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Elevation of Cytoplasmic Cytochrome *c* in Radiation-induced Apoptosis

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The signal transduction pathway involved in radiation-induced apoptosis remains unclear, especially as regards the site at which the primary effect of radiation occurs. In this study, we demonstrate that cytochrome *c* may be released from mitochondria into the cytosol after irradiation, but that direct irradiation of isolated mitochondria induces no elevation of cytochrome *c* release. These observations suggest that cytochrome *c* and mitochondria may be involved in the radiation-induced apoptotic pathway, but mitochondria might not be a target of radiation, and that a signal transduction pathway from an unknown target might exist upstream of mitochondria during radiation-induced apoptosis.

Key words: Apoptosis — Radiation — Cytochrome *c* — Mitochondria — HL-60

Apoptosis is a mode of cell death characterized by distinct morphological features and DNA fragmentation.[1)] Many types of cell death proceed via apoptosis, e.g., cell death in the context of cytotoxic T lymphocyte killing,[2)] the ligation of Fas receptors,[3)] after γ- or UV irradiation,[4,5)] and after exposure to cytotoxic drugs[6)] and many other stimuli.[7)] Some cell lines die without apoptotic morphological change after irradiation, but radiation-induced apoptosis is believed to be one of the major mechanisms of radiation-induced cell death.

Multiple lines of evidence indicate that apoptosis can be triggered by activation of caspases.[8)] Caspase-3, previously called CPP/Yama/Apopain, normally exists in the cytosolic fraction of cells as a 32 kDa inactive precursor that is converted proteolytically to a 20 kDa and 11 kDa active heterodimer when cells are signaled to undergo apoptosis.[9)] Activated caspase-3 cleaves the p115 death substrate poly(ADP-ribose)polymerase (PARP) to a specific 85 kDa form observed during apoptosis.[9)] Multiple apoptotic signals, including ionizing radiation, activation of Fas, treatment with granzyme B, and a variety of pharmacological agents, activate caspase-3.[9–13)] Addition of active caspase-3 to normal cytosol activates the apoptotic program.[14)] In a cell-free experimental system, cytosolic caspase-3 was cleaved and activated in a reaction that was first triggered by cytochrome *c*; then activated caspase-3 interacted with another cytosolic protein, DFF, inducing DNA fragmentation when added to isolated nuclei.[15–17)] This suggests that while characteristic events of apoptosis occur in the nucleus, the apoptotic program may be predominantly extranuclear and a direct signal transduction pathway may exist during apoptosis: cytochrome *c* to caspase-3 to DFF to DNA fragmentation.[17)]

As for radiation-induced apoptosis, we have demonstrated in a previous study that caspase-3 in HL-60 cells is activated after irradiation and that cytoplasmic extract prepared from irradiated cells has the ability to cause DNA fragmentation in a cell-free experimental system.[18)] But proof of cytochrome *c* being involved was lacking. Whether mitochondria and cytochrome *c* are involved in the radiation-induced apoptotic pathway remains to be clarified.

In the current study, we demonstrate that cytochrome *c* is released from mitochondria into the cytoplasm in HL-60 cells after irradiation, though direct irradiation of isolated mitochondria from HL-60 cells results in no obvious change in the level of released cytochrome *c*. These observations suggest that cytochrome *c* and mitochondria are involved in the radiation-induced apoptotic pathway, but mitochondria may not be the site at which the primary effect of radiation occurs.

MATERIALS AND METHODS

Anti-caspase-3 and anti-PARP antibodies used in this study were goat polyclonal antibodies reacting with the p11 subunit of caspase-3 of human origin and PARP of human origin, respectively. These antibodies and donkey anti-goat antibody conjugated with horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-cytochrome *c* antibody (clone 7H8.2C12) was purchased from Pharmingen (San Diego, CA) and sheep anti-mouse antibody conjugated with HRP was purchased from Amersham (Buckinghamshire, England).

