Combining radiotherapy with death ligands in cancer treatment: feasibility and molecular mechanisms
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Radiation and anti-cancer drugs facilitate mitochondrial bypass by CD95/Fas via c-FLIP downregulation

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Key words: CD95/Fas, apoptosis, c-FLIP, DNA damage, mitochondria, Type II, Bcl-2

Abbreviations: c-FLIP, cellular FLICE-like inhibitory protein; DISC, death-inducing signalling complex; FADD, Fas-associated protein with death domain; HDAC, histone deacetylase; JNK, Jun N-terminal kinase; L, ligand; mAb; monoclonal antibody; R, receptor; RNAi, RNA interference; TNF, tumor necrosis factor, TRAIL, TNF-related apoptosis-inducing ligand; TSA, Trichostatin A; UV, ultra violet; VPA, Valproic acid

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In many tumor cell types, ionizing radiation or DNA damaging anti-cancer drugs enhance sensitivity to death receptor-mediated apoptosis, which is of great clinical interest. APO010, a novel recombinant form of CD95/Fas ligand is currently in a Phase I dose-escalation trial in patients with solid tumors. To investigate the potential of combined modality treatment with APO010, we used p53-mutant Jurkat T leukemic cells, in which the mitochondrial pathway was blocked by Bcl-2 overexpression. These cells were largely resistant to APO010, but were effectively killed after pretreatment with ionizing- or UV radiation, etoposide, histone deacetylase- or proteasome inhibitors. In the combined setting, mitochondrial resistance imposed by Bcl-2 was not broken and membrane levels of CD95 were not increased. Instead, pretreatment with the different stimuli led in all cases to a large reduction in c-FLIP \(_{L,S}\) protein levels, suggesting a common mechanism by which Jurkat-Bcl-2 cells were sensitized for apoptotic execution by APO010. Upon deliberate c-FLIP \(_{L,S}\) downregulation by RNA interference, the mitochondrion-independent pathway for caspase activation was greatly facilitated and the capability of the sensitizing stimuli to increment apoptotic execution by APO010 was largely overruled. We conclude that c-FLIP downregulation is a common mechanism by which diverse anti-cancer regimens facilitate tumor cell execution by CD95/Fas via the direct pathway of caspase activation.

Introduction

Inhibition of apoptotic pathways critically contributes to cancer development. Consequently, tumor cells are often ‘addicted’ to overexpression of anti-apoptotic proteins such as Bcl-2 for their survival [1]. Targeting apoptosis regulators in cancer treatment is therefore interesting, as overcoming blocks in apoptosis may render cancer cells more susceptible to death than normal cells.

Ionizing radiation (IR) and DNA-damaging chemotherapeutics are effective in cancer treatment, because they hamper clonogenicity of tumor cells in a variety of ways. The DNA damage they induce triggers an -often p53-dependent- cell cycle arrest allowing for DNA repair. Failing this, irreversible cell cycle arrest, death due to mitotic catastrophe, or apoptotic cell death may ensue [2]. Apoptosis induction by DNA-damaging anti-cancer regimens is blocked by Bcl-2 overexpression, indicating that apoptosis signalling proceeds via the intrinsic, mitochondrial pathway [3]. In this pathway, pro-apoptotic members of the Bcl-2 family induce mitochondrial permeabilization and the subsequent release of Cytochrome c (Cyt c) and other mediators allows for Caspase-9 and effector caspase activation [4, 5].

In hematopoietic tumors, the apoptotic response to IR and DNA-damaging drugs is generally evident and apoptosis resistance can contribute to treatment resistance [6]. Solid tumors are less apoptosis prone when treated with conventional regimens. Therefore, the death receptor ligand TRAIL was of immediate clinical interest when it effectively induced apoptosis in many long-term established solid tumor cell lines [7, 8]. TRAIL proved non-toxic to normal tissue in animal models and moved rapidly towards the clinical testing phase [9, 10]. Currently, TRAIL receptor agonists are in Phase I and Phase II clinical trials, either as single modality treatment or in combination with conventional or novel anti-cancer drugs [10]. Combined modality treatment with death receptor agonists and DNA-damaging anti-cancer regimens was expected to be more effective than single agent treatment, given the distinct cell death pathways they engage. TRAIL-R1/2 and CD95 death receptors induce apoptosis by directly recruiting and activating caspases [11]. Upon ligand binding, they recruit FADD, inducer Caspase-8 and/or -10 and c-FLIP molecules [11, 12]. In the death-inducing signaling complex (DISC) thus formed at the cytoplasmic receptor tail, Caspase-8/10 are activated. Depending on their expression level, c-FLIP molecules can facilitate or inhibit this activation [13]. Upon self-processing by proteolysis, Caspase-8/10 are released into the cytosol, where they find effector caspsases as their targets. In this way, death receptors can in principle execute apoptosis in a mitochondrion-independent manner. However, by cleaving and activating BH3-only protein Bid, Caspase-8/10 also connect death receptors to the mitochondrial pathway [5].

