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Ionizing radiation induces blockade of c-Jun N-terminal kinase-dependent cell death pathway in a manner correlated with $p21^{Cip/WAF1}$ induction in primary cultured normal human fibroblasts

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Abbreviations: DCFH-DA, 2',7'-dichlorofluorescein diacetate; GPX, glutathione peroxidase; HSP, heat shock protein; IR, Ionizing radiation; JNK, c-Jun N-terminal kinase; PI, propidium iodide; ROS, reactive oxygen species; SOD, superoxide dismutase.

Abstract

During radiotherapy of cancer, neighboring normal cells may receive sub-lethal doses of radiation. To investigate whether such low levels of radiation modulate normal cell responses to death stimuli, primary cultured human fibroblasts were exposed to various doses of γ-rays. Analysis of cell viability using an exclusion dye propidium iodide revealed that the irradiation up to 10 Gy killed the fibroblasts only to a minimal extent. In contrast, the cells efficiently lost their viability when exposed to 0.5-0.65 mM H_2O_2. This type of cell death was accompanied by JNK activation, and was reversed by the use of a JNK-specific inhibitor SP600125. Interestingly, H_2O_2 failed to kill the fibroblasts when these cells were pre-irradiated, 24 h before H_2O_2 treatment, with 0.25-0.5 Gy of γ-rays. These cytoprotective doses of γ-rays did not enhance cellular capacity to degrade H_2O_2, but elevated cellular levels of $p21^{Cip/WAF1}$, a p53 target that can suppress H_2O_2-induced cell death by blocking JNK activation. Consistently, H_2O_2-induced JNK activation was dramatically suppressed in the pre-irradiated cells. The overall data suggests that ionizing radiation can impart normal fibroblasts with a survival advantage against oxidative stress by blocking the process leading to JNK activation.

Keywords: cell death; fibroblasts; hydrogen peroxide radiation, ionizing; JNK mitogen-activated protein kinases

Introduction

Ionizing radiation (IR) is a DNA-damaging agent that is widely used for cancer treatment. IR can induce multiple cellular responses such as DNA repair, growth arrest, and cell death. These actions of IR are controlled by numerous cellular components involved in the DNA damage checkpoint response (Iliakis *et al.*, 2003). P53 is one such component that responds to IR and regulates cell growth and death. It is well established that the mechanism of p53 action involves a transcriptional control of $p21^{Cip/WAF1}$ and various other target genes (Fei and El-Deiry, 2003).

An interesting feature of IR is that it is not always harmful to cells, but it can be cytoprotective under certain circumstances. For example, when cells are first exposed to relatively low, sub-lethal IR doses, they develop a resistance to subsequent challenges with higher harmful doses of the same agent (Wolff, 1992). This cytoprotective response has been characterized mostly by using untransformed normal, as opposed to transformed cancer, cell lines. This is partly based on the assumption that whereas cancer cells receive high IR doses during radiotherapy, neighboring normal cells have higher chances to be exposed to lower cytoprotective doses. Chromosome/nucleus stability assays have revealed that the pre-irradiation can reduce various genotoxic effects of challenging doses, such as mutations (Rigaud *et al.*, 1993; Rigaud and Moustacchi, 1996), chromosome aberrations (Wang *et al.*, 1991; Shimizu *et al.*, 1999), sister chromatid exchanges (Sasaki, 1995), and micronucleus formations (Azzam *et al.*, 1994). The induction of these protective responses required a time lag, which appears to reflect the need for accumulating IR-responsive protective proteins (Youngblom *et al.*, 1989; Wolff, 1992). Although the nature of these proteins is not clear yet, the chromosomal protection induced in Chinese hamster cells was accompanied by an enhanced cellular capacity to repair damaged DNA (Ikushima *et al.*, 1996). Therefore, the IR-responsive proteins may involve those

acting in the DNA repair pathway, which seems to confer the pre-irradiated cells with an increased ability to maintain their genomic stability.

