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Ionizing radiation suppresses FAP-1 mRNA level in A549 cells via p53 activation

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Abstract **Ionizing radiation (IR) is known to upregulate cell surface Fas through p53 activation in various cells. However, the signaling pathway intermediating between p53 activation and cell surface Fas upregulation remains to be elucidated. Recently, Fas-associated phosphatase-1 (FAP-1) has been reported to associate with Fas and inhibit cell surface Fas expression. We evaluated the expression of FAP-1 mRNA following IR in A549 cells. Ionizing radiation inhibited the expression of FAP-1 mRNA. Pretreatment with p53 inhibitor pifithrin α cancelled the IR-induced downregulation of FAP-1 mRNA. These results suggest that IR-induced p53 activation may upregulate cell surface Fas via the down-modulation of FAP-1.**

Keywords: Fas; Fas-associated phosphatase-1; Ionizing radiation; p53

1. Introduction

Radiation therapy is an important therapeutic modality in the treatment of lung cancer. Ionizing radiation (IR) has been shown to alter phenotype of target tissue, including upregulation of several classes of molecules on tumor cells that could potentially influence the susceptibility of the cells to apoptosis [1]. Of the upregulated molecules, the most characterized is Fas. Ionizing radiation induces cell surface Fas upregulation through the activation of p53 [2]. The increase in cell surface Fas induced by activated p53 involves transcription-dependent and transcription-independent mechanisms [3,4]. Müller and colleagues have demonstrated that induction of Fas gene transcription by p53 is mediated through a strong p53-responsive element located within the first intron of the gene [3]. Bennett et al. have found that p53 activation can regulate sensitivity to Fas-mediated apoptosis by allowing cytoplasmic Fas to redistribute to the cell surface [4]. However, the detailed molecular mechanism by which p53 activation increases surface Fas expression remains uncertain.

Fas-associated phosphatase-1 (FAP-1) is a protein tyrosine phosphatase, which was identified as a protein that associates with Fas [5]. It has been reported that FAP-1 is a negative regulator of Fas-mediated signal transduction and related to resistance against Fas-mediated apoptosis [6]. Ivanov and coworkers have shown that FAP-1 association with Fas inhibits Fas transport from Golgi complex to the cell surface [7]. Irie and colleagues have found that there is a potential binding site of p53 in the FAP-1 promoter, suggesting that activated p53 might suppress the transcription of FAP-1 gene [8].

In this study, using a human adenocarcinoma cell line A549, we found that IR suppressed the expression of FAP-1 mRNA via the activation of p53 pathway. Based on the evidence that FAP-1 inhibits cell surface Fas expression, our findings indicate that p53 activation following IR induces the upregulation of Fas on the cell surface through the suppression of FAP-1 expression.

2. Materials and methods

2.1. Reagents and antibodies

Pifithrin α(PFTα) was purchased from Sigma (Tokyo, Japan). Fluorescein isothiocyanate (FITC)-labeled mouse anti-human Fas mAb, clone DX2, and FITC-labeled mouse IgG1 were from DAKO JAPAN Co., Ltd. (Kyoto, Japan). Anti-p53 and anti-p21 antibodies were obtained from BD Biosciences (San Jose, CA).

2.2. Cell culture

The human adenocarcinoma cell line A549 was purchased from American Type Culture Collection (Manassas, VA). This cell line has wild-type p53 [9], and expresses both Fas and FAP-1 [6]. The culture medium was RPMI 1640 containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. The cells were grown in 60-mm tissue culture dishes at 37 °C in a 5% CO_2 humidified incubator.

2.3. X-ray irradiation

Reaching 50–70% confluence, cells were irradiated at room temperature with an X-ray source (dose rate: 5 Gy/min; MBR-15020R-3, Hitachi Medical, Hitachi, Japan).

2.4. Flow cytometric detection of Fas

Suspension of A549 cells (1×10^6 cells) were fixed with 4% paraformaldehyde for 15 min at room temperature, and washed twice. The cells were incubated with FITC-labeled anti-human Fas mAb, DX2, or FITC-labeled control mouse IgG1, in the dark for 30 min at 4 °C.

