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Glycerol restores heat-induced p53-dependent apoptosis of human glioblastoma cells bearing mutant p53

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Abstract

Background: We have previously reported that glycerol acts as a chemical chaperone to restore the expression of WAF1 in some human cancer cell lines bearing mutant p53. Since the expression of WAF1 is up-regulated by activated wildtype p53, glycerol appears to restore wtp53 function. The aim of the present study is to examine the restoration of heat-induced p53-dependent apoptosis by glycerol in human glioblastoma cells (A-172) transfected with a vector carrying a mutant *p53* gene (A-172/mp53 cells) or neo control vector (A-172/neo cells).

Results: A-172/mp53 cells showed heat resistance compared with A-172/neo cells but A-172/mp53 cells in turn became heat sensitive when pre-treated with glycerol before heat treatment. The accumulation of Bax in the A-172/mp53 cells was induced by heating with glycerol pre-treatment, but not without it, whereas the accumulation in the A-172/neo cells was induced in both cases. Furthermore, mp53 extracted from heated cells came to bind to the sequence specific region after heating combined with glycerol pre-treatment. The phosphorylation of mp53 at serine15 was suppressed by an inhibitor of the phosphatidylinositol 3-kinase (PI3-K) family.

Conclusion: These results suggest that glycerol is effective in inducing conformational change of phosphorylated p53 and restoring mp53 to wtp53 function, leading to enhanced heat sensitivity through the induction of apoptosis. This novel tool for enhancement of heat sensitivity in cancer cells bearing mp53 may be applicable for p53-targeted hyperthermia, because mutation or inactivation of p53 is observed in approximately 50% of human cancers.

Background

It is known that p53 induces cell growth arrest [1,2] or cell death [3] and the suppression of DNA replication [4] to suppress the initiation, progression or growth of tumor. p53 exhibits its function through the induction of downstream genes and/or protein interaction relating to tumor suppression. However, mutations in the *p53* gene cause conformational alterations in p53 protein and the majority of mp53 can no longer induce expression of downstream genes [5,6]due to sequence specific DNA binding inability. Heat up-regulates sequence specific DNA binding activity of wtp53 [7] and induces the expression of p53-regulated gene [8]. Thus, hyperthermia is regarded as a good tool for cancer therapy from the view of suppression of tumor growth. We have already reported that wt*p53*-transfected *p53*-knockout cells show higher inci-

dence of apoptosis by heat compared with m*p53*-transfected *p53*-knockout cells [9]. Furthermore, patients bearing wt*p53* show a high survival rate after radiotherapy resulting from Bax and Bcl-2 regulation [10,11]. Such hyperthermia/radiotherapy based on *p53* status, however, seems to have some difficulties for the treatment of cancer cells bearing mp53. To overcome this problem, we present here a new strategy for cancer therapy. In recent years, glycerol has been reported to act as a chemical chaperone to correct the conformation of proteins, which cause human diseases [12,13]. Consistent with this, we have reported that glycerol acts as a chemical chaperone to restore the expression of WAF1 in some human cancer cell lines bearing mp53[14] and to restore apoptosis in *p53*-knockout mouse fibroblast cells transfected with m*p53*[15]. Since the expression of WAF1 is up-regulated by activated wtp53, glycerol appears to restore wtp53 function. In the present study, we further examined the effect of glycerol on p53-dependent apoptosis induction through *bax* expression and whether the heat sensitivity of cells bearing mp53 is enhanced by glycerol. To enable a discussion of the results on the basis of *p53* status only, we transfected A-172 cells with the m*p53* gene, which had identical genetic backgrounds except for *p53* status, and demonstrated so-called "dominant negative effect" of mp53 protein [16,17].

Recently, it was reported that the PI3-K family such as ATM, ATR and DNA-PK contributes to the activation of p53 through the phosphorylation of serine 15 of p53 [18–22]. In the present study, to gain further insight into the mechanism of restoring mp53 to wtp53, we examined mediation of the phosphorylation of p53 by the PI3-K family to conformational change of mp53.

