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Calpain activation is upstream of caspases in radiation-induced apoptosis

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Abstract

The molecular events involved in apoptosis induced by ionizing radiation remain unresolved. In this paper we show that the cleavage of fodrin to a 150 kDa fragment is an early proteolytic event in radiation-induced apoptosis in the Burkitts' Lymphoma cell line BL30A and requires 100 μM zVAD-fmk for inhibition. Caspases-1, -3, -6 and -7 were shown to cleave fodrin to the 150 kDa fragment *in vitro* and all were inhibited by 10 μM zVAD-fmk. We also show that the *in vitro* cleavage of fodrin by calpain is inhibited by 100 μM zVAD-fmk as was the calpain-mediated hydrolysis of casein. We demonstrate that calpain is activated within 15 min after radiation exposure, concomitant with the cleavage of fodrin to the 150 kDa fragment whereas caspase-3 is activated at 2 h correlating with the cleavage of fodrin to the 120 kDa fragment. These results support a role for calpain in the early phases of the radiation-induced apoptosis pathway, upstream of the caspases.

Keywords: calpain, caspases, radiation, fodrin

Abbreviations: ICE, interleukin 1-β converting enzyme; hnRNP, heteronuclear ribonucleoprotein; Gy, Gray; PARP, poly(ADP-ribose)polymerase; U1-70 kDa, 70 kDa protein component of the U1 small ribonucleoprotein; DNA-PKcs, DNA dependent protein kinase catalytic subunit; D4-GDI, D4 GDP dissociation inhibitor; DTT, dithiothreitol; HEPES, N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid; TBE, Tris-Borate-EDTA; PS, phosphatidyl-serine; PAGE, polyacrylamide gel electrophoresis; DEVD-CHO, Ac-Asp-Glu-Val-Asp aldehyde; zVAD-fmk, Z-Val-Ala-Asp-CH_2F, FLICE, FADD like ICE; Mch, mammalian Ced-3 homologue; CPP32, cysteine protease protein of molecular mass 32 kDa; Caspase, cysteine protease cleaving at the carboxy terminal of aspartic acid

Introduction

Apoptosis is accepted as a form of cell death distinct from necrosis in that it is an active process in which the required machinery is already present in the cell (Vaux and Strasser, 1996). Although there are many stimuli and many pathways that can lead to apoptosis (Lazebnik *et al*, 1995; Wertz and Hanley, 1996), it is believed that these pathways have a point of convergence leading to the manifestation of events that are common to most if not all forms of apoptosis. These include activation of proteases, chromatin condensation, nuclear fragmentation, deregulation of cellular function and ultimately the formation of apoptotic bodies which are then rapidly phagocytosed, preventing the leakage of potentially fatal cytoplasmic material (Kerr *et al*, 1972; Earnshaw, 1995).

Previous studies on apoptosis identified three genes, *ced*-3, *ced*-4 and *ced*-9 as essential in the apoptotic process in *C. elegans* (Hengartner and Horvitz, 1994a). Interleukin 1β-converting enzyme (ICE)[1] was the first identified mammalian homologue of Ced-3 (Yuan *et al*, 1993) and a group of cysteine proteases homologous to ICE have now been characterised. These proteases have recently been termed caspases, since they cleave at the C-terminal of an aspartic acid residue (Alnemri *et al*, 1996). The caspases have been further categorised on the basis of their similarity to caspase-1 (ICE), caspase-3 (CPP32) or caspase-2 (Nedd2), (Kumar and Lavin, 1996). The Bcl-2 family of proteins have been shown to be mammalian homologues of the *ced*-9 gene product (Hengartner and Horvitz, 1994b) and they act upstream of the caspases (Perry *et al*, 1997). It is still not known how Bcl-2 protects cells from apoptosis. Ced-9 is known to interact with Ced-4 and it has been suggested that Bcl-X_L can complex with certain caspases removing them from their site of action. (Chinnaiyan *et al*, 1997). Different theories also suggest that Bcl-2 may act as an antioxidant, as a mitochondriotropic agent, or as a regulator of intracellular ion fluxes (Kroemer, 1997; Reed, 1997).

Several proteins have been shown to be cleaved by caspases during apoptosis. These include PARP (Lazebnik *et al*, 1994; Gu *et al*, 1995), hnRNP C1/C2 (Waterhouse *et al*, 1996), DNA-PKcs (Casciola-Rosen *et al*, 1995; Song *et al*, 1996), D4-GDI (Songqing *et al*, 1996), U1-70 kDa (Casciola-Rosen *et al*, 1994) and nuclear lamin (Lazebnik *et al*, 1995). Although it is possible that the caspases have overlapping functions it is generally believed that they are

effective as a highly regulated proteolytic cascade cleaving specific proteins at specific times during the apoptotic process (Kumar and Harvey, 1995). For example caspase-6 has been shown to specifically cleave lamin resulting in nuclear disintegration characteristic of apoptosis (Takahashi *et al*, 1996; Orth *et al*, 1996). The method of regulation of the caspases and the order in which they are activated remains unclear.

Peptide inhibitors of the caspases, based on the cleavage sequences in PARP and ICE (Thornberry *et al*, 1994), have been developed in an effort to better understand the role of these enzymes during apoptosis. Using these inhibitors and Fas-induced apoptosis as a model, three levels of proteolysis were predicted (Greidinger *et al*, 1995). In that model, the Fas receptor forms a trimer to which FADD and caspase-8 (MACH/FLICE/Mch5α) are recruited (Boldin *et al*, 1996; Fernandes-Alnemri *et al*, 1996; Muzio *et al*, 1996; Nagata, 1997). Caspase-8 is activated at this level and is responsible for the activation of the death pathway. zVAD-fmk inhibits apoptosis by preventing the processing of caspase-3 to its active form (Slee *et al*, 1996). In the case of Fas-induced apoptosis this inhibition may be at the level of caspase-8. In other forms of apoptosis, such as that induced by c-*myc* (McCarthy *et al*, 1997), zVAD-fmk can inhibit the appearance of the nuclear morphology of apoptosis but the cells eventually die by a process involving cytoplasmic blebbing. It is thus unclear as to how many levels of proteolysis exist during apoptosis induced by stimuli other than Fas. The understanding of these early events in non-Fas-induced apoptosis will help us understand the signaling pathways leading to the apoptotic phenotype.

