

Regular Article

Apoptotic Induction Mechanism of X-ray Irradiation Combined with Hydrogen Peroxide

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Kochi oxydol-radiation therapy for unresectable carcinomas (KORTUC) is a clinical therapy, that combines X-ray irradiation and localized hydrogen peroxide. However few studies have reported the basic biological mechanism of action of KORTUC. Therefore we examined the effects of using a combination of hydrogen peroxide and X-ray irradiation on cell death. Cell viability of DU145, SAS, and HL60 cells decreased upon X-ray irradiation combined with exposure to hydrogen peroxide, compared to that on X-ray irradiation alone. DNA fragmentation of HL60 cells increased when cells were exposed to the combination treatment in comparison to X-ray irradiation, and apoptosis was considered to have increased. Abasic sites by DNA oxidative damage were observed early during X-ray irradiation, but no such increase was observed with hydrogen peroxide treatment. Caspase-3 and caspase-9 activities increased with all the three types of treatment, whereas caspase-8 showed high activity on treatment with hydrogen peroxide and the combination. The appearance of Bax and cytochrome c on X-ray irradiation and western blot analysis indicated the involvement of caspase-9 activation. Thus, cytochrome c was released via Bax, and the pathway leading directly from caspase-8 to caspase-3 was inferred to be induced with hydrogen peroxide treatment. The results suggested that hydrogen peroxide treatment induces apoptosis through a cascade that is different from that induced by irradiation, and combination with hydrogen peroxide is considered to enhance the effect of irradiation.

Key words: radiation, hydrogen peroxide (H₂O₂), apoptosis, caspase, KORTUC (Kochi oxydol-radiation therapy for unresectable carcinomas)

1. Introduction

The primary treatment method used for radiation therapy is high energy irradiation by a linear accelerator (LINAC) device. Approximately 70% of the biological effect of the high energy radiation therapy on the tumor is considered to be caused by the indirect effect of the hydroxyl radical (OH radical) that is generated from water. The OH

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radicals play a major role in radiation-induced cell damage and induce apoptotic cell death by activating the pro-apoptotic pathways. Oxidation, which causes the radical reaction, is indispensable for cancer treatment, but the acid-deficiency caused to decrease the effect of radiation therapy. Radiation therapy has produced good aesthetic results compared to surgery, but the drop in the radical reaction is one of the reasons for low local control¹⁾.

Therefore, using a sensitizer in combination with radiation therapy was considered, and many drugs have been tried for this purpose. Hydrogen peroxide (H₂O₂) was one of the drugs tried, and KORTUC (Kochi oxydol-radiation therapy for unresectable carcinomas) therapy, which is a combination therapy involving localized injection of a hydrogen peroxide formulation is currently being used^{2, 3)}. Aoyama studied the effectiveness of KORTUC II for the treatment of chemotherapy-resistant supraclavicular lymph node metastases, recurrent breast cancer and stage IV primary breast cancer, and reported this treatment exhibited marked therapeutic effects with satisfactory treatment outcomes and an acceptable extent of adverse events³⁾. Because hydrogen peroxide generates OH radicals, it is considered to enhance the radical reaction when combined with radiation therapy, but only few studies have reported the biological mechanism of action. This study was conducted to elucidate the biological effects of hydrogen peroxide in combination with irradiation.

2. Materials and Methods

2.1. Materials

DU145 cells were purchased from RIKEN Quantitative Biology Center (Saitama, Japan), SAS cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and HL60 cells were purchased from Cosmo Bio (Tokyo, Japan). RPMI1640 medium and fetal bovine serum were purchased from Life Technologies (Grand Island, NY). Carbobenzoxy-IETD-fluoromethyl ketone (Z-IETD-FMK) and DNA oxidative damage kit were procured from Kamiya Biochemical (Seattle, WA). Z-Leu-Glu-His-Asp-FMK (LEHD-FMK) and Z-Asp-Glu-Val-Asp-FMK (DEVD-FMK) were purchased from Medical & Biological Laboratories Co., Ltd (Nagoya, Japan). Cellular DNA fragmentation ELISA kit was purchased from Roche Diagnostics (Mannheim, Germany). Polyclonal rabbit anti-human Bax antibody was obtained from PharMingen International (San Diego, CA), mouse anti-human cytochrome c monoclonal antibody from ProSci Inc. (Poway, CA), Horseradish peroxidase-conjugated sheep anti-mouse and donkey anti-rabbit Ig F(ab')₂ fragments were purchased from Amersham Pharmacia Biotech (Piscataway, NJ), and ECL western blotting detection reagents were from

Amersham Biosciences (Buckinghamshire, England). Block Ace, a blocking solution for immunoblotting, was purchased from Dainippon Pharmaceuticals (Osaka, Japan). Colorimetric assay kits of caspase-3, -8 and -9 activities were from BioVision (Palo Alto, CA). Diethylenetriaminepentaacetic acid (DETAPAC) was obtained from Wako Pure Chemical Industries (Osaka, Japan), and 5, 5-dimethyl-1-pyrroline N-oxide (DMPO) was from Labotec Corporation (Tokyo, Japan). Protein assay kits were obtained from Pierce (Rockford, IL) and Bio-Rad (Hercules, CA). All the other reagents were obtained from Wako Pure Chemical Industries and Sigma.

