Journal of Cellular Biochemistry 57:12–21 (1995)

Protein Kinase A Inhibitors Enhance Radiation-Induced Apoptosis

Duygu Findik, Qizhong Song, Hiroyoshi Hidaka, and Martin Lavin

Queensland Cancer Fund Research Unit, Queensland Institute of Medical Research, Brisbane, Queensland, Australia 4029 (D.F., Q.S., M.L.); Department of Pharmacology, Nagoya University School of Medicine, Showa-ku, Nagoya 466, Japan (H.H.)

Abstract In addition to a role for de novo protein synthesis in apoptosis we have previously shown that activation of a protein phosphatase or loss of activity of a kinase is also important in radiation-induced apoptosis in human cells [Baxter, and Lavin (1992): J Immunol 148:149–1954]. We show here that some inhibitors of protein kinases exacerbate radiation-induced apoptosis in the human cell line BM13674. The specific protein kinase A inhibitor isoquinoline sulfonamide (20 μM) gave rise to significantly increased levels of apoptosis at 2–6 h postirradiation compared to values after radiation exposure only. The same concentration of isoquinolinesulfonamide, which was effective in increasing apoptosis, reduced activity markedly. A 66% inhibition of cyclic AMP-dependent protein kinase A activity occurred in unirradiated cells at this concentration of H89 and activity was reduced to 58% in irradiated cells. Calphostin C, a specific inhibitor of protein kinase C, at a concentration of 0.1 μM, which caused 68% inhibition of enzyme activity in irradiated cells, failed to enhance the level of radiation-induced apoptosis. Other kinase inhibitors did not lead to an additional increase in apoptosis over and above that observed after irradiation. The results obtained here provide further support for an important role for modification of existing proteins during radiation-induced apoptosis.

Key words: programmed cell death, enzyme activity, DNA fragmentation, ionizing radiation, lymphoid cells

Evidence is accumulating for a role for phosphorylation/dephosphorylation of existing proteins in the process of apoptosis. Phorbol esters that activate protein kinase C (PKC) prevent DNA fragmentation and cell death in murine and human cells [McConkey et al., 1989; Rodriguez-Tarduchy et al., 1989]. Apoptosis induced by either heat treatment or by ionizing radiation exposure is accompanied by dephosphorylation of a limited number of specific proteins in human BM13674 cells [Baxter and Lavin, 1992], and okadaic acid, an inhibitor of protein phosphatases 1 and 2A [Fujiki et al., 1990] prevented apoptosis induced by either agent in these cells and the dephosphorylation of some proteins. We have recently extended these studies to show that okadaic acid prevented apoptosis induced by a variety of other damaging agents in both BM13674 cells and in cells from patients with leukemia [Song et al., 1992]. Furthermore, calyculin A, another specific inhibitor of phosphatases 1 and 2A, prevented apoptosis induced by ionizing radiation in BM13674 cells [Song and Lavin, 1993]. These results suggest that an interplay of phosphatase and kinase activities is of central importance in apoptosis in this system.

Ionizing radiation, an agent shown to cause apoptosis in different cell types, has been demonstrated to activate both a PKC-dependent signalling pathway [Hallahan et al., 1991] as well as a protein tyrosine–kinase cascade, which triggers apoptosis [Uckun et al., 1992]. While there is general agreement that apoptosis is an active process of cell death, the relative importance of de novo protein synthesis and modification of pre-existing proteins remains unclear. A requirement for RNA and protein synthesis has been demonstrated in murine thymocytes undergoing apoptosis [Wyllie et al., 1980; Duke and Cohen, 1986; McConkey et al., 1988]. On the other hand, inhibitors of macromolecular synthesis fail to arrest the process of apoptosis in human cells [Martin et al., 1990; Sellins and Cohen, 1987; Green and Cotter, 1993]. Indeed, in some cases, compounds such as cycloheximide and actinomycin D cause an induction of

Received November 22, 1993; accepted May 9, 1994.

Address reprint requests to Dr. Martin Lavin, Queensland Institute of Medical Research, Bancroft Centre, 300 Herston Road, Brisbane, Queensland, Australia 4029.

apoptosis [Baxter et al., 1989; Ijiri and Potten, 1987]. Specific gene expression has also been shown to be associated with or required for apoptosis [Owens et al., 1991; Shi et al., 1992; Strasser et al., 1992]. These data suggest that several pathways for apoptosis exist and in some cases all of the factors required are already present in the cell.

The purpose of the present study was to investigate the activity of various kinases in radiation-induced apoptosis and to examine the effects of inhibitors of these protein kinases on ionizing radiation-induced apoptosis in human cells.

MATERIALS AND METHODS

Reagents

Isoquinolinesulfonamide (H-89), calphostin C, and genistein were purchased from Calbiochem. KN-62, CKI-7, W-7, ML-9, and H-7 were kindly provided by Professor Hiroyoshi Hidaka, Nagoya University School of Medicine. Peptide substrate (P,L,R,R,T,L,S,V,A-NH_2) for calcium calmodulin-dependent protein kinase II (CaMKII) was purchased from Auspep (Parkville, Australia). α-Casein, myelin basic protein and Na_3VO_4 were purchased from Sigma. Polyclonal antibodies against the regulatory subunits of protein kinase A (PKA) were a gift from Dr. Tore Jahnsen, University of Oslo, Norway. Matrix-associated protein (MAP) kinase antibody was purchased from Pharmingen (San Diego, CA).

Cell Culture

A spontaneous Epstein-Barr virus negative Burkitt's lymphoma cell line, BM13674, was used in this study. These cells were established in this Institute by Professor Denis Moss. Cells were grown in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (FCS) at 37°C under 5% CO_2 in a humidified atmosphere. Exponentially growing cells were used for all experiments at a plating density of 1×10^6 cells/ml. Cell viability was determined using the MTT assay at a cell concentration of 1×10^6 cells/ml [Carmichael et al., 1987].

Detection of Apoptosis

Cell morphology was evaluated using microscopy. At the end of each incubation, cells were pelleted at 200*g* for 5 min and resuspended in fresh RPMI-1640 medium containing 10% FCS. The extent of apoptosis was determined using light microscopy with acridine orange as the number of cells undergoing micronuclear fragmentation and condensation.