Results and Discussion

To elucidate the effect of glycerol on the heat sensitivity of transformed A-172 cells, the clonogenic surviving fractions by heat after pre-treatment with or without glycerol (0.6 M) were measured. As shown in Fig. 1a, A-172 cells tranfected with mp53 (val to ala at codan 143) (A-172/m*p53*/143 cells) were more resistant to heat than the A-172/neo cells. By glycerol treatment, A-172/m*p53*/143 cells became about 1.5 times more heat-sensitive at D_{10} dose, whereas A-172/neo cells showed no enhanced heat sensitivity. In addition, A-172/m*p53*/143 and A-172/neo cells treated with glycerol alone (0.6 M) showed about 80% survival fractions, and thus the concentration of glycerol appeared to cause no serious cell damage regardless of *p53* status. These results suggest that glycerol affects the heat sensitivity of those cells in a way that it enhances heat sensitivity in m*p53* cells.

The change of cellular contents of Bax after heating was analyzed in A-172/mp53/143 cells with Western blot. As shown in Fig. 1b, Bax was accumulated after heating in the presence of glycerol at 0.6 M, although Bax accumulation was not induced after heating alone or treatment with 0.6 M glycerol alone in the cells. It is possible that mp53 might function as a transcriptional factor in heat-induced Bax accumulation under the presence of glycerol. In addition, A-172/m*p53*/143 cells only heated accumulated large amounts of p53 but no significant Bax, suggesting that heat treatment induces accumulation of mp53 as is the case in human glioblastoma A-7 cells [23]. The accumulation of mp53 is probably due to the elongation of its half-life by heat. Glycerol alone treatment may not be sufficient to convert mp53 into wtp53 because this treatment

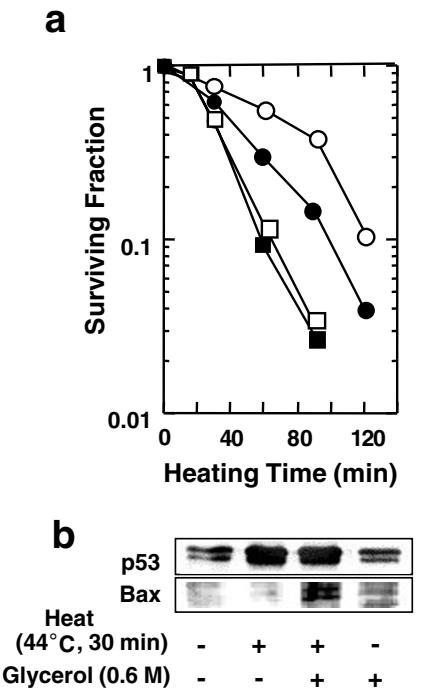

Figure 1
a, Clonogenic survival rates of cells after heating or combination treatments with glycerol. Two duplicate flasks were used per experiment, and two or more independent experiments were repeated for each survival point. Open square, A-172 neo contol cells; closed square, *neo* control cells pretreated with 0.6 M glycerol; open triangle, A-172/m*p53*/143 cells; closed circle, A-172/m*p53*/143 cells pretreated with 0.6 M glycerol. **b**, Restoration by glycerol of heat-induced Bax accumulation in A-172/m*p53*/143 cells. Western blotting samples were prepared from cells 10 h after heating at 44°C for 30 min. The glycerol was added 48 h before heating at a final concentration of 0.6 M. Glycerol was present in the medium during culture after heating.

