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Glycerol as a chemical chaperone enhances radiation-induced apoptosis in anaplastic thyroid carcinoma cells

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Abstract

Introduction: Anaplastic thyroid carcinoma, which is one of the most aggressive, malignant tumors in humans, results in an extremely poor prognosis despite chemotherapy and radiotherapy. The present study was designed to evaluate therapeutic effects of radiation by glycerol on *p53*-mutant anaplastic thyroid carcinoma cells (8305c cells). To examine the effectiveness of glycerol in radiation induced lethality for anaplastic thyroid carcinoma 8305c cells, we performed colony formation assay and apoptosis analysis.

Results: Apoptosis was analyzed with Hoechst 33342 staining and DNA ladder formation assay. 8305c cells became radiosensitive when glycerol was added to culture medium before X-ray irradiation. Apoptosis was induced by X-rays in the presence of glycerol. However, there was little apoptosis induced by X-ray irradiation or glycerol alone. The binding activity of whole cell extracts to *bax* promoter region was induced by X-rays in the presence of glycerol but not by X-rays alone.

Conclusion: These findings suggest that glycerol is effective against radiotherapy of *p53*-mutant thyroid carcinomas.

Background

Anaplastic thyroid carcinoma, which is a relatively uncommon malignancy comprising 5–14 % of thyroid cancers [1], is recognized as one of the most aggressive malignant tumor in humans and fails to respond to the available chemotherapeutic agents or radiation [2,3]. Most patients die within a year after diagnosis [4,5]. Thus, the improvement of therapeutic effects is needed to restore chemo- and radiosensitivity to anaplastic thyroid carcinomas which have a high frequency of mutant *p53* (m*p53*). Recently, *p53* gene therapy has been developed [6–8] and applied to patients carrying m*p53*[9–11]. However, overexpression of wild-type *p53* (wt*p53*) risks inducing suppression of proliferation in normal cells as well as targeted cancer cells. The suppression of proliferation of blood cells possibly gives negative effects on the physical condition of patients. Thus, there are still difficulties in the clinical application of *p53* gene therapy. We have reported a new cancer therapy strategy against mp53 cancer cells, i.e. chemical chaperon therapy. This therapy is based on glycerol-mediated conformational change of mp53 molecules. The restoration of the normal function in

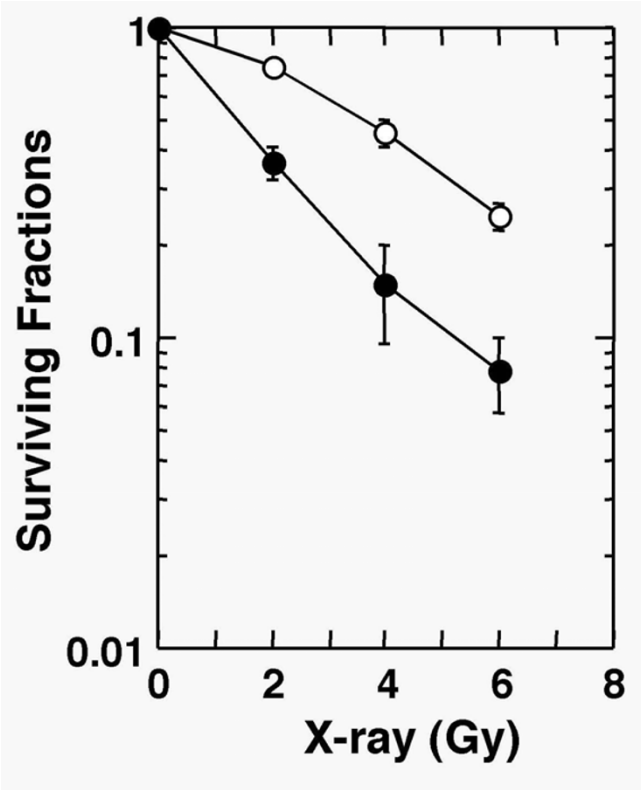

Figure 1
Clonogenic surviving fractions after X-ray. Opened circles, without glycerol; Closed circles, with 0.6 M glycerol.