2.2. Cell culture

DU145 cells (prostate cancer cell line) and SAS cells (squamous cell carcinoma cell line) as epithelial cells and HL60 cells (leukemia cell line) as blood cells were used.

DU145, SAS, and HL60 cells were grown on 100 mm culture dishes in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂/95% air incubator. Passaging was carried out every 2 days for DU145 and SAS cells when they became 70% to 80% confluent, whereas HL60 cells were passaged every third day, and the density in culture was not allowed to exceed 1×10⁶ cells/ml.

2.3. Exposure to X-rays and hydrogen peroxide

Dishes or microtiter plates containing cells were exposed to X-rays from a soft X-ray machine (SOFRON BST-1500CX; Sofron, Tokyo, Japan) under the following conditions: tube voltage, 120 kVp; tube current, 5 mA; FSD (focus-surface distance), 30 cm; dose rate, 2 Gy/min; room temperature. The dose and dose rate were estimated with a Fricke chemical dosimeter. For the treatment, hydrogen peroxide was added to the cells to obtain a final concentration of 0.1 mM. Cells were exposed to X-rays just after treatment with hydrogen peroxide for the combination treatment.

2.4. Cell viability analysis

HL60 cell concentration was adjusted to 5×10⁵ cells/ml for the X-ray exposure treatment. After 24 h, the cells were stained with 0.5% trypan blue for 1 min with an unstained cell suspension maintained in 1 ml growth medium as the control. The cells were then counted to evaluate the number of viable cells.

The viability of DU145 and SAS cells was determined by colony assay. To assess colony formation, cells that were 80% confluent were scraped from the dishes and the required number of cells were seeded. After seeding, the dish was agitated to distribute the cells uniformly, and was incubated for 2 h. X-ray irradiation and hydrogen peroxide -treatments were carried out and the cells were

incubated for approximately 1 week. The culture medium was removed, cells were washed with 2 ml of PBS, and colonies were fixed by addition of 1 ml of methanol. After the methanol was removed, the cells were air-dried, subjected to Giemsa staining, and then colonies consisting of more than 50 cells were counted using an inverted microscope⁴.

2.5. DNA fragmentation assay by ELISA

This assay is based on the quantification of accumulated DNA fragments in the cytoplasm of apoptotic cells. The accumulation was determined with a cellular DNA fragmentation ELISA kit according to the manufacturer's protocol. Exponentially growing HL60 cells (5×10^5) were incubated with $10 \mu\text{M}$ 5'-bromo-2'-deoxyuridine (BrdU) overnight at 37°C , and rinsed twice with BrdU-free medium by centrifugation at $200 g$ for 10 min. After 2 hours exposure to radiation, the cells were collected by centrifugation at $250 g$ for 10 min. The cells (1.5×10^4) were then lysed in 0.05% Triton X-100 for 30 min at 4°C and centrifuged at $12,000 g$ for 30 min at 4°C . The supernatant ($100 \mu\text{l}$) was added to the wells of a microtiter plate, which had been pre-coated with anti-DNA antibody, and incubated with peroxidase-conjugated anti-BrdU monoclonal antibody ($100 \mu\text{l}$) for 90 min at room temperature. The activity of the bound peroxidase was determined photometrically at 450nm with 3,3',5,5'-tetramethylbenzidine (TMB) as the substrate.

