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Enhancement of Radiation-induced Apoptosis by 6-Formylpterin

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Radiation-induced apoptosis and its possible enhancement in the presence of 6-formylpterin (6-FP), a metabolite of folic acid, were examined in human myelomonocytic lymphoma U937 cells. When cells were treated with 6-FP at a nontoxic concentration of 300 μM, and then exposed to X-rays at a dose of 10 Gy, significant enhancement of radiation-induced apoptosis as determined by nuclear morphological change, phosphatidylserine (PS) externalization and DNA fragmentation were observed. Flow cytometry for the detection of intracellular hydrogen peroxide (H_2O_2) revealed that 6-FP increased the formation of intracellular H_2O_2, which further increased when the cells were irradiated. Decrease of mitochondria *trans*-membrane potential (MMP), release of cytochrome c from mitochondria, and activation of caspase-3 were enhanced after the combined treatment. Remarkable activation of protein kinase C δ (PKC δ) and its translocation from cytosol to mitochondria were detected in combined treatment. Increase of intracellular Ca^{2+} concentrations ($[Ca^{2+}]$i) was also observed, however, neither calpain I nor calpain II could inhibit the apoptosis. In addition, c-Jun NH_2-terminal kinase (JNK) activation was not enhanced in the combined treatment. A protein involved in a caspase-independent apoptosis pathway, apoptosis inducing factor (AIF), remained unchanged even 3 h after treatment. These results indicate that intracellular H_2O_2 generated by 6-FP enhances radiation-induced apoptosis via the mitochondria-mediated caspase-dependent pathway, with the active involvement of PKC δ.

Keywords: Radiation; Apoptosis; 6-Formylpterin; ROS; PKC δ

INTRODUCTION

Oxidative stress is known to induce many forms of apoptosis, and as its common mediator, intracellular reactive oxygen species [ROS, such as hydroxyl radicals (•OH), superoxide anion radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2)] play a cardinal role in oxidative stress-induced apoptosis.[1] H_2O_2, as a species of ROS, is able to induce apoptosis in several types of cells leading to further investigation of extracellular H_2O_2 as an apoptosis inducer. In a previous study, extracellular H_2O_2 induced apoptosis in human myelomonocytic lymphoma U937 cells, which was associated with protein kinase C δ (PKC δ) activation and release of cytochrome c from mitochondria.[2] In another study, extracellular H_2O_2 induced apoptosis in rat kidney tubule epithelial NRK-52E cells, which was associated with activation and up-regulation of c-Jun NH_2-terminal kinase (JNK);[3] while extracellular signal-regulated protein kinase (ERK) involvement and Ca^{2+} signaling were suggested in H_2O_2-induced apoptosis on chondrocytes.[4] In contrast to apoptosis induced by extracellular H_2O_2, only a few studies have examined apoptosis induced by intracellular H_2O_2.[5]

On the other hand, ionizing radiation such as X-rays, intracellularly generates ROS which play important roles in the cell killing. This is the so called "indirect action" of ionizing radiation. Initially, ionizing radiation produces ˙OH which subsequently recombine with another ˙OH to generate H_2O_2, aqueous electrons which react with O_2 to form superoxide $O_2^{\cdot -}$, and hydrogen atoms to create $O_2^{\cdot -}$ in the presence of O_2. These ROS cause DNA and membrane damage to induce cell death and/or cellular dysfunction.[6]

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Here we used an intracellular H_2O_2 inducer, 6-formylpterin (6-FP), which is a member of the pterin family. Pterins, the 2-amino-4-hydroxy-pteridine derivatives, occur in conjugated or unconjugated form in a wide range of biological systems. Six-FP contains a CHO-residue in the 6-substituted conjugated pterin and this agent was found to be more abundant in some cancer cells.[7,8] Among the pterins, 6-FP contributes unique biological functions: (i) it is able to scavenge intracellular superoxide $O_2^{\cdot -}$ as an inhibitor of xantinoxidase, (ii) it also has the control function for inducible nitric oxide synthase[9,10] and, most importantly among the conjugated pterins, (iii) it has a unique property to transfer electron from NAD(P)H to oxygen and this property is considered to lead to intracellular H_2O_2 generation in living cells. A high concentration of 6-FP generates intracellular H_2O_2, which induces apoptosis of human promyelocytic leukemia HL-60 cells and suppresses proliferation of human panc-1 pancreatic cancer cells.[9] Since 6-FP is a good tool to produce H_2O_2 intracellularly, yielding an effect on the cells different from that of extracellular H_2O_2. The implied low toxicity of 6-FP and its ability to continuously generate intracellular H_2O_2 both suggest a possible potential of this agent to be an enhancer of apoptosis at lower nontoxic concentrations in combination with ROS associated physical stimuli such as ionizing radiation.

A human lymphoma U937 cell line lacks p53, making this a good model for research on p53-independent apoptotic pathway. Previously we have investigated and reported apoptosis of U937 cells induced by hyperthermia or by cadmium which involved the elevation of intracellular Ca^{2+} and the mitochondria-mediated caspase-dependent pathway. In this present study, we investigate the effects of non-lethal, continuous, and intracellular generation of H_2O_2, using 6-FP as an intracellular H_2O_2 generator, on radiation induced apoptosis of U937 cells.

METHODS

Cell Culture and Treatment

A human myelomonocytic lymphoma cell line U937 was obtained from Human Sciences Research Resources Bank, Japan Human Sciences Foundation, Tokyo, Japan. The cells were grown in RPMI 1640 culture medium containing 10% heat-inactivated fetal bovine serum. The cells were also screened and found to be free from mycoplasma contamination. Six-FP was initially dissolved in 0.1 M NaOH to make a 60 mM solution, and then diluted with RPMI 1640 medium containing 10% fetal bovine serum to a final concentration of 300 μM. The cells were irradiated at a dose of 10 Gy using an X-ray machine (MBR-1520-3, Hitachi Medical Technology Corporation, Tokyo, Japan), with or without pretreatment of 300 μM 6-FP for 1 h. After treatment, the cells were incubated at 37°C with 5% CO_2 and then harvested after 6 h for apoptosis evaluation.

