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Pretreatment with Interferon-α Radiosensitizes Daudi Cells Modulating Gene Expression and Biomarkers

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Abstract. *Background: Interferon (IFN) potentiates cytotoxicity by X-ray irradiation. To elucidate the mechanism of this potentiation, the biological markers related to DNA damage and cell survival were studied. Materials and Methods: IFN-α-sensitive Daudi and its resistant cells were used. Survival after treatment was assessed by clonogenic assays. DNA breaks were studied by pulse-field gel electrophoresis (PFGE). Production of reactive oxygen metabolites was measured using flow cytometry. Messenger RNA and protein were examined by RT-PCR and immunoblot, respectively. Results: IFN-α treatment for 24 h before irradiation potentiated the sensitivity of Daudi cells to X-rays. This combination induced 50 kb DNA fragmentation and activated caspase-3 in Daudi cells. Pretreatment with IFN-α inhibited the production of reactive oxygen species by irradiation. IFN-α pretreatment down-regulated most of the double-strand break (DSB) repair-related mRNAs, but did not affect the repair of DSBs studied by PFGE. The induction and phosphorylation of $p21^{Cip1/WAF1}$ (p21) was prominently suppressed in cells pretreated with IFN-α. Conclusion: Pretreatment with IFN-α potentiates the cytotoxic effects of X-rays. Inhibition of X-ray-induced p21 may cause the augmented sensitivity by IFN-α pretreatment.*

Interferon (IFN) is one of the cytokines with cytotoxic activity against cancer cells (1, 2). There are two types (type I and II) of IFN and each binds to a corresponding receptor. Binding of either IFN to its receptor activates receptor-associated kinases, which phosphorylate signal transducer and activator of transcription factors (STATs) (3). The transcription factors then translocate to the nucleus and activate specific target genes, including other transcription factors called IRFs (4). Some of the target genes of IFN have a role in inhibition of cellular proliferation and induction of apoptosis. For example, TRAIL is one of the TNF family cytokines induced by IFN and promotes apoptosis through binding to its receptor (5), while IFN decreases the expression of G1-regulating genes, resulting in G1 accumulation of the cell cycle (6-8). Accordingly, IFN has been used for the treatment of several cancers (9). Unfortunately, the cytotoxic activity of IFN is not sufficient to control cancer by a single agent in most cases. Thus, combined treatment with various other drugs has been examined (9-11).

Radiation is one of the modalities that has been used with IFN and a few studies have demonstrated a synergistic effect of this combination (12). However, the opposite result was recently reported, *i.e.*, that IFN pretreatment caused resistance to X-rays (13). Since IFN-induced radiosensitization and resistance, even radioresistance, are largely dependent on the cell types and biological effects, we studied the changes of biological markers during the co-treatment using IFN-sensitive Daudi cells.

Cell cycle regulation is one of the important factors that determine the sensitivity of tumor cells to anticancer agents. Cells can alter the cell cycle machinery in correspondence to the magnitude of DNA damage. If the damage can be repaired during the delay of the cell cycle, the cells can escape apoptosis. $p21^{Cip1/WAF1}$ (p21) is a well-known cell cycle inhibitor that is induced by DNA-damaging agents (14-16). Initially, it was considered to be a tumor suppressor gene because of its role as a Cdk inhibitor. However, several lines of evidence demonstrate an anti-apoptotic effect of p21 (17), suggesting an important role in the survival of DNA-damaged cells.

In this study, we determined the appropriate combination of IFN-α and X-ray irradiation. We also found that the induction and phosphorylation of p21 in response to X-ray irradiation was profoundly modified by IFN-α pretreatment.

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Materials and Methods

IFN and cell line. Daudi cells, derived from human Burkitt's lymphoma, were kindly provided by Sumitomo Pharmaceutical Co. (Osaka, Japan). IFN-resistant Daudi cells have been described elsewhere (18). The cells were cultured in RPMI 1640 medium supplemented with 10% FBS (Hyclone, Logan, UT, USA) in a humidified incubator at 37˚C with 5% CO_2. The colony forming assay was done using semi-solid culture medium that consisted of 0.8% methylcellulose, 10% FBS and 2 mM L-glutamine in RPMI 1640. Natural type of human IFN-α (Sumitomo Pharmaceutical Co., Osaka, Japan) was used at a concentration of 200 U/ml.

Irradiation. Cells, suspended in the culture medium at room temperature, were subjected to X-ray (150keV, *ca.*1 Gy/min, with an Al 2-mm filter) irradiation using an MBR-1520R (Hitachi Medical Co., Tokyo, Japan). After irradiation, the cells were cultured without any change of medium, or were immediately subjected to the colony formation assay, as described below.

