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Loss of drug-induced activation of the CD95 apoptotic pathway in a cisplatin-resistant testicular germ cell tumor cell line

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Abstract

Testicular germ cell tumors (TGCTs) are unusually sensitive to cisplatin. In the present study the role of the CD95 death pathway in cisplatin sensitivity of TGCT cells was studied in Tera and its *in vitro* acquired cisplatin-resistant subclone Tera-CP. Cisplatin induced an increase in CD95 membrane expression, which preceded the onset of apoptosis. Cisplatin-induced apoptosis was efficiently blocked by caspase-8 inhibitor zIETD-fmk in Tera cells, but only partially in Tera-CP cells. In addition, cisplatin induced FADD and caspase-8 recruitment to the CD95 receptor in Tera cells, which was not noticed in Tera-CP cells. Moreover, overexpression of vFLIP reduced apoptosis induction by cisplatin in Tera cells. CD95L-blocking experiments revealed the involvement of CD95/CD95L interactions in cisplatin-induced apoptosis of Tera cells as well as cisplatin-sensitive 833KE TGCT cells. Tera and 833KE cells, treated with low doses of cisplatin, were sensitive for an apoptosis-inducing anti-CD95 antibody. In contrast, CD95L blocking had no effect on cisplatin-induced apoptosis in Tera-CP or Scha, an intrinsic resistant TGCT cell line, nor did anti-CD95 antibody induce additional apoptosis in cisplatin-treated Tera-CP or Scha cells. Taken together, these results show that (1) cisplatin sensitivity of TGCT cells is dependent on the activation of the CD95 death pathway and (2) loss of cisplatin-induced activation of this CD95 signaling pathway may result in resistance to cisplatin.
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Keywords: CD95; cisplatin; resistance; apoptosis; caspases

Abbreviations: TGCT, testicular germ cell tumor; DISC, death inducing signaling complex; FADD, fas-associated protein with death domains; IC50, drug concentration inhibiting survival by 50%; $\Delta\Psi_m$, mitochondrial transmembrane potential; $DiOC_6(3)$, 3,3-dihexyloxacarbocyanine iodide; DMSO, dimethyl sulfoxide; PARP, poly (ADP-ribose)-polymerase; Ab, antibody; FLIP, FLICE-inhibitory protein; DED, death effector domain; GFP, green fluorescent protein; sCD95L, soluble CD95L; rhTRAIL, recombinant human tumor necrosis factor-related apoptosis-inducing ligand; FAP-1, Fas-associated phosphatase-1; MTT, 3-[4,5-dimethyl-thiazol-2yl]2,5-diphenyltetrazoliumbromide; PE, phycoerythrin; GAPDH, glyceraldehyde-3-phosphatase dehydrogenase; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HRP, horseradish peroxidase; DGGE, denaturating gradient gel electrophoresis; AU, arbitrary units.

Introduction

Testicular germ cell tumors (TGCT) represent one of the few solid tumor types that, when metastasized, are curable by cisplatin-containing chemotherapy, with an overall cure rate of about 80%.[1] Most human TGCT cell lines display an unusually high sensitivity to chemotherapeutic agents with cisplatin as key drug.[2,3] However, analysis of potentially relevant parameters in cisplatin sensitivity, including cellular detoxification mechanisms (e.g. the glutathione and the metallothionein systems), platinum accumulation, DNA platination and repair, p53 status, and expression of Bcl-2 family proteins have not been able, so far, to elucidate the identity of this inherent sensitivity of testicular tumors.[2,4,5] Since chemotherapeutic drugs commonly used in cancer therapy can induce tumor cell death by apoptosis,[6,7] and as inhibition of this apoptotic pathway can lead to chemotherapeutic drug resistance,[8,9] one might speculate that TGCTs are hypersensitive to drug-induced apoptosis.

It has been proposed that CD95 signaling plays an important role in chemotherapeutic drug-induced apoptosis in certain cell types.[10,11] Upregulation of the CD95 receptor (Fas, APO-1) and induction of its ligand CD95L (FasL, APO-1L) was observed after treatment of several tumor cell lines with chemotherapeutic drugs such as cisplatin.[12–16] Induction of CD95L expression mediates autocrine- or paracrine-cell death following binding to its receptor. Moreover, drug-induced upregulated CD95 expression may increase their sensitivity to physiological apoptotic signals. Triggering of the CD95 receptor with CD95L results in receptor trimerization and the formation of death-inducing signaling complex (DISC), including adaptor protein Fas-associated protein with death domains (FADD/MORT1) and initiator caspase-8 (FLICE).[17,18] Activated caspase-8 in the CD95/CD95L-initiated DISC activates effector caspases (e.g. caspase-3, -6, and -7), which in turn cleave intracellular substrates leading to a rapid cell death.[19,20] In some models, the CD95 death pathway can be triggered by CD95 trimerization in the

absence of CD95L in cells exposed to chemotherapeutic drugs.[21,22]

Interestingly, during normal spermatogenesis, spontaneous apoptosis occurs in the testis as an important physiological mechanism to limit the number of germ cells in the seminiferous epithelium.[23,24] Sertoli cells, which tightly regulate germ cell proliferation and differentiation, are implicated in the control of germ cell apoptosis.[25,26] The CD95–CD95L system between Sertoli cells and germ cells has been proposed as a crucial paracrine-signaling mechanism regulating the degree of germ cell apoptosis.[27–29] According to this model, CD95 expressed by germ cells and CD95L Sertoli cells respond to environmental conditions and initiate germ cell death.[30] Besides regulation of spontaneous apoptosis of germ cells, the CD95–CD95L system has also been implicated in maintaining the immune-privileged nature of the testis.[31]

Owing to the importance of the CD95 death pathway in spontaneous and chemically induced apoptosis of germ cells in normal testis, the present study was performed to evaluate the role of CD95-mediated cytotoxicity in cisplatin sensitivity and resistance in human TGCT cells. As a model for acquired cisplatin resistance served the cisplatin-sensitive TGCT cell line NTera2/D1 (Tera) and its cisplatin-resistant subclone Tera-CP.[32] The cisplatin-sensitive 833KE cells and cisplatin-resistant Scha cells were used as a model for intrinsic cisplatin sensitivity and resistance. To our knowledge, this is the first example of a loss of activation of the CD95-signaling pathway in cisplatin-induced apoptosis. This might be an important factor in the development of cisplatin resistance.

Results

Increased CD95 membrane levels after cisplatin treatment

To investigate the involvement of CD95 in cisplatin-induced apoptosis, the TGCT cell line Tera and its *in vitro* acquired cisplatin-resistant subline Tera-CP were studied. Figure 1 shows the survival curve of these cell lines after continuous incubation with cisplatin for 96 h. The IC_{50} (drug concentration reducing cell survival by 50%) for cisplatin was $0.77 \pm 0.12\ \mu$M (mean $\pm$ S.D.) in Tera and $2.13 \pm 0.21\ \mu$M in Tera-CP, resulting in a resistance factor of 2.8. Despite their different sensitivity to cisplatin, both cell lines showed a comparable upregulation of CD95 membrane expression upon cisplatin treatment (Figure 2a). The increase in CD95 membrane expression was accompanied by a cisplatin-induced upregulation of CD95 mRNA levels (Figure 2b).

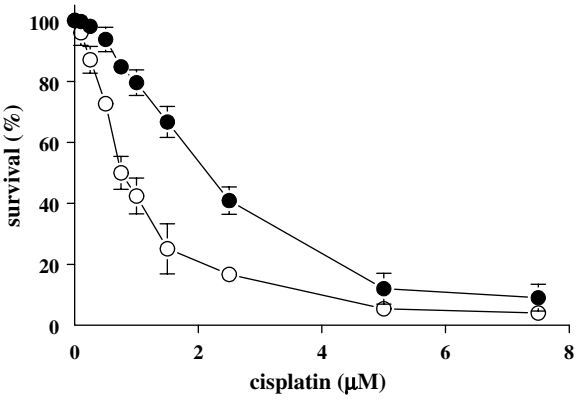

Figure 1 Survival of Tera and Tera-CP cells after cisplatin treatment. Tera (○) and Tera-CP (●) cells were treated with cisplatin for 96 h after which survival was measured by MTT assay (n=3, bars=S.D.)

Cisplatin-induced increase in CD95 membrane expression precedes onset of apoptosis

To evaluate whether the increase of CD95 membrane protein expression is required for apoptosis induction, the CD95 membrane expression after cisplatin addition was determined at different time intervals in comparison to the onset of apoptosis quantified by loss of the mitochondrial membrane potential ($\Delta\Psi_m$) assessed with the cationic dye 3,3-dihexyloxacarbocyanine iodide ($DiOC_6(3)$). Figure 3a shows that continuous treatment of Tera cells with a high cisplatin concentration (8 μM) for 9 h already increased the CD95 membrane expression. In contrast to the CD95 membrane expression, the onset of apoptosis induction was much slower and after 15 h of cisplatin treatment, only 23% of the cells were apoptotic. CD95 membrane expression was only highly elevated (eight-fold) in cells with intact mitochondria (high $DiOC_6(3)$ levels) and was moderately raised (two-fold) in

