INTERNATIONAL JOURNAL OF ONCOLOGY 28: 127-133, 2006

Anticancer agents sensitize osteosarcoma cells to TNF-related apoptosis-inducing ligand downmodulating IAP family proteins

PRISCO MIRANDOLA[1], IVONNE SPONZILLI[1], GIULIANA GOBBI[1], SANDRA MARMIROLI[2,3], LAURA RINALDI[1], ROBERTO BINAZZI[4], GABRIELLA GIULIANI PICCARI[5], GIULIA RAMAZZOTTI[5], GIAN CARLO GABOARDI[5], LUCIO COCCO[5] and MARCO VITALE[1,3]

[1]Department of Anatomy, Pharmacology & Forensic Medicine, Human Anatomy Section, University of Parma, Via Gramsci 14, 43100 Parma; [2]Department of Anatomy and Histology, University of Modena and Reggio Emilia, Largo del Pozzo 71, 41100 Modena; [3]ITOI-CNR, Unit of Bologna c/o IOR , via di Barbiano 1/10, I-40136 Bologna; [4]Department of Orthopaedic Surgery, University of Bologna, Istituto Ortopedico Rizzoli, Via Pupilli 1, 40136 Bologna; [5]Department of Anatomical Sciences, Cellular Signalling Laboratory, University of Bologna, via Irnerio 48, 40126 Bologna, Italy

Received July 29, 2005; Accepted September 5, 2005

Abstract. Although TNF-related apoptosis-inducing ligand (TRAIL) usually induces cell death in tumor cells, there are some tumor cell types that are resistant to its apoptogenic effects. Some chemotherapeutic drugs, however, can sensitize resistant cancer cells to TRAIL by either upregulating surface TRAIL death receptor expression or by modulating intracellular signalling pathways emanating from TRAIL receptors. U2OS human osteosarcoma cells express TRAIL-R2 but are resistant to TRAIL-induced apoptosis. However, the genotoxic drugs, Doxorubicin and Cisplatin, are able to sensitize U2OS cells to TRAIL, without affecting their surface expression of either death or decoy TRAIL receptors. We demonstrate that Doxorubicin and Cisplatin downmodulate X-IAP, while not affecting FLIP levels in U2OS cells. Selective downmodulation of X-IAP protein synthesis by specific small interference RNA transfection induced a sensitization of U2OS cells to TRAIL comparable to that induced by pharmacological treatment with genotoxic drugs. TRAIL-R2 downmodulation by siRNAs completely abolished the TRAIL-induced apoptosis of genotoxin-treated U2OS cells. Our findings demonstrate that Doxorubicin and Cisplatin do not sensitize U2OS osteosarcoma cells to TRAIL by surface receptor modulation but rather by the removal of the intracellular signalling inhibition generated by X-IAP, suggesting a foreseeable relevant advantage to the therapy of these tumors by the combined regimen of genotoxin-based chemotherapy and TRAIL.

Correspondence to: Dr Lucio Cocco, Department of Anatomical Sciences, Cellular Signalling Laboratory, University of Bologna, via Irnerio 48, 40126 Bologna, Italy
E-mail: lcocco@biocfarm.unibo.it

Key words: TNF-related apoptosis-inducing ligand, Doxorubicin, X-IAP, apoptosis, osteosarcoma

Introduction

Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family of cytokines, which are structurally related proteins playing important roles in regulating cell death, immune response and inflammation (1,2). The unique feature of TRAIL, compared with other members of the TNF family, is its ability to induce apoptosis in a variety of malignant cells, both *in vitro* and *in vivo*, displaying minimal toxicity on normal cells and tissue (3,4). TRAIL interacts with 4 high affinity transmembrane receptors belonging to the apoptosis-inducing TNF-receptor (R) family. TRAIL-R1 (DR4) and TRAIL-R2 (DR5) transduce apoptotic signals on binding with TRAIL whereas, although TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) are homologues to DR4 and DR5, they lack the intracellular death domain and apoptosis inducing capability. It has been proposed that TRAIL-R3 and TRAIL-R4 function as decoy receptors protecting normal cells from apoptosis (5,6).

Most normal human cell types that have been tested, including bone, epithelial, endothelial, fibroblastic, and smooth muscle cells, are refractory to TRAIL. Nevertheless, TRAIL can induce hepatocyte apoptosis (7) and cell damage in the human prostate (8) and brain (9). The earliest biochemical event following engagement of TRAIL death receptors by their ligand is the recruitment of proteins to the intracellular death domain of the receptor to form a structure known as the death-inducing signaling complex (DISC) (10). It is now established that, in untransfected cells, the TRAIL DISC resembles that of Fas in that the adaptor protein, Fas-associated death domain (FADD), and the apoptosis initiator, caspase-8, are recruited to TRAIL-R1 and/or TRAIL-R2 shortly after addition of TRAIL (1). TRAIL can trigger apoptosis in-dependently through TRAIL-R1 or TRAIL-R2. Once recruited to the DISC, caspase-8 autoactivates by proteolysis. In addition to the proteolytic caspase cascade, caspase activity is further regulated by the inhibitors of apoptosis (IAPs). The most well characterised protein of this family is X-IAP, which inhibits caspase-9 and caspase-3, not by interacting with their zymogens but through

binding to their intermediate and fully cleaved forms (11). Caspase-9 processes caspase-3 at the same cleavage site as caspase-8, so it may be redundant in caspase-3 activation. Full activation of the p24 form of caspase-3 is autocatalytic, a step that is blocked by X-IAP. Survivin is the smallest member of the IAP family proteins (12). Its expression is cell-cycle regulated at mitosis in normal tissue (13). However, there are also examples of survivin modulation independent of cell-cycle, like in cytokine-stimulated bone marrow $CD34^+$ cells (14) and endothelial cells (15). Tumors, on the contrary, mostly show a strong and deregulated expression of survivin. Like other IAPs, survivin can inhibit the intrinsic and extrinsic apoptotic pathways, as demonstrated by the inhibition of apoptosis *in vivo* in transgenic mice (16) and by the contrasting enhancement of apoptosis in all cases of survivin inhibition (17). c-FLICE-inhibitory protein (c-FLIP) has homologies to caspase-8 and caspase-10 but lacks protease activity (18). It is therefore believed that c-FLIP recruitment to the DISC, in place of the initiator caspases, blocks their activation. Some overexpression studies indicate c-FLIP as an inhibitor of caspase-8 activation at the TRAIL DISC (19) while others show correlations between FLIP levels and TRAIL resistance (20).