Regrowth colony assay. Exponentially growing cells were seeded in medium at 1.5 x 10^5/ml on day 0, and were treated with or without 200 U/ml IFN-α on day 1, and then were given the dose of X-rays (1, 3, or 5 Gy) on day 2. On day 4, the cells were washed three times with complete medium. After counting cell numbers, 2 or 6x10^3 viable cells were seeded in 1 ml of semi-solid culture medium per 35-mm diameter dish. Two 35-mm dishes and one dish of sterile water were placed in a 100-mm dish, and cultured in a CO_2 incubator. The number of colonies was counted under a microscope at 40x magnification on day 14. Clusters containing more than 50 cells were defined as colonies. Experiments were repeated independently three times. The mean±SEM was calculated and the results are shown as columns and bars.

Measurement of reactive oxygen species (ROS). To evaluate the hydrogen peroxide (H_2O_2) level, the conversion of 2',7'-dichlorofluorescin (DCFH) to fluorescent 2',7'-dichlorofluorescein (DCF) was measured by flow cytometry. First, 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Sigma, St.Louis, MO, USA) was dissolved in DMSO at a concentration of 5 mM and then this agent was added to the flask at a final concentration of 5 μM for 30 min. The cells were harvested, washed twice in PBS, and directly subjected to flow cytometry (FACS caliber; Becton Dickinson, San Jose, CA, USA). The fluorescence of viable cells (gated through the FSC *vs*. SSC panel) was measured in FL1. To monitor the production of superoxide anion (O_2^-), hydroethidine (HE) (Polysciences Inc, Warrington, PA, USA) was added at 10 μM and fluorescence was detected in FL3.

Measurement of DNA breaks. Pulse-field gel electrophoresis was performed to detect DNA double-strand breaks and apoptotic DNA breaks after irradiation. To measure breaks after irradiation, 1 x 10^6 cells were washed with PBS and embedded in a low melting agarose plug using a CHEF disposable plug mold (Bio-Rad Laboratories, Hercules, CA, USA). Each plug was equilibrated in lysis solution (100 mM EDTA, 1% N-laurylsarcosine, 10 mM Tris (pH 8)), and treated with 0.2 mg/ml Proteinase K at 50˚C overnight. The plug was washed with TE and stored at 4˚C until electrophoresis. Each plug was inserted into a well of 1.2% agarose gel and pulse-field gel electrophoresis was carried out for 20 h using a CHEF-DR II apparatus. The gel was stained with 0.5 μg/ml ethidium bromide and photographed with UV transillumination using a CCD camera.

Gene expression. The mRNA levels of repair-related genes were examined by RT-PCR. Total RNA was extracted by the Guanidinium CsCl-ultracentrifuge method. Single-strand cDNA was transcribed by M-MLV reverse transcriptase (Gibco BRL, Rockville, MD, USA) using a randam hexamer primer (19). cDNA, equivalent to 0.15 μg total RNA, was applied to one PCR, which was set to amplify the target message for 28 cycles. Primers were designed to yield a 300 to 500 bp product for each gene. The PCR products were separated in agarose gel, stained with ethidium bromide and photographed on the UV-transilluminator by a CCD camera. Information on the primer sequences is available on request.

Measurement of caspase activity. Caspase activity was measured using a Caspase colorimetric assay kit (BioVision, Palo Alto, CA, USA), according to the manufacturer's recommendations. One hundred and fifty micrograms of each protein was applied to assay DEVD-caspase activity.

Immunoblot analysis. The cells were lysed in RIPA buffer. Then 60 μg of protein was mixed with SDS loading buffer, boiled and separated by SDS-polyacrylamide gel electropheresis. Proteins were transblotted onto a PVDF membrane and incubated with each antibody, after which detection was done using an electrochemiluminescence system (Amersham Biosciences, Piscataway, NJ, USA). The following antibodies were used: anti-CPP32 mAb (Immunotech, France), cdc2, cdk2 and PCNA (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). Anti-Cip1/WAF1 was purchased from BD Transduction Laboratories (Lexington, KY, USA). p21 phosphorylation was studied by treating with phosphatases. Briefly, the frozen cell pellets were lysed in the reaction buffer (20 mM Hepes (pH7.4), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 30 mM 2-mercaptoethanol, 1 mM EGTA, 0.1% NP40) (20), and an equal amount of lysate was treated with Protein Phosphatase-1 (PP1) or PP2A (Upstate Biotechnology, NY, USA) at 30˚C for 1 h. Samples were applied to immunoblot analysis using anti-p21 anibody to detect the shift of bands.

