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Nuclear Translocation of DNase II and Acid Phosphatase during Radiation-induced Apoptosis in HL60 Cells

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DNase II is involved in DNA fragmentation induced by a variety of treatments. However, according to past reports DNase II does not directly generate TUNEL (*in situ* DNA end labeling)-positive cells. The purpose of this study was to investigate the participation of acid phosphatase in the generation of TUNEL-positive cells. DNase II-like proteins, whose molecular weights were 32-kDa, were detected in nuclear extracts of HL60 human myeloid leukemia cells post γ-irradiation by SDS-PAGE and immunohistochemistry. Acidic nuclease activity was especially active in 32-kDa bands. TUNEL assay was positive post γ-irradiation. From measurements of the activity of acid phosphatase, the activity in nuclear extracts increased remarkably post γ-irradiation. γ-irradiation can directly or indirectly activate DNase II. Once DNase II and acid phosphatase have been translocated from lysosomes into the nuclei, DNase II generates TUNEL reactive ends in combination with acid phosphatase.

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Apoptosis is a mode of cell death characterized by distinct morphological features and DNA fragmentation (1), and can be induced by a variety of treatments such as UV irradiation (2), γ-irradiation (3), Fas/APO-1 ligand (4) and cytotoxic chemotherapy (5). During apoptosis, endonucleases present in cells cleave DNA at sites located between nucleosomal units, thus resulting in the characteristic ladder of DNA fragments (6).

Several attempts have been made to characterize the nucleases that are responsible for this fragmentation. For instance, DNase I and NUC 18 are reported to be associated with apoptotic DNA cleavage (7–10). Recently, a new Ca^{2+}-dependent endonuclease (NUC 70) was purified from human hematopoietic cells (11), and shown to be a cytoplasmic protein translocated to nuclei early in apoptosis. DNase II has also been proposed to be involved in specific cases of apoptosis (12, 13). In addition, DNase γ (14) and caspase-activated DNase (CAD) (15, 16) have also been discovered.

Apoptosis signal transduction and execution require the coordinated action of the cascade of caspases (aspartate-specific cysteine proteases). Caspase activity is responsible, either directly or indirectly, for the cleavage of cellular protein, which is characteristically proteolyzed during apoptosis. In a former study, we demonstrated radiation-induced apoptosis in HL60 human myeloid leukemia cells that activated caspase-3 leading to the cleavage of PARP (poly(ADP-ribose)polymerase) (3). We also explored the radiation-induced release of cytochrome C from mitochondria. However, for endonuclease in apoptosis occurring in γ-irradiated HL60 cells, we have not as yet undertaken detailed research.

Recently, it has been shown that acidic nuclease, detected in HL60 cells upon activation by UV irradiation, is represented by four polypeptides of apparent molecular weights 56-, 48-, 45-, and 40-kDa (17). TUNEL assay was sensitive in detecting very early signs of apoptosis in HL60 cells at the various UV irradiation time periods tested (18). It has been demonstrated elsewhere that DNase II was involved in the DNA fragmentation induced by lovastatin, an HMG-CoA reductase inhibitor, in HL60 cells (19). It has also been proposed that CAD is the candidate for DNA cleavage in radiation-induced apoptosis (20). However, in the present investigation, DNase II-like protein, represented by the polypeptide with the apparent molecular weight 32-kDa, was detected in HL60 cells after γ-irradiation. DNA fragmentation was detected by the TUNEL assay but the results indicate an alternative pathway involving acid phosphatase in DNA cleavage.

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MATERIAL AND METHODS

Cell culture and induction of apoptosis

HL60 human myeloid leukemia cells were used and maintained in an alpha-minimum essential medium containing 10% fetal bovine serum (FBS), 5 U/ml penicillin G and 5 μg/ml streptomycin at 37°C in 2% CO_2. In order to induce apoptosis, HL60 cells were treated with 1, 5, 10, 20 and 30 Gy of ^{137}Cs of radiation (1.015 Gy/min; Gammacell 40, Atomic Energy of Canada Ltd., Canada), and then incubated in the same medium for various periods. All experiments were conducted when the cells were in an exponential growth phase.

Preparation of cytoplasmic and nuclear extracts

Nuclei were isolated as previously described (3) by resuspension of 1×10^8 cells in 1.5 mM $MgCl_2$ and three washings in phosphate buffered saline (PBS) (pH 7.4). Cells were then incubated in this solution at 4°C for 15 min and disrupted by 20 strokes of a tight-fitting Dounce homogenizer in order to burst open the cells and liberate the intact nuclei. The release of cytoplasm-free nuclei was monitored by phase-contrast microscopy after staining of a small aliquot of the lysate with trypan blue. Lysates were then centrifuged (400 g at 4°C) and the supernatants collected as cytoplasmic fractions. Pellets (nuclei fractions) were then washed three times by centrifugation/resuspension in the lysis buffer and resuspended in 10 mM Tris-HCl (pH 7.4), 200 mM sucrose, and 60 mM NaCl. Nuclei protein extracts were prepared by lysing isolated nuclei in 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM EDTA, and 50 mM Tris-HCl (pH 7.4) for 30 min at 4°C. After centrifugation at 14 000 g for 15 min, the supernatant (nuclear extract) was recovered. Before preparation of the cytoplasmic and nuclear extract, the cellular pH was checked.

