



The tyrosine kinase Lck is required for CD95-independent caspase-8 activation and apoptosis in response to ionizing radiation

C Belka^{*1}, P Marini¹, A Lepple-Wienhues², W Budach¹, A Jekle², M Los³, F Lang², K Schulze-Osthoff³, E Gulbins² and M Bamberg¹

¹Department of Radiation Oncology, University of Tuebingen (Germany), Hoppe Seyler Str. 3, 72076 Tuebingen, Germany;

²Department of Physiology, University of Tuebingen (Germany), Gmelinstrasse 5, 72076 Tuebingen, Germany; ³Department of Internal Medicine I, University of Tuebingen (Germany), Otfried Müller Str. 10, 72076 Tuebingen, Germany

Induction of apoptosis is a hallmark of cytostatic drug and radiation-induced cell death in human lymphocytes and lymphoma cells. However, the mechanisms leading to apoptosis are not well understood. We provide evidence that ionizing radiation induces a rapid activation of caspase-8 (FLICE) followed by apoptosis independently of CD95 ligand/receptor interaction. The radiation induced cleavage pattern of procaspase-8 into mature caspase-8 resembled that following CD95 cross-linking and resulted in cleavage of the proapoptotic substrate BID. Overexpression of dominant-negative caspase-8 interfered with radiation-induced apoptosis. Caspase-8 activation by ionizing radiation was not observed in cells genetically defective for the Src-like tyrosine kinase Lck. Cells lacking Lck also displayed a marked resistance towards apoptosis induction upon ionizing radiation. After retransfection of Lck, caspase-8 activation and the capability to undergo apoptosis in response to ionizing radiation was restored. We conclude that radiation activates caspase-8 via an Lck-controlled pathway independently of CD95 ligand expression. This is a novel signaling event required for radiation induced apoptosis in T lymphoma cells.

Keywords: caspase-8/FLICE; lymphoma; apoptosis; tyrosine kinase; Lck

Introduction

Apoptosis is the prevailing form of cell death in human lymphocytes and lymphoma cells responding to ionizing radiation and various cytostatic drugs. The induction of apoptosis can be arbitrarily divided into three different steps: First, the initial activation of apoptotic signals, second, the integration of pro- and antiapoptotic signals (apostat) and third, the terminal execution of apoptosis (Salvesen and Dixit 1997).

Activation of caspases is a key event during the intermediate and terminal phases of apoptosis (Salvesen and Dixit, 1997). Caspases implicated in apoptosis are currently divided into activator caspases and effector caspases. However, the exact order of activators and executioners and the involvement of individual caspases for specific death triggers is unknown. The group of activator caspases currently

includes caspase-8, -9, and -10 (Salvesen and Dixit, 1997; Hu *et al.*, 1998; Thornberry and Lazebnik, 1998), whereas caspase-3, -6 and -7 execute the final cell death (Salvesen and Dixit, 1998). The activator caspases are characterized by large N-terminal prodomains containing death effector domains (DED) or caspase recruitment domains (CARD) required for interaction with upstream molecules (Chou *et al.*, 1998). Activator caspases propagate the apoptotic signal by proteolytic activation of effector caspases. The activated effector caspases-3, -6 and -7 then execute the final cell death program through the degradation of vital proteins (Hirata *et al.*, 1998; Salvesen and Dixit, 1997).

A generally accepted pathway for the generation of initial apoptotic signals in response to DNA damage-induced apoptosis requires the activation of p53-controlled signals (Lowe *et al.*, 1993). A key element of p53-mediated apoptosis is the transcriptional activation of the Bax protein (Miyashita and Reed, 1995) which mediates the release of cytochrome c from mitochondria (Rosse *et al.*, 1998; Pastorino *et al.*, 1998). During the second phase cytochrome c together with Apaf-1 leads to a Bcl-2/Bcl_L controlled activation of the caspase-9 (Rosse *et al.*, 1998; Kuide *et al.*, 1998). Caspase-9 then activates caspase-3 which in turn executes cell death.

The involvement of individual activator caspases or effector caspases in radiation-induced cell death and their exact order within the apoptotic cascade are not analysed in detail. Furthermore mechanisms regulating their activity are not understood. Recent data from caspase-9 deficient mice suggest that the 'activator caspase' caspase-9 is involved in radiation-induced caspase-3 activation and apoptosis (Kuide *et al.*, 1998; Hakem *et al.*, 1998).

One of the best-defined apoptotic pathways is mediated by the death receptor CD95/Fas/APO-1. A key molecule of this pathway is the activator caspase-8. Rapidly after receptor crosslinking the adapter protein FADD/MORT1 is recruited to the receptor complex after via its death domain located at the COOH terminus (Chinnaiyan *et al.*, 1995). The NH₂ terminal region of FADD/MORT1 induces the proteolytic activation of the 'FADD like ICE protease' (FLICE/caspase-8) (Muzio *et al.*, 1996). Following activation of the 'activator caspase' caspase-8/FLICE, downstream 'effector caspases' including caspase-3 (CPP32) are activated which ultimately degrade various vital proteins (Hirata *et al.*, 1998).

Recently, BID was identified as an important caspase-8 substrate. BID is proteolytically activated by caspase-8 and mediates cytochrome c release from

*Correspondence: C Belka

Received 1 October 1998; revised 1 April 1999; accepted 1 April 1999

the mitochondria in response to CD95 triggering (Li *et al.*, 1998; Luo *et al.*, 1998).

Several observations suggested a role of CD95-induced pathways for the apoptotic response of lymphoma cells, neuroblastoma cells and keratinocytes to DNA damage. Ionizing radiation, cytostatic drugs and ultraviolet light were shown to induce the expression of CD95 receptor and ligand. Therefore, the interaction of the newly formed CD95 ligand (CD95-L) with CD95 may contribute to apoptosis induction at least in some cell types or apoptosis conditions (Friesen *et al.*, 1996; Leverkus *et al.*, 1997; Belka *et al.*, 1998). However, other reports showed that some of the apoptotic signals are common to CD95 and DNA damage (Los *et al.*, 1997) and that expression of CD95-L is not required for apoptosis induction by cytostatic drugs (Villunger *et al.*, 1997; Eischen *et al.*, 1997).

To further characterize the involvement of CD95 mediated pathways in radiation induced apoptosis, we focused on proximal steps of CD95 signaling. Therefore, we determined the pattern and mechanisms of caspase-8/FLICE activation in a Jurkat T-cell lymphoma model.

Results

Ionizing radiation induces the proteolytic activation of caspase-8 similar to CD95

After CD95 crosslinking FADD associates with the CD95 receptor and mediates the autoproteolytic activation of the procaspase-8 isoforms into mature caspase-8. We therefore determined caspase-8 activation using a monoclonal antibody which recognizes both procaspase-8 isoforms, the intermediate 44/42 kDa cleavage products and the active p18 caspase-8 subunit. Within 4 h after irradiation of Jurkat cells proteolytic activation of caspase-8 became detectable (Figure 1a). The procaspase-8 isoforms, the 44/42 kDa cleavage products and the active 18 kDa caspase-8 subunits were visible. In parallel, Jurkat cells were treated with an agonistic antibody (CH11, 100 ng/ml) against CD95. As expected, all proteolytic cleavage products of procaspase-8 were generated. Thus, procaspase-8 cleavage induced by ionizing radiation resembled the cleavage pattern induced by CD95 activation (Figure 1a).