Results

IFN-α potentiates sensitivity to X-ray irradiation. First, the effect of IFN-α on X-ray irradiation-induced cytotoxity was examined. As demonstrated in Figure 1A, 3 days of IFN-α treatment reduced the colony forming activity by nearly 40%, and X-ray irradiation caused a synergistic further reduction of colony formation. This synergistic effect was more prominent with higher doses of X-rays (3 and 5 Gy). To analyze the synergistic effect, 200 U/ml IFN-α and 3 Gy irradiation were examined alone and in combination (Figure 1B). The surviving fractions after IFN-α treatment or X-ray irradiation were 35% of the control in both cases, while the surviving fraction after the combination treatment was about 1% of the control cells (Figure 1B-sensitive). Next, we asked whether this synergistic effect was dependent on

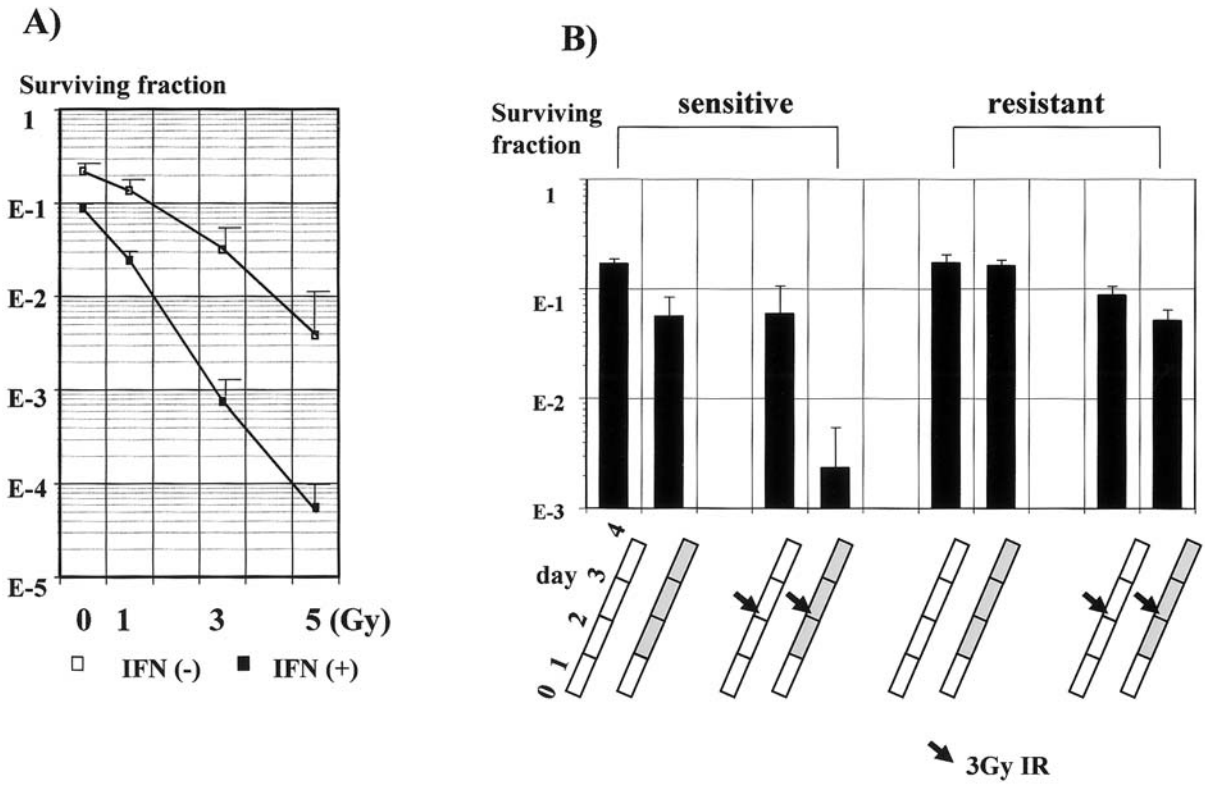

Figure 1. *Colony-forming activity of treated cells. A) Dose response curve of X-ray irradiation. Daudi cells were cultured with or without 200 U/ml IFN-α for 24 h and X-ray irradiated by each designated dose. Cells were further cultured for 48 h with (closed square) or without (open square) IFN-α, and then the colony assay was done. B) Synergistic effect of IFN-α and X-ray irradiation. Both IFN-α-sensitive and -resistant Daudi cells were treated with 200 U/ml IFN-α, X-rays and the combination of IFN-α and X-rays, and then the colony assay was done. Treatment conditions are demonstrated beneath the graph. The dark area of the experimental schedule denotes the period of IFN-α treatment. Closed arrows demonstrate the timing of X-ray irradiation.*

IFN, using IFN-α-resistant Daudi cells. The resistant cells were treated exactly as IFN-sensitive cells. As demonstrated in Figure 1B-resistant, there was no suppression of colony formation by IFN-α alone, and the combination with X-ray irradiation did not lead to synergism.