Detection of apoptosis

The TUNEL procedures were performed for the detection of apoptosis, using an in situ Apoptosis Detection Kit (TaKaRa BIOMEDICALS, Japan). Briefly, irradiated and unirradiated HL60 cells were rinsed in PBS and fixed for 30 min at room temperature with PBS containing 4% paraformaldehyde. After being washed with PBS, the endogenous peroxidase was inactivated by methanol with 3% hydrogen peroxide for 30 min at room temperature. After being washed with PBS, the cells were incubated with 100 μl permeabilization buffer (0.2% TritonX-100 in PBS) for 5 min on ice. After being washed with PBS, the cells were incubated for 90 min at 37°C with the TdT enzyme (dilution 1:9), and washed with PBS. The cells were incubated for 30 min at 37°C with anti-FITC HRP conjugate and washed with PBS. The cells were incubated for 15 min at room temperature with DAB (diaminobenzidine) and washed with distilled water. After counter staining with 3% methyl green, the cells were observed using an optical microscope. TUNEL-positive cells were stained brown and -negative cells green and the numbers of TUNEL-positive and -negative cells were counted.

Western blot analysis

For DNase II and G6PDH (glucose-6-phosphate dehydrogenase) detection, samples were boiled at 95°C for 5 min with equal volumes of sample buffer (100 mM Tris-HCl, pH 6.8, 5% SDS, 20% glycerol, 0.1% bromophenol blue and 200 mM DTT), and 15 μl aliquots were fractionated by SDS-PAGE; l0%–20% gradient polyacrylamide gels were used. Proteins in these gels were transferred to nitrocellulose membranes by semi-dry electroblotting. After blocking in TBS-T (10 mM Tris-HCI, pH 7.5, 100 mM NaCl and 0.1% Tween 20) containing 5% dry milk, DNase II and G6PDH proteins were detected using a 1/500 ~ 1/2 000 dilution of anti- DNase II (CHEMICON International Inc., California, USA) and anti-G6PDH antibodies (Sigma, Missouri, USA), respectively. Donkey anti-rabbit antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc., Santa Cruz, USA) was used to visualize immunoreactive proteins at a 1/2 000 ~ 1/4 000 dilution using enhanced chemiluminescence (ECL Ltd., Amersham, UK).

Immunohistochemistry

TOYOBO-ABC high-HRP Immunostaining Kit (TOYOBO, Japan) was used for immunohistochemistry. Briefly, irradiated and unirradiated HL60 cells were rinsed in PBS and fixed for 30 min at −20°C with acetone:ethanol (v:v). Then 1×10^6 cells were dropped on slides, and dried at room temperature. After the slides had been washed three times with PBS, the endogenous peroxidase was inactivated by methanol with 3% hydrogen peroxide for 10 min at room temperature. After the slides had been washed three times with PBS, the cells were blocked by PBS containing 2% normal goat serum for 60 min at room temperature. The cells were incubated overnight at 4°C with anti-DNase II antibody (1/25) in PBS containing 2% normal goat serum. After being washed with PBS, the cells were stained for 30 min with a biotinylated secondary antibody. Then after being washed again with PBS, an avidin-biotinylated peroxidase complex was dropped onto the cells. After further washing with PBS, the cells were incubated with tetramethylbenzidine for 60 min at room temperature and dyed blue where there was DNase II. After being washed with distilled water, the cells were enclosed with glycerol and observed under light microscopy. In addition, morphological analyses of the cells were performed under light microscopy after May-Grünwald-Giemsa staining (20).

Zymography assay for endonuclease activity

Zymography assays were done for endonuclease activity (17). Twelve percent of the polyacrylamide gels were co-polymerized with calf thymus DNA (0.2 mg/ml) and stored overnight at 4°C and 20 µl nuclear extracts were mixed with equal volumes of the sample buffer and separated at 100 mV for 90 min. After washing and renaturation of proteins, the gels were incubated in the activation buffer (50 mM sodium acetate, pH 5.2, 5 mM β-mercaptoethanol, 0.1% Triton X-100) for 12 h at 37°C. Acidic nuclease activity was detected by staining the gels with ethidium bromide. Active polypeptides showed up as black bands on a fluorescent background. In order to determine the apparent molecular weight of acidic nuclease, prestained standards of known molecular weights were used. Activation buffer (10 mM Tris-HCl, pH 7.8, 3 mM $CaCl_2$, 3 mM $MgCl_2$, 1 mM β-mercaptoethanol) was used as a control to check the Ca(2+)/Mg(2+)-dependent endonuclease activity.

Detection of acid phosphatase activity

The EnzChek Acid Phosphatase Assay Kit (Molecular Probes Inc., Oregon, USA) was used to detect acid phosphatase activity. Briefly, 50 µl of the 200 µM DiFMUP(6,8-difluoro-4-methylumbelliferyl phosphate) diluted with 0.1 M sodium acetate (pH5.0) was pipetted into each microplate well. Then 10 µl nuclear extracts and 1 U/mL potato acid phosphatase as a positive control were mixed with 100 µl 0.1 M sodium acetate (pH5.0), and 50 µl of each sample and the positive control was added to the DiFMUP-containing microplate wells; 50 µl of 0.1 M sodium acetate (pH5.0) was used as a negative control. The samples and controls were incubated at room temperature and protected from light for 60 min. The fluorescence was measured with a fluorescence microplate reader (Spectroan FL-2575, Towa Labo Co. Ltd., Japan) using excitation at 360 nm and emission detection at 460 nm. The data were converted into values in relation to the unirradiated sample (the unirradiated value was fixed at 1.0).

Lysosomal stability assay

Acridine orange (AO) (Sigma, St. Louis, MO) at a final concentration of 2 µg/ml PBS was added to unirradiated or γ-irradiated HL60 cells, and mixtures incubated at 37°C for 10 min before cytocentrifugation for 2 min at 450 × g, to wash away unincorporated dye. The number of cells was viewed by fluorescence microscopy. Following excitation with blue light, AO shows a metachromatic red (high concentration in lysosomes) and green (low concentration in nuclei and cytosol) fluorescence. Following excitation with green light, only concentrated intralysosomal AO gives any fluorescence; dark orange. The number of cells with or without fluorescence was counted.