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Abbreviations: FAP-1, Fas-associated phosphatase-1; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IR, ionizing radiation; MFI, mean fluorescence intensity; PFTα, pifithrin α

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After washing, the cells were analyzed on a flow cytometer (FACSCalibur; Becton Dickinson, Mountain View, CA). Ten thousand cells were examined for each determination. Values for mean fluorescence intensity index (MFI index) were calculated using the formula: MFI index = [(MFI of anti-Fas Ab-stained cells) − (MFI of control IgG1-stained cells)]/MFI of control IgG1-stained cells.

2.5. Western blot analysis

The expression of p53 and p21 were determined by immunoblot analysis. Whole cell lysate extracts were prepared, and the equal amount of cellular protein (30 μg/well) were separated on SDS–polyacrylamide gel (10%) and electrically transferred onto polyvinylidene fluoride membranes. After blockade of nonspecific binding, the blots were revealed by using primary antibodies, the corresponding secondary antibodies and ECL detection kit (Amersham Life Science, Tokyo, Japan).

2.6. RT-PCR

Total cellular RNA was extracted, and the first strand cDNA was synthesized using oligo(dT) primer and random primer. PCR was performed on cDNA using primers for the amplification of FAP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers were designed according to published sequences [10]: FAP-1-sense, 5′-GAATACGAGTGTCAGACATGG-3′; FAP-1-antisense, 5′-AGGTCTGCAGAGAAGCAAGAATAC-3′; GAPDH-sense, 5′-CAAAAGGGTCATCATCTCTG-3′; and GAPDH-antisense, 5′-CCTGCTTCACCACCTTCTTG-3′. Thermal cycling for FAP-1 and GAPDH was carried out as follows: 28 cycles, denaturation at 95 °C for 1 min, annealing at 58 °C for 2 min, and extension at 72 °C for 1 min. PCR products were analyzed by electrophoresis through 1.2% agarose gels and viewed under UV light after ethidium bromide staining.

2.7. Statistical analysis

We repeated each type of experiment at least three times and confirmed that similar data were obtained. All values are means ± S.E.M. Comparisons were made with one-way ANOVA with Fisher's *post hoc* test. A P value of <0.05 was considered significant.

3. Results

3.1. Upregulation of cell surface Fas by IR

The initial experiment was performed to determine whether IR was capable of inducing the upregulation of cell surface Fas on A549 cells. A549 cells were irradiated and the level of Fas

