

Glycerol restores p53-dependent radiosensitivity of human head and neck cancer cells bearing mutant p53

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Summary Mutation or inactivation of p53 is known to be present in approximately 50% of human cancers. We propose here a novel strategy for overcoming this problem in mutant p53-targeting cancer therapies. We examined the restoration of radiation-induced p53-dependent apoptosis by a chemical chaperone (glycerol) in human head and neck cancer cells (SAS cells, showing wild-type p53 phenotype). SAS cells transfected with mutant p53 (SAS/mp53) showed radioresistance compared with SAS cells (SAS/neo) transfected with neo vector as a control, but became radiosensitive when pre-treated with glycerol before X-ray irradiation. Apoptosis in the SAS/mp53 cells was induced by X-rays with glycerol pre-treatment, but not without glycerol pre-treatment, whereas apoptosis in the SAS/neo cells was induced in both cases. Gel mobility-shift assays showed that after X-ray irradiation combined with glycerol pre-treatment, mp53 was able to bind to the sequence-specific region upstream of the *bax* gene regulating apoptosis. These results suggest that glycerol is effective in inducing a conformational change of p53 and restoring normal function to mp53, leading to enhanced radiosensitivity through the induction of apoptosis. This novel tool for enhancement of radiosensitivity in cancer cells bearing mp53 may be useful for p53-targeted radiotherapy. © 2000 Cancer Research Campaign <http://www.bjcancer.com>

Keywords: p53; apoptosis; Bax; radiosensitivity; glycerol; chemical chaperone

Wild-type p53 (wt p53) has multi-functions, including suppression of cancer initiation and tumour growth through the induction of downstream genes and/or protein interaction (Caelles et al, 1994; Dulic et al, 1994; El-Deiry et al, 1994; Waga et al, 1994). In a recent clinical report, we showed that wtp53 patients show higher survival rate after radiotherapy, resulting from Bax and Bcl-2 regulation, compared with mutated p53 (mp53) patients (Harima et al, 1998). To trigger the multi-functions of p53, p53 is required to form the correct conformation which enables p53 to bind to specific DNA sequences regulating gene expression. Mutations in the p53 gene cause conformational alterations in the p53 protein, and the majority of mp53 proteins can no longer regulate expression of downstream genes (Bargonetti et al, 1991; Kern et al, 1991). Mp53 genes are frequently observed in human cancer cells (Hollstein et al, 1991; Jia et al, 1997). Thus, a strategy for inducing mp53 to fold correctly could be useful for cancer therapy. We have recently reported that heat stress induces WAF1 expression only when p53 protein is wtp53 using a human glioblastoma cell line (A-172, wtp53) and its transfectants with an mp53 vector (A-172/mp53) (Ohnishi et al, 1996, 1998). A-172 mp53 cells abolished heat-induced WAF1 expression due to the dominant negative nature of the mp53 protein over endogenous wtp53 (Kern et al, 1992; Unger et al, 1992). However, heat-induced WAF1 accumulation was restored in these mp53-transfected cells when the cells were pre-treated with glycerol (Ohnishi et al, 1999a). Glycerol thus appeared to confer wtp53 function on mp53. The function of glycerol as a chemical chaperone has been reported elsewhere (Thomas et al, 1995; Welch and Brown, 1996). Glycerol seems to correct the

conformation of some types of proteins which cause human diseases.

MATERIALS AND METHODS

Cells

Head and neck cell line of human squamous cell carcinoma, SAS cells (provided by JCRB, Tokyo, Japan) were cultured at 37°C in Dulbecco's Modified Eagle medium containing 10% (v/v) fetal bovine serum, penicillin (50 U ml⁻¹), streptomycin (50 µg ml⁻¹) and kanamycin (50 µg ml⁻¹) (DMEM-10) in a conventional humidified 5% CO₂ incubator.

Plasmids

SAS cells were transfected with plasmid pC53-248, which contains an mp53 gene (codon 248, from Arg to Trp) which encodes a dominant negative mp53, or with the control vector pCMV-Neo-Bam. These plasmids were provided by B Vogelstein, Johns Hopkins Oncology Center, MD, USA. The stable transfectants SAS/mp53 and SAS/neo were selected with G418 (200–400 µg ml⁻¹, Sigma Chemical Co, St Louis, MO), and used for the present experiments. Detailed procedure for transfection is described elsewhere (Ohnishi et al, 1998).

Glycerol treatment

Cells were treated with glycerol (at final concentration of 0.6 M) 48 h before X-ray irradiation and then were incubated at 37°C for 10 h in the presence of glycerol until sampling. X-ray irradiation was performed in the presence of glycerol. In the case of cell survival assay, the medium with glycerol was changed with

Received 15 March 2000

Revised 7 August 2000

Accepted 13 August 2000

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glycerol-free one after 10 h incubation and thereafter cells were incubated for 10 to 14 days at 37°C in glycerol-free medium.