The efficacy of TRAIL receptors and CD95 to kill cells via the direct pathway for effector caspase activation depends on the cell type. In so-called Type II cells, the mitochondrial contribution is required for apoptotic execution, whereas in Type I cells it is not, as ascertained by Bcl-2 overexpression. This relates to the efficacy of Caspase-8/10 activation in the DISC, which seems a cell-intrinsic phenomenon [14]. The mitochondrial bypass by death receptors is of interest, since it potentially overrules intrinsic apoptosis resistance in tumor cells, as e.g. imposed by Bcl-2 overexpression. Although TRAIL receptors and CD95/Fas induce
apoptosis by highly similar mechanisms [11, 12], CD95 agonists were not pursued for cancer therapy, due to severe hepatotoxicity upon systemic administration in mice [15]. Recently however, a Phase I clinical trial has been initiated at TopoTarget (ClinicalTrials.gov identifier: NCT00437736) with APO010 (MegaFas Ligand). This is a fusion protein of the extracellular domain of CD95L/FasL and the collagen domain of the serum protein ACRP30. The dimer of CD95L trimers that is thus formed can crosslink two adjacent CD95 trimers, rendering APO010 highly agonistic as compared to other available CD95 agonists [16]. It is therefore envisioned that combined modality treatment with low concentrations of this agent is effective and has acceptable toxicity, especially in settings of local therapy, such as radiotherapy of local perfusion with anti-cancer drugs. We have recently shown that in p53 mutant Jurkat cells engineered to overexpress Bcl-2 (J16-Bcl-2), as well as in solid tumor types APO010 and IR had a combined effect on cell death induction, both in short-term apoptosis assays and in clonogenic survival assays [17].

We demonstrate here that pre-treatment with IR, but also with UV radiation and various anti-cancer drugs sensitizes J16-Bcl-2 cells to APO010-mediated apoptosis, without breaking mitochondrial resistance imposed by Bcl-2 or upregulating CD95 membrane levels. The underlying mechanism concerns downregulation of c-FLIP long (L) and short (S) isoforms, which enables apoptotic execution by CD95 via the direct pathway for caspase activation. Since Jurkat cells are prototype Type II cells [18], this implies a conversion from Type II to Type I signaling by CD95.

Results

Combining APO010 with chemotherapy overcomes apoptosis resistance imposed by Bcl-2

IR and APO010 showed a combined effect on the apoptotic response of J16-Bcl-2 cells [17]. To investigate whether this also held true for DNA-damaging drugs, we examined combined effects of APO010 and the topoisomerase inhibitor etoposide. Cells were stimulated with different doses of APO010 in the presence or absence of etoposide and apoptosis was read out 48h later by nuclear fragmentation. As expected from the Type II nature of Jurkat cells [18], J16-Bcl-2 cells showed low sensitivity to APO010 alone, but the apoptotic response was significantly enhanced in combination with etoposide (Figure 1a).

To investigate whether etoposide pre-conditioned J16-Bcl-2 cells to more efficiently activate effector caspases upon APO010 stimulation, cells were first incubated with etoposide and 15h later stimulated with APO010. Whereas APO010 alone readily induced Caspase-3 activation in parental J16 cells, J16-Bcl-2 cells were relatively resistant. In etoposide-pretreated J16-Bcl-2 cells, Caspase-3 activation was significantly enhanced, approximating the response seen in J16 cells (Figure 1b).

Since effector caspases are directly activated by Caspase-8/10 in the mitochondrion-independent death receptor pathway, we investigated whether pre-treatment...
with etoposide improved the capacity APO010 to activate Caspase-8. In etoposide-treated J16-Bcl-2 cells, Caspase-8 activation -as read out by appearance of the p41/p43 and p18 cleavage products- was first observed at 2h after APO010 stimulation (Figure 1c). Caspase-3 activity was also apparent by proteolytic processing of its substrate PARP. At each subsequent time point, Caspase-8 activation and PARP processing were more pronounced in cells incubated with etoposide than in untreated cells.