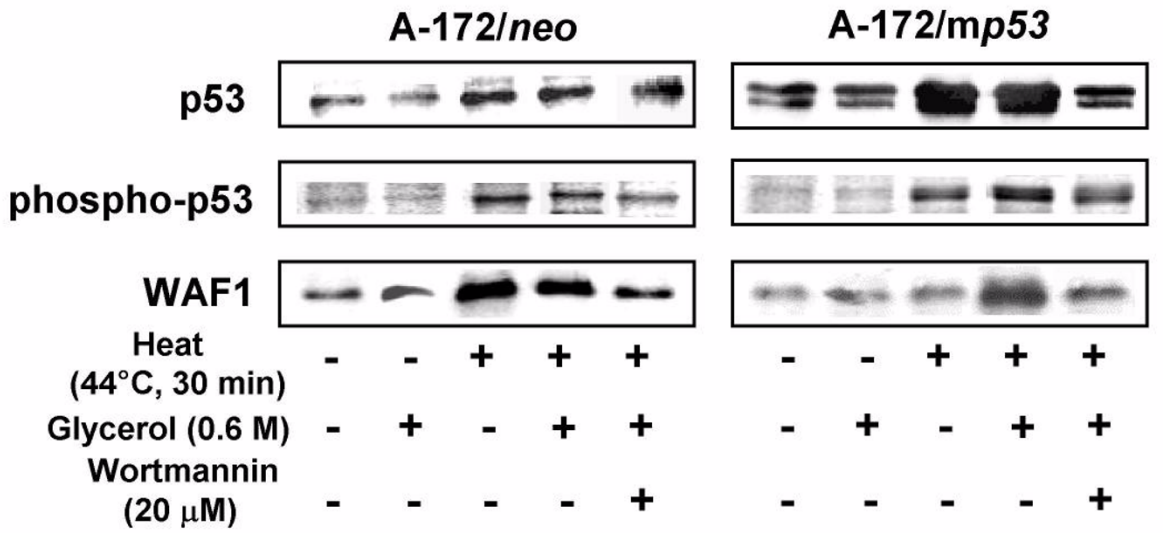

Figure 2
Suppression of heat-induced phosphorylation of serine15 of p53 and WAF1 accumulation by wortmannin. Western blotting samples were prepared from A-172/m*p53*/248 cells 6 h after heating at 44°C for 30 min. Wortmannin was added 2 h before heating at a final concentration of 20 μM.

did not lead to accumulation of endogenous latent wtp53 and did not induce Bax accumulation in A-172/m*p53*/143 cells. This also excludes the possible involvement of osmotic stress-induced signal transduction in the p53 pathway. We assumed that denaturation by heat stress may disrupt the aberrant conformation of the p53 mutant, and glycerol may exert its effect during renaturation to stabilize the transient wtp53 conformation that is otherwise very unstable. Subsequently, the conformation-stabilized p53 could be activated as a transcriptional factor by heat-induced signal transduction. Thus, the inability to induce Bax accumulation by glycerol alone may be due to mutant conformation of p53.

Glycerol treatment alone was insufficient to induce Bax accumulation (Fig. 1b). This result led us to assume that some initial signals evoked by heating are required to effectively induce signals leading to apoptosis. Thus, we next examined the activation of mp53 through phosphorylation after heat and glycerol treatments. It has been reported that the phosphorylation of serine 15 of p53 by the PI3-K family induces p53 activation [18–22]. Therefore, we estimated the activation of mp53 based on the induction of WAF1 which is one of downstream factors regulated by p53. As shown in Fig. 2, the phosphorylation of serine 15 of wtp53 was observed 6 h after heating without or with glycerol in A-172/neo cells (Fig. 2, the middle left column, line 3 and 4), whereas it was not observed after glycerol treatment alone (Fig. 2, the middle left column, line 2). The phosphorylation of serine 15 after heating with glycerol was suppressed by an inhibitor of wortmannin (20 μM) for PI3-K family (Fig. 2, the middle left column, lane 5). WAF1 was accumulated in relation to the level of serine 15 phosphorylation (Fig. 2, the lower left column). These results suggest that the PI3-K family such as ATM, ATR or DNA-PK contributes to heat-induced activation of p53 at serine 15 as reported in radiation-induced p53 activation [18–22]. In contrast to A-172/neo cells, A-172/m*p53*/248 cells did not show any significant increase of WAF1 6 h after heating (Fig. 2, the lower right column,

line 3), although the serine 15 was phosphorylated (Fig. 2, the middle right column, line 3). When A-172/m*p53*/248 cells were heated in the presence of glycerol, WAF1 was accumulated (Fig. 2, the lower right column, lane 4) with the phosphorylation of the serine 15 (Fig. 2, the middle right column, lane 4). Wortmannin suppressed WAF1 accumulation and serine 15 phosphorylation after heating in the presence of glycerol (Fig. 2, the middle and lower right columns, lane 4). These results mean that the phosphorylation of serine 15 of p53 is not sufficient for the heat-induced mp53 activation. It is suggested that the activation of mp53 demands both the reconstruction process by glycerol and the heat-induced phosphorylation process by the PI3-K family. In addition, it is suggested that the glycerol-enhanced heat sensitivity of mp53 cells is dependent on p53-dependent signal transduction and mediated by p53-regulated Bax induction. Glycerol may be universally effective against the expression of p53-regulated downstream genes in cells bearing mp53, because restoration of mp53 to normal p53 by glycerol is observed in Bax induction as well as WAF1 induction.