X-ray irradiation

For the X-ray irradiation treatment, subconfluent cells in 25-cm² flasks containing DMEM-10 were exposed to X-rays (2.5–10 Gy) using a 150 kVp X-ray generator (Model MBR-1520R, Hitachi, Tokyo, Japan) with a total filtration of 0.5 mm aluminium plus 0.1 mm copper filter, and then incubated at 37°C.

Cell survival assay

To measure radiosensitivity of the cells, cell survival after X-ray irradiation was quantitated by plating cells into 25 cm² flask containing the medium. Thereafter, cell colonies were rinsed with PBS, fixed with methanol, stained with 2% Giemsa solution (Merck, Woodbridge, NJ, USA). Colonies containing at least 50 cells were counted. The number of cells per colony was determined prior to experiments.

Analysis of apoptosis

Induction of apoptosis was analysed by detection of both DNA fragmentation and apoptotic bodies. To detect DNA fragmentation in the nucleosomal size range, cells were suspended in lysis buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 10 mM EDTA and 1% sodium N-lauroylsarcosinate, incubated with 100 µg ml⁻¹ proteinase K at 37°C overnight, and then centrifuged at 18500 g for 30 min. The resulting supernatants were incubated with 100 µg ml⁻¹ RNase A at 37°C for 1 h. The DNA in the solution was precipitated with ethanol at -20°C, dissolved in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, and electrophoresed for 30 min at 100 V through 3% NuSieve 3:1 gels containing 40 mM Tris-acetate, pH 7.8, 2 mM EDTA and 0.5 µg ml⁻¹ ethidium bromide. After electrophoresis, the gels were photographed under ultraviolet light. For detection of apoptotic bodies, cells were fixed with 1% glutaraldehyde (Nakalai Tesque, Kyoto, Japan) in PBS at 4°C, washed with PBS, stained with 0.2 mM Hoechst 33342 (Sigma Chemical Co), and then observed under a fluorescence microscope. At least 300 cells were counted in each experiment.

Gel mobility-shift assay

In *in vivo* samples, the cells (about 2 × 10⁷ cells) were irradiated with X-rays (6 Gy) in the presence or absence of glycerol (0.6 M). The nuclear extracts were prepared 6 h after X-ray irradiation according to the method described elsewhere (Ohnishi et al, 1998). In *in vitro* samples, whole cell extracts were prepared from intact cells (about 2 × 10⁷ cells) and treated with glycerol (0.6 M), X-rays (6 Gy) or a combination of glycerol and X-rays (6 Gy), and subsequently incubated for 30 min at 37°C. The p53-p53 consensus sequence (p53CON) binding activity was measured by a gel mobility-shift assay (Ohnishi et al, 1998) using a synthetic double-stranded DNA fragment encoding the p53CON (5'-GGACATGC-CCGGGCATGTCC-3', Japan Bioservice, Niiza, Saitama, Japan) based on a specific sequence located upstream of the *bax* gene as a probe.

RESULTS AND DISCUSSION

To examine the effect of glycerol on the radiosensitivity of SAS/mp53 and SAS/*neo* cells, the clonogenic surviving fractions after X-ray irradiation combined with pre-treatment with or without glycerol (0.6 M) were measured. As shown in Figure 1, the SAS/mp53 cells were about 1.5 times more resistant to X-rays than the SAS/*neo* cells at the D₁₀ dose. However, when pre-treated with glycerol, SAS/mp53 cells become about 1.5 times more X-ray-sensitive at the D₁₀ dose. Glycerol caused little enhancement of X-ray sensitivity in SAS/*neo* cells. On the other hand, SAS/mp53 and SAS/*neo* cells treated with glycerol alone (0.6 M) showed about 80% surviving fractions, and thus 0.6 M glycerol appeared to cause no serious cell damage regardless of p53 status. These results suggest that glycerol affects the radiosensitivity of these cells in a p53-dependent manner.

Furthermore, we examined the induction of apoptosis by detection of apoptotic bodies and DNA fragmentation to elucidate the mechanism of enhancement of radiosensitivity by glycerol in SAS/mp53 cells. We analysed the nuclear morphology with Hoechst 33342 staining. We scored the number of apoptotic bodies such as those shown by arrows in the insert of Figure 2. The incidence of apoptosis increased with time after X-ray irradiation (6 Gy) in SAS/*neo* cells (about 10% at 48 h), while there was almost no increase in the incidence of apoptosis up to 72 h after

