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Role of inducible heat shock protein 70 in radiation-induced cell death

Su-Jae Lee,[1] Sun-Ah Choi,[1] Kang-Hyun Lee,[2] Hee-Yong Chung,[3] Tae-Hwan Kim,[1] Chul-Koo Cho,[1] and Yun-Sil Lee[1]

[1]Laboratory of Radiation Effect, Korea Cancer Center Hospital, Seoul 139–706, Korea
[2]Department of Urology, Korea Cancer Center Hospital, Seoul 139–706, Korea
[3]Department of Microbiology, College of Medicine Hanyang University, Seoul 133–791, Korea

Abstract We previously demonstrated the protective effect of inducible heat shock protein 70 (Hsp70) against gamma radiation. Herein, we extend our studies on the possible role of Hsp70 to ionizing radiation-induced cell cycle regulation. The growth rate of inducible *hsp*70-transfected cells was 2–3 hours slower than that of control cells. Flow cytometric analysis of cells at G1 phase synchronized by serum starvation also showed the growth delay in the Hsp70-overexpressing cells. In addition, reduced cyclin D1 and Cdc2 levels and increased dephosphorylated phosphoretinoblastoma (pRb) were observed in inducible *hsp*70-transfected cells, which were probably responsible for the reduction of cell growth. To find out if inducible Hsp70-mediated growth delay affected radiation-induced cell cycle regulation, flow cytometric and molecular analyses of cell cycle regulatory proteins and their kinase were performed. The radiation-induced G2/M arrest was found to be inhibited by Hsp70 overexpression and reduced $p21^{Waf}$ induction and its kinase activity by radiation in the Hsp70-transfected cells. In addition, radiation-induced cyclin A or B1 expressions together with their kinase activities were also inhibited by inducible Hsp70, which represented reduced mitotic cell death. Indeed, *hsp*70 transfectants showed less induction of radiation-induced apoptosis. When treated with nocodazole, radiation-induced mitotic arrest was inhibited by inducible Hsp70. These results strongly suggested that inducible Hsp70 modified growth delay (increased G1 phase) and reduced G2/M phase arrest, subsequently resulting in inhibition of radiation-induced cell death.

INTRODUCTION

Heat shock proteins (Hsps) act as molecular chaperones, contributing to the folding of nascent polypeptides and also to protein transport and degradation. They protect cells and organisms from oxidative damage both in vivo and in vitro (Jacquier-Sarlin et al 1994; Plumier et al 1995) and often prevent cell death (Seo et al 1996). The cytoprotective effect of Hsps has been attributed to one of the major Hsps, Hsp70 (Jaattela and Wissing 1993), a member of the 70-kDa Hsp family, which is proposed to serve as a molecular chaperone (Gething and Sambrook 1992). Indeed, overexpression of Hsp70 protects cells, tissues, and organs from harmful assaults. However, under some conditions, the protective action of Hsp70 appears to be unrelated to its chaperoning activity. For example, tumor necrosis factor causes cell death by the activation of a signal transduction pathway, leading to apoptosis (Ichijo et al 1997), and this apoptotic process can be prevented by overproduction of Hsp72 (Jaattela et al 1992), seemingly appearing to interfere with the apoptotic program such as Jun N-terminal kinase (Gabai et al 1997), extracellular signal-regulated protein kinase, and p38 mitogen-activated protein kinase (Hung et al 1998).

On exposure to ionizing radiation, eukaryotic cells activate a variety of intracellular signaling pathways, thereby delaying cell cycle progression at ''checkpoints'' in the G1, S, or G2 phases and activating DNA repair. Cell cycle progression is controlled by specific Cdk-cyclin kinase activity that phosphorylates and activates proteins essential for execution of events that lead to cell cycle progression. Of the families of Cdk–cyclin kinase inhibitors, the $p21^{Waf}$ family includes the structurally related proteins, the $p21^{Waf}$, which are capable of inhibiting a variety of Cdk–cyclin kinases. In addition, Cdc2, a highly conserved mitosis-promoting Cdk, is thought to mediate in G2 pri-

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Correspondence to: Yun-Sil Lee, Tel: 82-2-970-1325; Fax: 82-2-977-0381; E-mail: yslee@mail.kcch.re.kr.

marily by phosphorylation and appears to operate in a variety of experimental systems. Inhibitory phosphorylation of Cdc2 is associated with the restriction of the kinase and its mitotic heterodimerization partner cyclin B1 (McDonald et al 1996). There is little direct information on the role of inducible Hsp70 in the cell cycle control; however, it is present both in the nucleus and cytoplasm in quiescent cells and begins to enter to the nucleus when the cells are stimulated to enter from G1 to S phase (Milarski and Morimoto 1986). Since our previous study suggested that inducible Hsp70 overexpression endowed cells with radioresistance (Park et al 2000), further study on the relationship between inducible Hsp70-mediated radioresistance and cell cycle regulation was carried out. Herein, we report that inducible Hsp70 decreased cell growth (increased G1 phase) and shortened radiation–induced G2/M phase arrest.

