoy receptor 1; (TRAIL-R3) and DcR2 (TRAIL-R4]. TRAIL-R1 and TRAIL-R2 share highly homologous cysteine-rich extracellular domains and intracellular domains that include a DD (death domain). The extracellular domains of DcRs are similar to the DRs, but TRAIL-R3 lacks a cytoplasmic DD and TRAIL-R4 has a truncated DD (Degli-Esposti et al., 1997a,b; Pan et al., 1997).

By binding to DRs, TRAIL induces receptor trimerization and a conformational change in the intracellular DD resulting in the recruitment of the FADD (Fas-associated DD) and pro-caspases 8 and 10 to the DISC (death-inducing signalling complex). The recruited caspases are self-activated and, in turn, activate downstream effector caspases, such as caspases 3 and 9, which transmit signals leading to apoptosis. In contrast, when TRAIL binds to DcRs, FADD cannot be recruited and, therefore, apoptosis is not triggered (Salvesen and Dixit, 1997; Bodmer et al., 2000; Kischkel et al., 2000). TRAIL is a potential therapeutic agent in cancer treatment due to its apoptotic activity in cancer cells and minimal cytotoxicity to normal cells.

Neuroblastoma is a common extra-cranial paediatric cancer arising from cells of the SNS (sympathetic nervous system) (Brodeur, 2003). Several types of chemotherapeutic agents are used to treat these tumours, one of which, etoposide, is an anti-neoplastic agent that has been reported to induce apoptosis in neuronal cells (Belani et al., 1994). There are a significant proportion of cases that do not respond to high-dose chemotherapy (de Cremoux et al., 2007) and that undergo relapses after completion of therapy (Kushner et al., 2004). Therefore the development of novel therapies is needed.

We have investigated whether etoposide treatment increases TRAIL cytotoxicity in neuroblastoma cells. Etoposide treatment increased TRAIL-R2 expression, which enhanced levels of TRAIL-induced apoptosis in caspase 8-expressing neuroblastoma cells. This observation supports the potential use of a combination of etoposide and TRAIL in future clinical trials for neuroblastoma treatment.

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Abbreviations used: AzaC, 5-aza-2′ deoxycytidine; BCA, bicinchoninic acid; DD, death domain; DcR, decoy receptor; DR5, death receptor 5; FADD, Fas-associated death domain; FBS, fetal bovine serum; IFNγ, interferon γ; NF-κB, nuclear factor κB; PARP, poly(ADP-ribose) polymerase; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand.

2. Materials and methods

2.1. Cells and reagents

The human neuroblastoma cell lines, IMR-32 and SK-N-MC, were obtained from the A.T.C.C. The cells were maintained in a humidified atmosphere containing 5% CO_2 and 95% humidified air at 37°C in DMEM (Dulbecco's modified Eagle's medium; Gibco – Invitrogen) supplemented with 4.5 g/l glucose, 10% FBS (fetal bovine serum; Gibco – Invitrogen), 10 mM Hepes (Gibco –Invitrogen) and 1 × antibiotic/antimycotic solution (Gibco – Invitrogen). The DR5:Fc fusion protein and the inhibitors zIETD-fmk, zLEHD-fmk and zDEVD-fmk were purchased from R&D Systems. Antibodies against caspase 8, caspase 9 and Bax were purchased from Santa Cruz Biotechnology; and antibodies against caspase 3, Mcl-1, Bcl-2 and Bid were purchased from Cell Signaling Technology. Recombinant IFNγ) was obtained from LG Life Sciences Ltd.

2.2. Cell viability assay

IMR-32 or SK-N-MC cells were seeded into 96-well plates (Corning) at 5×10^4 or 2.5×10^4 cells per well respectively in 100 μl of cell culture medium without Phenol Red. Following a 24 h incubation at 37°C to allow the cells to adhere, the medium was replaced with low serum (0.5% FBS) medium without Phenol Red. Cells were treated for 24, 48 or 72 h with vehicle or etoposide (Vepesid; Bristol-Myers Squibb) alone or in combination with recombinant human TRAIL (Invitrogen), which was added after the first 2 h of etoposide treatment. Alamar Blue was added for the last 3 h of the etoposide treatment, and absorbance at 570 and 600 nm was measured with an ELISA Reader (Molecular Devices).

