

## Interferon- $\gamma$ Sensitises Human Osteosarcoma Cells to Trail-mediated Apoptosis

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**Abstract.** *Background:* Nearly half of all patients with osteosarcoma are still not cured by the currently employed multimodal treatment. New strategies are therefore needed to further improve the outcome. Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2) is able to induce programmed cell death in transformed cells, while normal cells remain unaffected. *Materials and Methods:* We have investigated the effect of TRAIL in combination with IFN- $\gamma$  on four human osteosarcoma cell lines; Saos-2, U2OS, KPDXM and OHS, and on one normal human fibroblast cell line, MRC-5. *Results:* One of the four osteosarcoma cell lines was TRAIL-resistant, but was sensitised to TRAIL-mediated cell death upon pre-incubation with IFN- $\gamma$ . In two of the three TRAIL-sensitive cell lines, the effect of TRAIL was enhanced by IFN- $\gamma$ . The normal human fibroblast cell line MRC-5 was not affected by treatment with TRAIL and IFN- $\gamma$ . A caspase cascade involving the activation of caspase-8, caspase-7 and PARP was associated with the onset of apoptosis in the osteosarcoma cell lines. Apoptosis was partly inhibited by the addition of caspase inhibitors zVADfmk (general inhibitor) and zIETDFmk (selective caspase-8 inhibitor). These findings further emphasize the important role of the caspases in cell death signalling. *Conclusion:* Our results show that treatment with both IFN- $\gamma$  and TRAIL

efficiently induces cell death in osteosarcoma cell lines and should be further investigated as a potential future therapy.

Osteosarcoma (OS) is the most common primary malignant tumour of the bone in children and adolescents and is characterised by its high propensity to metastasise to the lungs and the skeleton (1,2). Currently, only half of the patients with OS are cured after multimodal therapy with surgery, high-dose combinatory chemotherapy and irradiation (3,4). The acquisition of resistant phenotypes of OS is also a major problem during and after the use of chemotherapy and radiation therapy (4-6).

TNF-related apoptosis-inducing ligand (TRAIL/Apo2) is a member of the tumour necrosis factor (TNF)-cytokine family. This ligand is a promising compound for the induction of cell death in malignant cell lines, including breast and haematological malignancies *in vitro*. Interestingly, this compound has not yet been found to have any cytotoxic side-effects on normal cells *in vitro* or *in vivo* (7,8). Also, repeated administration of recombinant TRAIL to non-human primates is considered to be safe and non-immunogenic (7). The use of TRAIL in combination with other anti-cancer drugs has resulted in marked apoptosis in osteosarcoma cell lines (8-11).

TRAIL exerts its selectivity through binding to receptors, of which five have been identified so far. The receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) are cell surface death domain receptors and ligand-dependent activation of these receptors recruits the intracellular death adaptor molecule FADD (Fas-associated death domain protein). FADD binds to caspase-8 and activates other caspases downstream, including caspase-3 and 7 (12,15-18). The other three receptors, DcR1 (TRAIL-R3), DcR2 (TRAIL-R4) and osteoprotegerin (OPG) are "decoy receptors" that act as antagonistic receptors and prevent TRAIL-induced apoptosis since they lack functional death domains (16,19,20).

*Abbreviations:* TRAIL: Tumour-necrosis factor-related apoptosis-inducing ligand; IFN- $\gamma$ : interferon gamma; PARP: poly(ADP-ribose) polymerase; zVADfmk: Z-V-A-D (OMe)-FMK, general caspase inhibitor; zIETDFmk: Z-I-E (OMe)-T-D(OMe)-FMK, caspase-8 inhibitor; OS: osteosarcoma.

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Interferons (IFNs) are natural glycoproteins that have antiviral, immunomodulatory and antiproliferative effects (21). They also have anti-tumour effects on a variety of tumour cells, both in clinical use and under experimental conditions (22,23), and have been shown to have anti-tumour effects in combination with chemotherapeutic drugs (24-26).

However, to our knowledge there are no studies addressing the combined treatment of IFN- $\gamma$  and TRAIL in osteosarcoma cell lines. In this study we have investigated the apoptotic potential of TRAIL alone or in combination with IFN- $\gamma$  on four different human osteosarcoma cell lines and on one normal, human fibroblast cell line. Our results show that a combined treatment with IFN- $\gamma$  and TRAIL efficiently induces cell death in osteosarcoma cells and should be further investigated for future therapy.

## Materials and Methods

**Cell lines.** The osteosarcoma (OS) cell lines U2OS (27) and Saos-2 (28), and the normal human fibroblast cell line MRC-5 (29) were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). OS cell lines OHS (30) and KPDXM (31) were obtained from The Norwegian Radium Hospital (DNR, Oslo, Norway). The cell lines were maintained in RPMI-1640 medium without antibiotics, supplemented with 10% FBS (Gibco BRL, Sweden) and 2 mM glutamine (Gibco BRL). The cells were incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere and all cell lines were regularly tested for the presence of *Mycoplasma* (ATCC Mycoplasma Detection Kit).