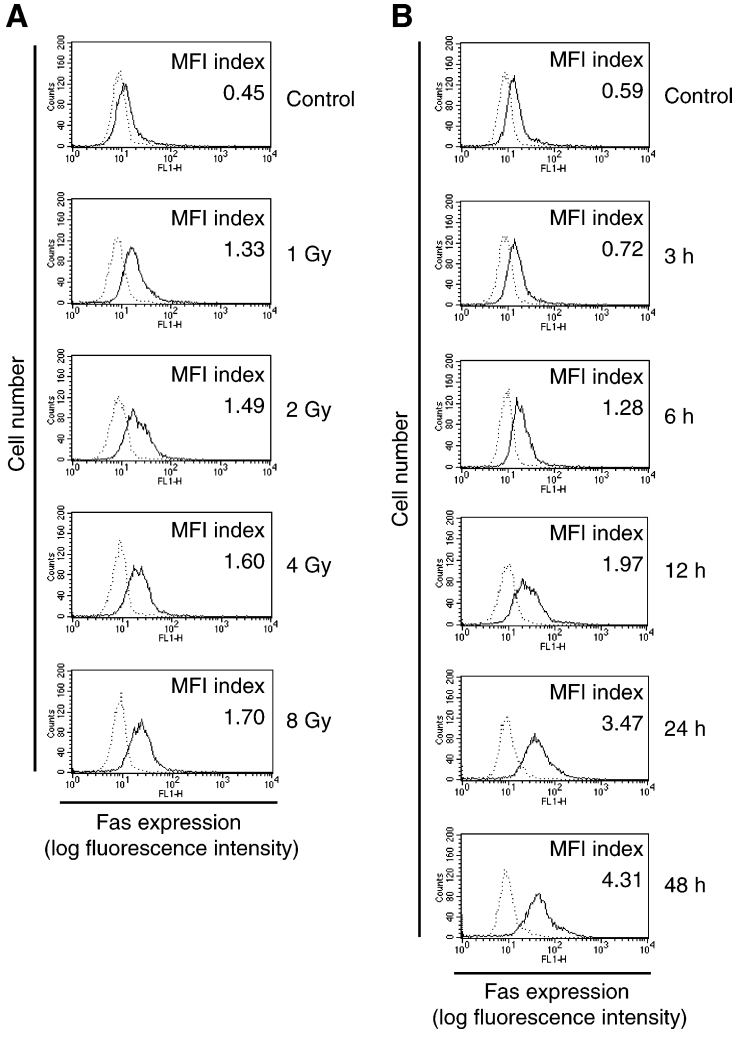

Fig. 1. Effect of ionizing radiation on the cell surface expression of Fas in A549 cells. (A) Dose-dependent effect of ionizing radiation on cell surface Fas expression. After the indicated doses of irradiation, A549 cells were incubated for 12 h, and then cell surface Fas expression was analyzed by a flow cytometer. (B) Time-dependent effect of ionizing radiation on cell surface Fas expression. A549 cells were exposed to irradiation (2 Gy) and cultured for the indicated time periods. Cell surface Fas expression was then analyzed by a flow cytometer. A solid line represents a histogram of cells stained with anti-Fas Ab. A dashed line denotes a histogram of cells stained with control mouse IgG1. The number of each panel represents mean fluorescence intensity index (MFI index).

was examined by flow cytometric analysis. Ionizing radiation induced dose-dependent and time-dependent increase in cell surface Fas (Figs. 1A and B).

3.2. Ionizing radiation downregulates the expression of FAP-1 mRNA in A549 cells

It is reported that FAP-1 association with Fas inhibits its export to the cell surface [7]. Hence, we evaluated the effect of IR on FAP-1 mRNA expression in A549 cells. The time-course of FAP-1 mRNA after exposure to IR is shown in Figs. 2A and B. Ionizing radiation downregulated FAP-1 mRNA expression. The kinetics of FAP-1 mRNA expression was biphasic after irradiation, with the first trough at 1.5 h and the second trough at 24 h.

3.3. Pifithrin α can inhibit the transcriptional activity of p53

Consistent with the knowledge that IR activates p53 [11,12], IR caused A549 cells to increase the levels of p53 biphasically, which seems to represent a mirror image of the time-dependent kinetics of FAP-1 mRNA (Fig. 3A). A p53 inhibitor PFTα suppressed the IR-induced accumulation of p21 protein in A549 cells (Fig. 3B), indicating that PFTα effectively inhibited the transcriptional activity of p53.

3.4. Involvement p53 in the downregulation of FAP-1 mRNA induced by IR

Using PFTα, we explored the involvement of p53 in the IR-induced downregulation of FAP-1 mRNA expression in A549 cells. Pretreatment with PFTα cancelled the suppression of FAP-1 mRNA expression by IR (Fig. 4). This result clearly shows that the level of FAP-1 mRNA expression is negatively modulated by p53 activity.

