The EMBO Journal Vol.16 No.20 pp.6200–6208, 1997

Activation of CD95 (APO-1/Fas) signaling by ceramide mediates cancer therapy-induced apoptosis

Ingrid Herr[1], Dagmar Wilhelm[2,3], Thomas Böhler[4], Peter Angel[2] and Klaus-Michael Debatin[1,4,5]

[1]Division of Molecular Oncology and [2]Division of Signal Transduction and Growth Control, Deutsches Krebsforschungszentrum, Heidelberg, [3]Forschungszentrum Karlsruhe, Institute of Genetics, Karlsruhe and [4]Hematology/Oncology, University Children's Hospital, Heidelberg, Germany

[5]Corresponding author

We report here that anticancer drugs such as doxorubicin lead to induction of the CD95 (APO-1/Fas) system of apoptosis and the cellular stress pathway which includes JNK/SAPKs. Ceramide, which accumulates in response to different types of cellular stress such as chemo- and radiotherapy, strongly induced expression of CD95-L, cleavage of caspases and apoptosis. Antisense CD95-L as well as dominant-negative FADD inhibited ceramide- and cellular stress-induced apoptosis. Fibroblasts from type A Niemann–Pick patients (NPA), genetically deficient in ceramide synthesis, failed to up-regulate CD95-L expression and to undergo apoptosis after γ-irradiation or doxorubicin treatment. In contrast, JNK/SAPK activity was still inducible by doxorubicin in the NPA cells, suggesting that activation of JNK/SAPKs alone is not sufficient for induction of the CD95 system and apoptosis. CD95-L expression and apoptosis in NPA fibroblasts were restorable by exogenously added ceramide. In addition, NPA fibroblasts undergo apoptosis after triggering of CD95 with an agonistic antibody. These data demonstrate that ceramide links cellular stress responses induced by γ-irradiation or anticancer drugs to the CD95 pathway of apoptosis.

Keywords: cellular stress/doxorubicin/γ-irradiation/JNK–SAPKs/Niemann–Pick

Introduction

Programmed cell death (apoptosis) is a genetically defined pathway of cellular suicide required for embryonal development, growth and differentiation of multicellular organisms. In addition, it serves as one of the pleiotropic mechanisms of cell killing by cytokines and cytotoxic T cells (Laster *et al.*, 1988; Oberhammer *et al.*, 1992; Dhein *et al.*, 1995; Nagata and Golstein, 1995) and appears to be the basic mechanism underlying the anti-oncogenic effect of most chemotherapeutic drugs and X-ray treatment (Haimovitz-Friedman *et al.*, 1994; Bose *et al.*, 1995; Ling *et al.*, 1995; Friesen *et al.*, 1996; Verheij *et al.*, 1996). Recent investigations provided evidence that ionizing radiation and anticancer drugs induce sphingomyelin signal transduction to mediate apoptosis (Haimovitz-Friedman *et al.*, 1994; Jayadev *et al.*, 1994; Wiegmann *et al.*, 1994; Bose *et al.*, 1995; Jaffrezou *et al.*, 1996). The sphingomyelin pathway is a ubiquitous signaling system that links specific cell surface receptors and environmental 'stresses' to cellular responses (Kolesnick and Golde, 1994; Hannun, 1996). This pathway is initiated by hydrolysis of sphingomyelin, which is a main lipid in the plasma membranes of mammalian cells. Sphingomyelin hydrolysis occurs within seconds to minutes after stimulation via the action of sphingomyelin-specific forms of phospholipase C, termed sphingomyelinases, to generate ceramide. Signaling can be initiated by an acid or a neutral form of sphingomyelinase, distinguishable by their pH optima (for review, see Kolesnick and Fuks, 1995; Testi, 1996). Ceramide then serves as a second messenger, leading to induction of the stress-activated protein kinases (JNK/SAPKs) (Verheij *et al.*, 1996). Details of the signal pathway downstream of JNK/SAPKs leading to apoptosis are not well understood. However, there is evidence that one of the known substrates of JNK/SAPKs, the transcription factor c-Jun (Hibi *et al.*, 1993), is involved in apoptosis (Estus *et al.*, 1994; Hallahan *et al.*, 1995; Pandey and Wang, 1995; Verheij *et al.*, 1996; Bossy-Wetzel *et al.*, 1997).

Recently, we demonstrated the involvement of the CD95 (APO-1/Fas) system in γ-irradiation- and drug-induced apoptosis of cancer cells (Friesen *et al.*, 1996; I.Herr *et al.*, submitted). The CD95 receptor–ligand system is a key regulator of apoptosis (Trauth *et al.*, 1989; Itoh *et al.*, 1991; Debatin *et al.*, 1993; Suda *et al.*, 1993; Krammer *et al.*, 1994). CD95 is constitutively expressed on the surface of many cells including activated T and B lymphocytes, colon epithelial cells, liver cells (Krammer, 1996) and several other tissues and tumors (Leithäuser *et al.*, 1993). The main function of CD95 appears to be the induction of apoptosis in sensitive cells triggered by the CD95 ligand (CD95-L) or by agonistic antibodies. CD95-L is a type II transmembrane molecule which is also found in a soluble form released from the cell membrane through proteolytic cleavage by metalloproteinases (Kayagaki *et al.*, 1995). Recently, critical elements of the CD95 pathway that link receptor–ligand interaction and downstream activation of caspases have been identified. After receptor multimerization, the death-inducing signaling complex (DISC) is formatted which contains the molecules FADD and FLICE. The chimeric protein FLICE contains an adaptor domain able to bind to FADD as well as a proteolytic domain similar to caspases. Thus, this Janus-type molecule links the death domain-associating proteins of the DISC to the proteolytic cascade that exerts the death signal (Muzio *et al.*, 1996; for review, see Debatin *et al.*, 1997). γ-Irradiation or treatment of tumor cells with anticancer drugs leads to enhanced CD95-L expression which initiates receptor–ligand interaction and activation

of caspases such as CPP32 (Friesen *et al.*, 1996; I.Herr *et al.*, submitted). One known substrate of CPP32 is PARP [poly(ADP-ribose) polymerase], which is also cleaved during drug-induced apoptosis (Kaufmann *et al.*, 1993; Nicholson *et al.*, 1995; Tewari *et al.*, 1995; I.Herr *et al.*, submitted).

In the present study, we provide different lines of evidence for a functional cross-talk between the sphingomyelin pathway and the CD95 system in anticancer drug- and γ-irradiation-induced apoptosis. Ceramide, the second messenger of the sphingomyelin pathway, strongly induced CD95-L expression. Failure to initiate CD95 signaling, either by inhibition through CD95-L antisense or by dominant-negative FADD, or in cells genetically deficient in ceramide synthesis, such as in fibroblasts from type A Niemann–Pick (NPA) patients, leads to significant reduction of apoptosis induced by ceramide. Exogenously added ceramide restored apoptosis and induction of CD95-L expression in NPA fibroblasts. In addition, NPA fibroblasts were sensitive to CD95-induced apoptosis using an agonistic antibody, suggesting that the CD95 pathway itself is intact in these cells.

Results

Activation of JNK/SAPKs during doxorubicin-induced apoptosis

Recently, we described that chemo- and radiotherapy induce expression of CD95-L which mediates apoptosis by binding to its receptor and activation of the CD95 pathway including cleavage of caspases (Friesen *et al.*, 1996; I.Herr *et al.*, submitted). However, the mechanism by which CD95-L expression is enhanced in response to drugs and γ-irradiation is still unknown. A characteristic feature of γ-irradiation is the induction of the cellular stress response including activation of JNK/SAPKs. Enhanced JNK/SAPK activity may be required for initiation of stress-induced apoptosis (Chen *et al.*, 1996; Verheij *et al.*, 1996), possibly through enhanced expression of c-Jun, a target of JNK/SAPKs (Hibi *et al.*, 1993) which seems to be important for stress-induced apoptosis (Hallahan *et al.*, 1995; Verheij *et al.*, 1996).