**Reagents and antibodies.** Human recombinant IFN- $\gamma$  and TRAIL were obtained from R & D Systems (London, UK) and were used at concentrations of 500 U/ml and 250 ng/ml. The pan-caspase inhibitor zVAD-fmk and the caspase-8 inhibitor zIETD-fmk (R & D Systems) were used at a final concentration of 5 mM in culture medium where indicated. Mouse anti-caspase-8 (1:1000 dilution), rabbit anti-caspase-3 (1:500), caspase-6 (1:1000), caspase-7 (1:1000), caspase-9 (1:1000), BID (1:1000), Bcl-2 (1:500) and PARP pAb (1:1000) were purchased from Cell Signalling Inc. (Abingdon, UK). Mouse anti-cFLIP mAb (1:500) was supplied from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit anti-actin and alkaline phosphatase (AP) conjugated anti-mouse IgG (1:30000) were purchased from Sigma (Sigma, St. Louis, MO, USA), whereas AP-conjugated anti-rabbit IgG (1:2000) was purchased from DAKO (DAKO, Denmark). All primary antibodies, except anti-actin, were incubated overnight at 4°C, before addition of the secondary antibody. All other incubations were performed at room temperature.

**Measurements of cell viability.** To determine the cytotoxicity mediated by TRAIL and IFN- $\gamma$ , 1x10<sup>4</sup> cells/100 ml were seeded into each well of a 96-well cell culture micro-titer plate (Becton Dickinson, USA) and incubated overnight at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Cells were then either treated for 48 h with IFN- $\gamma$ , or with TRAIL for 4 h. Cell viability was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoleum (MTT) (Sigma) assay (32). For analysis, 100  $\mu$ l MTT solution (0.15g MTT in 30ml 1X PBS, diluted 1:10 in cell growth medium) was added to each well of the microtiter plates, incubated for 2 h at

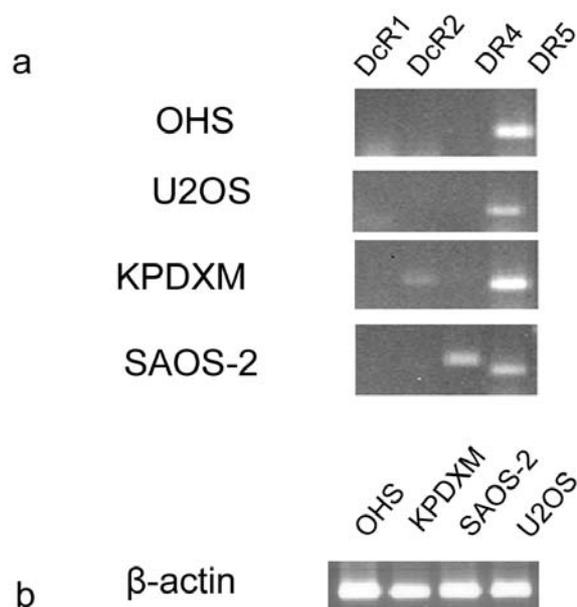


Figure 1. Presence of TRAIL death receptors in OS cell lines. a) DNA from OS cell lines was subjected to PCR to determine presence or absence of death-receptors (DcR1, DcR2, DR4 and DR5). PCR was performed using specific oligonucleotide pairs discriminating between the death receptors analysed. b) DNA from OS cells was subjected to PCR with the housekeeping gene  $\beta$ -actin to control equal DNA loading during PCR.

37°C in a humidified 5% CO<sub>2</sub> atmosphere, before termination of the reaction by adding 150  $\mu$ l "acidic" isopropyl alcohol (1 l isopropyl alcohol + 3 ml (concentrated) hydrochloric acid). Cell viability was measured spectrophotometrically at OD 570 (reference value OD 630) using a micro-culture plate reader.

**RT-PCR.** Total RNA was isolated from the cell lines using TRIZOL reagent (Invitrogen, Life Technologies, NY, U.S.). cDNA was made from 0.5 or 1.5 mg total RNA using Superscript<sup>TM</sup> II (Invitrogen) according to the protocol supplied by the manufacturer. All PCR products were analysed by agarose gel electrophoresis and visualised in UV-light after staining in EtBr.