2.3. Flow cytometry analysis

Expression of TRAIL receptors was analysed using anti-human TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4 antibodies (R&D Systems). Normal mouse IgG was used for the control. The fluorescence intensity of the samples was determined using a FACS-Calibur flow cytometer (Becton Dickinson) and analysed using the CellQuest software (Becton Dickinson).

2.4. Luminex assay

Neuroblastoma cells that were exposed to etoposide and/or TRAIL for 12 h were collected and lysed using the cell signalling universal lysis buffer (Upstate). The amount of protein was quantified with a BCA (bicinchoninic acid) protein assay kit (Pierce Biotechnology), and samples containing equal amounts of protein were incubated with 1 × bead suspension containing beads with capture antibodies specific for active caspase-3, cleaved PARP [poly(ADP-ribose) polymerase] and GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Upstate) for 2 h in the dark. The lysates were removed by vacuum filtration, and the beads were washed with assay buffer followed by incubation with 1 × biotinylated reporter (Upstate) and streptavidin-phycoerythrin (Strep-PE; Upstate). The fluorescence intensity was assessed using a Luminex 200 system (Luminex Corporation) and analysed using the MasterPlex CT and MasterPlex QT software (Miraibio). The fluorescence intensity of GAPDH was used as a control.

2.5. Western blot analysis

Cells were treated with etoposide and/or TRAIL, and cellular lysates were prepared using cold RIPA buffer (Invitrogen) containing protease inhibitors (Invitrogen) and phosphatase inhibitors (Invitrogen). Protein content was measured using the BCA protein assay reagent (Pierce), and equal amounts of protein from each cell lysate were dissolved in sample buffer (Invitrogen) for separation by denaturing gel electrophoresis under reducing conditions (Invitrogen). The separated proteins were electrophoretically transferred to PVDF (Amersham Biosciences) using an XCell II Blot™ apparatus (Invitrogen). The membrane was blocked with 2% BSA (Gibco – Invitrogen) in TBS (10 mM Tris, pH 7.5 and 100 mM NaCl) at 4°C overnight. The blot was incubated with primary antibody diluted in TBS-Tween 20 (0.1%, v/v; Sigma) for 4 h, and incubated with peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology) or anti-mouse IgG (Santa Cruz Biotechnology) for 2 h. After washing the membrane in TBS-Tween 20 (0.3% v/v; Sigma) 3 times for 5 min each time, the proteins were visualized by ECL® (enhanced chemiluminescence; Amersham Biosciences). The blot was reprobed for β-actin (Sigma) as a loading control.

2.6. Statistical analysis

All results are expressed as means ± S.D. $P<0.05$ was considered significant.

3. Results

3.1. Etoposide increases TRAIL-R2 expression in neuroblastoma cell

Etoposide treatment induced cell death in both IMR-32 and SK-N-MC cells in a dose- and time-dependent manner (Figures 1a and 1b). To explore the effect of etoposide treatment on the expression of TRAIL receptors in neuroblastoma cells, the expression levels of TRAIL receptors using flow cytometry were analysed. The number of TRAIL-R2 expressing cells gradually increased over 24 h of exposure to etoposide, while the number of cells expressing other TRAIL receptors slightly increased after 24 h treatment (Figure 1c).

3.2. Pre-treatment with etoposide enhances TRAIL cytotoxicity in SK-N-MC but not in IMR-32 cells

Treatment with TRAIL induced cell death in SK-N-MC cells in a dose-dependent manner, but had no effect on IMR-32 cells (Figure 2a). When SK-N-MC cells were pre-treated with etoposide for 2 h before TRAIL treatment, a significant increase in cell death occurred at 48 h after etoposide treatment compared with treatment with either etoposide or TRAIL alone. However,