Osteosarcoma is one of the most frequent malignant tumors of the bone. Chemotherapy usually represents the first-choice treatment for this malignancy and different drugs are used in the treatment protocols, including the genotoxic drugs, Doxorubicin (DOX) and Cisplatin (CDDP). Recently, the interest of several groups has focused on the effects of TRAIL on osteosarcoma cells. It is now generally accepted that DOX and CDDP sensitize tumor cells to the apoptogenic effects of TRAIL. The up-regulation of TRAIL-R1 and -R2 by these drugs is generally considered to be responsible for the increased sensitivity to TRAIL. However, Lacour *et al* (29) recently demonstrated that the genotoxic drug-induced sensitization of colon carcinoma cell lines to TRAIL is not mediated by the modulation of its death receptors but rather by the lowering of the signalling threshold required for TRAIL-induced procaspase-8 activation.

We have investigated here the mechanism behind osteosarcoma cell sensitization to the apoptogenic effects of TRAIL by the genotoxic drugs, DOX and CDDP, both at the level of TRAIL-receptor expression and downstream intracellular signalling.

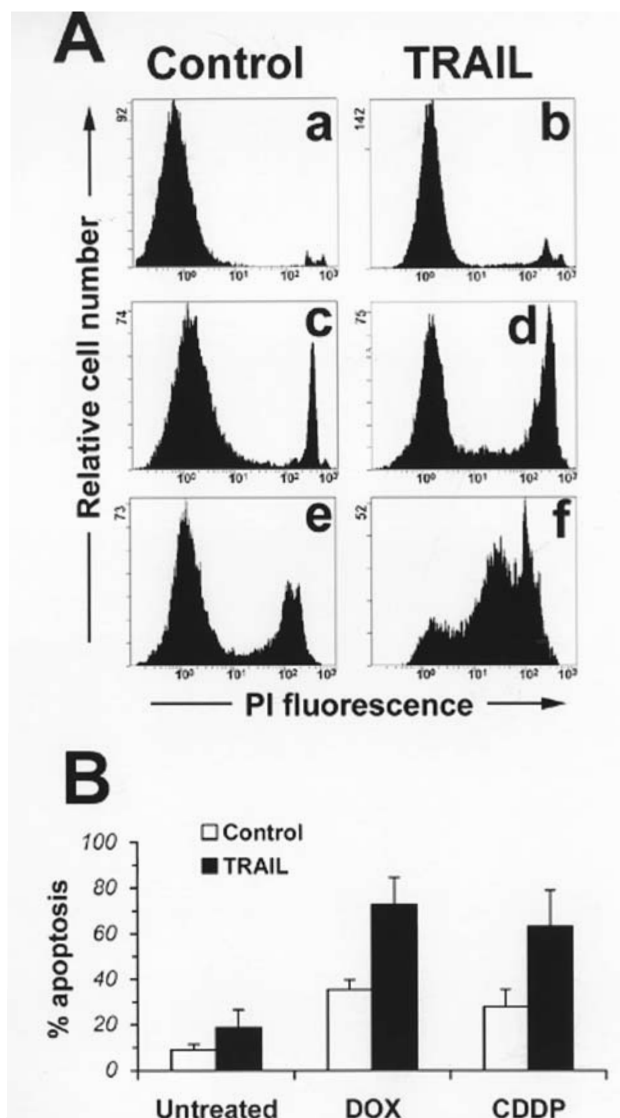

Figure 1. DOX and CDDP sensitize U2OS cells to TRAIL-induced apoptosis. A, flow cytometry quantification of cell death by Propidium Iodide staining (PI). While untreated cells are essentially resistant to TRAIL (histograms a and b), DOX (histograms c and d) and CDDP (histograms e and f) sensitize U2OS cells to TRAIL. Cells were treated for 24 h with 1-μM DOX or 40-μM CDDP and then assayed with 100 ng/ml TRAIL (histograms d and f). A representative of 5 independent experiments is shown. B, quantitative evaluation of TRAIL-induced apoptosis as in A. Data are expressed as means ± SD of 5 independent experiments.

Materials and methods

Cell lines and reagents. The U2OS human osteosarcoma cell line was grown in MEM (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS; Gibco) at an optimal cell density of $2x10^5$ to $6x10^5$ cells/ml.

Cell stocks were used between passages 30 to 65 and were re-established from cryopreserved stock cultures. The Jurkat cell line (American Type Culture Collection, Rockville, Maryland, USA), was maintained in RPMI-1640 (Gibco) supplemented with 10% FBS at 37˚C in 5% CO_2.

Doxorubicin (DOX) and Cisplatin (CDDP) were purchased from Sigma (St. Louis, MO, USA) and were used at 1- and 40-μM concentrations, respectively. Soluble TRAIL was purchased from Alexis Biochemicals (Laufelfingen, Switzerland). Cells were treated for 20 h with 100 ng/ml of TRAIL.

Cell viability and apoptosis. To measure TRAIL-mediated cytotoxicity, $5x10^5$ cells/well were seeded in 12-well microtiter plates (flat bottom) and allowed to adhere overnight. Cells were then treated for 20 h with 100 ng/ml of recombinant soluble TRAIL. Cell viability was determined by flow cytometry after propidium iodide (PI) staining. To assess the effects of chemotherapeutic agents on TRAIL-mediated apoptosis, cells were plated in 12-well plates and allowed to adhere for 24 h overnight. DOX and CDDP were added where appropriate, either alone or in combination with TRAIL, and incubated for an additional 24 h. Cell viability was again determined by staining with PI.