**Quality control of synthesised cDNAs.** The quality of synthesised cDNA was tested by PCR using oligonucleotides for the housekeeping gene  $\beta$ -actin. cDNA (10  $\mu$ l) was used in a PCR reaction with a total volume of 50  $\mu$ l. The PCR reaction consisted of 1xPCR buffer (Finnzymes Oy, Finland) 0.1 mM of each deoxynucleotide (Promega, Madison, USA) and 20 pmol of the oligonucleotide pair of  $\beta$ -actin (actin sense sequence 5' TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3', actin antisense sequence 5'-ACT CGT CAT ACT CCT GCT TGC TGA TCC A-3', product size 625 bp). 1.0 U of Dynazyme<sup>TM</sup> II (Finnzymes) was added after hot start (94°C for 5 min). PCR products were amplified by 25 cycles of 94°C at 30 sec, 65°C at 30 sec and 72°C at 1 min. Final extension were performed at 72°C for 10 min. PCR products were analysed by agarose gel electrophoresis and visualisation by UV-light after staining in EtBr.

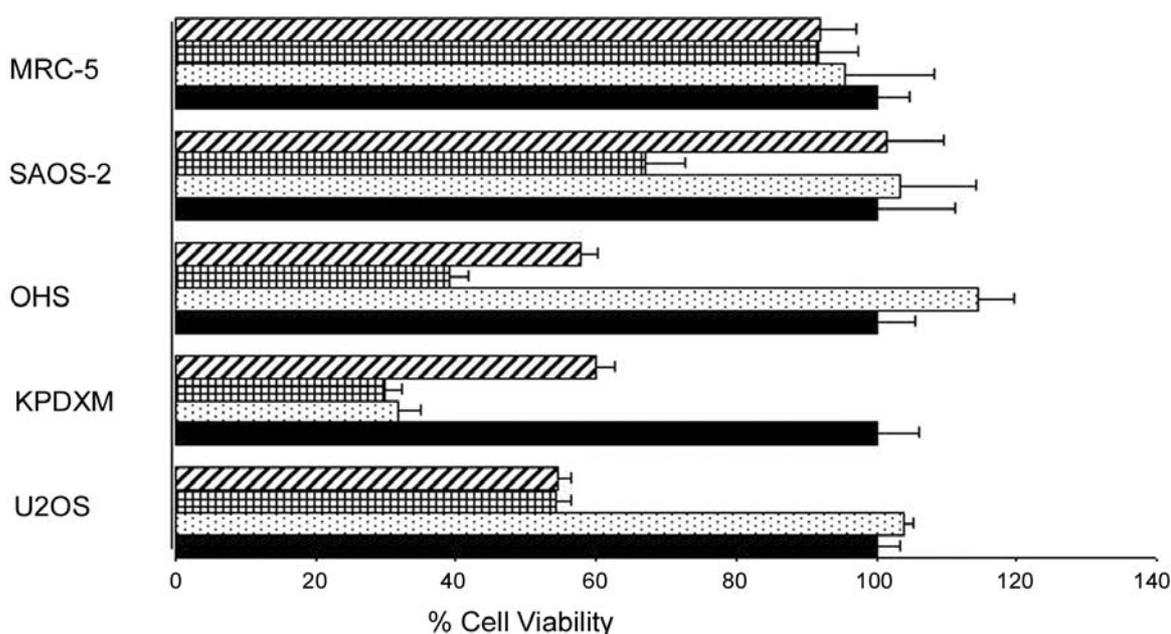


Figure 2. Responsiveness of OS cell lines to treatment with TRAIL and IFN- $\gamma$ . OS cell lines (Saos-2, OHS, KPDXM, U2OS) and the normal human fibroblastic cell line MRC-5 were incubated for 48h with IFN- $\gamma$  (dotted bars) followed by incubation with (cross-hatched bars) or without (crossed bars) 250ng/ml TRAIL. Black bars represent untreated cells. Values are means  $\pm$  SD of six measurements presented as % viability of cells.

**Analysis of death receptors.** Oligonucleotide pairs for TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4, previously described in Langaas *et al.*, (33) were used in PCR to analyse the presence of death receptor mRNA. The PCR reaction (total volume of 50  $\mu$ l) consisted of 1  $\mu$ l cDNA (corresponding to 25 ng total RNA), 1.0 U Dynazyme<sup>TM</sup> II (Finnzymes), 10 X PCR buffer (Finnzymes), 0.08 mM deoxynucleotide triphosphate mix (Promega) and 0.5  $\mu$ M of each oligonucleotide. PCR reactions were run at 93°C for 5 min (hot start) and then by 30 cycles at 93°C for 1 min, 52°C for 1.5 min and 72°C for 1 min. Final extensions were performed at 72°C for 10 min.