Assay of in vitro apoptosis

HL60 cells were exposed to 30 Gy γ-ray. The nuclear extract of the cells was prepared as before. The reaction mixture, containing 50 µl nuclear and cytoplasmic extracts of HL60 cells and anti-DNase II antibody or ICAD (Caspase Activated DNase Inhibitor, SIGMA) at a final concentration of 2 µM, was incubated at 23°C for 4 h in pH4 or 7. To assay for DNA fragmentation, 10 volumes of buffer D (100 mM Tris-Cl, pH 8.0, 5 mM EDTA, 0.2 M NaCl, 0.4% SDS, 0.2 mg/ml proteinase K) was added into each reaction and incubated at 37°C overnight. The DNA was prepared and loaded onto a 1.5% agarose gel, for electrophoresis.

RESULTS

Western blot analyses of DNase II proteins

Anti-DNase II antibody was used to identify DNase II-like proteins in both cytoplasmic and nuclear extracts prepared from irradiated or unirradiated HL60 cells. Cellular extracts were analyzed by Western blot. No cross-reactivity with DNase I was observed (data not shown). A time-dependent method was followed (Fig. 1), and after incubation at 37°C for various periods after exposure to 30 Gy of γ-rays, nuclear and cytoplasmic extracts were produced as mentioned above. The elements without irradiation, for example growth factor starvation, concerned apoptosis if low-dose γ-irradiation is performed. Therefore, 30 Gy of γ-rays is used in this study. We detected 32-kDa proteins in the cytoplasm of unirradiated cells but not in the nuclear extracts prepared from unirradiated cells. The levels of the cytoplasmic 32-kDa proteins were not subjected to any marked modification by either irradiation or a lack of it. Nuclear accumulation of the 32-kDa proteins was observed after 1 h of exposure to γ-rays. The cytosolic marker G6PDH was used to detect contamination of cytosolic proteins in the nuclear fraction (see below).

After 3-h incubation following exposure to various doses of γ-ray, nuclear and cytoplasm extracts were made as described above. The levels of the cytoplasmic 32-kDa proteins did not change remarkably as the dose of γ-rays increased. Nuclear accumulation of the 32-kDa proteins was also observed on exposure to $5 \sim 10$ Gy γ-ray. These results indicate that the detection of nuclear DNase II in HL60 cells on exposure to γ-ray might be caused by nuclear translocation of the protein at 32 kDa, as recognized by the anti-DNase II antibody.

Immunolocalization of DNase II in γ-irradiated HL60 cells

In situ immunohistochemical analyses were performed on whole cells to further prove the translocation of DNase II in nuclei of cells triggered to death by γ-rays. The immunolocalization of DNase II protein in unirradiated and irradiated HL60 cells is shown dose de-

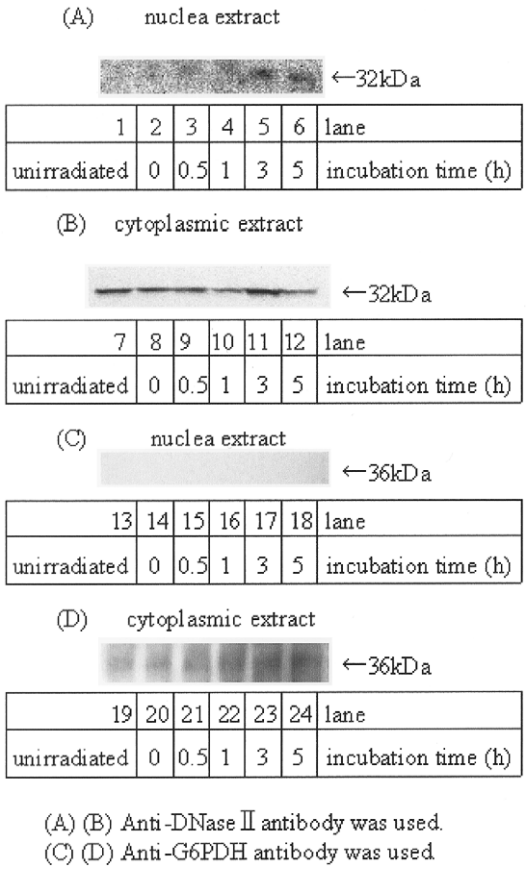


Fig. 1. Western blot analyses of DNase II proteins, assessed time-dependently, lanes 1, 7, 13, 19: unirradiated control, Western blot analysis was performed in the nuclear (A) and cytoplasmic (B) extracts of HL60 cells with anti-DNase II antibody. HL60 cells were incubated for various periods (lanes 2, 8: 0 min; lanes 3, 9: 30 min; lanes 4, 10: 1 h; lanes 5, 11: 3 h; lanes 6, 12: 5 h) after exposure to 30 Gy of ^{137}Cs-irradiation and then lysed. 62-kDa proteins were accumulated at 30 min ~ 1 h, and 32-kDa proteins at 3 h–5 h in nuclear extracts post 30 Gy γ-irradiation. In the cytoplasmic extract, 32-kDa proteins were not subjected to marked modification by either a lack of irradiation or exposure to γ-rays. The purity of the nuclear fraction was checked using an antibody that recognized a cytoplasmic enzyme, G6PDH (see 36-kDa C, D).

