J. RADIAT. RES., 39, 111–118 (1998)

Sensitivity to Ionizing Radiation in Saos-2 Cells Transfected with Mutant *p53* Genes Depends on the Mutation Position

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(*Received, September 24, 1997*)
(*Revision received, February 20, 1998*)
(*Accepted, February 27, 1998*)

Ionizing radiation/Mutant p53/Radiosensitivity/Transformant/Saos-2 cells

We have constructed an *in vitro* system to examine how *p53* mutants affect radiosensitivity. Mutations of *p53* were made using *in vitro* mutagenesis, and mutant cDNAs were introduced into the human osteosarcoma cell line, Saos-2, which is devoid of endogenous *p53*. For wild type *p53*, both the expression plasmid and a regulation plasmid (LacSwitch system) were transfected into the cells. The radiosensitivities of clones of mutant *p53* and wild type *p53* were examined. Transformants of wild type *p53* had increased radiosensitivity. The induction of wild type p53 protein by addition of IPTG did not significantly increase radiosensitivity. A mutation at codon 123 also increased radiosensitivity. Mutations at codons 143, 175, and 273 did not alter radiosensitivity.

INTRODUCTION

Mutation of the *p53* gene is the most common genetic alteration observed in human cancers[1,2]. *p53* is a tumor-suppressor gene and plays an important role in neoplastic development[3,4]. Normal *p53* gene product accumulates after ionizing irradiation (IR), and causes G1 arrest or promotes cell death through an apoptotic pathway[5–7]. *p53* in cells can either increase or decrease cell survival. Whether or not cancer cells are resistant or sensitive to IR is an important consideration in radiotherapy. It has been widely reported that cells with mutant *p53* are more resistant to IR or DNA-damaging agents[8–11]. On the other hand, there have been reports of cells with mutant *p53* that are sensitive to IR and anticancer drugs[12,13]. The specific mutations were not discussed in these reports. *p53* mutants have different properties according to the mutation positions. For example, Crook et al. reported that, using a large series of *p53* mutants, not all transcriptionally active mutants retained the ability to suppress transformation in primary rodent cells, but two tumor-derived point mutations displayed both transforming and transactivating activity[14]. Some

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mutant *p53* genes do not merely induce the functional equivalent of *p53* loss[15]. Radiosensitivity of cells may depend on the type of mutation of *p53*. To determine which mutations of *p53* affect radiosensitivity, we made four mutations of *p53* at codons 123, 143, 175 and 273. Mutations at codons 175 and 273 are hot spot mutations and localized in conserved regions IV and V, respectively. Codon 123 is in conserved region III and codon 143 is adjacent to this region. We transferred these mutant *p53* DNA to the human osteosarcoma cell line, Saos-2, which is null for *p53*, and obtained stable transformants. The effect of these mutations on radiosensitivity was examined.

MATERIALS AND METHODS

Cell culture

Saos-2 cell line (RCB0428: Riken Cell Bank) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The transformants were selected in medium containing 100 μg ml^{-1} of hygromicin or 400 μg ml^{-1} of G418.

Plasmids

The LacSwitch inducible mammalian expression system (Stratagene, La Jolla, CA., USA) was used. The expression plasmid was pOPI3CAT, and the *CAT* gene was replaced by genes of a mutant or wild type *p53* at a Not I site. Saos-2 cells were transfected with 2 μg of expression plasmid using the calcium phosphate precipitation method[16]. A plasmid of p3'SS, expressing the *lac I* gene coding the Lac repressor protein, was used for a regulation plasmid. For wild type *p53* transfection, both the expression plasmid and regulation plasmid were co-transfected into the cells. The expression of the wild *p53* gene was suppressed by the Lac repressor. Wild type *p53* transformants were induced to express *p53* by the addition of IPTG to the media.

In vitro mutagenesis

Mutagenesis was performed with the Sculptor system (Amersham, Buckinghamshire, England). The procedure was according to the manufacture's protocol. Wild type *p53* cDNA (pPro S*p53*: CD 104; Japanese Cancer Research Resources Bank) was introduced into the M13 vector. Oligonucleotides for the mutations at codon 123 (Thr $\rightarrow$ Ala), 143 (Val $\rightarrow$ Ala), 175 (Arg $\rightarrow$ His), 273 (Arg $\rightarrow$ His) were ordered from the Cruachem Kyoto Research Center in Japan. The mutant *p53* DNA sequences were checked with a Sequenase version 2.0 labeled dCTP kit (Amersham). The efficiency of mutagenesis was more than 80% (data not shown).