2.6. Quantitative analysis of DNA damage

Quantification of abasic (apurinic/aprimidinic, AP) sites resulting from oxidative damage to DNA was performed photometrically with a DNA oxidative damage kit. DNA samples were prepared according to the procedure of Sellins and Cohen.⁵ HL60 cells (5×10^7) were treated with 2 Gy irradiation and/or 0.1 mM hydrogen peroxide and harvested by gentle centrifugation for 10 min. The cells were incubated in a buffer containing 10 mM Tris, 1 mM EDTA, and 0.2% Triton X-100 for 20 min on ice, and then centrifuged at $10,000 g$ for 10 min. Proteinase K ($0.5 \mu\text{g/ml}$) was added to the supernatant, incubated for 30 min at 37°C , and precipitated overnight with an equal volume of isopropanol and 0.2 volumes of 5 M NaCl. After centrifugation, the pellets were air-dried and resuspended in 10 mM Tris/1 mM EDTA (TE) buffer. The concentration of DNA was determined by using a spectrophotometer and adjusted to $100 \mu\text{g/ml}$. Ten microliters of DNA and $10 \mu\text{l}$ of aldehyde reactive probe (ARP) were mixed and incubated for 60 min at 37°C . The ARP-labeled DNA was washed twice with TE buffer, and $2.26 \mu\text{g/ml}$ of purified ARP-labeled DNA was then transferred to each well of a microtiter plate (kit accessory). Horseradish peroxidase-conjugated streptavidin ($150 \mu\text{l}$) was added, and the plate was

incubated for 60 min at 37°C . The plate was washed, and treated with an accessory substrate solution for 60 min at 37°C . The reaction product of the ARP and the AP sites was determined at a wavelength of 650nm .

2.7. Caspase activity assay

Activities of caspase-3, -8 and -9 were measured using colorimetric assay kits according to the manufacturer's instructions. HL60 cells (6×10^6) were cultured for 6 hours after X-ray irradiation or hydrogen peroxide treatment, the cells were harvested, lysed in cell lysis buffer for 10 min on ice and centrifuged at $10,000 g$ for 1 min at 4°C . Fifty microliters of reaction buffer containing $200 \mu\text{g}$ protein and $5 \mu\text{l}$ of reaction reagent specific to caspase-3, caspase-8 or caspase-9 was added and incubated for 2 hours at 37°C . Specific substrates, such as DEVD-pNA for caspase-3, IETD-pNA for caspase-8 or LEHD-pNA for caspase-9 were added. The pNA (para-nitro aniline) light emission was quantified using a microtiter plate reader at 405nm .

2.8. Specific inhibitor of caspases

To determine the involvement of caspase-3, -8, and -9 in pro-apoptotic activity, a specific inhibitor of each caspase, DEVD-FMK, Z-IETD-FMK, and LEHD-FMK ($2 \mu\text{M}$), respectively, was added just before exposure to radiation or hydrogen peroxide. For the controls, a vehicle of DMSO was added at a final concentration of $0.1 \mu\text{M}$. The effects of these inhibitors were estimated by DNA fragmentation⁶.

2.9. Western blot analysis

Western blot analysis of Bax in mitochondria and cytochrome c released into the cytoplasm of HL60 cells, after 6 hours of X-ray irradiation and/or hydrogen peroxide treatment, was performed. Cells were collected by centrifugation and resuspended in $500 \mu\text{l}$ of isotonic lysis buffer (210 mM sucrose, 70 mM mannitol, 1 mM EDTA, 10 mM HEPES, pH7.4) containing 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail. After homogenization with a sonicator, cell lysates were centrifuged at $800 g$ to remove the cell debris. The supernatant was centrifuged at $1,000 g$ to pellet the nuclei, which was discarded. The supernatant was centrifuged at $10,000 g$ for 10 min at 4°C to pellet the mitochondria-enriched heavy membrane fraction. The supernatant was further centrifuged at $100,000 g$ for 20 min at 4°C to obtain the cytosolic fraction. The membrane fractions were resuspended in $30 \mu\text{l}$ of 0.5% Triton X-100 in isotonic lysis buffer containing protease inhibitors for 10 min at 4°C to release the membrane and the organelle-bound soluble proteins, including mitochondrial cytochrome c. Protein concentration was determined by a protein assay kit (Bio-Rad).

The proteins (10 µg/lane) were then loaded onto 10% SDS-polyacrylamide gels, and transferred onto polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) in a tank blotter. The membrane was blocked overnight at 4°C with Block Ace containing 0.1% Tween-20, and then incubated with primary antibody (1:1000 dilution in 10% Block Ace) for 2 hours at room temperature. The blots were rinsed with 10% Block Ace (5 times, 10 min each), incubated with horseradish peroxidase-conjugated secondary antibody (1:5000 dilution in 10% Block Ace) for 60 min at room temperature, and rinsed again. The immunoreactive bands were visualized with an ECL detection kit.

2.10. Statistical analysis

The data from three independent experiments are expressed as mean ± SD. Comparison between the two groups was performed using the Student's t-test for parametric variables and Mann-Whitey U test for non-parametric variables. Multiple comparisons were performed by one-way ANOVA. $P < 0.05$ was considered significant.