MATERIALS AND METHODS

Cell cultures and treatment

The mouse cells of NIH3T3 were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% antibiotic/antimycotic solution, and 2 mM L-glutamine (all from GIBCO BRL, Gaithersburg, MD, USA). The cell density was kept subconfluent, and the cells were passaged twice a week.

Vector construction

The detailed procedure to construct MFG.B7.puro vector, which was used for constructing MFG.Hsp70puro, has been published elsewhere (Kwak et al 1998; Park et al 2000). To establish cell line, 1 μg of MFG.Hsp70puro or the MFGpuro plasmids was introduced into cells by lipofection (Lipofectamine, GIBCO BRL) in serum-free media. Cells were propagated in DMEM supplemented with 10% FCS, 1% antibiotic/antimycotic solution, 2 mM L-glutamine, and 2 μg/mL of puromycin (all from GIBCO BRL). Twenty-four hours after transfection, media were changed and the cells were maintained in medium containing 10% serum and 2 μg/mL of puromycin. Control cells were transfected with MFGpuro alone. *hsp*70-transfected cells were frequently tested for the expression of Hsp70 by Western blot analysis and found to express high levels of inducible Hsp70 protein.

Irradiation

Cells were exposed to gamma rays with ^{137}Cs gamma ray source (Atomic Energy of Canada Ltd, Canada) with a dose rate of 3.81 Gy/min.

Cell counting

Cellular proliferation was monitored by counting cells using vital dye trypan blue (Sigma, St-Quentin, France), and the viable cells were counted using a photomicroscope.

[^{3}H]-thymidime incorporation assay

Cells were plated at a confluence of 70–80% in 10-cm Petri dishes. After irradiation, the cells were trypsinized, plated in 96-well plates (5×10^3 cells/well), and incubated for various periods. After adding 1 μCi of [^{3}H]-thymidine to the incubation medium, the cells were further incubated for the last 4 hours before harvest, and the radioactivity of the cells was determined with a scintillation counter (PACKARD, TRI-CARB 4530, Meriden, CT, USA).

G1-phase synchronization

Cells were plated 1 day before a 48-hour serum starvation. The medium was then changed to serum-containing medium, and cells were thereafter harvested at various time points to study cell cycle distribution.

Cell cycle analysis

For cell cycle analysis, cells were fixed in 80% ethanol at 4°C for at least 18 hours. The fixed cells were then washed once with phosphate-buffered saline (PBS)–ethylenediamine-tetraacetic acid (EDTA) (1 mM EDTA in PBS) and resuspended in 1 mL of PBS. After the addition of 10 μL of propidium iodide solution (5 mg/mL) and 10 μL of ribonuclease (10 μg/mL), the samples were incubated for 30 minutes at 37°C and analyzed with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Detection of apoptosis

For detection of apoptotic cells, cells were plated on glass slides and irradiated with 4 Gy. After 12 or 24 hours, the cells were fixed for 20 minutes in 3.7% formaldehyde, washed with PBS, and incubated with Hoechst 33258 solution in PBS (4 μg/mL) for 30 minutes at room temperature in the dark. Specimens were analyzed with a fluorescence microscope (Olympus, Japan) mounted on a Nikon Diaphot-TDM, and the percentage of apoptotic cells was determined.

Detection of mitotic index

For detection of mitotic index, cells were plated on glass slides and treated with nocodazole (400 ng/mL) combined with 4 Gy of radiation. After 6, 12, 24, or 48 hours,

