Synthesis of procaspases-3 and -7 during apoptosis in prostate cancer cells

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Abstract

Cells differ in the time required to execute cell death after receipt of a death signal. One reason may be the requirement for *de novo* synthesis of components of the death pathway. TSU-Pr1 prostate cancer cells treated with okadaic acid demonstrated activation of caspase-3, PARP cleavage, and nuclear fragmentation by 24 h and apoptosis by 72 h. Levels of procaspase-3 and procaspase-7, the precursor molecules of two effector caspases, were not depleted during apoptosis. Levels of procaspase-3 and -7 mRNA increased steadily in TSU-Pr1 cells up to 72 h after exposure to okadaic acid. Nuclear run-off experiments showed that the increase in mRNA was not due to transcriptional activation of caspase-3 and -7 mRNA. Antisense caspase-3 and caspase-7 oligodeoxynucleotides caused a depletion of procaspases-3 and -7 and a delay in apoptosis of TSU-Pr1 cells. Caspase antisense oligodeoxynucleotides inhibited apoptosis to a similar extent as peptide inhibitors of cysteine proteases. Synthesis of procaspases-3 and -7 was necessary to sustain programmed cell death in TSU-Pr1 prostate cancer cells.

Keywords: apoptosis; prostate cancer; caspase; antisense

Abbreviations: IMEM, improved minimal essential medium; ISEL, *in situ* end labeling; PARP, poly(ADP-ribose) polymerase; YVAD, N-acetyl-Tyr-Val-Ala-Asp-chloromethylketone; DEVD, N-acetyl-Asp-Glu-Val-Asp(OMe)-CH₂F; z-VAD, z-Val-Ala-Asp(OMe)-CH₂F; TLCK, Na-p-tosyl-L-lysine-chloromethylketone

Introduction

Apoptosis is the process of programmed cell death that can be induced by a variety of stimuli and is mediated by the activation of proteases. The cysteine proteases or caspases participate in a proteolytic cascade that terminates in the irreversible cellular changes that are characteristic of programmed cell death.¹ Caspases-3 and -7 are downstream

effector proteases in the proteolytic apoptosis cascade.^{1–3} Both of these closely related enzymes cleave poly(ADP)ribose polymerase (PARP) and activate endonucleases that induce DNA fragmentation.^{4–7} Caspase-3 is activated by either one of at least two separate proteolytic pathways. One pathway leading to caspase-3 activation transmits signals from cell surface death receptors and activates caspase-6 immediately upstream from caspase-3.² A second pathway results from egress of cytochrome c from mitochondria and leads to activation of caspase-9 immediately upstream from caspase-3.⁸ Caspase-7 is activated by caspase-3 and other caspases since it may be activated in some instances when caspase-3 is not.^{2,9} Caspases-3 and -7 are more closely related to each other by amino acid sequence than to any other members of the caspase family of proteins. They are both activators of nucleases that generate DNA fragmentation, an irreversible hallmark of cell death.

Procaspases reside in the cytoplasm as inactive precursor molecules that are activated by proteolytic cleavage. Cleavage of the amino terminal prodomain precedes cleavage into two subunits, p20 and p10. Two molecules each of p20 and p10 combine to form a tetramer that is the active caspase.¹⁰ The activation of death cell surface death receptors, such as FAS, can trigger activation of the caspase proteolytic cascade causing death of cells such as Jurkat T cells within a few hours after death ligand exposure.¹¹ Induction of apoptosis in other cell types may require an interval of hours or days before caspase activation. The implication of this delay between the death stimulus and caspase activation is that the cell is preparing components of the death pathway, perhaps by synthesizing components of the proteolytic cascade. In the course of our studies of apoptosis in prostate cancer cells, we observed that the induction of caspase activation was initiated no sooner than 24–48 h after exposure to agents that nevertheless induced cell death efficiently within 72 h of exposure. These cells were found to have detectable levels of caspase precursor proteins, but also increased levels of caspase mRNA within 12 h of exposure to a death-inducing agent, implying that *de novo* synthesis of effector caspases was necessary for cells to complete apoptosis. Using caspase antisense oligonucleotides to inhibit apoptosis we show that synthesis of procaspases-3 and -7 occurred during the initiation of apoptosis and was important for a rapid death response in TSU-Pr1 prostate cancer cells.