Assessment of Apoptosis

Quantitative DNA fragmentation assay was carried out according to the method of Sellins and Cohen.[11] The morphological changes in the cells were examined by Giemsa staining. To identify the apoptotic cells after exposure to radiation, the cells harvested after 6 h of incubation at 37°C were washed with Dulbecco's PBS and collected by centrifugation. The cells were fixed with methanol and acetic acid (3:1) and spread on glass slides. After drying, staining was performed with 3% Giemsa solution (pH 6.8) for 15 min. The apoptotic cells were determined by counting a total of 1000 cells per sample in randomly selected areas. Phosphatidylserine (PS) externalization of apoptosis was determined by analysis of Annexin V/fluorescein isothiocyanate (FITC) binding using flow cytometry (EPICS XL™, Beckman-Coulter, Miami, FL) according to the instructions of the manufacturer. In addition to Annexin V/FITC binding, propidium iodide (PI) uptake was also determined to assess secondary necrosis.[12]

Assessment of Intracellular H_2O_2

Evaluation of intracellular H_2O_2 in U937 cells was performed by flow cytometry using the fluorescence generated from cells loaded with the peroxide sensitive fluorescent probe, a H_2O_2 sensitive dye, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Eugene, OR).[13] DCFH-DA rapidly diffuses into the cytosol of cells where it is hydrolyzed to the non-fluorescent, oxidation sensitive DCFH. In the presence of cytosolic peroxide, DCFH is rapidly oxidized to the non-diffusible, fluorescent DCF. The cells were pretreated with 300 μM 6-FP for 40 min, then DCFH-DA was added at final concentration of 5 μM, 20 min after the cells were irradiated and incubated at 37°C for 30 min. Then the cells were harvested and the fraction of DCF fluorescence positive cells was measured by flow cytometry as the cells generating intracellular H_2O_2.

Mitochondria *Trans*-membrane Potential (MMP)

To examine the MMP, 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] was employed. The cells were harvested 6 h after irradiation with or without 6-FP

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and exposed to 40 nM $DiOC_6(3)$ (Wako Pure Chemical Industries, Ltd, Tokyo, Japan) in 1 ml of PBSF (PBS plus 1% fetal bovine serum) for 15 min at 37°C. Fluorescence intensities of $DiOC_6(3)$ were analyzed on a flow cytometer with excitation and emission settings of 484 and 500 nm, the fraction of cells showing low MMP was measured as detection of $DiOC_6(3)$ stained cells.

Changes in Intracellular Ca^{2+} Concentrations ($[Ca^{2+}]i$)

Six hours after treatment, cells were collected by centrifugation and washed with $[Ca^{2+}]$i-free HEPES-buffered Ringer solution (HR: pH 7.4, 118 mM NaCl, 4.7 mM KCl, 1.13 mM $MgCl_2$, 1.0 mM Na_2HPO_4, 5.5 mM glucose, and 10 mM HEPES). The buffer was supplemented with 0.2% bovine serum albumin Fraction V (Sigma), 2% minimal Eagle's essential amino acids (Flow Laboratories, Irvine, UK), and 2 mM L-glutamine. About 3×10^5 cells in 1 ml of HR were loaded with Fura-2/AM for 30 min at 37°C. Then an aliquot of cell suspension (10 μl) was transferred to a glass bottom dish coated with Cell-Tak TM (BD Biosciences, Bedford, MA) and left for 10 min. After addition of HR to the chamber, digital imaging by Fura-2 fluorescence (ratio of 340:380 nm at 510 nm) was carried out using an inverted microscope (TE300, Nikon, Tokyo, Japan) and a digital image processor (Argus 50 CA, Hamamatsu Photonics, Hamamatsu, Japan) as reported previously.[14,15]

Assessment of Intracellular $O_2^{\cdot -}$ Radical

To detect the cells with high $O_2^{\cdot -}$, we used hydroethidine (Molecular Probes, Eugene, OR), a dye that is oxidized within the cell and fluoresces when it intercalates into DNA. The levels of $O_2^{\cdot -}$ were measured using the method employed by Gorman *et al.*[16] Briefly, the cells were incubated with 4 μM HE for 15 min at 37°C. After washing twice with PBS, the fraction of HE positive cells was measured by flow cytometry as generation of the intracellular $O_2^{\cdot -}$.

Assessment of Intracellular Caspases Activity

The cell-permeable fluorogenic substrate (PhiPhiLux-G1D2) was used to monitor the intracellular caspase-3 activity according to the manufacturer's recommendations (OncoImmunin Inc., Gaithersburg, MD). Briefly, a sample with 50 μl of 10 μM PhiPhiLux-G1D2 substrate solution in RPMI 1640 was supplemented with 10% fetal bovine serum. After incubation for 1 h at 37°C in the dark the samples were washed once and diluted with 0.5 ml of ice-cold flow cytometry dilution buffer. The fraction of cells showing high caspase-3 activities was measured by flow cytometry according to the methods described elsewhere.[17]

To measure capase-8 activity, we employed FLICE/Caspase-8 Colorimetric Protease Assay Kit (MBL, Nagoya, Japan). The cells were harvested, lysed, and the protein collected. At 100 μg protein per sample, 50 μl of 10 mM dithiothreitol (DTT) was mixed to each sample and IETD-pNA substrate was added at a final concentration of 200 μM and then incubated at 37°C for 2 h before doing colorimetry at 400 nm using a spectrophotometer (Beckman Instruments Inc., CA).[18]

Flow Cytometric Detection of Fas on Cell Surface

Cells (2×10^5) were washed twice with PBS, resuspended in 50 μl washing buffer containing 2.5 μg/ml FITC-conjugated anti-Fas (MBL, Nagoya, Japan) and incubated for 30 min at room temperature. After staining, cells were washed twice and resuspended in PBS. Data acquisition and analysis were performed using the flow cytometer.