Preparation of Cell Lysates

For cyclic AMP-dependent PKA, PKC, CaMKII, casein kinases I and II, and $pp60^{c\text{-}src}$ assays, BM13674 cells were lysed in a buffer containing 20 mM Hepes, (pH 7.5), 0.2 mM EDTA, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 0.5% NP40. After incubating on ice for 10 min, the lysate was centrifuged at 12,000*g* for 10 min (4°C), and the supernatant was retained for the assay.

For PKC assays, 0.4 mM EGTA was added to the lysis buffer (see above) without the detergent, and cells were sonicated for 10 s with a Branson 250 sonifier (Duty cycle 30%, Output 2.5). The cell lysate was diluted with the lysis solution without EGTA and centrifuged at 12,000*g* for 10 min and the supernatant used in the assay.

To determine the MAP kinase activity BM13674 cells were resuspended in an extraction buffer containing 50 mM β-glycerophosphate (pH 7.3), 1.5 mM EGTA, 0.1 mM Na_3VO_4, 1 mM DTT, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotonin, 2 μg/ml pepstatin A. The cells were vortexed vigorously and kept on ice for 10 min. Following incubation the cell lysate was frozen, thawed once, and centrifuged at 12,000*g* for 10 min. The supernatant was employed in the assay.

Enzyme Assays

Kinase activities were assayed at 30°C for 5 min by measuring the transfer of ^{32}P from ATP[γ-^{32}P] to different substrates. PKA was assayed in a reaction mixture containing 50 mM Tris–HCl (pH 7.0), 10 mM magnesium acetate, 2 mM EGTA, 1–50 μM cAMP or absence of cAMP (as control), 500 μg/ml histone H2B, 4 μM ATP [γ-^{32}P] (50 Ci/mmol). PKC activity was assayed in a reaction mixture containing 40 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$, 10 mM DTT, 1 mM $CaCl_2$, or 1 mM EGTA (as control), 100 μg/ml phosphatidylserine, 500 μg/ml histone H_1, 5–10 μM ATP [γ-^{32}P] (50–100 Ci/mmol). Casein kinase I and II activities were assayed in a reaction mixture containing 50 mM β-glycerophosphate (pH 7.0), 10 mM magnesium acetate, 0.5 mM EGTA 1 mM DTT, 4 mg/ml α-casein or absence of casein (as control) and 10 μM ATP [γ-^{32}P] (22 Ci/mmol). CaMKII activities were

assayed in a reaction mixture containing 35 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mg/ml calmodulin or absence of calmodulin (as control), 0.1 mM peptide substrate (P, L, R, R, T, L, S, V, A-NH_2), 10 μM ATP (22 Ci/mmol). The incubations for these assays were performed at 30°C for 5 min, except that for CaMKII, which was carried out for 2 min.

MAP kinase activities were assayed in a reaction mixture containing 50 mM β-glycerophosphate (pH 7.3), 1.5 mM EGTA, 0.1 mM Na_3VO_4, 1 mM DTT, 10 μM calmidizolium, 10 mM $MgCl_2$, 3 μM H-89, 0.33 mg/ml myelin-basic protein or absence of myelin basic protein (as control), 0.1 mM ATP [γ-^{32}P] (0.4 Ci/mmol). The incubation was carried out at 30°C for 20 min.

$pp60^{c\text{-}src}$ protein tyrosine kinase activity was determined in a solid-phase assay as described by Presek et al. [1988], except that protein G–Sepharose was used instead of ar-Sepharose suspension. The reaction was carried out at 30°C for 20 min.

The phosphotransferase reactions contained 0.5–1.5 mg/ml of cell lysate protein. The reactions were terminated either by TCA precipitation or by spotting onto phosphocellulose paper strips (Whatman P81). In the case of TCA precipitation 1 ml of ice-cold 20% TCA and 100 μg of BSA as a carrier protein were added. The samples were centrifuged at 12,000*g* for 15 min. The pellet was washed twice with 10% and once with 5% TCA solutions. The final pellet was dissolved in 1 ml of 1 N NaOH. The phosphocellulose paper strips were washed once in 10 mM phosphoric acid, twice in 5 mM phosphoric acid, briefly rinsed in water and ethanol and air dried. Radioactivity was measured by scintillation counting.

Western Blotting

BM13674 cells were resuspended in a lysis buffer containing 20 mM Hepes (pH 7.5), 0.2 mM EGTA, 10 μg/ml leupeptin, and 1 μl aprotinin. The cell lysate was sonicated for 15 s and centrifuged at 12,000*g* for 10 min (4°C). The supernatant was taken as the cytoplasmic fraction. The pellet was dissolved in the same lysis buffer containing 0.5% NP40 and incubated on ice for 10 min. Following 5-s sonication, the lysate was centrifuged at 12,000*g* for 10 min. The supernatant was taken as the particulate fraction; 75 μg of protein was loaded on a 12% SDS–acrylamide gel. The proteins were blotted on a nitrocellulose membrane in bicarbonate buffer (3 mM Na_2CO_3, 10 mM $NaHCO_3$, 20% methanol, pH 9.9). The transfer was carried out for 16 h at 25-V constant voltage in a BioRad Western blotting apparatus. The ECL chemiluminescent kit from Amersham was used to visualise the immunoreactive bands.

DNA Extraction

DNA was extracted from cells using a modified form of the procedure described by Miller et al. [1988]. Briefly, cells were pelleted by centrifugation at 200*g* for 5 min at room temperature. Cell pellets were resuspended at 2×10^6 cells/ml in cell lysis buffer (10 mM NaCl, 1 mM EDTA, 1% SDS, 10 mM Tris–HCl, pH 7.8) containing 0.5 mg/ml proteinase K. The cell lysates were digested overnight at 37°C. After completion of digestion, saturated NaCl (~6 M) was added and the cell lysates were shaken vigorously. Each tube was then centrifuged at 1,800*g* on a benchtop centrifuge for 15 min. The supernatant was transferred to another tube, and an equal volume of absolute ethanol was added; the tubes were inverted several times until the DNA precipitated. The DNA precipitates were then transferred to 1.5-ml microcentrifuge tubes, washed with 70% ethanol three times, air-dried for 2 h at room temperature, and resuspended in TE buffer (10 mM Tris–HCl, 0.2 mM EDTA, pH 8.0). The DNA was allowed to dissolve for 2 h at 37°C and treated with RNase at a concentration of 0.2 mg/ml before quantitating.