Results

Induction of apoptosis in TSU-Pr1 cells

The phosphatase IB inhibitor, okadaic acid, efficiently induced apoptosis in TSU-Pr1 and other prostate cancer cells. This

was demonstrated by either ISEL or Hoechst dye staining which demonstrated the characteristic nuclear fragmentation seen in apoptosis (Figure 1A and B). Similar responses were seen with other prostate cancer cell lines, LNCaP, PC-3 and DU-145 (not shown). Apoptosis was accompanied by the appearance on Western blot of caspase-3 p17 and fragments of PARP that are characteristic of cleavage by caspases. We also noted that the 34 kDa procaspase-3 was not depleted and even appeared to increase in abundance (Figure 1C). The appearance of caspase-3 p17 and the activation of PARP cleavage coincided with an increase of caspase-3 activity in TSU-Pr1 cell extracts 24 h after exposure to okadaic acid (Figure 1D).

Because the amount of procaspase-3 was increased during apoptosis in TSU-Pr1 cells, we analyzed levels of caspase-3 mRNA by RNase protection analysis. We also assayed levels of caspase-7 mRNA since caspases-3 and -7 are closely related and both can mediate PARP cleavage, nuclease activation, and apoptosis in prostate cancer cells. Figure 2A shows the expression of caspases-3 and -7 mRNA after induction of apoptosis in TSU-Pr1 cells. During the 72 h period after exposure to 30 nM okadaic acid levels of caspase-3 and -7 mRNA increased 3.7 and 4.8-fold,

respectively, in TSU-Pr1 cells. Nuclear run-off assays were performed at 6 and 12 h after exposure to okadaic acid to determine if the increase in caspase mRNA levels were due to activation of caspase gene transcription. Data from one of two separate nuclear run-off experiments with similar results showed there was no appreciable change in either caspase-3 or -7 transcription within 12 h of exposure to okadaic acid (Figure 2B). Caspase-1, -3 and -7 mRNA transcription increased in the experiment due to addition of fresh culture medium at the time either vehicle or okadaic acid was added. However, the nuclear run-off data show no increase in caspase transcription during induction of cell death. These data suggest that the increase in caspase-3 and -7 mRNA during induction of apoptosis resulted from decreased RNA turnover.

Inhibition of apoptosis by caspase antisense oligodeoxynucleotides

To investigate the importance of procaspase-3 and 7 synthesis on apoptosis we first conducted experiments with chemical inhibitors of transcription and translation. However, actinomycin D at concentrations as low as 1 μ M

Figure 1 Okadaic acid induction of apoptosis in TSU-Pr1 cells. **(A)** Time course of appearance of ISEL-staining cells after addition of 30 nM okadaic acid. **(B)** Photomicrographs of representative cells at different times after addition of 30 nM okadaic acid to TSU-Pr1 cells. **(C)** Western blots of procaspase-3, caspase-3 p17 and PARP at different times after addition of okadaic acid to TSU-Pr1 cells. (NT, no determination of procaspase-3 at 12 h). **(D)** Fluorescence assay for caspase-3 activity at various times after addition of okadaic acid to TSU-Pr1 cells

Figure 2 Caspase-3 and 7 mRNA expression during apoptosis. **(A)** RNase protection analysis of TSU-Pr1 total RNA at difference times after addition of okadaic acid. Gels were analyzed by phosphorimager, normalized to *gapdh* and quantitated (see text). The far right lane shows undigested riboprobes, all designed to be somewhat larger than the region of the probe protected by mRNA. **(B)** Nuclear run-off analysis of nuclear RNA from TSU-Pr1 cells $\pm$ okadaic acid. Results were quantitated by phosphorimager and plotted after being normalized either for *GAPDH* or for β -actin

and cycloheximide at concentrations as low as 5 μ M were both toxic to TSU-Pr1 cells. Therefore, to inhibit the synthesis of procaspases-3 and -7 we used antisense oligodeoxynucleotides. Nonsense oligonucleotides with the identical length and nucleotide composition as the antisense oligonucleotides were used as controls. All cultures were treated with a total oligodeoxynucleotide concentration of 40 μ M made up of nonsense, antisense or both. Figure 3A shows that both antisense oligonucleotides to procaspase-3 and procaspase-7 mRNA each inhibited apoptosis and together had an additive effect. To maximize the effect of the antisense oligonucleotides in subsequent experiments antisense caspases-3 and -7 and nonsense caspases-3 and -7 oligonucleotides were used together, such that 'antisense' means caspase-3 antisense plus caspase-7 antisense and 'nonsense' means caspase-3 nonsense plus caspase-7 nonsense. Oligonucleotide treatment appeared to delay the onset of apoptosis, but at 72 h after okadaic acid exposure the fraction of dead cells had increased in cultures with both nonsense and antisense oligonucleotides (Figure 3B).