**Semi-quantitative RT-PCR (primer-drop).** A semi-quantitative RT-PCR of caspase-8 mRNA was performed on total RNA isolated from OS cells after pre-incubation with IFN- $\gamma$  (R&D Systems). cDNA was synthesised from 0.5  $\mu$ g of total RNA in a final volume of 50  $\mu$ l. RT-PCR was performed using oligonucleotides for caspase-8 and  $\beta$ -actin (caspase-8 sense 5'-GAT-ATT-GGG-GAA-CAA-CTG-GAC-3', caspase-8 antisense 5'-CAT-GTC-ATC-ATC-CAG-TTT-GCA-3', product size 386 bp). Each PCR reaction contained 1  $\mu$ l cDNA, 1XPCR buffer (Finnzymes) 0.1 mM of each deoxynucleotide (Promega) and 20 pmol of each starter oligonucleotide pair (caspase-8). 1.0 U of Dynazyme<sup>TM</sup> II (Finnzymes) was added to each reaction tube after the first denaturation at 98°C for 5 min, and equal aliquots (20 pmol) of a secondary oligonucleotide pair ( $\beta$ -actin) were added after 11 cycles of PCR. Each PCR cycle consisted of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, with final extension at 72°C for 10 min. Total number of cycles was 30. All PCR amplifications were performed on a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Massachusetts, USA).

**Flow cytometry.** OS cells were harvested for flow cytometric analysis by trypsinisation and fixation in 4% formaldehyde (buffered) for 24 h at room temperature. The formaldehyde was removed by adding 95% ethanol followed by a 1-hour rehydration in distilled water. Subtilisin Carlsberg solution (0.1% protease XXIV (Sigma) was added and the cells were then stained with DAPI-sulforhodamine solution (8  $\mu$ M DAPI, 50 mM sulforhodamine 101, 0.1 M Tris-HCl pH 7.5, 70 mM NaCl). Cell samples were analysed using PAS II PAS II flowcytometer (Partec, Münster, Germany) equipped with a 100 W mercury arc lamp HBO 100. Fluorescence from treatment with DAPI was measured at above 435 nm. For histogram analysis, a multicycle program for analysis of cell cycle was used (Phoenix Flow Systems, San Diego, CA, USA) and at least 10<sup>4</sup> nuclei were analysed from each cell sample.

**Immunoblotting.** Proteins were extracted from OS cell lines using a buffer containing 25 mM Tris pH 7.8, 2 mM EDTA, 20% glycerol, 0.1% NP-40, 1mM DTT and protease inhibitors (Roche Diagnostic, Mannheim, Germany). The resulting cell lysates were cleared by centrifugation at 16,000 x g for 5 min. The protein content of each lysate was measured by the Lowry method (BioRad, CA, USA). For SDS-PAGE analysis, equal amounts of protein were loaded on pre-cast 10% Tris-HCl polyacrylamide gels (BioRad) and separated at 150 V for 1.5 h. The separated proteins were transferred to nitrocellulose membranes by electro-blotting at 400 mA for 1.5 h. The membranes were probed with specific antibodies against caspase-8, caspase-3, caspase-6, caspase-7, caspase-9, cFLIP, BID, Bcl-2, PARP and  $\beta$ -actin. Alkaline phosphatase (AP) conjugated anti-mouse IgG or AP-conjugated anti-rabbit IgG were used as secondary antibodies. The CDP-Star system was used for detection (1:500, Roche Biochemicals, Germany). The antibodies were according to instructions by the manufacturer.

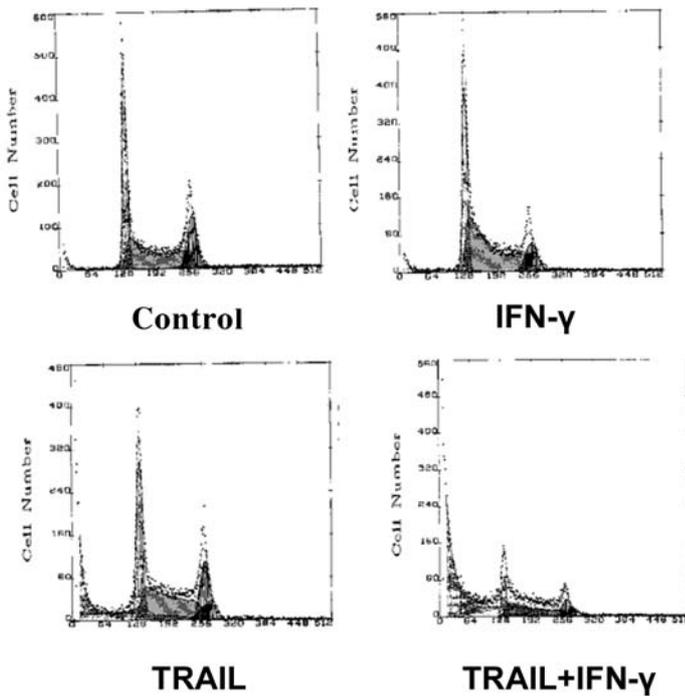


Figure 3. Flow cytometric analysis of OS cells after treatment with TRAIL and IFN- $\gamma$ . OS cell lines were subjected to FACS analysis after incubations with IFN- $\gamma$  (500 U/ml) and TRAIL (250 ng/ml). Histograms are presented for the TRAIL-responsive cell line U2OS. Histograms of DAPI-stained cells were analysed by a multicycle program and at least  $10^4$  nuclei were analysed from each cell sample.