Ionizing radiation induces cleavage of BID

Caspase-8 activation by CD95 induces proteolytic activation of BID. We therefore tested whether caspase-8 activation by ionizing radiation is also followed by BID cleavage. First signs of BID cleavage were detectable 10 h after irradiation of Jurkat cells with 10 Gy (Figure 1b). CD95 induced BID cleavage was used as control. This finding indicates that radiation induced activation of caspase-8 is followed by activation of its physiological target.

Caspase-8 is activated independently of CD95 receptor/ligand interaction

In a first set of experiments we analysed the effect of an inhibitory CD95-Fc decoy protein on CD95 and

radiation induced apoptosis. Whereas CD95 triggered apoptosis was clearly blocked, no influence of the CD95-Fc fusion protein was detectable on radiation induced apoptosis (Figure 2).

Since ionizing radiation induces the expression of CD95-L as detected by Western blotting (Figure 3a), we tried to determine whether CD95-L is functionally present at the time point of radiation induced caspase-8 activation. Cells expressing functionally active CD95-L should induce apoptosis in sensitive bystander cells. Therefore, Jurkat cells were irradiated and stained with the vital dye cell tracker green (CMK-fluorescein-AM) after 2–4 h. Stained cells were then incubated with untreated Jurkat cells and apoptosis in this unstained population was determined by flow cytometry after 6–8 h. No increase in apoptosis was detectable indicating that functionally active CD95-L was not present on the surface of irradiated Jurkat cells at this time (Figure 3b). As a positive control Jurkat cells were incubated with CD95-L transfected human fibroblasts or control transfected fibroblasts over 4 h. The CD95-L expressing fibroblasts induced apoptosis in approximately 45% of the Jurkat cells whereas no apoptosis was detectable in Jurkat cells after incubation with control transfected cells.

Additional evidence for the suggestion that CD95-L is not required for radiation induced apoptosis came from the observation that cyclosporin A (CsA) did not interfere with radiation induced caspase activation (not shown) and apoptosis (Figure 3c). CsA inhibits the

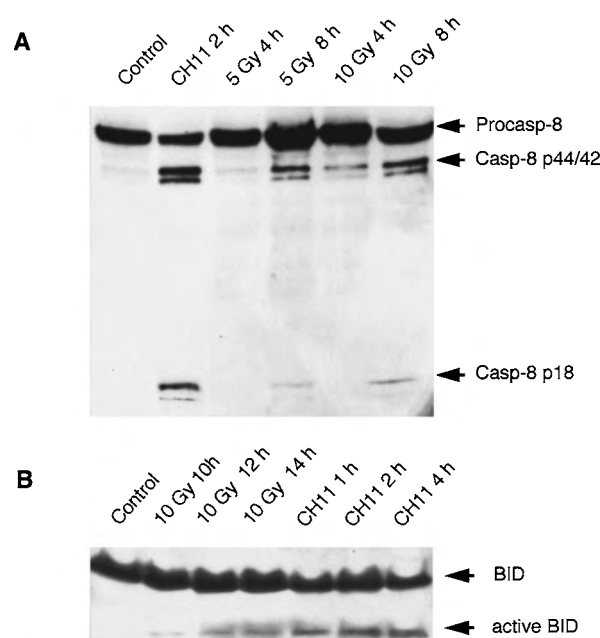


Figure 1 Ionizing radiation induces the proteolytic activation of procaspase-8 and BID. (a) Treatment of Jurkat cells with an agonistic CD95 antibody (100 ng/ml CH11) for 2 h induced procaspase-8 (Procasp-8) cleavage into the intermediate caspase-8 fragments (Casp-8 p44/42) and the mature p18 caspase-8 (Casp-8 p18). Essentially the same cleavage products became visible 4 h and 8 h after irradiation of Jurkat cells with 5 or 10 Gy, respectively. (b) Ionizing radiation induces BID cleavage. Jurkat cells were either irradiated with 10 Gy or treated with the anti-CD95 antibody CH11. After the indicated times, cell extracts were analysed by Western blotting with an anti-BID antibody. Both, irradiation and anti-CD95 induced the cleavage of the p26 full-length BID protein into the proapoptotic 15 kDa fragment

synthesis of CD95-L by interfering with the activation of NFAT transcription factors (Latinis *et al.*, 1997). Although 500 nM cyclosporin A clearly abrogated CD95-L mRNA synthesis upon stimulation with

PMA/ionomycin, no significant reduction of radiation induced apoptosis was detectable (Figure 3c).

In a second set of experiments, we used a CD95 resistant Jurkat subclone to analyse whether CD95 activation is required for radiation induced caspase-8 cleavage. Treatment of CD95 resistant cells with the agonistic CD95 antibody (100 ng/ml CH11) did neither activate caspase-8 (Figure 4a) nor induce apoptosis (not shown). In contrast, radiation activated caspase-8 in CD95-resistant as well as in normal Jurkat cells. Likewise, no differences in radiation induced apoptosis between CD95-resistant and CD95-sensitive Jurkat cells were observed (data not shown).

In addition, ionizing radiation clearly induced caspase-8 cleavage in BJAB lymphoma cells transfected with transdominant negative FADD (Figure 4b). As expected, CD95 crosslinking did not induce caspase-8 activation in these cells (Figure 4b).

Role of caspase-8 for radiation induced apoptosis

The previous data demonstrated that caspase-8 and its physiological substrate BID are activated by ionizing radiation. To determine the role of caspase-8 for radiation induced programmed cell death we used pool-transfected Jurkat cells expressing a T7 tagged dominant-negative caspase-8 mutant or the respective vector control. Expression of the caspase-8-DN mutant was verified by Western blotting using an antibody

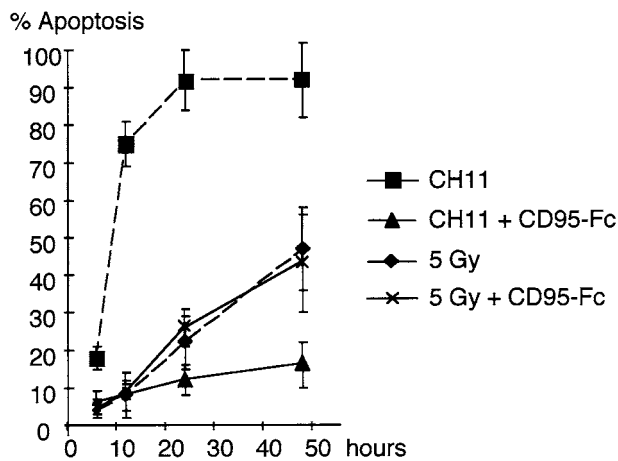


Figure 2 No effect of a soluble inhibitory CD95-Fc decoy protein on radiation induced apoptosis. In order to determine the involvement of CD95-L in radiation induced apoptosis, cells were preincubated with 3 μ g/ml CD95-Fc and then treated with either 100 ng/ml anti-CD95 antibody (CH11) or irradiated with 5 Gy. Whereas CD95-Fc significantly reduced anti-CD95 mediated apoptosis, no influence on radiation induced apoptosis was detectable