Electrophoresis of DNA

DNA (~10 μg) was loaded into each well and electrophoresis was carried out at 10 mA in 1.3% agarose gels with TBE buffer (89 mM Tris–HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). After electrophoresis, each gel was stained in H_2O containing 2 μg/ml ethidium bromide for 10 min and destained in H_2O for 10 min. The DNA was visualized by UV illumination.

RESULTS

Effect of Irradiation and Inhibitors on Protein Kinase Activities

We have previously shown that BM13674 cells undergo apoptosis after exposure to ionizing radiation and inhibitors of phosphatase activity prevent apoptosis 4–8 h postirradiation [Baxter and Lavin, 1992; Song et al., 1992; Song and Lavin, 1993]. Initially the effects of irradiation on the activities of various protein kinases were

determined. Irradiation caused a time-dependent decrease in the activities of PKA, casein kinases, and pp60^{src}, while an increase was observed with PKC, CaMKII, and MAP kinase activities remained unchanged (Table I). When irradiation was carried out in the presence of calphostin C, a potent and specific inhibitor of PKC [Kobayashi et al., 1989], the activity of the enzyme was inhibited by 68% compared to the irradiated value alone (Fig. 1). Irradiation alone increased PKC activity by 28%. A time-dependent increase in the percentage of apoptotic BM13674 cells was observed under these conditions (Fig. 2). By 6 h postirradiation, approximately 30% of the cells were undergoing apoptosis. The presence of calphostin C did not change the rate or extent of appearance of radiation-induced cell death (Fig. 2).

TABLE I. Effect of Ionizing Radiation on Activities of Various Protein Kinases in Cells Undergoing Apoptosis

	fmole P/min/mg cell lysate		
Enzyme	Untreated cells	30 min after irradiation	4 h after irradiation
PKA	397 ± 76[a]	224 ± 15	260 ± 27
PKC	816 ± 55	1,041 ± 210	1,671 ± 240
Calcium, calmodulin-dependent protein kinase II	2,650 ± 30	4,190 ± 300	3,500 ± 100
Casein kinase I, II	1,380 ± 56	630 ± 19	842 ± 50
MAP kinase	3,520 ± 360	3,720 ± 100	3,417 ± 70
pp60$^{c\text{-}src}$	45 ± 4.7	31 ± 4.5	27 ± 3.9

[a]Mean ±SD for three experiments. The radiation dose used was 10 Gy.

Protein Kinase Inhibitors in Apoptosis

Exposure of BM13674 cells to H89 a selective inhibitor of PKA [Chijiwa et al., 1990] caused a reduction in activity to 34 ± 9% in unirradiated cells and when combined with radiation was reduced to 4.5% of the untreated value (Fig. 3). Cellular PKA activity was found to be sensitive to radiation exposure, being reduced to approximately 56 ± 7% of the untreated value at a dose of 10 Gy (Fig. 3). It is not clear how this radiation dose reduces enzyme activity, but it might be explained by protein modification of the regulatory subunits or change in their expression levels. Under these conditions, a significant enhancement of apoptosis was observed over the period 2–6 h postirradiation (Fig. 4). At 4 h postirradiation, there was a 93% increase in apoptotic cells in the presence of H89 (20 μM); after 6 h, the value increased by 51% over and above that after irradiation. This enhancement of radiation-induced apoptosis was observed over the concentration range 10–30 μM for H89 (Fig. 5), but the extent of apoptosis at longer incubation times was the same in all cases. Thus inhibi-

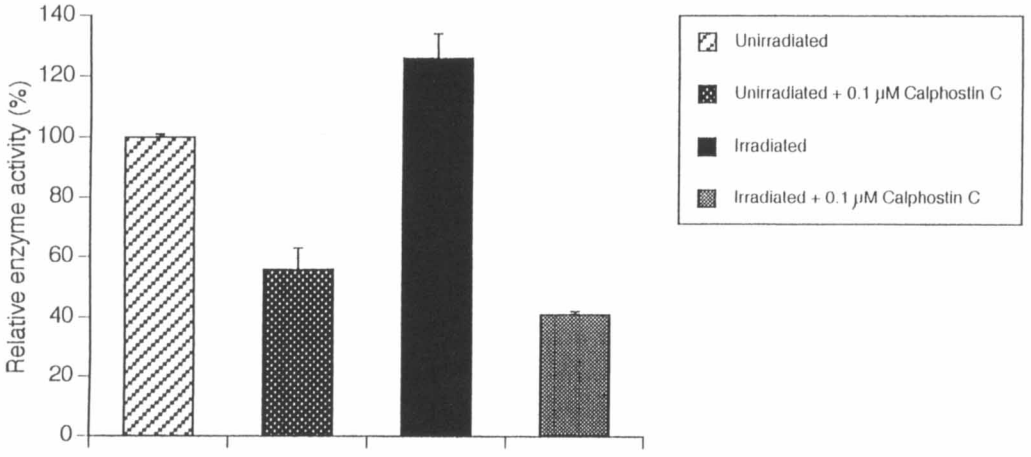

Fig. 1. Inhibition of PKC activity by calphostin C. PKC activity was assayed at 30 min postirradiation, as described under Materials and Methods using Histone H1, as a substrate. The reaction contained 0.5 mg/ml cell lysate. Phosphotransferase activity is expressed as a percentage of the enzyme activity in unirradiated cells in the absence of calphostin C. The radiation dose was 10 Gy.