mp53 leads to enhanced radiosensitivity and induction of apoptosis [12]. Glycerol appears to confer wtp53 function on mp53 [13]. The function of glycerol as a chemical chaperone has been reported elsewhere [14,15]. This novel tool for enhancing the radiosensitivity of cancer cells bearing mp53 is worth investing as p53-targeted cancer therapy, since *p53* mutations are frequently found in human cancer cells [16,17]. In the present study, we report the apoptotic effect of glycerol combined with radio-treatment in anaplastic thyroid carcinoma cells carrying m*p53*.

Results

To evaluate the effectiveness of glycerol in radiation induced lethality for anaplastic thyroid carcinoma 8305c cells, we examined colony formation assay. As shown in Fig. 1, when glycerol was added to culture medium before X-ray irradiation at a final concentration of 0.6 M, 8305c cells became about 3 times more X-ray-sensitive at the D_{10} dose. In contrast, there was no enhanced radiation sensitivity observed in 8305c cells after the addition of 0.3 M glycerol alone (data not shown). These results suggest that glycerol has an ability to enhance the radiosensitivity of 8305c cells. Figure. 2A shows the time-course of the frequency of X-ray induced apoptotic cells. Though the frequency did not increase after X-ray irradiation without the glycerol treatment, the frequency was augmented in a time-dependent manner in the presence of glycerol. Typical apoptotic bodies detected by Hoechst 33342 staining analysis are shown in Fig. 2C and 2D. The destruction of nuclei was observed 48 h after X-ray irradiation, especially with glycerol (Fig. 2D). Apoptotic bodies appeared at a higher frequency in the presence of glycerol (Fig. 2D) than in the absence of glycerol (Fig. 2C). Increased incidence of apoptotic body was not observed after glycerol treatment alone. We next examined the induction of DNA fragmentation after X-ray irradiation using agarose gel electrophoresis of DNA extracted from 8305c cells (Fig. 3). Clear DNA ladders in the nucleosomal size were observed 24–48 h after X-ray irradiation combined with glycerol (Fig. 3A). However, a faint DNA ladder was observed 48 h after X-ray irradiation alone (Fig. 3B). No DNA ladder was observed 48 h after glycerol treatment alone (Fig. 3A) and thereafter. These results indicate that glycerol sensitized radiation-induced apoptosis in anaplastic thyroid carcinoma in 8305c cells. DNA (p53 consensus sequence, p53CON) binding activity of whole cell extracts from X-ray (6 Gy) irradiated cells was analyzed with gel mobility-shift assay (Fig. 4). Whole cell extracts from non-irradiated cells showed no DNA binding activity. Similarly, the extracts from X-ray-irradiated cells showed no DNA binding activity 6 h after the irradiation. However, the extracts from glycerol-treated cells showed DNA binding activity and the binding activity was also observed in the extracts from the cells irradiated with X-rays 6 h after the irradiation.

Discussion

Recently, the mutation of *p53* has been reported to play an important role in anaplastic change in differentiated thyroid carcinomas [19,20]. Supporting this, 8305c cells show a point mutation at codon 273 of the *p53* gene. The *p53* gene plays a pivotal role in the pathway that controls cell growth, proliferation and apoptosis [21–24]. The lack of the p53-regulated pathway is a common feature in a large number of tumors, suggesting that it may be central to the pathogenesis of human cancers. We have recently reported that the effectiveness by radiotherapy or hyperthermic therapy depends on *p53*-status in human squamous cell carcinoma (SAS cells) [12,25]. That is, SAS cells transfected with m*p53* are more radiation or heat resistant than *neo* control cells. The similar p53-dependent radiosensitivity has also been reported elsewhere [26]. The present results may provide useful information for radiotherapy of m*p53* cancer cells.