To quantify apoptosis, cells were stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and PI using the annexin V kit (Beckman-Coulter, Miami, FL, USA). Samples

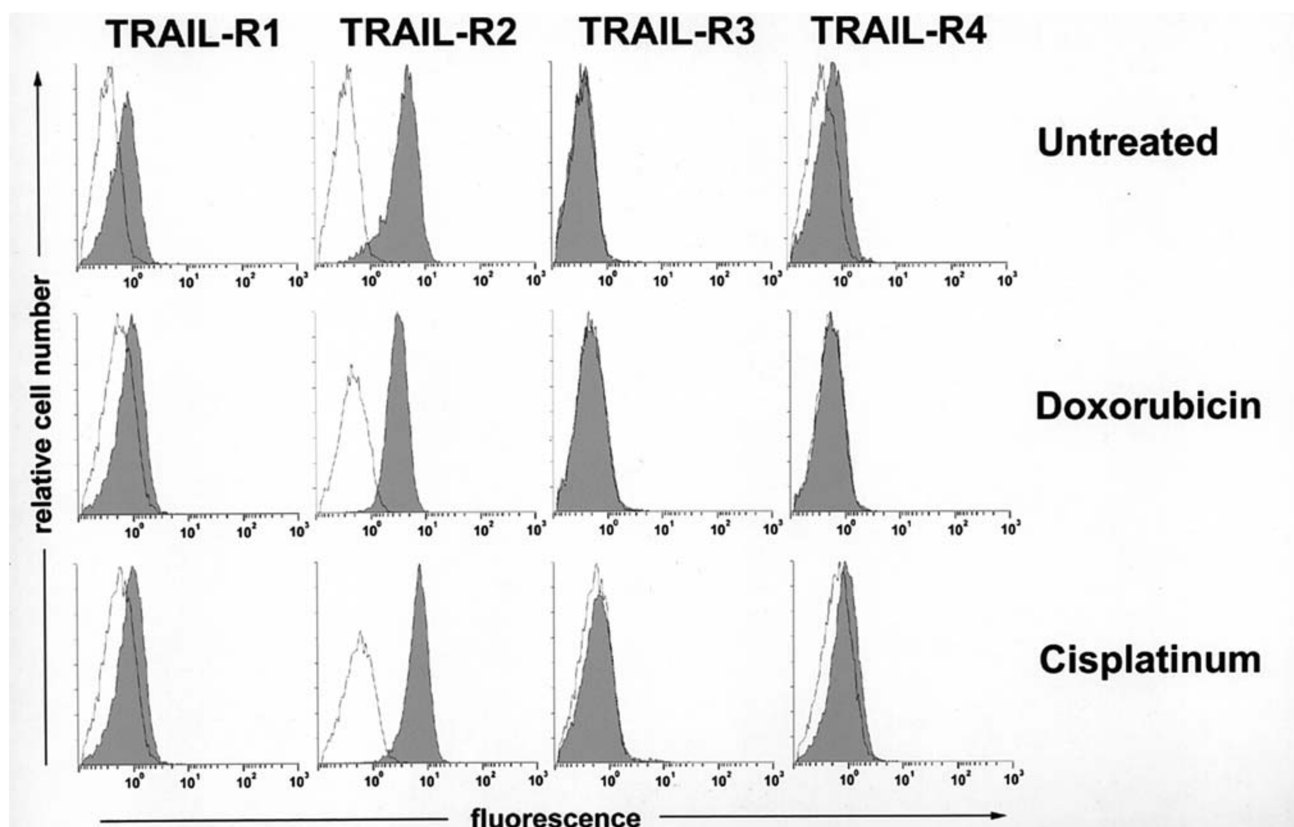

Figure 2. TRAIL-receptor phenotype of untreated and DOX or CDDP-treated U2OS cells. Cell were treated with DOX or CDDP for 24 h and surface TRAIL-R expression was analyzed by flow cytometry. Empty histograms, isotype-matched irrelevant mAb staining.

were then analyzed by an Epics XL flow cytometer (Beckman-Coulter) for the presence of viable (annexin V^- and PI^-), early apoptotic (annexin V^+, PI^-), and late apoptotic/secondary necrotic (annexin V^+ and PI^+) cells. At least 20,000 cells/sample were acquired for the flow cytometric analysis.

TRAIL-R phenotype. Aliquots of 0.5×10^6 cells/experimental point were single- or multiple-labeled by a panel of anti-TRAIL-R mAbs, as described previously (21). Expression of TRAIL-R1, -R2, -R3 and -R4 was analyzed by indirect staining using 1 μg of HS101 anti-human TRAIL-R1, HS201 anti-human TRAIL-R2, HS301 anti-human TRAIL-R3 and HS401 anti-human TRAIL-R4 monoclonal antibody, followed by PE-labeled goat anti-mouse IgG. A specific fluorescence was assessed by using isotype-matched irrelevant mAb followed by the same secondary reagent. Analysis was performed by an Epics XL flow cytometer (Beckman-Coulter) and the Expo ADC software (Beckman-Coulter). Data collected from 20000 cells are reported as either percentage of positive cells or mean fluorescence intensity (MFI) values.

SiRNA design and transfection. siRNA technology was used to silence both TRAIL-R2 and X-IAP expression. For TRAIL-R2, double-strand siRNAs (dsRNA) were designed to target sequences corresponding to nt 1222-1242, 1305-1325, 1393-1413 and 1439-1459 on human TRAIL-R2 mRNA (AFO16459). The target sequences were 5'-AAGTT GGGCC TCATG GACAAT-3', 5'-AAAGT GGGTC AACAA AACCGG-3', 5'-AAGAT TGAGG ACCAC TTGTTG-3', and 5'-AAGGT AATGC AGACT CTGCCA-3'. For X-IAP, double-strand siRNA were designed to target sequences corresponding to nt 53-73, 136-156, 807-827 and 989-1009 on human X-IAP mRNA (U45880). The target sequences were 5'-AAGGA TCTAA AACTT GTGTAC-3', 5'-AATTT TCCAA GTGGT AGTCCT-3', 5'-AAATC CATCC ATGGC AGATTA-3' and 5'-AACAT GCTAA ATGGT ATCCAG-3'. The selected sequences were screened against all known human genes using a BLAST search to confirm that only human TRAIL-R2 mRNA or X-IAP would be targeted. The respective sense and antisense RNA sequences were synthesized by Silencer siRNA construction kit (Ambion, Austin, Texas). Two non-specific siRNA duplexes containing the same nucleotides, but in irregular sequence (i.e. scrambled TRAIL-R2 siRNA and X-IAP siRNA), were prepared according to the manufacturer's protocol and used as controls.