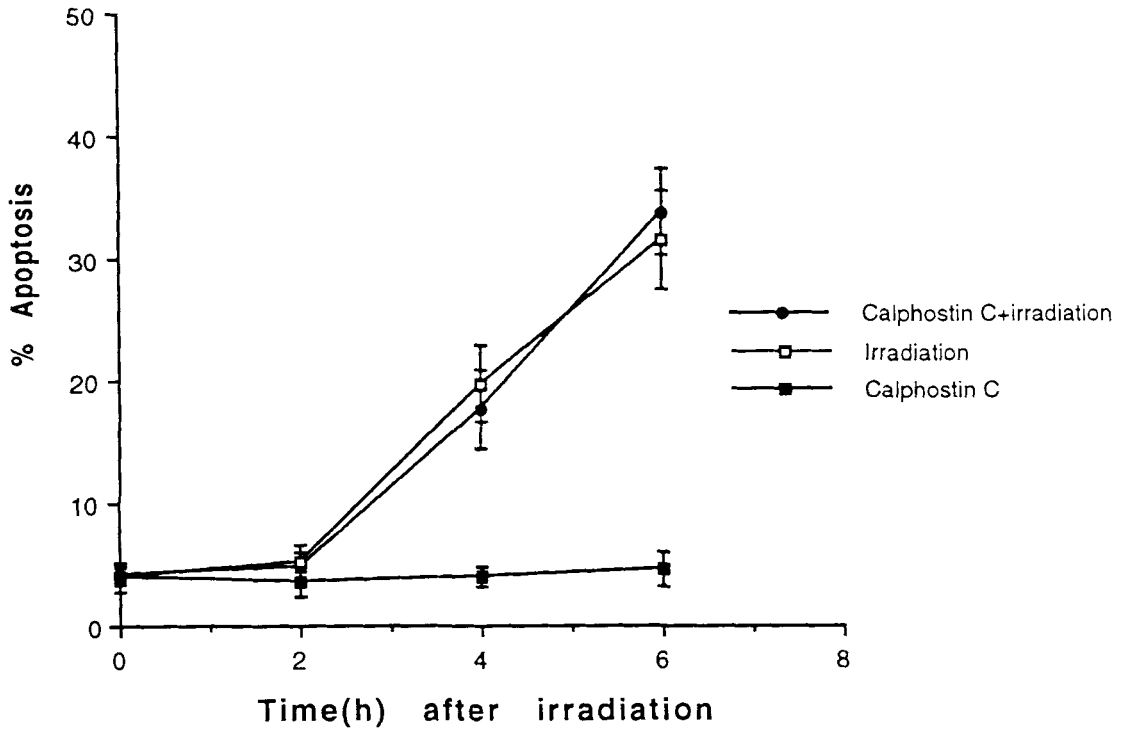

Fig. 2. Effect of calphostin C on γ-radiation induced apoptosis in BM13674 cells. Cells were pretreated with calphostin C (0.1 μM) for 1 h before exposure to γ-radiation (10 Gy). Percentage of apoptotic cells was determined by microcopic examination after 0-, 2-, 4-, and 6-h incubation. The results represent the mean ±SD of three separate experiments.

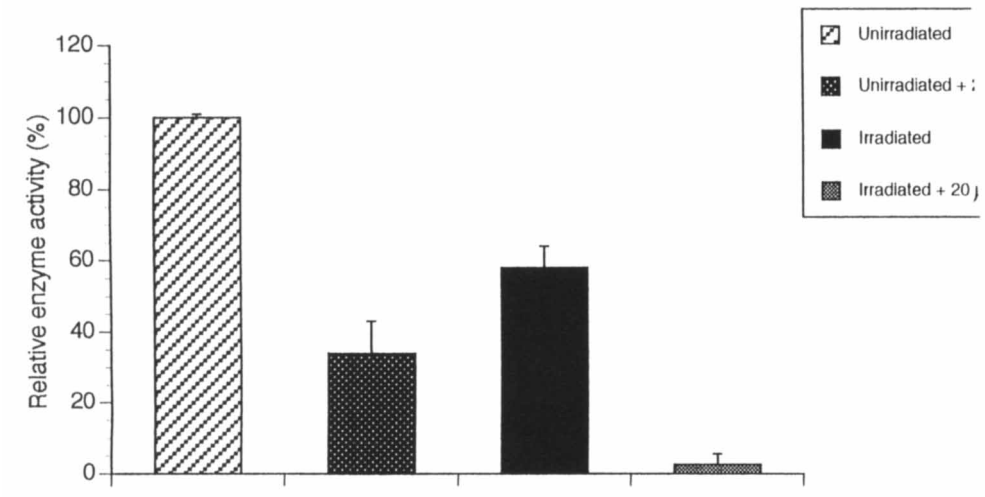

Fig. 3. Inhibition of PKA activity by H-89. PKA activity was determined at 30 mm post-irradiation using histone H2B as a substrate; 0.5 mg/ml cell lysate was employed in the reaction. All other conditions are as described under Materials and Methods. PKA activity is expressed as a percentage of the enzyme activity in unirradiated cells in the absence of H-89. The radiation dose used was 10 Gy.

tion of PKA appears to affect the rate of increase of apoptosis but not the final extent. H89 on its own did not appreciably increase the percentage of apoptotic cells above background levels.

As observed previously, DNA was fragmented into oligonucleosomal sized pieces 4 h after exposure of BM13674 cells to ionizing radiation (Fig. 6, lane 3) and the extent of fragmentation was increased in the presence of H89 (Fig. 6, lane 4). Western blot analysis revealed that the amount of PKA regulatory subunits (RIα and RIIα) in total cell extracts did not change appreciably