We conclude that etoposide treatment conditioned J16-Bcl-2 cells to more efficiently activate inducer and effector caspases upon APO010 stimulation.

Mitochondrial resistance imposed by Bcl-2 is not broken upon combined treatment
It was possible that in etoposide-treated cells, APO010 evoked effector caspase activation by overruling the mitochondrial resistance imposed by Bcl-2. Therefore, we monitored Cyt c release in parental J16 cells and in J16-Bcl-2 cells that were pretreated with etoposide or not. J16 cells showed Cyt c release upon APO010 treatment, but J16-Bcl-2 cells did not throughout the 8h time course, showing the effectiveness of Bcl-2 in preventing mitochondrial permeabilization (Figure 2a).

The primary data depicted in Figure 2b illustrate this point. Importantly, J16-Bcl-2 cells that were pretreated with etoposide showed no significant increase in Cyt c release after APO010 stimulation, even after 8h (Figure 2b). These were the same samples analyzed for Caspase-3 activation in Figure 1b, indicating that effector caspase activation in etoposide-treated J16-Bcl-2 cells was not a consequence of breaking Bcl-2-mediated mitochondrial resistance.

We conclude that after etoposide treatment, APO010 could more efficiently activate effector caspases via the direct, mitochondrion-independent pathway.

Etoposide downregulates c-FLIPL, S, and improves Caspase-8 activation in the DISC
Since Caspase-8 is activated in the DISC, we investigated whether DISC formation was improved in etoposide-treated cells. After 15h incubation in the presence or absence of etoposide, J16-Bcl-2 cells were stimulated with crosslinked CD95L. The combined treatment effect in this setting was verified by enhanced PARP processing in corresponding total cell lysates (Figure 2a).

The DISCs isolated from etoposide pretreated cells showed enhanced Caspase-8 activation as compared to the DISCs from control cells, which was most apparent at the 4h time point (Figure 3a). In the same samples, the DISCs from etoposide-treated cells showed a decrease in processed c-FLIP levels.

Immuno-blotting of total cellular lysates of control and etoposide-treated cells for c-FLIP levels showed that etoposide treatment strongly reduced protein expression of the FLIP L and FLIP S isoforms (Figure 3b). This was a post-translational effect, as c-FLIP mRNA levels were not reduced after etoposide treatment (Supplementary Figure 1a).

These results suggest that downregulation of c-FLIP protein levels by etoposide treatment enhanced the ability of CD95L to activate Caspase-8 in the DISC.
Chapter 5

JNK inhibition reveals relation between c-FLIP downregulation and sensitization to APO010

It was demonstrated previously that activation of the Jun N-terminal Kinase (JNK) pathway through activation of the E3 ubiquitin ligase Itch leads to c-FLIP downregulation [19]. Since etoposide is known to activate the JNK pathway (e.g. [20]), we tested whether JNK inhibition -using the chemical inhibitor SP600125 [21] or the cell permeable L-JNKi peptide [22]- reversed etoposide-induced c-FLIP downregulation and APO010 sensitivity. Etoposide activated JNK in J16-Bcl-2 cells, since both JNK and its target protein c-Jun were phosphorylated upon treatment (Supplementary Figure 2). JNK activity induced by etoposide was strongly reduced by SP600125 and to a lesser extent by L-JNKi peptide, validating the compounds as JNK inhibitors. In the same experiment, etoposide treatment downregulated c-FLIPL/S protein levels. SP600125 and L-JNKi peptide restored c-FLIP protein levels to an extent that was correlated with their inhibitory effect on JNK activity.