Since antisense oligonucleotides presumably blocked the synthesis of procaspases-3 and -7, we hypothesized that at some point after addition of okadaic acid the cells would have synthesized sufficient amounts of the procas-

pases to undergo cell death and the antisense oligonucleotides would no longer have an inhibitory effect on apoptosis. We treated TSU-Pr1 cells with okadaic acid and added combined antisense or combined nonsense oligonucleotides to the cultures at different times after addition of okadaic acid. All cultures were fixed at 72 h and stained for ISEL. As shown in Figure 3C the optimal effect of cysteine protease antisense oligonucleotides was seen upon simultaneous addition of the oligonucleotides and okadaic acid. When oligonucleotides were added 24 h after okadaic acid there was still an inhibitory effect on DNA end-labeling. By 48 h after treatment with okadaic acid, the effect of antisense oligonucleotides was very small and by 72 h the antisense oligonucleotides had no effect on okadaic acid-induced apoptosis.

To compare the potency of antisense caspase-3 and -7 oligonucleotides to peptide inhibitors of caspases and apoptosis, we treated TSU-Pr1 cells with okadaic acid and several different peptide inhibitors. Peptide inhibitors caused from 30–60% inhibition of okadaic acid-induced apoptosis of TSU-Pr1 cells at 48 h. DEVD, an inhibitor primarily of caspase 8¹² caused 30% inhibition. zVAD, a pancaspase inhibitor, caused approximately 60% inhibition. TLCK, and inhibitor of serine proteases,¹³ caused approximately 50% inhibition (Figure 3D). When DEVD and zVAD

or zVAD and TLCK were used together, the effects were additive, indicating that multiple proteases were involved in okadaic acid-induced cell death. DEVD, a caspase inhibitor blocked apoptosis comparably to Caspase-3 and -7 antisense oligonucleotides.

Effect of caspase antisense oligonucleotides on procaspase synthesis and caspase activation

The antisense oligonucleotides to caspases-3 and -7 inhibited synthesis of procaspases-3 and -7. This is shown in Figure

Figure 3 Caspase-3 and caspase-7 antisense oligonucleotides inhibit apoptosis in TSU-Pr1 cells. **(A)** Cultures were grown in charcoal-treated calf serum and treated with oligonucleotides at the time of addition of okadaic acid. Control cultures without okadaic acid showed that no combination of oligonucleotides induced apoptosis. Oligonucleotides were combined in equal amounts to a total of 40 μ M. After 72 h cells were fixed and subject to ISEL staining. **(B)** Okadaic acid and oligonucleotides were added to TSU-Pr1 cells in 5% CCS at time=0. Cells were sampled for ISEL assay at different time points. **(C)** Okadaic acid was added to TSU-Pr1 cells at time=0. Oligonucleotides were added at the times indicated. Cultures were all harvested at 72 h and assayed for apoptosis by ISEL. **(D)** Peptide protease inhibitors were added to cultures simultaneously with okadaic acid. Cultures were assayed at 48 h for apoptosis

4A, a panel of immunoblots of the precursor peptides sampled at 24 and 48 h after treatment with okadaic acid, oligonucleotides or peptide protease inhibitors. Procaspase-3 is seen at constant or increasing amounts after exposure of TSU-Pr1 cells to okadaic acid, regardless of the presence or absence of protease inhibitors. Procaspase-7 is slightly depleted despite increased levels of caspase-7 mRNA after addition

of okadaic acid (Figure 2A). Since procaspase-3 levels increased and procaspase-7 levels decreased during apoptosis, it is likely that under our experimental conditions there were differences in the activation and regulation of these proteins. Addition of antisense oligonucleotides to caspases-3 and -7 prevented the formation of new precursor protein. The cellular pools of both procaspases-3 and -7 were depleted