Isolation of the Cytosolic Fraction

Cells were suspended in an ice-cold solution containing 20 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 250 mM sucrose. The cells were disrupted by Dounce homogenization. After centrifugation at 1500*g* for 5 min at 4°C, the supernatants were centrifuged at 105,000*g* for 30 min at 4°C. The resulting supernatant was used as the soluble cytoplasmic fraction.

Isolation of the Mitochondrial Fraction

Cells were suspended in ice-cold solution containing 5 mM HEPES (pH 7.5), 210 mM mannitol, 1 mM EGTA, 70 mM sucrose, and 110 μg/ml digitonin. The cells were disrupted in a glass homogenizer (Pyrex No. 7727-07) and centrifuged at 2000*g* for 20 min at 4°C. The pellets were resuspended in the same buffer, homogenized again (Pyrex No. 7726), and centrifuged at 2000*g* for 5 min at 4°C. The supernatants (S1) were collected. The pellets were re-homogenized, centrifuged at 2000*g* for 5 min, and the resultant supernatants (S2) collected. Supernatants S1 and S2 were pooled and centrifuged at 11,000*g* for 10 min. The mitochondrial pellets were resuspended in lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium vanadate, 1 mM phenylmethylsulphonyl fluoride, 1 mM DTT, 10 μg/ml leupeptin, and 10 μg/ml aprotinin] for 30 min on ice and then centrifuged

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at 15,000*g* for 20 min. The supernatant was used as the soluble mitochondrial fraction. Protein concentration was determined using BioRad protein estimation kit (Bio-Rad laboratories, Hercules, CA).

Western Blot Analyses of Proteins

Cells were collected at indicated time and washed with cold PBS. Cells were lysed at a density of 10^6 cells/50 μl of lysis buffer (1 M Tris–HCl, 5 M NaCl, 1% Nonidet P-40 (v/v), 1% sodium deoxycholate, 0.05% SDS, 1 mM phenylmethylsulfonyl fluoride) for 20 min. After brief sonication, the lysates were centrifuged at 12,000 rpm for 10 min at 4°C, and protein content in the supernatant was measured using a Bio-Rad protein assay system. The proteins were boiled at 95°C for 10 min after mixing 1:1 with SDS-loading buffer (62.5 mM Tris–HCl, 20% SDS, 5% 2-mercaptoethanol, 10% glycine, 0.001% bromophenol blue) and applied with SDS polyacrylamide gel for electrophoresis (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). Western blot analyses of p-JNK, PKC δ, Bcl-2, Bcl-XL, Bax, Bid, AIF, cytochrome c and β-actin were performed using specific polyclonal or monoclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as described elsewhere.[19,20] For detection of those specific antibodies, chemiluminescence agent, ECL, was used following the manufacturer's instruction (Amersham Biosciences, Buckinghamshire, UK).

RESULTS

Enhancement of Radiation-induced Apoptosis by 6-FP

X-rays induced a dose and time dependent increase in apoptotic cells at the indicated doses and times, as measured by DNA fragmentation (Fig. 1a,b). The DNA fragmentation peaked at 6 h after irradiation at a dose of 10 Gy. Treatment of the cells with different concentrations of 6-FP for 6 h did not induce significant DNA fragmentation even up to 500 μM (data not shown); a significant but minimal increase in DNA fragmentation was observed only at 1000 μM. Interestingly, when cells were cotreated with irradiation at a dose of 10 Gy and 6-FP at 300 μM, radiation-induced DNA fragmentation (41.4%) was enhanced significantly (60.2%) (Table I). The observation on cell morphology illustrated in Fig. 1c, shows that no changes in the 6-FP-treated cells as in the control cells. However, when the cells were treated with both 6-FP and irradiation, typical features of apoptotic cells such as cytoplasmic aggregation, nuclear condensation and fragmentation were more prominent than in cells treated with X-rays alone (Fig. 1c, Table I). Flow cytometry using Annexin V/FITC and PI double staining shows that early apoptosis (only Annexin V/FITC staining) significantly increased in cells treated with 6-FP and radiation, than in cells treated with radiation alone. However, the % of secondary necrotic cells (Annexin V/ FITC and PI double stained cells) did not significantly change (Table I).

