Induction of WAF1 expression was investigated after heat treatment (44 °C, 30 min) in two human glioblastoma cell lines with the wild-type or a mutant p53 gene. WAF1 accumulation was induced by heat treatment in A-172 cells carrying the wild-type p53 gene but not in T98G cells carrying the mutant p53 gene. We examined whether this phenomenon was due to the induction of WAF1 expression. Northern blot analysis showed that heat treatment not only activated WAF1 but also up-regulated p53 expression only in A-172 cells carrying the wild-type p53 gene. Gel mobility shift assay indicated an increase in p53 DNA binding activity after heat treatment. These findings suggest that the WAF1 expression is heat-inducible in human glioblastoma cells and that this induction may be due to signal transduction mediated by p53 in response to heat stress.

WAF1, also known as p21/CIP1/sdi1, an inhibitor of cyclin-dependent kinases, can be induced by DNA-damaging agents such as ionizing radiation, growth factors such as platelet-derived growth factor and transforming growth factor β, or in the processes of cellular differentiation and senescence through p53-dependent or independent pathways (1–6, 8). Accumulated WAF1 inhibits the kinase activities of various cyclin-Cdk complexes, as a result of which cells are arrested in G1 (9, 10). Hyperthermia has recently been developed as a form of cancer therapy and has been applied to various types of malignant tumors. Thus, studies of the heat-induced signal transduction in cell cycle control are important. Following our previous report on p53 accumulation and its association with heat shock protein Hsp72/Hsc73 in human glioblastoma cells after hyperthermia (11), we investigated the responses of WAF1 in heat-treated human glioblastoma cells. Our results suggest that the induction of WAF1 by heat treatment may be due to signal transduction mediated by p53 in response to heat stress.

MATERIALS AND METHODS

Cell Culture and Reagents—Human glioblastoma A-172 cells bearing wt p53* gene and T98G cells bearing mp53 gene (provided by J CRB, Setagaya, Tokyo) were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, penicillin (50 units/ml), streptomycin (50 μg/ml), and kanamycin (50 μg/ml). Anti-human WAF1 monoclonal antibody (EA10) and anti-human p53 monoclonal antibody (PAB1801) were obtained from Oncogene Science Inc. (Uniondale, NY). Horseradish peroxidase-conjugated anti-mouse IgG antibody was purchased from Zymed Labs. Inc. (San Francisco, CA). Millipore Immobilon polyvinylidene difluoride membranes were purchased from Millipore (Bedford, MA). Megalabel y RNAzol was purchased from Zymed Labs. Inc. (San Francisco, CA). Millipore Immobilon polyvinylidene difluoride membranes were purchased from Millipore (Bedford, MA).

Western Blot Analysis—The cells were washed once with phosphate-buffered saline after trypsinization (0.0025%) and then loaded at 20 μg/well on an SDS-15% or 10% polyacrylamide gel containing 0.05% (w/v) SDS. After electrophoresis, proteins were transferred to Immobilon polyvinylidene difluoride membranes. The membranes were incubated with anti-human WAF1 or p53 antibodies (1/100 dilution with phosphate-buffered saline) for 2 h at room temperature and treated with horseradish peroxidase-conjugated anti-mouse IgG antibody (1/2000 diluted with phosphate-buffered saline containing 0.05% Tween 20). The sensitivity of visualization of WAF1 and p53 bands was enhanced using the BLAST system. The band densities were measured using a Macintosh (LC475) computer with the public domain NIH Image program.2 Other details were as described elsewhere (11).

Northern Blot Analysis—Total cellular RNA was prepared from the cells using the RNAzol® method according to the manufacturer's instructions.
Heat Shock Induces WAF1 Expression