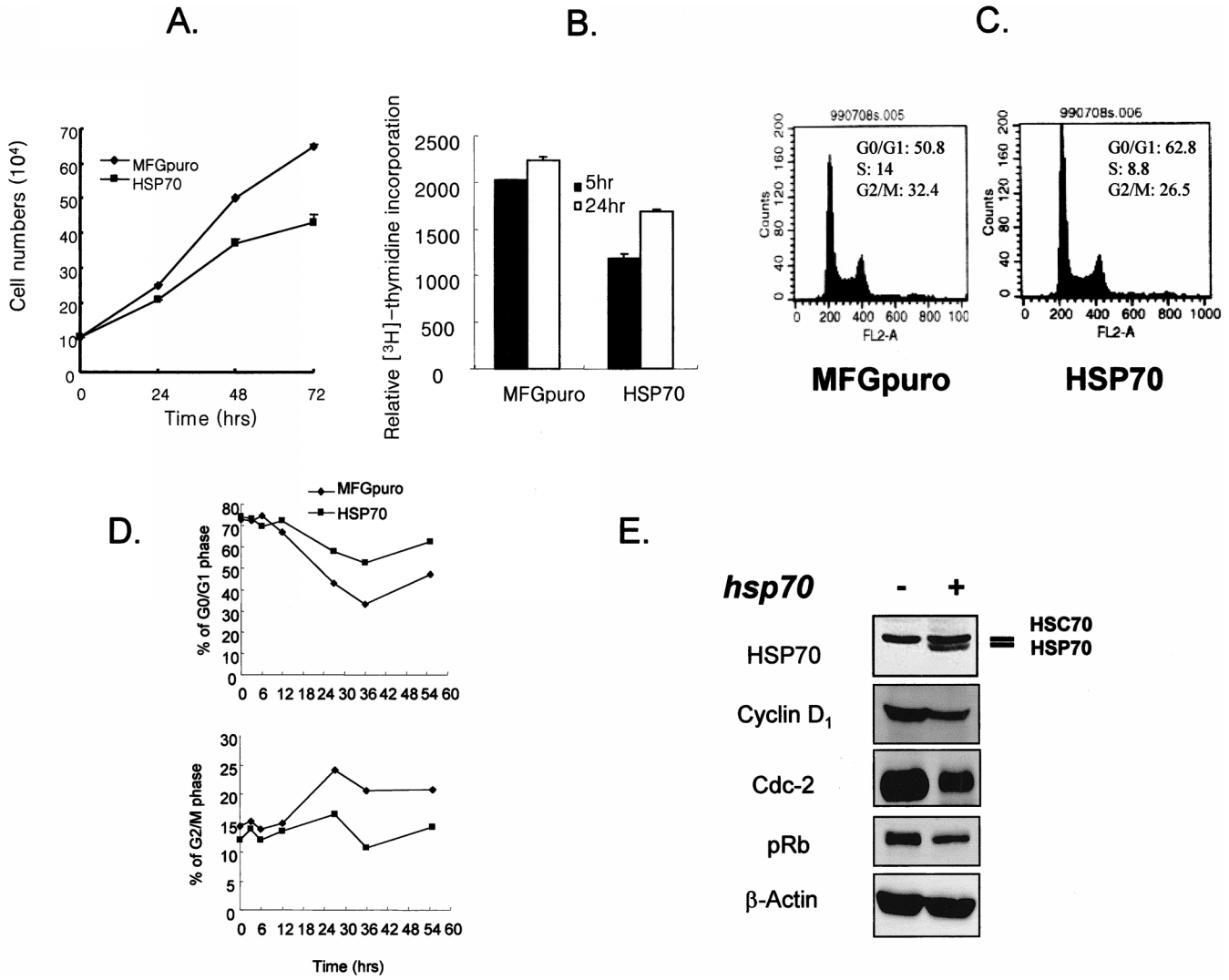

Fig 1. Comparison of cell growth. (A) Vector control and inducible *hsp70*-transfected cells were incubated for indicated times (0–72 hours). At each time point, viable cells were counted by trypan blue dye exclusion method. Error bar indicates mean ± SD from 3 independent experiments. (B) Vector control and inducible *hsp70*-transfected cells were incubated for indicated times (5 and 24 hours). At each time point, incorporation of [^{3}H]-thymidine was carried out for an additional 4 hours, and the radioactivity of the cells was determined with a scintillation counter. Error bar indicates mean ± SD from 3 independent experiments. (C) Cell cycle distributions of exponentially growing vector control and inducible *hsp70*-transfected cells were determined using flow cytometry after propidium iodide staining. (D) Cells were preincubated for 20 hours with 300 μM mimosine and harvested after incubation for the period indicated. Cell cycle distribution following release from mimosine treatment was analyzed after staining with propidium iodide by a flow cytometer. (E) Protein extracts from growing vector control and inducible *hsp70*-transfected cells were prepared, separated by SDS-PAGE, and analyzed by Western blot.

the cells were fixed in 70% ethanol. The rest of the procedures to obtain the percentage of mitotic cells are the same as described in the ''Detection of apoptosis'' section. Mitotic index was calculated by the percentage of mitotic arrested cells.

Polyacrylamide gel electrophoresis and Western blot

For polyacrylamide gel electrophoresis (PAGE) and Western blot, cells were solubilized with lysis buffer (120 mM sodium chloride, 40 mM Tris [pH 8.0], 0.1% NP40). Samples were denatured in sample buffer containing 25 mM Tris hydrochloride (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 10% 2-mercaptoethanol, and 0.002% bromphenol blue and boiled for 5 minutes. Then equal amounts of proteins (40 μg/well) were analyzed on 10% SDS-PAGE. After electrophoresis, proteins were transferred onto a nitrocellulose membrane and processed for immunoblotting. Blots were incubated with a 1:1000 dilution of antibodies to cell cycle–related proteins; the mouse monoclonal anti-p53 antibody (Calbiochem, Oncogene Research Products, Cambridge, MA, USA); anti–cyclin B1 and anti-Cdc2 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA); the rabbit polyclonal