Caspase-3 activity after IFN-α and irradiation. Since IFN-α pretreatment enhanced the cytotoxicity of irradiation, we studied the mechanism of this combination. First, 200 bp DNA ladder formation was investigated in treated cells, but we could not detect DNA fragmentation representative of apoptosis (data not shown). Then, we examined the emergence of large fragments of cellular DNA by treatment. Neither IFN-α nor irradiation alone generated DNA fragments (Figure 2A) and there were also no 2 Mb double-strand breaks after either treatment. However, the combination of IFN-α pretreatment and irradiation induced a 50 kb smear (Figure 2A), suggesting the occurrence of apoptosis. Next, we studied the changes of caspase activity using DEVD-AMF as a substrate. As demonstrated in Figure 3B, DEVD-caspase activity only showed a slight, but significant, increase after combined treatment. Since caspase-3 cleaves DEVD-AMF as a substrate, we then studied caspase-3 activation by detecting the cleaved fragment. Neither IFN-α nor irradiation alone produced the band of the cleaved fragment, but combined a faint band (Figure 2C).

Production of reactive oxygen species. To evaluate the mechanism through which the cytotoxicity of X-ray irradiation was enhanced by IFN-α pretreatment, we investigated whether IFN-α could modulate the production of ROS after irradiation. The formation of peroxides was observed 2 h after irradiation and continued until 48 h (data not shown). Accordingly, the effect of IFN-α-pretreatment was assayed at 24 h after irradiation. The cells were pretreated with IFN-α for 24 h and then were irradiated and cultured for a further 24 h. As demonstrated in Figure 3, the effect of IFN-α alone was not prominent, but ROS were produced after X-ray irradiation. Production of both H_2O_2