Next, we examined the effect of SP600125 on etoposide-mediated sensitization of J16-Bcl-2 cells to CD95L. J16-Bcl-2 cells were pre-treated with etoposide or not, in the presence or absence of the JNK inhibitor. In total cellular lysates and in the CD95L DISC, c-FLIP was downregulated...
by etoposide-treatment. However, co-treatment with SP600125 abrogated this effect, as seen at t=0 and at each successive time point after CD95L stimulation in total cell lysates and at 1h and 4h in the DISC (Figure 4a). Concomitantly, SP600125 reversed etoposide-induced sensitization to CD95L, since the increment in Caspase-8 activation and effector caspase activity (PARP cleavage) seen in etoposide-treated cells did not occur in cells that had been treated with etoposide and SP600125. The effect of the JNK inhibitors on etoposide-mediated sensitization of J16-Bcl-2 cells to APO010-induced Caspase-3 activation was validated by flow cytometry throughout an 8h time course. Etoposide strongly sensitized J16-Bcl-2 cells to APO010-induced Caspase-3 activation, which was efficiently reversed by SP600125 and also partially by L-JNKi peptide (Figure 4b). The ability of SP600125 and L-JNKi to reverse etoposide-mediated sensitization to APO010 correlated with their ability to inhibit c-Jun phosphorylation and to restore c-FLIP levels (Supplementary Figure 2). These results indicate that in J16-Bcl-2 cells, etoposide-mediated activation of the JNK pathway is (partly) responsible for c-FLIP downregulation. Moreover, they suggest that c-FLIP downregulation by etoposide is causally related to the drug’s ability to sensitize J16-Bcl-2 cells to APO010-induced apoptosis.

Downregulation of c-FLIP by RNA interference sensitizes J16-Bcl-2 cells to APO010
To test whether c-FLIP downregulation was sufficient to sensitize J16-Bcl-2 cells to APO010-induced apoptosis, we stably downregulated c-FLIP levels in these cells by RNA interference (RNAi), using retroviral constructs. The different short interfering (si) RNA constructs tested downregulated c-FLIP isoforms to a variable degree. The FLIP#1 construct strongly reduced expression of c-FLIPL and FLIPS, whereas the e.g. the FLIP#4 construct had a clear, but more modest effect (Figure 5a).

Upon treatment of FLIP#1 and FLIP#4 cell lines with APO010, Caspase-3 activation was enhanced as compared to empty vector-transduced cells to a degree that correlated with the extent of c-FLIP downregulation (Figure 5b). As tested in the FLIP#1 transduced cells, Caspase-3 activation at 8h was not accompanied by Cyt c release, indicating that mitochondrial resistance was not broken (Figure 5c).

We next investigated to which extent etoposide treatment could (further) sensitize control, FLIP#1- or FLIP#4 transduced J16-Bcl-2 cells to APO010-induced apoptosis. After pre-treatment with etoposide, cells were stimulated for 8h with APO010. Control cells were efficiently sensitized to APO010 by etoposide in a dose-dependent manner. FLIP#1-transduced cells were very sensitive to treatment with APO010 alone and etoposide treatment incremented the response to a limited degree. FLIP#4-transduced cells were sensitized to lesser extent by c-FLIP siRNA, allowing us to quantify the added effect of etoposide pretreatment (Figure 5d).

For this purpose, Caspase-3 activation upon combined treatment was normalized to Caspase-3 activation by APO010 alone and expressed as fold sensitization. This analysis revealed that etoposide strongly (>4-fold)
sensitized control cells to APO010. Etoposide-mediated sensitization to APO010 in FLIP#4 transduced cells was significantly reduced (Figure 5d).

These results indicate that modulation of c-FLIP levels is an important determinant for APO010 sensitivity in J16-Bcl-2 cells. c-FLIP downregulation greatly facilitated the mitochondrion-independent pathway for effector caspase activation, mimicking etoposide-mediated sensitization to APO010. Moreover, it largely overruled the capability of etoposide to increment apoptotic execution. We conclude that downregulation of c-FLIP protein expression is a major mechanism by which etoposide sensitizes J16-Bcl-2 cells to APO010-induced apoptosis.

Various stimuli that sensitize J16-Bcl-2 cells to APO010 downregulate c-FLIP

Next, we examined whether other stress stimuli could also facilitate APO010-induced apoptosis in J16-Bcl-2 cells. For this purpose, cells were pre-treated with IR, UV radiation, the HDAC inhibitors Trichostatin A (TSA) and Valproic Acid (VPA), or the proteasome inhibitors MG132 and lactacystin. In all cases, pretreatment significantly enhanced APO010-induced apoptosis (Figure 6a-d). In the time-frame of pre-sensitization, IR slightly increased CD95 membrane levels (Figure 6f), but etoposide and UV had no effect, while VPA and TSA significantly downregulated CD95. This ruled out CD95 upregulation at the plasma membrane as mechanism for increased APO010 sensitivity [23]. Interestingly, however, treatment of J16-Bcl-2 cells with all stimuli strongly downregulated c-FLIP protein levels (Figure 6e). In case of IR, UV and VPA, SP600125 could not overrule sensitization (Supplementary Figure 4), which correlated with an inability to restore c-FLIP levels (Supplementary Figure 3 and data not shown).