3. Results

The results of the cell viability assay are shown in Figure 1(A) and 1(B). Cell viability decreased significantly ($P < 0.05$) for DU145 and SAS cells treated with 0.1 mM hydrogen peroxide after X-ray irradiation, compared to cells exposed only to 2 Gy X-ray radiation. The results of the trypan blue exclusion method in HL60 cells are shown in Figure 1(C). Cell viability was significantly decreased ($P < 0.05$) when X-ray irradiation was combined with hydrogen peroxide, compared cells exposed to X-ray irradiation only.

The change in DNA fragmentation of HL60 cells is shown in Figure 2. DNA fragmentation progressed with time when the cells were subjected to treatment with X-ray irradiation, hydrogen peroxide, and a combination of X-ray irradiation and hydrogen peroxide. DNA fragmentation started earlier (at 2 hours) in the groups exposed to a combination of X-ray irradiation and hydrogen peroxide or hydrogen peroxide only, compared to the group exposed to X-ray radiation only. Overall, DNA fragmentation was highest in the combination treatment (X-ray irradiation and hydrogen peroxide) group, followed by the hydrogen peroxide-treated and X-ray-irradiated groups, respectively. There were statistically significant differences ($P < 0.05$) between the combination of X-ray irradiation and hydrogen peroxide, and X-ray radiation alone 4, 6, and 8 h after cells were exposed to X-ray radiation. The result for the quantification of DNA abasic (AP) site in HL60 cells is shown in Figure 3. There was a significant ($P < 0.05$) increase in DNA damage in the cells

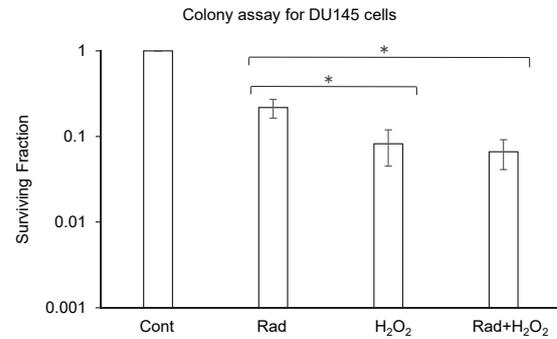


Fig. 1. (A) Colony assay for DU145 cells (1 week after exposure) For X-ray irradiation and hydrogen peroxide treatment, the number of colonies significantly decreased compared to that in the control. * $P < 0.05$

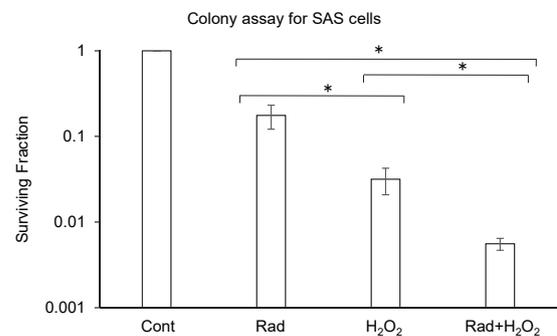


Fig. 1. (B) Colony assay for SAS cells (1 week after exposure) There was a significant decrease in cell viability for the group treated with X-ray irradiation in combination with hydrogen peroxide compared to the group treated with X-ray irradiation alone. * $P < 0.05$

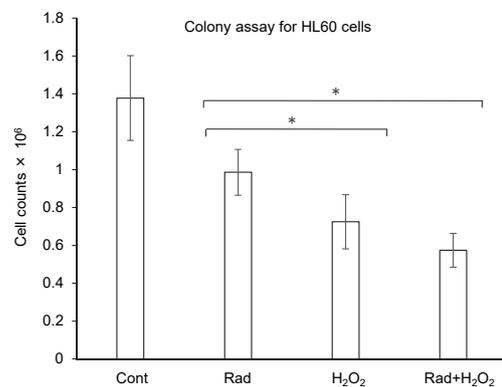


Fig. 1. (C) Cell viability of HL60 cells (24 hours after exposure) A significant decrease in cell viability after 24 hours was observed for the group treated with X-ray irradiation in combination with hydrogen peroxide. * $P < 0.05$

subjected to X-ray irradiation, and combination of X-ray irradiation and hydrogen peroxide, whereas DNA damage was not observed in the group treated with hydrogen peroxide only.

An increase in the amount of active caspase-3 was

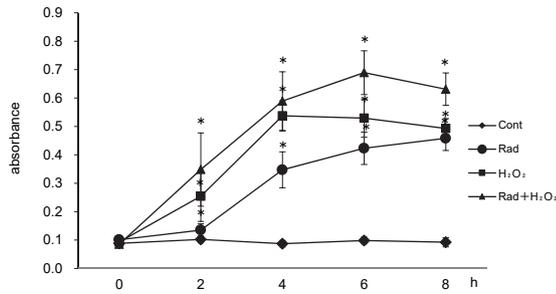


Fig. 2. Change in DNA fragmentation of HL60 cells. The vertical axis represents the amount of DNA fragmentation, and the horizontal axis represents time. In the HL60 cells subjected to X-ray irradiation, hydrogen peroxide treatment, and X-ray irradiation combined with hydrogen peroxide treatment, DNA fragmentation progressed over time and apoptosis was induced. * indicated a statistically significant difference ($P < 0.05$) between each point and the corresponding control value.