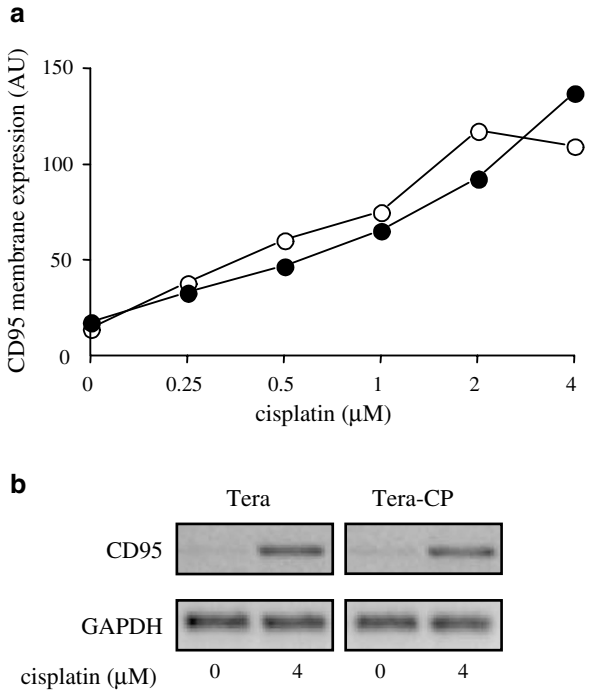

Figure 2 Effect of cisplatin on the CD95 expression in Tera and Tera-CP cells. (**a**) CD95 membrane expression of Tera (○) and Tera-CP (●) cells incubated with cisplatin for 24 h is plotted in arbitrary units (AU). (**b**) Tera and Tera-CP cells were incubated with or without cisplatin for 24 h. CD95 mRNA levels were determined by RT-PCR. Expression of GAPDH was used to control RNA integrity and quantity. A representative example of three independent experiments is shown

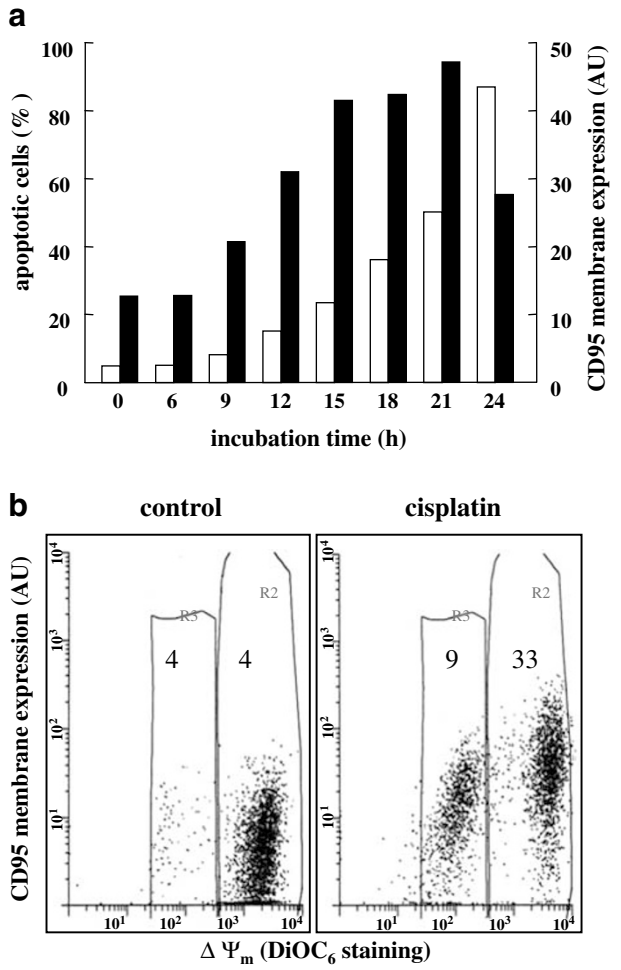

Figure 3 Cisplatin-induced increase in CD95 membrane expression precedes the onset of apoptosis. (**a**) Tera cells were incubated for several time intervals with 8 μM cisplatin and analyzed by flow analysis. The percentage of apoptotic cells (white bars) determined by loss of mitochondrial membrane potential ($\Delta\Psi_m$) is shown on the left *Y*-axis and the CD95 membrane expression (black bars) is represented on the right *Y*-axis. (**b**) Tera cells were incubated with 8 μM cisplatin for 24 h, followed by a double staining with $DiOC_6(3)$ (measuring $\Delta\Psi_m$) and anti-CD95 Ab. The numbers represent the CD95 membrane expression (in AU). A representative example of three independent experiments is shown

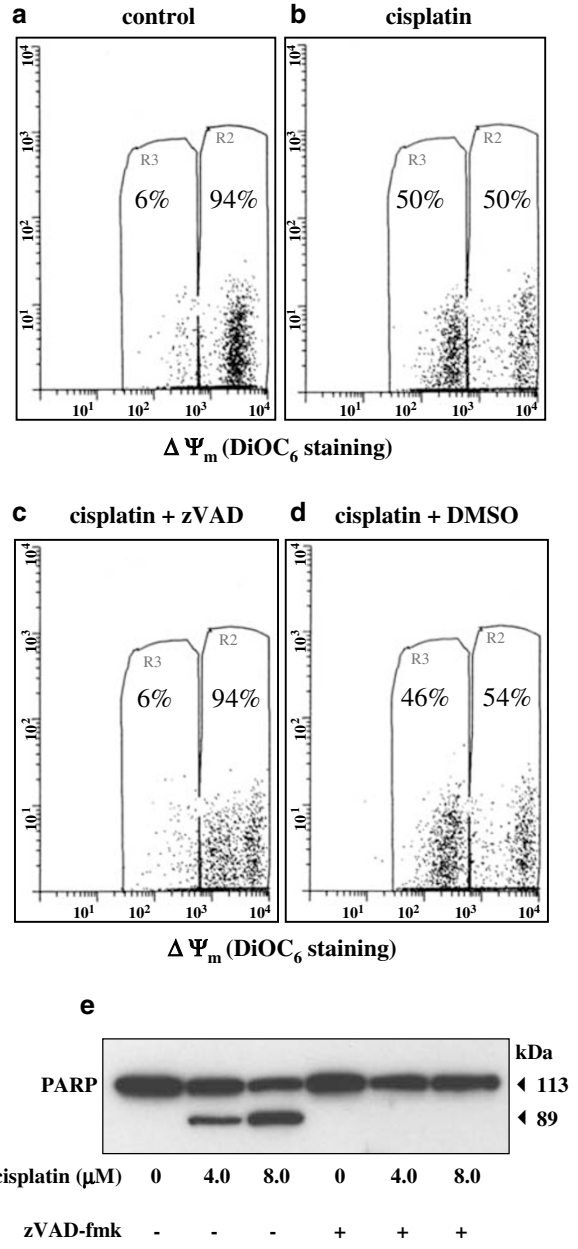

Figure 4 zVAD-fmk inhibits cisplatin-induced decrease in mitochondrial membrane potential and apoptosis. Tera cells were incubated for 24 h with cisplatin alone or plus 50 μM zVAD-fmk. Cells were then analyzed for apoptosis by flow cytometry (**a–d**) or immunoblotting for the processing of PARP (**e**). Apoptosis was assessed by $DiOC_6(3)$ to quantify the percentage of cells with decreased mitochondrial membrane potential ($\Delta\Psi_m$). Cells in R2 represent normal cells with high mitochondrial membrane potential and cells in R3 have a decreased mitochondrial membrane potential. The percentage of cells is indicated per region. A representative example of three independent experiments is shown

apoptotic cells with low $DiOC_6(3)$ levels (Figure 3b). These results suggest that the increase in CD95 membrane expression occurs before the onset of apoptosis induction.

Delay of onset of apoptosis enhances cisplatin-induced increase in CD95 membrane expression

To investigate the effect of inhibition of cisplatin-induced apoptosis on the CD95 membrane expression, Tera cells were incubated with the broad-spectrum caspase inhibitor zVAD-fmk prior to cisplatin exposure. As shown in Figure 4c, zVAD-fmk efficiently blocked the cisplatin-induced mitochondrial collapse. Although some cells showed intermediate $DiOC_6(3)$ levels, no complete loss of $\Delta\Psi_m$ was found, in contrast to cells treated with cisplatin only or in combination with the solvent dimethyl sulfoxide (DMSO) (Figure 4b and d, respectively). In addition, zVAD-fmk efficiently inhibited cisplatin-induced apoptosis, since the early apoptosis marker, poly (ADP-ribose)-polymerase (PARP), was not cleaved in the presence of the inhibitor (Figure 4e). The CD95 membrane expression was even more enhanced in these cells in comparison to cells treated with cisplatin only (Figure 5a). zVAD-fmk had no effect on the CD95 membrane

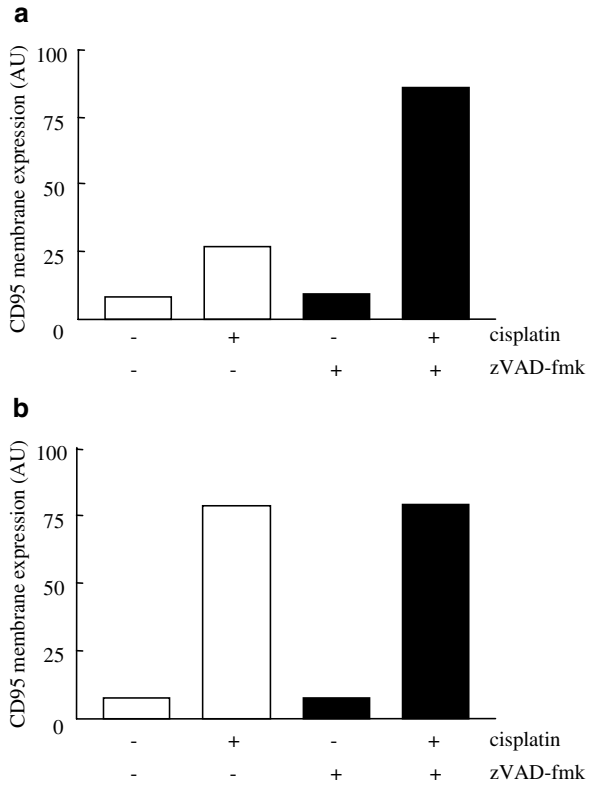

Figure 5 Delay of onset of apoptosis enhances the cisplatin-induced increase in CD95 membrane expression. Tera (**a**) and Tera-CP cells (**b**) were incubated 24 h with 8 μM cisplatin alone or in the presence of 50 μM zVAD-fmk. Cells were then analyzed for CD95 surface expression. A representative example of three independent experiments is shown

expression of cells that were not exposed to cisplatin. Tera cells exposed to cisplatin and zVAD-fmk showed comparable CD95 membrane expression as Tera-CP cells treated with the same concentration cisplatin alone or in combination with zVAD-fmk. These results suggest that a delay of onset of apoptosis, in the presence of zVAD-fmk in Tera cells, enhances the cisplatin-induced increase in CD95 membrane expression.