To examine whether JNK/SAPKs might also be activated by anticancer drugs, we treated leukemic T cells (CEM) with doxorubicin. The anthracycline doxorubicin acts on mammalian cells by multiple mechanisms involving cell membrane effects, intercalation into DNA, inhibition of topoisomerase II (Calabresi and Chabner, 1990) and generation of ceramide (Bose *et al.*, 1995; Jaffrezou *et al.*, 1996). Activation of JNK/SAPKs was measured by immune complex kinase assay using bacterially expressed GST–c-Jun as substrate. JNK/SAPK activity appeared already 2 h after induction, with a maximum between 8 and 12 h. Similar data were obtained by treatment of primary human fibroblasts with doxorubicin (data not shown, see also Figure 5). Under these conditions, the kinetics of induction of JNK/SAPKs precede the expression of CD95-L protein (data not shown, see also Figures 4 and 6).

Activation of the CD95 system by ceramide

γ-Irradiation and cytotoxic drugs like doxorubicin have been shown to activate the CD95 system of apoptosis

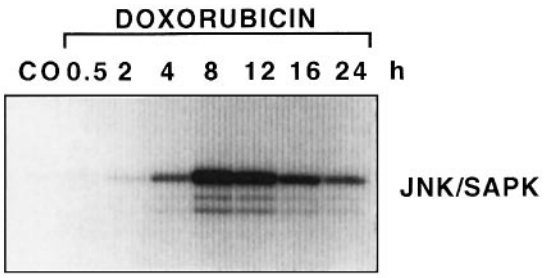

Fig. 1. Doxorubicin induces activity of JNK/SAPKs. CEM cells were treated with 500 ng/ml doxorubicin and lysed on ice after the indicated time points. The nuclei-free supernatant was normalized for protein content and immunoprecipitated with anti-JNK/SAPK antibody-conjugated Sepharose beads. GST–c-Jun 1–166 fusion protein was added to the immunocomplexes and incubated in kinase buffer in the presence of [γ-^{32}P]ATP. The phosphorylated fusion protein was resolved by 10% SDS–PAGE and visualized by autoradiograpy.

(Friesen *et al.*, 1996; I.Herr *et al.*, submitted). In addition, these inducers are described to enhance levels of ceramide and to activate JNK/SAPKs (Bose *et al.*, 1995; Jaffrezou *et al.*, 1996; Verheij *et al.*, 1996). Therefore, we asked whether generation of ceramide might critically influence CD95-mediated apoptosis. Treatment of leukemic T cells with C2-ceramide for 16 h strongly induced apoptosis at concentrations of 10–25 μM, as demonstrated by DNA fragmentation (Figure 2A). Prior to apoptosis mediated by C2-ceramide, expression of CD95-L was strongly upregulated (Figure 2B and D; and data not shown). CD95-L protein expression started to increase between 3 and 4 h. The highest levels of CD95-L protein expression were reached at 6–12 h. Cleavage of CPP32 and PARP was visible 6 h after treatment with C2-ceramide (Figure 2C) and maximal at 12 h when ~70% of the cells were already undergoing apoptosis (data not shown). Induction of increased CD95-L expression and apoptosis was specific for biologically active C2-ceramide since C2-dihydroceramide, which lacks the *trans* double bond at C4–C5 of the sphingoid base backbone, was ineffective. Furthermore, C2-ceramide led to a rapid and long-lasting induction of c-*jun* expression preceding the increase of CD95-L mRNA, as examined by RT–PCR (Figure 2D). These data are confirmed by treatment of primary human fibroblasts with ceramide (data not shown, see also Figure 5).

CD95 signaling is required for ceramide-induced apoptosis

To test the requirement for CD95 signaling in ceramide-induced apoptosis, we blocked CD95-L expression by an antisense approach and interrupted CD95 signaling by dominant-negative FADD. Leukemic T cells (CEM) were transiently transfected with empty expression vector, an antisense CD95-L construct or expression vectors encoding active or non-active dominant-negative FADD. The percentage of specific apoptosis induced by 10 μM C2-ceramide was determined by annexin staining and flow cytometry. Transfection with antisense CD95-L or expression of dominant-negative FADD strongly inhibited ceramide-, doxorubicin- and γ-irradiation-induced apoptosis (Figure 3A and data not shown). Transfection efficiency was at least 70% as determined by transfection of a green fluorescent protein (GFP) construct under the same conditions, which was analyzed by fluorescence micro-

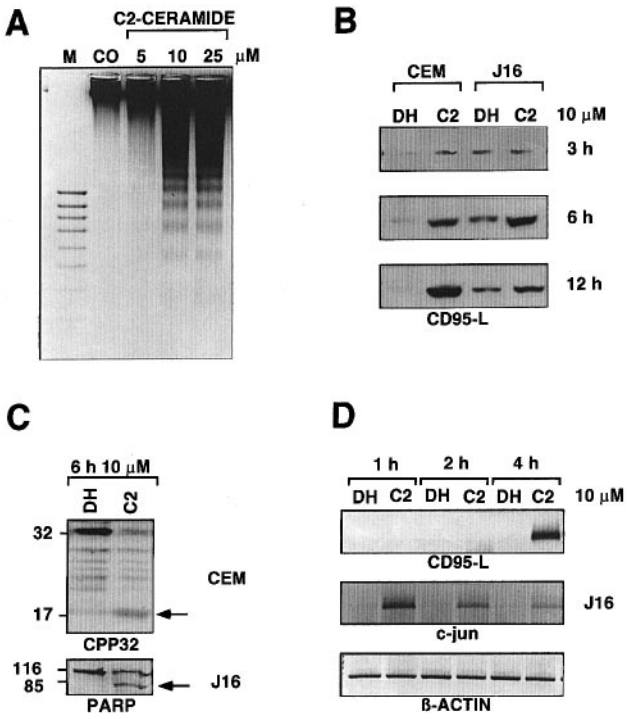

Fig. 2. Ceramide induces apoptosis, CD95-L expression and activation of caspases. (**A**) Serum-starved Jurkat-16 cells were treated with the indicated concentrations of C2-ceramide or with 25 μM biologically inactive C2-dihydroceramide in the control (CO); 16 h later cells were lysed, fragmented DNA was prepared and the DNA ladder detected as described in Material and methods. 'M' represents a 100 bp DNA marker. (**B**) C2-ceramide induces CD95-L protein expression. Serum-starved CEM and Jurkat-16 cells were stimulated with either 10 μM C2-ceramide (C2) or, as control, with 10 μM C2-dihydroceramide (DH) for the indicated time points. Protein expression was detected by Western blot analysis. The anti-CD95-L monoclonal antibody raised against the soluble part of human CD95-L recognizes in cell extracts the full-length CD95-L protein of 39 kDa. (**C**) Incubation of serum-starved CEM and Jurkat-16 cells with 10 μM C2-ceramide for 6 h induces cleavage of CPP32 and PARP. Proteolytic cleavage of the CPP32 precursor (32 kDa) to the active protease (17 kDa) was monitored by Western blot analysis using a specific monoclonal antibody. Cleavage of PARP (116 kDa) to the characteristic 85 kDa fragment was identified with a PARP-specific antiserum. (**D**) RT–PCR performed in Jurkat-16 cells treated for the indicated time points with either 10 μM C2-dihydroceramide (DH) or 10 μM C2-ceramide (C2). Expression of CD95-L, c-*jun* and β-actin mRNAs was examined by RT–PCR using sequence-specific primers for these genes.