Another protease, calpain, has been implicated in apoptosis in response to hypoxia in hepatocytes (Bronk and Gores, 1993), in neuronal degeneration (Saito *et al*, 1993) and in irradiation and dexamethasone treatment of murine thymocytes (Squier *et al*, 1994). Calpain is a calcium-dependent neutral protease with two isozyme forms, μ-calpain and m-calpain distinguished by their *in vitro* calcium requirements (Murachi, 1989; Croall and De Martino, 1991). Calpain stimulators such as ONO-3403 (Hiwasa, 1996) have been shown to cause apoptosis. Calpain inhibitors I and II (analogues of leupeptin) are known to protect against apoptosis in irradiated murine thymocytes and metamyelocytes (Squier *et al*, 1994) and in activation-induced apoptosis in HIV^+ donors (Sarin *et al*, 1994). However, these same inhibitors induce apoptosis in cultured Molt 4 and L5178Y cells (Shinohara *et al*, 1996) and in BL30A cells (unpublished data). Calpain inhibitors I and II may inhibit other proteases such as cathepsin B/L and the proteasome (Squier *et al*, 1994), thus it is difficult to predict calpain involvement in apoptosis using these inhibitors. However it has recently been reported that the inhibitor PD 150606, which binds to the calcium binding site of calpain and is not a proteasome inhibitor, is effective in inhibiting dexamethasone-induced apoptosis in thymocytes (Squier and Cohen, 1997). The importance of calpain in cell death has been reviewed recently (Squier and Cohen, 1996), however, several aspects of the specific role of calpain remain unanswered, in particular its position in the proteolytic cascade and its specific substrates. It is also not known whether calpain is involved in all forms of apoptosis.

One potential substrate for calpain during apoptosis is fodrin (Martin *et al*, 1995). Only the caspases seem to be involved in fodrin cleavage during Fas-mediated apoptosis (Cryns *et al*, 1996; Vanags *et al*, 1996), while both calpain and caspases appear to be involved in staurosporine and maitotoxin-induced apoptosis in neuronal cells (Nath *et al*, 1996) and in TNF-induced apoptosis in U937 cells 1996 (Vanags *et al*, 1996). In the latter paper it was suggested that calpain plays an important role in the later events induced by TNF in U937 cells.

In this paper we show that the early proteases involved in radiation-induced apoptosis are different from those involved in Fas-induced apoptosis. We also provide evidence that calpain is responsible for the initial cleavage of fodrin to the 150 kDa fragment and that calpain is upstream of caspases in radiation-induced apoptosis.

Results

Cleavage of fodrin to a 150 kDa fragment is the first proteolytic event in radiation-induced apoptosis

We have previously shown that 80–90% of BL30A cells exposed to 20 Gy of ionizing radiation die by apoptosis within 8 h (Waterhouse *et al*, 1996). In order to investigate this

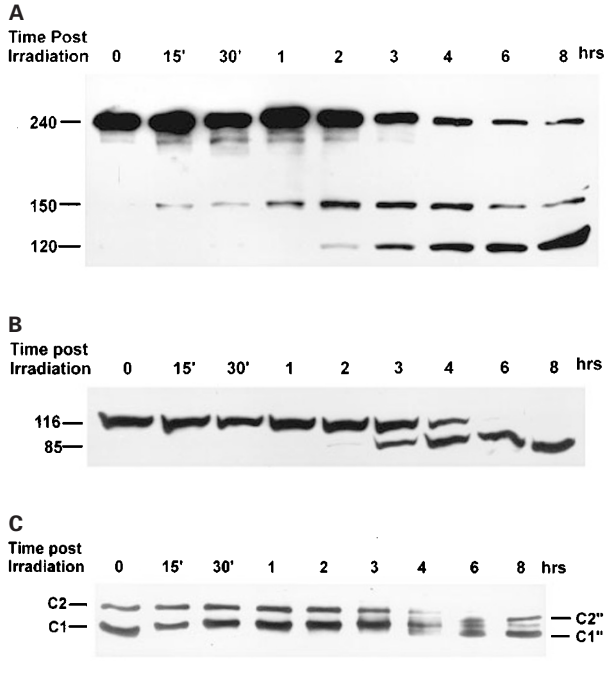

Figure 1 Time course of cleavage events during radiation-induced apoptosis. BL30A cells were treated with 20 Gy ionizing radiation and cells were harvested at the times indicated. Total protein was extracted and the cleavage of fodrin (100 μg/lane on a 4% gel) (**A**), PARP (30 μg/lane on a 4–15% gradient gel) (**B**) and hnRNP C1/C2 (5 μg/lane on a 10% slab gel) (**C**), was determined by Western blotting using appropriate primary antibodies

process in more detail, the time course of the cleavage of several proteins degraded during apoptosis was investigated. These experiments revealed the earliest cleavage event to be the degradation of fodrin to a 150 kDa fragment commencing within 15 min (Figure 1A), which precedes the cleavage of this protein to a 120 kDa fragment, first apparent by 2 h post-irradiation (Figure 1A). Cleavage fragments of PARP (Figure 1B) and the hnRNP C proteins (Figure 1C) also appear at 2–4 h in response to radiation damage.

The requirement of 100 μM zVAD-fmk to inhibit the cleavage of fodrin to a 150 kDa fragment is a feature distinguishing it from other proteolytic events in radiation-induced apoptosis

It has been shown that 10 μM zVAD-fmk is sufficient to inhibit the cleavage of fodrin during Fas-induced apoptosis in Jurkat cells (Greidinger *et al*, 1995). Since BL30A cells do not undergo Fas-induced apoptosis due to the absence of Fas-receptor (data not shown), we employed an EBV-transformed lymphoblastoid cell line, AT1ABR, to demonstrate the inhibition of fodrin cleavage by 10 μM zVAD-fmk during Fas-induced apoptosis (Figure 2A). In radiation-induced apoptosis in BL30A cells, 10 μM zVAD-fmk was sufficient to inhibit the cleavage of fodrin to a 120 kDa fragment, however ten times the concentration of zVAD-fmk was required to completely inhibit the cleavage of fodrin to a 150 kDa fragment (Figure 2B). This inhibition profile was also seen when BL30A cells were treated with 40 μM etoposide (Figure 2C) and when murine thymocytes were treated with 10 Gy radiation (Figure 2D).

Nuclear fragmentation as determined by DNA laddering on an agarose gel (Figure 3A), cleavage of proteins known to be degraded by caspases during apoptosis, PARP (Figure 3B) and hnRNP C1/C2 (Figure 3C), phosphatidylserine exposure as determined by annexin V binding (Figure 3D) and morphological changes as determined by electron microscopy (Figure 3E), were all prevented by the addition of 10 μM zVAD-fmk. This suggests that the protease responsible for the initial cleavage of fodrin to the 150 kDa fragment was unique in that 100 μM zVAD-fmk was required to fully inhibit the reaction.