The function of wtp53 is depressed by mp53 in a way that mp53 forms heterogeneous tetramer with wtp53. This effect of mp53 is the dominant negative effect [16,17]. From this, one possibility is that glycerol may depress the dominant negative effect of mp53 and p53-centered signal transduction may be restored by glycerol. However, we have already reported that WAF1 expression after heating was induced in Saos-2 cells (p53-null) transfected with m*p53* gene [14], when the cells were pre-treated with glycerol. WAF1 expression was not induced even after combined treatment with heat and glycerol in the cells transfected with *neo* vector alone without m*p53* gene. At least, mp53 is necessary for induction of *WAF1* gene expression by heat in glycerol-treated cells. These results strongly support that the conformation of mp53 was restored to normal type of p53 by glycerol.

Furthermore, to confirm the conformational change of mp53 to wtp53 suggested by Western blot analysis, the DNA binding activity of p53 for p53CON was measured in nuclear proteins extracted from A-172/neo or A-172/m*p53*/248 cells using the gel mobility-shift assay. It is known that wtp53 can bind to p53CON homologous to a specific DNA sequence located upstream of the *bax* gene which positively controls apoptosis [24]. In agreement of this, the binding activity of wtp53 significantly increased in A-172/neo cells treated with heat (44°C, 30 min) or combination of heat and glycerol (0.6 M) (Fig. 3, the upper column, lane 3 and 4). In contrast, a slight increase in the DNA binding activity of the nuclear proteins was observed when A-172/m*p53*/248 cells were treated with heat (44°C, 30 min) (Fig. 3, the lower column, lanes 3). The increase may be due to endogenous wtp53 in A-172/mp53/248 cells. The defective DNA binding ability of p53 from heat-treated A-172/mp53/248 cells may be due to the dominant negative nature of mp53 protein [16,17]. It is known that mp53 has no ability to induce apoptosis, because most mp53 can not bind to a specific DNA sequence [5,6]. On the other hand, when A-172/mp53/248 cells were heated in the presence of glycerol (0.6 M) before heating, they showed clear increased DNA binding activity of p53 to p53CON (Fig. 3, the lower column, lane 4). This result shows that mp53 underwent conformational change to wtp53. The enhanced heat sensitivity observed in glycerol-pretreated A-172/mp53/143 cells (Fig. 1a) might be induced through *bax* gene expression up-regulated by the activated mp53. No binding activity of p53 was observed when whole cell proteins extracted from intact A-172/neo or A-172/m*p53*/248 cells were treated with heat, heat plus glycerol or heat plus glycerol plus wortmannin (Fig. 3, both columns, lanes 8, 9 and 10). Thus, the acquisition of binding activity of wtp53 or glycerol-treated mp53 is likely to demand cellular signal transduction as described above. Furthermore, the increased DNA binding activity of nuclear proteins from A-172/neo or A-172/mp53/248 cell was suppressed by wortmannin (Fig. 3, lane 5). This suggests that the PI3-K family mediates glycerol-induced restoration of the DNA binding ability of wtp53 and mp53 after heating. On Western blot analysis (Fig. 2), phosphorylation of serine 15 seems to be required for this process.