Figure 4 Expression of procaspases and activated caspase-3 in TSU-Pr1 cells. TSU-Pr1 cells were treated with 30 nM okadaic acid and treated with peptide inhibitors or oligonucleotides. Cultures were sampled at the indicated times for Western blotting or for caspase-3 activity. **(A)** Western blots for procaspases-3 and -7. **(B)** Western blots for caspase-3 p20 and PARP. **(C)** Western blots for caspase-8 cleavage 24 and 48 h after exposure to okadaic acid in the presence of either antisense or nonsense caspase-3 and -7 oligonucleotides. **(D)** Assay for caspase-3 activity in TSU-Pr1 cells at different times after treatment with okadaic acid and oligonucleotides

within 48 h in the presence of antisense oligonucleotides. This result shows that both procaspases-3 and -7 were being actively synthesized during activation of cell death.

In cultures treated with caspase antisense oligonucleotides, active caspase-3 p17 and PARP cleavage were seen at 48 h, after depletion of caspase-3 precursor protein. In TSU-Pr1 cells treated with nonsense oligonucleotides, caspase-3 p17 and PARP cleavage were seen at 24 h. Taken with the data in Figure 4A, procaspase-3 and 7 synthesis occurs during the 48 h after exposure to okadaic acid at the same time as caspase-3 activation. Blocking the synthesis of procaspases-3 and -7 reduced caspase-3 activation, probably by limiting the amount of protease available for activation. In the presence of peptide inhibitors DEVD and zVAD, caspase-3 activation was blocked and only minimal PARP cleavage was seen. Two other protease inhibitors were tested as negative controls. As expected, neither YVAD, an inhibitor of caspases 1 and 4, nor TLCK effected caspase-3 activation (Figure 4B). As a control for inhibition of caspase synthesis and processing, we also analyzed caspase-8 by Western blotting and found caspase-8 levels and cleavage to be unaffected by the presence of antisense or nonsense oligonucleotides (Figure 4C).

In addition to gauging caspase-3 activity by PARP cleavage, we also assayed caspase-3 activity in cell extracts using a fluorogenic substrate. This assay showed that caspase-3 activity in the presence of antisense oligonucleotides required an additional 24 h to reach maximal activity comparable to control cultures (Figure 4D). This data demonstrates that TSU-Pr1 cells require *de novo* synthesis of caspases-3 and -7 to maximize the cell death response. Therefore, regulation of intracellular levels of caspases may be one way that cells modulate their responses to death-inducing agents.

Discussion

Many cell types undergo apoptosis quickly upon exposure to a variety of agents. It has been proposed that cells contain presynthesized precursor proteases that are rapidly activated by proteolytic cleavage in response to death signals.¹⁴ However, the propensity of cells to initiate programmed cell death varies widely as does the time between death signal and execution. Among cultured cell lines the time from exposure to an inducer of apoptosis and cell death may take from a few minutes for Jurkat T cells to 6 or more days for osteosarcoma cells.^{11,15} Our data show that in some instances the delay between death signal and response is due to the requirement for synthesis of caspases.

In vivo, neural cells undergo apoptosis in response to trauma or ischemia, but may require many days to complete the apoptotic program. During the time between ischemic or traumatic insult and cell death caspase-3 mRNA expression is increased and caspase-3 is synthesized.^{16,17} The lag time between injury and death may provide an opportunity for therapeutic intervention to preserve neuronal function after ischemic or traumatic injury.

Cancer cells also may have adapted to resist cell death. In other experiments with prostate cancer cells, we observed one cell line, DU-145, that had no detectable expression

procaspases-8, -6, -3 and -7.¹⁸ In fact, procaspase-3 expression in DU-145 cells could not be detected until 48 h after exposure to okadaic acid. TSU-Pr1 prostate cancer cells have baseline levels of procaspases, including procaspases-3 and -7. However, caspase activation was not seen until 24 h after exposure to okadaic acid. In the presence of antisense oligonucleotides to caspases-3 and -7, the respective procaspases were depleted by 48 h after exposure to okadaic acid. The inhibition of apoptosis caused by the antisense oligonucleotides showed that one of the factors responsible for the death response in these cells was the supply of procaspases-3 and -7. It is likely that the block in apoptosis induced by antisense oligonucleotides lasted only 24 h because the oligonucleotides were depleted in the culture. Clearly in TSU-Pr1 cells, continued presence of procaspases-3 and -7 were required to sustain a death response. If cancer cells are slow to respond to death signals because levels of caspases or other death effectors are rate-limiting, it may be important to coordinate the timing of administration of chemotherapeutic agents that inhibit transcription or translation with those that induce cell death.