Cisplatin-induced p53 accumulation and upregulation of CD95 membrane expression precedes activation of caspases

Since the cisplatin-induced augmentation in CD95 membrane expression precedes the onset of apoptosis, the time-dependent accumulation of p53 and processing of caspase-8, -9, and -3 during cisplatin treatment was evaluated (Figure 6). Accumulation of p53 occurred already after 6 h, prior to the increase of CD95 membrane expression and onset of apoptosis (Figure 3a). Processing of caspase-8, the most upstream caspase in the CD95 death pathway, was evident after 12 h of cisplatin treatment showing the intermediate cleavage products of approximately 40–45 kDa, corresponding to cleavage of procaspase-8 between the large and small subunits.[33] After 15 h of cisplatin incubation, a fragment of approximately 18 kDa (p18) appeared. Caspase-9, which is activated in the mitochondria-mediated death pathway,[34,35] was processed in a time-dependent manner into $\sim$35 and 37 kDa fragments (p35 and p37, respectively).[36,37] Processing of caspase-9 was first observed after 12 h of cisplatin exposure. Caspase-3 was present in control cells as a 32 kDa proform that was following cisplatin treatment cleaved into two fragments possibly p20/p19 and p17.[38] Cleavage products appeared 12 h after cisplatin treatment. Apoptosis induction by cisplatin resulted in PARP cleavage after 12 h of cisplatin treatment concurrently with the onset of loss of mitochondrial membrane potential (Figure 3a). Additional fluorimetric caspase activity assays were performed to obtain a more quantitative measurement of the caspase activities. As depicted in Figure 6b, enzyme activities of caspase-8, -9, and -3 increased simultaneously and is more pronounced after 11 h of incubation with cisplatin. Thus, cisplatin-induced apoptosis was preceded by an accumulation of p53 expression and was accompanied by simultaneous activation of caspases.

Inhibition of caspase-8 efficiently blocks cisplatin-induced apoptosis in Tera, but not in Tera-CP cells

To obtain more insight whether CD95-mediated activation of caspase-8 was responsible for initiation of the caspase cascade leading to cisplatin-induced apoptosis, we used peptide inhibitors specific for the different initiator caspases to inhibit apoptosis (Figure 7). Tera-CP cells were exposed to a higher concentration cisplatin in comparison to Tera cells to induce similar apoptosis levels as demonstrated by PARP cleavage. The caspase-8-specific inhibitor zIETD-fmk efficiently inhibited cisplatin-induced apoptosis of Tera cells, whereas it only partially inhibited apoptosis of Tera-CP cells. Inhibition of caspase-9 with zLEHD-fmk did not completely block apoptosis in both cell lines. As control, the broad-spectrum caspase inhibitor zVAD-fmk was used, which efficiently inhibited apoptosis in both cell lines. Acridine orange apoptosis assays confirmed the lack of apoptotic cells (defined as condensed and fragmented nuclei) in Tera cells pretreated with zIETD-fmk followed by cisplatin exposure (data not shown). These results suggest that caspase-8 activation is necessary for cisplatin-induced apoptosis of Tera cells and that the mitochondrial apoptotic pathway induced by cisplatin is not sufficient to initiate apoptosis. In contrast, apoptosis of the cisplatin-resistant Tera-CP cells is not completely depending on the activation of caspase-8, since zIETD-fmk could only partially block apoptosis. This might indicate that activation of a more complex network of caspases is necessary to eventually cause apoptosis in Tera-CP cells.

Cisplatin induces formation of DISC in Tera cells, but not in cisplatin-resistant Tera-CP cells

Since caspase-8 activation seems essential for the further activation of other caspases in cisplatin-induced apoptosis of Tera cells, it was evaluated whether cisplatin could cause

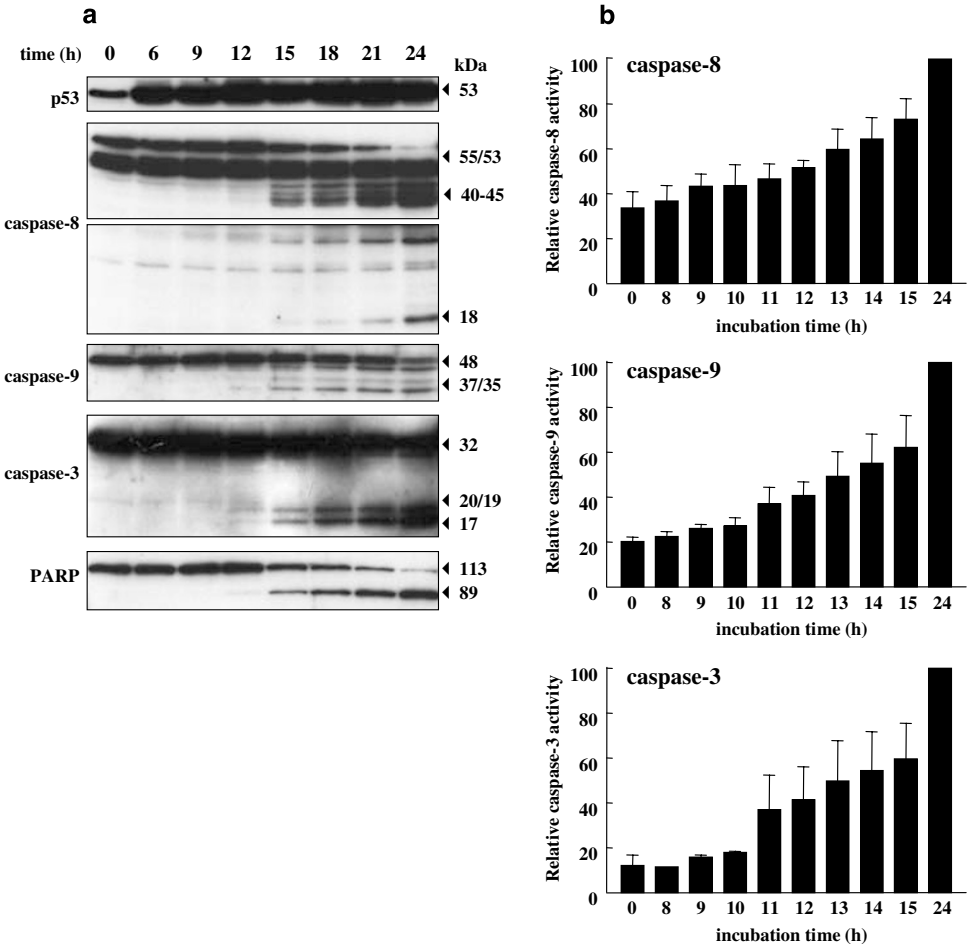

Figure 6 Time-dependent activation of caspases, accumulation of p53, and PARP cleavage by cisplatin. Tera cells were incubated with 8 μM cisplatin for the indicated periods. (**a**) Cells were analyzed by immunoblotting for p53, the processing of PARP, and activation of caspases. A shorter exposure time of the film was used to detect the different proforms of caspase-8. A longer exposure showed the cleaved products. A representative example of three independent experiments is shown. (**b**) Caspase-8, -9, and -3 activity was determined using fluorimetric caspase activity assays. Relative caspase activity was obtained by comparing the acquired enzyme activity with the maximum enzyme activity after 24 h of cisplatin incubation

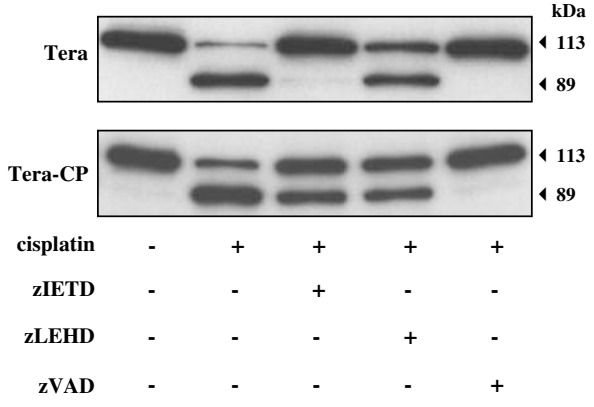

Figure 7 The caspase-8 inhibitor zIETD-fmk efficiently blocks cisplatin-induced apoptosis in Tera but not in Tera-CP cells. Tera and Tera-CP cells were incubated for 24 h with cisplatin (8 or 24 μM respectively) alone or in the presence of an inhibitor for caspase-8 (zIETD-fmk), caspase-9 (zLEHD-fmk), or zVAD-fmk (all 50 μM). Cells were then analyzed for apoptosis by immunoblotting for the processing of PARP. A representative example of three independent experiments is shown

trimerization of the CD95 receptor resulting in DISC formation and caspase-8 activation. Therefore, Tera cells were left untreated or were incubated for 24 h with 8 μM cisplatin alone or in combination with zVAD-fmk to inhibit cisplatin-induced apoptosis and analyzed for DISC immunoprecipitation using an antibody (Ab) directed against the CD95 receptor.