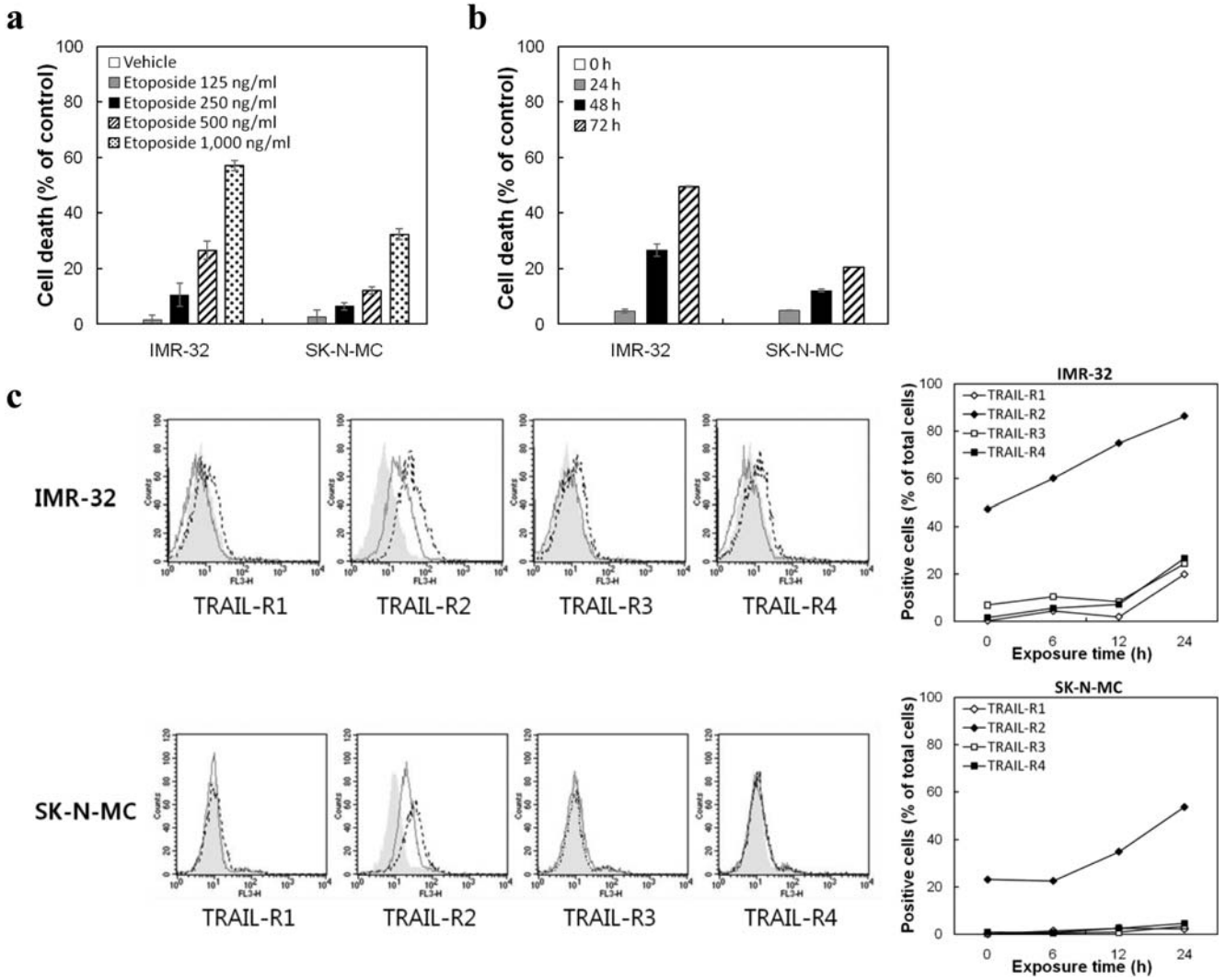

Figure 1 **Etoposide treatment induces cell death and increases expression of TRAIL-R2 in neuroblastoma cell lines**
IMR-32 and SK-N-MC cells were plated in 96-well plates and either (**a**) treated for 48 h with the indicated concentrations of etoposide or (**b**) exposed to 500 ng/ml etoposide for 24, 48 or 72 h. Cell viability was assessed using the Alamar Blue assay, which quantifies mitochondrial activity. The results are presented as cell death percentage against vehicle-treated control (means ± S.D. from three independent experiments). (**c**) Surface expression of TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4 in untreated cells (solid line) or cells treated for 6, 12 or 24 h with 500 ng/ml etoposide (broken line) was analysed by flow cytometry using receptor specific antibodies. Control antibody staining appears as shaded peaks.

treatment with TRAIL, in either the presence or absence of etoposide, had no effect on cell death in IMR-32 cells (Figure 2b). After consecutive treatment with etoposide and TRAIL, the increase in caspase 3 activity and PARP cleavage were observed in SK-N-MC cells, but not in IMR-32 cells (Figures 2c and 2d). Furthermore, treatment with DR5:Fc had no impact on cell viability in IMR-32 cells, but it completely inhibited the increase in cell death caused by consecutive treatment with etoposide and TRAIL in SK-N-MC cells (Figures 2e and 2f).