A clonogenic survival assay is an additional method that has been frequently used to characterize the IR-induced cytoprotective responses. In this case, the pre-irradiated cells were shown to withstand subsequent challenging doses of IR which would otherwise abolish their colony formation (Wolff, 1992; Iyer and Lehnert, 2002). This suggests that the pre-irradiation either enhanced cellular growth competence or reduced the challenge-induced cell death. Some investigators explored this latter possibility by using the TUNEL assay, a method widely used to diagnose DNA fragmentation induced during apoptosis (McGahon *et al.*, 1995). The reported results suggest that the pre-irradiation can reduce the challenge-induced accumulation of TUNEL-positive cells (Takahashi *et al.,* 2001; Sasaki *et al.*, 2002). While this data was interpreted in a way that the IR-induced cytoprotection can act against apoptosis, it should be noted that the TUNEL assay reflects genotoxic effects of IR, not necessarily apoptosis. Therefore, in order to confirm the possibility that IR can induce an anti-death response, this study directly analyzed cellular viability using an exclusion dye propidium iodide (PI). Primary cultured human dermal fibroblasts were used as the model. The data presented in this report suggests that when these cells are irradiated by relatively low, sub-lethal doses of γ-rays, they develop an ability to block the c-Jun N-terminal kinase (JNK)-dependent cell death pathway. The mechanism underlying this phenomenon is discussed.

Materials and Methods

Antibodies

The antibodies raised against JNK, HSP90, and HSP110 were purchased from PharMingen/Transduction Laboratories (San Diego, CA). The $p21^{Cip/WAF1}$, HSP60, and p53 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-HSP27, anti-HSP70, and anti-Cu/Zn superoxide dismutase (SOD) were supplied by Stress Bioreagents (Victoria, Canada). The anti-catalase was purchased from Biodesign International (Saco, ME). The anti-glutathione peroxidase (GPX) was a generous gift from Dr. Ho Zoon Chae (Chonnam National University, Korea).

Cell culture and treatments

Human dermal fibroblasts were isolated from normal adult foreskins, which were removed during circumcision and kept at 4°C in F-medium (1 part of Ham's F-12 medium and 3 parts of DMEM, 10 % FBS) containing gentamicin (50 μg/ml). To isolate cells, the foreskin samples were washed at least 8-fold in phosphate-buffered saline. Most subcutaneous tissues were removed from dermis with sterile surgical scissors, and the remaining skins were minced to fine pieces less than 1 mm. These tissue pieces were treated with dispase II solution (2.4 U/ml, Roche, Mannheim, Germany) at 37°C for 4 h. Dermal layers were separated from epidermal layers, washed, and further incubated in the presence of 0.35% collagenase (Roche, Mannheim, Germany) at 37°C for 2 h. Isolated cells were dissociated into single cell suspension by gentle pipetting, washed twice, and inoculated in F-medium at 2×10^4 /cm^2 in culture plates. The culture medium was changed two to three-fold a week. To treat cells, the cultured cells were trypsinized, plated in four well plates (5×10^4 cells/well), and grown to 70-80% confluence. The cells were then given the indicated treatments.

Analysis of viability

At the end of specified incubation times, attached fibroblasts were harvested by trypsinization, and pooled with floating cells. The cells were stained with PI (5 μg/ml) followed by flow cytometry analysis to monitor their staining intensity and size. The cells displaying either a high permeability to PI or a reduced size were understood to be dead, as previously defined (Mangan *et al.*, 1991; McGahon *et al.*, 1995).

Analysis of cellular levels of reactive oxygen species (ROS)

Treated and untreated control cells were harvested, and exposed to 50 μM 2',7'-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, Eugene, OR) for 5 min. This compound rapidly diffuses into cells, and once within cells, is hydrolyzed to oxidation-sensitive DCFH (Bass *et al.*, 1983). In the presence of ROS, DCFH is oxidized to the highly fluorescent DCF, which is retained by living cells. The cell-associated levels of DCF fluorescence were analyzed by flow cytometry (Bass *et al.*, 1983).

Western blot analysis

The cell lysates were prepared as described previously (Lee and Um, 1999). Equal amounts of the proteins were separated by SDS-PAGE, then electrotransferred to the Immobilon membranes (Millipore, Bedford, MA), which were subsequently blotted using the indicated antibodies and visualized by chemiluminescence (ECL; Amersham Pharmacia, Uppsala, Sweden).

JNK kinase assay

The lysate proteins (400 μg) were immunoprecipitated using anti-JNK, and resolved in 20 μl of a buffer defined previously (Kim *et al.*, 2001b). The kinase reactions were initiated by adding 2 μg of the recombinant c-Jun protein (New England Biolabs, Beverly, MA). After 30 min incubation, the reaction was quenched by adding a boiled sample buffer, and the proteins were subsequently separated by 12% SDS-PAGE. The gels were dried, and a PhosphoImager using Tina 2.0 software visualized the radioactive bands.