Caspases -1, -3, -6, -7 and calpain cleave fodrin *in vitro* to a 150 kDa fragment

Calpain has previously been shown to cleave murine fodrin between the tyrosine and glycine residues to create a 150 kDa fragment (Harris *et al*, 1988). There are also many potential caspase cleavage sites and one of these (DETD/S) is in close proximity to the calpain cleavage site. A

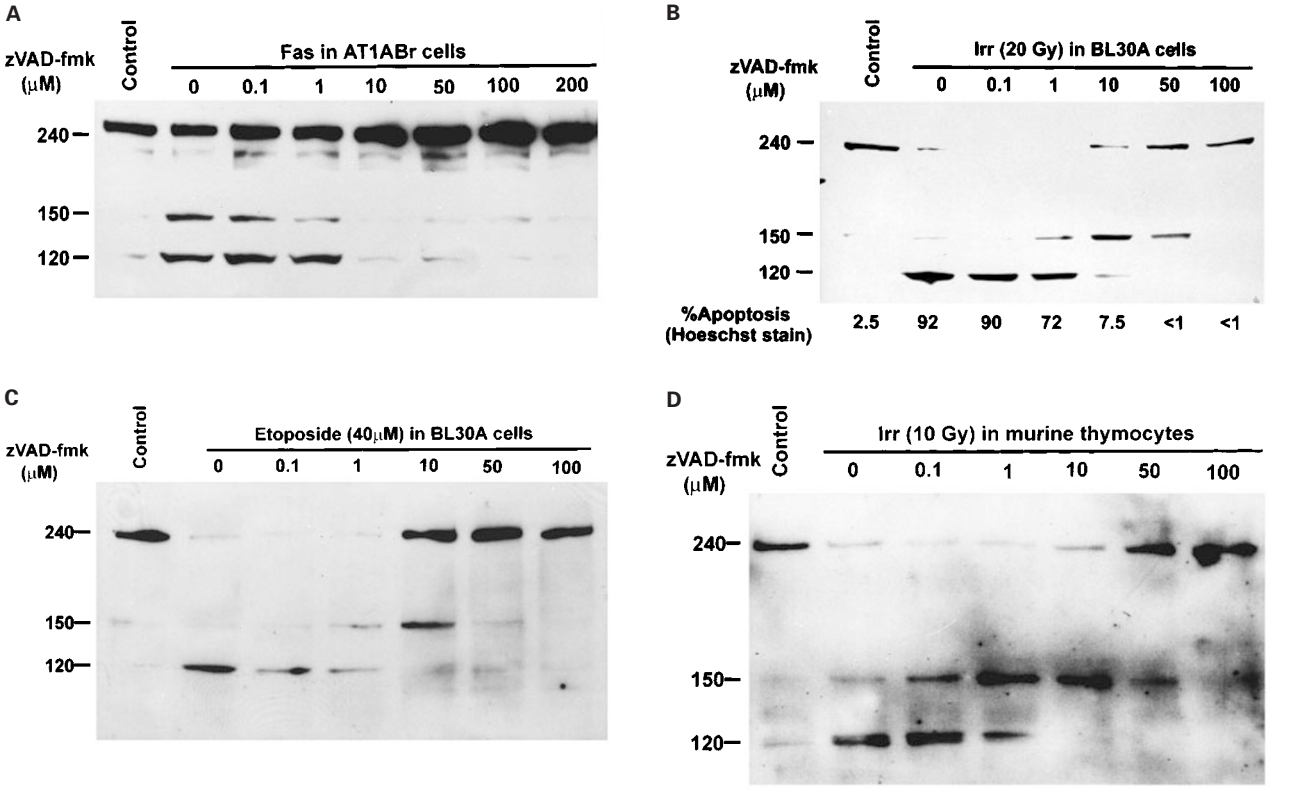

Figure 2 Inhibition of fodrin cleavage by zVAD-fmk. All cells were pre-treated for 15 min with the concentrations of zVAD-fmk indicated. The cells were treated with the apoptotic stimuli, incubated for 8 h and total protein was extracted. By this time the majority of cells in the treated populations without inhibitors were apoptotic. AT1ABr cells were treated with Fas antibody (**A**), BL30A cells were treated with 20 Gy ionizing radiation (**B**), BL30A cells were treated with 40 μM etoposide (**C**) and murine thymocytes were treated with 10 Gy ionizing radiation (**D**). 100 μg of protein was loaded per lane of a 4% SDS–PAGE gel and Western blotted using anti-fodrin primary antibodies