Experiments on all samples were performed identically 3 times.

Cell culture and induction of apoptosis HL-60 and HeLa S3 cell lines were used in this study because apoptosis is known to be induced by irradiation in HL-60 cells, but not in HeLa S3 cells. Both cell lines were maintained in α-MEM containing 10% fetal bovine serum, 5

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U/ml penicillin G and 5 μg/ml streptomycin at 37°C in 2% CO_2. To induce apoptosis, HL-60 cells were exposed to 30 Gy of ^{137}Cs γ-irradiation (1.115 Gy/min; gammacell 40, Atomic Energy of Canada, Ltd., Canada), followed by incubation in the same medium for various time periods. All experiments were conducted when these cells were in an exponential growth phase.

DNA fragmentation assay One million irradiated HL-60 cells and HeLa S3 cells were lysed in TE buffers (50 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA) containing 0.5% sodium lauroyl sarkosyl and 0.5 mg/ml proteinase K, followed by incubation at 50°C for 1 h. After the addition of RNase A to 0.1 mg/ml, lysates were incubated at 50°C for 1 h. One-sixth volume of running dye (10 m*M* EDTA, 0.25% bromophenol blue, 50% glycerol) was added and then lysates were subjected to 2% agarose gel electrophoresis. Separated DNA was stained with ethidium bromide and observed on a UV transilluminator.

Preparation of cytoplasmic extracts Cytoplasmic extracts were prepared as described previously.[19] Briefly, both HL-60 cells and HeLa S3 cells were incubated for various time periods after radiation treatment. Then the cells were washed twice with 15 ml of phosphate-buffered saline (PBS; 137 m*M* NaCl, 2.7 m*M* KCl, 8 m*M* Na_2HPO_4 and 1.5 m*M* KH_2PO_4) and washed with 5 ml of cell extract buffer (CEB; 50 m*M* piperazine-*N*, *N*′-bis(2-ethanesulfonic acid) (PIPES), pH 7.4, 50 m*M* KCl, 2 m*M* $MgCl_2$, 1 m*M* dithiothreitol (DTT), 5 m*M* ethylene glycol bis(2-aminoethyl ether)tetraacetic acid (EGTA), 10 μ*M* cytochalasin B and 1 m*M* phenylmethanesulfonyl fluoride (PMSF)). They were resuspended in a volume of CEB equal to that of the cell pellet and transferred to a Dounce homogenizer, followed by incubation on ice for 20 min, then gentle lysis (10–20 strokes). The cell lysate was transferred to a 1 ml Eppendorf tube and centrifuged at 1400*g* for 15 min at 4°C. The cytoplasmic extract was removed with care to avoid contamination from the nuclear pellet and diluted to 7.5–15.0 mg/ml with extract dilution buffer (EDB; 10 m*M* HEPES, pH 7.4, 50 m*M* NaCl, 2 m*M* $MgCl_2$, 5 m*M* EGTA, 1 m*M* DTT, 2 m*M* ATP, 10 m*M* phosphocreatine and 50 μg/ml creatine kinase). Extracts were immediately distributed and stored in aliquots of 20 μl at −80°C for later use.

Preparation of isolated mitochondria Mitochondria were isolated from HL-60 cells, which were harvested by centrifugation at 1000*g* for 10 min at 4°C and washed twice with ice-cold PBS and once with mitochondria buffer (MB; 20 m*M* HEPES-KOH, pH 7.5, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 m*M* EGTA, 1 m*M* EDTA, 1 m*M* DTT, 1 m*M* PMSF). The cells were resuspended in 5 volumes of MB with 250 m*M* sucrose, incubated on ice for 10 min, and homogenized with 10 strokes of a Dounce homogenizer. The lysate was centrifuged at 750*g* for 10 min at 4°C. The supernatant was centrifuged at 10,000*g* for 15 min at 4°C, and its precipitate was resuspended in an equal volume of MB with 250 m*M* sucrose as isolated mitochondria. Sequential experiments using this sample were performed immediately after preparation.