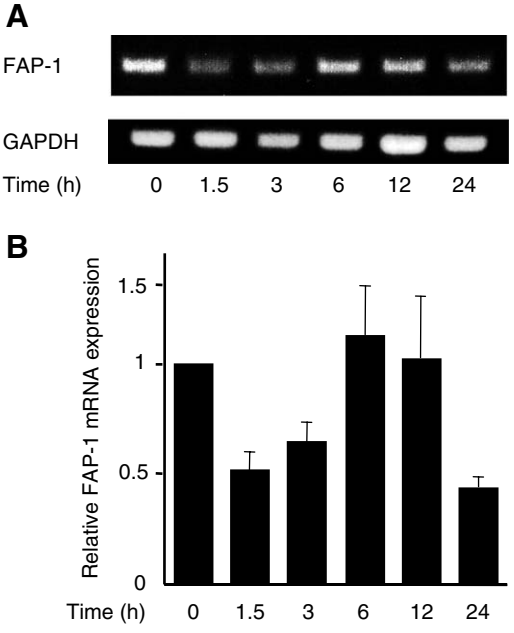

Fig. 2. Effect of ionizing radiation on FAP-1 mRNA expression in A549 cells. (A) Time-course of ionizing radiation-induced FAP-1 mRNA expression in A549 cells. After irradiation (2 Gy), cells were incubated for the indicated time periods, and then analyzed for FAP-1 mRNA by RT-PCR. (B) The ratio of FAP-1 to GAPDH mRNA was calculated and expressed relative to that of untreated A549 cells. The values are means ± S.E.M. of three independent experiments.

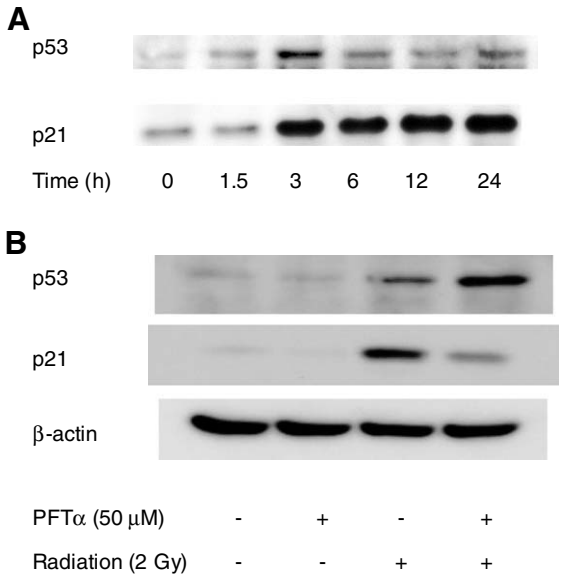

Fig. 3. Western immunoblot analysis of p53 and p21 in A549 cells. (A) Time-course of p53 and p21 accumulation after irradiation (2 Gy) in A549 cells. (B) Effect of p53 inhibitor PFTα on the accumulation of p21 in irradiated A549 cells. Cells were pretreated with PFTα (50 μM) for 3 h, and exposed to ionizing radiation (2 Gy). After 3 h, the accumulation of p53 and p21 was investigated.

4. Discussion

In the present study, we have demonstrated that IR increases the cell surface Fas and suppresses the expression of FAP-1 mRNA via p53 activation in A549 cells. On the basis of the observations that FAP-1 association with Fas inhibits its export to the cell surface [7], and that there is a potential binding site of p53 in the 5′ flanking region of the human FAP-1 gene [8], our findings imply that the downregulation of FAP-1 by activated p53 may play an important role in the IR-induced upregulation of cell surface Fas. Our study is the first report on the involvement of p53 activation in FAP-1 mRNA expression. Although a number of studies have reported that p53 is involved in the IR-induced cell surface Fas expression, the detailed mechanism is poorly understood. Our study provides one possible mechanism underlying this phenomenon.

A number of studies have provided evidence that genotoxic stimuli such as IR, etoposide and doxorubicin induces the expression of Fas in a p53-dependent manner [3]. p53 has been shown to modulate Fas expression with two mechanistically distinct forms: (a) transactivation of a p53 responsive element in the Fas promoter and subsequent increased Fas mRNA synthesis [3], and (b) the trafficking of Fas from intracellular storage vesicles to the cell membrane by a transcription-independent mechanism [4]. Several studies have demonstrated that cytoplasmic expression of Fas without surface expression directly affects the cell resistant to Fas-mediated apoptosis in pulmonary adenocarcinoma cells including A549 cells [13]. In order to restore cell sensitivity to Fas-mediated apoptosis in lung cancer cells, it is therefore crucial to elucidate the