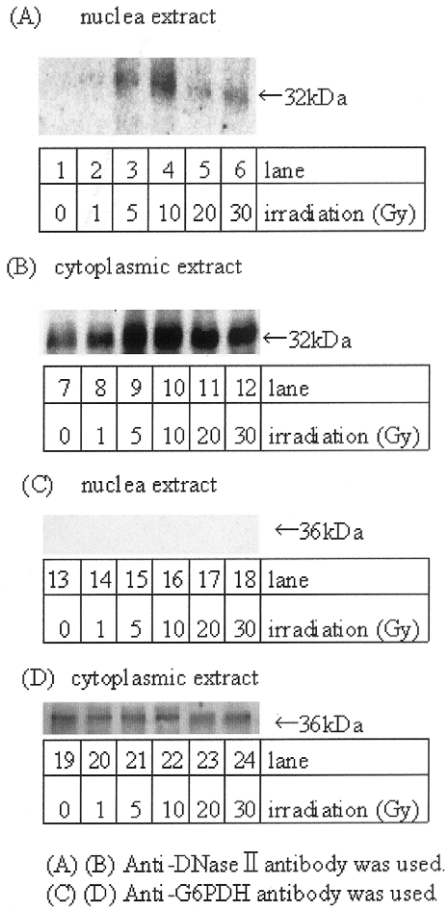


Fig. 2. Western blot analyses of DNase II proteins, assessed dose-dependently, lanes 1, 7, 13, 19: unirradiated control, Western blot analyses were performed in the nuclear (A) and cytoplasmic (B) extracts of HL60 cells with anti-DNase II antibody. HL60 cells were incubated for 3 h after exposure to various doses (lanes 2, 8: 1 Gy; lanes 3, 9: 5 Gy; lanes 4, 10: l0 Gy; lanes 5, 11: 20 Gy; lanes 6, 12: 30 Gy) of ^{137}Cs γ-irradiation and then lysed. 62-kDa and 32-kDa proteins were accumulated at 5 ~ 10 Gy in the nuclear extracts. In the cytoplasmic extract, 32-kDa proteins were not subjected to a marked modification by either a lack of irradiation or exposure to γ-rays. The purity of the nuclear fraction was checked using an antibody that recognized a cytoplasmic enzyme, G6PDH (see the 36-kDa C, D).

pendently in Fig. 3A. After incubating for 3 h following exposure to various doses of γ-rays, unirradiated and irradiated HL60 cells were stained as described above. In the unirradiated cells (Fig. 3A, (a)), the immunohistochemical analyses showed an intense cytoplasmic immunoreactivity (blue). On the other hand, nuclear staining was very weak, which revealed a very low localization of the DNase II. After γ-irradiation, an increase in the accumulation of blue staining in the nuclei was observed (Fig. 3A, (b), (c)). This suggests a redistribution of the proteins recognized by the anti-DNase II antibody to the nucleus. However, on exposure to 20 Gy γ-rays, the areas stained blue were diffused in the cells, and on exposure to 30 Gy γ-rays, apoptosis progressed too far. Thus the original form of the cells could not be maintained and the area was widely stained blue.

The morphology of the cells after May-Grünwald-Giemsa staining is shown in Fig. 3B. The characteristic features of apoptosis are observed in irradiated HL60 cells. It is interesting that these *in situ* experiments are in agreement with those obtained by immunoblot analyses (Fig. 2). They further support the supposition that γ-rays induce the accumulation of DNase II protein in the nuclei of irradiated cells.

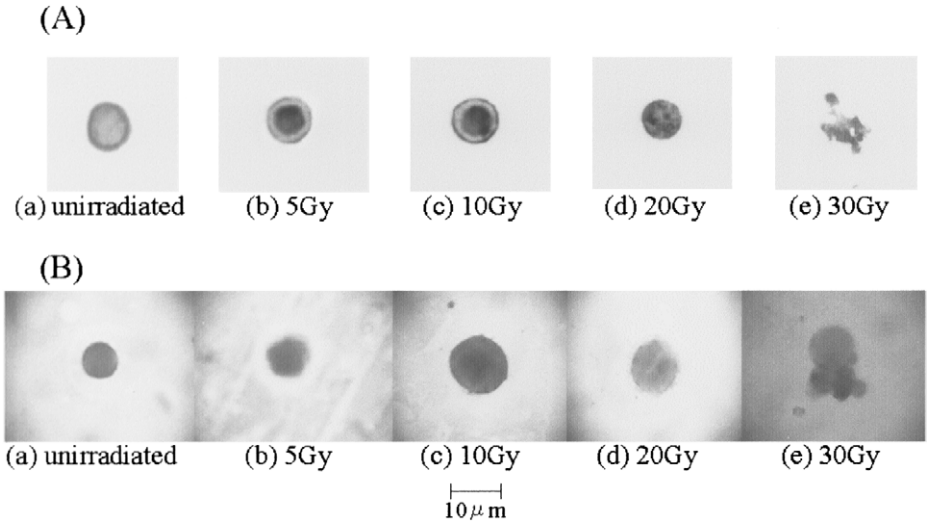


Fig. 3. Immunolocalization of DNase II in HL60 unirradiated and γ-irradiated cells. (A): Immunohistochemistry analyses; after γ-irradiation, cells were incubated with polyclonal antibody against DNase II. The antibody was visualized as described in Methods and Materials (blue). In control cells (a), cytoplasmic immunoreactivity was more intense than in nuclear cells. After γ-irradiation (5 ~ 10 Gy), an increase in the accumulation of blue staining in nuclei was observed (b, c). (B): Morphology of the cells after May-Grünwald-Giemsa staining (magnification; × 400).