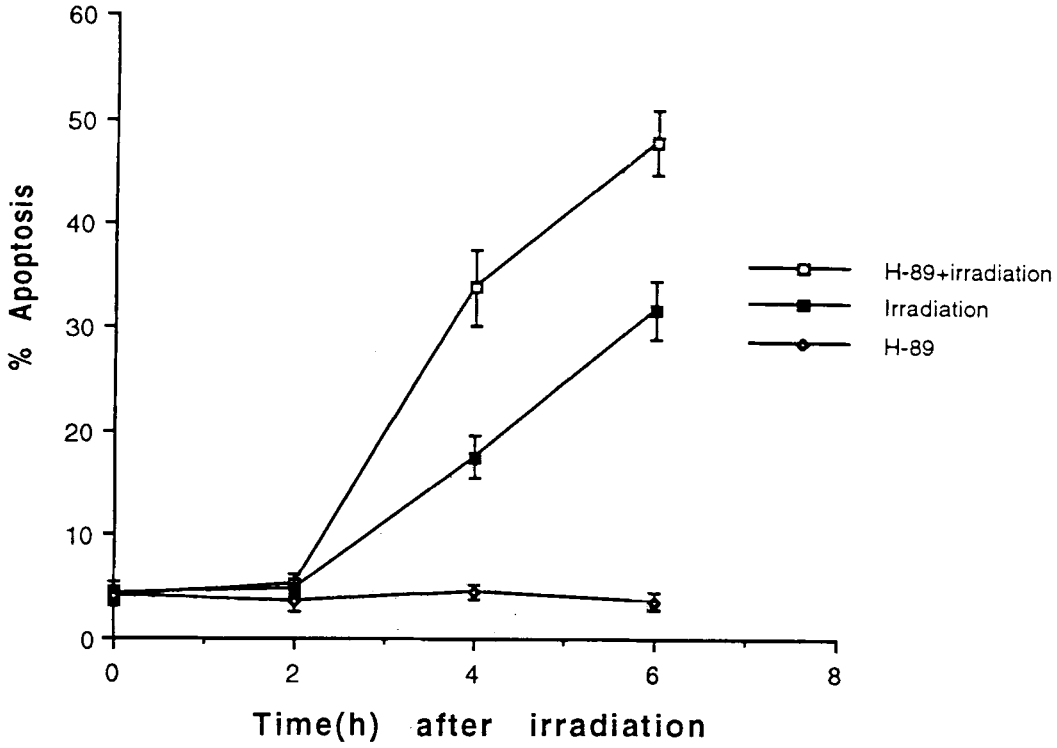

Fig. 4. Effect of H-89 on γ-radiation induced apoptosis in BM13674 cells. Cells were pretreated with H-89 (20 μM) for 1 h before exposure to γ-radiation (10 Gy). Percentage of apoptotic cells was determined by microcopic examination after 0-, 2-, 4-, and 6-h incubation. The results represent the mean ±SD of three separate experiments.

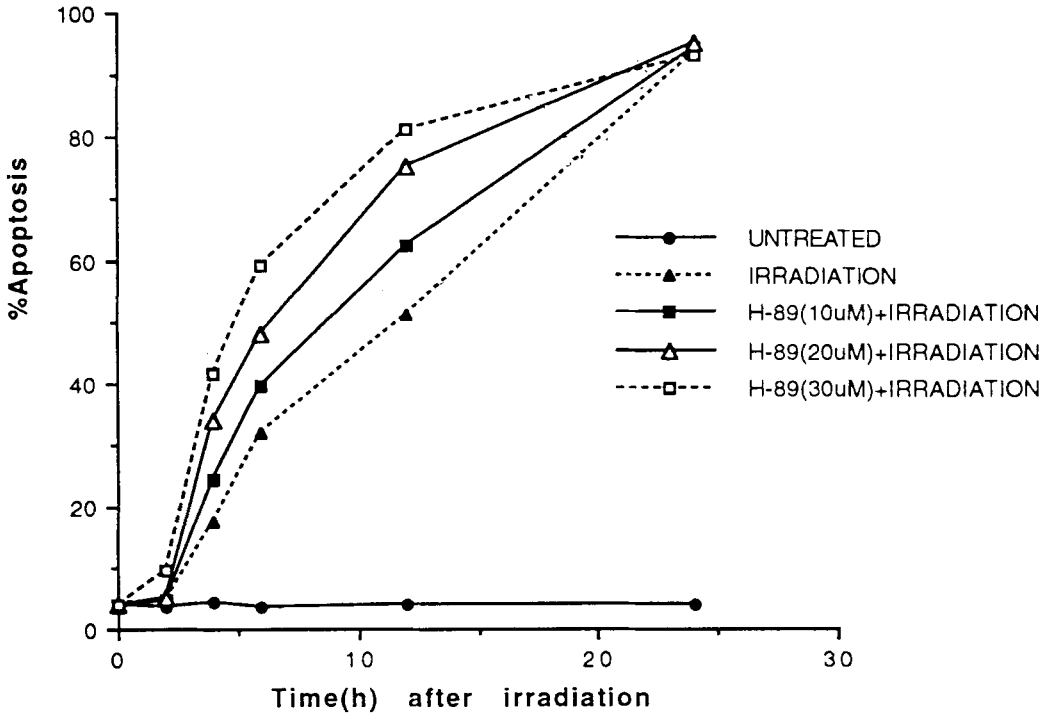

Fig. 5. Effect of increasing concentration of H-89 on rate and extent of apoptosis in BM13674 cells exposed to γ-radiation. Apoptosis was determined as described under Materials and Methods and points represent the means of two separate experiments.

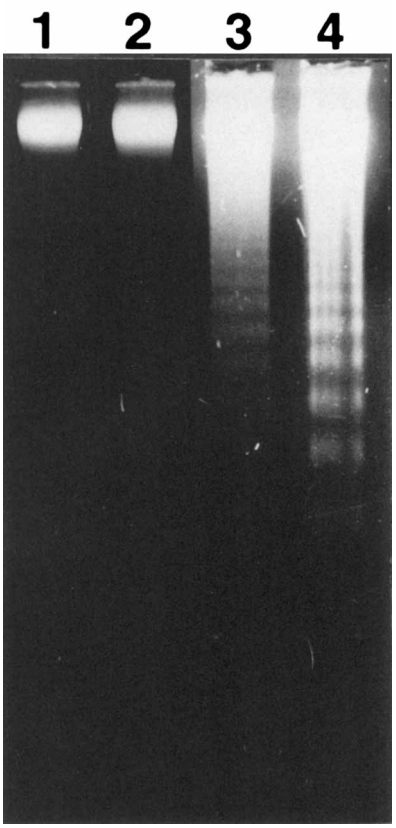

Fig. 6. Effect of H-89 on γ-radiation induced DNA fragmentation in BM13674 cells. Cells were treated to induced apoptosis with γ-radiation (10 Gy) in the presence or absence of H-89 (20 μM). DNA was extracted and electrophoresed in 1.3% agarose gels, after 4-h incubation. Lane 1, untreated; lane 2, untreated + H-89; lane 3, γ-radiation; lane 4, H-89 + γ-radiation.

during the process of radiation-induced apoptosis. This was also confirmed by examining particulate and cytosolic fractions separately (results not shown). We also employed another inhibitor, the isoquinolinesulfonamide H7 which inhibits both PKA and PKC. PKA activity was inhibited by 98% at 6 μM and PKC activity was reduced by 37% in irradiated cells (Table II). At 6 μM H7, apoptosis was significantly enhanced in irradiated cells, an increase of 108% above irradiation only (Fig. 7). A number of other protein kinase inhibitors were also employed. Each reduced the activity of the target enzyme but failed to accentuate the amount of apoptosis induced by ionizing radiation (Table II; Fig. 8).

No effect of calmodulin on extent of apoptosis was observed in BM13674 cells, and the inhibition of tyrosine phosphatases with Na_3VO_4 did not influence the rate of radiation induced apoptosis in these cells (Table II).