The present observed X-ray sensitivity enhanced by glycerol may result from the enhancement of *p53*-independent X-ray-induced apoptosis. However, this presumption is not possible from the following reason. We have reported

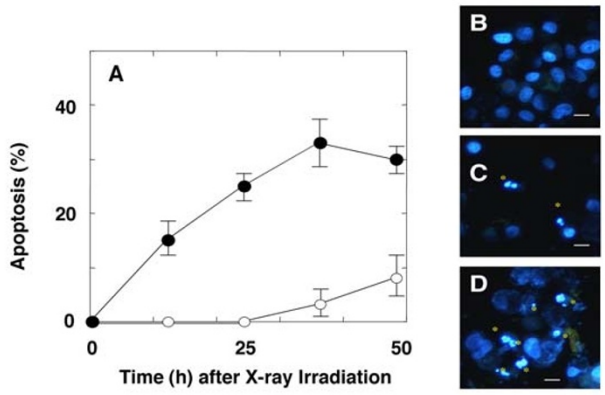

Figure 2
The time course of apoptotic body detected with Hoechst33342 staining after X-ray irradiation (6 Gy). **A,** 8305c cells were treated with (closed circles) or without (opened circles) 0.6 M glycerol. **B**-**D**, photographs of apoptotic body in 8305c cells. **B**, control; **C**, 48 h after X-ray irradiation alone; **D**, 48 h after X-ray irradiation combined with 0.6 M glycerol; Bars, 25 μm.

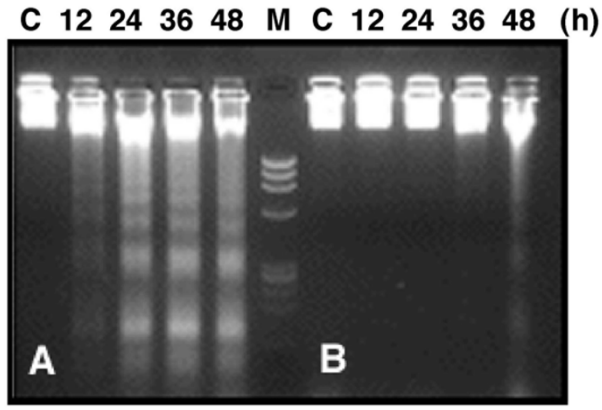

Figure 3
DNA fragmentation detected with agarose gel electrophoresis of DNA extracted from X-ray (6 Gy) irradiated cells. A and B represent with and without glycerol (0.6 M), respectively. C, no irradiation; Lanes 12, 24. 36 and 48, incubation periods (h) after X-ray irradiation. Lane M, øX174 DNA *Hae* III fragments as size markers.

that glycerol can restore wtp53 function to m*p53* cancer cells [12,13,27]. The present data in 8305c cells strongly supports the chemical chaperone effect of glycerol. Glycerol possibly enhanced X-ray-sensitivity in 8305c cells through restoration of mp53 to wtp53 functions. The present data from gel mobility-shift assay suggest that the restoration is brought through conformational change of mp53 to wtp53. The mp53 molecules from cells treated with glycerol and X-rays might affect the transactivation of p53-down stream genes, especially apoptosis-related genes such as *bax*. In relation to this, we have reported that glycerol has an ability to enhance *bax* expression in m*p53* human glioblastoma cells through phosphorylation of p53 at serine 15 by PI3-K family [28]. The conformational change of mp53 might be induced by glycerol alone because the DNA binding activity of mp53 was restored after glycerol treatment alone (Fig. 4). However, in this case, the transcriptional activity of mp53 is not likely to be restored. Possibly, the mp53 molecules from cells treated with glycerol alone is not modified by X-ray-induced phosphorylation or acetylation. Therefore, it seems that the cells treated with glycerol alone did not show apoptosis, as shown in Fig. 2 and 3. We have previously reported that the DNA binding activity of mp53 was almost not restored after glycerol treatment alone in *p53* mutated at codon 248 [12]. Therefore, the effect of glycerol on mp53 may depend on mutation site of *p53* and amino acid sequence affecting its three-dimensional conformation. This result suggests that the investigation of mutation site of *p53* is an important process for the cancer therapies based on *p53* status.