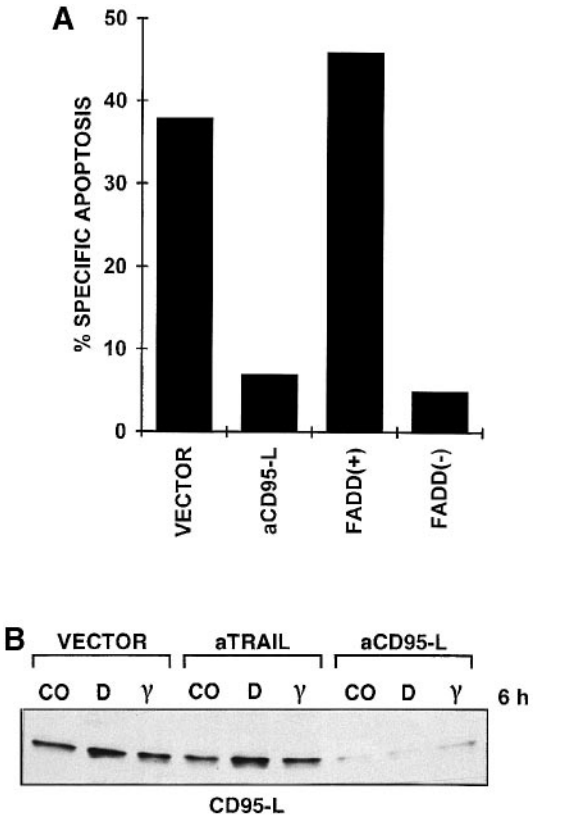

Fig. 3. Inhibition of CD95 signaling strongly reduces ceramide-induced apoptosis. (**A**) CEM cells were transiently transfected with empty vector (VECTOR), or an CD95-L antisense construct (aCD95-L), or expression vectors encoding active [FADD(–)] or inactive [FADD(+)] dominant-negative FADD protein as described in Materials and methods. After 20 h, cells were treated with 10 μM C2-ceramide or with 10 μM C2-dihydroceramide as control. Apoptosis was measured by annexin staining using flow cytometry. The percentage of specific apoptosis was calculated as followed: 100×[experimental apoptosis (%) – spontaneous apoptosis in the control (%)/100% – spontaneous apoptosis in the control (%)]. The constructs were tested in three separate experiments using CEM cells with very similar results. (**B**) Western blot analysis for CD95-L in CEM cells which were transiently transfected by electroporation with either empty vector (VECTOR) or antisense expression constructs against Trail (aTRAIL) or CD95-L (aCD95-L). At 20 h after transfection, cells were left either untreated (CO), treated with 500 ng/ml doxorubicin (D) or γ-irradiated with 10 Gy (γ), and proteins were harvested 6 h later.

scopy and flow cytometry (data not shown). Down-regulation of CD95-L expression was controlled by Western blot, which demonstrated that the antisense CD95-L construct, in contrast to the empty vector, or an unrelated antisense construct (TRAIL), nearly completely repressed basal and induced CD95-L protein (Figure 3B). Similar results were obtained in other leukemic T cell lines (Jurkat-16) and in the neuroblastoma cell line SHEP (data not shown).

Next we examined CD95-L expression in acid sphingomyelinase-deficient human fibroblasts derived from NPA patients and in fibroblasts derived from healthy individuals. NPA disease is caused by deficiency of the acid sphingomyelinase gene, as recently demonstrated by acid sphingomyelinase knock-out mice (Horinouchi *et al.*, 1995; Otterbach and Stoffel, 1995). Lymphoblasts from NPA patients fail to generate ceramide and to undergo apoptosis in response to ionizing radiation (Santana *et al.*, 1996). Consistent with these data, we found strongly reduced ceramide production and cell death in human fibroblasts from NPA patients after γ-irradiation or treatment with doxorubicin compared with normal fibroblasts (Figure 4A and data not shown). In contrast to the control fibroblasts (COFI) which strongly up-regulate CD95-L expression after treatment with therapeutic doses of doxorubicin from 6 to 24 h (Figure 4B and C), up-regulation of CD95-L protein in NPA fibroblasts was very weak (NPA 1) or totally absent (NPA 2). Correspondingly, doxorubicin-induced death in NPA 1 cells was higher (13%) than cell death of NPA 2 fibroblasts with completely blocked CD95-L expression (3%). The failure to increase CD95-L mRNA expression in NPA cells versus COFI was confirmed by RT–PCR. While normal fibroblasts strongly up-regulate CD95-L mRNA levels between 6 and 10 h after treatment with doxorubicin, CD95-L mRNA was undetectable in the NPA cells (Figure 4C). Similar results

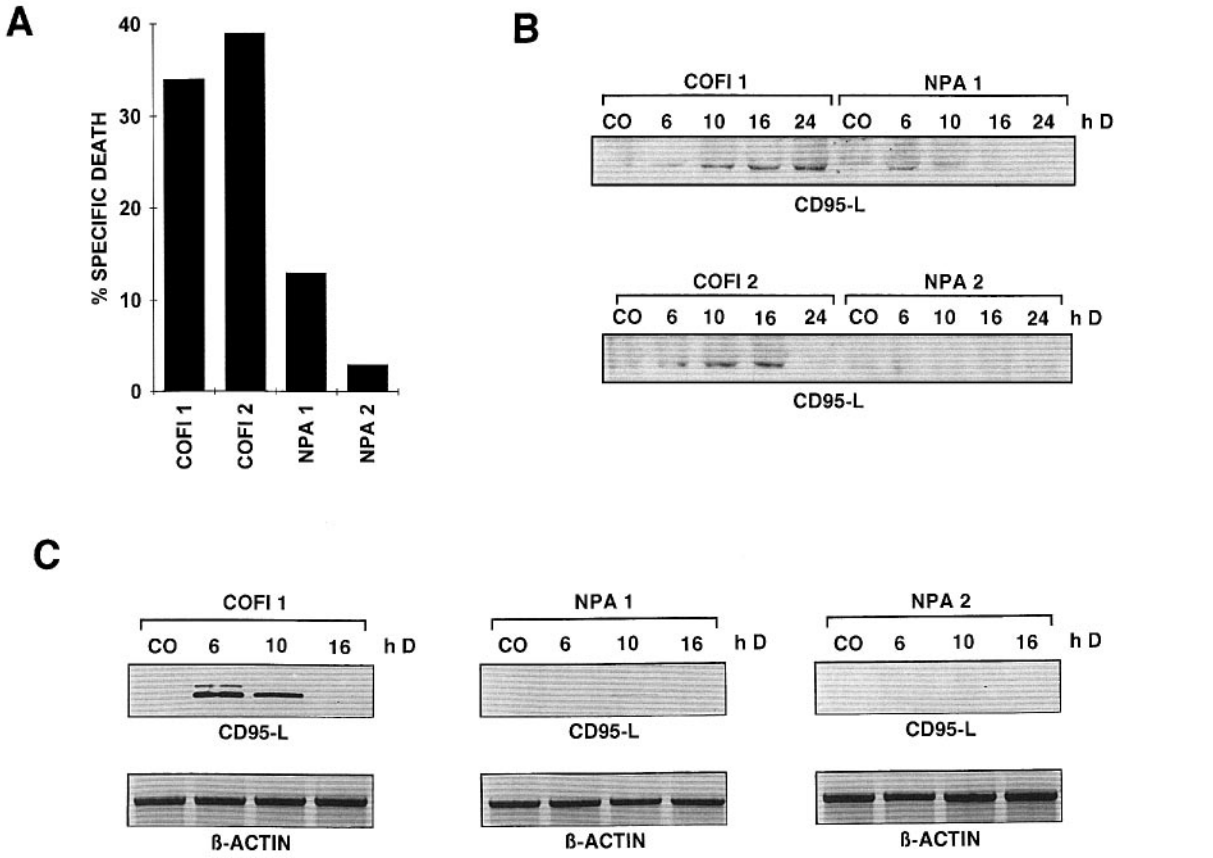

Fig. 4. Doxorubicin-induced apoptosis and CD95-L expression is strongly inhibited in fibroblasts of NPA patients. (**A**) Fibroblasts from healthy individuals (COFI 1 and COFI 2) and from NPA patients (NPA 1 and NPA 2) were treated with doxorubicin (500 ng/ml), and 36 h later cell death was determined by Trypan blue exclusion and calculated as described in Figure 3A. The experiment presented has been repeated three times with an essentially identical outcome. (**B**) Western blot analysis of CD95-L expression using the same experimental procedure as described in (A), except that the fibroblasts were lysed at the indicated time points after treatment with doxorubicin. (**C**) CD95-L mRNA expression of fibroblasts from a healthy control or NPA patients before and after treatment with doxorubicin as described in (A) and (B) was examined by RT–PCR. The same amounts of reverse-transcribed RNA from COFI 1, NPA 1 and NPA 2 were amplified in 38 PCR cycles using specific primers for CD95-L. The β-actin gene was amplified in 25 PCR cycles and serves as control for equal conditions. The 2% agarose gels containing ethidium bromide were visualized by UV transillumination and photographed using identical exposure times.

were obtained after γ-irradiation of control and mutant fibroblasts (data not shown). Although death and CD95-L expression are repressed in NPA fibroblasts, strongly induced activation of JNK/SAPKs and c-*jun* after treatment of control and mutant cells with doxorubicin was found (Figure 5 and data not shown).