To show that the isolated mitochondria were active, the oxidative phosphorylation activity of mitochondria prepared identically was assayed with an oxygen electrode (Yellow Springs Instrument Co.) at 28°C.[20] Briefly, 1.75 ml of reaction buffer (10 m*M* Tris-HCl pH 7.4, 20 m*M* phosphate buffer pH 7.4, 10 m*M* KCl, 5 m*M* $MgCl_2$, 0.2 m*M* EDTA and 250 m*M* sucrose) was placed in an air-shielded glass cell with an O_2-electrode, and 400 μl of isolated mitochondria, which had been incubated for 3 h at 0°C after preparation, was added. After addition of 30 μl of 0.6 *M* succinate, 9 μl and 11 μl of 40 m*M* ADP were added sequentially. The respiratory control ratio (RR) and ADP/O ratio (P/O) were calculated.

Western blot analysis HL-60 cells and HeLa S3 cells were suspended in equal volumes of PBS and used as samples. As another set of samples, HL-60 and HeLa S3 cytoplasmic extracts, and isolated mitochondria from HL-60 cells were prepared and diluted with EDB to 7.5 mg/ml using BCA Protein Assay Reagent (Pierce, Rockford, IL). All of the samples were boiled at 95°C for 5 min with an equal volume of sample buffer (100 m*M* Tris-Cl, pH 6.8, 5% sodium dodecyl sulfate (SDS), 20% glycerol, 0.1% bromophenol blue and 200 m*M* DTT), and 25 μl of these were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE). Fifteen percent polyacrylamide gels were used for detection of cytochrome *c* and caspase-3, and 7.5% polyacrylamide gel for PARP. Proteins in these gels were transferred to nitrocellulose membranes by semi-dry electroblotting. The membranes were blocked in TBS-T (10 m*M* Tris-Cl, pH 7.5, 100 m*M* NaCl and 0.1% Tween 20) containing 5% bovine serum albumin, and cytochrome *c*, caspase-3 and PARP were detected using 1/500, 1/500 and 1/200 dilutions of anti-cytochrome *c*, anti-caspase-3 and anti-PARP antibodies, respectively. To visualize immunoreactive proteins, anti-mouse antibody conjugated with HRP and donkey anti-goat antibody conjugated with HRP were employed at 1:30,000 and 1:15,000 dilution, respectively, using enhanced chemiluminescence (ECL, Amersham, Ltd.).

RESULTS

Radiation-induced DNA fragmentation and activation of caspase-3 in the whole cell After incubation for various time periods following 30 Gy of ^{137}Cs γ-irradiation, DNA was extracted from HL-60 cells and HeLa S3 cells. Subsequent agarose gel electrophoresis revealed that characteristic DNA ladder patterns appeared in a time-dependent manner in HL-60 cells. In HeLa S3 cells, no such ladder patterns were observed (Fig. 1).

To examine cleavage of caspase-3, HL-60 cells and HeLa S3 cells were incubated for various time periods after irradiation and lysed for SDS-PAGE. The inactive form of caspase-3 was detected in the lysate of unirradiated HL-60 cells. After irradiation, the level of p32 inactive caspase-3 gradually decreased and that of p11 cleaved product increased in the lysate of HL-60 cells. PARP in HL-60 cells was also cleaved gradually to the p85 product, which indicates specific activation of caspase-3. On the other hand, in the lysate of HeLa S3 cells, activated caspase-3 and cleaved products of PARP were not observed (Fig. 2).