We therefore conclude that in addition to etoposide, also IR, UV radiation, HDAC and proteasome inhibitors sensitize J16-Bcl-2 cells to APO010 apoptosis, potentially by the same mechanism of c-FLIP downregulation.

Downregulation of c-FLIP by RNAi impairs the ability of different stimuli to sensitize to APO010

Subsequently, we used c-FLIP RNAi to test whether c-FLIP downregulation was a common mechanism by which the various stress stimuli sensitized J16-Bcl-2 cells to APO010-induced apoptosis. Control or FLIP#4-transduced J16-Bcl-2 cells were pretreated with IR, UV, TSA, VPA, MG132, or lactacystin and next stimulated with APO010. After 8h, cells were harvested and analyzed for active Caspase-3 content by flow cytometry. All stimuli strongly sensitized control cells to APO010-induced Caspase-3 activation (Figure 7a). Sensitization was in the order of 4-fold (Figure 7b). However, in FLIP#4 transduced cells, that had intermediate c-FLIP levels (Figure 5a), sensitization was significantly reduced (Figure 7b).
These results indicate that c-FLIP downregulation is a common mechanism by which J16-Bcl-2 cells are sensitized to CD95-mediated apoptosis by a wide range of stimuli.

**Discussion**

Many reports describe combined effects of conventional- or targeted anti-cancer regimens and death receptor agonists in tumor cell killing. For TRAIL receptor agonists in particular, combination treatment proved effective in a variety of tumor types *in vitro* and *in xenograft settings in vivo* [9]. The mechanisms of action of death receptors and conventional anti-cancer regimens predict additivity in treatment outcome upon combination therapy. Moreover, upregulation of death receptors at the plasma membrane in response to DNA damaging regimens may lead to synergy. However, in various cell lines, such as Jurkat leukaemia [23], HT-29 colon carcinoma [24] and Hep3b hepatocellular carcinoma [25], combined effects of TRAIL and DNA damaging regimens could not be explained by changes in receptor membrane expression, raising the question of the underlying molecular mechanism. Exploring such mechanisms is of interest, since they may provide a guideline for rational combination therapy in the clinical setting, once molecular diagnosis in treatment guidance is firmly in place.

In this study, we show that pretreatment of J16-Bcl-2 cells with conventional anti-cancer regimens IR and etoposide, but also with UV radiation, HDAC inhibitors and proteasome inhibitors sensitized them to APO010-induced apoptosis, without breaking mitochondrial resistance imposed by Bcl-2. Rather, Caspase-8 activation was improved, allowing for execution via the direct pathway. Sensitization did not correlate with changes in CD95 plasma membrane levels, but with decreased c-FLIP protein levels. Restoring c-FLIP expression after etoposide treatment by JNK inhibition reversed sensitization to APO010. Moreover, c-FLIP downregulation by RNAi largely overruled the capability of all stimuli to sensitize J16-Bcl-2 cells to APO010-induced apoptosis. These data indicate that c-FLIP downregulation is an important mechanism by which a variety of cellular stressors sensitize cancer cells to CD95-induced apoptosis.

Most likely, the reduction in c-FLIP protein levels induced by these stimuli allowed fewer c-FLIP molecules to be recruited into the CD95L DISC, thereby altering the c-FLIP-to Caspase-8/10 ratio and allowing for more effective inducer caspase activation. c-FLIP plays a dual role in the DISC, since it inhibits Caspase-8/10 activation when present at high levels, but can promote Caspase-8/10 activation when present at low levels [12]. We found that c-FLIP downregulation facilitated Caspase-8/10 activation when present at high levels, but can promote Caspase-8/10 activation when present at low levels [12].

A number of studies report a correlation between c-FLIP downregulation by cellular stressors and increased sensitivity to TRAIL-induced apoptosis, although exact consequences at the level of the DISC have been examined only in a few cases [30]. In colon [24] and hepatocellular [25] carcinoma, c-FLIP downregulation by 5-FU was correlated with reduced levels of c-FLIP in...
the TRAIL DISC and with enhanced sensitivity to TRAIL-mediated apoptosis, in agreement with our present study on CD95. However, 10 Gy IR did not detectably alter c-FLIP levels in J16-Bcl-2 cells and did not sensitize them to APO010-induced apoptosis (data not shown), while it did improve Caspase-8/10 activation in the TRAIL DISC [23]. This suggests that CD95 and TRAIL receptors have partially distinct requirements to activate inducer caspases in the DISC. This may relate to differential receptor microdomain localization, or post-translational modifications [31].