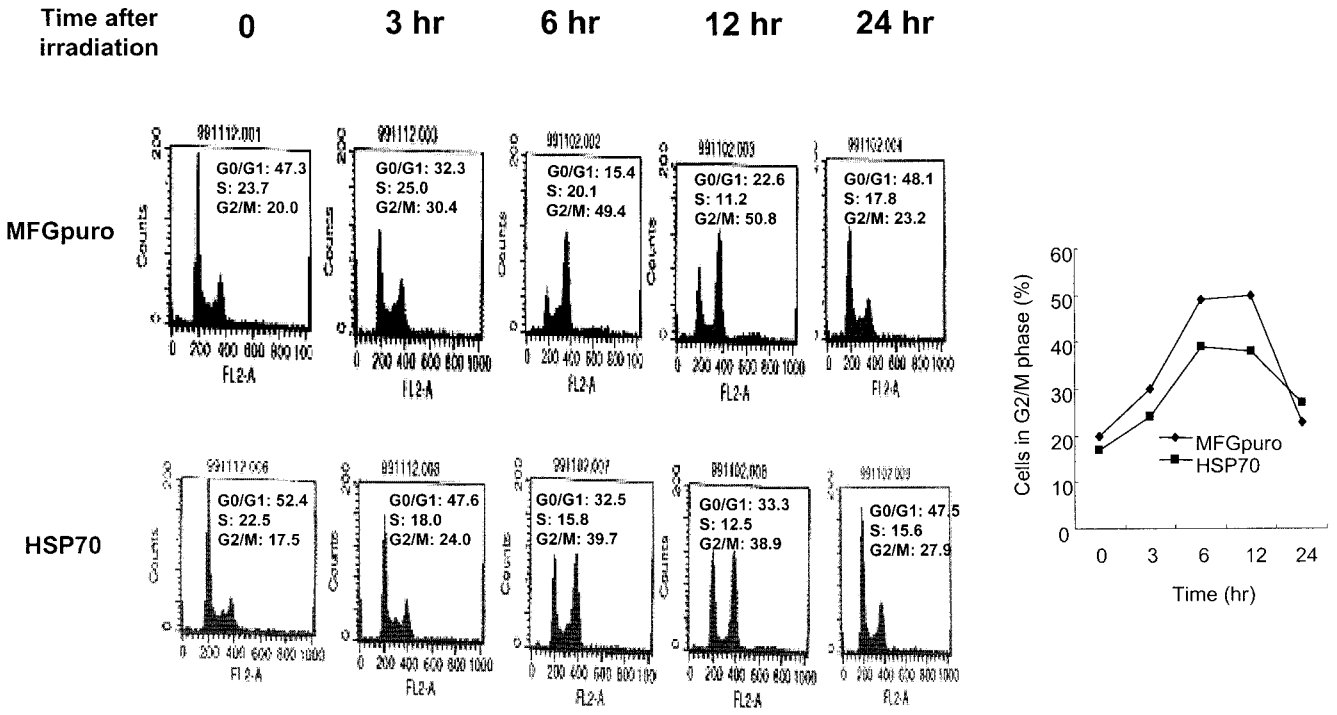

Fig 2. Cell cycle arrest. Cells were harvested following incubation after 4 Gy of radiation for the period indicated, and cell cycle distribution was analyzed after staining with propidium iodide by a flow cytometer.

anti-Cdk2, -Cdk4, –cyclin D1, and -p21^{Waf} antibodies (Santa Cruz Biotechnology); and –cyclin E antibody (Upstate Biotechnology Inc, Lake Placid, NY, USA). Blots were further incubated with horseradish peroxidase–conjugated secondary antibody diluted at 1:5000, and specific bands were visualized by chemiluminescence (ECL, Amersham International). Autoradiographs were recorded onto X-Omat AR films (Eastman Kodak Co).

Immunoprecipitation

Cell lysates were incubated with anti-CDK2, anti–cyclin B1 polyclonal antibody (Santa Cruz Biotechnology), or normal rabbit serum. The immunocomplex was collected on protein A–Sepharose (Sigma) and analyzed by SDS-PAGE using the ECL detection kit.

Immunoprecipitation and immune complex kinase assay

Cell lysates were incubated with a primary antibody, and immunocomplexes were collected on protein A–Sepharose beads and resuspended in kinase assay mixture containing [γ-^{32}P] adenosine triphosphate (ICN) and GST-pRB (Santa Cruz Biotechnology) or histone H1 (Life Technologies Inc) as substrates. The proteins were separated on SDS-polyacrylamide gels, and bands were detected by autoradiography.

Data analysis and statistics

The points shown in the figures are means of 3 independent determinations, and Student's *t*-test was used for statistical analysis.

RESULTS

Cell growth

When growth curves of control vector and inducible *hsp70*-transfected cells were examined by the trypan blue dye exclusion method, the growth rate of the transfected cells was 2 to 3 hours slower than that of vector control cells (Fig 1A). Relative rate of [^{3}H]-thymidine incorporation also revealed the growth arrest (Fig 1B), and flow cytometric analysis of DNA content also indicated elevated G1 (23.6%) and less S phase (37.1%) in the *hsp70*-transfected cells (Fig 1C). To examine whether the delay of cell growth by Hsp70 overexpression was due to an alteration of cell cycle, the control vector or *hsp70*-transfected cells were synchronized by serum starvation, then refed with fresh medium plus 10% fetal bovine serum, and cell cycle progression was monitored by measuring cellular DNA contents by flow cytometry. G1 arrest was induced with serum starvation both in the control and *hsp70*-transfected cells; however, the cell cycle of the transfected cells proceeded more slowly than the control cells in serum starvation (Fig 1D). To examine further