Results

Induction of fibroblast death using H_2O_2

In order to investigate whether IR can induce an

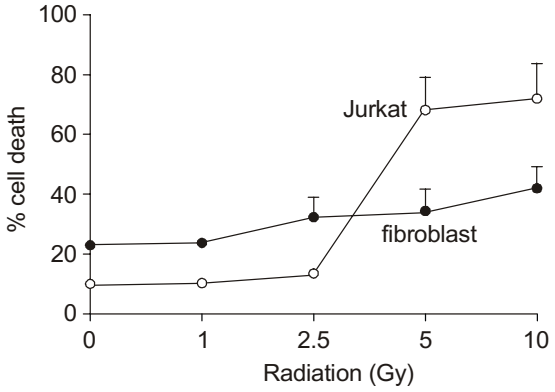
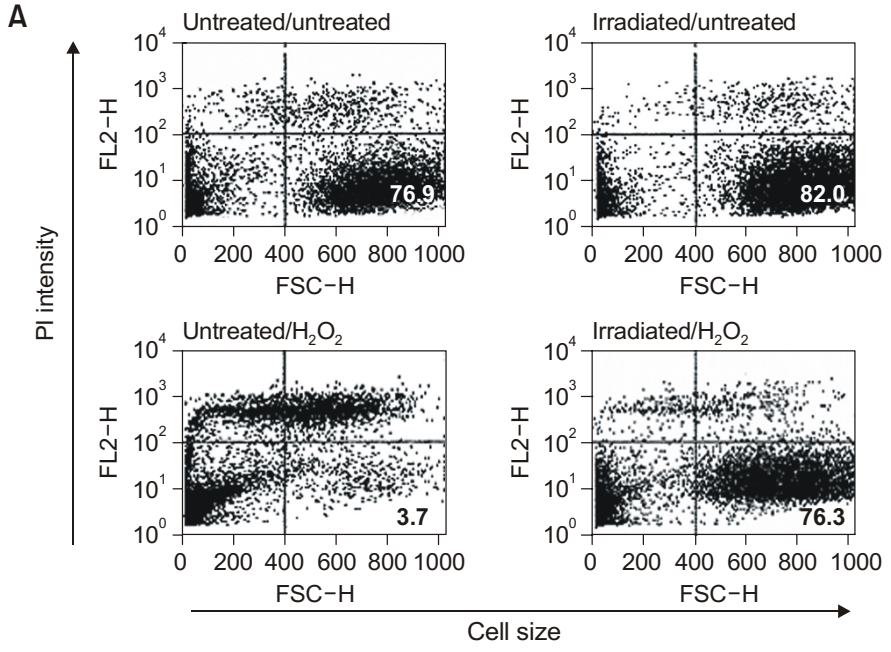


Figure 1. Ionizing radiation does not efficiently kill normal human fibroblasts. Either normal fibroblasts (closed circle) or Jurkat T leukemia cells (open circle) were irradiated with indicated doses of γ-rays. At the end of 48 h incubation, cellular viability was analyzed by flow cytometry. The values are the mean of five separate experiments with an error bar representing the standard deviations.



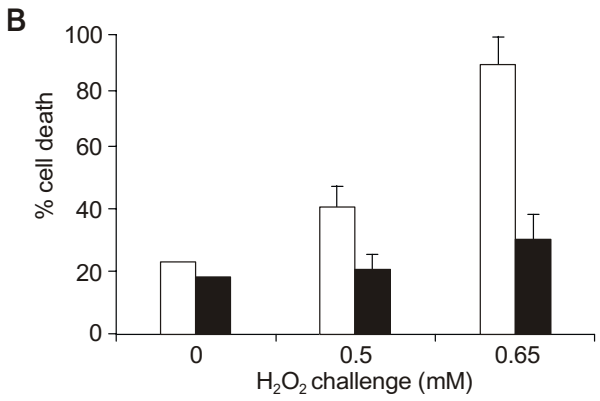


Figure 2. Radiation can induce a cellular resistance to H_2O_2. (A) The fibroblasts were irradiated with γ-rays at 0.5 Gy, and incubated for 24 h. The irradiated and untreated control cells were subsequently exposed to 0.65 mM H_2O_2 for 48 h, stained with propidium iodide, and then analyzed by flow cytometry. Lower right quadrans reflect viable cell populations. The results shown are representatives of four similar experiments. (B) The irradiated (closed bar) and untreated control cells (open bar) were challenged by indicated concentrations of H_2O_2. Cellular viability was analyzed 48 h after the challenge. Values are the means of four separate experiments with an error bar representing standard deviations.