To ensure that failure to up-regulate CD95-L expression and to undergo apoptosis in response to cellular stresses are due to a defect in ceramide generation, we bypassed this element of the signal transduction cascade by adding C2-ceramide exogenously to COFI and NPA fibroblasts. Using concentrations from 25 to 50 μM, C2-ceramide was found to induce apoptosis efficiently in COFI and fibroblasts from NPA patients (Figure 6A and B). NPA fibroblasts were even slightly more sensitive at lower concentrations of ceramide treatment compared with COFI. Correspondingly, ceramide treatment led to an even stronger up-regulation of CD95-L mRNA in the mutant cells than in the control fibroblasts. Treatment with biologically inactive C2-dihydroceramide had no effect on apoptosis and CD95-L expression (Figure 6C). Finally, COFI and fibroblasts from NPA patients displayed similar apoptosis sensitivity after direct triggering of the CD95 receptor with an agonistic antibody. Similar percentages

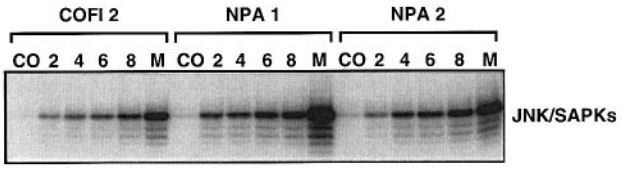

Fig. 5. Doxorubicin induces JNK/SAPK activity in NPA and COFI cells. Primary human control (COFI 2) and NPA fibroblasts (NPA 1 and NPA 2) were treated with 500 ng/ml doxorubicin and lysed on ice during a time period of 2–8 h as indicated. Untreated cells (CO) were used as negative control, and cells treated with the alkylating agent methyl methanesulfonate (M; 1 mM, 3 h) served as positive control for induction (Wilhelm *et al.*, 1997). The nuclei-free supernatant was normalized for protein content and immunoprecipitated with anti-JNK/SAPK antibody-conjugated Sepharose beads. GST–c-Jun 1–166 fusion protein was added to SAPK-bound beads in kinase buffer and incubated in the presence of [γ-^{32}P]ATP. The phosphorylated fusion protein was resolved by 10% SDS–PAGE and visualized by autoradiograpy.

of cell death were observed 24 h after stimulation of COFI and fibroblasts from NPA patients (Figure 7). Taken together, these data demonstrate that ceramide links the stress-induced sphingomyelin to the CD95 pathway of apoptosis.

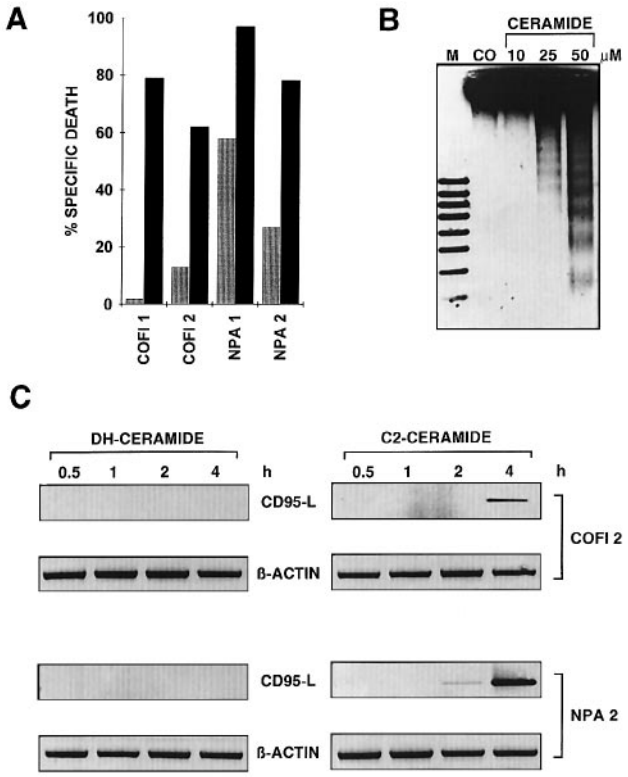

Fig. 6. Ceramide induces apoptosis and CD95-L expression in NPA and COFI cells. (**A**) Serum-starved control (COFI 1 and 2) and NPA (NPA 1 and 2) fibroblasts were treated with either 25 (gray bars) or 50 µM (black bars) C2-ceramide. Corresponding concentrations of C2-dihydroceramide were used in the controls. After 16 h, the percentage of specific death was determined by Trypan blue exclusion and calculated as described in Figure 3A. The experiment presented has been repeated three times with an essentially identical outcome. (**B**) DNA fragmentation 16 h after treatment of serum-starved NPA 2 cells with the indicated concentrations of C2-ceramide or with 25 µM C2-dihydroceramide in the control (CO). 'M' represents a 100 bp DNA marker. (**C**) CD95-L mRNA expression was examined by RT–PCR in control (COFI 2) and mutant fibroblasts (NPA 2) before and after treatment of the cells with 25 µM C2-dihydroceramide (DH-ceramide) or C2-ceramide for the indicated time points. Amplification of the β-actin gene serves as control for equal conditions. The 2% agarose gels containing ethidium bromide were visualized by UV transillumination and photographed using identical exposure times.

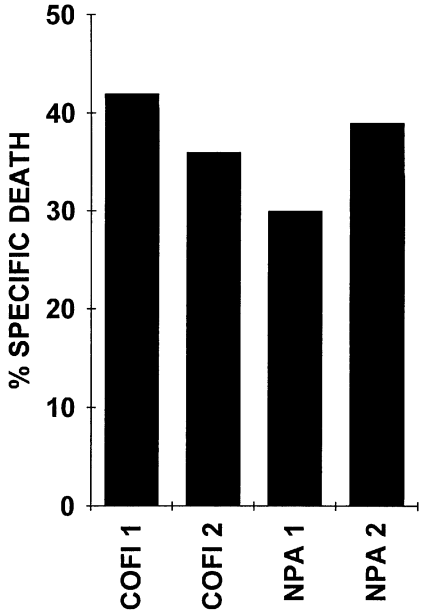

Fig. 7. Triggering of CD95 with an agonistic antibody induces apoptosis in NPA and COFI cells. Control (COFI 1 and 2) and NPA (NPA 1 and 2) fibroblasts were treated with 10 µg of anti-APO in the presence of 5 ng/mol protein A and 500 ng/ml cycloheximide. After 24 h, the percentage of specific death was determined by Trypan blue exclusion and calculated as described in Figure 3A. Cycloheximide or protein A alone had no influence on cell death at the concentrations used and during the examined time span. The experiment presented has been repeated three times with an essentially identical outcome.

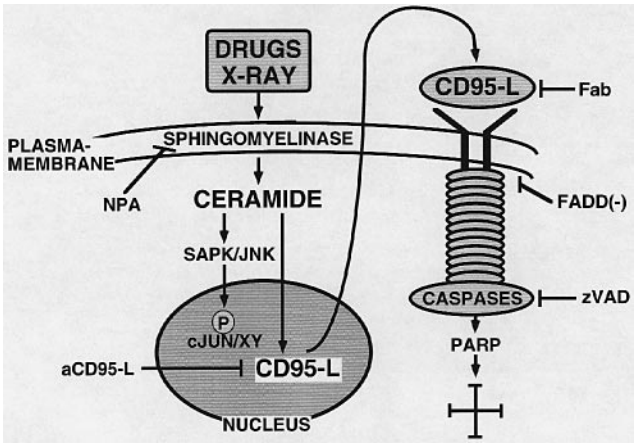

Fig. 8. Ceramide connects the sphingomyelinase/ceramide and the CD95 pathway of apoptosis. Drugs, doxorubicin; X-ray, γ-irradiation; aCD95-L, inhibition of CD95-L protein expression by transient transfection of an antisense-CD95-L construct; FADD(–), inhibition of CD95 signaling by transient transfection of a dominant-negative FADD construct; NPA, type A Niemann–Pick disease; Fab, F(ab)2 antibody fragments which specifically block the CD95 receptor; zVAD, inhibiting substrate for caspases.