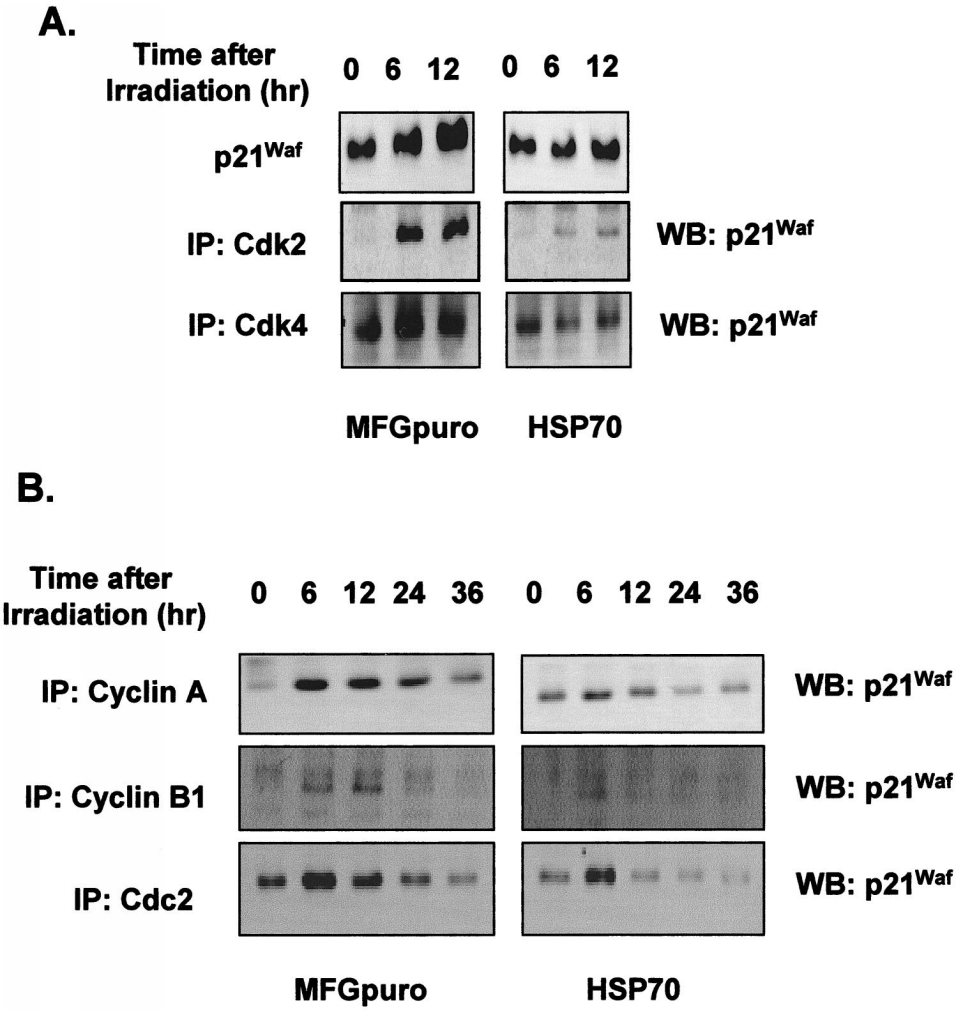

Fig 3. Expression of p21^{Waf}. At the indicated interval after 4 Gy of radiation, protein extracts (60 μg) from growing vector control and inducible *hsp70*-transfected cells were prepared, separated by SDS-PAGE, and analyzed by Western blotting for p21^{Waf}, or cell lysates (500 μg) were immunoprecipitated (IP) with anti-Cdk2 or anti-Cdk4 antibodies (A) and anti–cyclin A, cyclin B, or Cdc2 antibodies (B), separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with anti-p21^{WAF1} antibody followed by enhanced chemiluminescence detection.

whether the cell growth delay by inducible Hsp70 was due to altered expression of cell cycle–related proteins, Western blot analysis was performed. It was found that overexpression of inducible Hsp70 reduced the basal levels of Cdc2 and cyclin D1 proteins and increased dephosphorylation of retinoblastoma (Rb). There was no significant change in the levels of Cdk2, Cdk4, cyclin A, cyclin B1, cyclin E, p21^{Waf}, p27^{kip}, proliferating cell nuclear antigen, and p53 proteins (data not shown). Since cyclin D1–Cdk4 and cyclin A– or cyclin B1–Cdc2 complexes are known to be involved in the progression from G1 to S, S to G2, and G2 to M phase, respectively, and also pRb dephosphorylation is known to induce the growth arrest in G1 and S transition, it is highly likely that reduced expressions of Cdc2 and cyclin D1 and dephosphorylated pRb are responsible for the cell growth delay in the inducible *hsp70*-transfected cells.