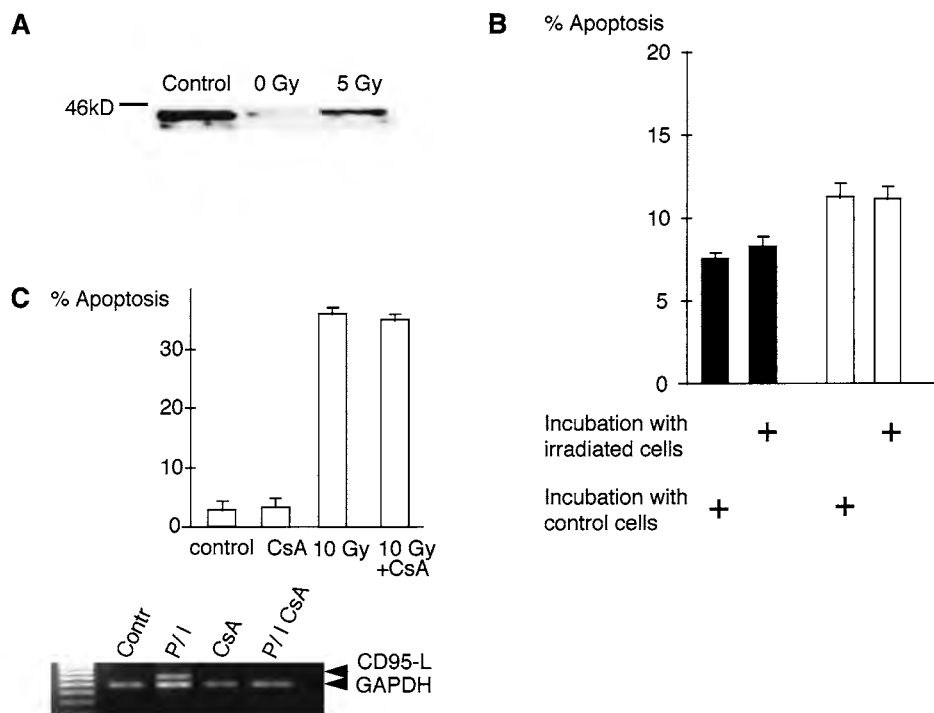


Figure 3 CD95-L is not required for radiation induced apoptosis. (a) Jurkat cells were irradiated and the expression of CD95-L was analysed by Western blotting 18 h later. An endothelial lysate as supplied by the manufacturer was used as control. Ionizing radiation clearly induced the expression of CD95-L as detected by the appearance of a 43 kDa protein. (b) Jurkat cells were irradiated and the expression of CD95-L was analysed by a functional test. Incubation of irradiated cells with control cells over 10 h did not induce apoptosis in the control population. A distinction between either population was made by staining with the vital dye cell tracker green. Irradiated Jurkat cells or control Jurkat cells were stained prior to irradiation and incubated with unstained Jurkat cells. Apoptosis was determined in unstained cells (gray bars). The same experiment was performed by incubating unstained cells, either irradiated or untreated, with stained cells (white bars). (c) Upper panel: Interfering with signaling pathways involved in CD95-L expression using cyclosporin A has no effect on radiation induced apoptosis. Lower panel: The inhibition of PMA/ionomycin (P/I, PMA 50 ng/ml and ionomycin 1 μ M) induced CD95-L expression was used as positive control for cyclosporin A (CsA) action. Although CsA (500 nM) abrogated CD95-L induction in response PMA/ionomycin, it had no effect on apoptosis