Discussion

The present studies establish a cross-talk between the cellular stress pathway initiated by the generation of ceramide and the CD95 pathway of apoptosis. Previously, ionizing radiation and some anticancer drugs have been shown to induce rapid generation of ceramide, followed by apoptosis (Haimovitz-Friedman *et al.*, 1994; Jayadev *et al.*, 1994; Wiegmann *et al.*, 1994; Bose *et al.*, 1995; Jaffrezou *et al.*, 1996). Elevation of ceramide by addition of exogenous ceramide analogs was sufficient for induction of apoptosis (Haimovitz-Friedman *et al.*, 1994; Jayadev *et al.*, 1994; Bose *et al.*, 1995; Jaffrezou *et al.*, 1996). Recently, we determined that intact CD95 signaling was required for chemo- and radiotherapy-induced apoptosis (Friesen *et al.*, 1996; I.Herr *et al.*, submitted).

We provide strong experimental evidence for a hierarchical organization of the sphingomyelin and the CD95 pathway in our system, as schematically summarized in Figure 8. Generation of ceramide by the anticancer drug doxorubicin or in response to γ-irradiation is functionally located upstream of CD95-mediated apoptosis. Ceramide rapidly and strongly induces expression of CD95-L protein, which interacts with CD95 receptor to activate CD95 signaling reflected by cleavage of caspases. Fibroblasts from two NPA patients with a gene defect in acid sphingomyelinase (Horinouchi *et al.*, 1995; Otterbach and Stoffel, 1995) do not up-regulate CD95-L expression

and are resistant towards γ-irradiation- and doxorubicin-induced apoptosis. In agreement with our findings, Santana *et al.* (1996) recently reported that lymphoblasts from NPA patients fail to respond to γ-irradiation with apoptosis due to the failure of ceramide generation. Correspondingly, stimulation of NPA fibroblasts with exogenous ceramide induces CD95-L expression and restores apoptosis sensitivity. Importantly, also direct triggering of CD95 with an agonistic antibody induces apoptosis in control and patient fibroblasts. We demonstrate that apoptosis mediated by doxorubicin, γ-irradiation (I.Herr *et al.*, submitted) or ceramide is strongly repressed in cells in which CD95-L synthesis or CD95 signaling was inhibited by an antisense approach or by dominant-negative FADD. However, under these conditions, apoptosis by antisense CD95-L or by a dominant-negative FADD construct was not totally blocked, suggesting that other systems independent of CD95 are activated during stress-induced apoptosis. Recent data demonstrate activation of TRAIL and tumor necrosis factor-α (TNF-α) after treatment of diverse cell lines with anticancer drugs and γ-irradiation (I.Herr *et al.*, submitted). The TRAIL system seems to act by a receptor distinct from CD95 and TNF receptors and to use signals independently of elements within the CD95 and TNF pathway such as FADD (Masters *et al.*, 1996; Pitti *et al.*, 1996). Thus, in addition to CD95-L, ceramide may activate expression of several apoptosis-inducing ligands which together may act as amplifiers for cell death following γ-irradiation or treatment with anticancer drugs (I.Herr and K.-H.Debatin, in preparation). These data are in line with recent results indicating that the loss of ceramide production confers resistance to γ-irradiation-induced apoptosis (Chmura *et al.*, 1997) and that CPP32 activation is required for ceramide-induced apoptosis (Mizushima *et al.*, 1996). We observed that phorbol ester-dependent inhibition of upstream events such as sphingomyelin hydrolysis and ceramide generation (Haimovitz-Friedman *et al.*, 1994) also blocked doxorubicin-induced CD95-L up-regulation, cleavage of CPP32 and cell death (I.Herr and K.-M.Debatin, unpublished data). These results are in agreement with recent findings demonstrating that apoptosis in response to γ-irradiation or CD95 triggering is strongly diminished upon co-treatment with phorbol esters (Haimovitz-Friedman *et al.*, 1994; Tepper *et al.*, 1995; Del Carmen Ruiz-Ruiz *et al.*, 1997), suggesting that PKC plays a negative role in CD95 signaling.

We found that the anticancer drug doxorubicin activates JNK/SAPKs, similarly to increased JNK/SAPK activity in response to γ-irradiation, ceramide or other forms of cellular stress (Chen *et al.*, 1996; Verheij *et al.*, 1996; Ichijo *et al.*, 1997). There is suggestive evidence that JNK/SAPK activation is associated with CD95-mediated apoptosis (Cahill *et al.*, 1996; Wilson *et al.*, 1996), and counteracting activation of JNK/SAPKs by sphingosine-1-phosphate inhibits CD95-induced cell death (Cuvillier *et al.*, 1996). Paradoxically, JNK/SAPK activation in response to various stimuli was also demonstrated to be dispensable for apoptosis or even to prevent this process (Liu *et al.*, 1996; Nishina *et al.*, 1997). These apparently opposing effects of JNK/SAPK activation on induction of apoptosis pathways are also reflected by the function of known JNK/SAPK substrates, such as the transcription factors ternary complex factor (TCF)/Elk-1 and c-Jun. Activation of TCF/Elk-1 by JNK/SAPKs mediates transcriptional activation of the c-*fos* gene in response to cellular stress, such as UV irradiation (Cavigelli *et al.*, 1995). However, c-*fos*-deficient cells, compared with their wild-type counterparts, exhibit UV hypersensitivity, due to enhanced apoptosis (Schreiber *et al.*, 1995). c-Jun, the most extensively studied substrate of JNK/SAPKs, seems to be required for both the apoptotic program and survival. In c-*jun*-deficient fibroblasts, the efficiency of cell death induced by DNA-damaging agents is strongly reduced (A.Kolbus, M.Schreiber, E.F.Wagner and P.Angel, unpublished). Transfection of a dominant-negative c-Jun mutant (TAM-67) was found to inhibit stress- and ceramide-induced apoptosis (Verheij *et al.*, 1996), whereas c-Jun seems to be required both for neuronal death and for neuronal survival and regeneration (Estus *et al.*, 1994; Ham *et al.*, 1995; Herdegen *et al.*, 1997). Thus, it is tempting to speculate that specific, c-Jun-containing AP-1 dimers might influence the CD95 system. For example, transcriptional activation of the CD95 and CD95-L genes, or the genes encoding metalloproteinases, which cleave the CD95-L protein from the cell surface (Kayagaki *et al.*, 1995), may be regulated directly or indirectly by c-Jun. On the other hand, activation of JNK/SAPKs and c-*jun* was unimpaired in NPA fibroblasts after stimulation with doxorubicin, suggesting that activation of the JNK/SAPK pathway may be required but is not sufficient for induction of apoptosis. Thus, additional mechanisms are necessary for induction of CD95 signaling during cellular stress-induced apoptosis. Activation of JNK/SAPKs in NPA fibroblasts also demonstrates that enhanced JNK/SAPK acitivity is a specific response rather than only a secondary effect of the apoptotic program. Efficient activation of JNK/SAPKs and c-*jun* expression in NPA fibroblasts may be explained by the existence of different and independent signal transduction pathways activating JNK/SAPKs, and some of them may be independent of ceramide production (Karin, 1995; Whitmarsh and Davis, 1996; Fanger *et al.*, 1997). Regardless of the exact role of JNK/SAPK activation in the CD95-dependent apoptotic program, enhanced expression of CD95-L, followed by binding of the ligand to its specific receptor, is required for cellular stress-induced apoptosis, as evidenced from the anti-apoptotic effect of (Fab′)2 anti-CD95 fragments (Friesen *et al.*, 1996; I.Herr *et al.*, submitted). Furthermore, cells from lpr mice deficient in surface CD95 showed much lower levels of apoptosis than control cells after γ-irradiation or heat shock (Reap *et al.*, 1997). Finally, interrupting CD95 signaling by dominant-negative FADD or preventing cleavage of caspases with zVAD blocked cell death induced by exogenously added ceramide, doxorubicin treatment or γ-irradiation (Mizushima *et al.*, 1996; I.Herr *et al.*, submitted). Therefore, these data suggest that activation of CD95 signaling during stress-induced apoptosis acts downstream of the sphingomyelin pathway.