As shown in Figure 8a, cisplatin induced recruitment of both FADD and caspase-8 to the CD95 receptor to form the DISC in Tera cells. Western blot analysis of the used lysates revealed that there were no cisplatin-induced changes in the expression level of FADD or caspase-8, except for cells treated with cisplatin only that showed reduced caspase-8 expression (Figure 8b). Interestingly, in contrast to the cisplatin-induced DISC formation observed in Tera cells, no caspase-8 could be coimmunoprecipitated with the CD95 receptor from the cisplatin-resistant Tera-CP cells. Although both cell lines revealed comparable CD95 membrane expression (Figure 5), the absence of FADD and caspase-8 in the precipitate demonstrates that in Tera-CP cells no formation of the DISC occurred upon cisplatin exposure. Loss

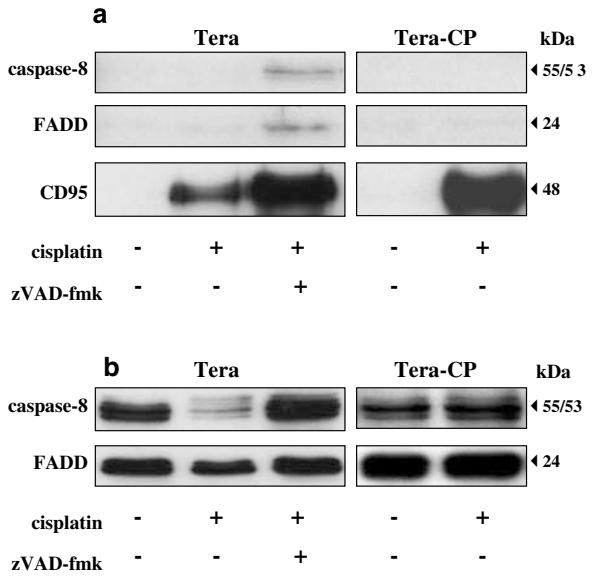

Figure 8 Cisplatin induces recruitment of FADD and caspase-8 to the CD95 receptor in Tera cells but not in Tera-CP cells. Tera and Tera-CP cells were treated with 8 μM cisplatin for 24 h alone or in the presence of 50 μM zVAD-fmk (Tera). Immunoprecipitation of the DISC was then performed as described under 'Materials and Methods'. CD95 immunoprecipitation (**a**) and used lysates (**b**) were stained for caspase-8, FADD, and CD95. A representative example of three independent experiments is shown

of DISC formation was not due to mutations in, for example, the CD95 death domain, since mutation analysis showed no abnormalities in exons 1–9 of the CD95 gene in Tera-CP cells.

Cisplatin-induced activation of the CD95 death pathway in Tera cells is dependent on CD95–CD95L interactions

To investigate whether CD95L mediates autocrine or paracrine cell death by crosslinking its receptor CD95, blocking experiments were performed using an anti-CD95L Ab (NOK-1) to inhibit cisplatin-induced CD95/CD95L interactions. Preincubation of Tera cells with NOK-1 (Figure 9a, black bars) markedly reduced cisplatin-induced cell death (approximately 60% at 4 and 8 μM cisplatin), while an isotype control Ab had no effect on apoptosis induction as determined by the acridine orange apoptosis assay (Figure 9a, grey bars). These results, also confirmed by PARP cleavage (Figure 9c), indicate that the CD95/CD95L interaction is involved in the cisplatin-induced cytotoxicity in Tera cells. However, although both cell lines revealed comparable CD95L protein expression, blocking of CD95/CD95L interactions by NOK-1 did not inhibit cisplatin-induced apoptosis in Tera-CP cells (Figure 9b and c). This confirms results described above, showing a lack of DISC formation in Tera-CP cells.

Overexpression of vFLIP in Tera cells inhibits cisplatin-induced apoptotis

To further investigate the importance of activation of the CD95-signaling pathway in cisplatin-induced cell death, a

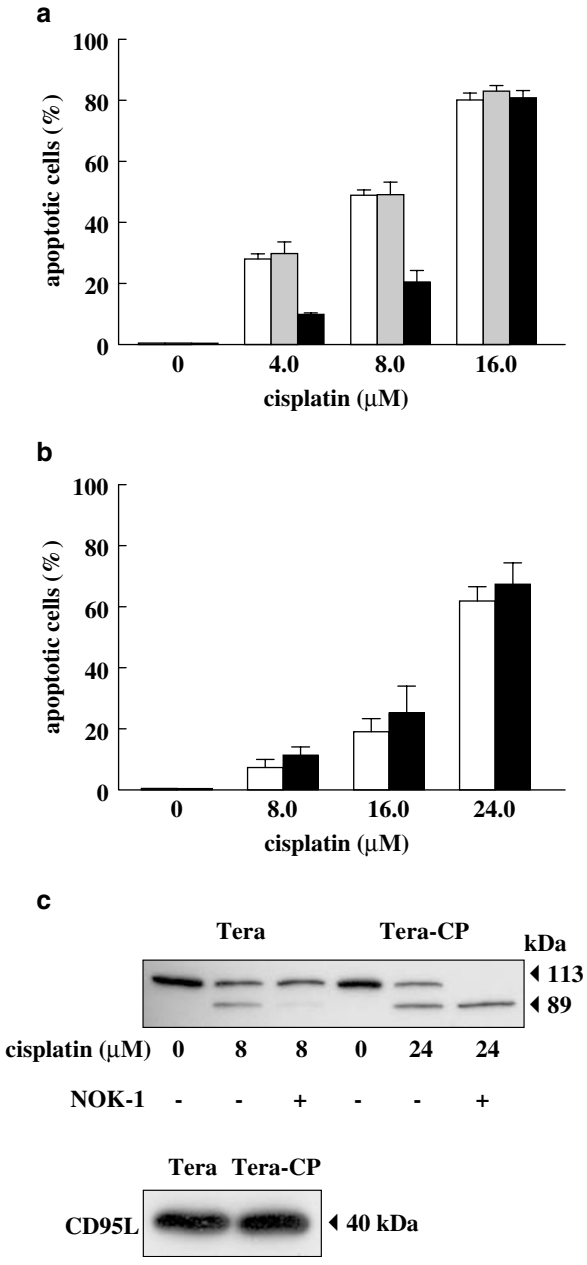

Figure 9 Reduction of cisplatin-induced apoptosis in Tera cells by anti-CD95L blocking antibody. Tera (**a**) and Tera-CP (**b**) cells were incubated with cisplatin only (white bars) or in the presence of 2 μg/ml control Ab (**A**, gray bars) or 2 μg/ml anti-CD95L NOK-1 Ab (black bars) for 20 h. Apoptosis was determined by acridine orange staining (**a,b**) or by PARP cleavage (**c**). Expression of CD95L protein was analyzed by immunoblotting (**c**). A representative example of three independent experiments is shown

specific caspase-8 inhibitor, viral FLICE-inhibitory protein (vFLIP), was overexpressed in Tera cells. The two death effector domains (DEDs) of vFLIP bind to cellular DED-containing proteins such as FADD and caspase-8, and thereby block the formation of the DISC and inhibit death receptor-induced apoptosis.[39,40] Whereas cisplatin-treated control Tera-GFP cells underwent apoptosis normally (Figure 10), overexpression of vFLIP reduced cisplatin-induced

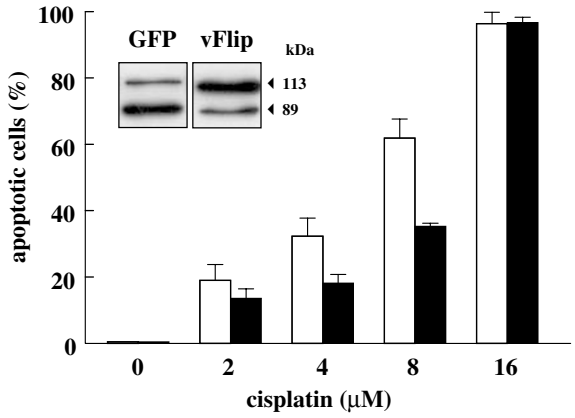

Figure 10 Overexpression of vFLIP in Tera cells inhibits cisplatin-induced apoptosis. Control Tera-GFP cells (white bars) or Tera cells expressing vFLIP (Tera-vFLIP, black bars) were incubated with different concentrations cisplatin for 20 h. Induction of apoptosis was assayed by acridine orange staining. Values shown are the mean ± S.D. of three independently performed experiments. Inset, Western blot analysis of PARP cleavage in Tera-GFP and Tera-vFLIP cells treated with 8 μM cisplatin for 20 h

apoptosis in Tera-vFLIP cells (approximately 50% at 4 and 8 μM cisplatin). This result further underscores the involvement of activation of the CD95 death pathway in cisplatin-induced cell death.

CD95-signaling pathway in Tera-CP cells is nonfunctional

To obtain more insight in the functionality of the cisplatin-induced elevation of the CD95 receptor in Tera-CP cells, both Tera and Tera-CP cells were incubated continuously with clinical achievable cisplatin concentrations for 72 h (0.25–4 μM). Under these conditions, cisplatin-induced apoptosis was relatively low, which enables detection of additional apoptosis induced by triggering the CD95 pathway using an agonistic anti-CD95 Ab, 7C11 (2 μg/ml). Figure 11a shows that cisplatin-treated Tera cells were sensitive for this agonistic anti-CD95 Ab, increasing apoptosis approximately two- to three-fold as compared to cisplatin only (range 0.25–1 μM cisplatin). Enhanced apoptosis induction by anti-CD95 cotreatment was already observed with low anti-CD95 Ab concentrations of 100 ng/ml (data not shown). Incubation with anti-CD95 Ab alone did not induce apoptosis in Tera cells. This is related to the low CD95 membrane expression, which is increased upon cisplatin treatment and thereby sensitizes the cells to anti-CD95 Ab-induced apoptosis. Despite comparable CD95 membrane expression levels, a combination of low doses of cisplatin and anti-CD95 Ab did not induce additional apoptosis in Tera-CP cells (Figure 11b). Moreover, no increase of apoptosis was observed when its natural ligand-soluble CD95L (sCD95L) was used to trigger the CD95-mediated apoptosis pathway in Tera-CP cells (Figure 12b). In contrast, both the agonistic anti-CD95 Ab and sCD95L efficiently increased the cisplatin-induced apoptosis approximately three-fold in Tera cells (Figure 12a).