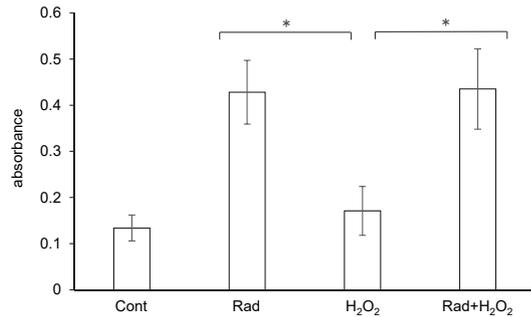


Fig. 3. Abasic sites of HL60 cells (10minutes after exposure). The damage to the DNA increased significantly in cells exposed to X-ray irradiation and combination treatment, compared to that in hydrogen peroxide-treated cells and control. * $P < 0.05$

observed in all the treatment groups after 6 hours as shown in Figure 4(A), but the amount of active caspase-3 was highest in the combination (X-ray irradiation and hydrogen peroxide) treatment group. The difference between the combination treatment, and the hydrogen peroxide-treated groups was significant ($P < 0.05$) compared to the X-ray-irradiated group. A similar trend was observed for active caspase-8 after 6hours (Fig. 4(B)). An increase in the amount of active caspase-9 was also observed in all the treatment groups after 6hours as shown in Figure 4(C); the increase was highest in the combination treatment group, which was significant ($P < 0.05$) when compared to that in the X-ray-irradiated group.

The results of DNA fragmentation upon treatment with hydrogen peroxide after the addition of the respective caspase inhibitors are shown in Figure 5(A). Addition of the caspase inhibitor to each treatment group, caspase-3, caspase-8, and caspase-9 decreased DNA fragmentation.

In case of DNA fragmentation upon treatment with

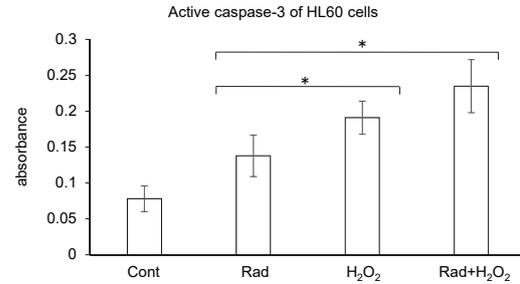


Fig. 4. (A) Active caspase-3 of HL60 cells (6hours after exposure). X-ray irradiation combined with hydrogen peroxide treatment showed the highest amount of active caspase-3, and a significant difference was observed between X-ray irradiation combined with hydrogen peroxide treatment and only X-ray irradiation. * $P < 0.05$

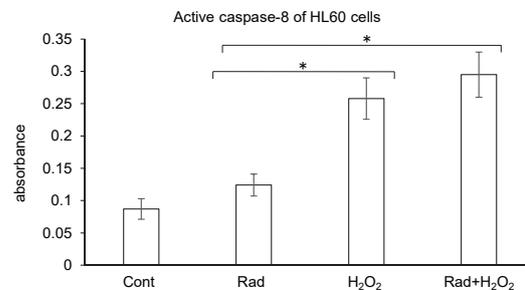


Fig. 4. (B) Active caspase-8 of HL60 cells (6hours after exposure). There was an increase in active caspase-8 after hydrogen peroxide treatment, and on X-ray irradiation combined with hydrogen peroxide treatment, but there was no increase with X-ray irradiation alone. No significant difference was observed between the treatment with hydrogen peroxide, and X-ray irradiation combined with hydrogen peroxide treatment. * $P < 0.05$

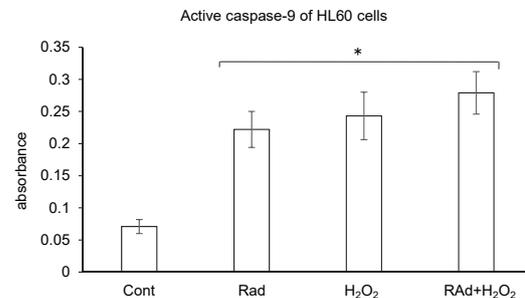


Fig. 4. (C) Active caspase-9 of HL60 cells (6hours after exposure). There was an increase in active caspase-9 in all the treatment groups; the maximum increase was observed on X-ray irradiation combined with hydrogen peroxide treatment, and a significant difference was observed for X-ray irradiation combined with hydrogen peroxide treatment, compared to X-ray irradiation. * $P < 0.05$

X-ray after the addition of the caspase inhibitors (Fig. 5(B)), DNA fragmentation decreased when caspase-3 and caspase-9 inhibitors were added. However, DNA fragmentation was observed even after the addition of caspase-8 inhibitor.