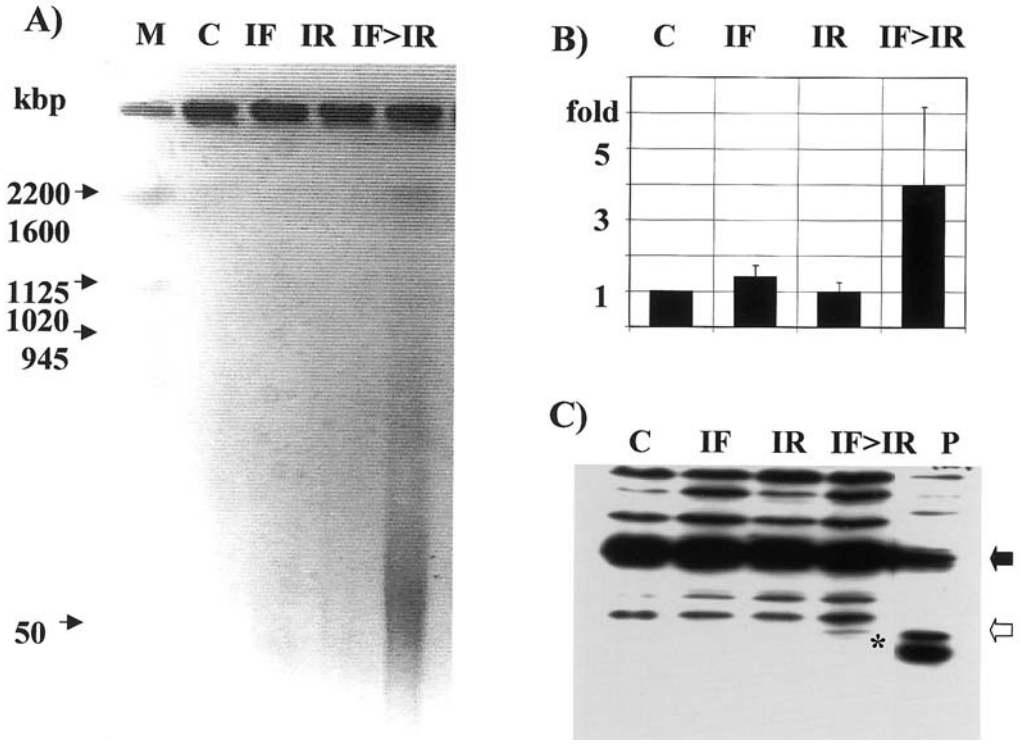

Figure 2. *Apoptosis by IFN-α and X-ray irradiation. A) Detection of apoptosis by pulse field gel electrophoresis. Nearly 50 kb smear DNA fragmentation was observed only in the IFN-α and X-ray-irradiated cells. B) DEVD-caspase activity. Each DEVD-caspase activity was compared to that of control cells, and mean and SD were demonstrated. Experiments were done three times independently. C) Cleavage of caspase-3. Caspase-3 activation was detected by the cleavage of caspase-3. Closed arrow indicates procaspase-3. Open arrow indicates the cleaved caspase-3. Asterisk indicates the cleaved band in the IFN-α- and X-ray-treated cells. P demonstrates the positive control of caspase-3 activation induced by TNF-α in U937 cells. M: marker, C: control, IF: 200 U/ml IFN-α-treated cells, IR: 3 Gy-irradiated cells, IF>IR: Cells first treated with IFN-α for 24 h and irradiated by 3 Gy, followed by a further 48-h treatment with IFN-α.*

and O_2^- after irradiation was suppressed by IFN-α pretreatment. These results suggested that IFN-α did not enhance the production of ROS by X-ray irradiation, but instead inhibited it.

DNA double-strand breaks and repair-related gene expression. Since irradiation induces DNA double-strand breaks, we studied whether IFN-α-pretreatment modulates the double-strand breaks (DSBs) induced by irradiation. Since 3 Gy of irradiation failed to induce the 2 Mb double-strand breaks (Figure 2A), we studied DSBs using a higher dose of X-ray irradiation. The cells were irradiated with 50 Gy, with or without IFN-α-pretreatment, and DSBs were studied by pulse-gel electrophoresis. Fifty Gy of irradiation induced a huge amount of DSBs immediately after irradiation. But, irradiation-induced double-strand breaks were mostly repaired within 24 h after regardless of IFN-α-pretreatment. Again, the smaller DNA fraction, less than 100 kb, emerged only in the IFN-α pretreated group (Figure 4A).

Because, one of the major functions of IFNs is alteration of gene expression, we studied the mRNA expression of double-strand repair-related genes by RT-PCR. Rad51, Rad54, DNA-PK, XRCC4 and Ligase IV were decreased by IFN-α treatment, while Ku80, hAPE-1 were not changed (Figure 4B). The combination treatment did not significantly affect the changes observed by IFN-α alone.

Induction of p21 is suppressed by IFN-α. One of the representative genes induced by irradiation is p21. We next studied whether IFN-α had an influence on the induction of p21 by irradiation. IFN-α did not affect the expression or mobility of p21 on electrophoresis, as shown in Figure 5A (a-d), while X-ray irradiation markedly induced p21 expression (Figure 5A e, f). This induction of p21 was partly inhibited by IFN-α pretreatment (Figure 5A g, h). Cdk2 expression was suppressed by IFN-α treatment, while X-ray irradiation increased it slightly. Moreover, the p21 induced by X-ray irradiation contained some slower migrating proteins (Figure 5A e, f). To investigate whether this slower migration resulted from the phosphorylation of p21, irradiation-induced p21 was treated with phosphatases (PP1 or PP2) before loading onto the gel and the pattern of