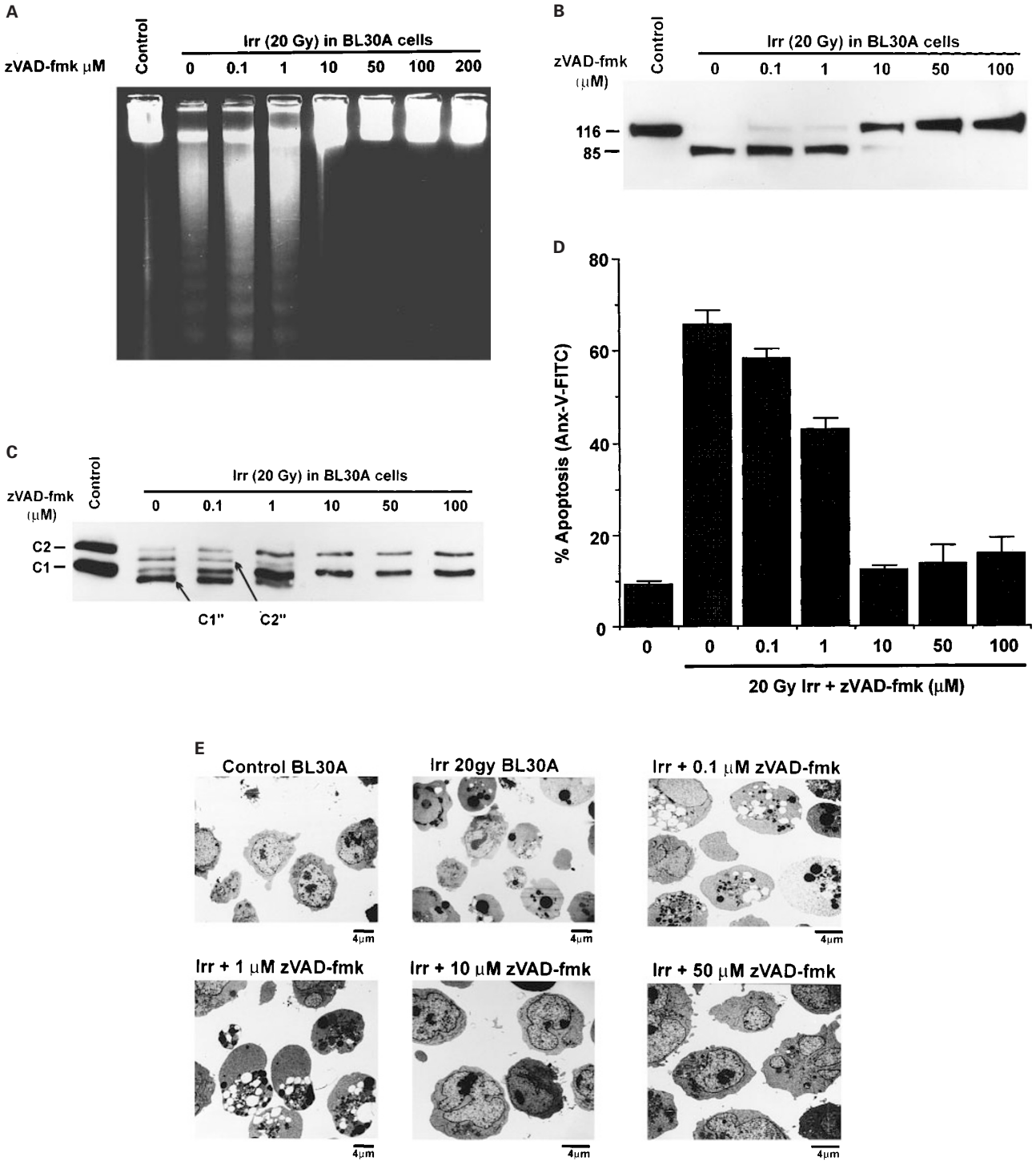

Figure 3 Inhibition of the hallmarks of apoptosis by zVAD-fmk. BL30A cells were pre-treated for 15 min with the concentrations of zVAD-fmk indicated followed by treatment with 20 Gy ionizing radiation. The cells were harvested after 8 h when 80–90% irradiated cells, with no inhibitor, were apoptotic. The DNA from 5×10^5 cells per sample was subjected to agarose gel electrophoresis and visualised by ethidium bromide staining (**A**), Total cellular protein was extracted and the cleavage of caspase specific substrates, PARP (30 μg of total protein per lane resolved on a 4–15% gel) (**B**) and hnRNP C1/C2 (5 μg of total protein resolved on a 10% slab gel) (**C**) was determined. The population of cells was stained with Annexin V and 5000 cells from each sample were assayed by flow cytometry to determine the extent of phosphatidylserine exposure (**D**) and the morphological changes associated with apoptosis as viewed by electron microscopy were shown (**E**). 10 μM of zVAD-fmk was sufficient to inhibit all of these apoptotic events

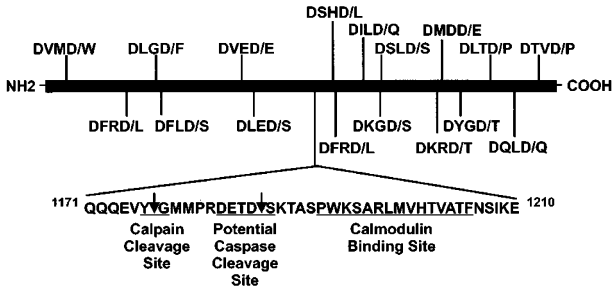

Figure 4 The amino acid sequence of human fodrin, residues 1171–1210 (Stabach *et al*, 1997) shows the calpain cleavage site and potential caspase cleavage site adjacent to the calmodulin binding domain. The position of the 18 potential caspase cleavage sites is also shown (not to scale)

similar situation exits in human fodrin (Stabach *et al*, 1997) (Figure 4). Thus there has been much debate as to whether caspases rather than calpain are responsible for the cleavage of fodrin during apoptosis *in vivo* (Greidinger *et al*, 1995; Cryns *et al*, 1996; Nath *et al*, 1996; Vanags *et al*, 1996). Since 100 μM zVAD-fmk can inhibit the cleavage of fodrin in radiation-induced apoptosis, we investigated which proteases caused cleavage of fodrin to a 150 kDa fragment *in vitro*. A panel of caspases was incubated with total cell extracts and assayed by Western blot for their ability to mediate fodrin cleavage. Of the caspases used, only caspases -1, -3, -6 and -7 had the ability to generate the 150 kDa fragment (Figure 5A). These reactions were all sensitive to inhibition by zVAD-fmk, caspases -1 and -6

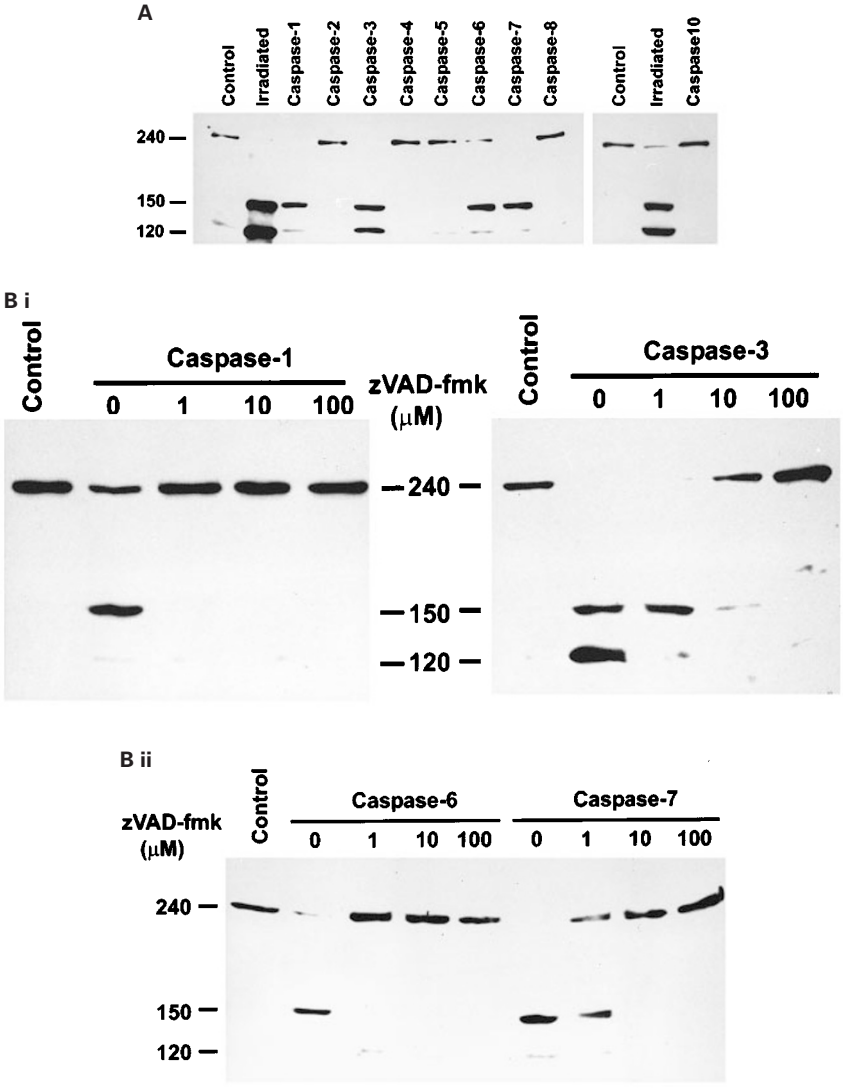

Figure 5 Caspases-1, -3, -6 and -7 can cleave fodrin to a 150 kDa fragment *in vitro*. Total cell lysates (100 μg) from BL30A cells were incubated at 37°C for 3 h with equal amounts of caspase activity (caspases-1, -8 and -10) as determined by assay of fluorogenic substrates. The samples were subjected to Western blotting using 4% SDS–PAGE gels and anti-fodrin antibodies (**A**). The cleavage of fodrin by recombinant caspases -1 and -3 (**B i** and caspases -6 and -7 (**B ii**) was inhibited by adding 10 μM zVAD-fmk to similar digests as described in Figure 5A

being inhibited by 1 μM zVAD-fmk and caspases -3 and -7 being inhibited by 10 μM zVAD-fmk Figure 5B i and ii). It is noted that there is a trace amount of caspase-3 activity remaining in the presence of 10 μM zVAD-fmk.