3.3. Cells lacking caspase 8 expression are resistant to TRAIL-induced apoptosis

Caspases 8, 9 and 3 activation and Mcl-1 cleavage were induced by TRAIL in SK-N-MC cells. Consecutive treatment with etoposide and TRAIL significantly increased the activation of caspases 8, 9 and 3, as well as Mcl-1 cleavage and Bid truncation, which all correlated with the increase in cell death (Figure 3a). Cell death decreased in the presence of caspases 8, 9 or 3 inhibitors in SK-N-MC cells (Figure 3b). In contrast, caspases 9 and 3 activation, Mcl-1 cleavage and Bid truncation were induced by etoposide alone in IMR-32 cells lacking caspase 8 expression. Moreover, etoposide-induced cell death decreased in the presence of caspase 9 or 3 inhibitors but was not affected by a caspase 8 inhibitor (Figure 3b). Consecutive treatment with etoposide and TRAIL did not increase activation of caspases, Mcl-1 cleavage, or Bid truncation compared with etoposide treatment alone. No substantial changes in Bcl-2 or Bax were detected under any of the treatment conditions in either cell line (Figure 3a).

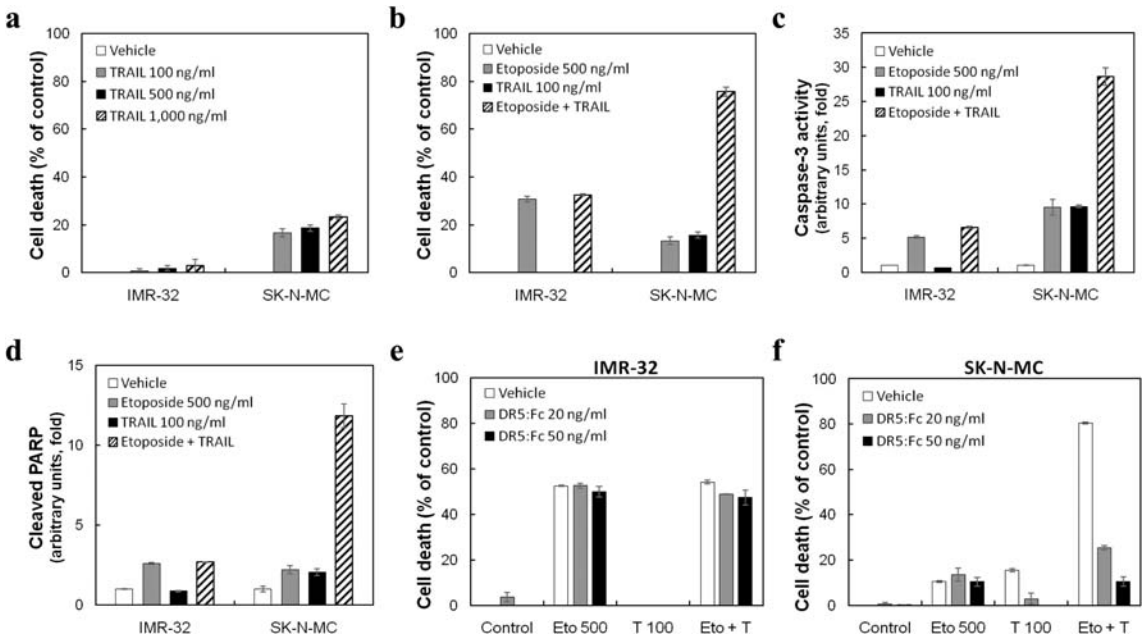

Figure 2 **Pretreatment with etoposide enhances TRAIL-induced cell death in SK-N-MC, but not in IMR-32 cells**
(**a**) Cell death was analysed 48 h after exposure to 100, 500 or 1000 ng/ml TRAIL. (**b**) Neuroblastoma cells were pretreated with etoposide (500 ng/ml) followed by exposure to TRAIL (100 ng/ml) treatment for 48 h. (**c**, **d**) Cells were cultured in 60 mm plates and exposed to 500 ng/ml etoposide and/or 100 ng/ml of TRAIL. After 12 h incubation, total cell lysates were obtained and analysed for caspase 3 activity and PARP cleavage using a luminex assay. (**e**, **f**) Cells were pretreated with the indicated concentrations of the DR5:Fc fusion protein for 1 h to block TRAIL binding to TRAIL-R2. Cell viability was assessed after consecutive treatment with etoposide (Eto; 500 ng/ml) and/or TRAIL (T; 100 ng/ml) for 48 h. The results are presented as cell death as a percentage against vehicle-treated control (means $\pm$S.D. from three independent experiments).