anti-death response in normal human fibroblasts, it was initially required to establish optimal conditions for killing these cells. Therefore, the fibroblasts were exposed to various doses of γ-rays. At the end of 48 h incubation, cellular viability was analyzed by their ability to exclude PI. However, the irradiation up to 10 Gy induced cell death only to a minimal extent (Figure 1). This did not appear to reflect a trivial technical problem, because 5 Gy and higher doses of γ-rays efficiently killed the Jurkat T leukemia cells. Therefore, the fibroblasts were alternatively treated with H_2O_2 (0.5-0.65 mM) for 48 h. In contrast to IR, this treatment resulted in a significant induction of cell death (Figure 2).

IR can induce a cellular resistance to H_2O_2

To explore the possibility that the H_2O_2-induced cell death can be modulated by IR, the fibroblasts received relatively low levels (0.5 Gy) of γ-rays. At the end of various times of incubation, the irradiated and unirradiated control cells were challenged by the lethal concentrations of H_2O_2. Cellular viability was analyzed 48 h after the challenge. The challenge-induced cell death was not significantly altered when the interval between pre-irradiation and H_2O_2 challenge was 8 h or less (data not shown). However, when the cells were challenged 24 h after the irradiation, H_2O_2-induced cell death was almost completely reversed (Figure 2). Similar protective effects of IR were observed when the cells were pre-irradiated with 0.25, but not 0.1, Gy. In contrast, the pre-irradiation with 1 Gy or higher doses resulted in an increase in the challenge-induced cell death (data not shown). Taken together, it appears that 0.25-0.5 Gy of γ-rays render human fibroblasts to be resistant to H_2O_2, and the induction of this protective response requires a time lag of ~24 h.

Cytoprotective IR doses do not enhance cellular antioxidant capacity

The time lag likely reflects the need for synthesis of macromolecules that can protect the cells from H_2O_2. It was reported that IR can elevate cellular ROS levels (Mikkelsen and Wardman, 2003), and oxidative stress can induce the synthesis of antioxidant enzymes (Lu *et al.*, 1993; Lee and Um, 1999). Therefore, it seemed possible that the pre-irradiation caused an increase in cellular antioxidant capacity by