μ-Calpain added to cell extracts also mediates fodrin cleavage to the 150 kDa fragment (Figure 6). Since the cleavage of fodrin during radiation-induced apoptosis is inhibited by 100 μM zVAD-fmk (Figure 2B) we tested whether calpain could be inhibited by zVAD-fmk. We show that 10 μM zVAD-fmk is not sufficient to inhibit the *in vitro* cleavage of fodrin by calpain, but 100 μM zVAD-fmk inhibits this cleavage completely (Figure 6). Since, in this reaction, it is possible that the inhibitor is blocking a calpain-activated caspase, we showed that zVAD-fmk also blocks the hydrolysis of casein by purified calpain in an *in vitro* assay, demonstrating that zVAD-fmk directly inhibits calpain (Table 1).

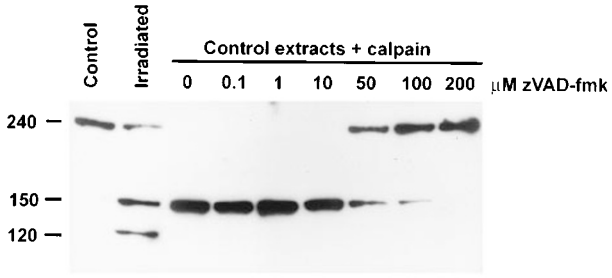

Figure 6 The μ-calpain cleavage of fodrin to a 150 kDa fragment *in vitro* is inhibited by zVAD-fmk. Calpain (0.256 U) was incubated with total cell lysate from BL30A cells and the indicated concentration of zVAD-fmk at 37°C for 20 min. The samples were Western blotted using 4% SDS–PAGE gels and anti-fodrin antibodies. Calpain can cleave fodrin to a 150 kDa fragment *in vitro* and this cleavage was completely inhibited by 100 μM zVAD-fmk

Calpain mediates the cleavage of fodrin to the 150 kDa fragment in radiation-induced apoptosis *in vivo*

We have shown that five proteases, μ-calpain and caspases-1, -3, -6 and -7 are capable of cleaving fodrin *in vitro*. Calpain and caspase-3 are less sensitive to inhibition by zVAD-fmk and as such, are possible candidates for the cleavage of fodrin to the 150 kDa fragment *in vivo*. To determine which enzyme is involved *in vivo*, we analyzed the time course of activation as evidenced by cleavage to their active fragments after 20 Gy radiation. Caspase-3 is only activated 2 h after irradiation in BL30A cells (Figure 7A) whereas calpain activation occurs within 15 min (Figure 7B). We also show that there is almost negligible activation of

Table 1 Inhibition of calpain activity by zVAD-fmk

[zVAD-fmk] μM	Rate (dA/min)
0	0.105
0.1	0.104
1	0.105
5	0.066
10	0.041
50	0.005
100	0.003

Calpain activity was measured as described (Jiang *et al*, 1997). 0.256 U of calpain was incubated with 1 mg/ml casein and the indicated concentration of zVAD-fmk at room temperature. The rate of hydrolysis was calculated for various zVAD-fmk concentrations. The experiment was performed several times with similar results each time. The standard deviation was between 5% and 15% in all cases. This shows that calpain is directly inhibited by zVAD-fmk

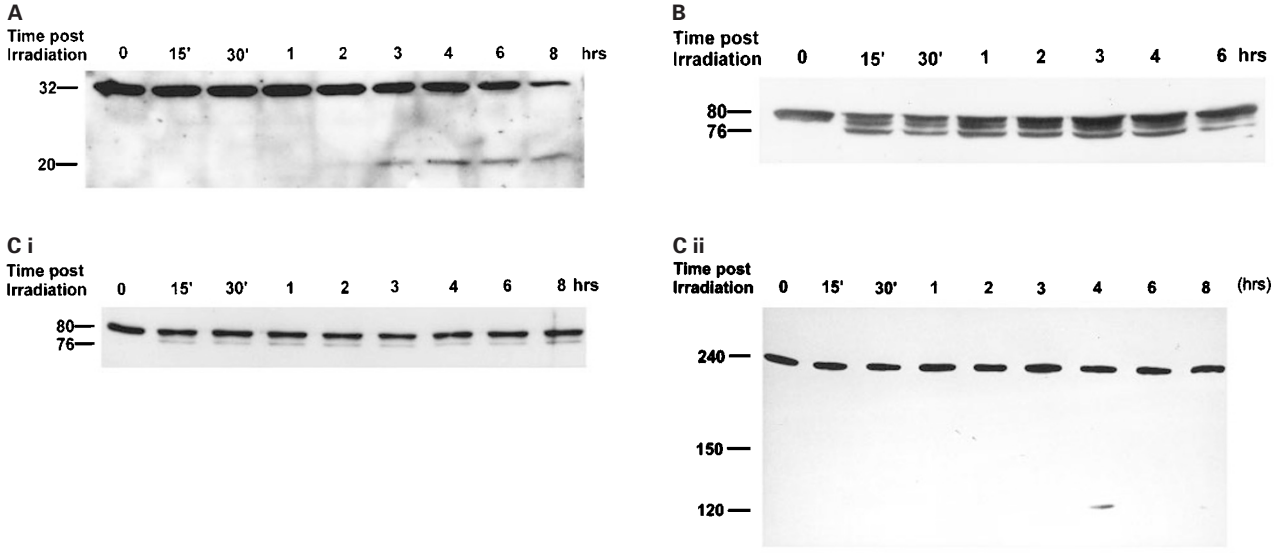

Figure 7 Time course of protease activation in BL30A and BL30K cells treated with 20 Gy ionizing radiation. The cells were treated with 20 Gy ionizing radiation and harvested at the times indicated. Total cell lysates from BL30A cells were extracted and caspase-3 activation was determined by Western blotting, 300 μg/lane on a 15% SDS–PAGE gel using anti-caspase-3 primary antibody (**A**). Calpain activation was determined by Western blotting. 10 μg/lane was loaded on an 8% SDS–PAGE gel and detected using anti-calpain antibodies (**B**). 10 μg/lane of BL30K lysates were run on an 8% SDS–PAGE gel and detected by Western blotting using anti-calpain primary antibodies. This shows that calpain is not activated in the radiation resistant cell line (**C i**). 100 μg/lane of the BL30K lysates were run on a 4% gel and analyzed by Western blotting to show that fodrin is not cleaved after radiation treatment in BL30K cells (**C ii**)