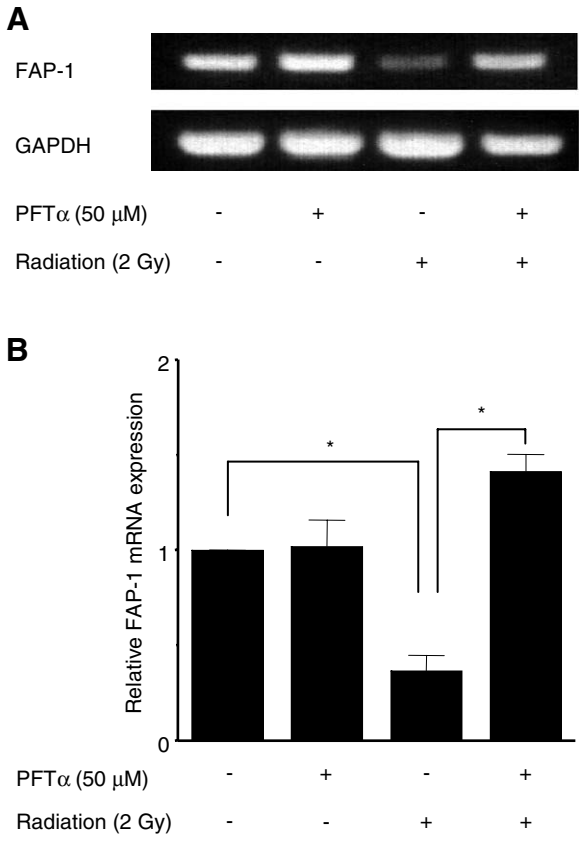

Fig. 4. Effect of p53 inhibitor PFTα on FAP-1 mRNA expression in irradiated A549 cells. (A) Cells were incubated with or without the pretreatment of PFTα (50 μM) for 3 h, and exposed to ionizing radiation (2 Gy). After 3 h, cellular RNA was harvested and analyzed for FAP-1 mRNA by RT-PCR. (B) The ratio of FAP-1 to GAPDH mRNA was calculated and expressed relative to that of untreated A549 cells. The values are means ± S.E.M. of three independent experiments. $^{*}P < 0.01$.

molecular mechanisms responsible for the trafficking of Fas to the cell surface.

FAP-1 expression is frequently observed in a variety of human tumor cells including lung cancer [14]. It is demonstrated that FAP-1 expression in cancer cells is related to the resistance to Fas-mediated apoptosis [15–17]. One of the reasons of this resistance can be due to FAP-1 function that prevents the trafficking of Fas from intracellular store to the cell surface by associating with Fas [7]. A recent report has provided evidence that FAP-1 is capable of dephospharylating phosphotyrosine 275 in the C-terminus of Fas in astrocytoma cells [18]. FAP-1 therefore seems to modulate Fas signaling on at least two levels: trafficking and signal downregulation.

Our results indicate that IR downregulates the expression of FAP-1 mRNA in A549 cells through the activation of p53. Mishima and coworkers have shown that a selective cyclo-oxygenase-2 inhibitor, etodolac, enhances carboplatin-induced apoptosis of human tongue carcinoma cells by downregulation of FAP-1 expression [19]. Based on the finding that cyclo-oxygenase-2 has potent inhibitory effects on p53 transcriptional activity [20], etodolac in combination with carboplatin may inhibit the expression of FAP-1 molecule through activation of p53. Various other stimuli have been reported to modulate the expression of FAP-1 in human tumor cells. Song et al. have shown that IL-2 enhances susceptibility of colon cancer cells to Fas-mediated apoptosis by upregulating Fas receptor level and downregulating FAP-1 expression [21]. Mundle and colleagues have demonstrated that TNF-α decreases the expression of FAP-1 mRNA in HL60 promyelocytic cells [22]. In addition to the consensus binding sequence for p53, potential binding sites for a variety of transcription factors including NF-IL-6, NF-κB, and EWS-FLI1 were found in the 5′-flanking region of the human FAP-1 gene [8,23]. The expression of FAP-1 may be regulated by the above agents through either transcription-dependent or transcription-independent mechanism.

In summary, this is the first evidence that IR is capable of modulating FAP-1 mRNA expression via the activation of p53 pathway in human lung tumor cells. Further studies are required to better understand the relationship between modulation of FAP-1 and Fas-mediated apoptosis, and its potential application to lung cancer therapy.

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