However, increased CD95-L expression in cells does not always lead to activation of the CD95 pathway. Thus, CD95-L is induced in resting T cells, e.g. by T cell receptor triggering or phorbol 12-myristate 13-acetate (PMA) stimulation, without induction of apoptosis (Klas *et al.*, 1993; Herr *et al.*, 1996), suggesting that the specific combination of pathways dictates the fate of the cell after a particular signal.

Taken together, our data demonstrate that molecules previously considered to become activated independently in response to cellular stresses culminate in activation of the CD95 pathway of apoptosis. The link between ceramide, stress pathways and the CD95 system provides a variety of molecular targets for therapeutic intervention and modulation of the sensitivity of tumor cells for induction of apoptosis.

Materials and methods

Cell culture

The human acute T-cell leukemia cell lines CEM and Jurkat-16 (J16) were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS; Conco, Wiesbaden, Germany), 100 U/ml penicillin, 100 mg/ml streptomycin, 25 mM HEPES and 2 mM L-glutamine (all from Gibco/Life Technologies, Paisley, UK). The primary fibroblasts COFI 1, 2 and NPA 1, 2 were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented as described for RPMI-1640 medium.

Measurement of apoptosis

Early apoptotic changes were identified by staining with fluorescein isothiocyanate (FITC)-conjugated annexin V (Bender Med Systems, Vienna, Austria) which binds to exposed phosphatidylserine on the surface of apoptotic cells (Koopman *et al.*, 1994). Cells were incubated in 200 µl of annexin buffer containing sterofundin solution (Braun, Melsungen, Germany) supplemented with 2% HEPES, pH 7.3 and 4 µl of annexin V–FITC (Bender Med Systems, Vienna, Austria) for 30 min at 4°C in the dark. Cells were analyzed by flow cytometry (FACScan, Becton Dickinson, Heidelberg, Germany) using the cell quest software.

Alternatively, cell death was determined by Trypan blue exclusion or apoptosis was detected by assessment of DNA fragmentation.

Agarose gel electrophoresis for DNA fragmentation

For analysis of DNA fragmentation, 1×10^7 cells were resuspended in 1.5 ml of lysis buffer containing 10 mM Tris–HCl, pH 7.5, 400 mM NaCl, 2 mM EDTA, pH 8.2, 0.7% SDS and 2 mg/ml proteinase K (Boehringer, Mannheim, Germany) and incubated at 37°C overnight. Protein was precipitated by addition of 0.5 ml of 5 M NaCl at room temperature for 1 h. Precipitated protein was pelleted by centrifugation at 3300 *g* for 30 min. DNA in the supernatant was precipitated with 2.5 volumes of 100% ethanol and washed in 70% ethanol after centrifugation for 30 min at 3300 *g*. The DNA pellet was dissolved in 100 µl of TE buffer containing 0.5 mg/ml RNase (Boehringer, Mannheim, Germany). After determination of the DNA content, 10 µg of DNA per lane were separated on 1.7% agarose gels containing ethidium bromide at 30 V for 6 h. The DNA fragmentation pattern was detected by UV trans-illumination.

Stimulation of cells

C2-ceramide (*N*-acetyl sphingosine) and C2-dihydroceramide (*N*-acetyl dihydrosphingosine) were obtained from Biomol, Hamburg, Germany. Stock solutions were prepared for C2-ceramide in dimethylsulfoxide (DMSO) and for C2-dihydroceramide in 100% ethanol. Stock solutions could be stored at –80°C for at least 4 weaks. Prior to treatment with ceramide, the cells were starved by culture for 12–16 h in medium containing 1% FCS. Cycloheximide (Sigma, Deisenhofen, Germany) stocks were dissolved in DMSO and aliquots were stored at –20°C. Doxorubicin (Sigma, Deisenhofen, Germany) was dissolved in sterile water, supplemented with 100% ethanol to a 90% ethanol stock solution which was stored in aliquots at –80°C. Anti-APO antibody was prepared as previously described (Dhein *et al.*, 1995). Protein A was obtained from Sigma, Deisenhofen, Germany. Stock solutions were dissolved in phosphate-buffered saline (PBS). Cells were γ-irradiated in their flasks using a cesium radiator. Final concentrations of the vehicles in medium were 0.1%, which had no influence on apoptosis or expression of the examined genes.

Transfections

Cells in medium were pelleted, resuspended at 1×10^8 cells/200 µl of PBS and transfected with 50 µg of expression plasmid by electroporation (925 µF, 200 V). After transfection, cells were resuspended in 10 ml of medium containing 10% FCS and seeded in a 6-well plate at 1×10^7 cells/well. Six hours later, medium was exchanged against fresh medium (in the case of ceramide stimulation the medium contained 1% FCS) and, after an additional 12 h, cells were stimulated.

Determination of caspase activity

Cleavage of CPP32 and PARP was detected by Western blotting. The cells (1×10^8) were washed in PBS, pelleted and lysed in one packed cell volume of a buffer containing 600 mM KCl, 20 mM HEPES–KOH, pH 7.9, 0.2 mM EDTA and 1 mM dithiothreitol (DTT) for 1 h on ice. After centrifugation for 30 min, the amount of proteins in the supernatant was determined as described by Bradford (1976), using the dye reagent purchased from Bio-Rad (München, Germany) and bovine serum albumin standards. Then 100 µg of lysate in Laemmli buffer were separated per lane on 12% SDS–PAGE under reducing conditions and transferred onto ECL membranes (Amersham, Braunschweig, Germany). Proteins were stained with specific antisera against CPP32 (Transduction Laboratories, Lexington, KY) or PARP (Enzyme Systems Products, Dublin, USA). Bound antibodies were detected with anti-mouse or anti-rabbit–horseradish peroxidase conjugate (Santa Cruz, CA). An enhanced chemiluminescence system (Amersham, Braunschweig, Germany) was used for detection.

Western blotting for CD95-L

A total of 5×10^6 cells were washed in PBS and lysed for 5 min on ice in 10 packed cell volumes of 30 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5% Na deoxycholate, 1 mM DTT, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml pepstatin, 2 µg/ml aprotinin, 2 µg/ml leupeptin. After centrifugation, 40 µg of protein/lane were separated on 12% SDS–PAGE and detected by Western blot as described for detection of ICE protease activity. Anti-CD95-L monoclonal antibody was obtained from Transduction Laboratories (Lexington, KY).

RT–PCR

RNA was harvested and RT–PCR was performed as described in Herr *et al.* (1996). Thirty eight cycles of amplification were used for CD95-L or *c-jun* and 25 cycles for β-actin. Primers used for amplification of CD95-L are described in Herr *et al.* (1996), whereas primers for amplification of the β-actin gene were obtained from Stratagene, Heidelberg, Germany. For amplification of base +1 (codon start) to base 254 of the c-*jun* cDNA, the following primer set was created: Jun 1, 5′-AGAGTTGCACTGAGTGTGGCTG-3′; Jun 2, 5′-TGTCAACAGCGC-CTGGGCAGCA-3′. Twenty µl of the PCR were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