DISCUSSION

The results described here provide further support for a role for pre-existing proteins in apoptosis. Failure of inhibitors of RNA and protein synthesis to prevent apoptosis induced by different agents in human cells [Baxter et al., 1989; Martin et al., 1990] raised the possibility that the full complement of factors necessary for apoptosis was already present in these cells. In the absence of protein synthesis a strong possibility was that the response was mediated by post-translational change such as phosphorylation/dephosphorylation, which are the major modifying influences in signaling pathways controlling cell growth and differentiation [Cohen, 1992].

It is clear that the process of apoptosis is complex involving several pathways which may be interactive or antagonistic. While McConkey et al. [1989] showed that PKC activation was important in preventing apoptosis in mouse thymocytes we did not find evidence for protection by TPA in the cell line used in this study [Baxter and Lavin, 1992]. Failure to exacerbate the extent of radiation-induced apoptosis by inhibitors of PKC in this study are supportive of the TPA data. The inhibitor calmidazolium also inhibits Ca^{2+} ATPase activities at 5 μM [Fischer et al., 1987]. Ca^{2+} ATPase is a transporter that facilitates transport of Ca^{2+} against the Ca^{2+} concentration gradient and is responsible for maintaining $[Ca^{2+}]$ near the basal value [Barritt, 1992], and Ca^{2+} is a positive regulator of PKC. The fact that calmidazolium had no effect on apoptosis in our cell lines is further proof that PKC is not involved in the apoptotic pathway in BM13674 cells. Dowd et al. [1991] have also shown that steady-state levels of calmodulin mRNA were increased up to 10-fold following a 4–6-h exposure of WEHI7.2 cells to 10^{-6} M dexamethasone and calmodulin inhibitors interfered with the death pathway. Various inhibitors of CaMKII and W-7 an inhibitor of calmodulin did not change the levels of apoptosis in BM13674 cells. Uckun et al. [1992] have reported that tyrosine phosphorylation appears to be an important proximal step in radiation-induced apoptosis since inhibitors of protein tyrosine kinases prevent apoptosis. Na_3VO_4, an inhibitor of tyrosine phosphatases, and genistein, an inhibitor of protein tyrosine kinases, had no influence on radiation-induced apoptosis in BM13674 cells. Further evidence for a multiplicity of pathways is

TABLE II. Effect of Inhibitors on Radiation-Induced Apoptosis*,†

Inhibitor	Concn.	Target	Enzyme activity in irradiated cells (%)	Increase in radiation-induced apoptosis
Calmidazolium	10 μM	Ca^{2+}/calmodulin-dependent protein kinase II	2	–
KN-62	5 μM	Ca^{2+}/calmodulin-dependent protein kinase II	73	–
CKI-7	50 μM	Casein kinase I	55	–
ML-9	50 μM	Myosin light chain kinase	ND	–
W-7	30 μM	Calmodulin		–
H-7	6 μM	PKA	2	+
		PKC	63	
$Na_3V_{O_4}$	100 μM	Tyrosine phosphatases	—	–
Genistein	30 μg/ml	Tyrosine kinases ($pp60^{c\text{-}src}$)	71	–

*The radiation dose used was 10 Gy; determinations were made 4 h postirradiation.
†KN-62, 1-[$n_3$0-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl- 4-phenylpiperazine; W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; CKI-7, N-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide; ML-9, 1-(5-chloronaphthalenesulfonyl)-1H-hexahydro-1,4-diazepine; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine.

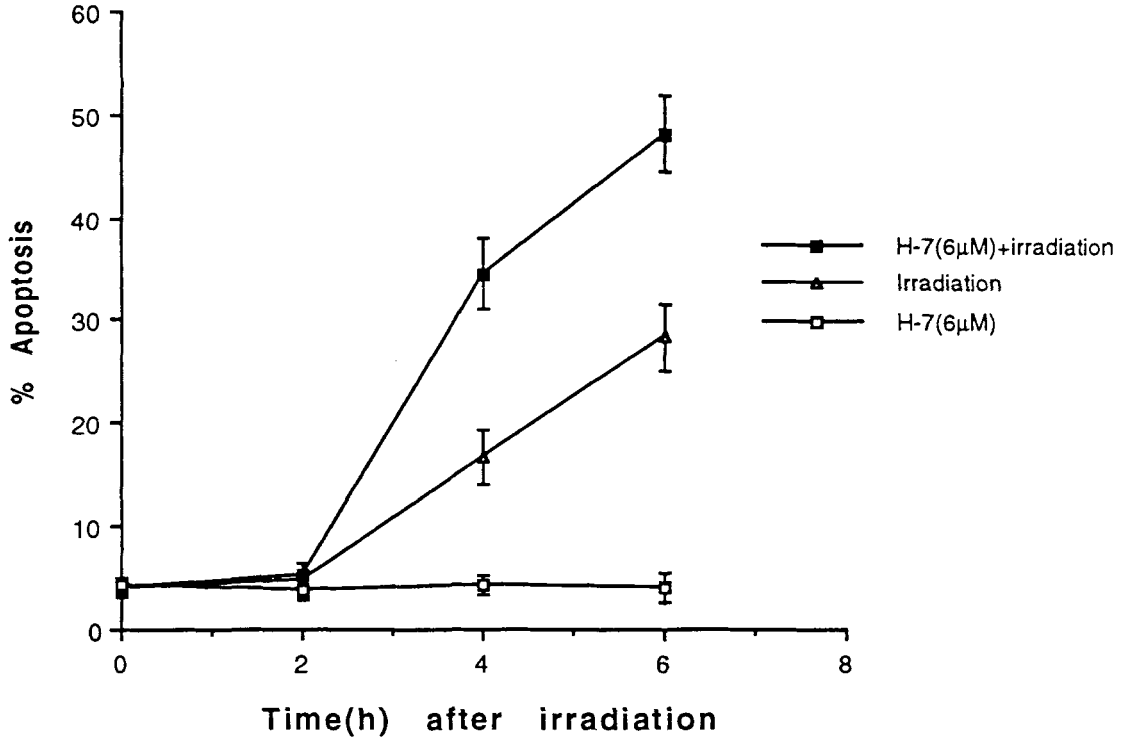

Fig. 7. Effect of H-7 on γ-radiation-induced apoptosis in BM13674 cells. Cells were pretreated with H-7 for 1 h before exposure to γ-radiation (10 Gy). Percentage of apoptotic cells was determined by microcopic examination after 0-, 2-, 4-, and 6-h incubation. The results represent the mean ±SD of three separate experiments.