New cancer therapies for patients with m*p53*-containing tumors are recently being developed. Especially, reports concerning to transfection of *p53* gene into tumor [25–27] and molecules which activate latent p53 [28], change the conformation of mp53 [29] or restore the function of mp53 [14,15,30,31]are on the increase. We have recently reported that glycerol has an ability to restore normal function to mp53, leading to WAF1 induction [14]. Thereafter, new compounds which rescue mp53 conformation and function have been reported by other laboratory [30]. Among the reported molecules, glycerol is easy to be recognized as the most useful molecule for cancer therapy, because it is widely used as a convenient reagent in clinical course already. Furthermore, as reported in this paper, glycerol has an ability to enhance *bax* expression in mp53 cells as a chemical chaperone through phosphorylation of p53 at serine 15 by PI3-K family and conformational change of mp53. Thus, it is expected that the cancer therapy combined hyperthermia and glycerol is efficient for patients with mp53-containing tumors, in which p53-dependent *bax* expression is less frequently induced.

As we have reported elsewhere [32], glycerol is also effective in inducing a conformational change of p53 and restoring normal function to mp53 (Fig. 4), leading to enhanced radiosensitivity. The enhanced radiosensitivity

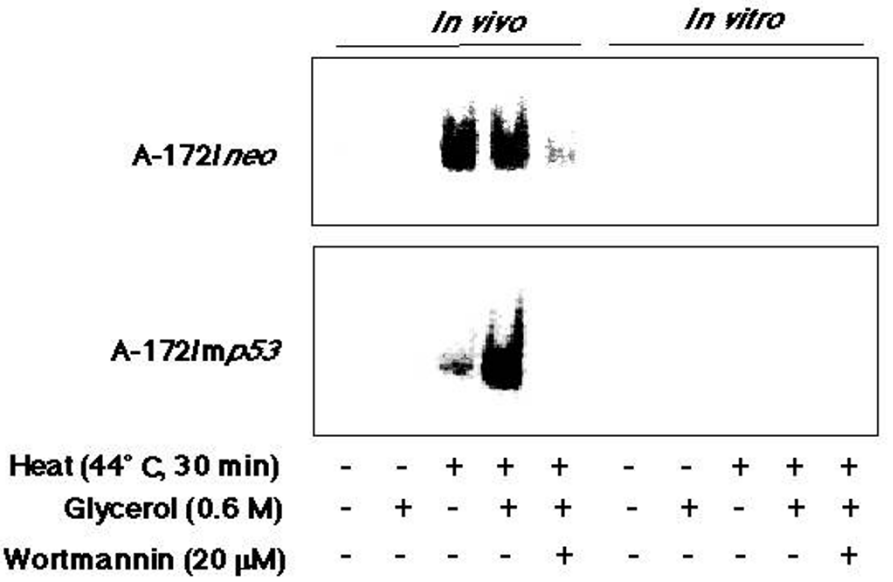

Figure 3
Gel mobility-shift assay of nuclear extracts or whole cell extracts from A-172/neo or A-172/mp53/248 cells to p53CON. ***in vivo***, the cells were treated with glycerol (0.6 M), wortmannin (20 μM) and heat (at 44°C for 30 min). The nuclear fraction was extracted from the treated cells 6 h after heating. ***in vitro***, the whole cell extracts prepared from intact cells, and then they were treated with glycerol, wortmannin and heat.

is closely related to the induction of p53-dependent apoptosis. Further, we have recently obtained results showing that glycerol functions as a chemical chaperone in p53-dependent CDDP sensitivity (unpublished data). Our results obtained from previous and the present studies show that glycerol is available for cancer therapy such as hyperthermia, radiotherapy or chemotherapy which induces the activation of p53 even in mp53 tumors.

Materials and Methods

Cells

Human glioblastoma A-172 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), streptomycin (50 μg/ml) and kanamycin (50 μg/ml) (DMEM-10).

Plasmids

A-172 cells were transfected with the plasmids pC53-SCX3, pC53-248 (m*p53*, point mutation from Val to Ala at codon 143 or Arg and neomycin resistance marker) or pCMV-Neo-Bam (neomycin resistance marker alone). Before transfection, these plasmids were digested with *Hin*dIII and linearized (plasmids were provided by Dr. B. Vogelstein, Johns Hopkins Oncology Center, MD, USA). A-172 cells were electroporated three times at 600 V with linearized DNA (10 μg/10 μl of pC53-SCX3, pC53-248 or pCMV-Neo-Bam). The transfectants (A-172/m*p53*/143, transfected with pC53-SCX3; A-172/m*p53*/248, pC53-248; A-172/*neo*, pCMV-Neo-Bam) were selected by G418 (200–400 μg/ml, Sigma Chemical Co., St. Louis, MO) and incubated at 37°C through all experiments.