PCR analysis for cDNA of p53

Primer pairs encompassed a 1,041 bp fragment (from Exon1 to Exon 9) specific for *p53*. Total DNA was isolated with a DNA extraction kit (Stratagene, La Jolla, CA, USA). One μg of DNA was added to the PCR reaction mixture. Thirty cycles of PCR amplification were performed with the parameters of 93°C, 55°C, and 72°C for 1 min, 1 min, and 2 min, respectively. The amplified products were analyzed by electrophoresis on a 1% agarose gel and detected by

ethidium bromide staining.

Western blotting for protein of p53

Total protein was extracted as described previously[17]. Thirty μg of protein was subjected to Western blotting analysis. Anti-p53 monoclonal antibody (Anti-Human p53 Oncoprotein; Upstate Biotechnology, Inc., Lake Placid, NY, USA) was used. Protein levels were analyzed using enhanced chemiluminescence (Amersham).

Radiation treatment and survival studies

Cells were irradiated with 200 kV X-rays (Toshiba X-ray machine; Tokyo, Japan) at a dose rate of 0.308 Gy/min at room temperature. Confluent cultures incubated in 10-cm tissue culture dishes were trypsinized immediately after irradiation and plated onto a 10-cm dish at densities expected to yield about 50 clonogenic cells. After 25–31 days, colonies were examined to calculate the surviving fraction. Wild type *p53* transfectants ware supplemented with IPTG in the medium 24 h prior to the experiments at a final concentration of 5 mM. After irradiation, cells were trypsinized and plated onto dishes with 5 mM IPTG. The medium was changed to remove IPTG 48 h after incubation, and colonies were examined four weeks later. Three replicate plates per experiment were used for each survival point, and the experiments were repeated at least three times. The results on survival were calculated as mean ±SE, and comparison between values were statistically analyzed by the Student's t-test.

RESULTS

Isolation of clones expressing mutant or wild p53

For mutant *p53* (mutations at codons 123, 143, 175, 273), *p53* expression plasmids were directly introduced into Saos-2 cells, and 12 clones were obtained for each mutant. As we were unable to create a wild type *p53* clone by directly introducing an expression plasmid, we first transferred a plasmid of p3'SS, which expresses the *lac I* gene encoding the Lac repressor protein, to Saos-2 cells to control the expression of wild type *p53*. Then, a wild type *p53* expression plasmid was introduced, and we obtained one clone. PCR was performed using primers from Exon 1 and Exon 7 in *p53* to check for wild type or mutant *p53* cDNA. Seventeen clones produced PCR products, one clone for 123A, 6 clones for 143A, 6 clones for 175H, and 3 clones for 273H (data not shown). The clone of wild type also produced PCR product. Western blotting was performed to determine p53 protein expression. Nine clones had *p53* cDNA and expressed p53 protein, one clone for 123A, 2 clones for 143A, 2 clones for 175H, and 3 clones for 273H (data not shown). The wild type clone also produced p53 protein. We further examined one typical clone of each mutant. Fig. 1 shows the results of PCR. PCR products of about 1 kbp were obtained from clones of mutants or wild type *p53* but not from the pOPI3 vector control clone. Fig 2-A shows the results of western blotting. The mutants of 175H and 273H had dense blots of p53 protein, while the mutats of 123A and 143A had only thin bands. Fig 2-B shows that wild type p53 protein was induced by the addition of 5 mM IPTG to the medium after a 24 h incubation. A

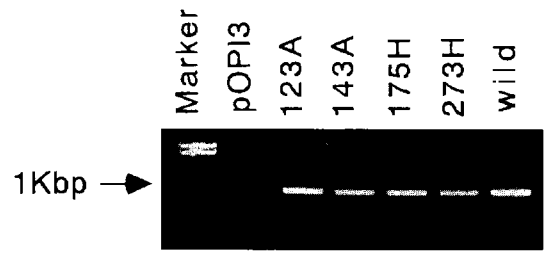

Figure 1. PCR analysis for cDNA of *p53*. The *p53* specific DNA fragment was amplified by PCR. The marker is Hind-III digest of λ DNA.