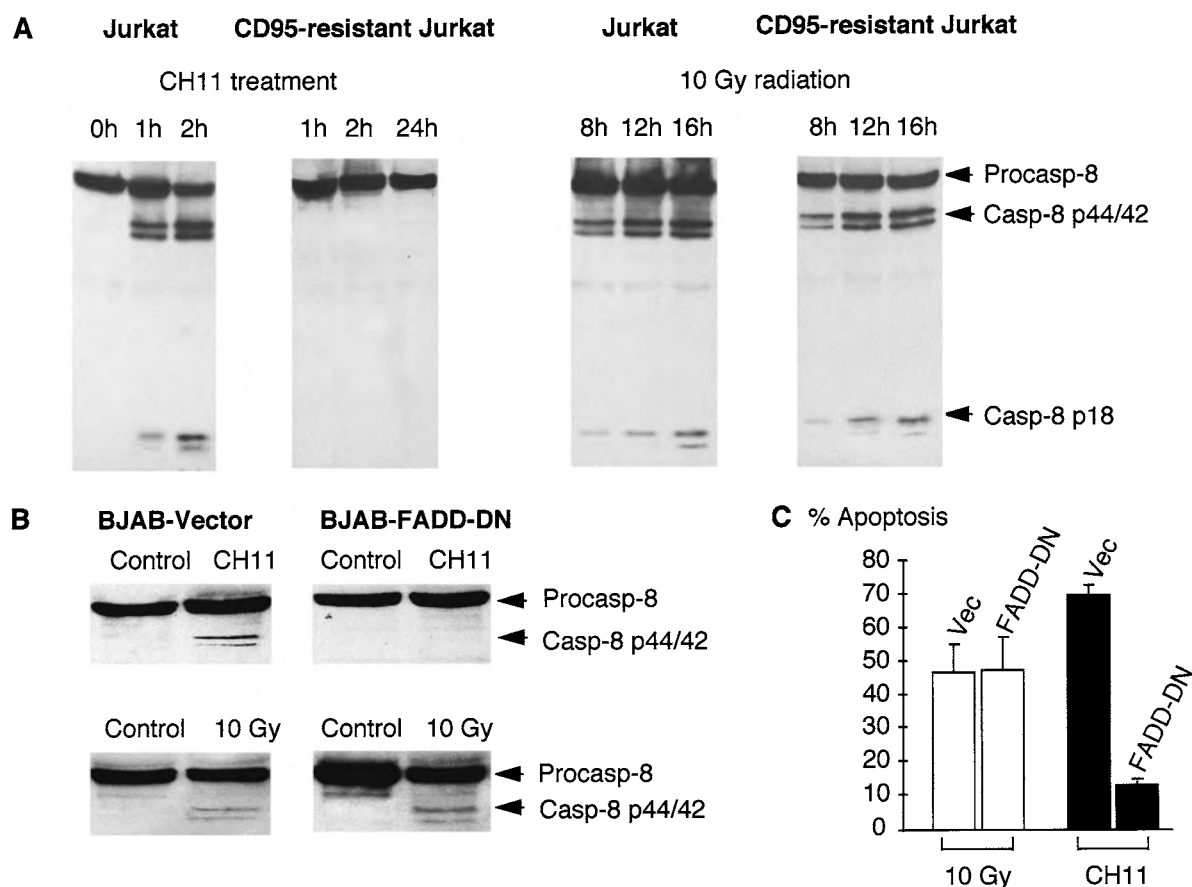


Figure 4 CD95 activation is not required for caspase-8 activation. (a) Left panel: Caspase-8 Western blot after treatment of normal Jurkat cells (2, 6 h) or CD95 resistant Jurkat cells (2, 6 and 24 h) with an agonistic CD95 antibody (CH11, 100 ng/ml). Whereas caspase-8 was rapidly activated in sensitive Jurkat cells, no activation was detectable in CD95 resistant cells even 24 h after treatment. Right panel: Caspase-8 Western blot 8, 16 and 24 h after irradiation of normal Jurkat cells or CD95 resistant Jurkat cells with 10 Gy. No differences in caspase-8 activation pattern were detectable. (b) Overexpression of FADD-DN abrogated anti-CD95 mediated, but not radiation induced caspase-8 activation. Both, irradiation and anti-CD95 treatment induced caspase-8 activation in vector transfected cells. In contrast, only irradiation induces caspase-8 activation in FADD-DN expressing cells (left). (c) CD95 and radiation induced apoptosis in BJAB cells expressing a dominant-negative FADD molecule. Apoptosis induction in response to 10 Gy radiation or 100 ng/ml CH11 was tested in BJAB cells expressing a dominant-negative FADD mutant (FADD-DN) or the vector control (Vec). Whereas CD95 induced apoptosis is blocked by FADD-DN, it had no influence on radiation induced apoptosis

directed against the T7-tag protein (Figure 5a). Overexpression of the dominant negative mutant significantly reduced radiation induced apoptosis (Figure 5a). In parallel, cells were treated with an agonistic CD95 antibody. As expected, the dominant negative mutant interfered with CD95 induced apoptosis. Apoptosis induction was not blocked completely suggesting that the dominant negative mutant was permissive to some degree (Figure 5b).

The tyrosine kinase Lck is required for radiation induced caspase-8 activation

The data presented above indicate that activation of caspase-8 occurred independently of CD95 receptor stimulation. However, the activation of caspase-8 by both CD95 stimulation and ionizing radiation points to the presence of overlapping pathways for the induction of apoptosis in response to either stimulus. In order to identify molecules involved in radiation induced caspase-8 activation, we focused on signaling pathways known to be shared by CD95 and ionizing radiation. Tyrosine kinases have been shown to be

crucial for radiation induced apoptosis in B-lymphoma cells and are also activated by CD95 (Uckun *et al.*, 1996; Schlottmann *et al.*, 1996; Eischen *et al.*, 1994).

The most abundant tyrosine kinase in human T cells is the Src-like kinase Lck. To test whether Lck is activated by radiation, Lck kinase activity was determined in Jurkat cells employing an autophosphorylation assay. Ionizing radiation induced the rapid activation of Lck kinase (Figure 6a).

To analyse whether Lck mediated signaling is essential for the cleavage of procaspase-8, we employed the Lck deficient Jurkat subclone JCaM1.6. This mutant Jurkat T cell line was originally isolated due to its failure to raise intracellular calcium levels following T-cell receptor stimulation. Analysis of the JCaM1.6 mutant revealed that those cells express a deleted and functionally inactive Lck kinase (Straus and Weiss, 1992).

Irradiation of JCaM1.6 cells with different doses did not induce caspase-8 activation. None of the characteristic cleavage products was detectable when total cell lysates from JCaM1.6 cells were subjected to Western blot analysis (Figure 6b).