G2/M block

Using flow cytometry to determine if altered cell growth by inducible *hsp70* can affect radiation-induced cell cycle arrest, G2 block in both cell lines was examined by measuring accumulation of cells in G2/M at approximately 3, 6, 12, and 24 hours after 4 Gy of radiation. The cell cycle arrest in the Hsp70-overexpressing cells was clearly decreased; G2/M arrest decreased by 20% and 24% at 6 and 12 hours after irradiation, respectively. These data clearly demonstrated that inducible Hsp70 released radiation-induced G2/M phase arrest (Fig 2).

p21^{Waf} induction

When cells were irradiated with 4 or 8 Gy, the increase of total level of p21^{Waf} protein in the inducible *hsp70*-overexpressing cells was much less than in the control cells. We, therefore, examined possible association of p21^{Waf} with Cdk2 or Cdk4. Binding activity of p21^{Waf} was increasingly associated with CDK2 and CDK4 at 6 and 12 hours after radiation, and these associations were also found to be inhibited in the inducible *hsp70*-overexpressing cells (Fig 3A). In the S and G2 phases, the binding activity of p21^{Waf} with cyclin A, cyclin B, or Cdc2 in-

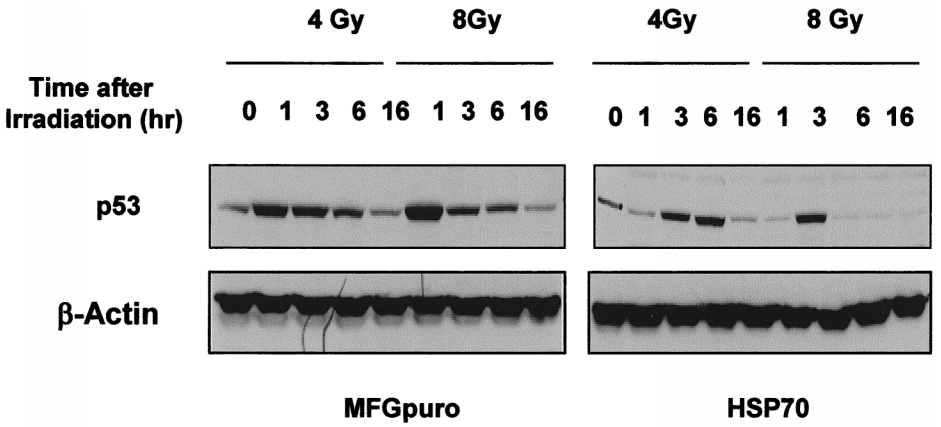

Fig 4. Expression of p53 protein. At the indicated interval after 4 or 8 Gy of radiation, protein extracts (60 μg) from growing vector control and inducible *hsp70*-transfected cells were prepared, separated by SDS-PAGE, and analyzed by Western blot for p53.

creased by radiation, whereas this activity decreased in the inducible *hsp70* transfected cells, suggesting a less radiation-induced cell cycle arrest by inducible *hsp70* than the control vector cells (Fig 3B).

p53 protein level

Since p53 protein is known to be an inducer of G1 or G2/M phase arrest, p53 protein expression after irradiation was also examined. Induction of p53 protein both in cell lines increased from the first 1 hour of irradiation and lasted for 6 hours, however, with less and delayed increase in the Hsp70-overexpressing cells (Fig 4). These results suggested that p53 protein expression correlated well with the radiation-induced cell cycle regulation, with less arrest in the inducible *hsp70*-overexpressing cells.

Cyclin A, cyclin B, and Cdc2 protein levels and their associated kinase activities

To elucidate the effects of perturbation of S or G2/M phase and premature entry to mitosis before completion of DNA synthesis (Tam and Schlegel 1997), cyclin A and B protein levels were examined. As shown in Figure 5A, irradiated cells showed elevated levels of cyclin A, cyclin B, and Cdc2 expressions all through the G2/M block and the elevated levels persisted for the duration of the block; however, reduced levels of these proteins were shown in the *hsp70*-transfected cells. When the protein levels of cyclin A, cyclin B, and Cdc2 were elevated, cyclin A– or cyclin B–associated kinase activities were also elevated (Fig 5B). Binding activity of cyclin A and Cdk2 or Cdc2 also increased, whereas it was inhibited in the *hsp70* transfected cells (Fig 5C).

Cell death

To study whether shortened G2/M phase arrest and altered response of cell cycle–related protein expressions by *hsp70* could affect radiation-induced cell death, Hoechst 33258 staining was performed. As shown in Figure 6A, 4 Gy of gamma rays induced an extensive nuclear condensation and fragmentation in the vector control cells, whereas overexpression of Hsp70 drastically diminished this phenomenon. The function of inducible Hsp70 in radiation-induced mitotic arrest was further studied by using nocodazole, a blocking agent in the M phase. As shown in Figure 6B, about 60% in the control vector cells and 40% in the transfected cells induced mitotic arrest by nocodazole treatment. In addition, when apoptotic cells were counted, nocodazole treatment reduced apoptosis and the reduction was greater in the control vector cells than the *hsp70*-transfected cell, suggesting the protection of the cells from mitotic cell death by Hsp70.

DISCUSSION

Although induction of Hsp70 protein synthesis may protect cells from lethal effects of various stimuli, the role of *hsp70* genes in the control of cell growth is not well defined. In the present study, we demonstrated that irradiation-induced cell death was inhibited by inducible Hsp70, and this inhibition resulted from abrogation of G2/M phase arrest mediated by irradiation.

Inducible *hsp70*-transfected NIH3T3 cells had delayed cell growth, shown by conventional trypan blue method and [^{3}H]-thymidine incorporation. Flow cytometric analysis also revealed increased G1 phase, slower cell release after synchronization with serum starvation, reduction of cell cycle–related proteins expression such as cyclin D1 and cdc2, and an increase of dephosphorylated pRb protein in the inducible *hsp70*-transfected cells (Fig 1). These results suggest that inducible Hsp70 modulates the expression levels of these proteins, which eventually lead to growth delay.