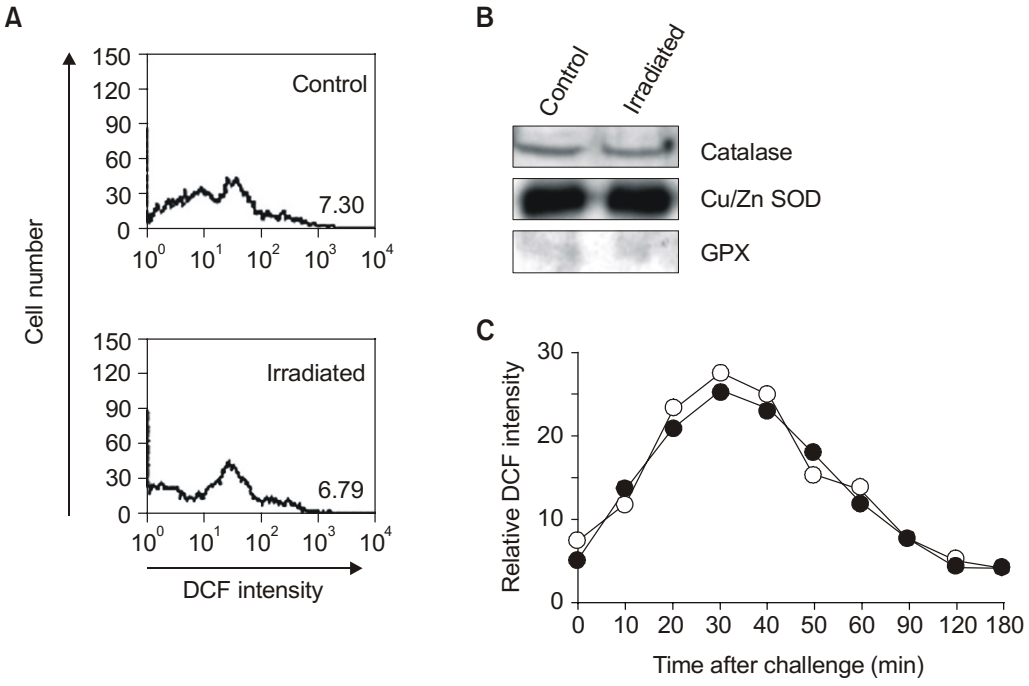


Figure 3. Cytoprotective radiation doses do not enhance cellular antioxidant capacity. Fibroblasts were irradiated with 0.5 Gy γ-rays, and incubated for 24 h. (A) The treated and untreated control cells were compared for their ROS levels using DCF fluorescence. (B) Levels of indicated antioxidant enzymes were compared by Western blot analysis. (C) The irradiated (closed circle) and untreated control cells (open circle) were challenged by 0.65 mM H_2O_2. At the indicated times after the challenge, cell-associated levels of ROS were analyzed using DCF fluorescence. Numbers indicated in (A) and (C) are the median values of DCF fluorescence, and representatives of three independent experiments.

generating ROS. To investigate this, the cells were irradiated with cytoprotective doses (0.5 Gy) of γ-rays. After various incubation times, the irradiated and unirradiated control cells were compared for their ROS levels using DCF fluorescence. However, the irradiation did not significantly enhance cellular ROS levels, as analyzed from 5 min (data not shown) up to 24 h after the treatment (Figure 3A). Western blot analyses performed 24 h after the irradition consistently revealed that 0.5 Gy γ-rays did not increase cellular levels of antioxidant enzymes, including catalase, Cu/Zn SOD, and GPX (Figure 3B). These observations suggested that the irradiation did not induce oxidative stress to the extent that could increase cellular antioxidant capacity. To further confirm this, the capacity of control and irradiated cells to consume H_2O_2 was compared. The cells were irradiated with 0.5 Gy γ-rays, incubated for 24 h, and then challenged by 0.65 mM H_2O_2. At the end of various times after the challenge, cell-associated levels of H_2O_2 were compared by DCF fluorescence. When the unirradiated control cells were analyzed, DCF fluorescence was rapidly elevated upon the exposure to H_2O_2, reflecting the association of exogenous H_2O_2 with the cells (Figure 3C). After a peak at ~30 min, the level of DCF fluorescence declined, reflecting the degradation of added H_2O_2. Importantly, a similar pattern of DCF fluorescence was obtained using the H_2O_2-resistant irradiated cells. This suggests that the irradiation did not significantly alter cellular capacity to degrade H_2O_2. Overall, the survival advantage of irradiated cells against H_2O_2 does not appear to reflect an enhanced cellular antioxidant capacity.

No induction of heat shock proteins (HSPs)

Given that H_2O_2-induced cell death can be suppressed by certain members of HSP family, such as HSP27 (Merendino *et al.*, 2002) and HSP70 (Gyrd-Hansen *et al.*, 2004), the possible accumulation of HSPs in the irradiated cells was next investigated. However, the cytoprotective doses of γ-rays did not significantly increase cellular levels of various HSPs, including HSP27, HSP60, HSP70, HSP90, and HSP110 (Figure 4). Therefore, these tested HSPs did not appear to be involved in the cytoprotection induced under this experimental condition.

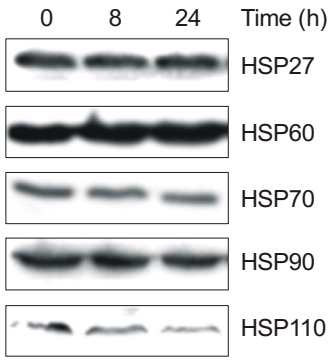


Figure 4. Cytoprotective radiation doses do not induce heat shock proteins. Fibroblasts were irradiated with 0.5 Gy γ-rays, and incubated for indicated periods. Cellular levels of indicated heat shock proteins were compared by Western blot analysis.