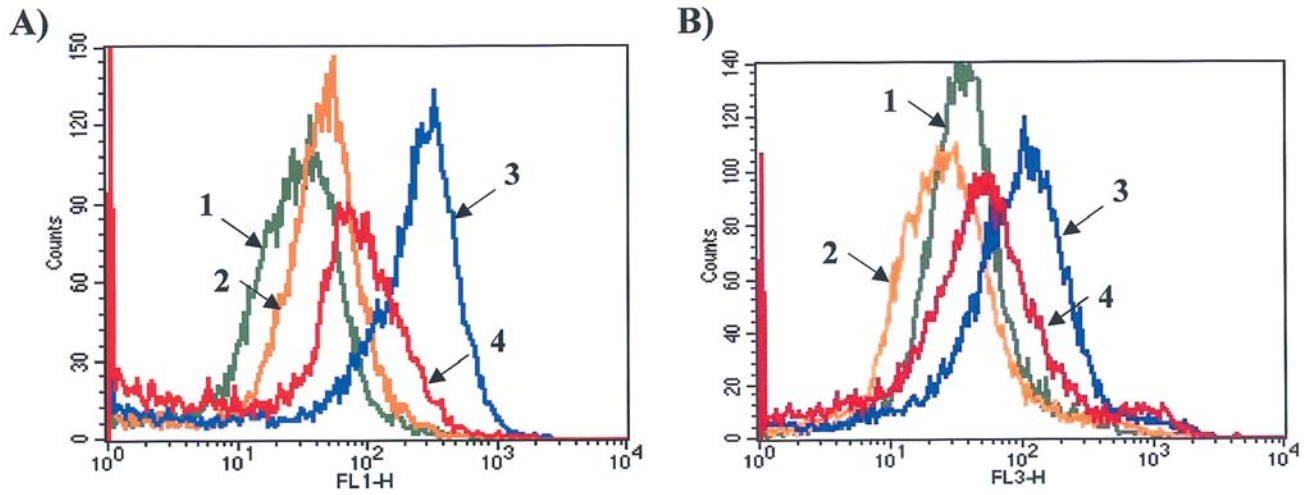

Figure 3. *Production of reactive oxygen species. A) Flow cytometry analysis of H_2O_2. B) Flow cytometry analysis of O_2^-. 1:control, 2: 200 U/ml IFN-α-treated, 3: X-ray (3 Gy) irradiation, 4: Pretreatment with 200 U/ml IFN-α for 24 h and then 3 Gy irradiation, followed by a further 24-h culture with IFN-α.*

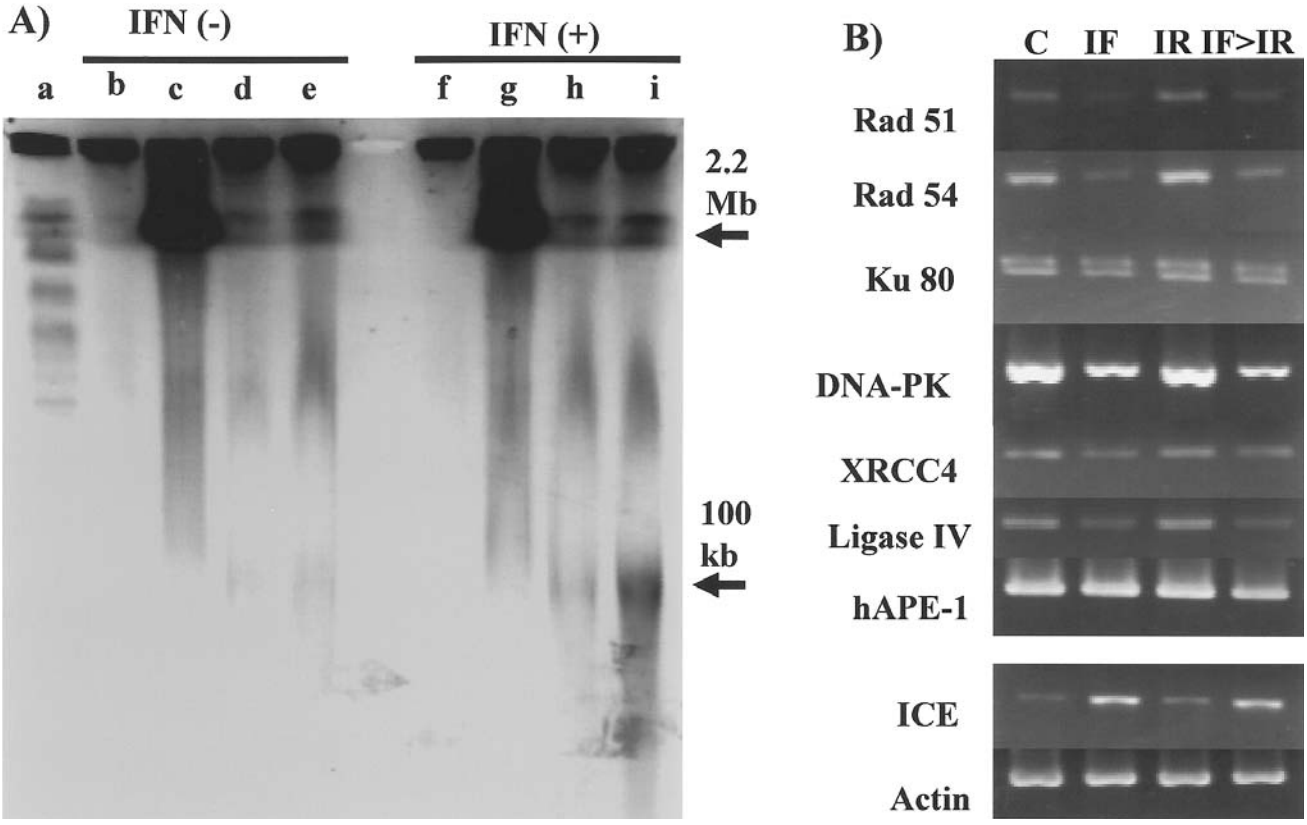

Figure 4. *DNA breaks. A) DNA double-strand breaks studied by pulse-field gel electrophoresis. Cells were cultured with or without 200 U/ml of IFN-α for 24 h, and then irradiated by 50 Gy followed by a further 24 or 48 h of culture with or without IFN-α. a: marker, b and f: without irradiation, c and g: immediately after irradiation, d and h: 24 h after irradiation, e and i: 48 h after irradiation. B) Expression of DNA double-strand breaks repair-related genes. mRNA levels were studied by RT-PCR. C: control, IF: 200 U/ml IFN-α-treated, IR: 3 Gy of X-ray-irradiated, IF>IR: Cells first treated with 200 U/ml IFN-α for 24 h, and irradiated by 3 Gy followed by a further 24-h treatment with IFN-α.*