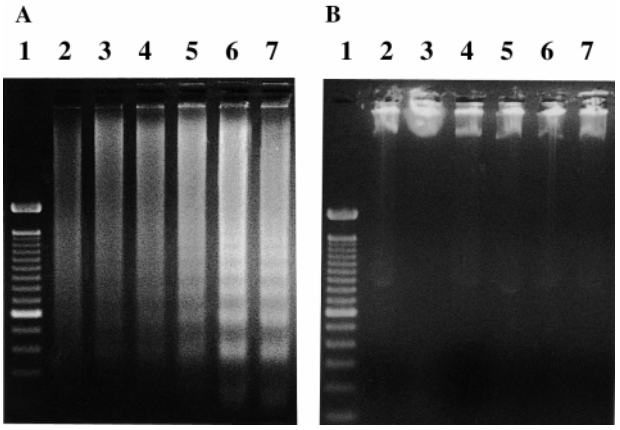

Fig. 1. Radiation-induced DNA fragmentation in HL-60 cells (A) and HeLa S3 cells (B). Cells were incubated for various time periods after 30 Gy of ^{137}Cs γ-irradiation, and lysed for 2% agarose gel electrophoresis. Lane 1: 100-bp marker, lane 2: unirradiated control, lanes 3–7: incubated for 0, 1, 2, 3 and 4 h after irradiation, respectively.

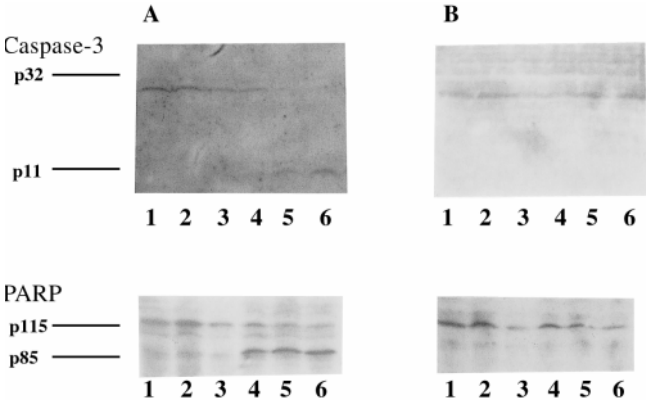

Fig. 2. Western blot analyses of caspase-3 and PARP in HL-60 cell lysates (A) and HeLa S3 cell lysates (B). Cells were incubated for various time periods after 30 Gy of ^{137}Cs γ-irradiation, and lysed for western blot analysis. Upper panels: caspase-3, lower panels: PARP. Lane 1: unirradiated control, lanes 2–6: incubated for 0, 1, 2, 3 and 4 h after irradiation, respectively.

Elevation of cytochrome *c* in the cytoplasmic extract

To determine whether cytochrome *c* is increased in cytoplasmic extract after radiation, we prepared cytoplasmic extracts from HL-60 cells and HeLa S3 cells. Cells of both lines were incubated for various time periods after irradiation and extracted to obtain samples for western blot analysis. Levels of cytochrome *c* in cytoplasmic extracts and in isolated mitochondria from irradiated HL-60 cells are shown in Fig. 3A, upper panel and lower panel, respectively. Compared with the weak band of cytochrome *c* in the extract from unirradiated HL-60 cells (Fig. 3A, upper panel, lane 1), those in the extracts from HL-60 cells incubated for 0, 2, and 4 h after radiation were increased (Fig. 3A, upper panel, lanes 2, 3 and 4, respectively). On the other hand, the level of cytochrome *c* in isolated mitochondria from irradiated HL-60 cells decreased in a time-dependent manner (Fig. 3A, lower