The activity of an acidic nuclease increased by exposure to γ-rays

Since γ-irradiation induces intracellular acidification and apoptosis in HL60 cells, we investigated the relationship between γ-irradiation-induced apoptosis and the activity of acidic nuclease. The activity of acidic nuclease present in nuclear extracts of cells undergoing apoptosis was analyzed by zymography performed in a time- and dose-dependent manner. Very low levels of acidic nuclease activity were observed in nuclear extracts isolated from unirradiated HL60 cells. Time-dependently, post γ-irradiation nuclease activity was readily detected at 3 h after exposure to 30 Gy. Nuclease activity increased, reaching a maximum level at 5 h post irradiation (Fig. 4A). Dose-dependently, after 3 h post γ-irradiation, the band began to become clearer from 10 Gy. The molecular weights of the bands were at 32-kDa (data not shown). The intensity of bands was also pH-dependent. Bands became visible when gels were incubated for 12 h at acidic pH. It can be inferred from these results that an acidic nuclease is associated with the majority of the nucleolytic activity, especially on the 32-kDa band in HL60 cells.

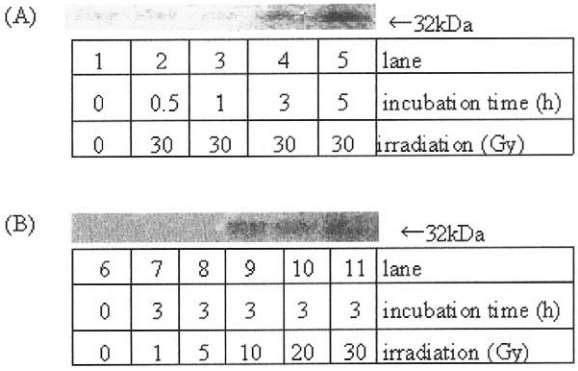


Fig. 4. Zymography carried out at pH5.2 in nuclear extracts isolated from HL60 unirradiated and γ-irradiated cells. (A): Time-dependent manner, lane 1: unirradiated cells; lane 2–5: HL60 cells were incubated for various periods (lane 2: 30 min; lane 3: 1 h; lane 4: 3 h; lane 5: 5 h) after exposure to 30 Gy of ^{137}Cs γ-irradiation and then lysed. (B): Dose-dependent manner, lane 6: unirradiated cells; lane 7 ~ 11: HL60 cells were incubated for 3 h after exposure to various doses (lane 7: 1 Gy; lane 8: 5 Gy; lane 9: l0 Gy; lane 10: 20 Gy; lane 11: 30 Gy) of ^{137}Cs γ-irradiation and then lysed. Very low levels of acidic nuclease activity (32-kDa) were observed in nuclear extracts isolated from unirradiated HL60 cells (lanes 1, 6). Post γ-irradiation, 32-kDa bands became more intense (lanes 4–5, 9 ~ 11). However, the 62-kDa band was not detected.

γ-irradiation-induced TUNEL-positive apoptosis in HL60 cells

One thousand cells were counted, and cells stained brown or black were judged TUNEL-positive cells. First, TUNEL procedures in HL60 cells were performed in a time-dependent manner (Fig. 5A). After incubation at 37°C for various periods on exposure to 30 Gy γ-irradiation, the cells were dealt with using the processes described above. Control cells (unirradiated cells, Fig. 5A (a)) were stained green with methyl green. As the time of incubation became longer, cells were stained brown or black and morphological changes, which are characteristic of apoptosis in cells, appeared (Fig. 5A (e), (f)). TUNEL-positive cells began to increase from 1 h post γ-irradiation, and the fraction of TUNEL-positive cells was larger than 90% 3 h post γ-irradiation (Table 1, (A)).