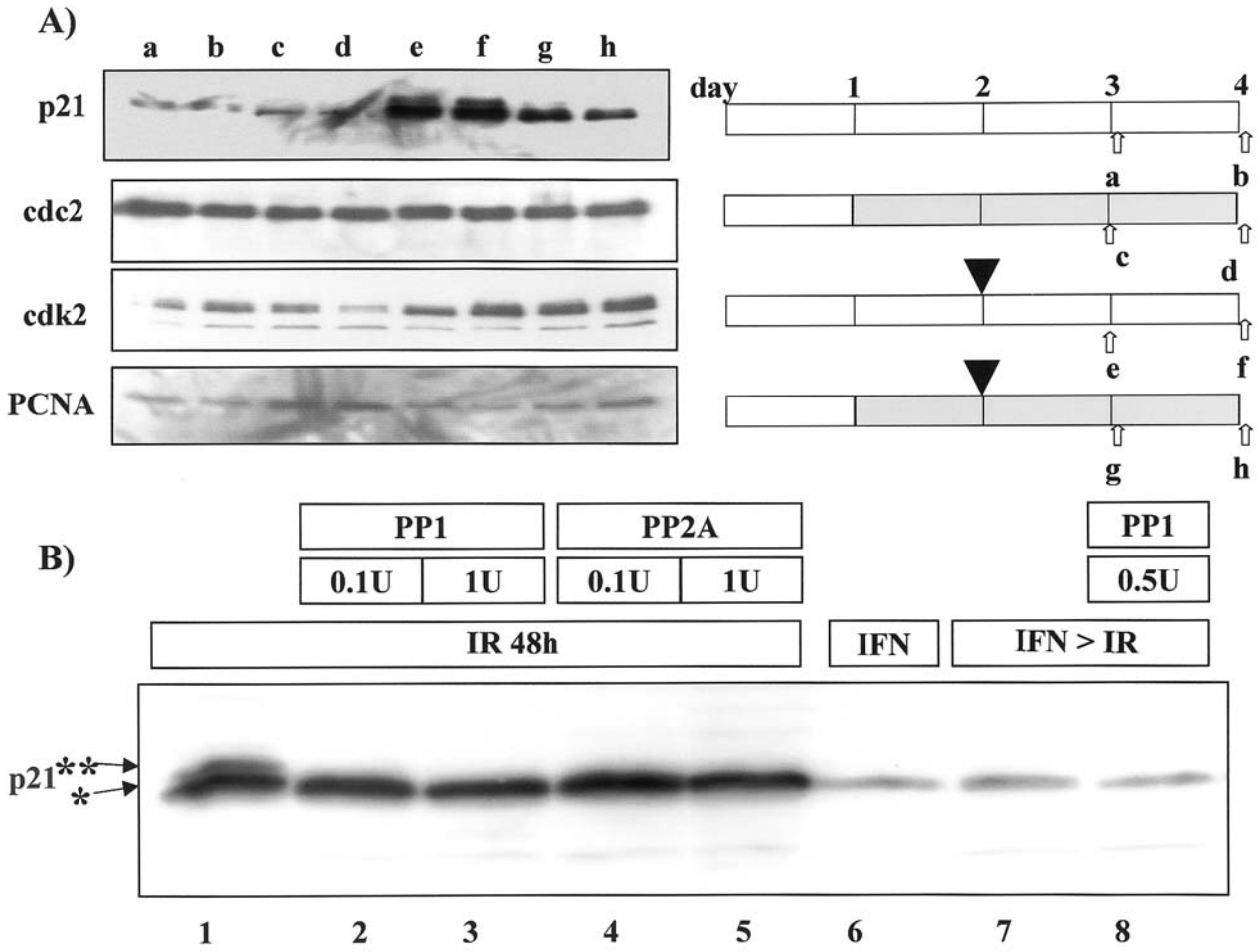

Figure 5. *IFN-α inhibits p21 induction and phosphorylation by X-rays. A) Immunoblotting of cell cycle-related genes. Cell-harvesting points were demonstrated schematically next to the immunoblot pictures. The dark area of the experimental schedule denotes the period of IFN-α treatment. Closed arrows demonstrate the timing of X-ray irradiation. Open arrows indicate the harvesting points. B) Phosphorylation of p21 induced by X-rays. Cell lysates used for 1 to 5 lanes were harvested at f-point of the experiment A), and 6 at d. Samples of 7 and 8 were collected at h-point. These samples were pretreated with indicated phosphatases (PP1 or PP2A), as mentioned in the text. * and ** denote p21 and phospho-p21, respectively.*