Since radiation-induced DNA damage activates cell cycle checkpoints responsible for growth arrest or cell death (Bates and Vousden 1992) and inducible Hsp70 protects

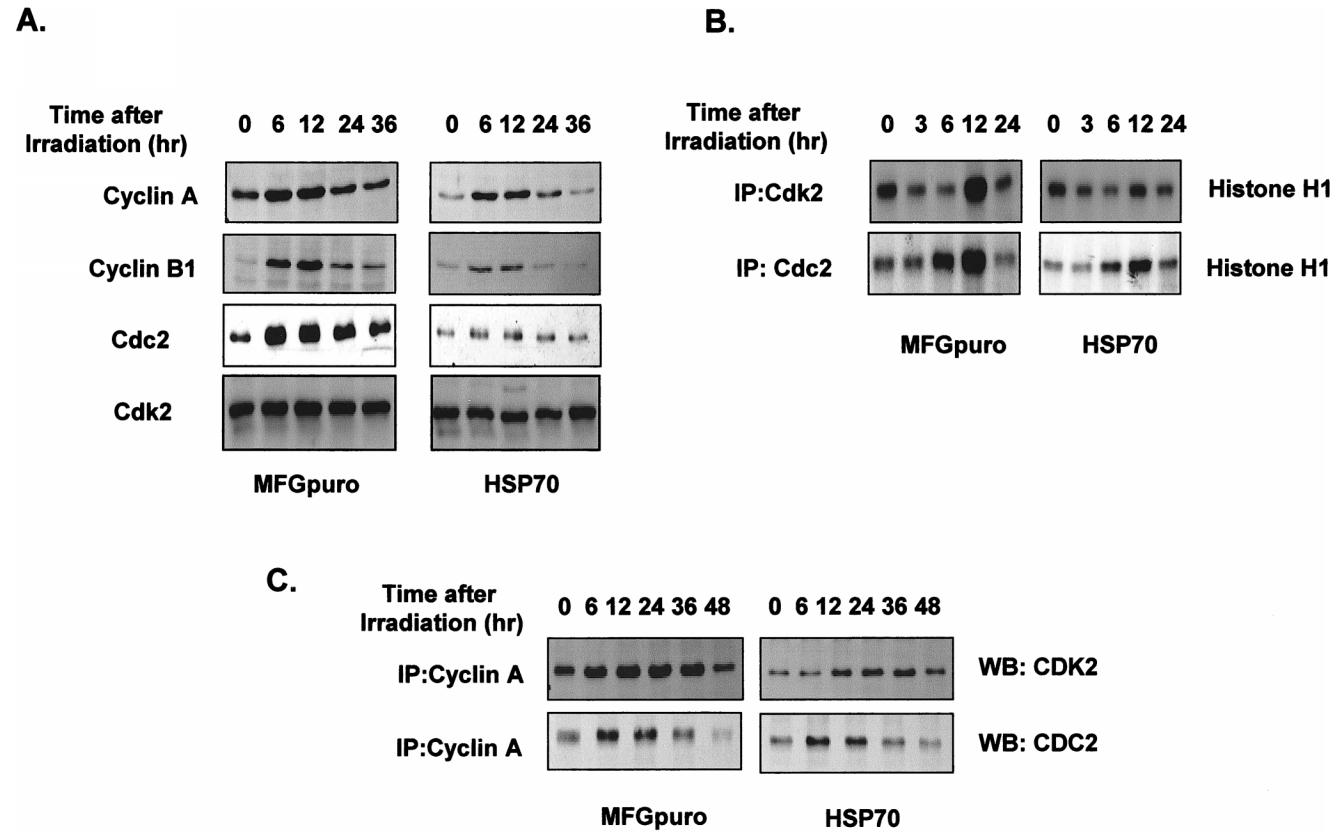

Fig 5. Cyclin A, cyclin B, and Cdc2 protein levels and their associated kinase activities. At the indicated interval after 4 Gy of radiation, protein extracts (60 μg) from growing vector control and inducible *hsp70*-transfected cells were prepared, separated by SDS-PAGE, and analyzed by Western blotting for cyclin A, cyclin B, Cdc2, and Cdk4 (A). Cell lysates (200 μg) were immunoprecipitated (IP) with anti-Cdk2 or Cdc2 antibodies, and kinase activity was assayed using histone H1 as a substrate (B). Cell lysates (500 μg) were immunoprecipitated (IP) with anti–cyclin A antibody, separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with anti-Cdk2 or anti-Cdc2 antibodies followed by enhanced chemiluminescence detection (C).