calpain in BL30K cells (Figure 7C i), nor is fodrin cleaved (Figure 7C ii), correlating with the relative resistance of these cells to radiation-induced apoptosis. The strong correlation between the time of activation of calpain (Figure 7B) and the time of the initial cleavage of fodrin to a 150 kDa fragment (Figure 1A) coupled with the inhibitor data suggests that, in radiation-induced apoptosis, calpain is responsible for the initial cleavage of fodrin to the 150 kDa fragment. The time course of activation of caspase-3 also correlates with the time of cleavage of fodrin to a 120 kDa fragment and inhibitor data suggests that caspase-3 is responsible for the second cleavage to the 120 kDa fragment. Caspase-2 (Nedd-2/ICH-1) did not cleave fodrin in our experiments, however it has been reported that caspase-2 cleaved fodrin to a 150 kDa fragment *in vitro* (Nath *et al* 1996). Caspase-2 was only activated in BL30A cells 2 h after radiation treatment (data not shown), suggesting that even if caspase-2 can cleave fodrin to a 150 kDa fragment *in vitro*, it does not mediate this cleavage *in vivo* in radiation-induced apoptosis.

Cleavage of caspase-3 to its active form is inhibited by 100 μM zVAD-fmk but not by 10 μM zVAD-fmk

In order to determine whether calpain activation is instrumental in the activation of the caspases or whether it lies on another pathway, we investigated the proteolytic cleavage (activation) of caspase-3 by Western blotting after irradiation in the presence of 10 μM zVAD-fmk. This concentration should have minimal effect on calpain activity but will completely block caspase activity. The results of this experiment are shown in Figure 8. Caspase-3 is still proteolytically processed in the presence of the 10 μM zVAD-fmk thus the apoptotic signal from radiation through calpain to caspase processing is still functional, only the downstream events relying on activity of caspases are inhibited (Figure 3). Caspase-3 is not processed when the concentration of zVAD-fmk is increased to 100 μM, the concentration required to inhibit calpain. This provides strong support for calpain being placed upstream of the caspases in radiation-induced apoptosis.

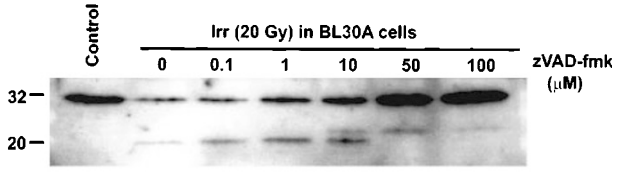

Figure 8 Cleavage of caspase-3 to its active form is inhibited by 100 μM zVAD-fmk but not by 10 μM zVAD-fmk. BL30A cells were pre-treated for 15 min with the concentrations of zVAD-fmk indicated, followed by treatment with 20 Gy γ-radiation. The cells were harvested after 8 h when 80–90% of the irradiated cells were apoptotic in the absence of inhibitor. Total cell lysates were extracted and 300 μg/lane of each sample was run on a 15% SDS–PAGE gel. Caspase-3 activation was determined by Western blotting using anti-caspase-3 primary antibody. The 17 kDa band represents the active fragment

Discussion

Our results show that the first proteolytic event in radiation-induced apoptosis of BL30A cells is the cleavage of fodrin to a 150 kDa fragment appearing within 15 min, followed by further cleavage to a 120 kDa fragment which is evident by 2–4 h, concomitant with the cleavage of PARP and the hnRNP C proteins (Figure 1). In human and murine fodrin there is a caspase cleavage site within 1 kDa of the calpain cleavage site (Figure 4) (Harris *et al*, 1988). Both caspases and calpain have been shown to cleave fodrin to a 150 kDa fragment *in vitro*, however it is still debatable as to which enzyme is responsible for cleavage *in vivo* (Cryns *et al*, 1996; Nath *et al*, 1996; Vanags *et al*, 1996).

It has been reported that in Fas-induced apoptosis of Jurkat cells, both cleavage events in fodrin are inhibited by 10 μM zVAD-fmk (Greidinger *et al*, 1995). This is also true in our hands in AT1ABR cells (Figure 2A). The level at which zVAD-fmk (10 μM) inhibits Fas-induced apoptosis may be caspase-8, which has been linked directly to the Fas-initiated death-inducing complex. In radiation- and etoposide-induced apoptosis of BL30A cells and in radiation-induced apoptosis of murine thymocytes, the initial cleavage of fodrin to a 150 kDa fragment is mediated by a protease inhibited by high concentrations of zVAD-fmk (100 μM) whereas the protease responsible for the cleavage of fodrin to the 120 kDa fragment is more sensitive to inhibition by zVAD-fmk (10 μM) (Figure 2D).

zVAD-fmk is categorised as a caspase inhibitor and is likely to have different specificity for different caspases, however its inhibitory profile for individual caspases is not known. In radiation-induced apoptosis, 10 μM zVAD-fmk is sufficient to inhibit all the hallmarks of apoptosis:- nuclear fragmentation, cleavage of PARP, cleavage of hnRNP C1/C2, phosphatidylserine exposure, apoptotic morphology (Figure 3) and the formation of the 120 kDa fragment of fodrin, but not the appearance of the 150 kDa band (Figure 2B). Thus zVAD-fmk at 10 μM stops the proteolytic cascade at a point further downstream in the radiation pathway. While it delays apoptosis for up to 8 h (Figure 3), the cells have lost their ability to proliferate (data not shown) and may eventually die.

In all systems studied in this paper, a single 150 kDa fragment of fodrin was observed, in contrast to the SH-SY5Y neuronal model (Nath *et al*, 1996) where a doublet was observed at 150/145 kDa. In irradiated murine thymocytes, but not in irradiated human lymphoma cells, we observed a doublet at 120 kDa. This may indicate that different proteases are responsible for the cleavage of fodrin to the two fragments in different models of apoptosis.

In order to determine which enzyme could be responsible for the cleavage of fodrin to the 150 kDa and 120 kDa fragments in radiation-induced cell death, we used a panel of recombinant caspases and μ-calpain *in vitro*. The only proteases with the ability to form similar fragments to those seen *in vivo* were caspases -1, -3, -6, -7 and μ-calpain (Figures 5A and 6). In our experiments, caspases -1 and -6 were inhibited by 1 μM zVAD-fmk (Figure 5B). This suggests that caspases -1 and -6 are not involved in, or upstream of any of the apoptotic events assayed here

(Figure 3) as fodrin was still fully cleaved and all apoptotic events were completed in the presence of 1 μM zVAD-fmk. Caspase-6 has been implicated in the cleavage of lamin, which has been shown to be a late event in Fas-induced apoptosis (Greidinger *et al*, 1995). Caspase-7 is inhibited by 10 μM zVAD-fmk and therefore cannot be responsible for the cleavage of fodrin to the 150 kDa fragment during radiation-induced apoptosis, however it may be involved in the cleavage of fodrin to the 120 kDa fragment. Caspase-3 is also inhibited by 10 μM zVAD-fmk, although trace activity is still observed when treated with this concentration of inhibitor (Figure 5B i). Nevertheless, it is unlikely that caspase-3 cleaves fodrin to the 150 kDa fragment *in vivo*, since it is not activated in BL30A cells until 2 h after treatment with 20 Gy ionizing radiation (Figure 7A) and the 150 kDa fragment is evident at least 1.5 h prior to this activation.