To determine whether cisplatin increased the susceptibility of Tera cells specifically to the CD95 death pathway and not to death receptor-mediated apoptosis in general, recombinant

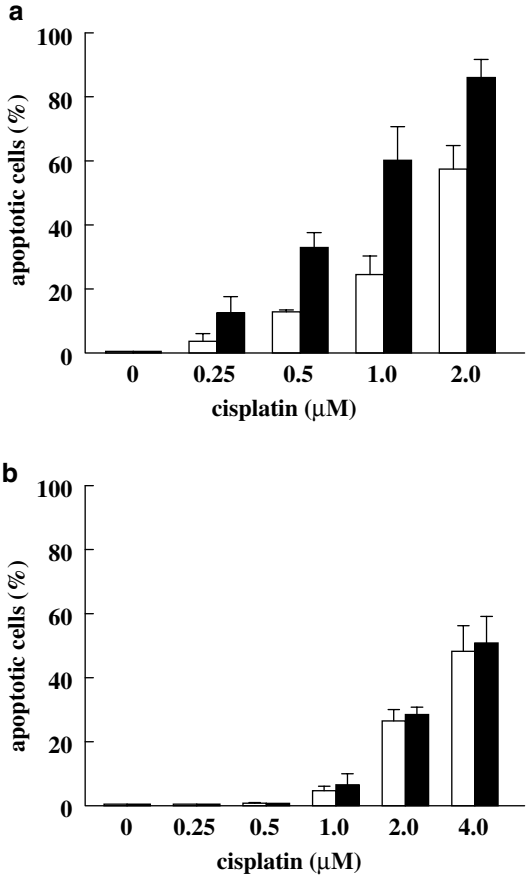

Figure 11 The CD95-signaling pathway in Tera-CP cells is not functional. Apoptosis of Tera (**a**) and Tera-CP (**b**) cells induced by cisplatin only (white bars) or in combination with 2 μg/ml agonistic anti-CD95 Ab, 7C11 (black bars) determined by acridine orange staining. Values shown are the mean ± S.D. of three independently performed experiments

human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL) was added to cisplatin-treated Tera cells (Figure 12c and d). Interestingly, although even low concentrations of rhTRAIL (0.01 and 0.1 μg/ml) induced massive apoptosis in the rhTRAIL-sensitive colon carcinoma cell line SW948, rhTRAIL could not further increase the degree of apoptosis in cisplatin-treated Tera cells (black and white bars, respectively, in Figure 12c). No increase in apoptosis was observed in Tera cells treated with increasing concentrations of rhTRAIL (up to 5.0 μg/ml) or cisplatin (range 0.25–2.0 μM) (Figure 12c and d, respectively). These results further underscore the importance of the CD95 death pathway in these cells.

Defective CD95-induced caspase activation in Tera-CP cells

Western blot analysis of caspase activation and PARP cleavage (Figure 13a) confirmed the results obtained by acridine orange assays (Figures 11 and 12). Tera cells treated with cisplatin and the agonistic anti-CD95 Ab revealed more PARP cleavage compared to cells treated with cisplatin only.

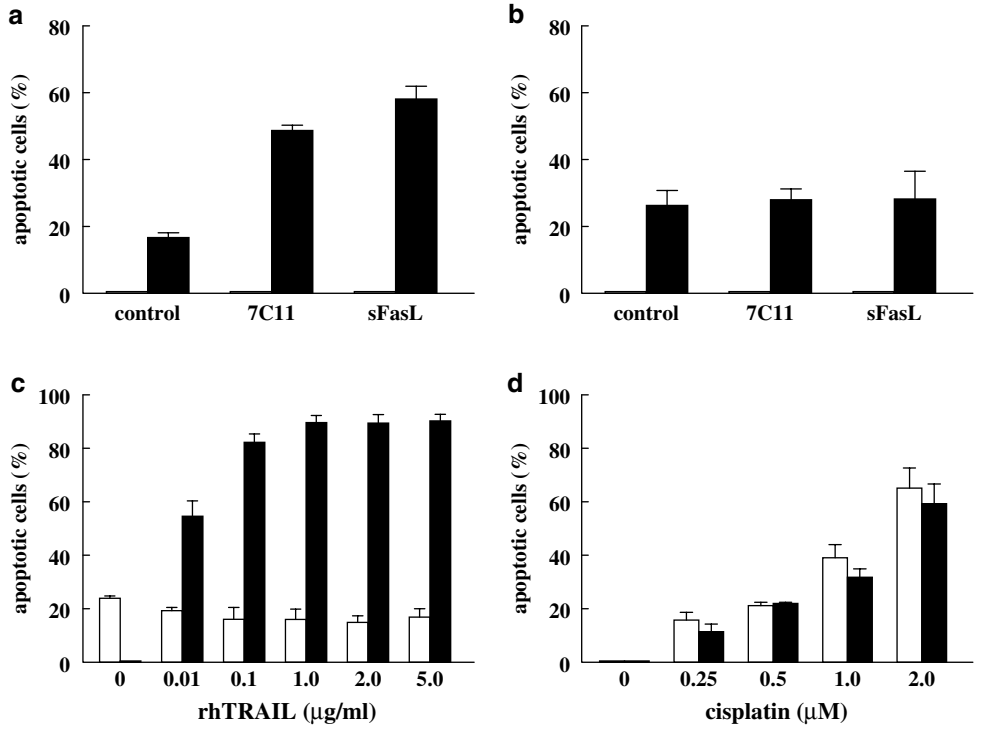

Figure 12 Cisplatin sensitizes Tera cells only to the CD95- but not to the TRAIL-death pathway. Tera (**a**) and Tera-CP (**b**) cells were incubated with 0.5 or 2.0 μM cisplatin respectively (black bars) or in control medium (white bars) for 72 h. After 48 h, an agonistic anti-CD95 Ab, 7C11 (2 μg/ml) or sCD95L (1 μg/ml) was added to the medium. (**c**) Tera cells pretreated with 0.5 μM cisplatin (white bars) and SW948 (black bars) were incubated with different concentrations rhTRAIL (range 0.01–5.0 μg/ml). (**d**) Tera cells were treated with different concentrations cisplatin (0.25–2.0 μM) only (white bars) or in combination with 1 μg/ml rhTRAIL (black bars). Apoptosis was determined by acridine orange staining. Values shown are the mean $\pm$ s.d. of three independently performed experiments

Again, no increase in PARP cleavage was observed in Tera-CP cells coincubated with cisplatin and anti-CD95 Ab in comparison to cisplatin alone. Combination of cisplatin and anti-CD95 Ab revealed more caspase-8 cleavage into the p18 fragment and more caspase-3 activation into the p20/19 and p17 fragments. In Tera-CP cells, only a marginal increase in caspase-8 and -3 was observed, suggesting that the CD95-signaling pathway in the cisplatin-resistant subline Tera-CP is indeed inactive. Western blot analysis was performed to examine the relative expression levels of several potential apoptosis-inhibiting proteins (Figure 13b). Interestingly, protein levels of the long isoform of cellular FLIP ($cFLIP_L$) were slightly increased in Tera-CP cells in comparison to Tera cells. However, more Bcl-2 protein was present in Tera cells than in Tera-CP cells. The protein levels of Fas-associated phosphatase-1 (FAP-1), Bcl-X_L, and X-linked inhibitor of apoptosis (XIAP) were comparable in both cell lines.

CD95 death pathway in two other TGCT cell lines

To investigate whether the observations found in the Tera/Tera-CP model also apply to other TGCT cell lines, additional experiments were performed with 833KE and Scha, two TGCT cell lines with different cisplatin sensitivities.[2] While 833KE is a cisplatin-sensitive cell line, Scha cells are resistant to cisplatin with an IC50 for continuous cisplatin incubation of 1.0 ± 0.2 and 3.3 ± 1.0 μM, respectively.[2] In both cell lines, cisplatin induced a concentration-dependent upregulation of Fas membrane expression.[41] As shown in Figure 14a, the blocking anti-CD95L Ab NOK-1 reduced cisplatin-induced apoptosis of 833KE cells approximately 50% at 8 μM cisplatin. In contrast, NOK-1 did not inhibit cisplatin-induced apoptosis in Scha cells (Figure 14b). Addition of an isotype control Ab had no effect on apoptosis induction (data not shown). These results indicate that upon cisplatin exposure, interactions of CD95L with the CD95 receptor activate the CD95 death pathway in the cisplatin-sensitive 833KE cells, while CD95/CD95L interactions are not involved in cisplatin-induced apoptosis in the cisplatin-resistant Scha cells. In addition, an apoptosis-inducing anti-Fas Ab could not induce additional apoptosis in cisplatin-pretreated Scha cells (Figure 14d), suggesting that the CD95-signaling pathway is nonfunctional. Cisplatin-pretreated 833KE cells, however, were sensitive for this anti-Fas Ab (Figure 14c), increasing apoptosis approximately three- to four-fold as compared with cisplatin only (range 0.25–4 μM cisplatin). These results do not only underscore the importance of the CD95 death pathway in cisplatin-sensitive TGCT cells, but also imply that lack of a functional CD95 signaling may result in cisplatin resistance in TGCT cells.