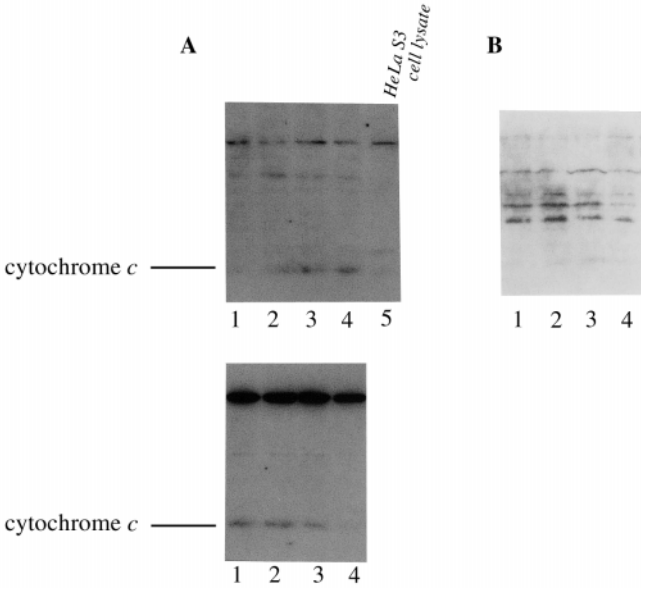

Fig. 3. Western blot analyses of cytochrome *c* in cytoplasmic extracts from irradiated HL-60 cells (A, upper panel), in mitochondria prepared from irradiated HL-60 cells (A, lower panel) and in cytoplasmic extracts from irradiated HeLa S3 cells (B). Cells were incubated for various time periods after 30 Gy of ^{137}Cs γ-irradiation, then the cytoplasmic extract and isolated mitochondria were prepared for western blot analysis. Lane 1: unirradiated control, lanes 2–4: 0, 2 and 4 h after radiation, respectively. Lane 5 contains HeLa S3 total cell lysate as a positive control.

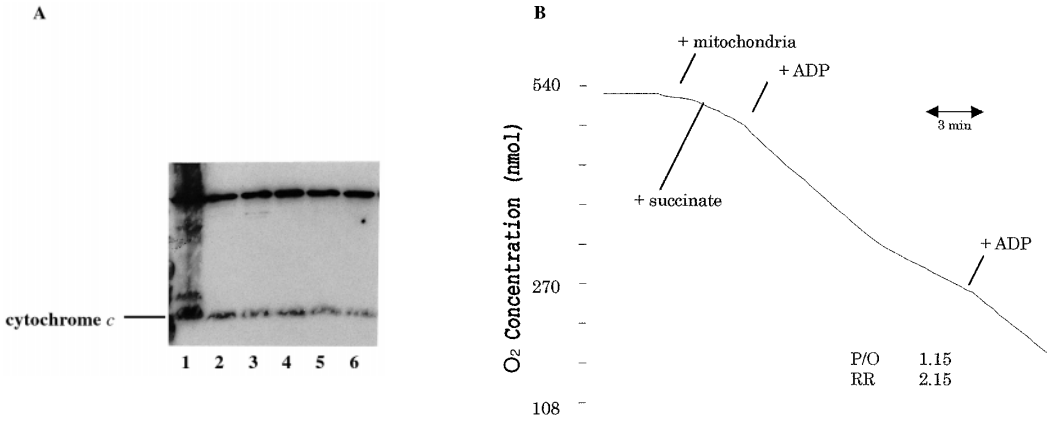

Fig. 4. Cytochrome *c* release from isolated mitochondria and oxygen consumption of isolated mitochondria. A: Mitochondria were isolated from unirradiated HL-60 cells, and exposed to ^{137}Cs γ-rays. After incubation for various time periods, the supernatants were subjected to western blot analysis. Lane 1: HeLa S3 total cell lysate as a positive control, lane 2: unirradiated control, lanes 3–6: incubated for 0, 15, 30 and 60 min after radiation, respectively. B: Oxygen consumption of isolated mitochondria was evaluated at 28°C with an oxygen electrode.

panel). These observations may indicate that cytochrome *c* is released from mitochondria into the cytosol in irradiated HL-60 cells. As for cytochrome *c* in HeLa S3 cytoplasmic extract, no increase was seen after irradiation (Fig. 3B).