U2OS cells, plated into 6-well plates at a density of 5×10^5 cells/well, were transfected with 400 nM of siRNA and 10 μl of SuperFect transfection reagent (Qiagen, Hilden, Germany) in 760 μl of MEM medium. After a 3-h culture at 37˚C, the transfection mixture was aspirated and substituted with complete growth medium. The cells (floating plus attached cells) were harvested for semiquantitative RT-PCR and flow cytometric protein expression analysis 6, 24 or 48 h after transfection. The transfection efficiency was assayed by delivering a green fluorescence protein (GFP) expression vector, obtaining a number of GFP^+PI^- cells >95% of total cultures.

Semiquantitative RT-PCR analysis. Total RNA was isolated using the RNeasy mini kit (Qiagen). Total RNA (1 μg) was

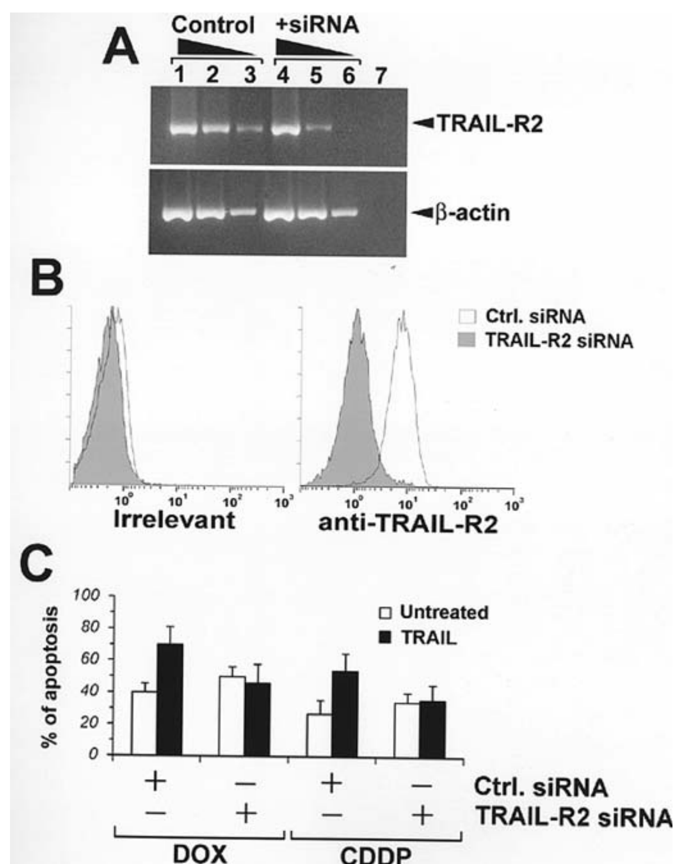

Figure 3. siRNA inhibition of TRAIL-R2 synthesis and expression in U2OS cells. A, semiquantitative RT-PCR of TRAIL-R2 mRNA in TRAIL-R2 siRNA transfected cells. Control, cells transfected with scrambled TRAIL-R2 siRNA; +siRNA, cells transfected with TRAIL-R2 siRNA. cDNA dilutions were 1:10 (lanes 1 and 4), 1:100 (lanes 2 and 5) and 1:1000 (lanes 3 and 6). Lane 7, negative control. B, surface indirect immunophenotype by anti-TRAIL-R2 mAb and anti-Ig-PE second step reagent. Grey histograms, TRAIL-R2 siRNA-treated U2OS cells. Cells were transfected with siRNAs and surface expression of TRAIL-R2 was analyzed by flow cytometry. Empty histograms, scrambled TRAIL-R2 siRNA, negative control (Ctrl. siRNA). C, prevention of TRAIL-induced apoptosis by anti-TRAIL-R2 siRNA transfection. DOX and CDDP pre-treated U2OS cells were cultured for 24 h with or without TRAIL (filled or empty bars, respectively). TRAIL-induced apoptosis was prevented by anti-TRAIL-R2 siRNA transfection (TRAIL-R2 siRNA) but not by scrambled siRNAs (Ctrl. siRNA). The percentage of propidium iodide (PI)-positive cells is shown on the vertical axis as mean ± SD for 4 independent experiments.

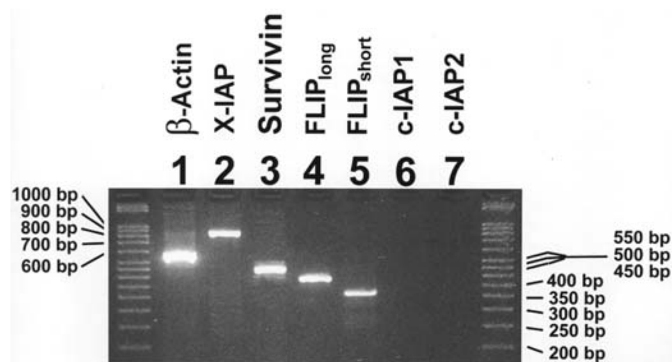

Figure 4. RT-PCR analysis of inhibitor of apoptosis (IAP) expression in U2OS cells. Equal amounts of total cDNA were amplified by PCR to detect the indicated genes. c-IAP-1 and c-IAP-2 are not expressed in this cell line.

