

## p27Cip/Kip Is Involved in Hsp25 or Inducible Hsp70 Mediated Adaptive Response by Low Dose Radiation

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### Adaptive response/Low dose radiation/Hsp70/Hsp25/p27CIP/KIP/G1 arrest.

Thermoresistant (TR) clone of radiation-induced fibrosarcoma (RIF) cells have been reported to show adaptive response to 1cGy of low dose radiation, and hsp25 and inducible hsp70 are involved in this process. In the present study, to further elucidate the mechanism of how hsp25 and inducible hsp70 regulate the adaptive response, hsp25 or inducible hsp70 overexpressed RIF cells were irradiated with 1cGy and cell cycle was analyzed. Hsp25 or inducible hsp70 overexpressed cells as well as TR cells showed increase of G1 phase population after  $\gamma$ -irradiation at 1cGy, while the parent RIF cells did not. [<sup>3</sup>H]-Thymidine and BrdU incorporation also indicated that both hsp25 and inducible hsp70 were involved in G1 arrest after 1cGy irradiation. Molecular analysis revealed upregulation of p27Cip/Kip protein in hsp25 and inducible hsp70 overexpressed cells, and cotransfection of p27Cip/Kip antisense abolished the induction of adaptive response and 1cGy-mediated G1 arrest. The above results indicate that induction of adaptive response by hsp25 and inducible hsp70 is mediated by upregulation of p27Cip/Kip protein, resulting in low dose radiation-induced G1 arrest.

### INTRODUCTION

Adaptive responses, which reduce harmful effects of post-irradiation at high dose radiation by pre-irradiation at low dose,<sup>1)</sup> have been demonstrated in chromosome aberration,<sup>2)</sup> cell survival,<sup>3)</sup> sister chromatid exchanges,<sup>4)</sup> micronucleus induction,<sup>5)</sup> mutation<sup>6)</sup> and neoplastic transformation.<sup>5)</sup> The mechanisms and conditions for the adaptive response to radiation have not yet been clarified, although continuous production of free radicals induced after irradiation and other sources has been shown to stimulate cells to evolve a repair system for chromosome breaks.<sup>7)</sup> An alteration of DNA molecule triggers the repair system, cell cycle regulation systems,<sup>8)</sup> antioxidant defense systems,<sup>9)</sup> and molecular chaperone or stress-response systems.<sup>10)</sup> Our previous data showed that cells showed an adaptive response, when they were preirradiated with 1cGy, and a reduction of apoptosis by low-dose preirradiation was a potential mechanism.<sup>11)</sup>

It has been well established that members of the heat shock protein (Hsp) family function as molecular chaper-

ones and assist intracellular folding of newly synthesized proteins,<sup>12)</sup> and several studies showed that a member of the hsp70 protein family is induced during adaptive response to oxidative stress and radiation,<sup>13)</sup> and this induction occurs during pretreatment of cells with a low concentration of H<sub>2</sub>O<sub>2</sub>. Low doses of X-rays were found to activate the promoter of inducible hsp70 gene: Transcription was silent under control conditions, but was highly induced by heat shock element,<sup>14)</sup> and the low dose of 4 cGy radiation that induced the adaptive response also increased inducible hsp70 mRNA.<sup>15)</sup> The induction of an adaptive response by low dose radiation also involved induction of PBP74/mortalin/Grp75, a member of the hsp70 family.<sup>16)</sup> However, how hsp70s contribute to the induction of adaptive response is not well defined. We previously demonstrated that mouse RIF cells, which did not induce hsp25 and inducible hsp70, did not exhibit an adaptive response after 1cGy preirradiation,<sup>17)</sup> whereas its thermoresistant TR cells, which expressed hsp25 and inducible hsp70, showed a response. Moreover, when inducible hsp70 or hsp25 was transfected to RIF cells, the cells acquired the adaptive response.

In the present study, in order to elucidate the mechanisms involved in the induction of adaptive response by hsp25 and inducible hsp70, we compared cell cycle distribution of hsp25 or inducible hsp70 transfected cells after low dose radiation and found that p27Cip/Kip was responsible for the induction of adaptive response.