Cytochrome *c* release from isolated mitochondria In the previous experiment, we found that cytochrome *c* is released from mitochondria into the cytoplasm after radiation. To determine whether radiation directly causes mitochondria to release cytochrome *c*, we exposed isolated mitochondria to γ-rays. After various periods of incubation at 37°C, mitochondria were removed by centrifugation of 1000*g* for 5 min, and 15 μl aliquots of supernatants were subjected to western blot analysis. As shown in Fig. 4A, weak bands of cytochrome *c* released from the isolated mitochondria were observed in these supernatants, but the intensities of the bands showed no increase within 60 min after direct exposure. This suggests that mitochondria may not be the site at which the primary change occurs upon irradiation.

As for the oxidative phosphorylation activity of the isolated mitochondria, the calculated values of P/O and RR were 1.17 and 2.15, respectively, which indicated that the isolated mitochondria used in our study are active. A record of the oxygen electrode measurement is shown in Fig. 4B.

DISCUSSION

We have shown here that cytochrome *c* in cytoplasmic extract from irradiated HL-60 cells is increased, that cytochrome *c* in mitochondria isolated from irradiated HL-60 cells is decreased and that isolated mitochondria do not release cytochrome *c* after direct radiation. These observations suggest that cytochrome *c* is released from mitochondria in HL-60 cells after irradiation and that mitochondria may not be the site at which the primary effect of radiation occurs. In this report, we assayed oxidative phosphorylation activity in only one sample of isolated mitochondria, but it is reasonable to consider that all samples of isolated mitochondria had the activity because all isolated mitochondria were identically prepared.

We have reported previously that caspase-3 in HL-60 cells is activated after irradiation, and that *in-vitro* DNA fragmentation is induced by cytoplasmic extract from irradiated HL-60 cells in which caspase-3 was activated.[18] Others have reported that cytosolic caspase-3 is activated in a reaction triggered by cytochrome *c*.[15–17] Our studies and previous reports suggest that radiation-induced apoptosis involves an extranuclear signal transduction pathway: mitochondria to cytochrome *c* to caspase-3 to apoptotic execution.

We have shown that mitochondria may be involved in the radiation-induced apoptotic pathway, but may not be the site at which the primary effect of radiation occurs. This may imply that some unknown pathway upstream of mitochondria exists. Previous studies have indicated that ionizing radiation induces production of ceramide and that ceramide induces apoptosis.[7, 21] Our observations and the results of previous studies lead us to the speculation that the ceramide-induced pathway might pass to mitochondria and might result in the release of cytochrome *c* from mitochondria. However, it remains unclear whether mitochondria are involved in the ceramide-induced pathway.

In summary, we have found that cytochrome *c* is released from mitochondria in HL-60 cells after irradiation, but that isolated mitochondria do not release cytochrome *c* after direct irradiation. The radiation-induced apoptotic pathway may involve cytochrome *c* and mitochondria, but mitochondria may not be the site at which primary change caused by irradiation occurs.