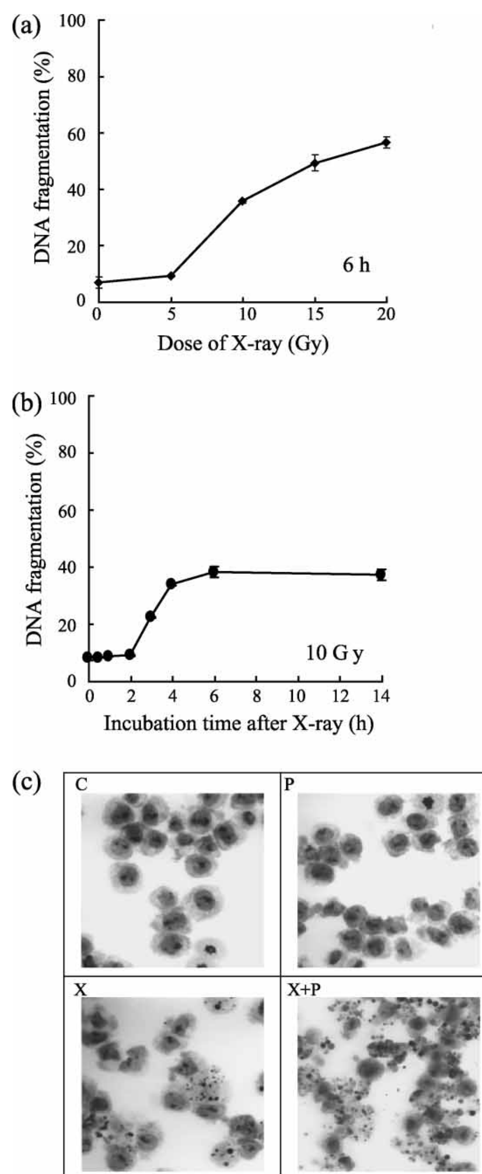

FIGURE 1 DNA fragmentation induced by radiation and 6-FP. (a) U937 cells were irradiated at various doses and harvested at 6 h. (b) U937 cells were irradiated (10 Gy) and harvested at the indicated time. DNA fragmentation assay was carried out according to the method of Sellins and Cohen. The data presented as the mean ± SD ($n = 4$). (c) U937 cells were treated with radiation (10 Gy) with or without pre-incubation of 6-FP (300 μM) for 1 h, and cells were harvested 6 h after irradiation. Signs of apoptosis were detected by Giemsa staining and then examined under a microscope at a magnification of 10 × 40. (C, control; P, 6-FP alone; X, radiation alone; X + P, combined treatment with radiation and 6-FP.)

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TABLE I Radiation-induced apoptosis enhanced by 6-FP

	Control (%)	6-FP (%)	X-rays (%)	X-rays + 6-FP (%)
Fraction of apoptosis	1.5 ± 0.7	2.0 ± 0.8	30.6 ± 4.5	57.1 ± 3.2*
DNA fragmentation	6.9 ± 1.6	9.5 ± 1.0	41.4 ± 3.4	60.2 ± 2.4**
Early apoptosis	0.6 ± 0.1	1.9 ± 0.6	22.6 ± 3.5	46.1 ± 5.2**
Secondary necrosis	0.7 ± 0.5	0.6 ± 0.3	0.8 ± 0.2	0.6 ± 0.2***

Data are mean ± SD; $n = 3$; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p > 0.05$ vs. X-ray groups.

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Generation of H_2O_2 and $O_2^{\cdot -}$

It is known that 6-FP is able to generate intracellular H_2O_2.[9] This could be an important factor involved in the enhancement of apoptosis induced by irradiation. U937 cells were treated with 300 μM of 6-FP for 1 h before irradiation. DCFH-DA, a H_2O_2 sensitive dye, was added to the cells 20 min before irradiation. The treated cells were incubated at 37°C for 30 min and then harvested for flow cytometry. The results showed that about 60.4% of 6-FP-treated cells generated intracellular H_2O_2 while irradiation also induced H_2O_2 generation in about 41.1% of the cells. Cotreatment with 6-FP and irradiation revealed H_2O_2 generation in almost 100% of the cells (Fig. 2). No detectable H_2O_2 generation was observed in the control cells. Using an HE probe to detect intracellular $O_2^{\cdot -}$ radical,[16] the cells were harvested at 6 h after irradiation according to a protocol used in H_2O_2 detection. Intracellular $O_2^{\cdot -}$ increased in cells treated with irradiation alone. Cotreatment with 6-FP cells revealed total inhibition of radiation-induced $O_2^{\cdot -}$ production (data not shown). This result is consistent with a previous report describing that 6-FP consumes oxygen and generates H_2O_2 with $O_2^{\cdot -}$ as an intermediate.[9]

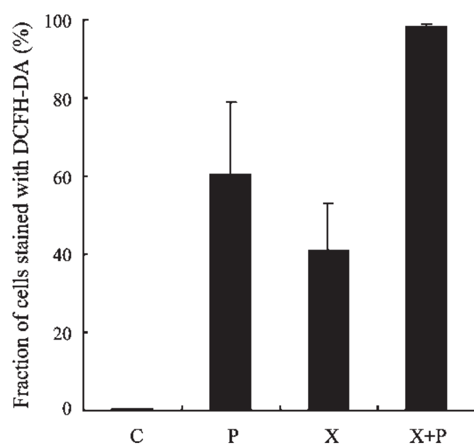

FIGURE 2 Intracellular H_2O_2 induced by 6-FP in U937 cells. The cells were pre-incubated for 40 min with or without 300 μM 6-FP, then 5 μM of DCFH-DA was added to the cells and incubated for 20 min before irradiation (10 Gy). After irradiation, cells were incubated for 30 min before measurement of the intracellular H_2O_2 by flow cytometry. The bars in the figure indicate the means ± SD ($n = 3$). (C, control; P, 6-FP alone; X, radiation alone; X + P, combined treatment with radiation and 6-FP.)

Detection of Mitochondria Mediated Caspase Dependent Pathway

Apoptosis is a highly regulated form of cell death distinguished by the activation of caspases that cleave various proteins, resulting in cell death.[21] Mitochondria plays a central role in activating caspases by releasing cytochrome c to the cytoplasm.[22,23] To determine whether the mitochondria mediated caspase-dependent pathway is involved in 6-FP-enhanced radiation-induced apoptosis, we used a mitochondria voltage-dependent dye, $DiOC_6(3)$,[24] to monitor MMP; activation of caspases was also studied. At 6 h after irradiation, cells with low MMP were increased significantly, and increased further when the cells were cotreated with 6-FP (Fig. 3a). Six-FP alone did not affect the MMP. At 6 h incubation after treatment, the activity of caspase-8 increased by X-irradiation and more increase was observed by cotreatment with 6-FP (Fig. 3b). Caspase-3 activity was measured using the phiphilux G1D2 kit. The results revealed that caspase-3 activity was significantly increased by irradiation alone and a further increase was observed in the cells cotreated with 6-FP (Fig. 3c). The changes in the expression of cytochrome c were determined by western blot. The result showed that there was a release of cytochrome c from the mitochondria to the cytosol when cells were irradiated and more cytochrome c was released when cotreatment with 6-FP was done (Fig. 4a). These results suggest that the enhancement of apoptosis by 6-FP involves the mitochondria-caspase dependent pathway.