In our previous study, in which J16-Bcl-2 cells were pre-sensitized with IR, improved effector Caspase-8/10 activation in the DISC allowed apoptosis-induction by TRAIL via the direct pathway for effector caspase activation [23]. Now we show that c-FLIP downregulation in the same cells allows APO010 to bypass the mitochondrial pathway for apoptotic execution. These findings imply a ‘switch’ from Type II to Type I signalling in these cells for both TRAIL receptors and CD95. J16-Bcl-2 cells are not the only Type II cells for which this apparent ‘switch’ takes place upon combined treatment. Also in case of Type II CEM leukemia cells, cycloheximide pretreatment allowed CD95-mediated apoptosis via the direct pathway [32]. However, in other Type II cells (mesothelioma and carcinoma cell lines), an intact mitochondrial amplification loop was required for apoptotic execution upon combined treatment with DNA-damaging regimens and TRAIL, even though inducer caspase activity was enhanced [33-35]. Therefore, enhanced inducer caspase activation may be sufficient in some cell types for execution via the direct pathway, while in other cell types -perhaps particularly in solid tumors- the mitochondrial pathway may still be required.

We have demonstrated that in one cell type, IR, UV radiation, etoposide, HDAC- and proteasome inhibitors all downregulate c-FLIP protein levels. c-FLIP expression is tightly regulated, both at the transcriptional and at the protein level. The NF-kB pathway can induce c-FLIP transcription, while the ubiquitin-proteasome pathway mediates c-FLIP degradation [36, 37]. c-FLIP molecules have a short half life and are subject to proteasomal degradation at the steady state. In addition, they are ubiquitinated in response to death receptor stimulation, via a defined ubiquitin ligase that is under control of the JNK pathway [19, 38]. In agreement with this, short-term incubation with proteasome inhibitor rescued the disappearance of c-FLIP after addition of the stress stimuli, but upon prolonged incubation, c-FLIP was still downregulated (results not shown). This can be explained by the fact that proteasome activity is required for NF-kB activation, which in turn directs c-FLIP transcription.

In case of etoposide, we found that c-FLIP downregulation was impeded by JNK inhibition. This concurs with the report that implicated JNK in c-FLIP ubiquitination [19] and with the finding that JNK inhibition reversed etoposide-mediated sensitization to TRAIL in mesothelioma cells [20]. In case of UV radiation, however, which potently activated the JNK pathway and downregulated c-FLIP, the JNK inhibitors -while functional- could not rescue c-FLIP protein expression. Only in case of IR, c-FLIP levels were partially restored upon JNK inhibition (Supplementary Figure 3). The extent of c-FLIP restoration correlated with the extent to which sensitization to APO010 was reversed (Supplementary Figure 4). We conclude that JNK activation may contribute to, but is not a general mechanism by which c-FLIP is downregulated in response to stress stimuli and that JNK activation per se does not sensitize cells for APO010-induced apoptosis. In conclusion, c-FLIP downregulation is an important common mechanism by which various stress inputs sensitize J16-Bcl-2 cells to CD95-mediated apoptosis via direct pathway for caspase activation. The apparent ability of death receptors to ‘switch’ from Type II to Type I signalling following c-FLIP downregulation may be of more general relevance for sensitizing tumor cells to death receptor-mediated apoptosis.