During Fas-induced apoptosis, 100 μM of the specific caspase-3 inhibitor, DEVD-CHO, inhibits the cleavage of fodrin to the 120 kDa fragment but not to the 150 kDa fragment (Greidinger *et al*, 1995). This is also evident in radiation-induced apoptosis (data not shown). Caspase-3 is most likely to be responsible for the cleavage of fodrin to the 120 kDa fragment since DEVD-CHO inhibits this cleavage *in vivo* and since *in vitro*, caspase-3 appears to cleave fodrin to the 120 kDa fragment more efficiently than caspase-7 (Figure 5A). There are 18 potential caspase cleavage sites in fodrin (Figure 4) however the actual cleavage giving rise to the 120 kDa fragment is not yet known.

Since none of the caspases tested appeared to be responsible for the cleavage of fodrin to the 150 kDa fragment, this suggests that either calpain activates a caspase as yet undescribed in the literature, or that zVAD-fmk is not a specific caspase inhibitor and can inhibit calpain. There is no evidence to suggest that calpain can activate the caspases in a cell free system. The use of peptides has revealed no definite recognition sequence for calpain as reviewed in (Takahashi, 1990). Amino acids with an aromatic or large aliphatic side chain are preferred in positions P1, P2 and P3 and basic or large aliphatic amino acids in the P′1 position. Nonetheless, we observed that 100 μM zVAD-fmk could inhibit the cleavage of fodrin by calpain *in vitro* (Figure 6). We have also demonstrated that zVAD-fmk can directly inhibit the hydrolysis of casein by calpain in an environment free of caspases (Table 1). The data shows that 100 μM zVAD-fmk was sufficient to inhibit the reaction completely, whereas 10 μM was only sufficient to reduce the rate of hydrolysis. This inhibition profile correlates strongly with the inhibition profile for the calpain cleavage of fodrin *in vitro* and the cleavage of fodrin to a 150 kDa fragment *in vivo*. zVAD-fmk is supplied with an OMe block on the Asp-β-carbonyl to enhance cell permeability (Enzyme Product Systems Catalogue). The Asp-OMe may sterically appear like a leucine and in this way act as a calpain inhibitor (anonymous reviewer's comments). Our results provide the first direct evidence that zVAD-fmk inhibits calpain as well as caspases. Since zVAD-fmk also inhibits apoptosis inducing factor (Susin *et al*, 1996), conclusions from experiments using zVAD-fmk as a caspase specific inhibitor should be made with caution.

The time course of activation of calpain (Figure 7B) also correlates with the time course of cleavage of fodrin to a 150 kDa fragment (Figure 1A). This combined with the strong correlation between the inhibition profiles of calpain by zVAD-fmk *in vitro* and the inhibition profile of the cleavage of fodrin to the 150 kDa fragment *in vivo* and also the fact that none of the caspases assayed can be responsible for fodrin cleavage *in vivo*, is compelling evidence for the involvement of calpain in the cleavage of fodrin to a 150 kDa fragment during radiation-induced apoptosis in BL30A cells.

The reasons for the two step cleavage of fodrin are still unclear. It has been suggested that the cleavage of fodrin may result in phosphatidylserine exposure (Martin *et al*, 1995, 1996; Vanags *et al*, 1996). We show that in radiation-induced apoptosis, since 10 μM zVAD-fmk is sufficient to inhibit phosphatidylserine exposure, the cleavage of fodrin to a 150 kDa fragment is not likely to induce this event. Since all of the other assayed apoptotic events are also inhibited by 10 μM zVAD-fmk, it is also probable that the cleavage of fodrin to a 150 kDa fragment does not result in any of these phenomena. The initial cleavage of fodrin to a 150 kDa fragment may just be a consequence of calpain activation, however this appears unlikely since two distinct proteolytic pathways, one in Fas-induced apoptosis and one in radiation-induced apoptosis, involve the cleavage of fodrin to a 150 kDa fragment. It is possible that the first cleavage makes fodrin more susceptible to the second caspase-mediated

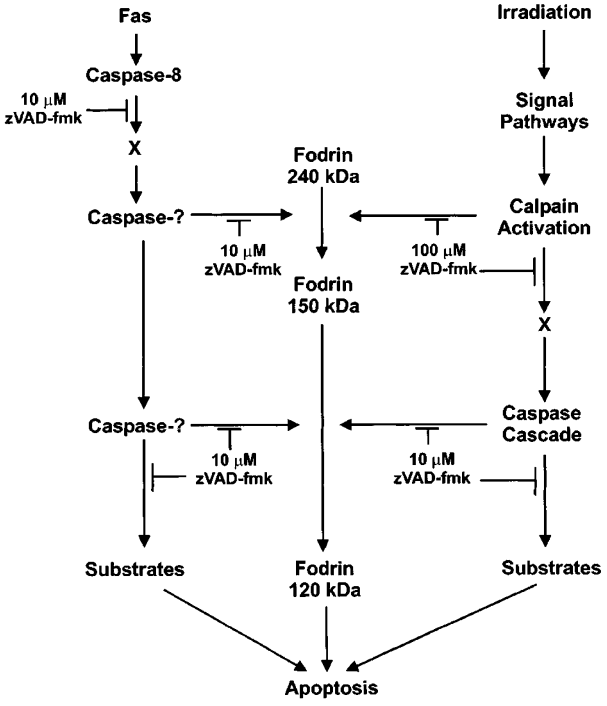

Figure 9 A schematic representation of the possible molecular ordering of the apoptotic pathway in radiation-induced cell death

cleavage giving rise to the 120 kDa fragment. However, this remains to be established.

The evidence provided in Figure 8, places calpain upstream of the caspases, however the mode of activation of calpain is presently unknown. Studies in our laboratory have shown that levels of ceramide increase after ionizing radiation in the sensitive BL30A cell line, but not in the resistant BL30K cell line (Michael *et al*, 1997). It is also known that ceramide can lead to activation of calpain in differentiated PC12 cells (Xie and Johnson, 1997). To further address the role of calpain as an important component of the radiation-signalling pathway leading to apoptosis, it will be important to determine the signals leading to calpain activation and the subsequent steps leading to activation of the caspases.

We propose a model (Figure 9) for the proteolytic cascade in radiation-induced apoptosis. The data presented in this study predict that calpain is responsible for the cleavage of fodrin to a 150 kDa fragment and that calpain is activated upstream of the caspases.