migration was studied. Slowly migrating p21 disappeared after the treatment of phosphatases, demonstrating that p21 induced in Daudi cells by X-ray irradiation was phosphorylated (Figure 5B). These results demonstrated that pretreatment with IFN-α not only suppressed the induction of p21, but also its phosphorylation by irradiation.

Discussion

Radiation is one of the fundamental therapeutic modalities for cancer, but radiosensitivity is not sufficient to improve the clinical outcome in some tumors. To overcome the resistance, one strategy is to combine irradiation with anticancer agents. This strategy has been studied using either chemotherapeutic agents or biological response modifiers. Thus, we studied the effect of combined therapy with IFN-α and irradiation.

Our data demonstrated that pretreatment with IFN-α markedly enhanced the radiosensitivity of Daudi cells. A synergistic effect was not observed in IFN-resistant Daudi cells, so this effect was considered to depend on the biological activity of IFN-α. If cells are treated with radiation alone, seeding cells immediately after irradiation is the most effective method for colony inhibition. Moreover, an administration of IFN-α prior to irradiation enhanced the radiosensitivity, but not *vice versa* (our unpublished data).

These facts indicate that intracellular recovery processes after irradiation are impaired by IFN-α. Since IFN-α alters gene expression at transcription and translation stages, we

focused on cell cycle regulation and DNA damage repair genes. We found that the expression of mRNA for RAD51, RAD54, DNA-PK, XRCC4 and DNA Ligase IV, all of which are essential for the repair of DSBs by non-homologous end joining (NHEJ), was suppressed by IFN-α, while other genes were little affected. However, no obvious DSBs were observed after 3 Gy of irradiation by our pulse-gel electrophoresis assay. Moreover, the main DSBs induced by 50 Gy of irradiation were promptly repaired irrespective of prior treatment with IFN-α. These results suggest that cells are at least capable of repairing the majority of DSBs for a few days after IFN-α treatment. A longer pretreatment of IFN-α may alter the repair of DSBs induced by X-rays, since mRNAs of most NHEJ-related genes are down-modulated by IFN-α. Further studies are required to answer this issue.

The cell cycle status of cancer cells at the time of irradiation might play an important role in the effect of X-rays, because cancer cells must arrest the cell cycle to repair DNA damage. We found that X-rays extensively induced p21 in Daudi cells, and it was markedly suppressed in IFN-α-pretreated cells. Originally, p21 was discovered as one of the prototype cell cycle inhibitors (14-16), and it is mainly regulated by the tumor suppressor gene p53 (14). But even in cells lacking p53, p21 can be induced by several treatments (21-23). Several lines of evidence have recently suggested that p21 is involved in the control of apoptosis due to γ-irradiation (24, 25). Tian *et al.* revealed that the inhibition of p21 by antisense oligo-nucleotide sensitized cells to radiation both *in vivo* and *in vitro* (25). Waldman *et al.* demonstrated that p21-deficient cells were sensitive to radiation *in vivo*, since these cells were unable to arrest in the G2-phase and DNA synthesis continued without mitosis (26). It has further been demonstrated that the localization of p21 is also important for its function, since p21 in the nucleus mainly acts as a cell cycle inhibitor, while p21 in the cytoplasm has an anti-apoptotic effect (27). Phosphorylation of p21 regulates its cellular location in cancer cells, *i.e.*, phosphorylation of serine 147 by Akt suppresses translocation of p21 into the nucleus and promotes cell survival (28-30). In this study, we found that IFN-α pretreatment prevented the induction and phosphorylation of p21 after exposure to X-ray irradiation, but the mechanism involved remains obscure. IFN-α also inhibited the production of reactive oxygen species by irradiation (Figure 3). H_2O_2 can induce p21 (31) and p21 mediates the accumulation of ROS (32). Thus, the inhibition of ROS production may have contributed to the suppression of p21 induction by X-ray irradiation, and the inhibition of p21 production may render cells susceptible to X-ray irradiation.

In conclusion, the pretreatment with IFN-α is able to potentiate the cytotoxicity of X-rays and this synergistic effect may depend on the inhibition of p21 by IFN-α. These results may be useful for the clinical application of IFN-α with irradiation as well as other DNA-damaging agents.

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