Heat shock was quantified by spectrophotometric methods, and 10 μg of total cellular RNA was loaded per lane on a 1% agarose gel containing 17% formaldehyde in MOPS buffer. RNA was subsequently transferred onto GeneScreen membranes in 20x3 SSC. After baking for 2 h at 80°C, the membranes were prehybridized for 3 h at 42°C in a solution containing 50% formamide, 0.1 M sodium-Pipes (pH 6.8), 0.65 M NaCl, 0.1% SDS, 5 mM EDTA, and 100 μg/ml denatured salmon sperm DNA. Filters were then hybridized with appropriate probes labeled with [γ-32P]ATP using Megalabel according to the manufacturer's instructions. For WAF1/sdi1 mRNA, the 2.1-kilobase pair BamHI fragment of the WAF1/sdi1 cDNA of plasmid 2D10–5 (provided by Dr. A. Noda, Meiji Cell Technology Center, Odawara, Japan) was used. For p53 mRNA, the 1.5-kilobase pair XbaI/XhoI fragment of the p53 cDNA of plasmid pCMV5C (provided by Dr. M. Oren, Weizmann Institute of Science, Rehovot, Israel) was used.

Preparation of Nuclear Extracts—All steps in the preparation of nuclear extracts were carried out at 4°C. The cells were washed once with phosphate-buffered saline immediately after heating and then washed once more in washing buffer. The cells were homogenized on ice in hypotonic buffer with 20 strokes of a hand-driven Dounce homogenizer. The nuclei were precipitated by centrifuging the homogenates at 1000 × g for 10 min and resuspended in extraction buffer and stirred for 1 h. The resulting nuclear suspensions were centrifuged at 20,000 × g for 30 min to remove the chromatin. The nuclear extracts were collected and dialyzed against binding buffer. The protein concentration of each extract was quantified using a Bio-Rad Protein Assay Kit.

Gel Mobility Shift Assay—The p53 binding activity was measured by a gel mobility shift assay using a synthetic double-stranded p53 oligonucleotide as a probe. This

![Figure 1](image1.png) Fig. 1. Induction of WAF1 and p53 accumulation by heat treatment. A, WAF1 accumulation by heat treatment. The arrowhead indicates the WAF1 band. B, relative levels of WAF1 protein at different time points after heat treatment as compared with that in non-heated A-172 cells. C, control sample cultured at 37°C. D, A-172; T98G. C, p53 accumulation by heat treatment. The arrowhead indicates the p53 band. D, relative levels of p53 protein at different time points after heat treatment as compared with that in non-heated cells. Symbols are the same as B.

![Figure 2](image2.png) Fig. 2. Induction of WAF1 and p53 expression after heat treatment. A, WAF1 expression induced by heat treatment. B, relative levels of WAF1 mRNA at different time points after heat treatment as compared with that in non-heated cells. C, control sample cultured at 37°C. D, A-172; T98G. C, p53 expression induced by heat treatment. D, relative levels of p53 mRNA at different time points after heat treatment as compared with that in non-heated cells. Symbols are the same as B.

(RJX Photo Film Co., Ltd., Tokyo) for 72 h at room temperature with intensifying screens. The gels were stained with ethidium bromide before blotting to confirm equal loading by 28S rRNA bands. The band densities were measured as described above.
probe was labeled with \( ^{32} \text{P} \)ATP using Megalabel \(^{32} \text{P} \) and then incubated with nuclear extracts (5 \( \mu \)g of protein/1 - 3 \times 10^{5} \text{cpm probe}) at 25 °C for 30 min in the presence of poly(dI-dC)-poly(dI-dC) (1 \( \mu \)g) in a total reaction volume of 15 \( \mu \)l adjusted with binding buffer. After incubation, 1.5 \( \mu \)l of dye solution was added to the reaction mixture, which was then subjected to electrophoresis on a 5% (w/v) polyacrylamide gel for 1 h at 150 V using Tris acetate-EDTA buffer. Subsequently, the gel was dried and then observed on a Fujix BAS1000 (Fujji, Tokyo, Japan) and photographed on a Pictography 3000 (Fuji, Tokyo) connected to the Fujix BAS1000. The densities of the p53-specific bands were measured as described above.