Discussion

In the present study, we have demonstrated that cisplatin sensitivity of the TGCT cell line Tera is dependent on the

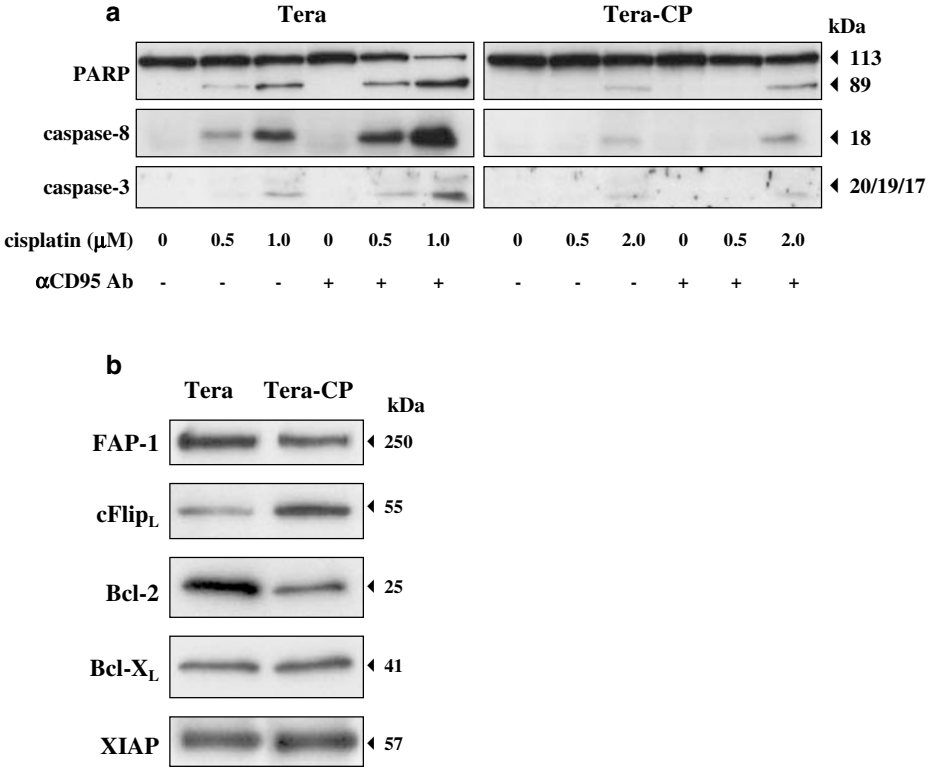

Figure 13 Defective CD95-induced PARP cleavage and caspase activation in Tera-CP cells. Tera and Tera-CP cells were incubated for 72 h with cisplatin. After 48 h of incubation, medium (controls) or 2 μg/ml agonistic anti-CD95 Ab, 7C11, was added. After treatment, cells were lysed and analyzed by immunoblotting for PARP cleavage and activation of caspase-8 and -3 (**a**). Expression of several apoptosis inhibitory proteins, FAP-1, cFLIP$_L$, Bcl-2, Bcl-X$_L$ and XIAP, was analyzed by immunoblotting (**b**). A representative example of at least three independent experiments is shown

activation of the CD95 death pathway. This conclusion is supported by results from several experiments. First, the cisplatin-induced increase in CD95 membrane expression preceded the onset of apoptosis. Secondly, cisplatin-induced apoptosis of Tera cells was effectively inhibited by the caspase-8 inhibitor zIETD-fmk indicating an important role of this caspase in the initiation of apoptosis. Thirdly, cisplatin induced FADD and caspase-8 recruitment to the CD95 receptor in Tera cells. Fourthly, cisplatin-induced apoptosis was inhibited by the blocking anti-CD95L antibody NOK-1, indicating CD95L involvement in the activation of the CD95 death pathway upon cisplatin treatment. Fifthly, overexpression of vFLIP reduced cisplatin-induced apoptosis in Tera cells. This underscores a functional involvement of CD95 signaling during cisplatin-induced cell death. Finally, in contrast to Tera, the CD95-signaling pathway was not functional in the cisplatin-resistant Tera-CP.

Several other studies have shown that activation of the CD95 death pathway by anticancer drugs is involved in drug-induced apoptosis in certain cell types.[12–16] Induction of CD95L and upregulation of the CD95 receptor is observed after exposure of different tumor cell lines with several chemotherapeutic drugs at therapeutic concentrations. The presence of functional wild-type p53 has been identified as an important mediator CD95 gene activation in response to DNA damage.[15,16] Moreover, p53 appears to mediate apoptosis through CD95 transport from cytoplasmic stores to the cell membrane.[42] Interestingly, although the p53 gene is the most frequently mutated gene in human cancers,[43] almost no p53 mutations have been detected in human TGCTs.[44] In Tera cells, the cisplatin-induced increase in CD95 membrane expression was preceded by the accumulation of wild-type p53 upon cisplatin exposure (Figures 3a and 6). In addition, CD95 mRNA levels were elevated after treatment with cisplatin (Figure 2b). These results suggest a possible role for p53 in the increased CD95 levels and transport of CD95 to the cell membrane.

DISC formation or aggregation of CD95 is often used as an explanation and a method to demonstrate that drugs induce activation of the CD95 death pathway.[14,21,22,45,46] Doxorubicin-induced apoptosis of human T leukemia cells was shown to involve CD95 aggregation, which is associated with the recruitment of FADD and caspase-8 to the CD95 receptor.[14] In our model, cisplatin induced DISC formation in the cisplatin-sensitive Tera cells indicating that the CD95 pathway is triggered upon cisplatin exposure. In addition, more CD95 was precipitated from cells treated with cisplatin compared to untreated cells. This further supports a role of CD95 in the cisplatin-mediated effects. The increase in CD95 precipitation upon cisplatin exposure was directly related to the cisplatin-induced increase in CD95 mRNA levels (Figure 2b) and CD95 membrane expression (Figures 2 and 5).

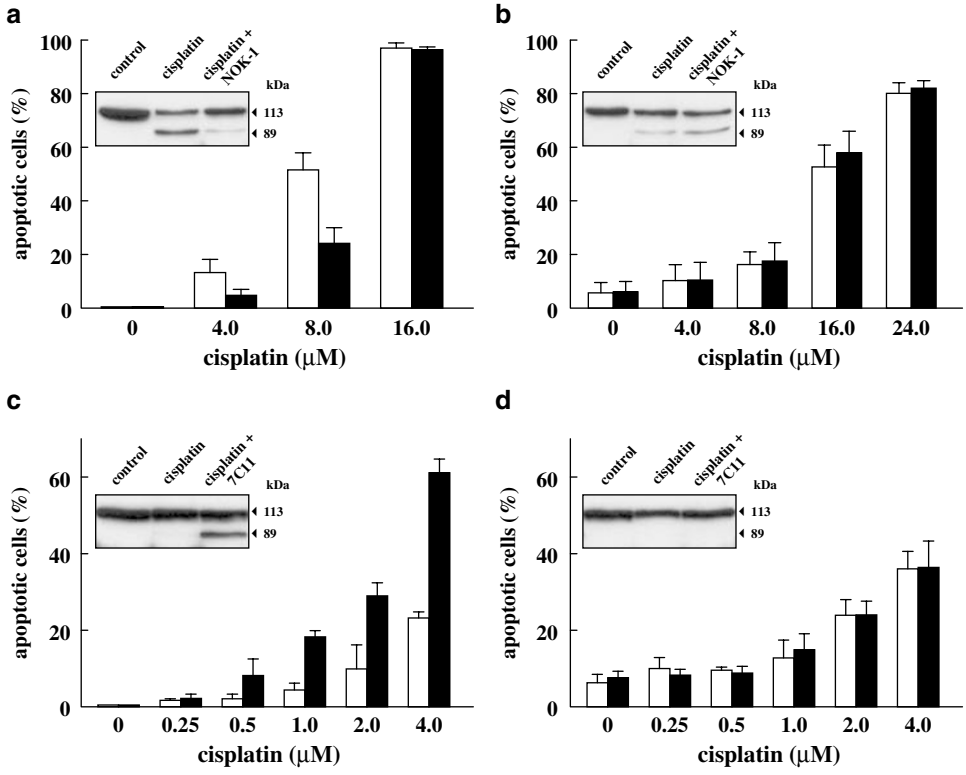

Figure 14 CD95 signaling in two other TGCT cell lines. 833KE (**a**) and Scha (**b**) cells were incubated with cisplatin only (white bars) or in the presence of 2 μg/ml anti-CD95L NOK-1 Ab (black bars) for 20 h. Apoptosis was determined by acridine orange staining. Inset, Western blot analysis of PARP cleavage in 833KE (**a**) and Scha (**b**) cells treated for 20 h with 8 or 16 μM cisplatin, respectively, in the presence or absence of 2 μg/ml anti-CD95L NOK-1 Ab. Apoptosis of 833KE (**c**) and Scha (**d**) cells induced by cisplatin only (white bars) or in combination with 2 μg/ml agonistic anti-CD95 Ab, 7C11 (black bars) determined by acridine orange staining. Inset, Western blot analysis of PARP cleavage in 833KE (**c**) and Scha (**d**) cells treated for 72 h with 2 μM cisplatin in the presence or absence of 2 μg/ml anti-CD95 7C11 Ab