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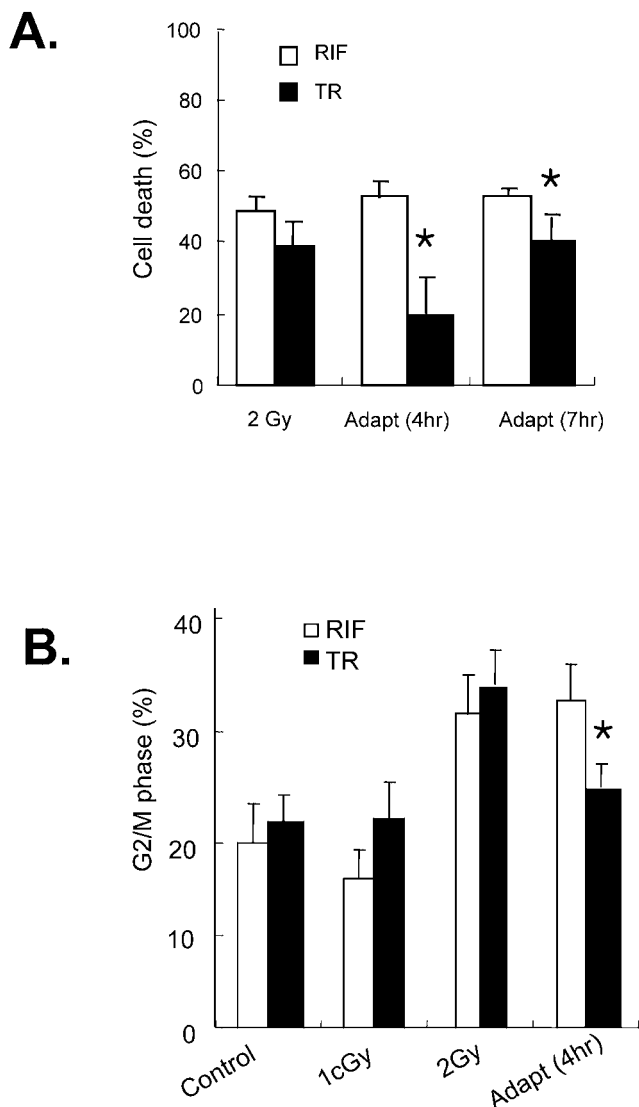
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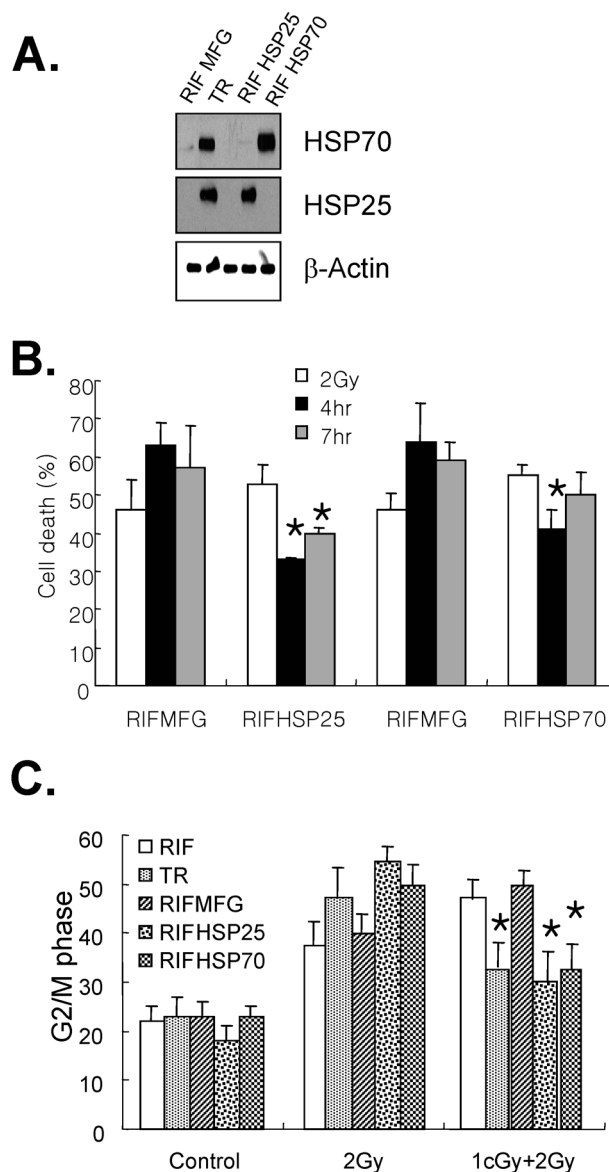
## MATERIALS AND METHODS