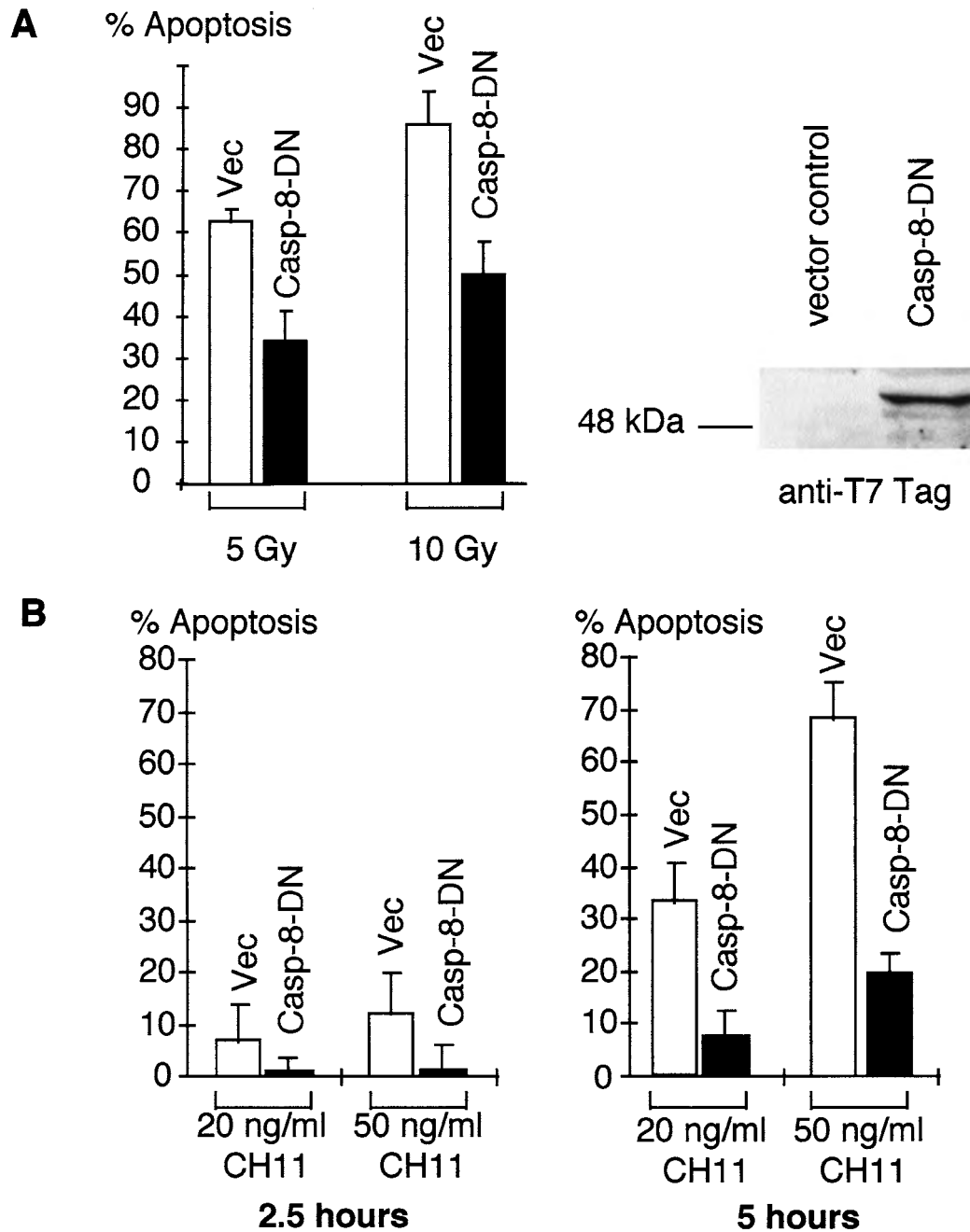


Figure 5 Activation of caspase-8 is involved in radiation induced apoptosis. (a) To determine the involvement of caspase-8 activation in radiation induced apoptosis, a T7 tagged dominant-negative caspase-8 mutant (casp-8-DN) or the vector control (Vec) was overexpressed in Jurkat cells. Overexpression of the dominant negative mutant was monitored by immunoblotting with a T7 antibody (right panel). The dominant negative mutant significantly reduced radiation induced apoptosis (left panel). (b) Apoptosis induction in response to CD95 treatment was tested in casp-8-DN expressing cells. The overexpression of casp-8-DN significantly reduced CD95 induced apoptosis

To prove that Lck is required for caspase-8 processing, we employed JCaM1.6 cells reexpressing the Lck cDNA (JCaM1.6/Lck⁺). Immunoblotting revealed that JCaM1.6/Lck⁺ cells activated caspase-8 in response to ionizing radiation (Figure 6b). Thus, reexpression of Lck completely restored the capability to activate caspase-8 in response to ionizing radiation.

In parallel, we analysed whether the activation of effector caspases were abrogated in Lck negative cells. Ionizing radiation induced proteolytic activation of caspase-3 was not detectable in the JCaM1.6 cells,

whereas irradiation clearly activated caspase-3 in Lck expressing cells (Figure 6b).

Western blotting confirmed that JCaM1.6/Lck⁺ cells expressed and activated p56Lck in response to irradiation, whereas no expression and activation of p56Lck was observed in JCaM1.6 cells (Figure 6c).

Requirement of Lck for radiation-induced apoptosis

These data suggested a major role of Lck associated signaling pathways in radiation induced caspase-8

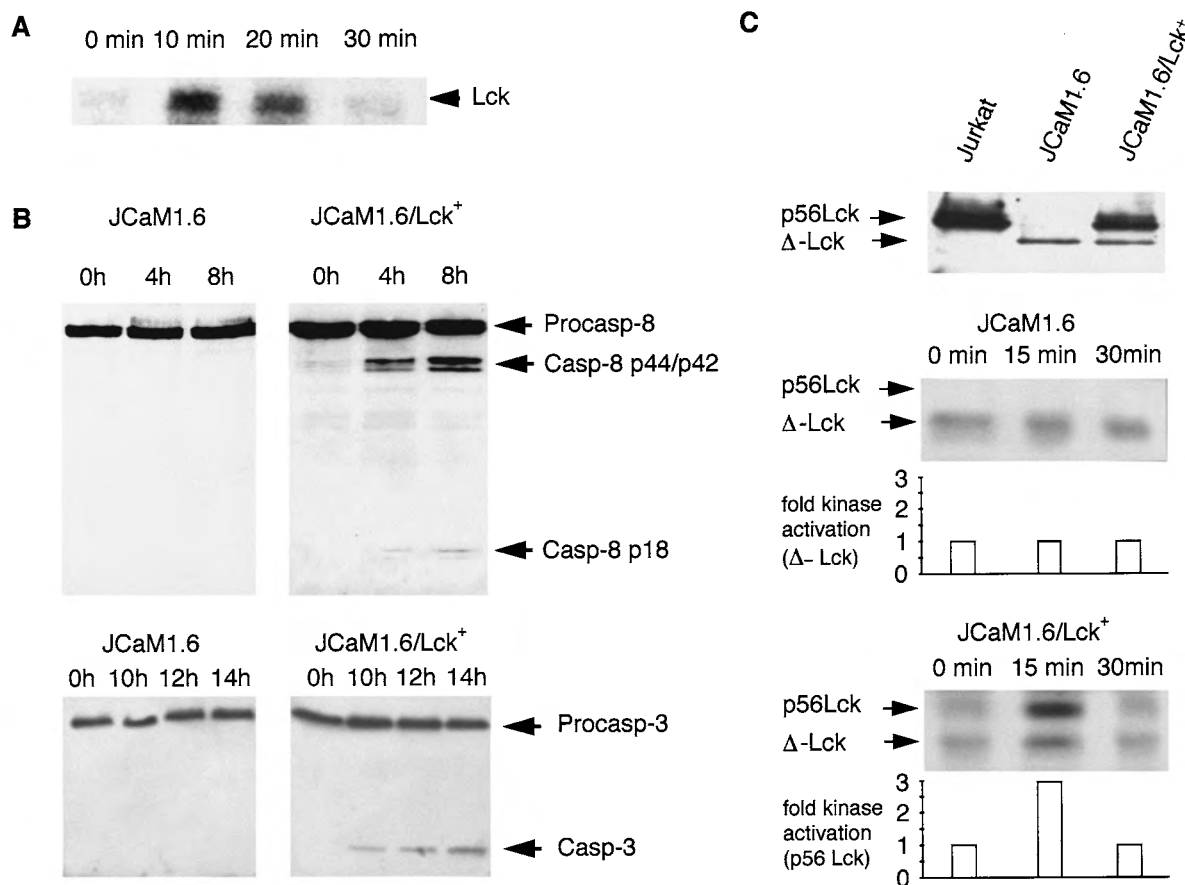


Figure 6 Involvement of the Src-like protein kinase Lck in radiation induced caspase activation. (a) Ionizing radiation induces Lck activation. Lck kinase activation was analysed using an *in vitro* autophosphorylation assay. After irradiation with 10 Gy maximal Lck activation occurred after 10 min declining thereafter. (b) The Lck deficient Jurkat clone JCaM1.6 was irradiated with 10 Gy and caspase-8 cleavage was determined by Western blotting. Ionizing radiation did not induce the activation of caspase-8 in JCaM1.6 (upper left panel), however expression of Lck (JCaM1.6/Lck⁺) restored the capacity to activate caspase-8 (upper right panel). In parallel, caspase-3 activation by ionizing radiation was tested in JCaM1.6 and JCaM1.6/Lck⁺ cells. Whereas no activation was detectable in the JCaM1.6 cells (lower left panel) ionizing radiation clearly induced caspase-3 activation in the Lck expressing cells (lower right panel). (c) Expression and activation of p56 Lck in Jurkat, JCaM1.6 and JCaM1.6/Lck⁺ cells. Western blotting revealed that only the deleted Lck molecule is detectable JCaM1.6 cells (Δ-Lck), whereas Jurkat cells and JCaM1.6/Lck⁺ cells expressed almost equal amounts of the non-deleted p56 Lck kinase (upper panel). In accordance, no activation of p56 Lck or increased phosphorylation of Δ-Lck in response to radiation (10 Gy) was detectable in JCaM1.6 cells (middle panel). Reexpression of Lck restored the p56 Lck kinase activation in response to radiation (lower panel)

activation. Therefore, we determined the role of Lck for apoptosis induction by radiation. Jurkat cells, JCaM1.6 and JCaM1.6/Lck⁺ cells were irradiated with 2, 5 and 10 Gy and apoptosis was determined after 24, 36 and 48 h. In contrast to parental Jurkat cells, JCaM1.6 cells were highly resistant towards radiation-induced apoptosis. Only minimal apoptotic changes were detectable after 24 or 48 h even after irradiation with 10 Gy (Figure 7a). Retransfection of Lck (JCaM1.6/Lck⁺ cells) restored the sensitivity towards ionizing radiation almost completely.