**Materials and Methods**

**Cell lines and reagents**

The J16 Jurkat clone stably overexpressing Bcl-2 and luciferase (J16-Bcl-2) was generated by retroviral transduction as described [39]. J16 cells are heterozygous mutant for p53 and the DNA damage-induced apoptosis pathway is p53-independent [39]. J16-Bcl-2 cells and derivatives were cultured in Iscove’s modified Dulbecco’s medium (IMDM) with 8% fetal bovine serum (FBS) and antibiotics. APO010 in native and biotinylated form was kindly provided by TopoTarget, produced as described [17] and kept frozen in storage buffer at -80°C. SP600125 was from ENZO Life Sciences International Inc. The cell permeable L-JNKi peptide (sequence NGRRKKRRQRRRRPPKRPTTLNLFPQVPRSDQ with carboxy-terminal amide) was produced at the Division of Cell Biology at the Netherlands Cancer Institute, according to standard procedures. The product was purified by HPLC and its sequence was verified by mass spectrometry. TSA, VPA, anti-Caspase-8 monoclonal antibody (mAb, clone C15) and soluble human FLAG-tagged CD95L were obtained from Alexis (Temecula, CA). AlexaFluor 647-conjugated goat anti-mouse Ig and goat anti-rabbit Ig were from Molecular Probes (Leiden, The Netherlands). Biotinylated anti-FLAG mAb (clone M2), etoposide and puromycin were from Sigma-Aldrich (St Louis, MO). Anti-Cyt c mAb (clone 6H2.B4) and rabbit anti-active Caspase-3 antibody were from BD Biosciences (San Jose, CA), anti-actin mAb (clone C4) was from Chemicon International (Temecula, CA). AlexaFluor 647-conjugated goat anti-mouse Ig and goat anti-rabbit Ig were from Molecular Probes (Leiden, The Netherlands). Lactacystin and MG132 were from Calbiochem (Darmstadt, Germany). Horseradish peroxidase-conjugated rabbit anti-mouse Ig and swine anti-rabbit Ig were from DAKO A/S (Glostrup, Denmark) and the enhanced chemiluminescence kit was from Pierce Biotechnology (Rockford, IL). Rabbit anti-PARP polyclonal Ab (pAb) 9542 was from Cell Signaling Technologies (Danvers, MA). Streptavidin-conjugated sepharose beads were from Zymed (San Francisco, CA).

**Retroviral gene transduction**

Stable knock down of c-FLIP in J16-Bcl-2 cells...
with siRNA (complementary sense and antisense oligonucleotides FLIP#1: 5'-GAATAGACCGTGGAGCAGCAGG-3'; FLIP#2: 5'-GATGACAGATGGAGGCAAGCAGG-3'; FLIP#3: 5'-GCATGATGCTCAGAAGGAAT-3'; FLIP#4: 5'- GGAGCAGGCAAGTGTTAC-3'; FLIP#5: 5'-GCAAGGAGAGTGTTGC-3').

DISC isolation

J16-Bcl-2 cells were stimulated with 2 μg/ml etoposide and 15h later seeded at 10^6 cells/sample in 30 ml medium in a 15-cm dish. Cells were stimulated with 20 ng/ml FLAG-CD95L pre-coupled to 100 ng/ml biotinylated anti-FLAG M2 mAb for the indicated periods of time. DISC isolation was performed as described [23], with some modifications of the original protocol [12].

Immunoblotting

Total cellular protein was determined by Bio-Rad protein assay (München, Germany) and was separated at 40 μg per lane on 4-12% NuPage Bis-Tris gradient gels (Invitrogen) in MES buffer, according to the manufacturer's instructions. Subsequent immunoblotting was performed as described ([23], and references therein). For some blots, the iBlot™ system (Invitrogen) was used according to the manufacturer's instructions. Where appropriate, autoradiography signals were quantified using a Fluorchem8000 chemiluminescence imager (Alpha Innotech Corp., San Leandro, CA).

Flow cytometric detection of CD95

For flow cytometric detection of CD95 surface levels, cells were incubated for 15h with the indicated stimuli at 37°C, 5% CO₂. After incubation, cells were stained with biotinylated APO010 and subsequently with streptavidin-conjugated allophycocyanine (BD Pharmingen). Samples were gated on live, PI negative cells, and data were analyzed using FCS Express. Mean Fluorescence Intensity (MFI) values of stimulated cells were normalized to those of unstimulated (Control) cells.

Statistics

Unpaired Student’s t-tests and were considered significant when p < 0.05. Analyses were performed using GraphPad Prism version 4 for Windows (Graph Pad Software, San Diego, CA).

References


Supplementary Information

Supplementary Figure 1. c-FLIP downregulation by etoposide is post-transcriptionally regulated. J16-Bcl-2 cells were either left unstimulated (Control) or treated with 2 µg/ml etoposide (Etop) for 15h. mRNA levels of Actin, GAPDH, PGK (controls), and c-FLIP (test) were determined in duplicate by qualitative real-time (qRT)-PCR. Values obtained were normalized to those of Actin, GAPDH and PGK in control samples. c-FLIP levels of control- and etoposide treated samples were normalized to the average expression levels of Actin, GAPDH and PGK. c-FLIP protein levels as detected by immunoblotting in total cell lysates as shown in the right panel were from the same samples that were used for qRT-PCR. These data were also shown in Figure 3b.