Upon treatment with a combination of X-ray irradiation

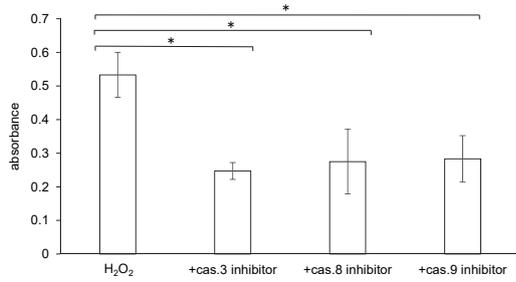


Fig. 5. (A) DNA fragmentation of HL60 cells after treatment with hydrogen peroxide (6hours after exposure) When caspase inhibitors were added to hydrogen peroxide, there was a reduction in fragmentation of cells for all the treatment groups. **P*<0.05

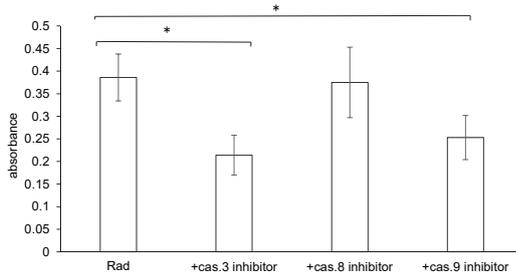


Fig. 5. (B) DNA fragmentation of HL60 cells after X-ray irradiation (6hours after exposure) When caspase-3 and caspase-9 inhibitors were added to X-ray irradiation, DNA fragmentation of the cells decreased. **P*<0.05

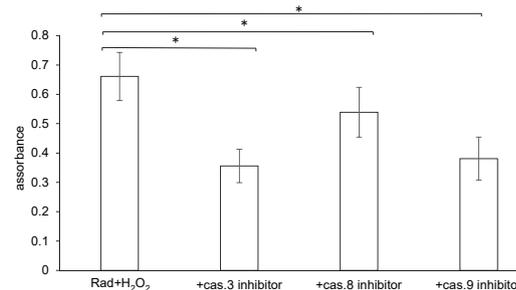


Fig. 5. (C) DNA fragmentation of HL60 cells when X-ray irradiation was combined with hydrogen peroxide (6hours after exposure) On combination treatment with X-ray irradiation and hydrogen peroxide, DNA fragmentation decreased when caspase-3 and caspase-9 inhibitors were added, and a slight decrease was observed on addition of caspase-8 inhibitor. **P*<0.05

and hydrogen peroxide after the addition of the caspase inhibitors, a decrease in DNA fragmentation was observed in the presence of caspase-3, caspase-8, and caspase-9 inhibitors (Fig. 5(C)), although the decrease was comparatively less when caspase-8 inhibitor was added.

The results of western blotting are shown in Figure 6. Although the expression of mitochondrial Bax increased in the cells treated with X-ray irradiation and hydrogen peroxide, the expression was found to be notably higher

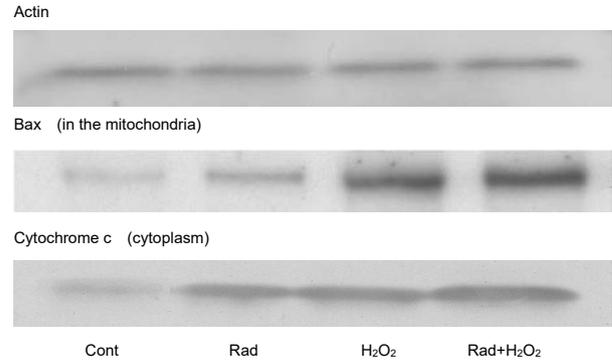


Fig. 6. Western blotting for HL60 (6hours after exposure) Bax expression increased in the mitochondria of cells treated with both X-ray irradiation and hydrogen peroxide. For cytoplasmic cytochrome c, the expression was similar in all the treatment groups, compared to the control.