Materials and Methods

Reagents

Etoposide, β-glycerophosphate and Nonidet P-40 (NP-40) were purchased from Sigma, (St Louis MO, USA). Leupeptin, aprotinin and EGTA were from ICN, (Costa Mesa, CA, USA). Calpain-I and Triton X-100 were from Calbiochem (La Jolla, CA, USA) and Pepstatin was from Auspep (Parkville, Australia). z-Val-Ala-Asp-CH_2F (zVAD-fmk) was obtained from Enzyme Products Systems, (Dublin, CA, USA). Complete inhibitor, Proteinase K, DNAse free RNAse A and PARP antibody were from Boehringer Mannheim GmBH. Annexin V was from Biowhittaker, (Walkersville, MD, USA). Caspase-3 (CPP32) antibody was from Santa Cruz (CA, USA). The monoclonal antibody to hnRNP C (4F4) has been described previously (Choi and Dreyfuss, 1984) and was a kind gift from Dr. Gideon Dreyfuss (Philadelphia, USA). The antibody to human calpain-I has been previously described (Samis *et al*, 1987). Monoclonal antibody to Fas (clone CH11) was from Immunex (Seattle, Washington, USA.). Electrophoresis materials were from BIO-RAD and all other reagents were analytical grade.

Cell culture

The isogenic Burkitt's lymphoma cell lines used in this study, (BL30A, radiation sensitive and BL30K, radiation resistant) have been described previously (Waterhouse *et al*, 1996). AT1ABr, a lymphoblastoid cell line from a patient with ataxia-telangiectasia, has also been previously described (Watters *et al*, 1997). Murine thymocytes were removed from 4–6 week old outbred mice (Quackenbush). Cells were maintained at 37°C in Roswell Park Memorial Institute (RPMI)1640 medium supplemented with 10% heat-inactivated foetal calf serum (FCS) in a 5% CO_2 atmosphere. BL30A cells were maintained under similar conditions using 20% FCS. For the induction of apoptosis, BL30A cells were irradiated with 20 Gy ionizing radiation using a ^{137}Cs source at 3 Gy/min or exposed to 40 μM etoposide. AT1ABr cells were treated with anti-Fas antibody (250 ng/ml). Thymocytes were exposed to 10 Gy ionizing radiation. Inhibitors were added 15 min prior to the apoptotic stimulus.

Protein isolation for Western blot, caspase digestion and calpain digestion

Cells were pelleted and washed twice in cold PBS. The pellet was then lysed in modified Universal Immunoprecipitation Buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 25 mM β-glycerophosphate, 0.2% Triton X-100, 0.3% NP40 and 1 × complete inhibitor^{TM}) and rocked at 4°C for 10 min. This was then microfuged at 10 500 g for 10 min and the supernatant was stored at −70°C. If lysates were to be digested by caspases or calpain, the complete inhibitor^{TM} was not added. For calpain digestion, EDTA and EGTA were also left out of the lysis buffer and 0.2 mM $CaCl_2$ and 5 mM β-mercaptoethanol were added.

Western blotting

For Western blotting, equal quantities of protein were resolved by standard Laemmli SDS-polyacrylamide gel electrophoresis. The gel was electroblotted to Hybond-C nitrocellulose using Towbins Buffer (125 mM Tris, 95 mM glycine, 0.02% SDS, 20% methanol). The membrane was washed in PBS for 1 h and then blocked in 5% skim milk powder in PBS (SMP-PBS) for 1 h. Primary antibody in 5% SMP-PBS was added and rocked gently overnight at 4°C. The membrane was washed in PBS (3 × 20 min), the horseradish peroxidase-conjugated secondary antibody in 5% SMP-PBS was added and incubation continued for 2 h at room temperature. The membrane was washed again in PBS (3 × 20 min). Proteins were detected using the DuPont Rennaissance^{TM} chemiluminescence kit with Reflection^{TM} film.

Detection of DNA fragmentation

5×10^5 cells were microfuged for 3 min and the pellet was lysed in 25 μl lysis buffer (0.5% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0, 50 μg/ml Proteinase K) and incubated at 50°C for 1 h. 10 μl of DNAse-free RNAse A was added and incubation continued for a further hour at 50°C. Samples were heated in loading buffer (10 mM EDTA, 0.25% bromophenol blue, 40% sucrose, 1% agarose (low melting point) to 70°C and were resolved on a 2% agarose TBE gel.

Annexin V binding

The cells were pelleted by centrifugation at 500 × *g*. The media was aspirated and the cells were resuspended in 50 μl of 0.5 μg/ml Annexin V in HEPES buffer (10 mM HEPES-NaOH pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) as described (Koopman *et al*, 1994). This was incubated at room temperature for 5 min and the volume was made up to 200 μl with HEPES buffer. Propidium iodide was added to a final concentration of 10 μg/ml before analysis. The annexin V binding was determined by flow cytometry.

Caspase digestion

Caspases were expressed in *E. coli* as previously described (Song *et al*, 1996). Activities of various caspases in bacterial extracts were estimated using fluorogenic peptide substrates, YVAD-AMC for caspase-1, -4 and -5 and DEVD-AMC for all others. Aliquots containing equivelent molar amounts of active caspases (usually in 2–10 μl volume) were incubated with 100 μg of total cell lysate from BL30A cells at 37°C for 3 h. The digestion reactions were carried out in a total of 20 μl, consisting of 2 μl of total cell lysate (50 μg/ml) from BL30A cells, 2 μl of 10 × caspase assay buffer [250 mM HEPES, pH 7.5, 50 mM EDTA, 20 mM DTT and 1% CHAPS] and the caspase. The

reaction was terminated by boiling the samples in 5 × SDS – PAGE loading dye for 5 min. The samples were then subjected to Western blot analysis from a 4% SDS – PAGE gel using anti-fodrin antibodies.

Calpain Digestion

100 μg of total protein extracted from BL30A cells was placed in each of several microfuge tubes. 5 μl of the appropriate inhibitor was added to each tube followed by 2 μl (0.256 U) of calpain. The sample volume was made to 20 μl with lysis buffer and incubated at 37°C for 20 min. The reactions were terminated by adding 5 μl of 5 × SDS – PAGE loading dye and boiling for 5 min.

Calpain activity assays

In order to determine the effect of zVAD-fmk on calpain activity, a spectrophotometric assay (Jiang *et al*, 1997) was used. Briefly, calpain (0.256 U) and the appropriate concentration of zVAD-fmk were diluted to 95 μl in casein/imidazole buffer (50 mM imidazole-HCl (pH 7.5), 10 mM β-mercaptoethanol, 1 mM sodium azide and 1 mg/ml casein) and incubated for 5 min at room temperature. Calcium chloride was then added to a final concentration of 5 mM to initiate the calpain hydrolysis reaction. The spectrophotometer was immediately blanked and the absorbance at 500 nm was measured at 30 s intervals. Since the hydrolysis reaction was sigmoidal, the rates were measured at the steepest point of the curve.

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