To determine the integrity of other apoptotic pathways in JCaM1.6, cells were treated with 100 ng/ml CH11 over 24 h. As published previously (Latinis and Koretzky, 1996), no significant differences in CD95 induced apoptosis between Jurkat cells and JCaM1.6 cells were detectable (Figure 7b).

Since the alterations in apoptosis induction do not always reflect loss of clonogen tumor cells (Dewey *et al.*, 1995), we analysed whether Lck mediated caspase-8-activation is important for radiation-induced clonogenic cell death. Clonogenic survival of lymphoma cells

was determined 8 weeks after irradiation. As shown in Figure 7c, a significant difference was observed in clonogenic survival of JCaM1.6 and Lck-retransfected cells. The apoptosis prone JCaM1.6/Lck⁺ cells were nearly twice as sensitive as compared to the Lck-deficient cells (Figure 7c). Thus an Lck controlled signaling pathway is crucial for the activation of caspase-8, apoptosis and clonogen cell kill in response to ionizing radiation.

Discussion

The data presented here support the following model for radiation induced apoptosis in human T-lymphoma cells: Irradiation induces a rapid activation of the 'activator caspase' caspase-8 (FLICE). The cleavage of procaspase-8 following ionizing radiation resembles the cleavage induced by CD95 triggering. Nevertheless, this does not require CD95-L expression and occurs independently of CD95 activation. Cells lacking the tyrosine kinase Lck were resistant to radiation-induced

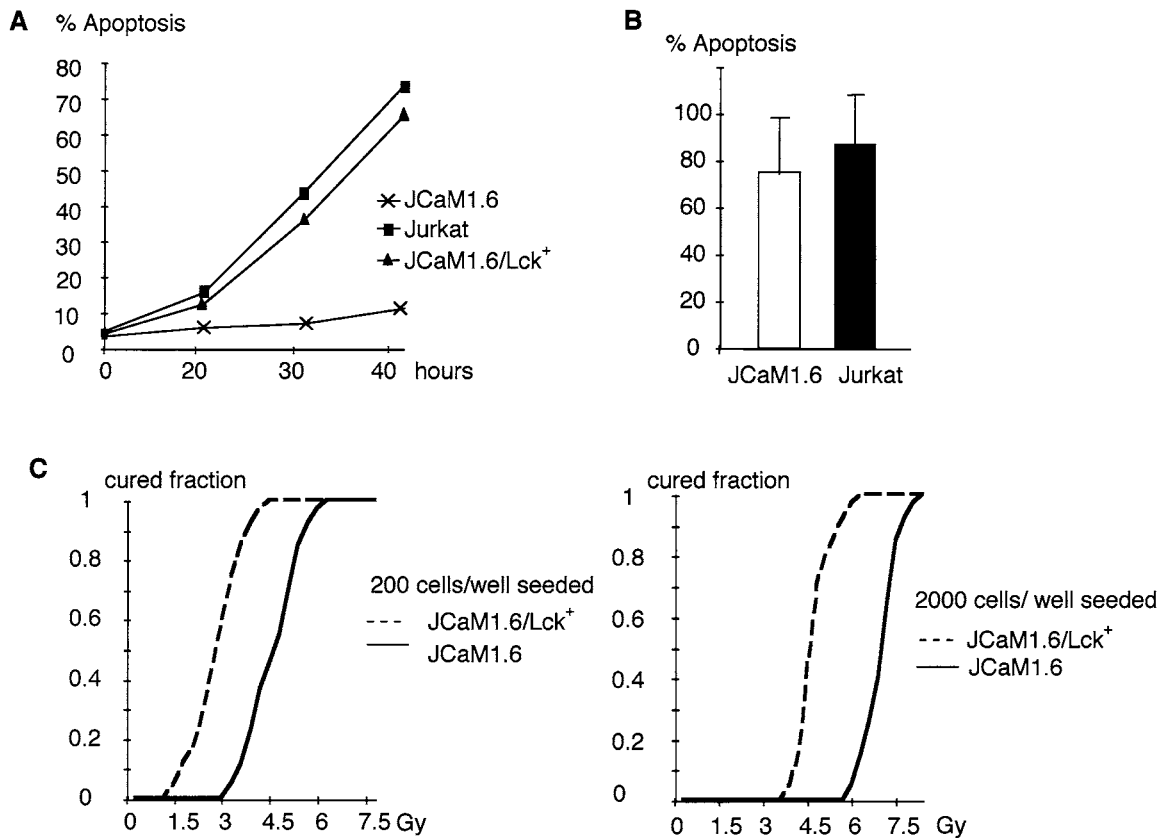


Figure 7 Radiation induced apoptosis requires Lck. (a) Apoptosis induction in Jurkat cells, JCaM1.6 cells and JCaM1.6/Lck⁺ cells was determined by morphology (morphology data not shown) and flow cytometric analysis 24, 36 and 46 h after irradiation with 10 Gy. Lck deficient JCaM1.6 cells were found to be highly resistant towards radiation induced apoptosis. The reexpression of Lck restored the capacity to undergo apoptosis in response to ionizing radiation. (b) No differences in CD95 induced apoptosis in JCaM1.6 and Jurkat cells. Cells were treated with 100 ng/ml CH11 for 24 h. Apoptosis was determined by FACS analysis. (c) Radiation induced clonogen cell kill was analysed comparing the colony formation of JCaM1.6 and JCaM1.6/Lck⁺ cells 8 weeks after irradiation (left panel: seeding of 200 cells/ml and right panel 2000 cells/ml). The different rate of apoptosis induction is clearly reflected by high differences in clonogen cell kill

caspase-8 cleavage and apoptosis, suggesting that Lck plays an important role for radiation induced apoptosis.

The proposed model of a CD95-L-independent activation of caspase-8 is supported by several observations: First, caspase-8 activation by irradiation occurred prior to CD95-L expression. Second, ionizing radiation induced caspase-8 activation in CD95 resistant cells as well as in cells expressing dominant-negative FADD. Third, a soluble CD95-Fc fusion protein blocked CD95 induced apoptosis but had no influence on radiation induced apoptosis. In addition, cyclosporin A, which inhibits CD95-L expression, did not inhibit apoptosis following ionizing radiation.

Lck requirement for radiation induced caspase-8 activation is supported by the finding that procaspase-8 cleavage is not detectable in Lck deficient human JCaM1.6 cells, and retransfection of Lck restored caspase-8 activation.