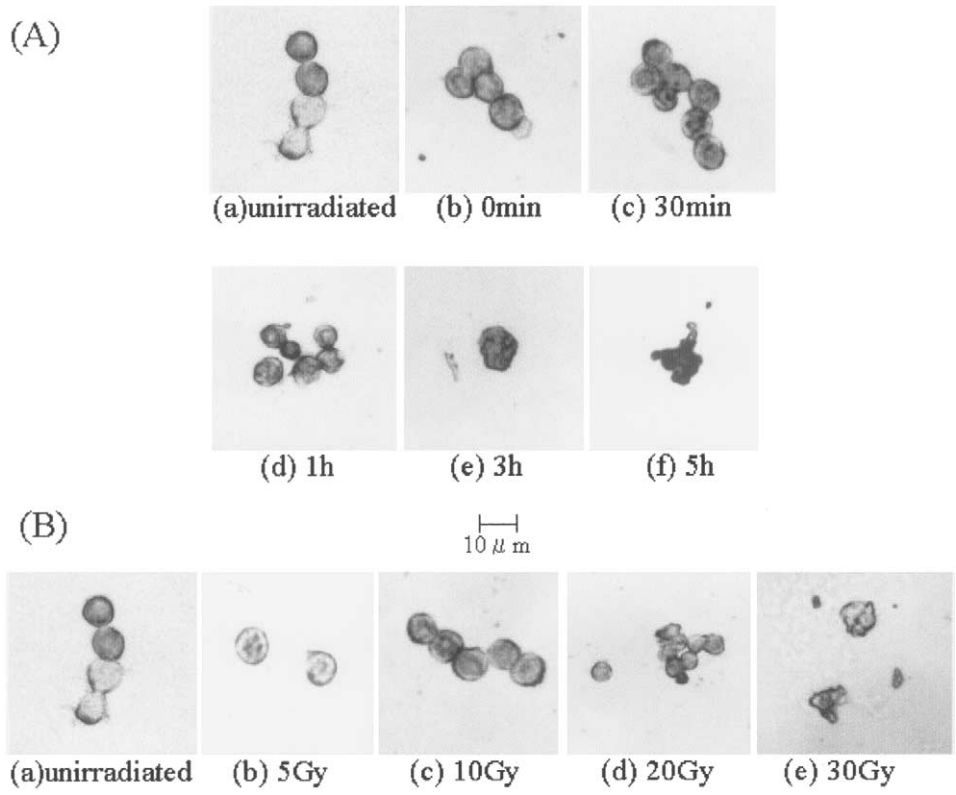


Fig. 5. TUNEL procedure carried out in HL60 unirradiated and γ-irradiated cells. (A): Time-dependent manner; (a): unirradiated cells, (b)–(f): HL60 cells were incubated for various periods (b: 0 min; c: 30 min; d: 1 h; e: 3 h; f: 5 h) after exposure to 30 Gy of ^{137}Cs γ-irradiation and the TUNEL procedure was then performed. (B): Dose dependent manner; (a): unirradiated cells, (b) HL60 cells were incubated for 3 h after exposure to various doses (b: 5 Gy; c: 10 Gy; d: 20 Gy; e: 30 Gy) of ^{137}Cs γ-irradiation and then the TUNEL procedure was performed. Control cells (unirradiated cells, A (a), B (a)) were stained green with methyl green. Post γ-irradiation, cells were stained brown or black and morphological changes appeared (A (e) (f), B (e)) (magnification: × 400).

Second, again in a dose-dependent manner (Fig. 5B), as the dose of γ-rays increased, the stained cells changed from green (Fig. 5B (a)) to brown (Fig. 5B (d), (e)). TUNEL-positive cells began to increase after exposure to 20 Gy γ-irradiation, and the fraction of TUNEL-positive cells was greater than 90% after 30 Gy γ-irradiation (Table 1, (B)). These results revealed that exposure to γ-irradiation induced apoptosis in HL60 cells and a subsequent increase in DNA fragmentation was detected by an increase in the number of TUNEL-positive cells.

Table 1

TUNEL-positive apoptosis cells induced by γ-irradiation

(A) Time-dependent manner						
Incubation time	Unirradiated	0 min	30 min	1 h	3 h	5 h
TUNEL positive	3.6%	6.7%	10.9%	47.2%	92.5%	95.7%
(B) Dose-dependent manner						
Radiation dose (Gy)	Unirradiated		5	10	20	30
TUNEL positive	3.6%		9.8%	25.1%	87.8%	92.5%

(A) Cells were incubated at 37°C for various periods after exposure to 30 Gy of γ-irradiation. (B) After exposure to various doses of γ-irradiation, cells were incubated at 37°C for 3 h. TUNEL-negative cells stained green with methyl green. TUNEL-positive cells stained brown or black.

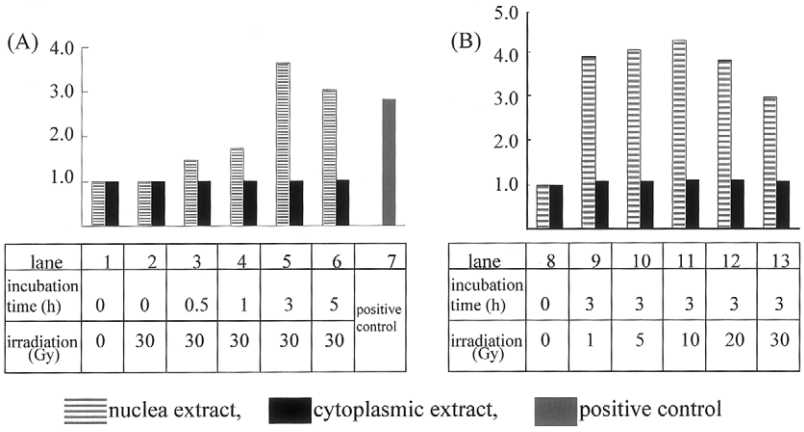


Fig. 6. Acid phosphatase activity assay in the nuclei and cytoplasm of HL60 cells. (A): Time-dependent manner; lane 1: unirradiated cells, lane 2: HL60 cells were incubated for various periods (lane 2: 0 min; lane 3: 30 min; lane 4: 1 h; lane 5: 3 h; lane 6: 5 h) after exposure to 30 Gy of ^{137}Cs γ-irradiation and then lysed. (B): Dose-dependent manner; lane 8: unirradiated cells, lane 9 ~ 13: HL60 cells were incubated for 3 h after exposure to various doses (lane 9: 1 Gy; lane 10: 5 Gy; lane 11: l0 Gy; lane 12: 20 Gy; lane 13: 30 Gy) of ^{137}Cs γ-irradiation and then lysed. Lane 7: potato acid phosphatase (0.1 U/mL) used as a positive control. The acid phosphatase activity in nuclear extracts increased markedly post γ-irradiation. In contrast, the activity in cytoplasm hardly changed at all.

Acid phosphatase activity in the nuclei and cytoplasm of HL60 cells

Acid phosphatase activity in the nuclei and cytoplasm of HL60 cells was measured using the EnzChek Acid Phosphatase Assay Kit. The value of irradiated cells was compared with that of non-radiation cells. The non-radiation value was fixed at 1.0 (Fig. 6A, lane 1 and B, lane 8). The value of 0.1 U/mL potato acid phosphatase as a positive control is indicated in Fig. 6A, lane 7. Also in a time-dependent manner, the acid phosphatase activity in cytoplasm hardly changed at 3 ~ 5 h after exposure to 30 Gy γ-irradiation. On the other hand, acid phosphatase activity in nuclear extracts increased remarkably after exposure to 30 Gy γ-irradiation, and peaked at 3 h (Fig. 6A, lane 5). In a dose-dependent manner, the acid phosphatase activity in cytoplasm slightly increased at 3 h after exposure to 1 ~ 30 Gy γ-irradiation. However, the acid phosphatase activity in nuclear extracts increased remarkably at 3 h after exposure to 1 ~ 30 Gy γ-irradiation, and peaked at 10 Gy (Fig. 6B, lane 11).