3.4. Re-expression of caspase 8 sensitizes cells to TRAIL-induced apoptosis

By treating IMR-32 cells with IFNγ for 48 h, the expression of caspase 8 gradually increased (Figure 4a). Restoration of caspase 8 sensitized IMR-32 cells to TRAIL cytotoxicity and etoposide potentiated this TRAIL cytotoxicity. Additional treatment with a caspase 8 inhibitor or the DR5:Fc fusion protein significantly suppressed TRAIL-induced cell death (Figures 4b–4d). Increased caspase activity, Mcl-1 cleavage and Bid truncation were observed in response to consecutive treatment with etoposide and TRAIL in caspase 8 restored IMR-32 cells (Figure 4e).

4. Discussion

TRAIL induces apoptosis in cancer cells, but it does not affect normal cells (Walczak et al., 1999). However, a significant proportion of cancer cells exhibit resistance to the cytotoxic effect of this ligand, suggesting that the use of TRAIL alone may not be enough to treat cancers (Wajant et al., 2002). TRAIL-resistance is due to de-regulated expression of the TRAIL receptors or the intracellular components acting downstream of the receptors. Previous reports have shown that conventional chemotherapeutic agents (Gibson et al., 2000; Singh et al., 2003), irradiation (Shankar et al., 2004b; Marini et al., 2005) and HDAC (histone deacetylase) inhibitors (Singh et al., 2005) enhance the cytotoxicity of TRAIL via up-regulation of TRAIL receptors. Up-regulation of DRs' expression by the chemotherapeutic agent is dependent on the activity of NF-κB (nuclear factor κB; Mendoza et al., 2008) or p53 (Shankar et al., 2004a; Seitz et al., 2010), which transcriptionally regulate the expression of DRs by binding to sites in the promoter (Yoshida et al., 2001; Liu et al., 2004). Overexpression of the NF-κB p65 subunit up-regulates TRAIL-R2 expression in epithelial-derived cell lines (Shetty et al., 2002). We have shown that etoposide treatment significantly increases the surface expression of TRAIL-R2 in the neuroblastoma cell lines IMR-32 and SK-N-MC. Our preliminary data demonstrated that etoposide treatment increased NF-κB p65 activity (data not shown), suggesting the possibility that etoposide increases TRAIL-R2 expression via an NF-κB pathway in neuroblastoma cells. This hypothesis still requires examination.

Treatment with etoposide prior to TRAIL treatment significantly enhanced cell death in SK-N-MC cells, compared with etoposide or TRAIL treatment alone. Moreover, the enhanced cell death was completely inhibited by treatment with the fusion protein DR5:Fc, which acts as a dominant-negative by competing with endogenous DR5 on the cell surface. Although etoposide treatment dramatically increased TRAIL-R2 expression and slightly increased expression of TRAIL-R1, -R3 and -R4, cell death induced by serial treatment with etoposide and TRAIL did not increase in comparison with etoposide alone in IMR-32 cells. This result may reflect the deregulation of intracellular components rather than the slight increase in DcRs.

Caspase 8 is an essential mediator of the initiation of DR-induced apoptosis (Varfolomeev et al., 1998) and is frequently