Our data show for the first time that ionizing radiation activates a key element of the CD95 signaling pathway independently of CD95/FADD activation. Hence the expression of CD95-L or an activation of CD95 is not essential for the initial triggering of apoptotic cascades by ionizing radiation. Recent work on the initial activation of caspase networks suggest that a complex of Apaf-1, cytochrome c and caspase-9 is a key step for the induction of apoptosis (Kuida *et*

al., 1998). Since caspase-8 was also shown to associate with Apaf-1 *in vivo* and is capable of autoproteolytic activation similar to caspase-9 (Hu *et al.*, 1998), the activation of caspase-8 might constitute a parallel pathway or alternative pathway for the initial induction of apoptosis.

The dominant negative mutant inhibited CD95 induced apoptosis stronger than radiation induced apoptosis. Since all experiments were performed from the same batch of bulk transfected cells, this finding might indicate that caspase-8 activation is an absolute requirement for CD95 induced apoptosis, while it might be bypassed by other caspases in case of radiation induced apoptosis. A similar observation was reported using Jurkat cells genetically lacking caspase-8. Whereas CD95 induced apoptosis was abrogated in cells lacking caspase-8, the cells were only partially deficient in cell death induced by ultraviolet irradiation, adriamycin and etoposide (Juo *et al.*, 1998).

Our data provide evidence for a unknown function of Src-like kinases for activation of the initial phase of apoptosis in response to ionizing radiation in human T lymphoma cells. The observation that Lck which is involved in the signaling pathways of many T-cell surface receptors is a key step for the modulation of apoptosis is paralleled by the finding that the B-cell receptor associated kinase BTK is essential for

radiation induced apoptosis in B-cells (Uckun *et al.*, 1996). In this case, the kinase domain of BTK was found to be crucial for the apoptotic response to ionizing radiation. In addition to the findings on BTK, it was shown that activation of Lck by CD19 crosslinking promoted radiation induced apoptosis in a radiation resistant B-lymphoma cell line (Waddick *et al.*, 1993).

Similarly, the kinase Lyn was shown to be required for the induction of apoptosis in response to UV light in DT40 B-cells (Qin *et al.*, 1997). However, no suggestion regarding the temporal relation of kinase activation to the activation of the apoptotic cascade has been given.

The implication of Src-like kinases in the activation of the initial apoptotic phase is also supported by the observation that the addition of peptides containing the SH2 domains of Lck, Src or Abl interfered with the early stages of apoptosis in a cell free system (Farschon *et al.*, 1997). Since it was shown that the Lck SH2 domain is required for interaction of Lck with tyrosine phosphorylated protein structures (Couture *et al.*, 1996), the addition of the soluble Lck SH2 domain might act in a dominant negative fashion by blocking the interaction of Lck with yet unknown target structures which are involved in the regulation of apoptosis.

It is tempting to speculate how Lck is involved in controlling early apoptosis signal generation. Similar to the tyrosine kinase Abl which is also involved in apoptosis regulation, Lck was shown to phosphorylate the phosphatidylinositol 3 kinase (PI3K) SH2 domain.

However, the kinase activity of PI3K was not influenced, but the phosphorylation of the PI3K SH2 domain induced dissociation from upstream signaling molecules, for example the insulin receptor (von Willebrand *et al.*, 1998). Furthermore it was shown that Lck phosphorylates and binds to tyrosine-phosphorylated paxillin thereby influencing the cytoskeleton (Ostergaard *et al.*, 1998). Lck is also required for the activation of GTPases of the Rho family which were shown to be involved in the control of apoptosis (Han *et al.*, 1997; Jimenez *et al.*, 1995).

Another important function of Lck is the regulation of several ion transport systems. After activation of the T-cell receptor, Lck triggers the generation of inositoltriphosphate which in turn mediates the release of calcium from endoplasmatic stores. The observation that human T-cells not expressing the inositoltriphosphate receptor-1, which is located downstream of Lck, are highly resistant to ionizing radiation (Jayaram and Marks, 1997), suggests that the modulation of calcium signals might be involved in the initial apoptotic phase. In addition, other ion fluxes were shown to be regulated by Lck in response to ceramide and CD95 crosslinking apoptotic stimuli (Gulbins *et al.*, 1997; Szabo *et al.*, 1998).

Taken together we suppose that the influence of Lck on the cytoskeleton architecture or ion fluxes might contribute to its role in radiation induced apoptosis.

A critical question is the correlation between radiation induced apoptosis and long term clonogenic survival (Dewey *et al.*, 1995). Experimental data show that the increase of radiation-induced apoptosis by forced overexpression *c-myc* did not alter the survival of clonogenic cells (Aldridge *et al.*, 1995). In contrast,

restoring Bax expression in breast cancer cells increased apoptosis and decreased clonogen survival in response to ionizing radiation (Sakakura *et al.*, 1996). By using a genetic model with restoring the lack of a single apoptosis regulating gene, the question in how far apoptosis and clonogen survival are related can be answered. Our model demonstrates that the induction of apoptosis is strongly correlated to the loss of clonogenicity. Cells not prone to apoptosis (JCaM1.6) have a nearly doubled radioresistance when compared with the retransfected counterpart (JCaM1.6/Lck⁺).

Materials and methods

Materials and reagents

All biochemicals were from Sigma chemicals (Deisendorf, Germany) unless otherwise specified. Cyclosporin A (Calbiochem, Bad Soden, Germany) was dissolved in ethanol in 10 µg/µl stocks. Hoechst 33342 was purchased from Calbiochem and dissolved in distilled water as 1.5 mM stock solution. Cell tracker green[®] was purchased from Mobitech (Goettingen, Germany). The recombinant inhibitory CD95-Fc fusion protein kit was from Alexis Biotech (Grünberg, Germany) and used according to the manufacturer's guidelines.

Cell culture

Jurkat T-lymphoma cells were from ATCC (Bethesda, MD, USA). Cells were grown in RPMI 1640 medium (Gibco Life Technologies, Eggenstein, Germany) and maintained in a humidified incubator at 37°C and 5% CO₂. The Lck deficient Jurkat clone (JCaM1.6) and the Lck cDNA expressing JCaM1.6/Lck⁺ clone were a kind gift from A Weiss (University of California, CA, USA). FADD-DN and vector control transfected BJAB cells were a kind gift of V Dixit (University of Michigan, MI, USA). CD95 resistant Jurkat clones were prepared after prolonged treatment with 100 ng/ml CH11 and limited dilution to generate individual clones.

Radiation

Cells were irradiated with 6 MV photons from a linear accelerator (LINAC SL25 Phillips) at a dose rate of 4 Gy/min at room temperature.