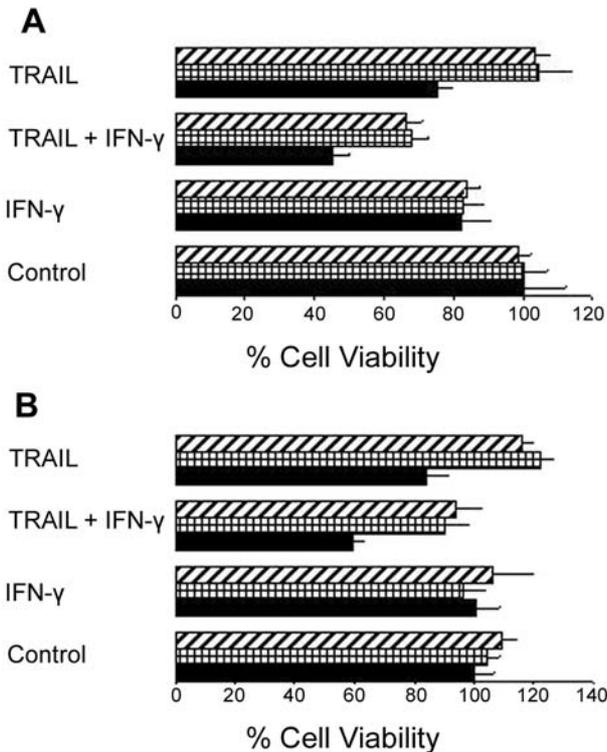


Figure 4. Effect of caspase inhibition on TRAIL and IFN- $\gamma$ -treated cells. Inhibition of caspase-activated cell death, analysed after 1h pre-incubation in 5 mM of general caspase inhibitor zVADfmk (zVAD) (cross-hatched bars) and caspase-8 specific inhibitor zIETDfmk (zIETD) (crossed bars). Black bars represent cells grown without presence of inhibitors, but with treatments as indicated. OS cell lines: A) Saos-2 and B) KPDXM.

### Results

*Expression of death and decoy receptors in OS cell lines.* TRAIL-receptor mRNAs for the four OS cell lines are shown in Figure 1a. The mRNA corresponding to TRAIL receptor DR5 (R2) was detected in all OS cell lines. Cell line Saos-2 expressed detectable mRNA for TRAIL-receptor DR4 (R1). Antagonistic TRAIL-receptor DcR1 (R3) mRNA was not found in any of the cell lines investigated, but the cell line KPDXM had a detectable level of mRNA for the other antagonistic receptor, TRAIL DcR2 (R4). Amplification products for the housekeeping gene  $\beta$ -actin were used as controls for equal loading (Figure 1b).

*TRAIL-induced cell death in OS cells alone or in combination with IFN- $\gamma$ .* It has previously been shown that IFN- $\gamma$  modulates apoptosis in, among others, Ewing sarcoma cells by converting cell lines resistant to therapy sensitive through the STAT/IRF1 pathway (26). All four OS cell lines were therefore tested for their responsiveness to TRAIL (250 ng/ml) alone or in combination with IFN- $\gamma$  (500 U/ml) using the MTT-test. The human fibroblast cell line MRC-5 was also tested as a control for this effect in normal cells. OS cell lines were also tested for the ability of IFN- $\gamma$  to sensitise for TRAIL-induced apoptosis. A 48-h pre-incubation with IFN- $\gamma$  (500 U/ml), was followed by a 4-h exposure to TRAIL (250 ng/ml). The cell line U2OS was resistant to TRAIL-induced cell death after the 4-h incubation with TRAIL alone (Figure 2), while the other three OS cell lines were sensitive to this treatment (Figure 2). U2OS was