TGCTs often express both the CD95 receptor and its ligand, CD95L.[47,48] In normal testis, Sertoli cells have been proposed to regulate apoptosis of germ cells via the CD95-CD95L system.[25,26] The coexpression of both CD95 and CD95L suggests that TGCTs can bypass the requirement of CD95L from Sertoli cells and are able to induce apoptosis upon cisplatin treatment via an autocrine or paracrine activation of the CD95 death pathway by CD95L. In our model, antagonistic Abs specific for CD95L (NOK-1) reduced cisplatin-induced apoptosis in Tera cells (Figure 9), indicating the involvement of CD95/CD95L interactions in activation of the CD95 death pathway upon cisplatin treatment. However, the suppression of apoptosis induction was not complete. In addition, preincubation with NOK-1 had no effect when cisplatin induced more than 90% apoptosis. In our hands, the efficacy of antagonistic Ab directed against CD95L is dependent on the degree and duration of apoptosis induction. Moreover, as postulated by Poulaki and colleagues,[49] the lack of effect of antagonistic Ab directed against CD95L or CD95 receptor (e.g. ZB4 or $F(ab')_2$ anti-CD95 fragments) may also be explained by inaccessibility of the targets for the inhibitory Ab.[49,50] However, the partial (50–60%) reduction of cisplatin-induced apoptosis by NOK-1 Ab or overexpression of vFLIP suggests that mechanisms independent of the CD95 death pathway are also involved in cisplatin-induced apoptosis. As described, DNA-damaging agents can activate parallel pathways such as the intrinsic mitochondria-mediated and the extrinsic death receptor-mediated apoptotic pathway.[10,11,14,45] For example, both the receptor and mitochondrial pathway are activated in the B-cell lymphoma cell line BJAB upon drug treatment, since blockade of either pathway only partially inhibited apoptosis.[45] At higher drug concentrations or at later stages of drug exposure, DNA-damaging drugs may eventually bypass the requirement for CD95-mediated amplification of the death signal and trigger apoptosis in a CD95-independent way.[14] Therefore, the involvement of the death receptor pathway in chemotherapy-induced apoptosis will most likely depend on several factors and not only on the cell line used, but also on the type of drug, drug concentrations, and the kinetics of the experiments. A number of studies could not demonstrate the involvement of the CD95 system in drug- and irradiation-induced apoptosis.[51–55] These studies show that only a death receptor-independent mitochondrial pathway is activated upon exposure to DNA-damaging agents. Besides activation of the CD95 death pathway, cisplatin treatment of Tera cells might also activate the mitochondrial death pathway. DNA damage can lead to the p53-induced increase in proapoptotic proteins such as Bax, Noxa, and PUMA.[56] These proteins can permeabilize the mitochondrial outer membrane leading to the release of apoptotic factors such as cytochrome *C*, Smac/DIABLO, and apoptosis-inducing factor (AIF) from the

mitochondria into the cytosol followed by apoptosis induction.[57] Whether these mechanisms are involved in cisplatin-induced apoptosis in Tera cells needs to be resolved in future research.

Interestingly, cisplatin-treated Tera cells displayed a different response to anti-CD95 Ab and rhTRAIL. Whereas cisplatin sensitizes Tera cells to triggering of the CD95 death pathway, cisplatin-treated Tera cells were resistant to rhTRAIL-induced apoptosis (Figure 12c and d). Although both CD95 and DR4/DR5 death receptors are considered to use the same signaling pathway, differences in sensitivity to CD95L- or TRAIL-induced apoptosis were also found in other studies.[58,59] Both caspase-8 and -10 have been shown to function independent of each other in initiating CD95 and TRAIL-receptor-mediated apoptosis.[60,61] However, caspase-10 has been shown to be the most apical caspase in TRAIL-induced apoptosis in thyroid carcinoma cells.[58] In addition, another study showed that TRAIL-induced apoptosis of rhabdomyosarcoma cell lines may require the presence of caspase-10 to amplify the apoptotic signal.[59] Besides differences in requirement for caspase-10, triggering of death receptors has also been shown to result in activation of a nuclear factor-κB (NF-κB)-dependent survival program.[62,63] Therefore, further exploration of the involvement of initiator caspases and NF-κB survival signals upon ligation of death receptors by rhTRAIL or anti-CD95 Ab is needed.

In contrast to Tera cells, Tera-CP cells have a dysfunctional CD95-signaling pathway and do not use the CD95-mediated route to undergo apoptotic death quickly upon cisplatin exposure. This defect in the CD95-signaling pathway might be caused by different mechanisms. Besides direct down-regulation of the CD95 receptor, several mutations in the CD95 gene or splicing defects in the CD95 mRNA have been described, which result in nonfunctional CD95 receptors.[64,65] However, mutation analysis showed no mutations in the CD95 gene of Tera-CP cells. In addition, the CD95 receptor was not down regulated in the resistant cells and showed a comparable cisplatin-induced increase in membrane expression as observed in the sensitive Tera cells. The presence of a functional intact CD95 receptor protein implicates that other mechanisms downstream the CD95 signaling route are responsible for the nonfunctional activation of the CD95 death pathway. Examination of potential apoptosis inhibitors revealed a slightly elevated expression of cFLIP in Tera-CP cells. In contrast, Bcl-2 protein levels were higher in Tera cells compared to Tera-CP cells. The precise involvement of these inhibitors in the defect apoptotic route in Tera-CP cells needs further exploration.

Current interest is focused on possible cross-resistance for anticancer drugs and CD95-induced apoptosis.[12,13,66,67] For example, CD95- and doxorubicin-resistant leukemia and neuroblastoma cells were shown to display cross-resistance for cell death induction.[12] However, others have demonstrated that multidrug resistant cells are not necessarily resistant to CD95-mediated apoptosis.[67] Moreover, several studies have shown that treatment of drug-resistant tumor cell lines with anticancer drugs could sensitize them to anti-CD95-induced apoptosis.[68–70] For instance, the cisplatin-resistant bladder cancer cells and ovarian tumor cell lines were sensitive to treatment with a combination of anti-CD95 Ab and cisplatin.[68,69] Therefore, the relative contribution and significance of CD95 resistance in the sensitivity to anticancer drugs has still to be established and will probably depend on tumor type and drug used. Besides the Tera/Tera-CP model, additional experiments were performed with two other TGCT cell lines to obtain more insight into the importance of CD95 resistance in cisplatin sensitivity of germ cell tumors. Like Tera-CP, the CD95 death pathway was nonfunctional in the cisplatin-resistant Scha cells. In contrast, the cisplatin-sensitive 833KE cells were also sensitive to CD95-induced apoptosis. Furthermore, CD95/CD95L interactions were involved in cisplatin-induced apoptosis in 833KE cells but not in Scha cells, underscoring the importance of the CD95 death pathway in cisplatin sensitivity of TGCT cells.

In conclusion, the present study shows in two TGCT models of acquired and intrinsic cisplatin resistance that the loss of cisplatin-induced activation of the CD95 death pathway may result in cisplatin resistance. Therefore, the presence of a functional CD95-signaling pathway in human TGCTs may be an important factor determining their unique sensitivity for cisplatin.

Materials and Methods

Cell lines

The human embryonal carcinoma cell line Tera and its cisplatin-resistant subline Tera-CP were described previously.[32] Both cell lines express wild-type p53. Tera-CP has increased detoxifying capacity shown by a 1.4-fold increased glutathione (GSH) level, 1.5-fold increased glutathione *S*-transferase (GST) activity, and 1.4-fold increased glutathione *S*-transferase-π (GSTπ) expression compared with Tera. No differences have been observed between the two lines in DNA topoisomerase I or II activities and c-*myc* mRNA or protein expression. Platinum accumulation is equal in both cell lines, whereas platinum-DNA binding is lower in Tera-CP as compared to Tera.[32]

In addition to this model, two unrelated human TGCT cell lines, 833KE and Scha,[4] and the colon carcinoma cell line SW948[71] were used. Compared to Tera 833KE and Scha are 1.3- and 4.3-fold resistant to cisplatin. Both cell lines express wild-type p53. It was observed that 833KE cells have a 2.9-fold increased GSH level, comparable GST activity, and a 1.6-fold decreased GSTπ expression. Scha has a 5.2-fold increased GSH level, 2.6-fold decreased GST activity, and comparable GSTπ expression. In both 833KE and Scha, platinum accumulation is increased whereas platinum-DNA binding is lower compared to Tera.

All TGCT cell lines grew as monolayers in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) (both from Life Technologies, Breda, the Netherlands). The SW948 cell line was cultured in Leibovitz L15-RPMI 1640 (1 : 1) enriched with 10% FCS, 0.05 M pyruvate, 0.1 M glutamine, and 0.025% β-mercaptoethanol. All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Cytotoxicity assay

The microculture tetrazolium assay was used to measure cytotoxicity. Treatment consisted of continuous incubation with cisplatin (Pharmachemie BV, Haarlem, the Netherlands). After a 4-day culture period, 3-[4,5-dimethyl-thiazol-2yl]2,5-diphenyltetrazoliumbromide (MTT)-solution (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) was added and formazan production was measured as described previously.[32]

Controls consisted of media without cells (background extinction) and cells incubated with medium instead of cisplatin. Cell survival was defined as the growth of treated cells compared to untreated cells. Resistance was indicated by a resistance factor: the ratio IC50 Tera-CP over IC50 Tera. The mean IC50 $\pm$ S.D. was determined in three experiments, each performed in quadruplicate.