reverse transcribed with MMV reverse transcriptase and progressive dilutions (1:50, 1:100, 1:1000) were subjected to PCR amplification to generate specific sequences for ß-actin, TRAIL-R1, -R2, -R3, and -R4, (22,23) c-IAP-1, c-IAP-2, X-IAP, survivin (24), and $FLIP_{Long}$ and $FLIP_{Short}$ (25). Reverse transcription was performed using a thermal programme of 25˚C for 5 min, 42˚C for 45 min and 70˚C for 15 min. PCR was performed under the following reaction conditions: 94˚C for 1 min, 52˚C (c-IAP-1), 56˚C (XIAP), 58˚C ($FLIP_{Long}$ and $FLIP_{Short}$) or 60˚C (c-IAP-2 and survivin) for 1 min, 72˚C for 1 min and a final extension at 72˚C for 5 min. We used 35 cycles of amplification for ß-actin and IAPs. The sequences of primers used for PCR were as follows: c-IAP-1, 5'-ATGAA CATAC TAGAA AACAGC-3' (sense) and 5'-CCTGT CCTTT AATTC TTATCA-3' (antisense); c-IAP-2, 5'-TGACT TTTCC TGTGA ACTCT-3' (sense) and 5'-GCCTT TCATT CGTAT CAAGA-3' (antisense); X-IAP, 5'-GCAGG GTTTC TTTAT ACTGG-3' (sense) and 5'-TGTCC CTTCT GTTCT AACAG-3' (antisense); survivin, 5'-GCATGG GTGCC CCGAC GTTG-3' (sense) and 5'-GCTCC GGCCA GAGGC CTCAA-3' (antisense); ß-actin, 5'-TGACG GGGTC ACCCA CACTG TGCCC ATCTA-3' (sense) and 5'-CTAGA AGCAT TTGCG GTGGA CGATG GAGGG-3' (antisense); $FLIP_{Long}$, 5'-CGAGG CAAGA TAAGC AAGGA-3' (sense) and 5'-TGACT GGTTC TTGTT GAGCG-3' (antisense); $FLIP_{Short}$, 5'-TCAGG AACCC TCACC TTGTT-3' (sense) and 5'-ATCAG GACAA TGGGC ATAGG-3' (antisense); TRAIL-R1, 5'-CTGAG CAACG CAGAC TCGCT GTCCAC-3' (sense) and 5'-TCCAA GGACA CGGCA GAGCC TGTGC CAT-3' (antisense); TRAIL-R2, 5'-GCCTC ATGGA CAATG AGATA AAGGT GGCT-3' (sense) and 5'-CCAAAT CTCAA AGTAC GCACA AACGG-3' (antisense); TRAIL-R3, 5'-GAAGAA TTTGG TGCCA ATGCC ACT G-3' (sense) and 5'-CTCTT GGACTT GGCTGG GAGAT GTG-3' (antisense); and TRAIL-R4, 5'-CTTTT CCGGCG GCGTTC ATGTC CTTC-3' (sense) and 5'-GTTTC TTCCA GGCTG CTTC CCTT TGTAG-3' (antisense). PCR-amplified products were resolved and electrophoresed on 2% agarose gel and visualised with ethidium bromide.

Results

U2OS osteosarcoma cells express TRAIL-R2 and die by TRAIL-induced apoptosis. We used Propidium Iodide staining and flow cytometry to assess apoptosis induced by soluble TRAIL on cultured U2OS cells. Cultured, healthy U2OS cells are virtually insensitive to 24-h treatment with 100 ng/ml of soluble TRAIL (Fig. 1A, a and b). On the contrary, the treatment with 1-μM DOX or 40-μM CDDP for 24 h before TRAIL administration, sensitizes U2OS cell cultures to the apoptogenic effects of TRAIL (Figs. 1A, c-f, and 1B), with DOX showing a stronger effect than CDDP.

Cultured U2OS cells dimly express TRAIL-R1 and TRAIL-R4, while showing a neat surface expression of TRAIL-R2. TRAIL- R3 is not expressed at all by U2OS cells (Fig. 2, top line). Neither DOX nor CDDP treatment altered the expression pattern of TRAIL receptors on U2OS cells (Fig. 2, middle and bottom lines).

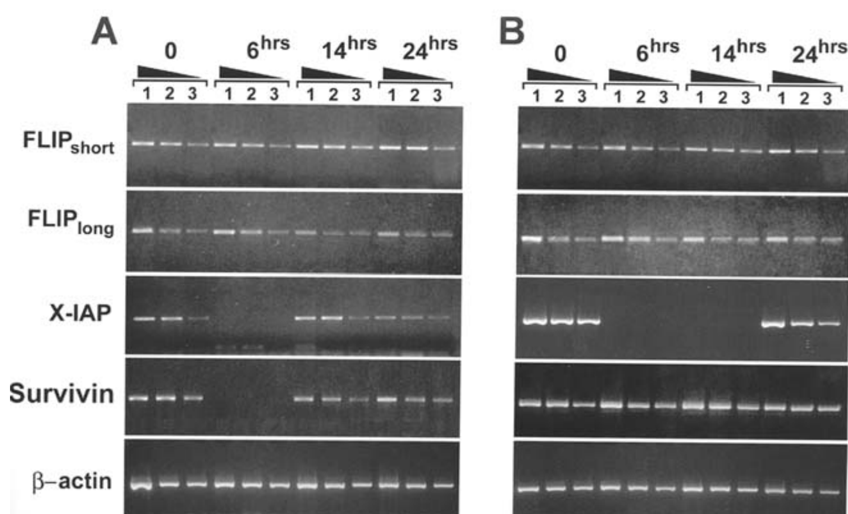

Figure 5. Semiquantitative RT-PCR analysis of FLIP, X-IAP and survivin in U2OS cells after 6, 14 and 24 h of treatment with 1-μM DOX (A) or 40-μM CDDP (B). cDNA was diluted 1:10 (lane 1), 1:100 (lane 2), or 1:1000 (lane 3). X-IAP and survivin are downmodulated by DOX at 6 h treatment, while only X-IAP is downmodulated by CDDP at 6 and 24 h.