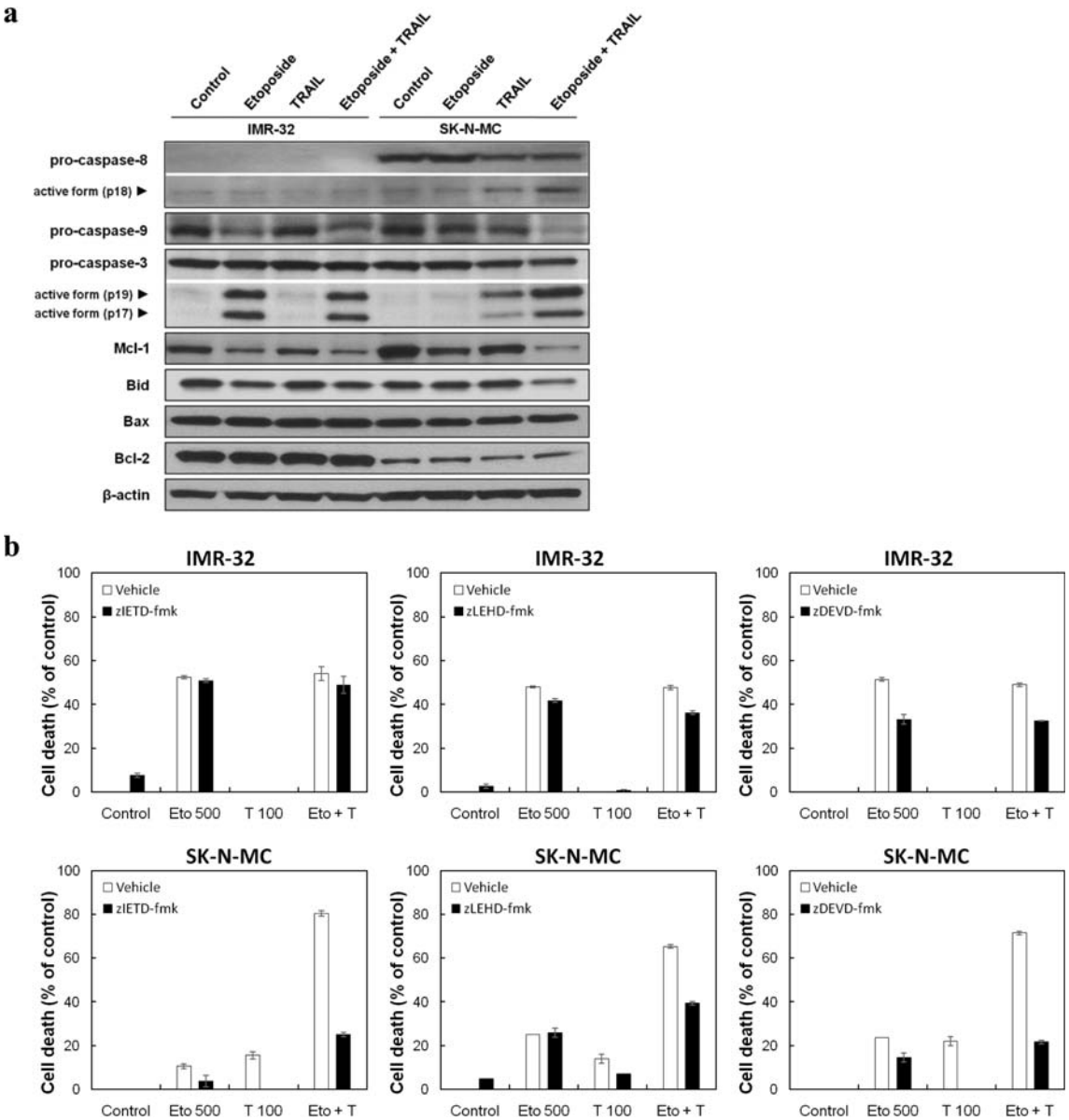

Figure 3 **Cells lacking caspase 8 expression are resistant to TRAIL-induced cell death**
(**a**) The expression of caspases (8, 9 and 3) and Bcl-2 family members (Mcl-1, Bid, Bax and Bcl-2) in IMR-32 and SK-N-MC cells after treatment with etoposide (500 ng/ml) and/or TRAIL (100 ng/ml) for 12 h. Equal loading was assessed by staining with anti-β-actin. (**b**) Cells were treated for 48 h with etoposide (Eto; 500 ng/ml) and/or TRAIL (T; 100 ng/ml) in the absence or presence of 10 μM zIETD-fmk (a caspase-8 inhibitor), 50 μM zLEHD-fmk (a caspase-9 inhibitor), or 50 μM zDEVD-fmk (a caspase-3 inhibitor). The results are presented as cell death as a percentage against vehicle-treated control (means $\pm$ S.D. from three independent experiments).