Supplementary Figure 2. Inhibiting etoposide-induced JNK signaling restores c-FLIP protein levels. J16-Bcl-2 cells were incubated with 10 µM L-JNKi peptide for 24h or with 10 µM SP600125 for 30 min, 5% CO2 and next stimulated with 2 µg/ml etoposide (Etop). 15h later, c-FLIP, p-cJun and p-JNK protein levels were determined in total cellular lysates by immunoblotting. Actin and total JNK levels served as controls.

Supplementary Figure 3. Inhibiting JNK signaling only effectively restores c-FLIP levels in etoposide-treated cells. J16-Bcl-2 cells were incubated with 10 µM L-JNKi peptide for 24h or with 10 µM SP600125 for 30 min, after which cells were stimulated with etoposide (Etop, 2 µg/ml), 30 Gy IR or 60 J/m² UV (UV). 15h after treatment with Etop and IR or 2 and 4 h after treatment with UV, c-FLIP, p-cJun and p-JNK protein levels were determined in total cellular lysates by immunoblotting. Actin, total cJun and total JNK levels served as controls.

Supplementary Figure 4. JNK inhibition only effectively reverses sensitivity to APO010 in etoposide-treated cells. J16-Bcl-2 cells were incubated in the presence or absence of 10 µM SP600125 (SP) for 30 min, or 10 µM L-JNKi peptide for 24h at 37°C, 5% CO2. Subsequently, these cells were stimulated with (A) etoposide (Etop, 2 µg/ml), (B) UV (60 J/m²), (C) IR (30 Gy) or (D) VPA (6,4 mM). Directly after stimulation (UV) or after 15h incubation at 37°C, 5% CO2 (Etop, IR and VPA), cells were stimulated for the indicated time periods with 0.4 ng/ml APO010 and analyzed for Caspase-3 activation by flow cytometry. Data represent mean ± SD of at least 3 independent experiments. Statistically significant differences between values for ‘sensitized’ cells in the presence or absence of JNK inhibitors are indicated for *P<0.05, **P<0.01. Note that (A) is the same graph as Figure 4b and is shown here only for comparison.

Supplementary Materials and Methods

Reagents
Rabbit anti-phospho-JNK pAb 9251 and rabbit anti-phospho-c-Jun pAb 9261 were from Cell Signaling Technologies (Danvers, MA). Rabbit anti-c-Jun pAb (sc-1694) was from Santa Cruz Biotechnology (Santa Cruz, CA) and cycloheximide was from Sigma.

mRNA isolation, cDNA synthesis and qualitative real-time (qRT)-PCR
mRNA was isolated as described [23]. One µg of total RNA was used to generate first strand cDNA by using SuperScript II and random hexamers (Invitrogen). RT-PCR was performed using serially diluted cDNA (in 8 µl H2O), 10 µl Fast SYBR® Green Master Mix (Applied Biosystems, Nieuwekerk a/d IJssel, the Netherlands) and 1 µl of 10 µM Forward and Reverse primer. PCR reactions were done following standard procedures using the 7500 Fast real-time PCR System (Applied Biosystems). Primer sequences GAPDH FW: 5′-GACCCCTTCATTGACCTC-3′, GAPDH RV: 5′-GCCGATCCACACGAGTACT-3′, c-FLIP L (targeting only c-FLIP L) FW: 5′-GACAGAGCTTCTTCGAGACAC-3′, c-FLIP L RV: 5′-CCTGAGTGAGTCTGATCCACAC-3′, c-FLIP L+S (targeting both c-FLIP L and c-FLIPs isoforms) FW: 5′-GCCGAGGCAGGATAAGCAAG-3′, c-FLIP L+S RV: 5′-GTTGAGCGCCAAGCTGTTC-3′.

Sample preparation for immunoblotting JNK/cJun levels
For immunoblotting JNK/cJun proteins, cells were lysed in RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP40, 1% deoxycholate, 0.1% SDS and proteasome inhibitors) for 15 min on ice. Subsequently, DNA was sheared by sonification and lysates clarified by centrifugation (20,000 x g, 10 min, 4°C). Protein concentration in lysates was determined by the BCA protein assay (Pierce) according to the manufacturer’s instructions. Subsequent gel electrophoresis and immunoblotting were performed as described in the Materials and Methods section.