Lysosomal stability assay

If lysosomes are disrupted by radiation, the nuclear translocation of acid phophatase will not occur normally. In order to investigate this, the lysosomal stability assay was performed. In cells examined by fluorescence microscopy after AO staining, the number of intact lysosomes was found to decline slightly after 3 h following exposure to 30 Gy γ-irradiation (Fig. 7D arrow), and after 5 h the cells that had no intact lysosomes increased (Fig. 7E arrow). In a dose-dependent manner, the numbers of intact lysosomes declined when exposed to 20 ~ 30 Gy γ-irradiation. These results suggest that lysosome disruption by exposure to γ-irradiation occurred at the latter period of apoptosis and necrosis (Table 2).

Effects of anti-DNase II antibody or ICAD on DNA fragmentation

To investigate the effects of DNA fragmentation, we added anti-DNase II antibody or ICAD at a final concentration of 2 μM to our in vitro apoptosis-inducing system consisting of a mixture of irradiated or non-irradiated nuclear extracts and intact nuclei. The results indicated that anti-DNase II antibody inhibited the formation of DNA ladder in pH 4 (Fig. 8, lane 6). However, the inhibition of DNA fragmentation by ICAD was only partial in pH 7, and in pH 4 the formation of DNA fragmentation was not inhibited by ICAD (Fig. 8, lane 7).

DISCUSSION

Apoptosis is known to be a cell-death process that occurs cell autonomously, that is, cells have a mechanism for committing suicide or are programmed to die. Various apoptotic stimuli activate caspase cascades that lead to cleavage of >60 death-related substrates and to activation of the CAD DNase that causes DNA fragmentation (15). Oxidative stress, growth factor starvation, and activation of the Fas/APO-1/CD95 receptor all induce apoptosis in a variety of cell types, including the established human Jurkat T-cell line. In these cells, leakage of lysosomal enzymes from the normal vacuolar compartment may then induce apoptosis.

As we have seen in this study, it was indicated that cytoplasmic nuclease was translocated to the nucleus after γ-irradiation. Zymography assays demonstrated that the 32-kDa protein is likely to be the nuclease responsible for generation of TUNEL-positive cells after γ-irradiation.

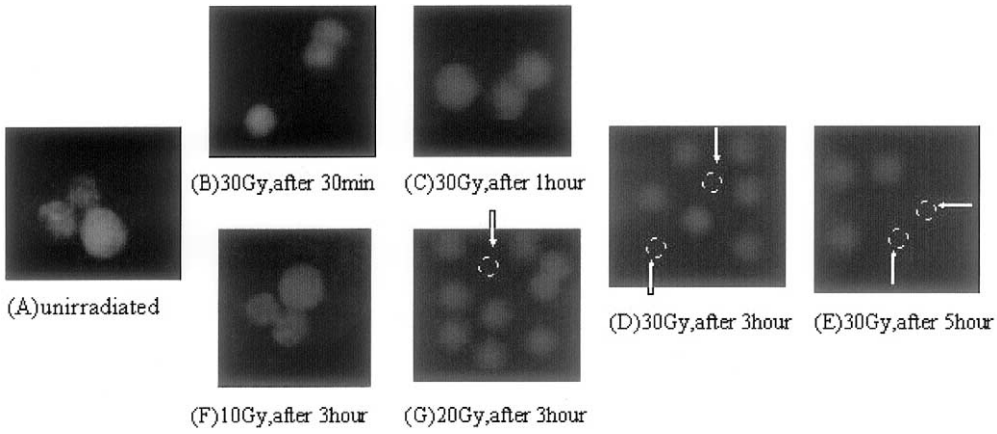


Fig. 7. Lysosomal stability assay was performed. In cells examined by fluorescence microscopy after acridine orange, the number of intact lysosomes was found to decline slightly at 3 hs after exposure to 30 Gy γ-irradiation (D, arrow), and at 5 h the cells which had no intact lysosomes increased (E, arrow). Dose dependently, the number of intact lysosomes declined on exposure to 20 ~ 30 Gy irradiation.

These results resemble those in UV(C)-irradiation of HL60 cells (17). However, when HL60 cells were irradiated with a high dose of γ-rays, and/or incubated after irradiation, there was a tendency for DNase II and acid phosphatase in the nuclear extract to decrease. However, too much progress in apoptosis results in partially degraded cells, which could affect separation of nuclear and cytoplasmic components. Therefore the acid phosphatase activity in the nuclear extract was not parallel to the acid phosphatase activity in the cytoplasmic extract.