### Plasmids

The method of constructing the MFG retroviral vector by replacing the *GFP* sequence of MFG.GFP.IRES.puro was also used to construct MFG-HSP25puro or MFG-hsp70puro. The MFG.GFP.IRES.puro itself was used as a



**Fig. 1.** Induction of adaptive response by low dose radiation. RIF and TR cells were irradiated with 1cGy, and a high challenge dose of 2 Gy was administered with 4 or 7 hrs intervals. **(A)** At 48hrs of high dose radiation, cell death was determined using PI staining. **(B)** At 12 hrs after radiation, flow cytometry analysis was performed after PI staining. Each point represents mean  $\pm$  SD for three independent experiments. \* $P < 0.05$  compared to cells irradiated with 2 Gy alone. Adapt (4hr): cells incubated for 4 hrs between 1cGy and 2Gy. Adapt (7hr): cells incubated for 4 hrs between 1cGy and 2Gy.



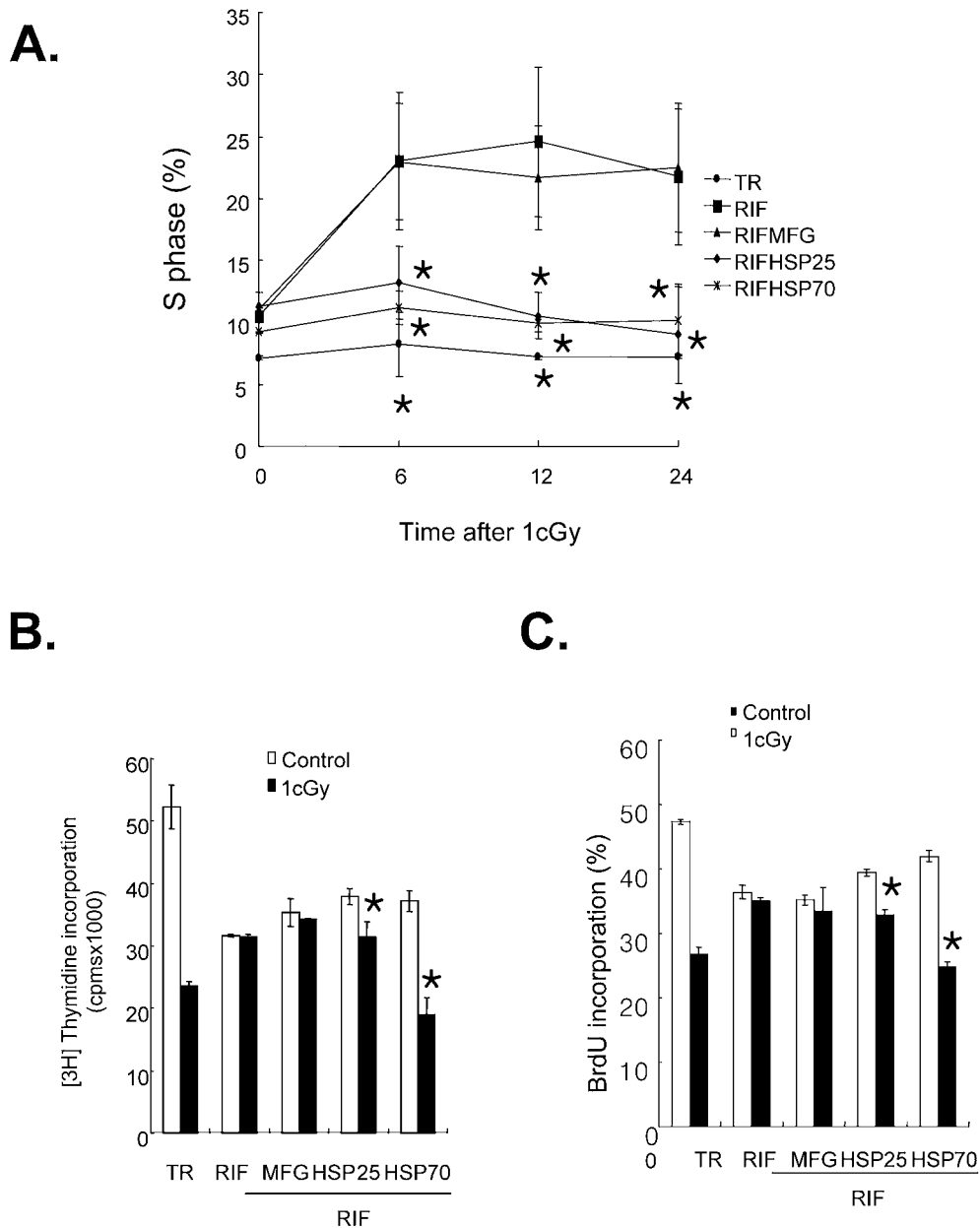
**Fig. 2.** Involvement of HSP25 and inducible HSP70 in adaptive response. **(A)** Protein extracts of TR cells, vector control (MFG) RIF cells and RIF cells with HSP25 and inducible HSP70 transfection were prepared and assessed by Western blot analysis. **(B)** Vector control RIF cells and RIF cells with HSP25 and inducible HSP70 transfection were irradiated with 1cGy, and a high challenge dose of 2 Gy was administered with 4 or 7 hrs intervals. At 48hrs of high dose radiation, cell death was determined using PI staining. **(C)** TR cells, vector control RIF cells and RIF cells with HSP25 and inducible HSP70 transfection were irradiated with 1cGy, and a high challenge dose of 2 Gy was administered with 4 hrs intervals. At 12hrs of high dose radiation, flow cytometry analysis was performed after PI staining. Each point represents mean  $\pm$  SD for three independent experiments. \* $P < 0.05$  compared to cells irradiated with 2 Gy alone. Adapt (4hr): cells incubated for 4 hrs between 1cGy and 2Gy. Adapt (7hr): cells incubated for 4 hrs between 1cGy and 2Gy. Relative inhibition of cell death was shown as % of unirradiated control cells.