The Changes of PKC δ and Bcl-2 Family

According to a previous study, PKC δ can be activated by extracellular H_2O_2 treatment in U937 cells to enhance release of cytochrome c from mitochondria.[2] Over-expression of PKC δ enhances radiation sensitivity in NIH3T3 cells.[25] In this study the level of PKC δ in the cytosol was not significantly changed by irradiation or by 6-FP treatment. In the combined treatment, however, apparent activation of PKC δ by cleavage from 78 kD to an active form, ~40 kD protein, and subsequent translocation of the cleaved form to the mitochondria were observed (Fig. 4a).

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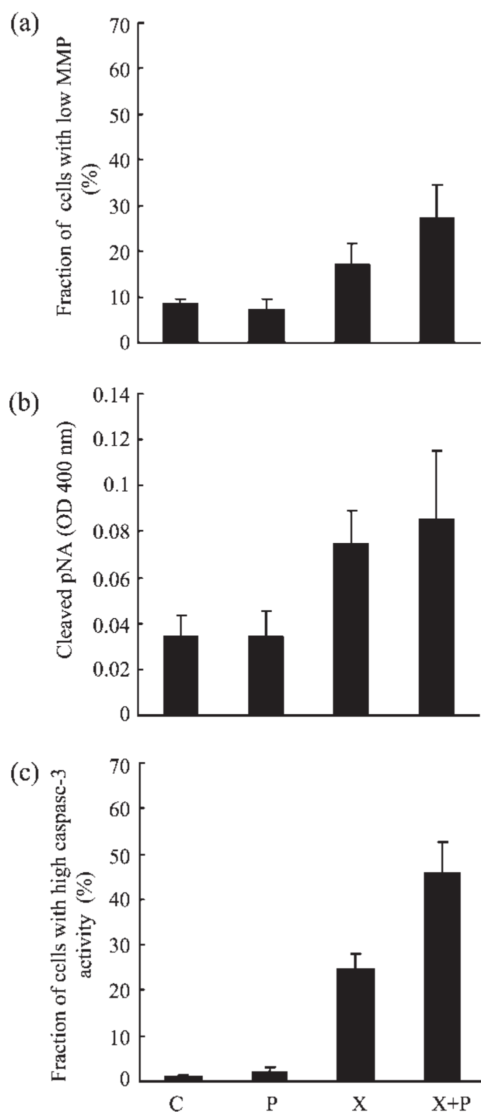

FIGURE 3 Loss of MMP and activation of caspase-8 and -3. U937 cells were pre-incubated with 6-FP (300 μM) for 1 h before irradiation (10 Gy), then the cells were harvested 6 h after irradiation. (a) Loss of MMP was noted in X-irradiated cells and was enhanced in the presence of 6-FP as measured by flow cytometry using $DiOC_6(3)$ staining. (b) Activation of caspase-8 was measured by FLICE/Caspase-8 Colorimetric Protease Assay Kit. (c) Radiation-induced activation of caspase-3 was enhanced by 6-FP as measured by flow cytometry using PhiPhiLux-G1D2 staining. All results are presented as the means ± SD ($n = 5$). (C, control; P, 6-FP alone; X, radiation alone; X + P, combined treatment with radiation and 6-FP.)

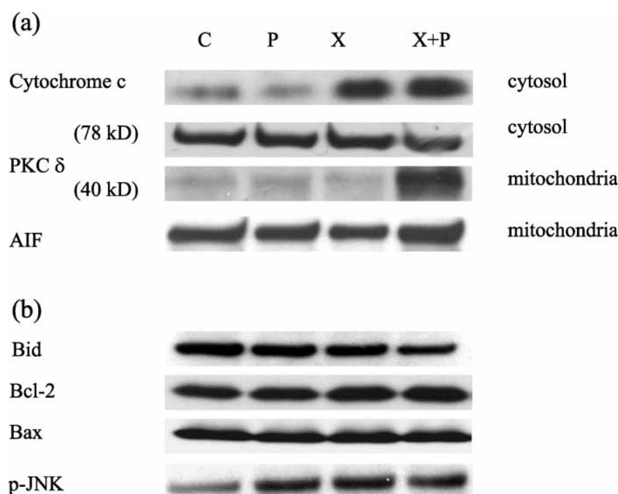

FIGURE 4 The changes in the expression of proteins. U937 cells were pre-incubated with 300 μM 6-FP for 1 h before the 10 Gy irradiation and the cells were harvested 3 h later for protein analysis by Western blot method. (a) Cleavage of PKC δ in the cytosol and its translocation to the mitochondria and expression of AIF in the mitochondria. (b) The changes of Bcl-2 family proteins and expression of p-JNK. (C, control; P, 6-FP alone; X, radiation alone; X + P, combined treatment with radiation and 6-FP.)

The Bcl-2 family of proteins that has anti-apoptotic or pro-apoptotic functions can control the release of mitochondrial apoptosis factors including cytochrome c and apoptosis inducing factor (AIF).[26] In this study, we used western blot analysis to determine the expression of Bcl-2 and two of its members, Bid and Bax. No decrease in the expression of Bid was noted in the cells treated with radiation, but a significant decrease was observed in the combined treatment (Fig. 4b). No changes in the expression of Bcl-2 and Bax were observed. The results suggest that PKC δ and Bid activation are upstream to mitochondrial activity in the apoptotic pathway.