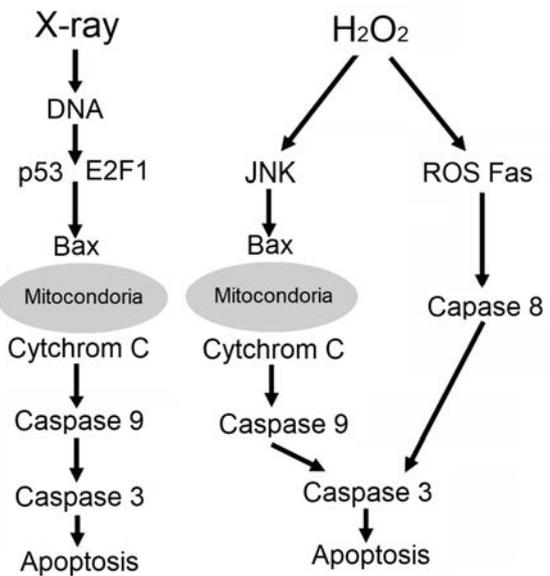


Fig. 7. Summary of the signaling pathways of radiation and hydrogen peroxide -induced apoptosis. Left: Radiation induces DNA injury, then p53 recognizes the DNA injury, causing mitochondria to release cytochrome c and activating caspase-9. Right: The two signaling pathways for hydrogen peroxide-induced apoptosis. The first is dependent on caspase-8 activation, which then activates caspase-3 via the transfer/relocalization of Bax to the mitochondria in a similar way to the radiation signaling pathway.

in the group treated with the combination of X-ray irradiation and hydrogen peroxide. The expression of cytochrome c in the cytoplasm was higher in all the treatment groups when compared to the control.

4. Discussion

Since OH radicals generated by hydrogen peroxide are known to induce apoptosis in cancer cells, hydrogen peroxide was used in combination with irradiation therapy

to enhance the effect of radiation therapy⁷. In the current exposure experiments, viability of DU145, SAS, and HL60 cells decreased when exposed to X-ray irradiation in combination with hydrogen peroxide, compared to X-ray irradiation only. Since few studies have examined the mechanism of action for X-ray irradiation combined with hydrogen peroxide, we investigated their effects with a focus on apoptosis. We used HL60 cells to assess the levels of apoptosis because the 2 Gy X-ray irradiation dose used in conventional radiotherapy does not induce typical apoptosis in solid tumors⁸.

When HL60 cells were subjected to X-ray irradiation and hydrogen peroxide individually, and in combination, a decrease in cell viability was observed after 24 hours of exposure. It is well known that OH radicals generated by hydrogen peroxide play a major role in cell damage, and induce apoptotic cell death by activating the pro-apoptotic pathways⁹. The exposure conditions used to examine the apoptosis-inducing effect have been reported to be in the range of 10 μ M to 8 mM¹⁰. According to a previous study, when compared to control HL60 cells, DNA fragmentation appeared with hydrogen peroxide concentrations in the range 10 μ M to 5 mM, and ultrastructural necrotic changes appeared at concentrations of 3 mM or more¹¹. In this study, the highest concentration of hydrogen peroxide used for exposure was 0.1 mM; it was obtained by referring to the concentration of hydrogen peroxide that is used in clinics¹². When DNA fragmentation was quantified under this condition, it showed a significantly higher value with X-ray irradiation in combination with hydrogen peroxide, compared to only X-ray irradiation, and apoptosis was considered to have increased.

It is known that AP (apurinic/aprimidinic) sites are generated in DNA at an early stage due to the generation of active oxygen radicals. AP sites are a type of lesions on the DNA, and considered as one of the factors that helps in the estimation of the oxidative damage caused to the genomic DNA¹³. Our results indicated that AP sites were observed at an early stage when only X-ray irradiation was used and increase in AP sites was not observed on treatment with only hydrogen peroxide. The fact that DNA fragmentation that occurs just after 2 hours of hydrogen peroxide treatment was higher than that on X-ray irradiation suggests that the effect of hydrogen peroxide differs from that of X-ray irradiation, and that hydrogen peroxide induces DNA damage at an earlier stage.

Our results indicated that caspase-3 is involved in cell death by X-ray irradiation treatment as well as that by hydrogen peroxide treatment, and hydrogen peroxide induces apoptosis through the pathway mediated by caspase-8 activation, but apoptosis caused by X-ray irradiation is induced through caspase-9 and does not depend on caspase-8. Caspases are broadly classified

into two types, initiator caspases that are involved at relatively early stages of apoptosis induction, and executioner (effector) caspases that are involved in the actual execution of apoptosis¹⁴. Executioner caspases in the downstream of the cascade are activated through the activation of initiator caspases, such as caspase-8 during receptor-mediated apoptosis, and caspase-9 during mitochondria-mediated apoptosis¹⁵. Caspase-8 is the most upstream enzyme in the death receptor apoptotic pathway and directly activates caspase-3. Furthermore, caspase-8 can cleave the BH3 interacting-domain death agonist (Bid). The cleaved Bid interacts with the apoptosis-promoting protein Bax and accumulation of Bax in the mitochondria promotes the release of cytochrome c^{16–18}. A pathway in which caspase-8 directly activates caspase-3 to induce apoptosis is also known to be present.