negative control throughout the experiment. The retroviral plasmids were introduced into 293pgp retrovirus packaging cell line by transient transfection with Lipofectamine (Gibco/BRL). After 72 hrs, the supernatants were harvested and used for retroviral infection. The virus titers, measured in NIH3T3 cell line by puromycin-resistant colony formation, were between  $10^5$  and  $5 \times 10^5$ /ml. The infection and selection of the target cells by puromycin were performed as

described previously.<sup>18)</sup>

#### Cell culture

RIF (radiation-induced fibrosarcoma cells) and TR (a thermoresistant clone of RIF) which showed wild type p53 status, were cultured in Dulbeccos minimal essential medium (DMEM) (GIBCO, Gaithersburg, MD), supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO)



**Fig. 3.** Increase of low dose radiation mediated G1 arrest by HSP25 and inducible HSP70. (A) Flow cytometric analysis of PI stained cells at indicated time points after 1cGy radiation, (B)  $^3\text{H}$  thymidine incorporation and (C) BrdU incorporation at 24 hrs of 1cGy radiation were performed in TR cells, vector control RIF cells and RIF cells with HSP25 and inducible HSP70 transfection at indicated time points after 1cGy radiation. Each point represents mean  $\pm$  SD for three independent experiments. \* $P < 0.05$  compared to unirradiated control cells.

and antibiotics, at 37°C in a 5% CO<sub>2</sub> humidified incubator.

**Irradiation**

Cells were plated in 3.5, 6, or 10 cm dishes and incubated at 37°C under 5% humidified CO<sub>2</sub>-95% air in culture medium until 70–80% confluent. Cells were then exposed to γ-rays with <sup>137</sup>Cs γ-ray source (Atomic Energy of Canada, Ltd., Canada) with dose rate of 3.81 Gy/min. For low dose irradiation, dose rate of 0.143 cGy/min was used.