derived from the results with rat thymocytes where increased levels of cAMP, by activating PKA, stimulate DNA fragmentation characteristic of apoptosis [McConkey et al., 1990]. On the contrary, in BM13674 cells inhibition of PKA caused an enhancement of radiation-induced apoptosis. Furthermore, forskolin failed to cause apoptosis or enhance the extent of apoptosis in BM13674 cells exposed to heat or ionizing radiation [Baxter and Lavin, 1992]. Our results suggest that radiation-induced apoptosis proceeds regardless of the status of PKA since inhibition of PKA on its own does not result in apoptosis. However, when PKA is reduced in irradiated cells, an additional component of protection is removed, resulting in increased levels of apopto-

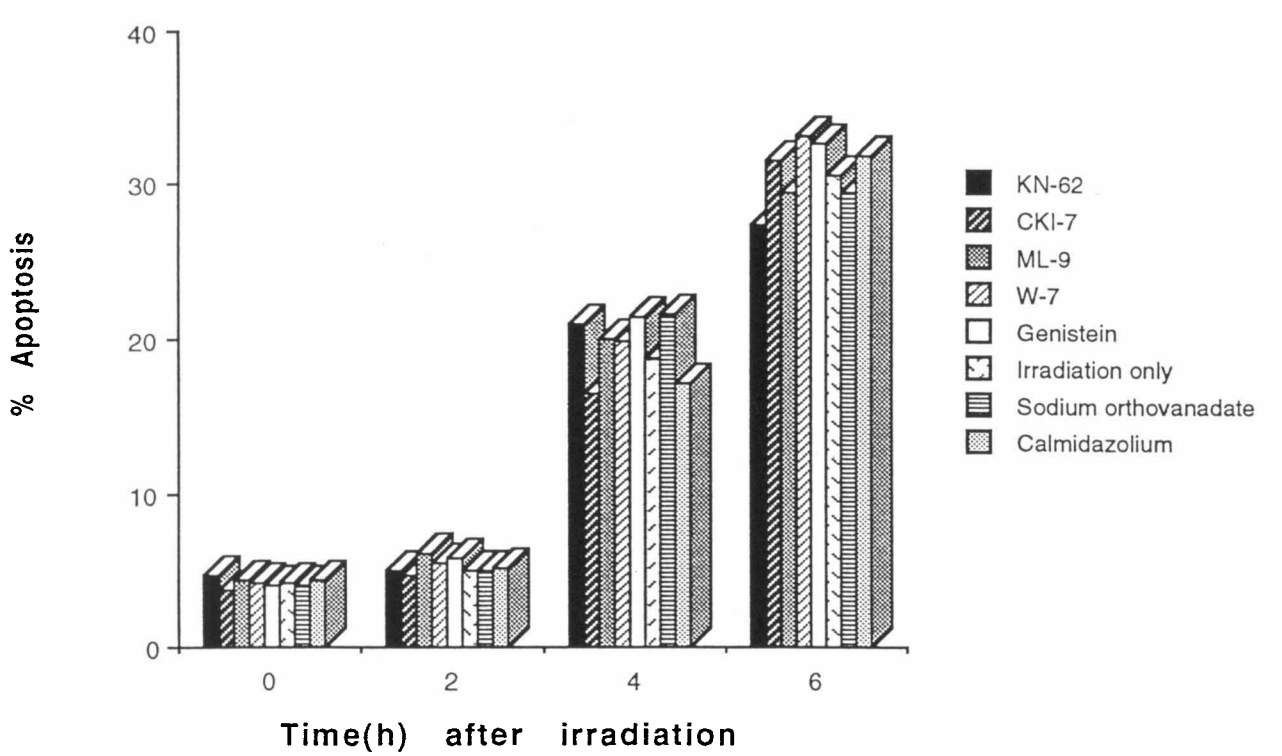

Fig. 8. Effect of a variety of inhibitors on γ-radiation induced apoptosis in BM13674 cells.

sis. This suggests that a residual level of PKA activity has an opposing effect to a radiation driven pathway of apoptosis, and, given the observations described here and by Baxter and Lavin [1992], increasing the level of PKA with cAMP or forskolin would not be expected to alter the process. The results obtained here are in good agreement with those supporting a role for serine/threonine phosphatases in apoptosis [Baxter and Lavin, 1992; Song and Lavin, 1993], since PKA regulates the activity of these phosphatases by phosphorylating targeting subunits and specific inhibitors [Cohen, 1992]. However, since it is likely that several pathways are involved in apoptosis, the generality of these observations requires testing in other cell lines.

The overall picture emerging is that apoptosis can be induced without de novo protein synthesis in human cells and protein kinases and phosphatases participate in the pathways involved. The signal transduction pathways are complex and display differences between various cell types and tissues. This might explain the different effects seen at the gene regulation and posttranscriptional levels by the same agents causing apoptosis in these systems. In the study described here ionizing radiation was the inducing agent. Hallahan et al. [1991] and Uckun et al. [1992, 1993] have shown that tyrosine phosphorylation is an important proximal step in the radiation-induced activation of the PKC signaling cascade in lymphocyte precursors. It appears that ionizing radiation triggers one or more tyrosine kinases that initiate a cascade involving serine/threonine kinases and ultimately changes at the level of DNA. There is also evidence for the activation of a phosphatase in *Schizosaccharomyces pombe,* which after exposure to ionizing radiation appears to activate weel kinase to bring about mitotic delay by phosphorylating cdc2 kinase [Rowley et al., 1992]. No doubt there exist parallels between the radiation-activated pathways in cells undergoing apoptosis and in those where death is characterized by more necrotic type changes. A more detailed understanding of radiation activation of existing proteins as well as gene induction will be of value not only in describing mechanisms of radiosensitivity but also in the changes that occur during apoptosis.

ACKNOWLEDGMENTS

The first two authors made equal contributions to the work carried out in this manuscript. We thank the Queensland Cancer Fund and the University of Queensland Cancer Research Fund for generous support. We thank Dr. Tore

Jahnsen for the antibodies against the regulatory subunits of PKA. We also thank Dr. Peter Presek for TBR antisera and Dr. Susan Hamilton for her advice regarding PKC assays. Mrs. Lu Hong and Mr. Steven Harrison are also acknowledged for assistance with some of the experimental work. Thanks to Hazel Dunn, Philippa Cox, and Tracey Stimson for typing the manuscript.