Correlation between the cytoprotection and $p21^{Cip/WAF1}$ induction

It was shown that $p21^{Cip/WAF1}$, a well defined target of IR-activated p53 (Fei and El-Deiry, 2003), can attenuate H_2O_2-induced cell death (Asada *et al.*, 1999; Kim *et al.*, 2001a). Therefore, Western blot analyses were performed to determine the relationship between the $p21^{Cip/WAF1}$ induction and H_2O_2 resistance. As shown in Figure 5, 0.5 Gy γ-rays increased cellular levels of p53, reflecting its stabilization/activation (Fei and El-Deiry, 2003). This was optimally detected 3 h after the irradiation (Figure 5A). Consistent with this observation, $p21^{Cip/WAF1}$ protein levels were enhanced in response to the same treatment. This was most evident 24 h after the irradiation, when the cells displayed the resistance to H_2O_2. Similar results were obtained using 0.25 Gy (Figure 5B). Therefore, it was clear that the cytoprotective doses (0.25-0.5 Gy) of γ-rays induced the p53-$p21^{Cip/WAF1}$ pathway in this system. The induction of both p53 and $p21^{Cip/WAF1}$ was also observed using the doses (1-20 Gy) higher than the cytoprotective range. In contrast, 0.1 Gy γ-rays which failed to

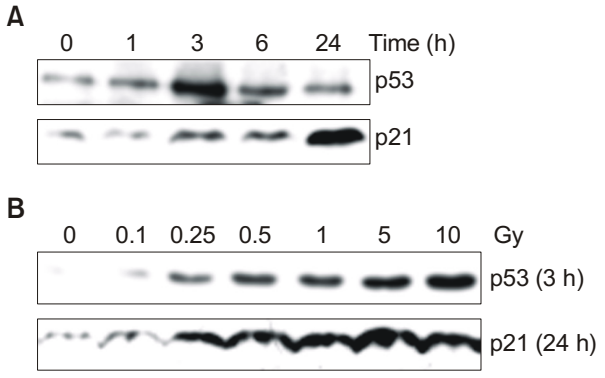


Figure 5. Radiation-induced cytoprotection is accompanied by the induction of p53 and $p21^{Cip/WAF1}$. (A) The cells were irradiated with 0.5 Gy γ-rays. At the indicated times of incubation, cellular levels of p53 and $p21^{Cip/WAF1}$ were analyzed by Western blotting. (B) The cells were irradiated with indicated doses of γ-rays. Cellular levels of p53 and $p21^{Cip/WAF1}$ were analyzed 3 and 24 h, respectively, after the treatment by Western blotting.