Determination of apoptosis and clonogen survival

Apoptosis was quantified after staining of the cells with Hoechst 33342 and subsequent fluorescence microscopy. In brief 1 × 10⁵ cells were centrifuged (1000 r.p.m., 4°C 5 min) and transferred into HBS buffer. Hoechst 33342 was added to a final concentration of 1 µM and incubated for 60 min at 37°C. Cell morphology was then determined by fluorescence microscopy (Zeiss Axiovert 135, Carl Zeiss, Jena, Germany) using an excitation wavelength filter of 380 nm. A total of 250 cells was counted and cells with intense chromatin clumping were considered as apoptotic. Characteristic sections were documented by a video imaging device. Cell death was also quantified by FACS analysis after propidium iodide staining and scatter characteristics employing FACS Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany).

In order to determine the clonogen survival after irradiation with different doses, 200 and 2000 cells of the respective cell line were seeded in 1 ml culture medium into eight wells on a 48-well plate. Eight weeks after irradiation the proportion of wells with homogenous cell growth was determined.

Immunoblotting

Cells (1×10^5) were lysed in a modified lysis buffer containing 25 mM HEPES, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 10 mM EDTA, 10 mM NaF and 125 mM NaCl. After removing insoluble material by centrifugation for 30 min at 13 000 r.p.m., 20 μ g lysate was separated by SDS-PAGE. Blotting was performed employing a semidry blotting apparatus (Hoefer Scientific, Freiburg, Germany) with 0.8 A/cm² for 30 min onto Hibond C membranes (Amersham, Braunschweig, Germany). Equal protein loading was confirmed by Ponceau S staining (Sigma). Blots were blocked in PBS buffer containing 0.05% Tween 20 and 5% bovine serum albumin at 4°C overnight. Caspase-8 was detected using a mouse monoclonal antibody directed against the p18 subunit (Ferrari *et al.*, 1998) used as a 1:10 dilution of the hybridoma supernatant. After repeated washings with PBS/Tween 20 (0.05%) the membrane was incubated with the secondary antibody (anti IgG-AP 1:10 000, Santa-Cruz-Biotech, Heidelberg, Germany) in PBS/Tween for 3 h at room temperature and washed three times with PBS/Tween. The detection of antibody binding was performed employing enhanced chemoluminescence. The anti-BID antibody was a kind gift from J Yuan (Boston, USA) and was used in a dilution of 1:3000. The anti-Lck antibody used for Western blotting (3A5) was from UBI/Biomol (Hamburg, Germany), the anti-caspase-3 and anti-CD95-L antibodies were from Transduction Labs (Dianova, Hamburg, Germany).

In vitro kinase assay, autophosphorylation of Lck

1.5×10^6 cells per sample were irradiated in 100 μ l HEPES buffered saline (HBS) consisting of 20 mM HEPES, 132 mM NaCl, 1 mM CaCl₂, 700 μ M MgCl₂, 800 μ M MgSO₄ and 5.4 mM KCl at 37°C for the indicated time periods. After cell lysis in a buffer containing 0.1% SDS, 0.5% deoxycholate, 1.0% Triton X-100, 25 mM HEPES, 10 mM EDTA, 10 mM NaPP, 10 mM NaF, 125 mM NaCl, 10 mM vanadate, aprotinin (100 μ g/ml) and leupeptin (100 μ g/ml) for 10 min on ice, supernatant was cleared by centrifugation for 15 min at 4°C. The kinase was immunoprecipitated by adding 2 μ g of anti-Lck mAb (Santa Cruz) at 4°C for 4 h, followed by addition of 20 μ l of protein A/G plus agarose (Santa Cruz) for 1 h. Agarose pellets were washed twice with lysis buffer and twice with kinase buffer consisting of 12.5 mM MOPS, 12.5 mM β -glycerophosphate, 0.5 mM EGTA, 7.5 mM MgCl₂, 0.5 mM NaF and 10 mM vanadate. Thirty μ l of kinase buffer containing 10 μ M dATP and 0.1 μ Ci γ -[³²P]ATP was added. Kinase reactions were performed at 37°C for 20 min and stopped by adding a buffer containing 2% SDS, 40% glycerol and 0.1% bromophenolblue. Immunoprecipitates were separated electrophoretically by 10% SDS-PAGE, transferred to a PVDF-membrane and visualized by exposure to a Kodak X-omat film. The fold kinase activation was determined using NIH-image software.

Detection of CD95L mRNA expression by RT-PCR

Expression of CD95L mRNA was examined by reverse transcription (RT)-PCR essentially as described previously (Bauer *et al.*, 1998). Total cellular RNA was extracted from

1×10^6 Jurkat cells by the acidic guanidinium thiocyanate phenol-chloroform method. One μ g of total RNA was reverse transcribed after heat denaturation (3 min, 60°C) and annealing with 2.5 μ M random hexamer primers (Perkin Elmer, Weiterstadt, Germany) in the presence of 50 U MnLV RT (Perkin Elmer), 5 mM MgCl₂ and 1 mM of each dNTP in 20 μ l for 30 min at 42°C. The reaction was stopped by heat inactivation for 5 min at 95°C. Aliquots of 10 μ l of the cDNA were then amplified in a DNA thermocycler (Stratagene, Heidelberg, Germany) with 1.25 U of AmpliTaq DNA-polymerase (Perkin Elmer), 100 pM of both upstream and downstream CD95L primers and 2 mM MgCl₂ in a volume of 50 μ l. Each of the PCR cycles consisted of a denaturation step (94°C, 1 min), an annealing step (54°C, 1 min) and an elongation step (72°C, 1 min). For GAPDH mRNA expression, which was analysed as a control for sample loading and integrity, 2 μ l cDNA were amplified (1 min 94°C, 1 min 62°C, 1 min 72°C). The PCR products (498-bp human CD95L fragment, 397-bp human GAPDH fragment) were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. Primers used for amplification were human CD95L sense primer corresponding to nucleotides 386–410 (5'-ATGTTT-CAGCTCTTCCACCTACAGA-3') and antisense primer complementary to nucleotides 884–858 (5'-CCAGAGA-GAGCTCAGATACGTTGACA-3') and GAPDH sense (5'-ATGGCACCGTCAAGGCTGAGA-3') and antisense primer (5'-GGCATGGACTGTGGTCATGAG-3').

Transfection of caspase-8-DN and selection for stable expression

The expression vector for the T7 tagged caspase-8 dominant-negative mutant (casp-8-DN) was a kind gift from E Alnemri (Philadelphia, PA, USA). Electroporation was performed using a Gene pulser II electroporation device (Biorad, Munich, Germany). In brief, 10^8 Jurkat cells were washed twice with TBS buffer pH 7.4 prior to transfection. Twenty-five μ g DNA was electroporated with 125 μ F and 500 V. Cells were selected with 800 μ g/ml G418 (Clontech, Heidelberg, Germany). Expression of the dominant negative mutant was monitored by immunoblotting using an antibody directed against the T7 tag epitope (Novabiochem/Calbiochem, Bad Soden, Germany).

Acknowledgements

We highly acknowledge the expert technical help from H Faltin and G Turan and supply of reagents by Drs E Alnemri, V Dixit, A Weiss and J Yuan. The work presented here was supported by a grant from the fortune project (311/333 University of Tuebingen) to C Belka, E Gulbins and W Budach.

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