REFERENCES

Barritt GJ (ed) (1992): "Communication Within Animal Cells." New York: Oxford University Press, pp 193–229.

Baxter GD, Lavin MF (1992): Specific protein dephosphorylation in apoptosis induced by ionizing radiation and heat shock in human lymphoid tumour lines. J Immunol 148: 1949–1954.

Baxter GD, Collins RJ, Harmon BV, Prentice RL, Smith PJ, Lavin MF (1989): Cell death by apoptosis in acute leukaemia. J Pathol 158:123–129.

Carmichael J, De Graff WG, Gazdar AF, Minna JD, Mitchell JB (1987): Evaluation of a tetrazolium-based semiautomated colormetric assay: Assessment of radiosensitivity. Cancer Res 47:943–946.

Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T, Hidaka H (1990): Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[Z-p-Bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide (H-89), of PC12D phenochromocytoma cells. J Biol Chem 265:5267–5272.

Cohen P (1992): Signal integration at the level of protein kinase, protein phosphatases and their substrates. TIBS 17:408–413.

Dowd DR, MacDonald PN, Komm BS, Haussler MR, Miesfeld R (1991): Evidence for early induction of Calmodulin gene expression in lymphocytes undergoing glucocorticoid-mediated apoptosis. J Biol Chem 266:18423–18426.

Duke RC, Cohen JJ (1986): IL-2 addiction: Withdrawal of growth factor activates a suicide program in dependent T cells. Lymph Res 5:289–299.

Fischer TH, Campbell KP, White GC (1987): An investigation of functional similarities between the sarcoplasmic reticulum and platelet Calcium-dependent adenosine triphosphatases with the inhibitors quercetin and calmidazolium. Biochemistry 26:8024–8030.

Fujiki H, Suganuma M, Nishiwaki S, Yoshizawa S, Winger B, Sugimura T, Schmitz FJ (1990): A new pathway of tumor promotion by the okadaic acid class compounds. Adv Second Messenger Phosphoprotein Res 24:340–344.

Green DR, Cotter TG (1993): Macromolecular synthesis, c-myc and apoptosis. In Lavin M, Watters D (eds): "Programmed Cell Death: The Cellular and Molecular Basis of Apoptosis." Chur: Switzerland: Harwood Academic Publishers, pp 153–166.

Hallahan DE, Sukhatme VP, Sherman ML, Virudachalam S, Kufe D, Weichselbaum RR (1991): Protein kinase C mediates x-ray inducibility of nuclear signal transducers EGRI and JUN. Proc Natl Acad Sci USA 88:2156–2160.

Ijiri K, Potten CS (1987): Further studies on the response of intestinal crypt cells of different hierarchical status to eighteen different cytotoxic agents. Br J Cancer 55:113–123.

Kobayashi E, Nakano H, Morimoto M, Tamaoki T (1989): Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. Biochem Biophys Res Commun 159:548–553.

McConkey DJ, Hartzell P, Duddy SK, Hakansson H, Orrenius S (1988): 2, 3, 7, 8 tetrachlorodibenzo-p-dioxin (TCDD) kills immature thymocytes by Ca^{2+} mediated endonuclease activation. Science 242:256–259.

McConkey DJ, Hartzell P, Jondal M, Orrenius S (1989): Inhibition of DNA fragmentation in thymocytes and isolated thymocyte nuclei by agents that stimulate protein kinase C. J Biol Chem 264:13399–13402.

McConkey DJ, Orrenius S, Jondal M (1990): Cellular signalling in programmed cell death (apoptosis). Immunol Today 11:120–121.

Martin SJ, Lennon SV, Bonham AM, Cotter TG (1990): Induction of apoptosis (programmed cell death) in human leukaemic HL-60 cells by inhibition of RNA or protein synthesis. J Immunol 145:1859–1867.

Miller A, Dykes DD, Polesky HH (1988): A Simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215.

Owens GP, Hahn WE, Cohen JJ (1991): Identification of mRNAs associated with programmed cell death in immature thymocytes. Mol Cell Biol 11:4177–4188.

Presek P, Reuter C, Findik D, Bette P (1988): High-yield purification of a $pp60^{c\text{-}src}$ related protein-tyrosine kinase from human platelets. Biochim Biophys Acta 969:271–280.

Rodriguez-Tarduchy G, Lopez-Rivas A (1989): Phorbol esters inhibit apoptosis in IL-2 dependent T lymphocytes. Biochem Biophys Res Commun 164:1069–1075.

Rowley R, Hudson J, Young PG (1992): The wee 1 protein kinase is required for radiation-induced mitotic delay. Nature 356:353–355.

Sellins KS, Cohen JJ (1987): Hyperthermia induces apoptosis in thymocytes. J Immunol 139:3199–3206.

Shi Y, Szalay MG, Paskar L, Boyer M, Singh B, Green DR (1992): Role of c-myc in activation induced apoptotic cell death in T cell hybridomas. Science 257:212–214.

Song Q, Baxter GD, Kovacs EM, Findik D, Lavin MF (1992): Inhibition of apoptosis in human tumour cells by okadaic acid. J Cell Physiol 153:550–556.

Song Q, Lavin MF (1993): Calyculin A, a potent inhibitor of phosphatase 1 and 2A prevents apoptosis. Biochem Biophys Res Commun 190:47–55.

Strasser A, Harris AW, Cory S (1992): bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. Cell 67:889–899.

Uckun FM, Tuel-Ahlgren L, Song CW, Waddick K, Myers DE, Kirihara J, Ledbetter JA, Schieven GL (1992): Ionizing radiation stimulates unidentified tyrosine-specific protein kinases in human B-lymphocyte precursors, triggering apoptosis and clonogenic cell death. Proc Natl Acad Sci USA 89:9005–9009.

Uckun FM, Schieven GL, Tuel-Ahlgren LM, Dibirdik I, Myers DE, Ledbetter JA, Song CW (1993): Tyrosine phosphorylation is a mandatory proximal step in radiation-induced activation of the protein kinase C signaling pathway in human B-lymphocyte precursors. Proc Natl Acad Sci USA 90:252–256.

Wyllie AH, Kerr JFR, Currie AR (1980): Cell death: The significance of apoptosis. Int Rev Cyt 68:251–305.