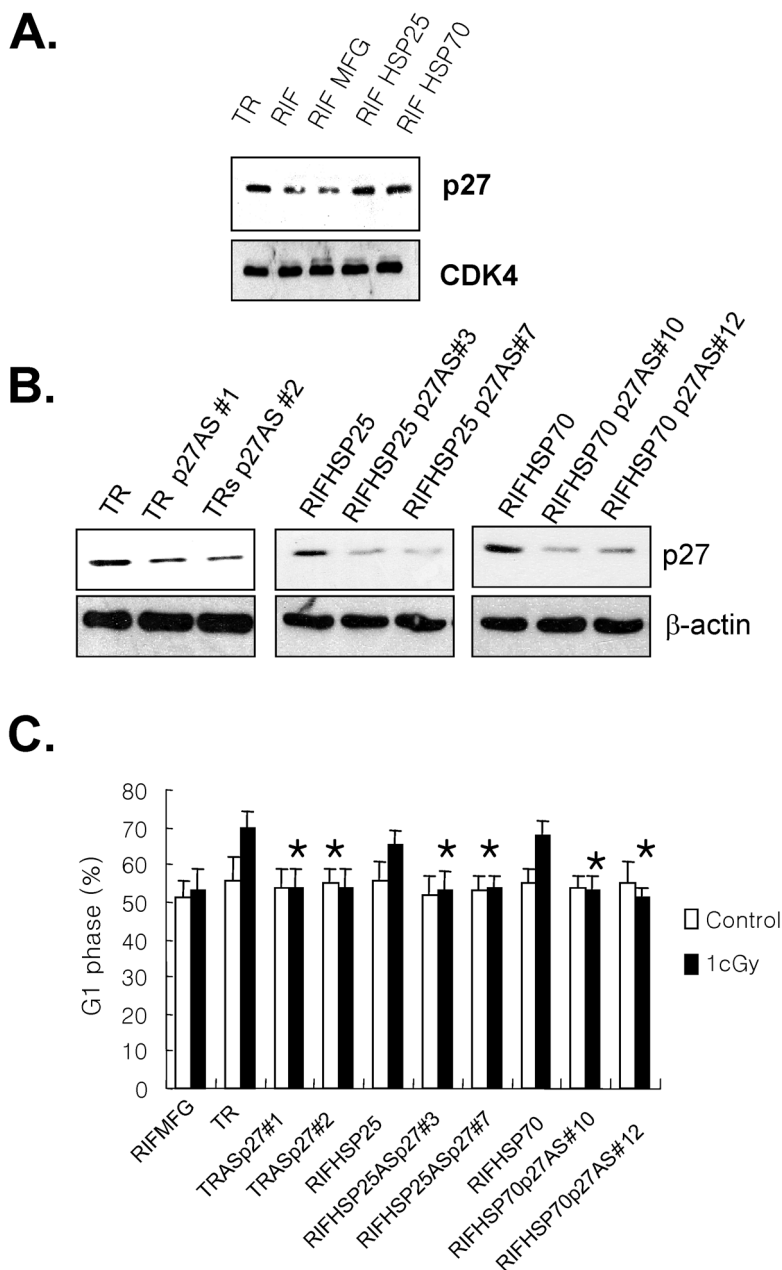
**Cell cycle analysis**

For cell cycle analysis, cells were fixed in 80% ethanol for

at least 18 hrs at 4°C. The fixed cells were then washed once with PBS-EDTA and resuspended in 1 ml of PBS. After the addition of 10 μl each of propidium iodide (PI, 5 mg/ml) and RNase (10 mg/ml), the samples were incubated for 30 min at 37°C and analyzed with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

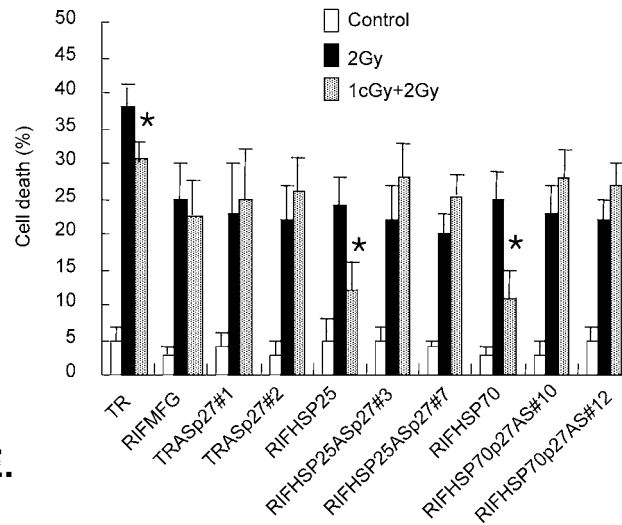
**[<sup>3</sup>H] thymidine incorporation assay**

Cells were plated at confluence of 70–80% in 10 cm diameter petri dishes. After irradiation, the cells were trypsinized, plated in 96 well plates (5 × 10<sup>3</sup> cells/well) and incubated for various periods. The cells were incubated