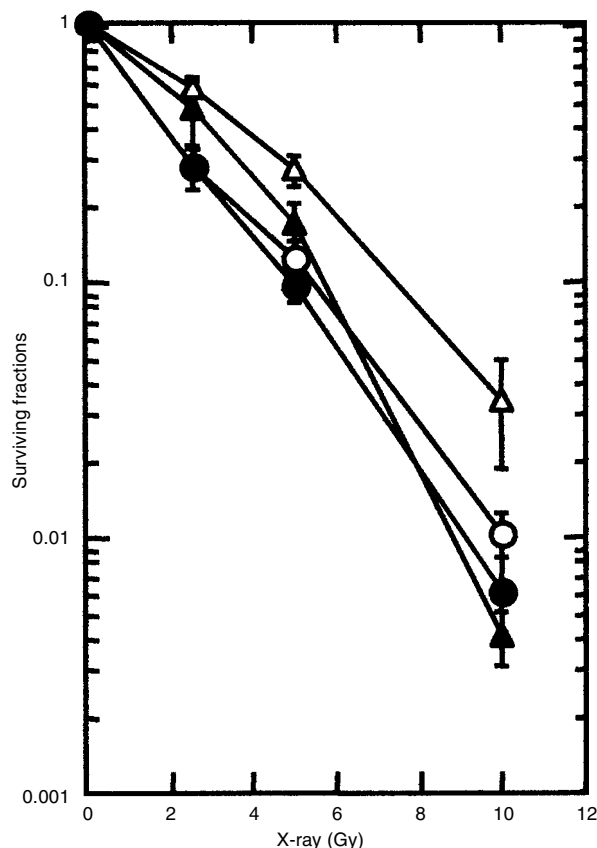


Figure 1 Clonogenic surviving fractions of transformant SAS cells after X-ray irradiation measured by the colony formation assay. Open circles, SAS/*neo* cells; closed circles, SAS/*neo* cells pre-treated with 0.6 M glycerol for 48 h before X-ray irradiation; open triangles, SAS/mp53 cells; closed triangles, SAS/mp53 cells pre-treated with 0.6 M glycerol. Surviving fractions were measured in 3 independent duplicate experiments

lanes 2 and 3 of *in vivo*). The defective DNA binding ability of p53 from SAS/*mp53* cells may be due to the dominant negative nature of this mp53 protein (Kern et al, 1992; Unger et al, 1992). In contrast, when SAS/*mp53* cells were treated with glycerol (0.6 M) before X-ray irradiation, nuclear proteins prepared from the cells showed a clear increase of DNA binding activity, probably due to binding of p53 to p53CON (Figure 4 lane 4 of *in vivo*). When unlabelled p53CON probe ($\times 100$) was added to the reaction mixture containing ^{32}P -labelled p53CON and the nuclear proteins from SAS/*mp53* cells treated with X-rays combined with glycerol, the binding of p53 to p53CON disappeared (data not shown). This indicates that the observed bands are p53-p53CON specific bands. From these results, the apoptosis observed in SAS/*mp53* cells (Figures 2 and 3) might be induced through *bax* gene expression which is up-regulated by the activated mp53, which underwent a conformational change to wtp53 as reported elsewhere (Brown et al, 1997). The acquisition of binding activity of glycerol-treated mp53 is likely to demand cellular signal transduction induced by X-rays, because no binding activity of p53 was observed when whole cell proteins extracted from intact SAS/*neo* or SAS/*mp53* cells were treated with X-ray (Figure 4, lane 3 of *in vitro*) or X-ray combined with glycerol (Figure 4, lane 4 of *in vitro*).

We showed that X-ray or glycerol treatment alone was insufficient to convert the conformation of mp53 to that of active wtp53, because neither treatment led to an increase in the DNA binding activity of p53 (Figure 4). Therefore, we assume that conformational change of mp53 to that of wtp53 induced by glycerol is not sufficient to trigger apoptosis, and some initial signals evoked by X-ray irradiation are required for effective signals leading to apoptosis. Conformation-stabilized p53 produced by glycerol treatment is probably activated through phosphorylation by X-ray-activated protein kinases such as ATM (Banin et al, 1998; Canman et al, 1998; Khanna et al, 1998; Nakagawa et al, 1999), ATR (Tibbetts et al, 1999), PKC (Takenaka et al, 1995) or other kinases (Hall et al, 1996; Jamal and Ziff, 1995). The present results suggest at least that the apoptosis induced by X-rays reported here depends on p53-dependent signal transduction. Glycerol may be generally effective against p53-regulated downstream genes through the restoration of the function of mp53, because restoration of mp53 function to normal by glycerol is observed in WAF1 induction as well (Ohnishi et al, 1999a).

Possible other explanations can be proposed for the increased DNA binding activity of nuclear extracts from SAS/*mp53* cells after combined X-ray and glycerol treatments. Overexpressed mp53 may obtain the ability to enhance the DNA binding activity of endogenous wtp53 due to reactions triggered by the combined treatments. Unknown signal transduction pathways may contribute to the modification of mp53 function.

Some investigators have vigorously searched for molecules which have an ability to induce normal function of p53 in inactive p53 or mp53. The latent sequence-specific DNA-binding function is activated by small peptides (Hupp et al, 1995). As already described, glycerol induces conformational change of mp53 to wtp53 (Brown et al, 1997) and restores the ability of mp53 to induce WAF1 expression (Ohnishi, 1999a,b). Similar chaperone-like function has been also found in other molecules such as a synthetic peptide derived from p53 C-terminal domain (Selivanova et al, 1997) and a small compound (Foster et al, 1999). Such manipulation of mp53 or inactive p53 by these molecules may provide new cancer therapies effective for patients carrying mp53 tumours.

ACKNOWLEDGEMENTS

This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

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