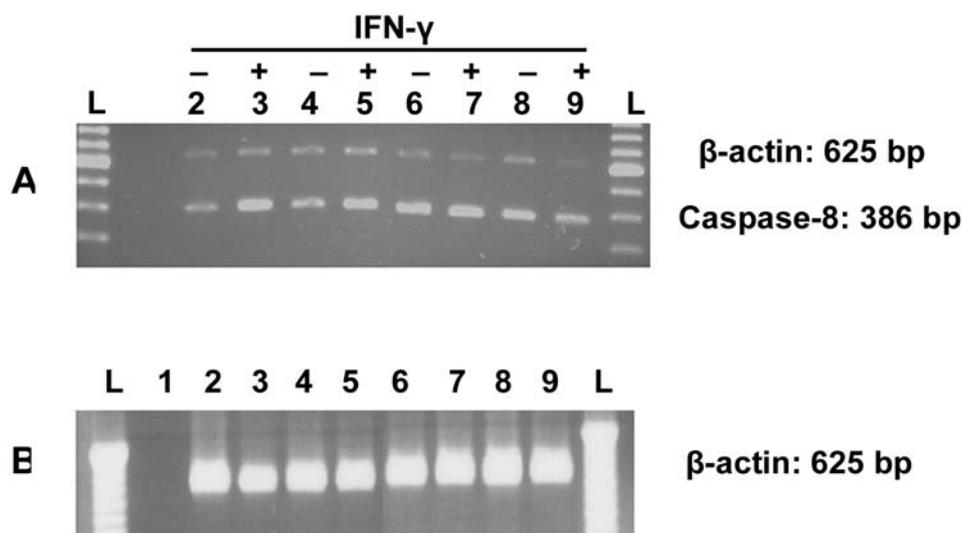


Figure 5. Influence of IFN- $\gamma$  on caspase-8 mRNA level. OS cell lines were pre-incubated with IFN- $\gamma$  (500 U/ml) before isolation of total RNA and RT-PCR of caspase-8 mRNA. A): PCR-product size: caspase-8=386 bp,  $\beta$ -actin=625 bp. L= 100 bp DNA ladder, Lane 1: negative PCR control (dH<sub>2</sub>O). Lanes 2-3: Cell line OHS. Lanes 4-5: KPDXM. Lanes 6-7: Saos-2. Lanes 8-9: U2OS. Cells were pre-incubated with (+) or without (-) IFN- $\gamma$ . B):  $\beta$ -actin PCR was used as quality control of mRNA. Lanes loaded with samples as in A).

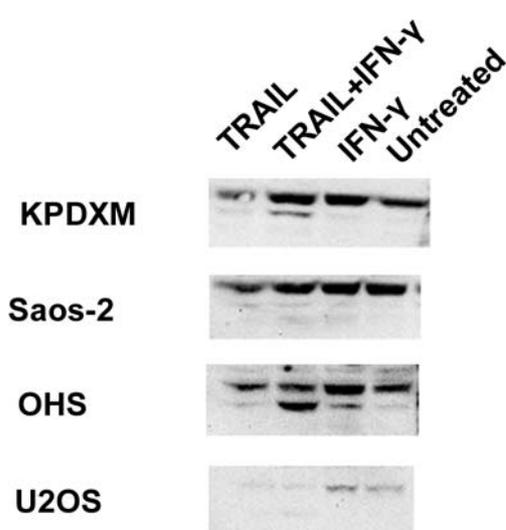


Figure 6. Activation of caspase-8 in OS cell lines by IFN- $\gamma$  and TRAIL. OS cells were treated with TRAIL (250 ng/ml) and / or IFN- $\gamma$  (500 U/ml) or left untreated. Western blot analysis (SDS-PAGE) for detection of caspase-8 (full length and active/short form) was then performed using specific antibodies. Expression of  $\beta$ -actin was used as a control of equal loading (figure not shown). Results are representative of 2-3 separate OS cell incubation experiments.

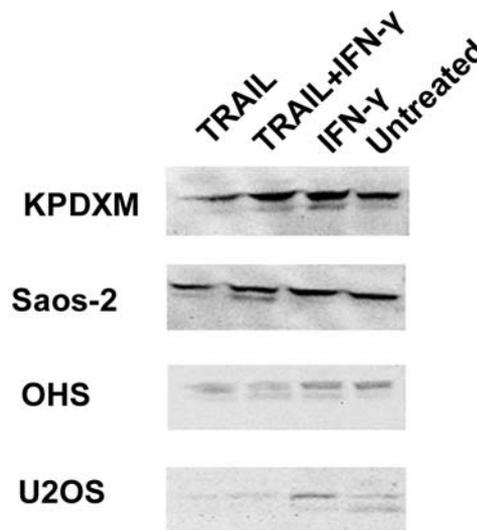


Figure 7. Activation of caspase-7 in OS cell lines by IFN- $\gamma$  and TRAIL. OS cells were treated with TRAIL (250 ng/ml) and/ or IFN- $\gamma$  (500 U/ml) or left untreated. Western blot analysis was performed using specific antibodies for detection of caspase-7 (full length and active/short form). Expression of  $\beta$ -actin was used as a control of equal loading (figure not shown). Results are representative of 2-3 separate OS cell incubation experiments.

sensitised by the pre-treatment with IFN- $\gamma$  and showed increased susceptibility to TRAIL-induced apoptosis following the 48-h pre-incubation (Figure 2). The normal, human fibroblast cell line MRC-5 was resistant to treatment with both IFN- $\gamma$  and TRAIL (Figure 2). Cells that responded to TRAIL analysed by MTT also demonstrated morphological changes

typical of apoptosis, such as rounded cell bodies and nuclear condensation, as observed by phase contrast microscopy (data not shown). Cell death was further verified by the results from flow cytometric analysis of DAPI-stained OS cells. These data indicate that IFN- $\gamma$  pre-treated TRAIL-resistant cell lines, here represented by Saos-2, were induced to cell death (Figure 3).