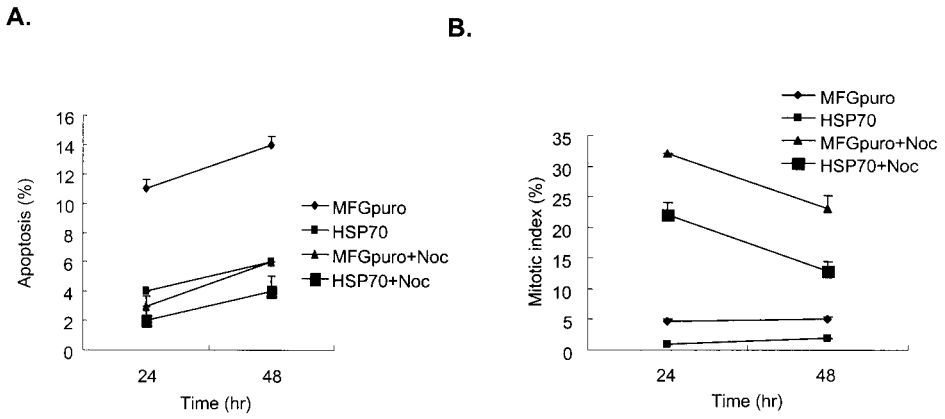

Fig 6. Cell death. At 24 or 48 hours after 4 Gy of radiation, DNA fragmentation of vector control and inducible *hsp70*-transfected cells was obtained by Hoechst 33258 staining (A). Immediately after gamma rays, both vector control and inducible *hsp70*-transfected cells were treated with nocodazole (400 ng/mL), and the mitotic index was determined at indicated times of post-irradiation as described in Materials and Methods (B). Error bar indicates the mean ± SD from 3 independent experiments.

cells from radiation (Park et al 2000), we explored a question of whether cell growth delay mediated by inducible Hsp70 was correlated with radioresistance and found that excessive expression of inducible Hsp70 resulted in an alleviation of the radiation-induced G2/M block (Fig 2), which could be explained by the fact that fewer Hsp70-overexpressed cells were arrested in the G2/M phase and they were released from the G2/M block sooner. To investigate molecular mechanisms, we first checked the $p21^{Waf}$ expression that is known as a broad range CDK inhibitor. Radiation-induced $p21^{Waf}$ expression was inhibited by inducible Hsp70 (Fig 3), and its binding efficiency with cyclin A, cyclin B, or cdc2 was also reduced by inducible Hsp70. Also, expressions of cyclin D and E were

well correlated with $p21^{Waf}$ induction (data not shown). On the other hand, radiation-induced expressions of cyclin A and B and their kinase activities, which were associated with inductions of premature mitosis and apoptosis, were also inhibited by inducible Hsp70 (Fig 3). In fact, mitotic index was much less in the *hsp70* transfected cells when treated with nocodazole. In addition, radiation-induced apoptosis was inhibited by nocodazole treatment, and this inhibition was greater in the control vector cells (Fig 6). These results suggest that inducible Hsp70 protects the cells from the radiation-induced cell death, especially mitotic cell death, resulting in reduced radiation-induced G2/M phase arrest. In addition, radiation-induced p53 protein induction was also inhibited in *hsp70* transfected cells when compared with vector control cells (Fig 4). Expression of p53 after radiation showed G2/M phase arrest and induction of apoptosis (Schwartz et al 1997), and Hsp70 attenuated these phenomena.

The fact that radiation blocks cell cycle is well known (Hartwell and Kastan 1994), and delays of cells at G1 and G2/M phases and a small but quantifiable radiation-induced S-phase delay have been demonstrated (Wang and Iliakis 1992). Usually, G2/M delay has been suggested to be due to radioresistance (Warenius et al 1996). However, as shown in the present study, reduction of radiation-induced G/M phase arrest occurred in the *hsp70* transfected cells, which are usually due to $p21^{Waf}$ induction. There are reports to suggest a correlation between radiosensitivity and G2/M phase delay (McKenna et al 1990; Warenius et al 1996) and a longer delay in cells of more radiosensitive cell line with the same radiation dose (Li et al 1998), which are associated with premature mitotic entry before completion of DNA synthesis. However, there exists another possibility that increased levels of the stress proteins are responsible for development of resistance to stress agents (Li and Werb 1982; Subjeck et al 1982), because protein kinase C activity increased in the *hsp70*-transfected cells (Park et al 2000). Therefore, it is likely that induced stress proteins can protect cells from stress-induced damage by preventing protein denaturation and/or by repairing such damages (Schroeder et al 1993).

In conclusion, we attempted to define the role of inducible Hsp70 in radiation-induced cell cycle arrest. Radiation may alter signaling mechanisms, control the cell cycle regulation, especially G2/M phase regulators, and consequently promote kinase activity even in the presence of DNA damage. Alternatively, inducible Hsp70 may alter the membrane signaling cascade, which relays the DNA damage signal to the control mechanisms that block the irradiated cells in G2/M, thus effectively preventing the cells to progress through G2 and mitosis. These data may suggest that the Hsp70 induction could enhance tumorigenesis and limit the efficacy in cancer therapy, because Hsp70 is highly expressed in many tumor cells (Mivichi and Rossi 1990; Kaur and Ralhan 1995), transgenic mice expressing the *hsp70* gene develop T-cell lymphomas (Seo et al 1996), and the expression of Hsp70 is an indicator of poor therapeutic outcome in breast cancer (Ciocca et al 1993).

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