lacking in cancers, such as neuroblastoma, medulloblastoma, rhabdomyosarcoma, small cell lung cancer and melanoma (Teitz et al., 2000; Fulda et al., 2001; Pingoud-Meier et al., 2003). Loss of caspase 8 expression correlates with low sensitivity to TRAIL cytotoxicity. Caspase 8 expressing cancer cells are sensitive to TRAIL cytotoxicity, whereas cells lacking caspase 8 are TRAIL-resistant. Moreover, cells lacking caspase 8 are sensitized to TRAIL cytotoxicity when caspase 8 expression, which can be induced by AzaC (5-aza-2′ deoxycytidine) or IFNγ, is restored (Hopkins-Donaldson et al., 2000). The loss of caspase 8 expression is the result of gene silencing by aberrant methylation and can be restored by demethylating agents such as AzaC. However, the clinical use of demethylating agents has been limited by the toxic side effects of these drugs (Hopkins-Donaldson et al., 2000; Teitz et al., 2000; Michalowski et al., 2008). IFNγ restores caspase 8 expression through transcriptional activation, which involves a Stat1/IRF1 pathway (Ruiz-Ruiz and Lopez-Rivas, 2002; Fulda and Debatin, 2002, 2006). We have shown that TRAIL treatment increases caspase 8 activity in SK-N-MC cells, but not in IMR-32 cells, because the gene was not expressed in IMR-32 cells. Treatment with IFNγ was used to restore caspase 8 expression in IMR-32 cells. Caspase 8 expression gradually increased in

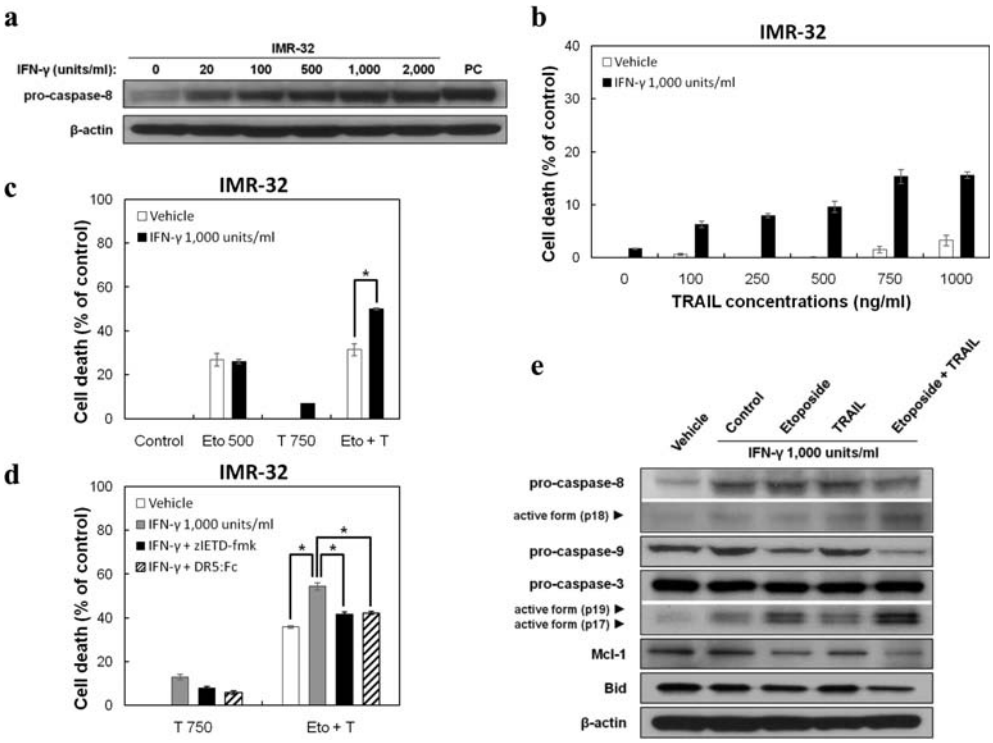

Figure 4 **Re-expression of caspase 8 sensitizes IMR-32 cells to TRAIL cytotoxicity**
(**a**) Expression of caspase 8 in IMR-32 cells after treatment with the indicated concentrations of IFNγ for 48 h. Caspase 8 expression in SK-N-MC cells was used as a positive control (PC). Equal loading was assessed by staining with anti-β-actin. (**b–d**) Cells were cultured in 100 mm plates and exposed to 1000 units/ml IFNγ. After a 24 h incubation, cells were replated in 96 well plates, cultured for 24 h, and treated with etoposide (Eto; 500 ng/ml) and/or TRAIL (T; 100, 250, 500, 750, or 1000 ng/ml) in the absence or presence of 10 μM zIETD-fmk or 50 ng/ml DR5:Fc. The results are presented as cell death percentage against vehicle-treated control (means $\pm$ S.D. from three independent experiments). Statistically significant differences are specified by asterisks (*$P<0.05$). (**e**) IMR-32 cells were exposed to 1000 units/ml IFNγ for 48 h, followed by consecutive treatment with etoposide (500 ng/ml) and/or TRAIL (750 ng/ml) for 12 h. Total cell lysates were used to detect the expression and cleavage of caspases 8, 9, 3, Mcl-1 and Bid. Equal loading was assessed by staining with anti-β-actin.