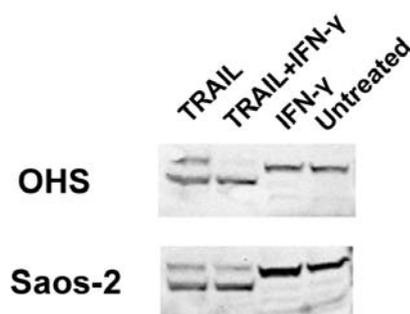


Figure 8. Activation of PARP in OS cell lines by IFN- $\gamma$  and TRAIL. OS cells were treated with TRAIL (250 ng/ml) and/ or IFN- $\gamma$  (500 U/ml) or left untreated. Western blot analysis was performed using specific antibodies for detection of PARP (full length and active/short form). Expression of  $\beta$ -actin was used as a control of equal loading (figure not shown). Results are representative of 2-3 separate OS cell incubation experiments.

*The effects of caspase inhibitors on TRAIL-mediated cell death in OS cells.* The ability of TRAIL and IFN- $\gamma$  to induce apoptosis was tested in the presence of the broad-spectrum caspase inhibitor zVADfmk and the specific caspase-8 inhibitor zIETDfmk for all five OS cell lines. Both caspase inhibitors blocked TRAIL-mediated cell death to the same extent. The OS cells however, when pre-incubated with IFN- $\gamma$  exhibited a variable response, showing that other factors than the activation of caspases alone are involved in the execution of cell death promoted by IFN- $\gamma$ . The caspase-8 specific inhibitor, zIETDfmk, was able to prevent most of the cell death induced by TRAIL treatment, indicating that activation of caspase-8 is important for the mediation of the TRAIL effect (Figure 4a and 4b). Similar results were obtained for the other two cell lines investigated (results not shown).

*IFN- $\gamma$  and the level of caspase-8 mRNA.* IFN- $\gamma$  induces changes in an array of genes associated with the apoptotic pathway, including genes coding for the death receptors, caspases and the pro-apoptotic member of the Bcl-2 family of proteins, Bak (24,34,35). We analysed the level of caspase-8 mRNA by a semiquantitative RT-PCR in the OS cell lines after incubation with IFN- $\gamma$ . All four OS cell lines had detectable levels of caspase-8 mRNA, but there was no significant increase in the level of caspase-8 mRNA in any of the OS cell lines analysed after pre-treatment with IFN- $\gamma$  (Figure 5).

*Mechanisms of TRAIL-induced apoptosis in OS cell lines.* Treating IFN- $\gamma$  pre-incubated cells with TRAIL resulted in a cleavage of caspase-8 in Saos-2, OHS and U2OS cell lines (Figure 6). Caspase-8 cleavage was barely detectable in the cell line KPDXM. Caspase-7 was activated and cleavage was

detectable after treatment with IFN- $\gamma$  alone or in combination with TRAIL for all OS cell lines (Figure 7). Elevated levels of FLIP have been associated with inhibition of signals from FasL- and TRAIL-mediated apoptosis (35-38). We therefore compared the levels of this protein in the four OS cell lines, after incubations with or without IFN- $\gamma$ , TRAIL or both. We found no difference in the levels of FLIP in any of the cell lines after any of the pre-incubations (data not shown). This may indicate that FLIP has no inhibitory effect in the analysed OS cell lines. The involvement of mitochondria in the caspase-cascade was also investigated, and caspase-9 and BID were analysed by Western blotting. The results indicate that caspase-9 was not activated (no cleavage products were detected), neither was the level of BID changed (data not shown). Further analysis of the downstream effector caspases, detected no cleavage of caspase-3 nor caspase-6 (data not shown). However, the effector caspase substrate PARP had detectable cleavage products present after TRAIL and IFN- $\gamma$  treatment indicating activation in all cell lines, here represented by the OHS and Saos-2 cell lines (Figure 8).

## Discussion

In this study we have analysed 4 different human OS cell lines and our data demonstrate that 3 out of 4 cell lines (OHS, Saos-2 and KPDXM) are sensitive to TRAIL-mediated cell death. In two recent studies 1 out of 6 (8) and 2 out of 6 (9) OS cell lines were TRAIL-sensitive to some degree. This reflects the diversity of OS cell lines and thus the variable TRAIL-responsiveness. The reasons for this variable sensitivity of different tumour cell lines to TRAIL is currently not understood, but the presence and level of the death-and-decoy receptors have been suggested as a possible explanation to this variability (39,40). However, there are still no studies that demonstrate such a correlation.