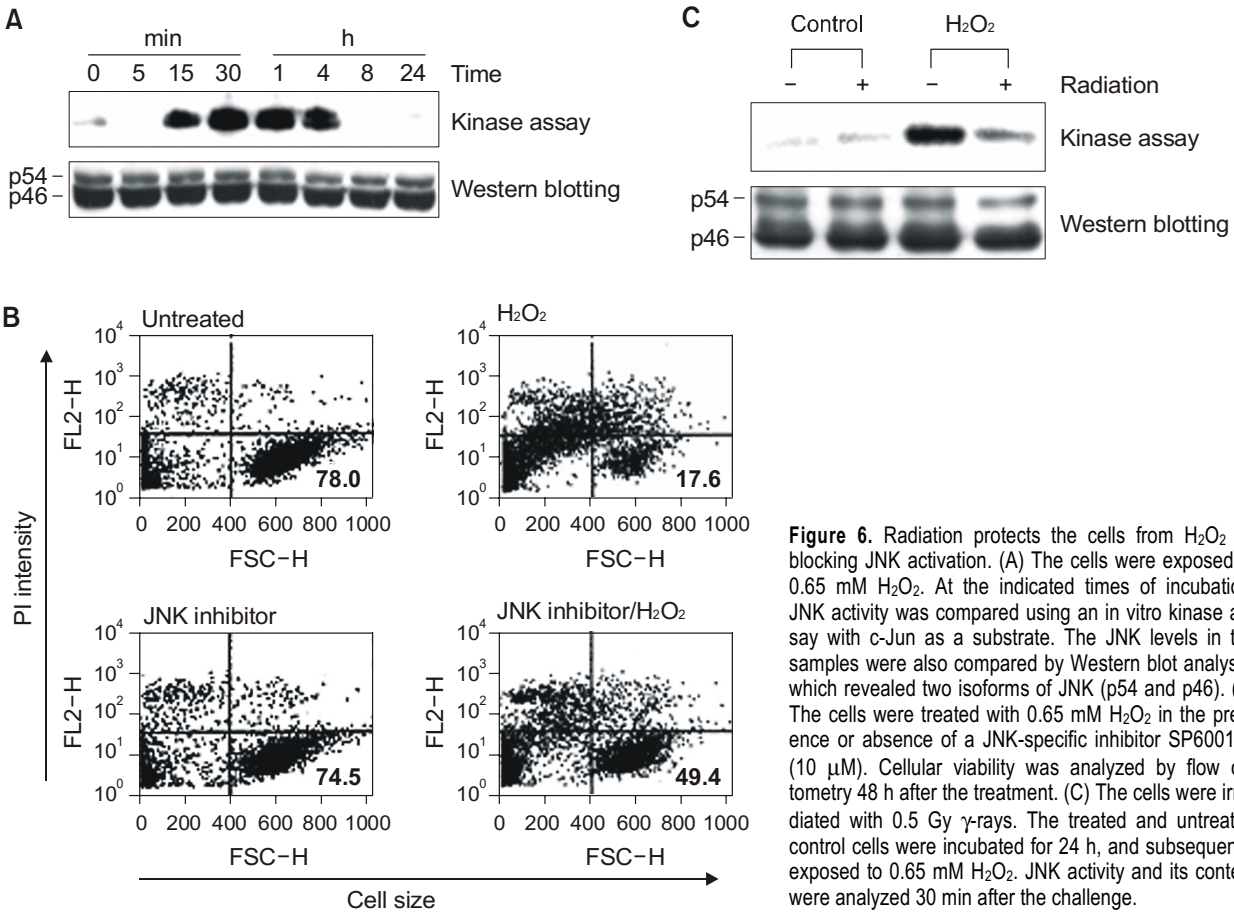


Figure 6. Radiation protects the cells from H_2O_2 by blocking JNK activation. (A) The cells were exposed to 0.65 mM H_2O_2. At the indicated times of incubation, JNK activity was compared using an in vitro kinase assay with c-Jun as a substrate. The JNK levels in the samples were also compared by Western blot analysis, which revealed two isoforms of JNK (p54 and p46). (B) The cells were treated with 0.65 mM H_2O_2 in the presence or absence of a JNK-specific inhibitor SP600125 (10 μM). Cellular viability was analyzed by flow cytometry 48 h after the treatment. (C) The cells were irradiated with 0.5 Gy γ-rays. The treated and untreated control cells were incubated for 24 h, and subsequently exposed to 0.65 mM H_2O_2. JNK activity and its content were analyzed 30 min after the challenge.

induce the H_2O_2 resistance, significantly influence neither p53 nor $p21^{Cip/WAF1}$ levels.

JNK mediates the H_2O_2-induced fibroblast death

It was reported that JNK acts as a mediator of H_2O_2-induced cell death (Verheij *et al.*, 1996; Kim *et al.*, 2001a; b; Lee *et al.*, 2003), and up-regulation of $p21^{Cip/WAF1}$ suppresses this type of cell death by blocking H_2O_2-induced JNK activation (Asada *et al.*, 1999; Kim *et al.*, 2001a). Given the induction of $p21^{Cip/WAF1}$ by cytoprotective γ-ray doses in this system, it seemed possible that the pre-irradiation influenced the H_2O_2-induced JNK activation. To confirm the ability of H_2O_2 to activate JNK in fibroblasts, these cells were exposed to 0.65 mM H_2O_2. JNK activity was analyzed by an in vitro kinase assay various times after the treatment. As shown in Figure 6A, the H_2O_2 treatment activated JNK, which was evident from 15 min to 4 h after the treatment. This sustained pattern of JNK activation was proposed to support a cell death (Guo *et al.*, 1998; Davis, 2000; Lee *et al.*, 2003). Indeed, the addition of SP600125, a JNK-specific inhibitor, efficiently attenuated H_2O_2-induced fibroblast death (Figure 6B). Therefore, it was clear that H_2O_2 kills fibroblasts in a JNK-dependent manner.

Pre-irradiation suppresses the JNK activation induced by H_2O_2 challenge

To explore whether the H_2O_2-induced JNK activation was suppressed in the pre-irradiated cells, the cells were irradiated with 0.5 Gy γ-rays, incubated for 24 h, and then challenged by 0.65 mM H_2O_2. JNK activity was analyzed 30 min after the challenge. The pre-irradiation itself did not significantly influence the levels of JNK activity analyzed from 5 min (data not shown) up to 24 h (Figure 6C). However, it did significantly reduce the challenge-induced JNK activation. Therefore, IR appears to impart the cells with a resistance to oxidative stress by blocking the JNK activation.