Activation of SAPK/JNK, AIF and Expression of Fas

According to previous reports, SAPK/JNK and Fas are involved in the induction of ROS related apoptosis.[27,28] Since it has been shown that 6-FP generates intracellular H_2O_2, we investigated phosphorylation of JNK (p-JNK) as the activation of SAPK/JNK and expression of Fas in the enhancement of radiation-induced apoptosis by 6-FP. Activation of JNK was measured by the expression of p-JNK using western blot 1 h after X-irradiation, the results showed that p-JNK was increased in U937 cells treated with 6-FP or irradiation alone, but no enhancement was detected in the cells cotreated with radiation and 6-FP (Fig. 4b). Expression of Fas was noted in 5.6% of cells treated by radiation alone, 6.8% in the cells cotreated with radiation and 6-FP, and 4.9% in the control cells. To determine the possible involvement of a caspase independent pathway, AIF was investigated. AIF induces apoptosis by translocating to the nucleus from mitochondria by a process independent of caspases.[29] The result showed that the level of AIF in mitochondria is not changed in any of the treatments, and no observable translocation to the nucleus was detected (Fig. 4a). The results suggest p-JNK, AIF and Fas are not involved in the enhancement of radiation-induced apoptosis by 6-FP.

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Elevation of $[Ca^{2+}]i$ Concentration

ROS can be used as a messenger in normal cell functions. However, at oxidative stress levels they can disrupt normal physiological pathways and cause cell death, such a switch being largely mediated through Ca^{2+} signaling.[30] In this study, changes of $[Ca^{2+}]i$ concentration in cells were observed by fluorescence microscopy using Fura-2 AM stain. U937 cells treated with radiation, 6-FP, or both were harvested after an incubation time of 6 h. The $[Ca^{2+}]i$ concentration increased in irradiated cells, and cotreatment with 6-FP significantly enhanced the radiation-induced $[Ca^{2+}]i$ concentration increase (Fig. 5). Previously, we reported that calcium-dependent protease calpain take part in the $[Ca^{2+}]i$-dependent pathway of apoptosis induced by cadmium in U937 cells, but in this experiment, unexpectedly neither calpain inhibitor I nor II influenced the enhancement effects of 6-FP on radiation-induced apoptosis (data not shown). Whether or not the changes in $[Ca^{2+}]i$ are involved in the enhancement needs further investigation.

DISCUSSION

In this study, we have shown that 6-FP-generated intracellular H_2O_2 enhanced the radiation-induced apoptosis in U937 cells. The mechanism of this enhancement points to the involvement of the mitochondria-mediated caspase-dependent pathway.

Radiation induces transient intracellular H_2O_2 production that contributes to the induction of apoptosis of cells while 6-FP generates intracellular H_2O_2 continuously and at high doses is also able to induce apoptosis.[9,31] Our data show that both radiation and 6-FP induced intracellular H_2O_2 production, which was significantly increased in the combined treatment. Although H_2O_2 generated by 300 μM 6-FP alone was not capable of inducing apoptosis or initiating signal transduction for apoptosis, in combination with radiation, the sustained high level of H_2O_2 in a greater number of cells resulted in increased apoptosis. Therefore, we consider that this sustained high level of intracellular H_2O_2 by 6-FP in the combined treatment is likely responsible for the enhancement of radiation-induced apoptosis.

Caspases, a family of intracellular cysteine proteases, are the central executioners of apoptosis. Activation of the caspase cascade is associated with proteolytic cleavage of diverse structural and regulatory proteins that collectively contribute to the apoptotic phenotype.[32] Among the caspases, caspase-3 is the predominant executor of apoptosis. Caspase-8 is known to be located at upstream in the caspase cascade, this enzyme can be immediately activated in X-irradiated cells. This is capable of inducing the release of cytochrome c from the mitochondria.[33] This study affirmed the previous findings that irradiation activates caspases-3, -8 and induces loss of MMP associated with the release of cytochrome c to the cytosol. Caspase-3, but not caspase-8, was significantly enhanced when cells were cotreated with 6-FP at a dose not capable of inducing any of these events with 6-FP alone. These increased apoptotic activities in the combined treatment are consistent with the enhancement of radiation-induced apoptosis, suggesting that the mitochondria-caspase dependent pathway is involved in the enhancement of radiation-induced apoptosis by 6-FP.

To verify the role of H_2O_2 in the enhanced effect, we assayed p-JNK expression, Fas expression and PKC δ expression; all of these proteins are commonly associated with oxidative stresses on cells.[2,3,28] Fas and p-JNK are known to be up-regulated when cells are treated with extracellular H_2O_2,[34,35] while PKC δ is said to be activated in irradiated cells.[36] Our study showed that Fas expression is not changed on the membrane in the treated cells, suggesting its non-involvement in intracellularly generated H_2O_2 in contrast to the apoptosis induced by external H_2O_2. On the other hand, p-JNK was similarly increased in 6-FP or radiation treated cells but no enhancement was observed in the combined treatment. The increase of p-JNK suggests that oxidative stress was induced into the cells treated with either 6-FP or radiation but that p-JNK is not necessarily involved in the apoptosis induction.

A more important observation in this study is that PKC δ was activated in cells treated with both radiation and 6-FP. PKC δ is a member of the protein kinase C (PKC) family, a family of serine/threonine protein kinases, involved in intracellular signals that regulate growth, differentiation, and apoptosis.[37] High doses of radiation can activate PKC δ by cleavage from a 78 kD protein to a 40 kD active form.[36] PKC δ is also activated by H_2O_2 through its phosphorylation of threonine and was said to be followed by translocation to the mitochondria which would result in cytochrome c release leading to the induction of apoptosis in U937 cells.[2] In our results, activation of PKC δ was detected when cells were irradiated at 20 Gy, but not observed at 10 Gy (data not shown); a dose used in a previous study showing PKC δ activation. In the combined treatment, cytosolic 78 kD PKC δ was significantly decreased without increase of cytosolic 40 kD PKC δ. In the mitochondria however, 40 kD PKC δ was significantly increased but no 78 kD PKC δ was detected. The above data strongly suggest that in the combined treatment there was activation of PKC δ by cleavage into 40 kD protein; and this activated 40 kD PKC δ subsequently translocated to the mitochondria. In short, radiation activates PKC δ by cleavage and