We found detectable levels of death receptor DR5 (R2) in all 4 of the OS cell lines analysed (Figure 1). This is in agreement with a study performed on Ewing sarcoma cells showing that 80% of the analysed cells were found to express DR4, while 100% of them expressed DR4.41 This indicates a susceptibility to TRAIL. Also, some of the cell lines expressed decoy receptor Dcr2 (R4) to a variable degree, which should indicate that they might have a variable response to TRAIL. Our results did not, however, reveal any direct correlation between the presence of receptors analysed by RT-PCR and the measured susceptibility to TRAIL. Differential expression and regulation of these receptors have also been described in a study by Zhang *et al.* (42) This might indicate that the mRNA expression pattern of receptors is not the full explanation to the observed TRAIL sensitivity and resistance, which also has been suggested by other studies (8,9).

In this study we observed that recombinant, human TRAIL was able to induce cell death in 3 out of 4 analysed OS cell lines. IFN- $\gamma$  was able to induce sensitivity to TRAIL in TRAIL-resistant cell lines and increase the sensitivity in 2 out of 3 TRAIL-sensitive cell lines. Hence, IFN- $\gamma$  seems to be a promising agent for combined therapy with TRAIL in order to trigger cell death in TRAIL-resistant OS cell lines. However, a study performed by Yang *et al.* (25) on human neuroblastoma cell lines indicates that IFN- $\gamma$  not is able to sensitise these cell lines to TRAIL-mediated apoptosis. The action of TRAIL may thus be cell line and death-receptor expression-dependent. The normal, human fibroblast cell line MRC-5, was unaffected by both TRAIL and IFN- $\gamma$  at the concentrations used in this study, indicating little or no effect on normal cells (Figure 2).

The augmentation of apoptosis by IFN- $\gamma$  has also been described for other tumour cell lines, such as cell lines derived from Ewing sarcoma, medulloblastoma, neuroblastoma (25,26), colon carcinoma (33) and lung epithelial cells (43). Methylation of sequences in the promoter of the caspase-8 gene has been suggested as a possible regulation of gene-expression, as IFN- $\gamma$  has been shown to induce the expression of caspase-8 in breast carcinoma and colon carcinoma cell lines (34). A study by Fulda *et al.*, (26) shows that combined treatment with IFN- $\gamma$  and other compounds such as APO1, TNF- $\alpha$  or chemotherapeutics (doxorubicin, cisplatin or VP-16) augment the action of the compound alone, and more pronounced apoptosis was observed in Ewing sarcoma, neuroblastoma and medullablastoma cell lines. This is in agreement with our observations using IFN- $\gamma$ .

The IFN- $\gamma$  mediated sensitisation to cell death by different compounds has been suggested to be mediated through a STAT1/IRF1-dependent pathway (26). This was concluded since it was established that IFN- $\gamma$  induces the phosphorylation of STAT1 and increase the expression of IFR1.

We did not observe any change in the level of caspase-8 mRNA after pre-incubation of the OS cells with IFN- $\gamma$ . But, the protein level of caspase-8 was increased after the pre-incubation with IFN- $\gamma$ . The selective inhibition of caspase-8-mediated cell death performed by the caspase-inhibitor zVADfmk also indicates the central role for caspase-8 as an initiator caspase in the OS cell lines after treatment with IFN- $\gamma$  and TRAIL.

The activation of death receptors results in the induction of a caspase cascade involving caspase-8 (44,45). Our results show a caspase-cascade involving the activation of caspase-8, caspase-7 and PARP, inducing cell death. We were not able to detect any activation of caspase-3 although the recent report by Evdokiou *et al.* (8) showed that the activity of caspase-3 increases during combined treatment with different chemotherapeutics (doxorubicin, cisplatin) in other OS cell lines. Neither did our cells have detectable levels of proteolytically processed caspase-9,

indicating that the mitochondria probably are not involved in mediating cell death by TRAIL. Expression of the intracellular caspase inhibitory protein, FLICE-inhibitory protein or FLIP, was detected in the analysed OS cell lines, but the levels did not change during incubation with TRAIL or TRAIL and IFN- $\gamma$ , and could not be correlated to the observed resistance to TRAIL in these cells. The level of Bcl-2 was not up-regulated (data not shown), and the truncated form of BID was not detectable (data not shown). Thus, truncated BID (tBID)-induced apoptosome formation involving cytochrome C, APAF-1 and pro-caspase-9 (46-49), do not seem to be involved in mediating cell death during treatment with TRAIL and IFN- $\gamma$  in these OS cell lines.

Our results indicate that IFN- $\gamma$  and TRAIL act synergistically to kill OS cells without any effect on normal human fibroblasts, which has exciting implications for future therapy of osteosarcoma.

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