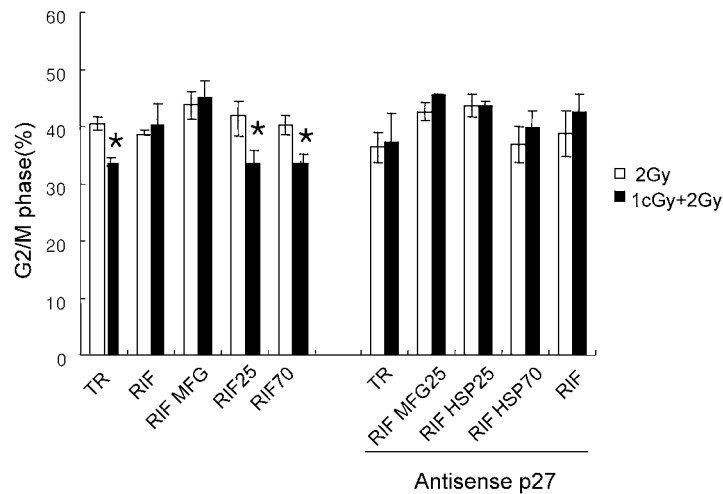


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D.



E.



**Fig. 4.** Increased expression of p27Cip/Kip in HSP25 and inducible HSP70 overexpressed cells. (A, B) Protein extracts of TR cells, vector control (MFG) RIF cells and RIF cells with HSP25 and inducible HSP70 transfection were prepared and assessed by Western blot analysis. Two stable clones after p27Cip/Kip antisense transfection were isolated from TR cells, vector control (MFG) RIF cells and RIF cells with HSP25 and inducible HSP70 transfection, and protein extracts were prepared and assessed by Western blotting analysis. Flow cytometry analysis at 12 hrs of 1cGy radiation was performed (C). Cells were irradiated with 1cGy and a high challenge dose of 2 Gy was administered with 4 or 7 hrs intervals. At 48hrs of high dose radiation, cell death was determined using PI staining (D). At 12 hrs after radiation, flow cytometry analysis was performed after PI staining (E). Each point represents mean  $\pm$  SD for three independent experiments. \* $P < 0.05$  compared to cells irradiated with 2 Gy alone.

with [ $^3$ H] thymidine (37 kB1/well) for 4 hrs before harvest, and the radioactivity of the cells was determined with a scintillation counter (Parkard, TRI-CARB 4530, Meriden, DT).

#### BrdU incorporation assay

BrdU incorporation assay was carried out using a com-

mercially available ELISA kit (Roche, Nutley, NJ) according to manufacturer's instruction.

#### Detection of cell death

Cells were cultured, harvested at indicated times, and stained with propidium iodide (PI), according to manufacturer's protocol, and were then analyzed by a FACScan flow

cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

#### *Polyacrylamide gel electrophoresis and Western blot*

For polyacrylamide gel electrophoresis (PAGE) and Western blot, cells were solubilized with lysis buffer [120 mM NaCl, 40 mM Tris (pH 8.0), 0.1% NP40], the samples were boiled for 5 min, and equal amount of protein (40 µg/well) was analyzed on 10% SDS-PAGE. After electrophoresis, proteins were transferred onto a nitrocellulose membrane and processed for immunoblotting. Blots were further incubated with horseradish peroxidase-conjugated secondary antibody diluted at 1:5,000, and specific bands were visualized by chemiluminescence (ECL, Amersham International, Uppsala, Sweden). Autoradiographs were recorded onto X-Omat AR films (Eastman Kodak Co., Rochester, NY, USA).

## RESULTS

#### *Induction of adaptive response by low dose radiation*

When cells were preirradiated with 1cGy before a high challenging dose of radiation (2 Gy), cell death induced by high dose radiation was significantly inhibited in TR cells, but not in the parental RIF cells (Fig. 1A). When radiation-induced G2/M phase arrest was examined, 2Gy radiation induced 32–37% G2/M phase arrest, which is a typical radiation induced cell cycle arrest. However, low dose preirradiated cells with 4 hrs interval between low and high challenging irradiation significantly reduced this G2/M phase arrest to 25% in TR cells, but not in RIF cells (Fig. 1B). The fact that the expression of hsp25 and inducible hsp70 has been shown to be different in these two cell lines led us to speculate that hsp25 and inducible hsp70 might be involved in the induction of adaptive response such as reduction of cell death and G2/M arrest induced by high challenging dose radiation

#### *Involvement of hsp25 and inducible hsp70 in adaptive response*

Since expressions of hsp25 and inducible hsp70 were increased in TR cells, hsp25 or inducible hsp70 was transfected to RIF cells (Fig. 2A) and a question of whether there was any link between hsps and the induction of an adaptive response was examined. Cell death data revealed that hsp25 or inducible hsp70 overexpression acquired adaptive response, whereas their parent RIF cells did not show an adaptive response (Fig. 2B): Pretreatment of 1cGy radiation attenuated cell death by high dose radiation in hsp25 or inducible hsp70 transfected cells. Increased induction of G2/M arrest at 6 hrs after 2Gy high dose radiation was significantly reduced by priming of 1cGy radiation when time interval between low and high dose was 4 hrs (Fig. 2C). From the data, hsp25 and inducible hsp70 appear to be involved in the induction of adaptive response.