Irradiation-induced apoptosis has been explained using the mitochondria-mediated apoptotic cascade¹⁹. When the instruction for irradiation-induced apoptosis is sent, mitochondria mediate the activation of apoptotic protease activating factor 1 (Apaf-1) by releasing cytochrome c that binds to Apaf-1²⁰. It is known that Apaf-1-cytochrome c complex subsequently promotes the activation of pro-caspase-9 and causes apoptosis²¹. Even in this study, Bax and cytochrome c were observed by western blot analysis during irradiation-induced apoptosis of HL60 cells, indicating the involvement of caspase-9 activation after release of cytochrome c. Activation of caspase-8 was not observed even after 6 hours of X-ray irradiation, and irradiation-induced apoptosis was considered to have been triggered because of the activation of caspase-9 with the release of cytochrome c from active mitochondria without activation by caspase-8. High expression of Bax was observed on treatment with hydrogen peroxide. While enhancement of the pathway leading to caspase-3 through the release of cytochrome c and caspase-9 is considered, the existence of a pathway where the transmission of a series of signals starting from the activation of caspase-8-releasing cytochrome c through Bax, or the existence of a pathway where caspase-8 directly activates caspase-3 can also be inferred²².

The expression of wild-type p53 is a key mediator of the initial cellular responses to ionizing radiation-induced DNA damage²³. Increased p53 expression coupled with various post-translational modifications (e.g., phosphorylation (P) and acetylation (Ac)), is a critical step. Accelerated senescence can result from the p53-dependent induction of p21/waf1 or the upregulation of other cell cycle inhibitory proteins (e.g., p16 INK4a). p53 activation also triggers the de novo synthesis of pro-apoptotic molecules that mediate intrinsic (e.g., Bax, Puma) and extrinsic (e.g., Fas) apoptotic cell death²⁴. Besides p53, the key upstream regulators of these cascades following DNA damage are unclear in p53

deficient cells such as HL60 cells. Putzer reported that E2F1-induced apoptosis occurs independently of p53 in tissue culture and in transgenic mice. The apoptotic targets of E2F1 in the absence of p53 include the p53 homolog protein p73 and apoptosis protease-activating factor 1 (Apaf1) both of which are transcriptionally regulated by E2F1. This initiates the assembly of Apaf1 with cytochrome c, procaspase-9 activation and the successive initiation of proapoptotic effector caspases including Caspase-3, -6 and -7²⁵. Interestingly, Paliwal showed that ARF-induced C-Terminal Binding Protein (CtBP) degradation and CtBP small interfering RNA both led to p53-independent apoptosis in colon cancer cells²⁶.

Regarding the apoptotic cascade of hydrogen peroxide, Marzo reported that high intracellular hydrogen peroxide levels induces the activation of c-Jun NH2-terminal kinase (JNK) and the release of mitochondrial cytochrome-c. The significant increase in the concentration of cytochrome-c in the cytosol then induces the caspase-9-mediated activation of caspase-3, and the definitive execution of the apoptotic process²⁷. Conversely, many reports have proposed that oxidants such as hydrogen peroxide can regulate Fas-mediated cell death²⁸. Mechanistic studies have suggested that both exogenously and endogenously generated ROS promote the activation of initiator caspases such as caspase-8, however the apoptotic cascades initiated by ionizing radiation and hydrogen peroxide require further study.

In this study, viability of cancer cells was lower when X-ray irradiation was combined with hydrogen peroxide treatment compared to that on X-ray irradiation only, and efficacy of the combination therapy was confirmed. When the apoptotic mechanism was examined with X-ray irradiation or hydrogen peroxide treatment, the result indicated that caspase-8 was not activated by X-ray irradiation, but was activated by hydrogen peroxide treatment, and a relative increase in the expression of Bax was also observed. On combining X-ray irradiation with hydrogen peroxide treatment, in addition to the apoptosis cascade induced by X-ray irradiation, the apoptosis cascade induced by hydrogen peroxide treatment that may be similar to or different from the apoptosis cascade induced by X-ray irradiation is considered to increase cell death.

KORTUC treatment demonstrated clinically marked therapeutic effects with satisfactory treatment outcomes and acceptable adverse effects though patient numbers were small^{2, 3}. The combined treatment of hydrogen peroxide and X-ray irradiation was shown to be reasonable biologically by our study and we are anticipating further randomized clinical trials to establish the therapeutic efficacy of KORTUC.

Conflict of Interest

The authors declare that they have no conflict of interest.

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