Discussion

This study demonstrated that IR can induce an anti-death response. To do so, we showed, using an

exclusion dye-based cell viability assay, that pretreatment with IR protects human fibroblasts from subsequent lethal doses of H_2O_2. This cytoprotective response was optimally induced when the cells were irradiated with γ-rays at 0.25-0.5 Gy. In contrast, it was reported that the DNA/chromosomal protection was optimally induced when various mammalian cell types were pre-irradiated below ~0.1 Gy (Ikushima *et al.*, 1996; Shimizu *et al.*, 1999; Sasaki *et al.*, 2002). Therefore, the optimal dose range for IR-induced cytoprotective responses appears to vary depending on experimental settings.

IR could induce the resistance to oxidative stress either by enhancing cellular antioxidant capacity or by blocking the lethal signaling triggered by oxidative stress. Our data suggests that the former is not the case. First, no evidence was obtained supporting that the cytoprotective doses of γ-rays induced oxidative stress and antioxidant enzymes, including catalase, Cu/Zn SOD, and GPX. Moreover, a direct comparison of control and pre-irradiated cells for their H_2O_2-consuming ability revealed no significant difference. The sum of the data suggests that IR induces the H_2O_2 resistance without enhancing cellular capacity to degrade H_2O_2. It was similarly proposed that the IR-induced chromosomal protection was unrelated to cellular levels of Cu/Zn SOD in mouse embryonic fibroblasts (Wolff, 1996). To investigate the alternative possibility that IR rescued cells from the H_2O_2 challenge by blocking H_2O_2-induced cell death pathway, we focused on JNK. This was based on the previous reports that JNK can act as a mediator of H_2O_2-induced cell death (Verheij *et al.*, 1996; Kim *et al.*, 2001a; b; Lee *et al.*, 2003). This role for JNK was also supported by the present observation that H_2O_2-induced fibroblast death was accompanied by JNK activation, and the inhibition of JNK activity by its specific inhibitor reversed the fibroblast death. Interestingly, the ability of H_2O_2 to activate JNK was dramatically reduced when the cells were pre-irradiated with the cytoprotective doses of γ-rays. This suggests that the pre-irradiation imparts cells with the resistance to H_2O_2 by inducing a blockade of JNK activation. To best of our knowledge, this is the first report to demonstrate that IR-induced cytoprotective response can be accompanied by a blockade of cell death signaling. In contrast to the JNK-suppressing effect of IR in this system, relatively high doses of IR killed certain cancer cell lines by activating JNK (Verheij *et al.*, 1996; Dent *et al.*, 2003). Therefore, IR appears to regulate JNK in two opposing modes depending on experimental conditions.

The mechanism whereby IR suppresses JNK activation is currently unclear. However, the time lag required for the induction of cytoprotective response might reflect accumulation of a protein(s) that can inhibit JNK activation. $P21^{Cip/WAF1}$ is one such protein that can inhibit the JNK activation induced under various experimental conditions (Shim *et al.*, 1996). Moreover, it was shown that up-regulation of $p21^{Cip/WAF1}$ can suppress H_2O_2-induced cell death by blocking JNK activation (Asada *et al.*, 1999; Kim *et al.*, 2001a). We could not directly confirm this role for $p21^{Cip/WAF1}$ using primary cultured normal fibroblasts because of their low tranfection efficiency and limited growth capacity. However, the authors confirmed that IR can elevate levels of both p53 and $p21^{Cip/WAF1}$ in the fibroblasts. This was true using the protective (0.25-0.5 Gy), but not lower unprotective, γ-ray doses. Moreover, the accumulation of $p21^{Cip/WAF1}$ protein also required a time lag, being evident when the cytoprotective effect of IR was materialized (24 h after irradiation). Given this tight correlation between the $p21^{Cip/WAF1}$ induction and H_2O_2 resistance, IR seems to induce the H_2O_2 resistance by elevating cellular $p21^{Cip/WAF1}$ levels. In this regard, it should be mentioned that 1 Gy and higher doses of γ-rays also elevated levels of both p53 and $p21^{Cip/WAF1}$ in fibroblasts, but failed to induce the H_2O_2 resistance. Pre-irradiation with these relatively high γ-ray doses instead enhanced the H_2O_2 challenge-induced cell death. Therefore, relatively high γ-ray doses appear to induce an additional harmful event(s).

In summary, this study showed that IR can induce a blockade of JNK-dependent cell death pathway. This action of IR appears to be tightly associated with the induction of p53-$p21^{Cip/WAF1}$ pathway. These findings provide new insights into the mechanism whereby IR modulates cellular functions, particularly in normal, as opposed to cancer, tissues.

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