TRAIL-R2 is responsible for TRAIL-induced U2OS cell apoptosis. To formally prove that the apoptogenic effects of soluble TRAIL on U2OS cells were mediated by TRAIL-R2, we designed and generated 4 siRNAs targeting TRAIL-R2 mRNA sequences corresponding to nucleotides 1222-1242, 1305-1325, 1393-1413 and 1439-1459, respectively.

Transfection of these siRNAs to proliferating U2OS cells strongly reduced TRAIL-R2 mRNA expression, as demonstrated by semiquantitative RT-PCR (Fig. 3A). The expression of surface TRAIL-R2 was consequently abolished (Fig. 3B). Kinetic experiments showed that the downmodulation of TRAIL-R2 mRNA and protein generated by specific siRNAs lasted for 48 h, after which the levels of the protein started to increase again (data not shown). During the period of surface TRAIL-R2 downmodulation, DOX or CDDP-treated U2OS were completely resistant to TRAIL-induced apoptosis (Fig. 3C), clearly showing that TRAIL-R2 is responsible for the apoptogenic effects of TRAIL.

DOX and CDDP induce a selective down-modulation of X-IAP. Given that TRAIL-R2 is responsible for TRAIL-induced apoptosis, while its expression is not modified by genotoxic drugs, we asked whether intracellular components of the signalling cascade downstream TRAIL-R2 could be responsible for the observed sensitizing effects of DOX and CDDP to TRAIL-induced apoptosis.

We therefore analyzed, using RT-PCR, the expression of the major known apoptosis inhibitors in U2OS cells. Fig. 4 shows the expression profile at the mRNA level of c-FLIP (long and short forms), X-IAP, c-IAP-1, c-IAP-2 and survivin. We found that U2OS cells do not express c-IAP-1 and c-IAP-2, while they constitutively express both forms of c-FLIP, X-IAP and survivin. Semiquantitative RT-PCR analysis of c-FLIP, X-IAP and survivin (Fig. 5A and B) showed that the expression of neither form (long or short) of c-FLIP is affected by DOX or CDDP. On the contrary, both genotoxic drugs completely abrogated the mRNA expression of X-IAP after 6 h of treatment, a time frame that is compatible with the subsequent sensitization to the apoptogenic effect of TRAIL. Survivin mRNA was downmodulated by DOX but not by CDDP treatment (Fig. 5B). We therefore decided to selectively downmodulate X-IAP by specifically designed siRNA in U2OS cells to see if we were able to generate an enhancement of their sensitivity to TRAIL. Transfection of U2OS cells with X-IAP siRNA completely abrogated X-IAP protein synthesis, as shown in Fig. 6A.

In the absence of X-IAP, U2OS cells became sensitive to the apoptogenic effects of TRAIL 6 h after transfection and were still sensitive 24 h later (Fig. 6B and C)

Discussion

A major goal of pharmacological tumor therapy is the optimization of response to chemotherapy by increasing drug selectivity. Since TRAIL is mostly effective in tumor cells while sparing normal cells, it offers great promise for the pharmacological treatment of cancer. Although the identification of TRAIL 'decoy' receptors, R3 and R4, initially led to the conclusion that their expression might represent the only mechanism for a cell that also expresses the TRAIL death receptors to escape apoptosis, accumulating evidence has shown that the modulation of the intracellular signalling emanating from death receptors may be, at least, as relevant for cell survival. It is now clear that tumor cells have several possible strategies for escaping TRAIL-induced apoptosis, ranging from the modulation of TRAIL receptor expression to the modulation of the intracellular key mediators of caspase activation. Tumor cells that are resistant to TRAIL can, however, be sensitized to its apoptogenic effects by associated chemotherapeutic agents active on the cellular mechanisms of resistance.

Our data essentially demonstrate that: i) only TRAIL-R2 is expressed on the surface of U2OS osteosarcoma cells that, however, are resistant to the apoptogenic effect of soluble TRAIL; the genotoxic drugs DOX and CDDP, which sensitize U2OS cells to the apoptogenic effects of TRAIL, do not