RESULTS AND DISCUSSION

To examine the effects of heat treatment on WAF1 accumulation, Western blot analysis was performed. Briefly, A-172 and T98G cells were plated (2 \times 10^{5}/25-cm⁴ flask) 12 h before heat treatment (44 °C, 30 min). After heat treatment, cells were harvested at various times, and then total proteins were extracted. 20-\( \mu \)g aliquots of protein were loaded on an SDS-polyacrylamide gel for Western blot analysis. As shown in Fig. 1, A and B, WAF1 accumulation (1.4-fold as compared to that of control sample cultured at 37 °C) was observed in A-172 cells at 3 h after heat treatment. This elevated level was maintained until 24 h (3.8-fold). The level of WAF1 mRNA in T98G cells did not increase after heat treatment. In contrast, it began to decrease 6 h after heat treatment and continued this decreasing tendency until 24 h, dropping to the level half that in non-heated cells. The WAF1 mRNA level in T98G cells was higher than that in A-172 cells under normal culture conditions as shown in lanes 1 and 6 of Fig. 2A. However, the level of WAF1 as determined by Western blotting was much lower in T98G cells than that in A-172 cells under normal culture conditions (Fig. 1A, lanes 1 and 6). We interpret these contradictory results as suggesting that there might be unknown mechanisms for the regulation of WAF1 synthesis at the post-transcriptional level. T98G cells may possess other p53-independent pathways that account for the high level of basal expression of WAF1. It is well known that p53 accumulation in cells exposed to ionizing radiation or UV light is a post-transcriptional event resulting from prolongation of the biological half-life of the protein (13), which may be due to stabilization by binding to other proteins such as Hsp72 (14, 17). However, we found evidence of p53 expression by heat treatment in human glioblastoma cells in a wt p53-dependent manner. As shown in Figs. 2, C and D, an apparent increase in p53 mRNA appeared 3 h (2.1-fold) and peaked 10 h (5.1-fold) after heat treatment in A-172 cells, while the level of p53 mRNA in T98G cells remained unchanged throughout the observation period. Thus, we conclude that the accumulation of p53 in heat-treated cells was achieved in part through induction of p53 and that p53 induction may be regulated by p53 itself or by an unknown upstream signal since it has been reported that there is a p53 consensus sequence in the promoter region of p53 (15). Agarwal and co-workers (16) observed increased expression of wt p53 in response to genotoxic stress. We also observed this response in \( \gamma \)-irradiated glioblastoma cells (data not shown). Therefore, we considered that the p53-dependent activation of p53 expression may be a general mechanism for cells in response to both non-genotoxic and genotoxic stress by a process of autoregulation. In summary, our results of Northern blotting analysis clearly demonstrated that heat-induced WAF1 accumulation was a consequence of WAF1 expression through a p53-dependent pathway and that heat...
treatment not only activated WAF1 but also up-regulated p53 expression only in A-172 cells carrying the wtp53 gene.

To demonstrate the DNA binding activity of heat-induced p53, an experiment using gel mobility shift assay was carried out. Briefly, nuclear extracts were prepared from the cells before or immediately after heat treatment or γ-irradiation. The nuclear extracts were then incubated with the labeled p53CON. After electrophoresis, the gel was dried, and the signals were visualized on a BAS1000. As shown in Fig. 3, the p53-specific bands became denser in A-172 cells after heat treatment (6.4-fold as compared to controls cultured at 37 °C) as well as after γ-ray irradiation (17-fold), indicating that wtp53 binding activity increased after heat treatment or γ-irradiation. This result was different from another report in which it was demonstrated that the accumulated p53 in RKO cells after heat treatment (43°C, 1 h) lost its p53CON binding activity (17). We consider that this difference may have been due to cell type. Our results from gel mobility shift assay provided evidence that heat treatment could activate the DNA binding activity of wtp53 in human glioblastoma cells, which in turn might lead to the induction of WAF1 expression.

Likewise, wtp53 can be accumulated by various non-genotoxic cellular stressors such as hypoxia (17), heat shock (11, 17), osmotic shock, heavy metals (cadmium), blockers of the cellular respiratory system (NaN₃), amino acid analogues (azetidine and canavanine), an inhibitor of protein synthesis (puromycin), and oxygen free radicals (H₂O₂), all of which evoke the heat shock response (18). Taken together with our previous observation that p53 associates with Hsp72/Hsc73 (11), we suggest that p53 may act as a stress-responsive protein and play an important role in protection of cells from harmful stressors. Heat-induced p53 accumulation and the resultant WAF1 induction may contribute to the recovery process of the cells after heat injury, such as protein denaturation, as does wtp53 through interaction with or induction of other factors (7, 19, 20) in the cells after exposure to genotoxic stress.

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