JNK/SAPK assay

A total of 1×10^7 cells were washed in ice-cold PBS and lysed in 400 µl of lysis buffer (20 mM Tris pH 7.5, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, 25 mM β-glycerophosphate, 2 mM Na pyrophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 10 µg/ml pepstatin, 10 µg/ml aprotinin, 10 µg/ml leupeptin) at 0°C. Lysates were cleared by centrifugation for 20 min at 2°C and 13 000 r.p.m., and nuclei-free supernatant was normalized for protein content. Then 1.5 µl of polyclonal antibodies raised against JNK1 (C-17, Santa Cruz) and JNK2 (FL, Santa Cruz) were added to the supernatant, together with 50 µl of a 50% suspension of protein A–Sepharose beads. After 2 h incubation at 4°C, the beads were washed twice with lysis buffer and once with kinase buffer. The immune complex kinase assays were performed in 25 µl of kinase buffer consisting of 25 mM HEPES, pH 7.4, 25 mM $MgCl_2$, 25 mM β-glycerophosphate, 0.1 mM Na_3VO_4 and 5 µCi of [γ-^{32}P]ATP containing 2 µg of GST–Jun 1/166 (described in van Dam *et al.*, 1995). After 20 min at 30°C, the reaction was terminated by addition of Laemmli buffer and the products were resolved by 12% SDS–PAGE and visualized by autoradiography.

cDNA constructs

A 360 bp cDNA sequence encoding the 5′ end of human CD95-L was isolated by RT–PCR from CEM-derived mRNA and cloned in the antisense direction in front of the cytomegalovirus (CMV) promoter into the eukaryotic expression vector pcDNA3 (Invitrogen, NV Leek, The Netherlands). The GFP expression construct was obtained from Clontech (Heidelberg, Germany). FADD dominant-negative expression vectors are described in Chinnaiyan *et al.* (1995, 1996).

Acknowledgements

We thank Dr K.Harzer for providing fibroblasts from type A Niemann–Pick patients and from healthy individuals, Dr V.M.Dixit for dominant-

negative FADD constructs, Dr P.Roncaioli for confirming low ceramide levels in fibroblasts from NPA patients and H.Lattisch for technical assistance. This work was supported by 'Deutsche Leukämieforschungshilfe'.

References

Bose,R., Verheij,M., Haimovitz-Friedman,A., Scotto,A., Fuks,Z. and Kolesnick,R.N. (1995) Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals. *Cell*, **82**, 405–414.

Bossy-Wetzel,E., Bakiri,L. and Yaniv,M. (1997) Induction of apoptosis by the transription factor c-Jun. *EMBO J.*, **16**, 1695–1709.

Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.*, **72**, 248–254.

Cahill,M.A., Peter,M.E., Kischkel,F.C., Chinnaiyan,A.M., Dixit,V.M., Krammer,P.H. and Nordheim,A. (1996) CD95 (APO-1/Fas) induces activation of SAP kinases downstream of ICE-like proteases. *Oncogene*, **13**, 2087–2096.

Calabresi,P. and Chabner,B.A. (1990) Chemotherapy of neoplastic diseases. In Gilman,A.G., Rall,T.W., Nies,A.S. and Taylor,P. (eds), *Pharmacologic Basis of Therapeutics*. Eighth edn. Pergamon Press, New York, pp. 1202–1263.

Cavigelli,M., Dolfi,F., Claret,F.-X. and Karin,M. (1995) Induction of c-fos expression through JNK-mediated TCF/Elk-1 phosphorylation. *EMBO J.*, **14**, 5957–5964.

Chen,Y.-R., Meyer,C.F. and Tan,T.-H. (1996) Persistent activation of c-Jun N-terminal kinase 1 (JNK1) in γ-radiation-induced apoptosis. *J. Biol. Chem.*, **271**, 631–634.

Chinnaiyan,A.M., O'Rourke,K., Tewari,M. and Dixit,V.M. (1995) FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell*, **81**, 502–512.

Chinnaiyan,A.M., Tepper,C.G., Seldin,M.F., O'Rourke,K., Kischkel,F.C., Hellbardt,S., Krammer,P.H., Peter,M.E. and Dixit,V.M. (1996) FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. *J. Biol. Chem.*, **271**, 4961–4965.

Chmura,S.J., Nodzenski,E., Beckett,M.A., Kufe,D.W., Quintans,J. and Weichselbaum,R.R. (1997) Loss of ceramide production confers resistance to radiation-induced apoptosis. *Cancer Res.*, **57**, 1270–1275.

Cuvillier,O., Pirianov,G., Kleuser,B., Vanek,P.G., Coso,O.A., Gutkind,S. and Spiegel,S. (1996) Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature*, **381**, 800–803.

Debatin,K.-M., Goldmann,C.K., Waldmann,T.A. and Krammer,P.H. (1993) CD95 induced apoptosis of leukemia cells from patients with adult T-cell leukemia. *Blood*, **81**, 2972–2977.

Debatin,K.-M., Beltinger,C., Böhler,T., Fellenberg,J., Friesen,C., Fulda,S., Herr,I., Los,M., Scheuerpflug,C. and Sieverts,H. (1997) Signalling and cell death. *Biochem. Soc. Trans.*, **25**, 405–410.

Del Carmen Ruiz-Ruiz,M., Izquierdo,M., de Murcia,G. and Lopez-Rivas,A. (1997) Activation of protein kinase C attenuates early signals in Fas-mediated apoptosis. *Eur. J. Immunol.*, **27**, 1442–1450.

Dhein,J., Walczak,H., Bäumler,C., Debatin,K.-M. and Krammer,P.H. (1995) Autocrine T-cell suicide mediated by APO-1 (Fas/CD95) *Nature*, **373**, 438–441.

Estus,S., Zaks,W.J., Freeman,R.S., Gruda,M., Bravo,R. and Johnson,E.M.,Jr (1994) Altered gene expression in neurons during programmed cell death: identification of c-*jun* as necessary for neuronal apoptosis. *J. Cell. Biol.*, **127**, 1717–1727.

Fanger,G.R., Gerwins,P., Widmann,C., Jarpe,M.B. and Johnson,G.L. (1997) MEKKs, GCKs, MLKs, PAKs, TAKs and tpls: upstream regulators of the c-Jun amino-terminal kinases? *Curr. Opin. Genet. Dev.*, **7**, 67–74.

Friesen,C., Herr,I., Krammer,P.H. and Debatin,K.-M. (1996) Involvement of the CD95(APO-1/Fas) receptor/ligand system in drug-induced apoptosis in leukemia cells. *Nature Med.*, **2**, 574–577.

Haimovitz-Friedman,A., Kan,C., Ehleiter,D., Persaud,R., McLoughlin,M., Fuks,Z. and Kolesnick,R.N. (1994) Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. *J. Exp. Med.*, **180**, 525–535.

Hallahan,D.E., Dunphy,E., Virudachalam,S., Sukhatme,V.P., Kufe,D.W. and Weichselbaum,R.R. (1995) c-*jun* and Egr-1 participate in DNA synthesis and cell survival in response to ionizing radiation exposure. *J. Biol. Chem.*, **270**, 30303–30309.

Ham,J., Babij,C., Whitfield,J., Pfarr,C.M., Lallemand,D., Yaniv,M. and Rubin,L.L. (1995) A c-Jun dominant-negative mutant protects sympathetic neurons against programmed cell death. *Neuron*, **15**, 927–939.

Hannun,Y.A. (1996) Functions of ceramide in coordinating cellular responses to stress. *Science*, **274**, 1866–1859.

Herdegen,T., Skene,P. and Bähr,M. (1997) The c-Jun transcription factor—bipotential mediator of neuronal death, survival and regeneration. *Trends Neurosci.*, **20**, 227–231.

Herr,I., Balemans,L., Boehler,T., Walczak,H. and Debatin,K.-M. (1996) Monitoring of CD95(APO-1/Fas) ligand expression in human T-cells by quantitative RT–PCR. *Cell Death Differ.*, **3**, 299–305.

Hibi,M., Lin,A., Smeal,T., Minden,A. and Karin,M. (1993) Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the cjun activation domain. *Genes Dev.*, **7**, 2135–2148.

Horinouchi,K., Erlich,S., Perl,D.P., Ferlinz,K., Bisgaier,S.L., Sandhoff,K., Desnick,R.J., Stewart,C.L. and Schuchman,E.H. (1995) Acid sphingomyelinase deficient mice: a model of types A and B Niemann–Pick disease. *Nature Genet.*, **10**, 288–293.

Ichijo,H. *et al*. (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science*, **275**, 90-93.

Itoh,N., Yoneharea,S., Ishii,A., Yonehara,M., Mizushima,S., Sameshima,M., Hase,A., Seto,Y. and Nagata,S. (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell*, **66**, 233–243.