New strategy based on the functional restoration of mp53 is recently being developed. Recent studies have reported new compounds which rescue mp53 conformation and function [29,30]. The compounds can induce apoptosis in m*p53* human tumor cells through restoration of the transcriptional transactivation function to mp53. The compounds may serve as anticancer drugs targeting mp53. Compared with these compounds, glycerol seems to be the most hopeful molecule for cancer therapy, because it has been widely used as a clinical reagent already.

Conclusions
The present study suggests that chemical chaperones, such as a glycerol, are useful for radiotherapy of *p53*-mutant thyroid carcinomas.

Methods
Cells
The cells used in this study were a human anaplastic thyroid carcinoma-derived cell line, 8305c bearing a point mutation at codon 273 in the *p53* gene [18]. 8305c cells were obtained from JCRB (HSRRB, Osaka, Japan). Cells were maintained in continuous culture at 37°C in a 5 % CO_2 humidified atmosphere. Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS. Penicillin (100 U/ml) and streptomycin (100 μg/ml) were added to the media.

Glycerol treatment
Glycerol was added into the culture medium at a final concentration of 0.6 M at 48 h before irradiation by X-ray

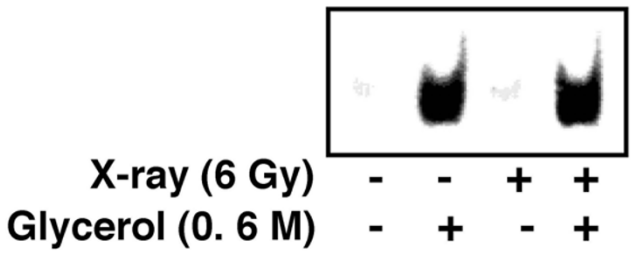

Figure 4
DNA (p53CON) binding activity of whole cell extracts from X-ray (6 Gy) irradiated cells analyzed with gel mobility-shift assay. Non-irradiated or glycerol-treated cells were collected with X-ray-irradiated cells 6 h after the irradiation.

source (MBR-1520R, Hitachi, Tokyo, Japan). Thereafter the cells were incubated at 37°C in the presence of glycerol until sampling of apoptosis assay. For survival assay, the medium with glycerol was changed to glycerol-free medium after 10 h incubation and thereafter cells were incubated for seven to ten days at 37°C in glycerol free-medium.

Cell survival assay

To measure the radiosensitivity of the cells, cell survival after X-ray irradiation was measured by plating cells into 25 cm^2 flasks containing medium. Thereafter, 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. Surviving fractions were measured in 3 independent duplicate experiments. Bars (standard errors) are shown when they exceed the symbols.

Analysis of apoptosis

We performed the Hoechst 33342 staining and the agarose gel electorophoresis for analyses of apoptosis, using the 8305c cells. Two or more independent experiments were repeated. Induction of apoptosis was analyzed by detection of apoptotic bodies and DNA fragmentation. To detect apoptotic bodies, cells were fixed with 1% glutaraldehyde (Nacalai 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. Apoptotic bodies were counted at 3 different fields of the microscopic observation. One hundred cells were judged under one field. For detection of 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 protease K at 37°C overnight, and then centrifuged at 18,500 × *g* for 30 min. The resulting supernatants were incubated with 100 μg/ml 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 ethidium bromide. After electrophoresis, the gels were photographed under ultraviolet light.

Gel mobility-shift assay

Whole cell extracts were prepared from X-ray-irradiated or non-irradiated cells (about 2×10^7 cells) according to the method described elsewhere [12]. The binding activity of p53 to p53 consensus sequence (p53CON) was measured by a gel mobility-shift assay [12] using a synthetic double-stranded DNA fragment encoding the p53CON (5'-GGACATGCCCGGGCATGTCC-3', Japan Bioservice, Niiza, Saitama, Japan) based on a specific sequence located upstream to the *bax* gene as a probe.

Authors' contributions

KY, AT and KY participated in the colony formation assay. IO, ME and HH participated in apoptosis assay. KO participated in the design of the study, performed the all analyses and wrote the manuscript. TO conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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