#### *Increased G1 arrest by low dose radiation in hsp25 and inducible hsp70 overexpressed cells*

To examine mechanisms involved in the induction of adaptive response, cell cycle distribution was studied after low dose irradiation (1cGy). As seen in Fig 3A, dramatically reduced G1 phase from 6 hrs after low dose radiation was observed in control and control vector (RIFMFG) alone transfected cells, while no difference was observed in hsp25 and inducible hsp70 overexpressed cells as well as TR cells. [<sup>3</sup>H]-thymidine and bromodeoxyuridine (BrdU) incorporation data also revealed that control cells and vector alone transfected cells did not show any significant change of DNA synthesis by low dose radiation, whereas reduced DNA synthesis (Fig. 3B and 3C) was observed in the hsp25 and inducible hsp70 overexpressed cells, suggesting increased G1 phase arrest by 1cGy radiation in hsp25 and inducible hsp70 overexpressed cells.

#### *Increased expression of p27Cip/Kip in hsp25 and inducible hsp70 overexpressed cells and involvement of adaptive response*

To elucidate the mechanisms of G1 phase arrest by 1 cGy involved in hsp25 and inducible hsp70 overexpressed cells and whether G1 phase arrest was related to the induction of adaptive response, we examined expression levels of proteins which are related to G1 arrest in cell cycle regulation. Figure 4A shows that the expression of p27Cip/Kip was increased by hsp25 and inducible hsp70, while the expression of p21Waf protein did not change (data not shown). Next, in order to ascertain direct involvement of p27Cip/Kip in G1 phase arrest by low dose radiation, hsp25 and inducible hsp70 overexpressed cells as well as TR cells, were treated with antisense of p27Cip/Kip, and 2 clones each whose expression of p27Cip/Kip was downregulated (Fig. 4B) were isolated. As shown in Fig. 4C, low dose radiation-mediated G1 phase arrest was abolished by antisense p27Cip/Kip treatment in hsp25 and inducible hsp70 overexpressed cells. The induction of adaptive response by low dose radiation was determined. As shown in Fig. 4D, the reduction of high dose radiation-induced cell death by low dose preirradiation in hsp25 or inducible hsp70 transfected cells was restored to the control cell level by cotransfection of p27Cip/Kip antisense. Moreover, the inhibition of high dose radiation-induced G2/M phase arrest by low dose pre-treatment was also restored by antisense p27Cip/Kip treatment (Fig. 4E). The data, therefore, indicate that increased expression of p27Cip/Kip is responsible for the induction of adaptive response, and that p27Cip/Kip mediated G1 arrest is related to the induction of adaptive response by hsp25 and inducible hsp70.

## DISCUSSION

Adaptive response to ionizing radiation is a phenomenon

by which the harmful effects of a high dose exposure to ionizing radiation can be mitigated, when cells were first exposed to a low dose of radiation. This radioresistance can occur even after radiation with doses as low as 0.5–10 cGy, and it requires 3–4 hrs for full induction.<sup>16)</sup> The radioadaptive response was first described by Olivieri *et al* in 1984<sup>19)</sup> in cultured human lymphocytes and was later confirmed by others in a wide variety of animal and plant cells. It has been characterized as follows:<sup>1)</sup> 1) The adaptation is a rapid process, being fully expressed 4–6 hrs after irradiation and persists for more than 20 hrs.<sup>20)</sup> 2) It has a dose limitation below ~0.1 Gy for an optimal expression. 3) In some systems, higher doses are incapable of inducing adaptation and rapidly destroy an adapted state that was previously induced by lower doses.<sup>3)</sup> In other systems, relatively high doses delivered at a low dose rate induced an adaptive response.<sup>21)</sup> However, the molecular mechanisms and signaling pathways involved in the regulation of such a response remain unknown. The present study demonstrated that hsp25 and inducible hsp70 were responsible for the induction of an adaptive response, and that increased G1 phase arrest by high expression of p27Cip/Kip in hsp25 and inducible hsp70 overexpressed cells may be a key modulator of these phenomena.