Detection of CD95 membrane expression

Cisplatin-treated or untreated cells were stained with a phycoerythrin (PE)-conjugated Ab against CD95 (DX2 from Becton Dickinson, Erembodegem-Aalst, Belgium) for 30 min on ice. Subsequently, cells were washed and analyzed by flow cytometry (Epics Elite, Coulter-Electronics, Hialeah, FL, USA). The mean fluorescence intensity was determined by comparison of the fluorescence intensity of unlabeled cells.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated by lysing cisplatin-treated or untreated cells in 500 μl guanidine thiocyanate buffer (4 M guanidine thiocyanate, 0.5% n-lauroyl sarcosine, 25 mM sodium citrate (pH 7.0), 0.1 M 2-β-mercaptoethanol) according to Wisman *et al*.[72] The quality of the samples was checked by agarose gel electrophoresis. Prior to cDNA synthesis, RNA was treated with DNase I (Roche Diagnostics, Almere, the Netherlands). cDNA was synthesized from 5 μg total RNA as described by the manufacturer's protocol (Life Technologies) using oligo dT primers and mM LV transcriptase. A 339 bp CD95 fragment was amplified in 36 cycles using Taq DNA polymerase (Life Technologies) with the primers 5′-CATGGCTTAGAAGTGGAAAT-3′ and 5′-ATTTATTGCCACTGTTTCAGG-3′ (sense and antisense, respectively) amplifying the transmembrane coding region of the CD95 gene. Human glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) was used as a control to normalize the amount of template RNA. Primer sequences for GAPDH were 5′-CACCACCATGGAGAAGGCTGG-3′ and 5′-CCAAAGTTGTCATGGATGACC-3′ (sense and antisense, respectively) which resulted in a 200 bp fragment after 22 cycles. PCR products were electrophorized in a 2% agarose gel in 1 $\times$ Tris-borate EDTA buffer.

Western blot analysis

After treatment with cisplatin alone or in combination with several apoptosis inhibitors or an agonistic anti-CD95 Ab (7C11 from Immunotech, Marseille, France), cells were harvested at indicated time points and washed twice with cold phosphate-buffered saline (PBS: 6.4 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 0.14 mM NaCl; 2.7 mM KCl; pH=7.2). Cells were lysed with standard Western blot sample buffer (50 mM Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-β-mercaptoethanol) and boiled for 5 min. Protein concentration was determined according to Bradford.[73] Total cell lysates were size fractionated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto activated polyvinylidene difluoride membranes (Millipore, Bedford, UK). Equal protein loading was confirmed by Ponceau red staining of membranes. After blocking for 1 h in Tris-buffered saline supplemented with 5% milk powder (Merck, Darmstadt, Germany) and 0.05% Tween-20 (Sigma-Aldrich Chemie BV), immunodetection of p53, CD95, CD95L, FADD, caspase-8, caspase-9, caspase-3, PARP, $cFLIP_L$, FAP-1, Bcl-2, Bcl-X_L or XIAP was done using the following Abs: mouse anti-p53-DO-1, mouse anti-Bcl-2, rabbit anti-Bcl-X_L, goat anti-FAP-1, and goat anti-CD95 Abs, purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-PARP Ab was obtained from Roche Diagnostics. Rabbit anti-caspase-9 and anti-caspase-3 Abs were purchased from Becton Dickinson. Mouse anti-FasL and mouse anti-XIAP Abs were obtained from Transduction Laboratories (Lexington, KY, USA). Mouse anti-caspase-8 Ab C15[74] was kindly provided by Dr. P Krammer (DKFZ, Heidelberg, Germany). Mouse anti-cFLIP Ab NF6[75] was a kind gift from Dr. M Peter (University of Chicago, IL, USA). Binding of these antibodies was determined using horseradish peroxidase (HRP)-conjugated secondary Abs (all from DAKO, Glostrup, Denmark) and visualized with the ECL-chemiluminescence kit of Roche Diagnostics.

Determination of apoptosis

Measurement of mitochondrial membrane potential

Changes in the inner mitochondrial transmembrane potential ($\Delta\Psi_m$) were determined by incubating cells with 50 nM $DiOC_6(3)$ (Calbiochem, Breda, the Netherlands) for 30 min at 37°C. Apoptotic cells have damaged mitochondria with lost membrane integrity that are no longer able to maintain their transmembrane potential, resulting in a decreased binding of $DiOC_6(3)$.[76] As a positive control, cells were treated with 50 μM carbonyl cyanide *m*-chlorophenylhydrazone (Sigma-Aldrich Chemie BV), an agent known to disrupt $\Delta\psi_m$. Stained cells were directly analyzed on an Epics Elite flow cytometer.

Acridine orange apoptosis assay

Cells in 96-well tissue-culture plates were incubated with cisplatin alone or in combination with several apoptosis inhibitors or an agonistic anti-CD95 antibody, sCD95L (Alexis Co., San Diego, CA, USA) or rhTRAIL (made according to Ashkenazi *et al*.).[77] After 20 or 72 h of incubation, acridine orange (10 μg/ml) was added to each well to distinguish apoptotic cells from vital cells. Staining intensity was determined by fluorescence microscopy and apoptosis was defined by the appearance of apoptotic bodies and/or chromatin condensation. Apoptosis was expressed as percentage apoptotic cells in a culture.

Caspase enzyme activity assay

Activity of caspase-8, -9 and -3 was assayed according to the manufacturer's instructions using the caspase-specific fluorescence peptide substrates Ac-IETD-AFC (Calbiochem), Ac-LEHD-AFC, and Ac-DEVD-AFC, respectively (Biomol Tebu-bio, Heerhugowaard, the Netherlands). Fluorescence from free 7-amino-4-trifluoromethyl coumarin (AFC) was monitored in a FL600 Fluorimeter Bio-tek plate reader (Beun de Ronde, Abcoude, the Netherlands) using 380 nm excitation and 508 nm emission wavelengths. Caspase activity was calculated by converting the fluorescence units to pmol of AFC using a standard curve generated with free AFC and normalized to 1 μg of protein. Relative caspase activity was obtained by comparing the acquired caspase activity to the maximum caspase activity after 24 h defined as 100%.

Inhibition of cisplatin-induced apoptosis

At 1 h prior cisplatin treatment, cells were incubated with 50 μM broad-spectrum caspase inhibitor zVAD-fmk, caspase-8 inhibitor zIETD-fmk, or caspase-9 inhibitor zLEHD-fmk (all from Calbiochem). Stock solutions of inhibitors were prepared in DMSO. The final concentration of DMSO solvent in the culture medium never exceeded 0.1% (v/v), which was nontoxic to the cells and did not inhibit cisplatin-induced apoptosis. To

block CD95/CD95L interactions, cells were incubated with 2 μg/ml anti-CD95L Ab NOK-1 (Becton Dickinson).

DISC immunoprecipitation

DISC formation upon cisplatin treatment was detected by immunoprecipitation of the CD95 receptor according to Micheau *et al*.[21] and Scaffidi *et al*.[78] Briefly, cells were treated with cisplatin (8 μM) with or without zVAD-fmk (50 μM). After 24 h, cisplatin-treated cells (both apoptotic and attached cells) were washed once in cold PBS and incubated with the cleavable cross-linker 3,3′-dithiobis[sulfosuccinimidyl-propionate] (Pierce Chemical Co., Rockford, IL, USA) for 15 min at 4°C. The reaction was stopped by incubation in PBS containing 10 mM ammonium acetate for 5 min at 4°C. Cells were harvested using a rubber policeman, washed twice in PBS, and lysed in lysis buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitors (Roche Diagnostics)) for 15 min on ice. After centrifugation at 2500 $\times$ *g* at 4°C for 10 min, protein concentration was determined of the supernatant and equal amounts of proteins were used for the immunoprecipitation. An excess concentration of 2 μg/ml mouse anti-human CD95 Ab (APO1–3, Alexis Co.) was added to the lysates and reacted at 4°C for 1 h. Immune complexes were precipitated using 30 μl protein A-sepharose (Amersham, Pharmacia Biotech, Roosendaal, the Netherlands) and washed three times in 1.5 ml lysis buffer. The precipitate was resuspended in standard Western blot sample buffer and boiled for 5 min. Immunoprecipitated proteins were separated on 12% SDS-PAGE. Western blot for FADD, caspase-8, and CD95 was performed as described above. Goat HRP-conjugated secondary Ab specific for mouse IgG1 or mouse IgG2b were used for detection of FADD or caspase-8, respectively (both from Southern Biotechnologies, Birmingham, AL, USA). HRP-conjugated Protein G (Sigma-Aldrich Chemie BV) was used for detection of CD95 Ab.

Denaturating gradient gel electrophoresis (DGGE)

Genomic DNA from Tera and Tera-CP cells was isolated by proteinase K (Merck) digestion and phenol–chloroform extraction. Mutations in the CD95 gene were detected by PCR amplification of genomic DNA using 10 sets of primers described by Grønbæk *et al*.[79] A standard 35-cycle amplification was performed followed by a heteroduplexing step, involving denaturation at 96°C for 5 min and reannealing for 45 min at 55°C. An aliquot of 10 μl of the amplified product was transferred to a 0.75 mm thick 9% polyacrylamide gel (acrylamide : bisacrylamide, 37.5 : 1) containing a 20–60% denaturing gradient of urea–formamide (100% urea–formamide contains 7 M urea and 40% deionized formamide). DGGE was performed in 1 $\times$ TAE (40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA, pH 8.0), at 58°C for 12 h (105 V). The separation pattern was visualized by ethidium bromide staining for 10 min and UV transilluminator of the gel.

Retrovirus-mediated transfection

The retroviral LZRS-based vector,[80] containing the equine herpes virus type 2 E8-FLIP gene[39] and the enhanced green fluorescent protein (GFP) gene separated by an internal ribosomal entry site, was kindly provided by Dr. J Borst (The Netherlands Cancer Institute, the Netherlands). Retroviral transduction was performed as follows: Tera cells were mixed with supernatant of amphotropic Phoenix packaging cells producing the empty control LZRS-GFP-virus or retroviruses carrying the E8-FLIP gene and plated at a density of 6×10^5 cells per 35-mm culture dish. The production of GFP from the bicistronic cDNA was used to collect GFP-positive cells from E8-FLIP-transduced cells (Tera-vFLIP) or control-virus-transduced cells (Tera-GFP) on a MoFlo cytometer (Fort Collines, USA).

Acknowledgements

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