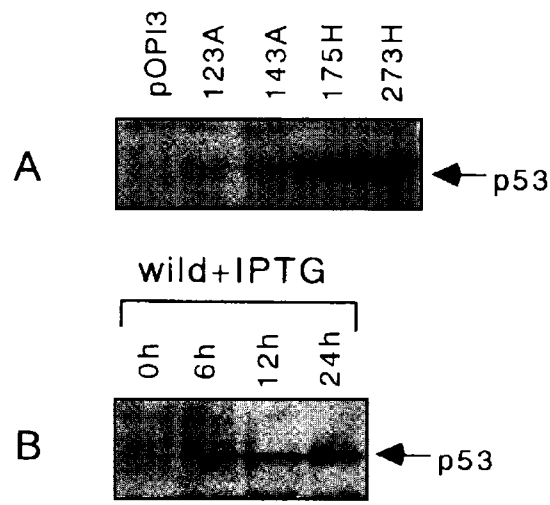

Figure 2. Western blot analysis for p53 protein in the transformants. (A) Transformants of control plasmid (pOPI3) and mutant *p53* (123A, 143A, 175H, and 273H). (B) Induction of wild type p53 protein by the addition of 5 mM IPTG to the medium.

small amount of p53 protein was observed at 0 h incubation. This indicates that the LacSwitch system is leaky and a small amount of p53 protein is produced in uninduced wild type *p53* transformants. The doubling time of the wild type *p53* transformant (58 h) was longer than that of Saos-2 (41 h). This may also be caused by low levels of leaky wild type p53 protein expression. After the addition of 5 mM IPTG to the medium, the doubling time of the wild type *p53* transformant almost doubled (106 h) (data not shown).

Radiosensitivity in each transformant

Confluent cells were used for X-ray irradiation. The doubling time of the wild type *p53* transformants were longer than that of Saos-2, and wild type *p53* transformants had a reduction in S phase (from 18% to 8%) and a increase in G0/G1 phase (from 68% to 79%) after 24 h

incubation with 5 mM IPTG (data not shown). Then, the confluent condition seems to be useful to adjust the condition of cells for the X-ray irradiation. Figure 3 shows the IR dose-survival curves of the parental Saos-2 cells, control pOPI3 vector transformants, wild type, and transformants of 123A, 143A, 175H, and 273H. Wild type *p53* transformants were more sensitive to IR than parental cells both with or without induction by IPTG ($p < 0.005$ at 6 Gy and $p < 0.01$ at 4 Gy). There was no significant difference in the survival rate with or without IPTG induction. The transformants of 123A were more sensitive to IR than parental cells ($p < 0.005$ at 6 Gy and $p < 0.025$ at 4 Gy). Each transformant of 143A, 175H, and 273H exhibited similar radiosensitivities as parental Saos-2 cells or the control transformants. We also examined the radiosensitivities of other *p53* mutant clones (one clone for 143A, one clone for 175H and two clones for 273H), and they also exhibited similar radiosensitivities as mutant *p53* transformants ($p > 0.05$ at 6 Gy, data not shown).