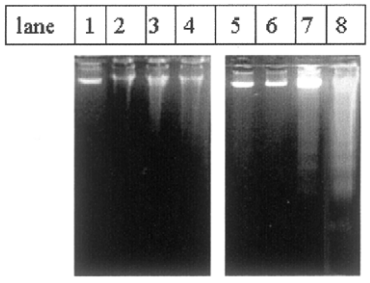
At the beginning of apoptosis, however, the translocation of DNase II and acid phosphatase occurred normally. Furthermore, terminal transferase used for TUNEL reaction extends 3′-hydroxyl ends of DNA (21). Caspase-activated DNase (CAD), which is active at neutral pH (22) and cleaves DNA leaving 5′-phosphate and 3′-hydroxyl ends, is responsible for the cells autonomous generation of TUNEL-reactive DNA fragments. An assay of in vitro apoptosis indicates that anti-DNase II antibody can prevent DNA fragmentation in a nuclear extract cell-free system in pH4 and DNase II is the main DNase concerning radiation-induced apoptosis in HL60 cells. Therefore in our study, the most likely candidate for the acid DNase in lysosomes that causes DNA fragmentation is DNase II (12, 23, 24). However, it is unlikely that the action of DNase II directly generates TUNEL-positive cells, because cleavage of DNA by DNase II generates 5′-hydroxyl and 3′-phosphate ends that cannot be used as substrates for terminal transferase. Lysosomes are rich in acid phosphatases that can remove the phosphate group from the 3′ end of DNA fragments. As a result of these above findings, acid phosphatase activity in the nuclei of HL60 cells increased time- and dose-dependently after γ-irradiation, and a lysosomal stability assay demonstrated that the numbers of intact lysosomes decline at the latter period of apoptosis and necrosis. These results suggest that acid phosphatase was translocated from lysosomes into the nuclei at the beginning of γ-radiation-induced apoptosis. However, cytoplasmic damage occurred

Table 2

The rate of cells without fluorescence by acridine orange

(A) Time-dependent manner (after 30 Gy irradiation)						
Incubation time	Unirradiated	0 min	30 min	1 h	3 h	5 h
AOP	98.8%	98.6%	97.7%	97.4%	84.5%	58.2%
WF	1.2%	1.4%	2.3%	2.6%	15.5%	41.8%
(B) Dose-dependent manner (for 3 h incubation after irradiation)						
Radiation dose (Gy)	Unirradiated		5	10	20	30
AOP	98.8%		97.3%	96.9%	88.3%	84.5%
WF	1.2%		2.7%	3.1%	11.7%	15.5%

Abbreviations: AOP = rate of acridine orange positive cells; WF = rate of cells without fluorescence.



lane	1	2	3	4	5	6	7	8
Gy	0	30	30	30	0	30	30	30
Additive	null	AD II	ICAD	null	null	AD II	ICAD	null
pH	7				4			

abbreviation : AD II ; Anti-DNase II

ICAD; Caspase Activated DNase Inhibitor

Fig. 8. Effects of anti-DNase II antibody or ICAD (caspase-activated DNase) on the formation of DNA fragmentation. Intact nuclei of HL60 cells and irradiated or non-radiated nuclear extracts were mixed and anti-DNase II antibody or ICAD was added to them: lane 1: non-radiation nuclear extract in pH7; lane 2: 30 Gy irradiated nuclear extract and anti-DNase II in pH7; lane 3: 30 Gy irradiated nuclear extract and ICAD in pH7; lane 4: 30 Gy irradiated nuclear extract without additives in pH7; lane 5: non-radiation nuclear extract in pH4; lane 6: 30 Gy irradiated nuclear extract and anti-DNase II in pH4; lane 7: 30 Gy irradiated nuclear extract and ICAD in pH4; lane 8: 30 Gy irradiated nuclear extract without additives in pH4; anti-DNase II antibody inhibited the formation of DNA ladder in pH 4 (lane 6). However, the inhibition of DNA fragmentation by ICAD was only partial in pH 7 (lane 3), and in pH 4 the formation of DNA fragmentation was not inhibited by ICAD (lane 7).

by leakage of acid phosphatase at the latter period of apoptosis and necrosis when the rupture of lysosomes occurred. Thus, it is possible to assume that DNase II, in combination with acid phosphatase in lysosomes, generates TUNEL reactive ends.

A hypothetical mechanism for TUNEL-positive apoptosis in HL60 cells is proposed in Fig. 9; γ-irradiation would directly or indirectly activate DNase II by way of acidification. This hypothesis is supported by the recent results of several papers set out below. Intracellular acidification, caused by agents such as UV(C), Fas receptor activation, treatment with ceramide, etoposide (25), withdrawal of growth factors or protein kinase C inhibition (26), is suitable for the release and the activation of DNase II. Further research will be necessary to study the association between DNase II and sensitivity to γ-rays using various cell lines during γ-irradiation-induced apoptosis.

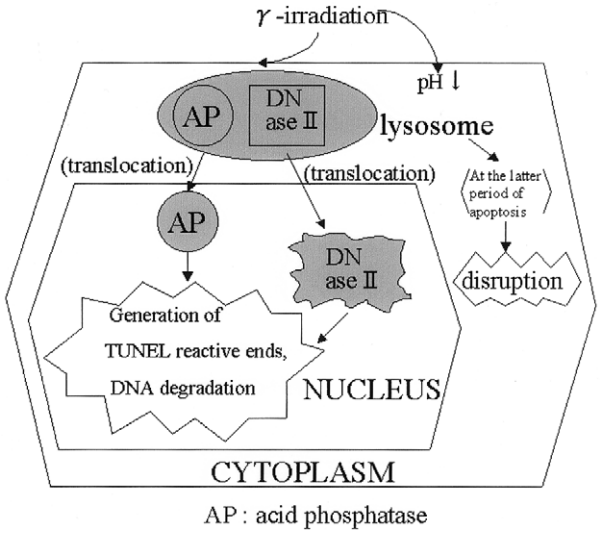


Fig. 9. Hypothetical role of DNase II and acid phosphatase (AP) in γ-ray-induced apoptosis. DNase II and AP are localized in the cytoplasm (lysosome) of living cells. γ-irradiation induces their nuclear translocation. DNase II is responsible for DNA degradation, and generates TUNEL reactive ends in combination with AP.

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