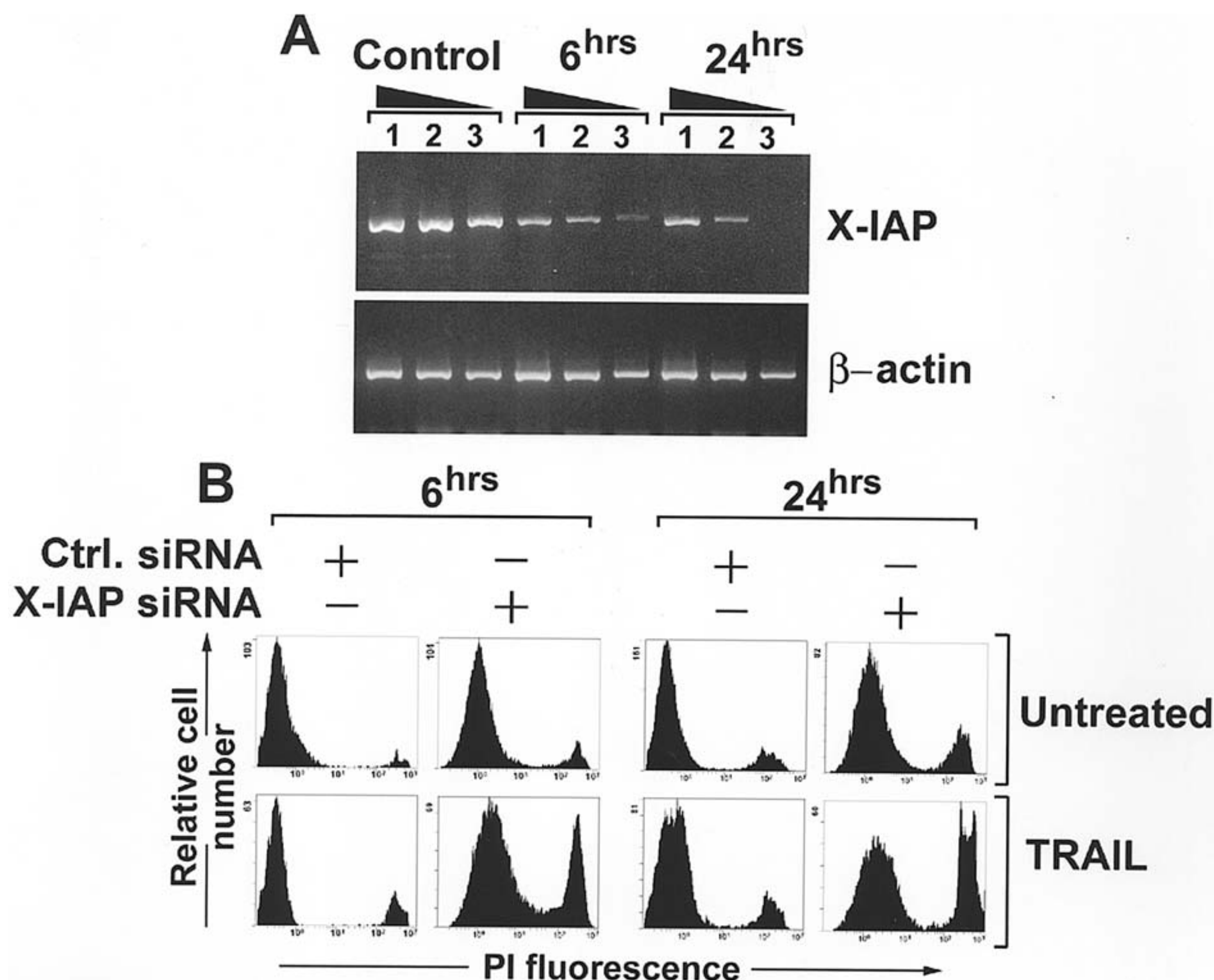

Figure 6. X-IAP siRNA transfection sensitizes U2OS cells to TRAIL-induced apoptosis. A, semiquantitative RT-PCR analysis of X-IAP mRNA after X-IAP siRNA transfection (6 and 24 h post transfection) or scrambled X-IAP siRNA transfection (Control). X-IAP mRNA is already downmodulated after 6 h. cDNA dilutions were 1:10 (lane 1), 1:100 (lane 2), and 1:1000 (lane 3). B, flow cytometry evaluation of TRAIL-induced apoptosis of U2OS cells 6 and 24 h after X-IAP siRNA transfection or scrambled X-IAP siRNA transfection (Ctrl. siRNA). Cells were stained with Propidium Iodide (PI), the amount of incorporated dye is shown on the horizontal axis. A representative of 3 independent experiments is shown.

modulate the surface expression of TRAIL receptors; ii) only TRAIL-R2 is responsible for the induction of apoptosis of drug-sensitized U2OS cells; and iii) DOX and CDDP down-modulate the expression of X-IAP, one major intracellular inhibitor of caspase activation downstream TRAIL receptors, which is responsible for the sensitization to TRAIL of U2OS cells.

Several osteosarcoma cell lines can be sensitized to TRAIL by anticancer drugs such as Etoposide, Doxorubicin and Cisplatin, but not by Methotrexate or Cyclophosphamide. The reported mechanism that accounts for the acquired sensitivity to TRAIL is the up-regulation of death receptors (26), while the up-regulation of decoy receptors accounts for the acquisition of TRAIL resistance (27). We show that the surface expression of TRAIL receptors is not affected by DOX and CDDP. These data parallel those obtained by van Valen and co-workers on human osteosarcoma cells in which no change of TRAIL-receptor gene expression profile was detected by real-time PCR (28). On the contrary, the intracellular levels of X-IAP are deeply affected by both drugs with a kinetics that is anticipated with respect to the biological effects observed. Similar findings were also recently obtained in colon cancer cell lines (29), where DOX and CDDP have been reported to lower the threshold required for the activation of procaspase 8, without affecting TRAIL receptor expression.

In human lung carcinoma cells, DOX has been reported to induce the NF-kB-dependent expression of both IAPs and TRAIL-R2 (30). It is therefore conceivable that tumors of different origins may respond with different and even opposing modalities to the same drug. Osteosarcomas, however, can be rendered sensitive to the apoptogenic effects of TRAIL by genotoxic drugs, not by surface receptor modulation but by the removal of the intracellular signalling blockage downstream TRAIL death receptors. This implies that future combined regimens of genotoxin-based chemotherapy concurrent with TRAIL will have relevant consequences for a more effective treatment of osteosarcoma.

Acknowledgements

This work was supported by CARIPARMA, Italian Ministry of University and Research FIRB and Cofin grants and CARISBO Foundation. We are grateful to Instrumentation Laboratory, Italy, for their technical support.