Jaffrezou,J.-P., Levade,T., Bettaieb,A., Andrieu,N., Bezombes,C., Maestre,N., Vermeersch,S., Rousse,A. and Laurent,G. (1996) Daunorubicin induced apoptosis: triggering of ceramide generation through sphingomyelin hydrolysis. *EMBO J.*, **15**, 2417–2424.

Jayadev,S., Linardic,C.M. and Hannun,Y.A. (1994) Identification of arachidonic acid as a mediator of sphingomyelin hydrolysis in response to tumor necrosis factor α. *J. Biol. Chem.*, **269**, 5757–5763.

Karin,M. (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. *J. Biol. Chem.*, **270**, 16483–16486.

Kaufmann,S.H., Desnoyers,S., Ottaviano,Y., Davidson,N.E. and Poirier,G.G. (1993) Specific proteolytic cleavage of poly(ADP-ribose)polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res.*, **53**, 3976–3985.

Kayagaki,N., Kawasaki,A., Ebata,T., Ohmoto,H., Ikeda,S., Inoue,S., Yoshino,K., Okumura,K. and Yagit,H. (1995) Metalloproteinase-mediated release of human Fas ligand. *J. Exp. Med.*, **182**, 1777–1783.

Klas,C., Debatin,K.-M., Jonker,R.R. and Krammer,P.H. (1993) Activation interferes with the apoptotic pathway in mature human T cells. *Int. Immunol.*, **5**, 625–631.

Kolesnick,R. and Fuks,Z. (1995) Ceramide: a signal for apoptosis or mitogenesis? *J. Exp. Med.*, **181**, 1949–1952.

Kolesnick,R. and Golde,D.W. (1994) The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. *Cell*, **77**, 325-328.

Koopman,G., Reutelingsperger,C.P.M., Kuijten,G.A.M., Keehnen, R.M.J., Pals,S.T. and van Oers,M.H.J. (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood*, **84**, 1415–1420.

Krammer,P.H. (1996) The CD95(APO-1/Fas) receptor/ligand system: death signals and diseases. *Cell Death Differ.*, **3**, 159–160.

Krammer,P.H. *et al*. (1994) The role of APO-1 mediated apoptosis in the immune system. *Immunol. Rev.*, **142**, 175–191.

Laster,S.M., Wood,J.G. and Gooding,L.R. (1988) Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis. *J. Immunol.*, **141**, 2629–2634.

Leithäuser,F. *et al*. (1993) Constitutive and induced expression of APO-1, a new member of the NGF/TNF receptor superfamily, in normal and neoplastic cells. *Lab. Invest.*, **69**, 415–429.

Ling,C.C., Guo,M., Hu Chen,C. and Deloherey,T. (1995) Radiation-induced apoptosis: effects of cell age and dose fractionation. *Cancer Res.*, **55**, 5207–5212.

Liu,Z.-G., Hsu,H., Goeddel,D.V. and Karin,M. (1996) Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. *Cell*, **87**, 565-576.

Masters,S.A., Pitti,R.M., Donahue,C.J., Ruppert,S., Bauer,K.D. and Ashkenazi,A. (1996) Activation of apoptosis by Apo-2 ligand is independent of FADD but blocked by CrmA. *Curr. Biol.*, **6**, 750–752.

Mizushima,N., Koike,R., Kohsaka,H., Kushi,Y., Handa,S., Yagita,H. and Miyasaka,N. (1996) Ceramide induces apoptosis via CPP32 activation. *FEBS Lett.*, **395**, 267–271.

Muzio,M. *et al*. (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell*, **85**, 817–827.

Nagata,S. and Golstein,P. (1995) The Fas death factor. *Science*, **267**, 1449–1456.
Nicholson,D.W. *et al*. (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature*, **376**, 37–43.
Nishina,H. *et al*. (1997) Stress-signalling protects thymocytes from apoptosis mediated by CD95 and CD3. *Nature*, **385**, 350–353.
Oberhammer,F.A., Pavelka,M., Sharma,S., Tiefenbacher,R., Purchio,A.F., Bursch,W. and Schulte-Hermann,R. (1992) Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor beta 1. *Proc. Natl Acad. Sci. USA*, **89**, 5408–5412.
Otterbach,B. and Stoffel,W. (1995) Acid sphingomyelinase-deficient mice mimic the neurovisceral form of human lysosomal storage disease (Niemann–Pick disease). *Cell*, **81**, 1053–1061.
Pandey,S. and Wang,E. (1995) Cells en route to apoptosis are characterized by the upregulation of c-*fos*, c-*myc*, c-*jun*, cdc2 and RB phosphorylation, resembling events of early cell-cycle traverse. *J. Cell Biochem.*, **58**, 135–150.
Pitti,R.M., Masters,S.A., Ruppert,S., Donahue,C.J., Moore,A. and Ashkenazi,A. (1996) Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J. Biol. Chem.*, **271**, 12687–12690.
Reap,E.A., Roof,K., Maynor,K., Borrero,M., Booker,J. and Cohen,P.L. (1997) Radiation and stress-induced apoptosis: a role for Fas/Fas ligand interactions. *Proc. Natl Acad. Sci. USA*, **94**, 5750–5755.
Santana,P. *et al*. (1996) Acid sphingomyelinase-deficient human lymphoblasts and mice are defective in radiation-induced apoptosis. *Cell*, **86**, 189–199.
Schreiber,M., Baumann,B., Cotten,M., Angel,P. and Wagner,E.F. (1995) Fos is an essential component of the mammalian UV response. *EMBO J.*, **14**, 5338–5349.
Suda,T., Takahashi,T., Golstein,P. and Nagata,S. (1993) Molecular cloning and expression of the Fas ligand: a novel member of the tumor necrosis factor family. *Cell*, **75**, 1169–1178.
Tepper,C.F., Jayadev,S., Liu,B., Bielawska,A., Wolff,R., Yonehara,S., Hannun,Y.A. and Seldin,M.F. (1995) Role for ceramide as an endogenous mediator of Fas-induced cytotoxicity. *Proc. Natl Acad. Sci. USA*, **92**, 8443–8447.
Testi,R. (1996) Sphingomyelin breakdown and cell fate. *Trends Biochem. Sci.*, **21**, 468–471.
Tewari,M., Quan,L.T., O'Rourke,K., Desnoyers,S., Zeng,Z., Beidler,D.R., Poirier,G.G., Salvesen,G.S. and Dixit,V.M. (1995) YAMA/CPP32β, a mammalian homolog of Ced3, is a crmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose)polymerase. *Cell*, **81**, 801–809.
Trauth,B.C., Klas,C., Peters,A.M.J., Matzku,S., Möller,P., Falk,W., Debatin,K.-M. and Krammer,P.H. (1989) Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science*, **245**, 301–305.
Van Dam,H., Wilhelm,D., Herr,I., Steffen,A., Herrlich,P. and Angel,P. (1995) ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-*jun* induction in response to genotoxic agents. *EMBO J.*, **14**, 1798–1811.
Verheij,M. *et al*. (1996) Requirement for ceramide initiated JNK/SAPKs signalling in stress-induced apoptosis. *Nature*, **380**, 75–79.
Whitmarsh,A.J. and Davis,R.J. (1996) Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J. Mol. Med.*, **74**, 589–607.
Wiegmann,K., Schutze,S., Machleidt,T., Witte,D. and Kronke,M. (1994) Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. *Cell*, **78**, 1005–1015.
Wilhelm,D., Bender,K., Knebel,A. and Angel,P. (1997) The level of intracellular glutathione is a key regulator for the induction of stress-activated signal transduction pathways including JNK/SAPKs and p38 kinase by alkylating agents. *Mol. Cell. Biol.*, **17**, 4792–4800.
Wilson,D.J., Fortner,K.A., Lynch,D.H., Mattingly,R.R., Macara,I.G., Posada,J.A. and Budd,R.C. (1996) JNK, but not MAPK, activation is associated with Fas-mediated apoptosis in human T cells. *Eur. J. Immunol.*, **26**, 989–994.

Received on June 4, 1997; revised on July 25, 1997