We have observed that TSU-Pr1 prostate cancer cells and three other cell lines, LNCaP, DU-145 and PC-3 (data not shown) all increased the mRNA levels of caspases-3 and -7 within 6 h of exposure to okadaic acid. In TSU-Pr1 cells the RNA increase resulted from decreased turnover since no increase in transcription was seen. Cells have diverse mechanisms to generate the proteins required for programmed cell death. Both stabilization and synthesis of caspase mRNA has now been reported. Enhanced mRNA expression is a general mechanism by which cells negotiate programmed cell death. For example rat prostate epithelial cells increased mRNA levels of a number of genes including *myc*, *hsp27* and *fos* during of apoptosis after androgen ablation.¹⁹ It may be that prostate epithelial cells depend on increased mRNA levels of a number of cofactors of cell death to mediate apoptosis. Unlike some lymphocytic cell lines, which appear to have the protein machinery for apoptosis available at necessary levels for a rapid death response, prostatic epithelial cells are not kept at the same level of readiness and therefore require new protein synthesis as an initial response to a death signal.

Specific inhibition of protein synthesis with antisense mRNA or oligonucleotides has not been often used to probe the function of caspases. A cell line stably transfected with antisense to caspase-2 has been shown to have increased resistance to apoptosis, demonstrating that reducing the background levels of a procaspase can slow programmed cell death.²⁰⁻²³ Similar findings were reported after reduction of procaspase-3 by antisense transcripts.^{24,25} Antisense oligonucleotides to caspase-1 were used to inhibit apoptosis consistent with our results with caspase-3 and caspase-7 antisense oligonucleotides.²⁶

We found that inhibition of either caspases-3 or -7 reduced cell death and that combined use of antisense for both proteases gave a larger response. In TSU-Pr1 cells treated with okadaic acid both effector caspases contribute to the death response. Caspases-3 and -7 are more closely related than other cysteine protease mediators of cell death

and both are able to cleave PARP and activate death nucleases.^{2,4,5,9} The RNase protection analysis showed that caspase-7 and caspase-3 RNA were coordinately regulated during the death response. The levels of these two proteases were comparable, and both the procaspase-3 and -7 levels were depleted at about the same time in antisense-treated cells. All these observations imply a coordinated regulation of caspase synthesis during programmed cell death. This coordinated regulation suggests a much more complex process than the activation cascade of latent precursor proteases whose presence is sufficient to consummate the death response.

Materials and Methods

Cell Culture. TSU-Pr1 human prostate cancer cells were cultured in improved minimal essential medium (IMEM) supplemented with 5% fetal bovine serum (Biofluids Inc, Rockville, MD, USA). Apoptosis was induced by incubating the cultures in the presence of 30 nM okadaic acid (Sigma, St Louis, MO, USA). Cells incubated in eight-well glass chamber slides were fixed with 10% neutral buffered formalin (Sigma, St Louis, MO, USA) for 10 min and washed twice in PBS for *in situ* end labeling (ISEL). Caspase-3 and caspase-7 oligonucleotides were added to cultures to a total concentration of 40 μ M. One half of cells growing on 8-well chamber slides in IMEM with 5% charcoal-stripped calf serum or serum-free media were treated with 20 μ M of each phosphodiester oligodeoxynucleotides.²⁷ The following sequences were used for the oligonucleotides: caspase-3 antisense: 5'GAG TTT TCA GTG TTC TCC AT 3'; caspase-7 antisense: 5'TCA TCT GCC ATC CCA CAA GG 3'; caspase-3 nonsense: 5'TGT TGC TAT GCT ATT GTC AC3'; caspase-7 nonsense: 5'CTC GTA CCT CAA CTG ACA GC 3'.