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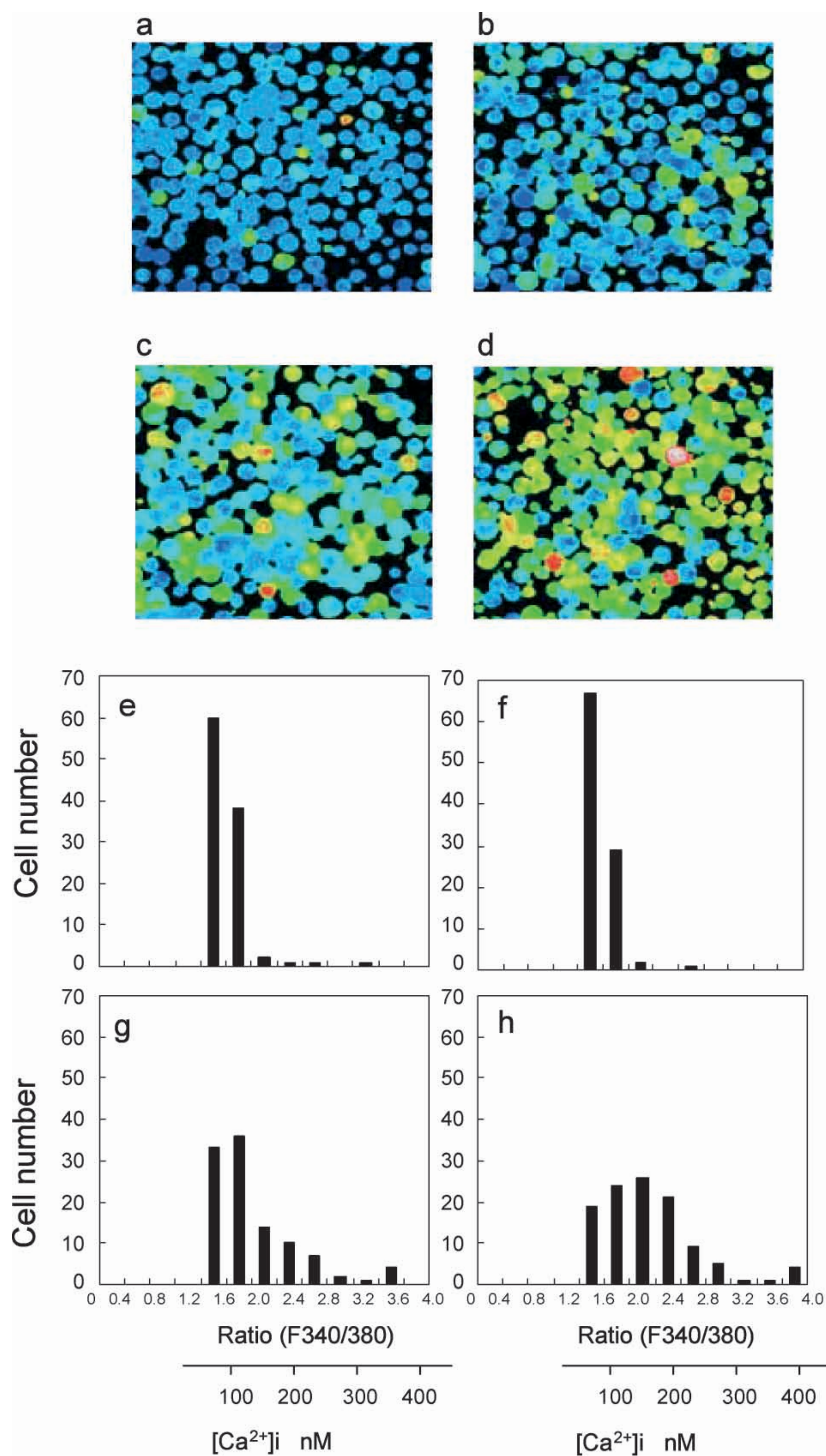

FIGURE 5 Elevation of $[Ca^{2+}]$i concentration. Concentration of $[Ca^{2+}]$i was increased by 10 Gy irradiation (c,g) and was enhanced in the presence of 300 μM 6-FP (d,h). The cells were stained with Fura-2/AM as described under "Material and Methods", section, before measurements of $[Ca^{2+}]$i and imaging (a to d, digital images of Fura-2 fluorescence; e to h, histograms of $[Ca^{2+}]$i).

H_2O_2 generated by 6-FP may be involved in the translocation of the activated PKC δ to the mitochondria. This is likely how 6-FP plays its role in the enhancement of radiation-induced apoptosis.

Another family of proteins, the Bcl-2 family, is involved in pro- or anti-apoptotic processes by interacting with the mitochondria.[22] Among them, Bcl-2 and Bcl-XL are known to be anti-apoptosis while Bid and Bax are known to be pro-apoptosis. Overexpression of Bcl-2 or Bcl-Xl in U937 cells is able to inhibit apoptosis induced by several environmental stimuli.[38] In this study, Bcl-2 and Bax were unchanged as shown by western blot analysis. Bid however was significantly decreased by radiation when cells were

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cotreated with 6-FP. Bid, a proapoptotic Bcl-2 family member containing BH3 domain, can be cleaved by caspase-8, and the cleaved Bid, the carboxyl-terminal fragment, translocates to the mitochondria to induce the release of cytochrome c, which is 500 times more numerous than Bax.[39,40] This downregulation of Bid strengthens involvement of mitochondria-caspase dependent pathway in the enhancement.

The above data point to the role of mitochondria in caspase-dependent pathway of apoptosis. To verify the possible role of mitochondria in the caspase-independent pathway, which usually involves activation of the AIF and eventual translocation of AIF to the nucleus; we assayed AIF expression in the mitochondria and its presence at the nucleus. Neither the changes of AIF expression at the mitochondria nor translocation to the nucleus were observed, thus downplaying the role for a caspase-independent pathway in apoptosis.