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REFERENCES

1) Kerr, J. F. R., Wyllie, A. H. and Currie, A. R. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer*, **26**, 239–257 (1972).
2) Duke, R. C., Chervenak, R. and Cohen, J. J. Endogenous endonuclease-induced DNA fragmentation: an early event in cell-mediated cytolysis. *Proc. Natl. Acad. Sci. USA*, **80**, 6361–6365 (1983).
3) Laster, S. M., Wood, J. G. and Gooding, L. R. Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis. *J. Immunol.*, **141**, 2629–2634 (1988).
4) Sellins, K. S. and Cohen, J. J. Gene induction by γ-irradiation leads to DNA fragmentation in lymphocytes. *J. Immunol.*, **139**, 3199–3206 (1987).
5) Martin, S. J. and Cotter, T. G. Ultraviolet B irradiation of human leukemia HL-60 cells *in vitro* induces apoptosis. *Int. J. Radiat. Biol.*, **59**, 1001–1016 (1991).
6) Lennon, S. V., Martin, S. J. and Cotter, T. G. Dose-dependent induction of apoptosis in human tumor cell lines by widely diverging stimuli. *Cell Prolif.*, **24**, 203–214 (1991).
7) Obeid, L. M., Linardic, C. M., Karolak, L. A. and Hannun, Y. A. Programmed cell death induced by ceramide. *Science*, **259**, 1769–1771 (1993).
8) Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W. and Yuan, J. Human ICE/CED-3 protease nomenclature. *Cell*, **87**, 171 (1996).
9) Schlegel, J., Peters, I., Orrenius, S., Miller, D. K., Thornberry, N. A., Yamin, T. T. and Nicholson, D. W. CPP32/apopain is a key interleukin 1 beta converting enzyme-like protease involved in Fas-mediated apoptosis. *J. Biol. Chem.*, **271**, 1841–1844 (1996).
10) Datta, R., Banach, D., Kojima, H., Talanian, R. V., Alnemri, E. S., Wong, W. W. and Kufe, D. W. Activation of the cpp32 protease in apoptosis induced by 1-beta-D-arabinofuranosylcytosine and other DNA-damaging agents. *Blood*, **88**, 1936–1943 (1996).
11) Datta, R., Kojima, H., Banach, D., Bump, N. J., Talanian, R. V., Alnemri, E. S., Weichselbaum, R. R., Wong, W. W. and Kufe, D. W. Activation of a CrmA-insensitive, p35-sensitive pathway in ionizing radiation-induced apoptosis. *J. Biol. Chem.*, **272**, 1965–1969 (1997).
12) Hasegawa, J., Kamada, S., Kamiike, W., Shimizu, S., Imazu, T., Matsuda, H. and Tsujimoto, Y. Involvement of CPP32/Yama(-like) proteases in Fas-mediated apoptosis. *Cancer Res.*, **56**, 1713–1718 (1996).
13) Martin, S. J., Amarante-Mendes, G. P., Shi, L., Chuang, T. H., Casiano, C. A., O'Brien, G. A., Fitzgerald, P., Tan, E. M., Bokoch, G. M., Greenberg, A. H. and Green, D. R. The cytotoxic cell protease granzyme B initiates apoptosis in a cell-free system by proteolytic processing and activation of the ICE/CED-3 family protease, CPP32, via a novel two-step mechanism. *EMBO J.*, **15**, 2407–2416 (1996).
14) Enari, M., Talanian, R. V., Wong, W. W. and Nagata, S. Sequential activation of ICE-like and cpp32-like proteases during Fas-mediated apoptosis. *Nature*, **380**, 723–726 (1996).
15) Liu, X., Kim, C. N., Yang, J., Jemmerson, R. and Wang, X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell*, **86**, 147–157 (1996).
16) Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P. and Wang, X. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science*, **275**, 1129–1132 (1997).
17) Liu, X., Zou, H., Slaughter, C. and Wang, X. DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell*, **89**, 175–184 (1997).
18) Kurihara, H., Torigoe, S., Omura, M., Saito, K., Kurihara, M. and Matsubara, S. DNA fragmentation induced by cytoplasmic extract from irradiated cells. *Radiat. Res.*, (1998), in press.
19) Lazebnik, Y. A., Cole, S., Cooke, C. A., Nelson, W. G. and Earnshaw, W. C. Nuclear events of apoptosis *in vitro* in cell-free mitotic extracts: a model system for analysis of the active phase of apoptosis. *J. Cell Biol.*, **123**, 7–22 (1993).
20) Lessler, M. A. Oxygen electrode measurements in biochemical analysis. *Methods Biochem. Anal.*, **17**, 1–29 (1969).
21) Haimovitz-Friedman, A., Kan, C.-C., Ehleiter, D., Persaud, R. S., McLoughlin, M., Fuks, Z. and Kolesnick, R. N. Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. *J. Exp. Med.*, **180**, 525–535 (1994).