Apoptosis assays

In situ end labeling (ISEL) and Hoechst 33342 dye staining have been described previously.^{18,27}

Caspase-3 assay

Caspase-3 activity was assayed fluorometrically using Apopain/CPP32 Fluorometric Substrate (Upstate Biotechnology, Lake Placid, NY, USA). 10^7 cells were placed in lysis buffer (100 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 1 mM PMSF, 10 μ g/ml pepstatin, 10 μ g/ml leupeptin, and 1 mM EDTA) on ice for 30 min. Lysates were centrifuged ($10\,000 \times g$, 15 min, 4°C) and supernatants were stored at -80°C. Cell lysates (50 μ g protein) were incubated in the above lysis buffer for 1 h at 37°C with 50 μ M of apopain/cpp32 fluorometric substrate in a total volume of 0.5 ml. Samples were then read on a Hitachi F-4500 Spectrophotometer (Hitachi Instruments, Inc., Naperville, IL, USA) at an excitation/emission wavelength of 360/450 nm.

RNase protection assay

This was performed as previously described. Total cellular RNA was isolated by TRIzol reagent (Life Technologies, Gaithersburg, MD, USA). 30 μ g total cellular RNA was hybridized with 1×10^5 c.p.m. of each probe overnight at 50°C. Following RNase digestion with 40 μ g RNase (Sigma, St. Louis, MO, USA) at 25°C for 30 min, samples were extracted with phenol/chloroform and ethanol-precipitated. Samples were electrophoresed on 6% polyacrylamide/7M urea gels and autoradiographed. Antisense RNA probes were generated from

cDNA fragments subcloned into pGEM7. The caspase-7 probe was a 446 bp *SmaI*-*Apal* fragment. The caspase-3 probe was a 203 bp *SfuI*-*HindIII* fragment. The GAPDH control was a 104 bp *HindIII*-*EcoRI* fragment.

Nuclear Run-off Assay

For each experimental condition TSU-Pr1 cells were plated in 5 T150 flasks. Culture medium was changed and either okadaic acid or vehicle was added when cells were 70–80% confluent. Cells were harvested at 0, 6 and 12 h after addition of culture medium with or without okadaic acid. Cells were lysed with 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 2 mM MgCl₂, 0.5% NP-40. Nuclear pellets were resuspended in 500 μ l 20 mM HEPES pH 7.7, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM Pefabloc SC (the serine protease inhibitor AEBSF, Boehringer Mannheim, Indianapolis, IN, USA), 50% glycerol. 500 μ l of 50 mM HEPES, 6 mM MgCl₂, 4 mM MnCl₂, 0.4 M (NH₄)₂ SO₄, 1.6 mM each of ATP, CTP, and GTP, 10 mM DTT, 6 mM spermidine was mixed with 500 μ l isolated nuclei. A total of 0.5 mCi α -³²P-UTP was added (0.25 mCi of 3000 Ci/mmol and 0.25 mCi of 800 Ci/mmol, Amersham, Arlington Heights, IL, USA) was added to each sample and run-off was incubated for 1 h at room temperature. mRNA was purified by RNAzol (Tel-TEST, Inc. Friendswood, TX). RNA was resuspended in 200 μ l 20 mM NaOAc, 10 mM EDTA). 0.5×10^7 d.p.m. of RNA was hybridized to nitrocellulose filters containing 3 μ g of each plasmid. After hybridization for 3 days at 42°C, the filters were washed four times with $2 \times$ SSPE, 0.1% SDS at 25°C and digested with 40 μ g/ml RNase A for 30 min at 25°C in 10 mM Tris-HCl pH 7.5, 0.2 M NaCl, 1 mM EDTA, 0.1 mM LiCl. The filters were finally washed four times for 30 min at 42°C. Hybridization was determined by phosphorimager analysis and normalized to either GAPDH or β -actin.

Immunoblotting

Protein extracts were made by resuspending cell pellets in a lysis buffer of 50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 0.5 mM PMSF and 0.5 mM DTT. After rocking at 4°C for 30 min, DNA was sheared by passing the lysate through a 26 gauge needle. Cell supernatants were obtained following centrifugation at $30\,000 \times g$ for 10 min. 50 μ g of protein was loaded on a 12% SDS/polyacrylamide gel. After electrophoresis blots were treated with blocking buffer containing 5% dry milk for 1 h at room temperature and sequentially incubated with 1:1000 mouse monoclonal anticaspase-3 antibody (Transduction Laboratories, Lexington, KY, USA) or antiserum to caspase-7 peptide at room temperature for 1 h. 1:5000 secondary horseradish peroxidase-conjugated anti-mouse antibody (Pierce, Rockford, IL, USA) was added for 45 min. Protein bands were revealed using Supersignal[®] substrate (Pierce). Caspase-8 antiserum was from the laboratory of Dr. Peter Krammer.

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