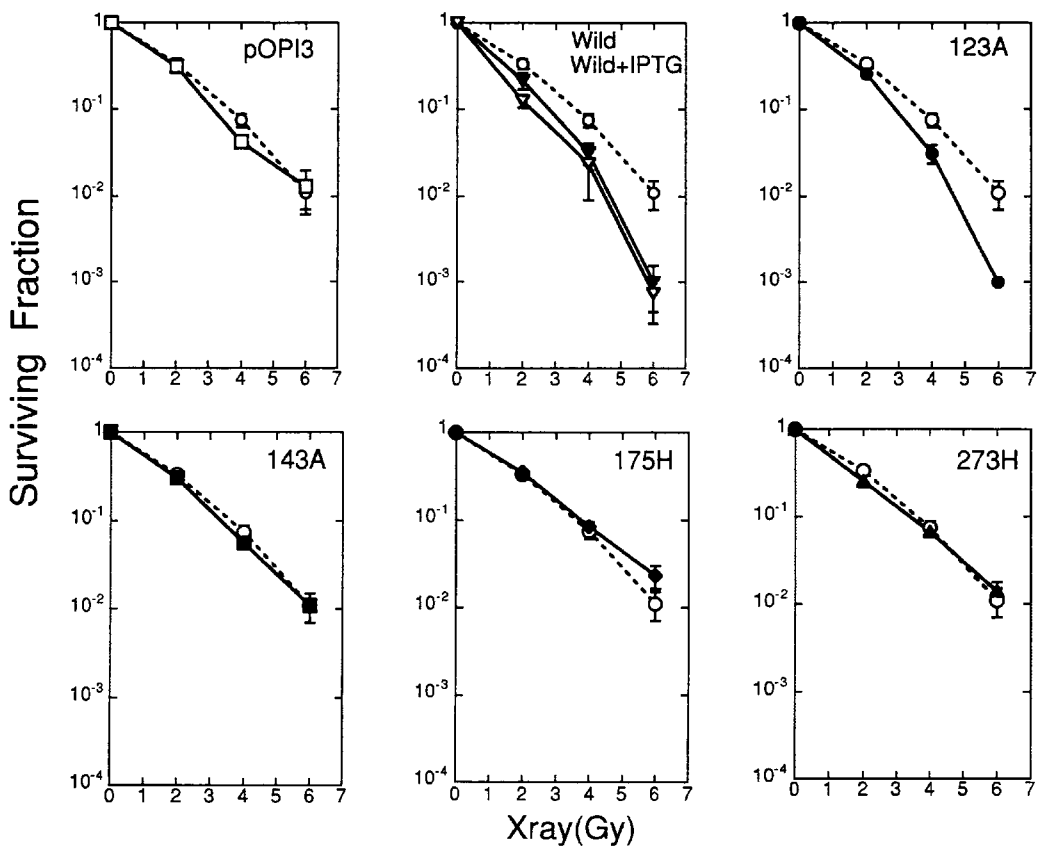

Figure 3. IR dose-survival curves of the pOPI3 transformant (□), wild type *p53* with (▽) or without (▼) induction of p53 protein by IPTG, 123A (●), 143A (■), 175H (◆) and 273H (▲) compared with parental Saos-2 (○, broken line). Error bars represent standard deviations.

DISCUSSION

p53 plays an important role in the sensitivity of tumor cells to IR. Normal p53 acts as guardian of the genome by promoting G1 arrest and repairing DNA damage[18], and also kills cells by apoptosis[19]. p53-dependent signal transduction is induced by stress including IR[20]. IR induced tumors and mutation of *p53*[21]. The *p53* gene is commonly mutated in human cancer, and the mutation points are widespread throughout the gene. Of about 6.5 million cancer cases world wide each year, 2.4 million tumors are estimated to contain a *p53* mutation[22]. It is important to determine which mutations affect the sensitivity of tumor cells to IR, because sensitivity of tumor cells to IR has substantial clinical consequences in the treatment of cancer.

We made stable transformants of wild type *p53* and four kinds of *p53* mutants at codons 123, 143, 175 and 273 for determination of which *p53* mutations affect radiosensitivity. Wild type *p53* transformants were more sensitive to IR than parental cells (Fig. 3), which may indicate that wild type *p53* cells are sensitive to IR and the cells with *p53* loss are resistant to IR. There was no significant difference in sensitivities between parental cells, Saos-2 and transformants with the 143A, 175H, or 273H mutation (Fig. 3), which indicates that the effect of the loss of *p53* is identical for all of these mutations. These results coincide with reports where cells with mutant *p53* were more resistant to IR than wild type *p53* cells[8,10,11]. The 123A mutant was more sensitive to IR than parental cells (Fig. 3), and had a similar degree of sensitivity as wild type *p53* cells. There is no significant difference in the survival rates of wild type *p53* transformants with or without IPTG induction. This may mean that the small amount of p53 producied by leaky expression in the wild type transformant (Fig. 2-B) were enough to alter radiosensitivity.

The density of the p53 protein blots (Fig. 2-A) may depend on the half life of the protein. These results are in accord with reports that p53 protein with mutations at 175H or 273H have long half lives (3.6–6.4 h for 175H and 7 h for 273H), and the p53 protein with a 143A mutation has a shortened half life (1.5–2 h)[23].

We examined 4 mutants of *p53* (123A, 143A, 175H, and 273H) for radiosensitivity and found their radiosensitivities differed. We previously examined the sensitivity of wild type *p53* and mutant *p53* transformants (123A, 143A, 175H and 273H) to commonly used anticancer drugs, nimustine (ACNU), cisplatin (CDDP), adriamicin (ADR) and bleomicin (BLM)[24]. Transfectants with wild type *p53* or mutant 123A were significantly more sensitive to ACNU than transfectants with pOPI3. This tendency corresponds well with our results on IR irradiation. Mutation of 123A seems to alter the sensitivity to anticancer drugs or IR irradiation as compared with mutations at 143A, 175H and 273H. The 123A mutant resembles wild type *p53* in sensitivity to CDDP, ACNU, BLM[24] and IR irradiation.

As the positions of *p53* mutations are spread widely throughout the gene, and the radiosensitivity of cells depends on the position of *p53* mutation, it seems to be important to check the mutation position of *p53* in tumor cells for predictive assays for radiotherapy.

ACKNOWLEDGEMENTS

This work was supported in part by Grants-in Aid for Scientific Research (No. 09680528 and No. 09255245) from the Ministry of Education, Science, Sports and Cultures, Japan. This work was also financially supported in part by the Radiation Effects Association, Japan and Sasakawa Health Science Foundation.

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