References

1. Wiley SR, Schooley K, Smolak PJ, Din WS, Huang CP, Nicholl JK, Sutherland GR, Smith TD, Rauch C, Smith CA, *et al*: Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 3: 673-682, 1995.
2. LeBlanc HN and Ashkenazi A: Apo2L/TRAIL and its death and decoy receptors. Cell Death Differ 10: 66-75, 2003.
3. Smyth MJ, Takeda K, Hayakawa Y, Peschon JJ, van den Brink MR and Yagita H: Nature's TRAIL - on a path to cancer immunotherapy. Immunity 18: 1-6, 2003.
4. Takeda K, Smyth MJ, Cretney E, Hayakawa Y, Kayagaki N, Yagita H and Okumura K: Critical role for tumor necrosis factor-related apoptosis-inducing ligand in immune surveillance against tumor development. J Exp Med 195: 161-169, 2002.
5. Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D, Ramakrishnan L, Gray CL, Baker K, Wood WI, Goddard AD, Godowski P and Ashkenazi A: Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science 277: 818-821, 1997.
6. Ashkenazi A and Dixit VM: Apoptosis control by death and decoy receptors. Curr Opin Cell Biol 11: 255-260, 1999.
7. Jo M, Kim TH, Seol DW, Esplen JE, Dorko K, Billiar TR and Strom SC: Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. Nat Med 6: 564-567, 2000.
8. Nesterov A, Ivashchenko Y and Kraft AS: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) triggers apoptosis in normal prostate epithelial cells. Oncogene 21: 1135-1140, 2002.
9. Nitsch R, Bechmann I, Deisz RA, Haas D, Lehmann TN, Wendling U and Zipp F: Human brain-cell death induced by tumour-necrosis-factor-related apoptosis-inducing ligand (TRAIL). Lancet 356: 827-828, 2000.
10. MacFarlane M: TRAIL-induced signalling and apoptosis. Toxicol Lett 139: 89-97, 2003.
11. Liston P, Young SS, Mackenzie AE and Korneluk RG: Life and death decisions: the role of the IAPs in modulating programmed cell death. Apoptosis 2: 423-441, 1997.
12. Altieri DC: Survivin, versatile modulation of cell division and apoptosis in cancer. Oncogene 22: 8581-8589, 2003.
13. Kobayashi K, Hatano M, Otaki M, Ogasawara T and Tokuhisa T: Expression of a murine homologue of the inhibitor of apoptosis protein is related to cell proliferation. Proc Natl Acad Sci USA 96: 1457-1462, 1999.
14. Fukuda S and Pelus LM: Regulation of the inhibitor-of-apoptosis family member survivin in normal cord blood and bone marrow CD34(+) cells by hematopoietic growth factors: implication of survivin expression in normal hematopoiesis. Blood 98: 2091-2100, 2001.
15. Harfouche R, Hassessian HM, Guo Y, Faivre V, Srikant CB, Yancopoulos GD and Hussain SN: Mechanisms which mediate the antiapoptotic effects of angiopoietin-1 on endothelial cells. Microvasc Res 64: 135-147, 2002.
16. Grossman D, Kim PJ, Blanc-Brude OP, Brash DE, Tognin S, Marchisio PC and Altieri DC: Transgenic expression of survivin in keratinocytes counteracts UVB-induced apoptosis and cooperates with loss of p53. J Clin Invest 108: 991-999, 2001.
17. Williams NS, Gaynor RB, Scoggin S, Verma U, Gokaslan T, Simmang C, Fleming J, Tavana D, Frenkel E and Becerra C: Identification and validation of genes involved in the pathogenesis of colorectal cancer using cDNA microarrays and RNA interference. Clin Cancer Res 9: 931-946, 2003.
18. Tschopp J, Irmler M and Thome M: Inhibition of fas death signals by FLIPs. Curr Opin Immunol 10: 552-558, 1998.
19. Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE and Tschopp J: Inhibition of death receptor signals by cellular FLIP. Nature 388: 190-195, 1997.
20. Cretney E, Takeda K, Yagita H, Glaccum M, Peschon JJ and Smyth MJ: Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. J Immunol 168: 1356-1361, 2002.
21. Mirandola P, Secchiero P, Pierpaoli S, Visani G, Zamai L, Vitale M, Capitani S and Zauli G: Infection of CD34(+) hematopoietic progenitor cells by human herpesvirus 7 (HHV-7). Blood 96: 126-131, 2000.
22. Secchiero P, Mirandola P, Zella D, Celeghini C, Gonelli A, Vitale M, Capitani S and Zauli G: Human herpesvirus 7 induces the functional up-regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) coupled to TRAIL-R1 down-modulation in CD4(+) T cells. Blood 98: 2474-2481, 2001.
23. Secchiero P, Gonelli A, Mirandola P, Melloni E, Zamai L, Celeghini C, Milani D and Zauli G: Tumor necrosis factor-related apoptosis-inducing ligand induces monocytic maturation of leukemic and normal myeloid precursors through a caspase-dependent pathway. Blood 100: 2421-2429, 2002.
24. Notarbartolo M, Cervello M, Dusonchet L, Cusimano A and D'Alessandro N: Resistance to diverse apoptotic triggers in multidrug resistant HL60 cells and its possible relationship to the expression of P-glycoprotein, Fas and of the novel anti-apoptosis factors IAP (inhibitory of apoptosis proteins). Cancer Lett 180: 91-101, 2002.
25. Hopkins-Donaldson S, Bodmer JL, Bourloud KB, Brognara CB, Tschopp J and Gross N: Loss of caspase-8 expression in highly malignant human neuroblastoma cells correlates with resistance to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis. Cancer Res 60: 4315-4319, 2000.
26. Evdokiou A, Bouralexis S, Atkins GJ, Chai F, Hay S, Clayer M and Findlay DM: Chemotherapeutic agents sensitize osteogenic sarcoma cells, but not normal human bone cells, to Apo2L/TRAIL-induced apoptosis. Int J Cancer 99: 491-504, 2002.
27. Bouralexis S, Findlay DM, Atkins GJ, Labrinidis A, Hay S and Evdokiou A: Progressive resistance of BTK-143 osteosarcoma cells to Apo2L/TRAIL-induced apoptosis is mediated by acquisition of DcR2/TRAIL-R4 expression: resensitisation with chemotherapy. Br J Cancer 89: 206-214, 2003.
28. Van Valen F, Fulda S, Schafer KL, Truckenbrod B, Hotfilder M, Poremba C, Debatin KM and Winkelmann W: Selective and nonselective toxicity of TRAIL/Apo2L combined with chemotherapy in human bone tumour cells vs. normal human cells. Int J Cancer 107: 929-940, 2003.
29. Lacour S, Hammann A, Wotawa A, Corcos L, Solary E and Dimanche-Boitrel MT: Anticancer agents sensitize tumor cells to tumor necrosis factor-related apoptosis-inducing ligand-mediated caspase-8 activation and apoptosis. Cancer Res 61: 1645-1651, 2001.
30. Spalding AC, Jotte RM, Scheinman RI, Geraci MW, Clarke P, Tyler KL and Johnson GL: TRAIL and inhibitors of apoptosis are opposing determinants for NF-kappaB-dependent, genotoxin-induced apoptosis of cancer cells. Oncogene 21: 260-271, 2002.