Glycerol treatment

Cells were treated with glycerol (at final concentration of 0.6 M) 48 h before heating (44°C, 30 min) and then were

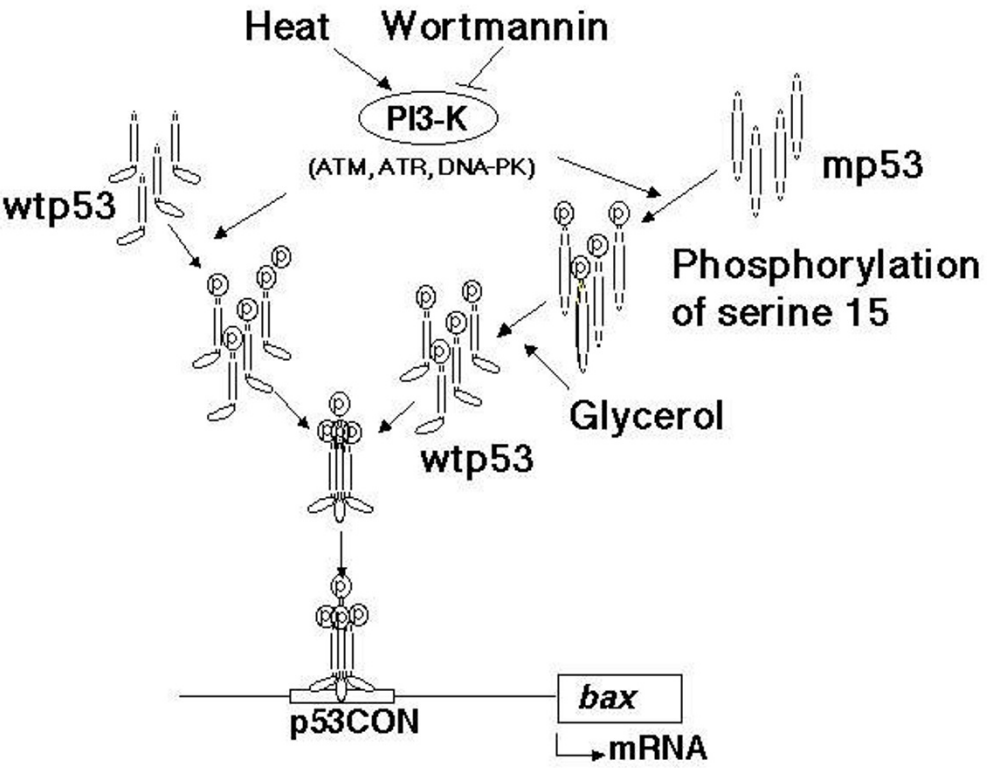

Figure 4
A schema showing restoration of normal p53 function by glycerol in mp53. Serine 15 of wtp53 and mp53 are phosphorylated by the PI3-K family. Glycerol is effective in inducing conformational change of misfolding mp53 and renaturing mp53 to wtp53 which mediates heat sensitivity through *bax* expression.

incubated at 37°C for 6 or 10 h in the presence of glycerol until sampling. In the case of cell survival assay, the medium with glycerol was changed with glycerol free one after 10 h incubation and thereafter cells were incubated for ten to fourteen days at 37°C in glycerol free medium. In *in vitro* treatment, whole cell extracts from intact cells were treated with glycerol (at final concentration of 0.6 M) for 30 min at 37°C.

Wortmannin treatment

Cells were treated with wortmannin (Nacalai tesque, Inc., Kyoto, Japan, at final concentration of 20 μM) 2 h before heating (44°C, 30 min) and then were incubated at 37°C in the presence of wortmannin until sampling. In *in vitro* treatment, whole cell extracts from intact cells were treated with wortmannin (at final concentration of 20μM) during heating.

Cell survival assay

Cell survival after heating at 44°C for 0, 15, 30, 60, 90 or 120 min was quantitated by plating cells into 25 cm^2 flask containing the medium. Ten to fourteen days later, 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 experiment.