It was previously suggested that oxidative stress causes a rise in $[Ca^{2+}]i$ concentration in the cytoplasm, which induces $[Ca^{2+}]i$ influx into mitochondria and nuclei to control cell death.[4] Our result showed that $[Ca^{2+}]i$ concentration was elevated 6 h after irradiation in U937 cells. Treatment with 6-FP alone did not affect the $[Ca^{2+}]i$ concentration, but when the cells were cotreated with 6-FP and irradiation, a significant increase of $[Ca^{2+}]i$ was observed. Thus we suspect that elevation of $[Ca^{2+}]i$ concentration is involved in the induction and enhancement of apoptosis. In previous study, IP3Rs subtype IP3R1 receptor and calpain participated in the $[Ca^{2+}]i$ dependent pathway as in cadmium-induced apoptosis of U937 cells.[19,41] To verify further the role of $[Ca^{2+}]i$, we employed calpain inhibitors I and II, but the results showed that neither calpain inhibitor I nor II inhibits apoptosis induced by radiation and the combined treatment. Western blot analysis also revealed no changes in IP3R1 expression (data not shown). These results suggested that the $[Ca^{2+}]i$ pathway involving calpain and IP3R1 did not play an important role in our case, although other pathways involving $[Ca^{2+}]i$ are said to exist. The active involvement of PKC δ, a known calcium independent PKC, further denies role for $[Ca^{2+}]i$ in the 6-FP enhanced radiation-induced apoptosis. However, further study may be necessary to elucidate the possible role of $[Ca^{2+}]i$ in apoptosis.

In summary, 6-FP provided a more sustained level of nonlethal H_2O_2 intracellularly at the time when cells were X-irradiated. The irradiation resulted in an increased level of intracellular H_2O_2 leading to the enhanced activation of the mitochondria-mediated caspase-dependent pathway, with the significant involvement of PKC δ; the ultimate result was enhanced radiation-induced apoptosis (Fig. 6). This finding suggests that agents capable of generating

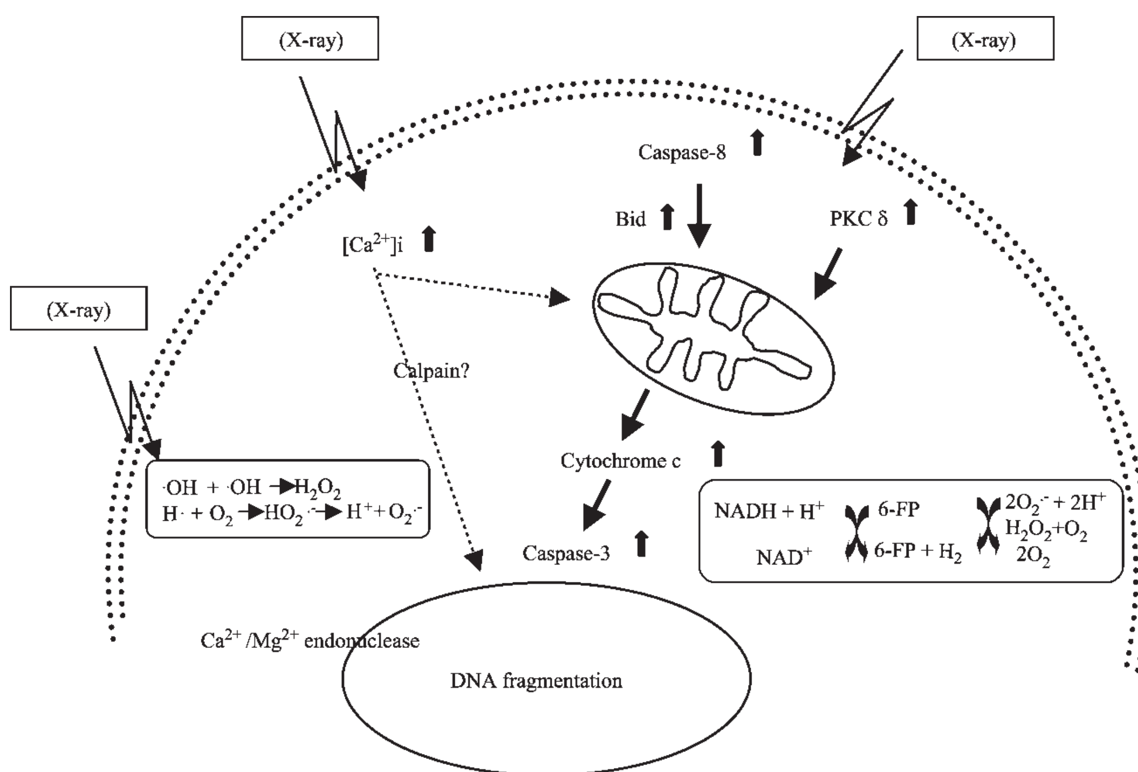

FIGURE 6 Schematic presentation of the mechanism of enhancement of radiation-induced apoptosis by 6-FP. X-ray activates caspase-8 and PKC δ by increasing the loss of MMP and the release of cytochrome c from mitochondria to the cytosol. Cytochrome c activates caspase-3 resulting in induction of apoptosis. 6-FP enhances this pathway by generating H_2O_2 intracellularly. Concentration of $[Ca^{2+}]i$ was elevated by radiation and enhanced by 6-FP. However, the role of $[Ca^{2+}]i$ in the enhancement effect is not clear.

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H_2O_2 intracellularly, such as 6-FP, can potentiate radiation-induced apoptosis.

Future studies to investigate the effects 6-FP on other cell lines, especially tumor cells may be required. Defense systems of the cells against oxidative stress in response to the treatments, such as those involving catalase, also need further scrutiny.

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