response to IFNγ treatment and IMR-32 cells became sensitized to TRAIL. As a result, treatment with etoposide prior to TRAIL treatment enhanced cell death. In addition, treatment with a caspase 8 inhibitor or the dominant negative DR5:Fc decreased etoposide and TRAIL-induced cell death, indicating that etoposide potentiated the TRAIL-induced cell death in caspase 8 restored IMR-32 cells.

SK-N-MC is non-MYCN amplified neuroblastoma cells, whereas IMR-32 contains 25 copies of the MYCN gene per cell (Reynolds et al., 1988). A subset of neuroblastoma with amplification of the oncogene MYCN has a particularly poor prognosis (Hopkins-Donaldson et al., 2000). The aberrant caspase 8 methylation has been found exclusively in neuroblastoma patient biopsies or cancer cell lines with MYCN amplification in several studies (Fulda et al., 1999; Hopkins-Donaldson et al., 2000). The inactivation of caspase 8 by hypermethylation has become a hallmark of defective apoptosis in advanced disease, suggesting that caspase 8 may act as a tumour suppressor gene in neuroblastoma (Fulda et al., 1999, 2001; Gonzalez-Gomez et al., 2003). These studies focused on the association between MYCN amplification and the methylation status of caspase 8 gene rather than caspase 8 expression. However, Fulda et al. (2001) found no correlation between MYCN amplification and caspase 8 mRNA or protein expression. In addition, another study in neuroblastoma cell line models clearly showed that MYCN has no direct effect on caspase 8 expression (van Noesel et al., 2003). Thus, the correlation between MYCN amplification and caspase 8 expression needs further investigation.

TRAIL-induced apoptosis has been correlated with the expression of Bcl-2 family members (Walczak et al., 2000). Mcl-1 is an anti-apoptotic Bcl-2 family protein that can bind to BH3-only proteins such as Bid and thereby inhibits tBid (truncated Bid)-mediated cytochrome *c* release from mitochondria (Clohessy et al., 2006; Adams and Cory, 2007). A decrease of Mcl-1 expression leads to cytochrome *c* release and the activation of caspases 9 and 3, which results in cells undergoing apoptosis (Clohessy et al., 2006). DNA damage (Arbour et al., 2008) and activated caspase 8 (Han et al., 2004; Weng et al., 2005; Han et al., 2006) can induce Mcl-1 cleavage and initiate the mitochondrial cascade. Caspases 8, 9 and 3 activation, Mcl-1 cleavage and Bid truncation increased in response to consecutive treatment with etoposide and TRAIL in SK-N-MC cells. The combined etoposide and TRAIL treatment increased caspase activation, Mcl-1 cleavage and Bid truncation in caspase 8 restored IMR-32 cells. The data suggest that etoposide-potentiated TRAIL-induced cell death is mediated by intrinsic cell death signalling pathways.

Our results indicate that etoposide treatment can enhance TRAIL cytotoxicity in neuroblastoma cells by up-regulating TRAIL-R2 expression. Furthermore, TRAIL cytotoxicity requires caspase

8 expression. Combined treatment with etoposide and TRAIL may be useful as a clinically applicable strategy for the treatment of neuroblastoma.

Author contribution

Hye Ryung Kim, Myoung Woo Lee, Ki Woong Sung and Hong Hoe Koo designed the study; Hye Ryung Kim, Dae Seong Kim and Ha Yeong Jo performed the experiments; Myoung Woo Lee, Soo Hyun Lee, Hee Won Chueh, Hye Lim Jung, Keon Hee Yoo and Hong Hoe Koo analysed the results; and Hye Ryung Kim, Myoung Woo Lee, Ki Woong Sung and Hong Hoe Koo wrote the paper.

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