RIF cells, in which hsp25 and inducible hsp70 are not expressed, did not exhibit an adaptive response to low-dose preirradiation with 1 cGy, while its thermoresistant TR cells, which express hsp25 and inducible hsp70, did (Fig. 1). When hsp25 and inducible hsp70 were transfected to RIF cells (Fig. 2A), the cells acquired inhibition of cell death (Fig. 2B), and decreased radiation-induced G2/M phase arrest by low dose preirradiation (Fig. 2C), suggesting that the expression of both hsp25 and inducible hsp70 is important for the induction of the adaptive response. When we examined the mechanisms of adaptive response by low dose radiation, the cell population at G2/M phase was found to be reduced by low dose preirradiated cells with 4 hrs interval between low and high challenging irradiation. Since G2/M phase arrest is a typical radiation induced cell cycle arrest and G2/M arrested cells usually go to cell death,<sup>22)</sup> reduced G2/M phase arrest by low dose radiation might be the key mechanism of adaptive response by hsp25 and inducible hsp70.

To examine the role of 1 cGy radiation in hsp25 and inducible hsp70 overexpressed cells, cell cycle distribution was determined. Significantly increased G1 phase arrest was observed in hsp25 and inducible hsp70 overexpressed cells (Fig. 3A), and [<sup>3</sup>H]-thymidine and BrdU incorporation data also indicated reduced DNA synthesis in these cells (Fig. 3B and 3C), suggesting that 1 cGy radiation preferentially affected more hsp25 and inducible hsp70 overexpressed cells which resulted in increased G1 phase arrest by low dose radiation. Since the expression of p27Cip/Kip was increased in hsp25 and inducible hsp70 overexpressed cells (Fig. 4A) and p27Cip/Kip is an inhibitor of broad range of

cell cycle, the role of p27Cip/Kip in the induction of adaptive response was examined. When p27Cip/Kip antisense was transfected and 2 stable clones were examined (Fig. 4B), increased G1 phase by hsp25 and inducible hsp70 found to be abrogated (Fig. 4C). In our experiment, the expression of p21Waf, another broad spectrum inhibitor of cell cycle, was not altered by hsp25 and inducible hsp70 overexpression. In addition, the induction of adaptive response by hsp25 and inducible hsp70 was abolished by cotransfection of p27Cip/Kip antisense, indicating that increased expression of p27Cip/Kip by hsp25 and inducible hsp70 is responsible for the adaptive response (Fig. 3D). Moreover, the reduction of radiation-induced G2/M phase arrest by hsp25 and inducible hsp70 overexpressed cells was restored by antisense cotransfection of p27Cip/Kip (Fig. 4). We do not exactly know how increased G1 arrest by p27Cip/Kip is involved in reduced G2/M phase arrest by high dose radiation in hsp25 or inducible hsp70 overexpressed cells, when they were pretreated with 1 cGy. One possibility is that increased G1 arrest might affect G2/M phase arrest.

Cell cycle progression depends on the activity of a series of cyclin dependent kinase (CDK) complexes.<sup>26)</sup> The activity of the CDKs depends on the phosphorylation state, binding of cyclins, and the presence of CDK inhibitors (CDKIs).<sup>24,25)</sup> At least, two families of CKDIs are able to modulate CDK activity during G1/S phase transition, the p27Cip/Kip family and the INK4 family<sup>26,27)</sup>. p27Cip/Kip family are able to bind and regulate cyclin A, D, and E-dependent kinases,<sup>26,27)</sup> and p27Cip/Kip is directly involved in cell cycle restriction point control. Various antimetabolites such as radiation induce p27Cip/Kip protein, allowing it to associate with and inhibit the cyclin E/CDK complex.<sup>28)</sup> p27Cip/Kip knockout mice exhibit multiple organ hyperplasia and have 2-fold larger testes than their wild-type littermates.<sup>29,30)</sup> Furthermore, a higher pituitary tumor incidence was reported in p27Cip/Kip knockout mice,<sup>31,32)</sup> indicating that the loss of p27Cip/Kip may contribute to oncogenesis and tumor progression. Our present data demonstrated that induction of adaptive response in cells carrying hsp25 and inducible hsp70 is due to an increase of p27CIP/Kip through reduction of apoptosis and G2/M phase arrest. However, further study will be needed to elucidate the mechanism of how hsp25 and inducible hsp70 regulate p27Cip/Kip.

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