Western blotting analysis

Detailed procedure of Western blotting is described elsewhere(El-Deiry et al., 1994). Aliquots (20 μg) of whole cell extracts were used for Western blotting analysis of Bax and p53. After electrophoresis on 15% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS and electrophoretic transfer onto Poly Screen PVDF membranes (DuPont/NEN Research Products, Boston, MA), the proteins on each membrane were incubated with the anti-human Bax polyclonal antibody Ab-1 (Oncogene Science Inc., Union-

dale, NY), anti-human p53 monoclonal antibody DO-1 (Oncogene Science Inc.), anti-human phosphorylated p53 polyclonal antibody Phospho-p53 (Ser15) or anti-human WAF1 monoclonal antibody EA10 (Oncogene Science Inc.). The bands were visualized using horseradish peroxidase-conjugated goat anti-rabbit (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or anti-mouse IgG antibody (Zymed Labs. Inc., San Francisco, CA) and the BLAST®: Blotting Amplification System (DuPont/NEN Research, Boston, MA).

Preparation of nuclear or whole cell extracts for gel mobility-shift assay

Nuclear extracts were prepared from A-172 transformed cells 6 hr after heat treatment, heat and glycerol treatments or no treatments as *in vivo* treatment samples. As *in vitro* treatment samples, whole cell extracts were prepared from intact A-172 transformed cells suspended in extraction buffer (20 mM HEPES-KOH, pH 7.6, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA-NaOH, pH 8.0, 0.5 mM Dithiothreitol(DTT), 0.5 mM phenylmethyl-sulfanylfluoride (PMSF), 25% (v/v) glycerol, 1.2 μM spermidine) and were treated with glycerol (0.6 M), heat (44°C, 30 min) or combination of glycerol and heat, and subsequently incubated for 30 min at 37°C. The procedures of nuclear protein extraction are described previously[2]. Shortly, the cells were washed with PBS and suspended in washing buffer (10 mM Tris-HCl pH 7.5, 130 mM NaCl, 5 mM KCl, 8 mM MgCl2) and then homogenized on ice in hypotonic buffer (20 mM HEPES-KOH, pH 7.6, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethyl-sulfanylfluoride (PMSF) with a hand-driven Dounce homogenizer. The homogenates were centrifuged to precipitate the nuclei, which were resuspended in extraction buffer (20 mM HEPES-KOH, pH 7.6, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA-NaOH, pH 8.0, 0.5 mM DTT, 0.5 mM PMSF, 25% (v/v) glycerol, 1.2 μM spermidine). The resulting nuclear suspensions were centrifuged to precipitate the chromatin and the nuclear extracts were collected and dialyzed against binding buffer (20 mM HEPES-KOH, pH 7.6, 0.5 mM EDTA-NaOH, pH 8.0, 50 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 10% (v/v)glycerol). The protein concentration of each extract was quantified using a BIO-RAD Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard.

Gel mobility-shift assay

The p53-p53CON binding activity was measured by a gel-shift assay using a synthetic double-stranded DNA fragment encoding the p53CON (5'-GGACATGCCCG-GGCATGTCC-3') on the upstream of *bax* gene (Japan Bioservice, Niiza, Saitama, Japan) as a probe. Detailed procedure is described elsewhere [2]. The probe was labeled with [γ-^{32}P]ATP using Megalabel (Takara Shuzo Co., Ltd., Ohtsu, Shiga, Japan) and the required nuclear extract (5 μg as protein) was incubated at 25°C for 30 min with the labeled p53CON probe (1~3 × 10^5 cpm) and poly [dIdC]-poly [dIdC] (1 μg) (Pharmacia Biotech, Uppsala, Sweden) diluted with binding buffer to a final volume of 15 μl. After this incubation, the samples were electrophoresed on a 5% (w/v) polyacrylamide gel for 1 h at 150 V using Tris-acetate-EDTA buffer. Subsequently, the gel was dried and observed with Fujix BAS 1000 Imaging Analyzer (Fuji) and photographed on Pictrography 3000 (Fuji) connected to the BAS 1000.

Acknowledgments

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