Downregulation of ERK2 is essential for the inhibition of radiation-induced cell death in HSP25 overexpressed L929 cells

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Abstract

We previously reported that overexpression of HSP25 delayed cell growth, increased the level of p21^waf, reduced the levels of cyclin D1, cyclin A and cdc2, and induced radioresistance in L929 cells. In this study, we demonstrated that HSP25 induced-radioresistance was abolished by transfection with plasmids containing antisense *hsp25* cDNA. Extracellular regulated kinase (ERK) and MAP kinase/ERK kinase (MEK) expressions as well as their activation (phospho-forms) were inhibited by *hsp25* overexpression. Furthermore, when control vector transfected cells were treated with PD98059, MEK inhibitor, they became resistant to radiation, suggesting that inhibition of ERK1/2 activities was essential for radioresistance in L929 cells. To confirm the relationship between ERK1/2 and *hsp25*-mediated radioresistance, ERK1 or ERK2 cDNA was transiently transfected into the *hsp25* overexpressed cells and their radioresistance was examined. HSP25-mediated radioresistance was abolished by overexpression of ERK2, but not by overexpression of ERK1. Alteration of cell cycle distribution and cell cycle related protein expressions (cyclin D, cyclin A and cdc2) by *hsp25* overexpression were also recovered by ERK2 cDNA transfection. Increase in Bcl-2 protein by *hsp25* gene transfection was also reduced by subsequent ERK2 cDNA-transfection. Taken together, these results suggest that downregulation of ERK2 is essential for the inhibition of radiation-induced cell death in *HSP25* overexpressed cells.
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Introduction

Cellular response to biological stresses is to produce heat shock proteins (HSP). HSPs are divided into high-molecular weight and low-molecular weight HSPs according to their apparent molecular sizes. It is recognized that low molecular weight HSPs such as HSP25, HSP27, and αB-crystallin act as chaperones,[1] and that HSP27 protein participates in mediating physiological processes other than the stress response, including cellular differentiation and regulation of apoptosis.[2] We earlier reported that over-expression of *hsp25* gene conferred radioresistance and induced growth delay in L929 cells, and these alterations were probably mediated by inhibiting expressions of cyclin D1, A, and Cdc2, and increasing Bcl-2 expression,[3] thus leading us to the conclusion that the intracellular signal transduction pathways associated with cell growth were altered by HSP25 overexpression. This alteration might have resulted in cell growth delay and radioresistance.

The mitogen-activated protein kinases (MAP kinases: ERK1 and ERK2) are common intermediates in intracellular signaling cascades involved in diverse cellular functions, including growth and differentiation.[4,5] The regulatory network controlling MAP kinase in mammalian cells is complex and consists of at least two pathways which converge just upstream of MAP kinase at MAP kinase kinase (MEK). One pathway involves the c-*raf* proto-oncogene, while the other appears to be linked to serpentine receptors via G protein and protein kinase C (PKC).[6] It is generally accepted that the activation of the ERK cascade leads to cell proliferation.[7,8] However, recent investigations have attempted to clarify situations in a number of cells where ERK mediates cell cycle arrest,[9] antiproliferation,[10] apoptotic[11] and nonapoptotic death.[12] Although it still remains unknown how the MAP kinase pathway affects cellular survival or death in response to ionizing radiation, our previous observations suggest that the MAP kinase cascade is important for cellular response to ionizing radiation. In the present paper, we observed that the HSP25 overexpressed cells downregulated ERK1/ 2 expression and that subsequent transfection of ERK2 cDNA, but not ERK1 cDNA, into the *hsp25* gene transfected cells eliminated HSP25 mediated-radioresistance and cell growth delay.

Results

Overexpression of hsp25 induced radioresistance

To investigate relationship between HSP25 and radioresistance, we performed a clonogenic survival assay. As shown in Figure 1A, *hsp25*-transfected cells (clone #8)

(A)

(B)

(C)
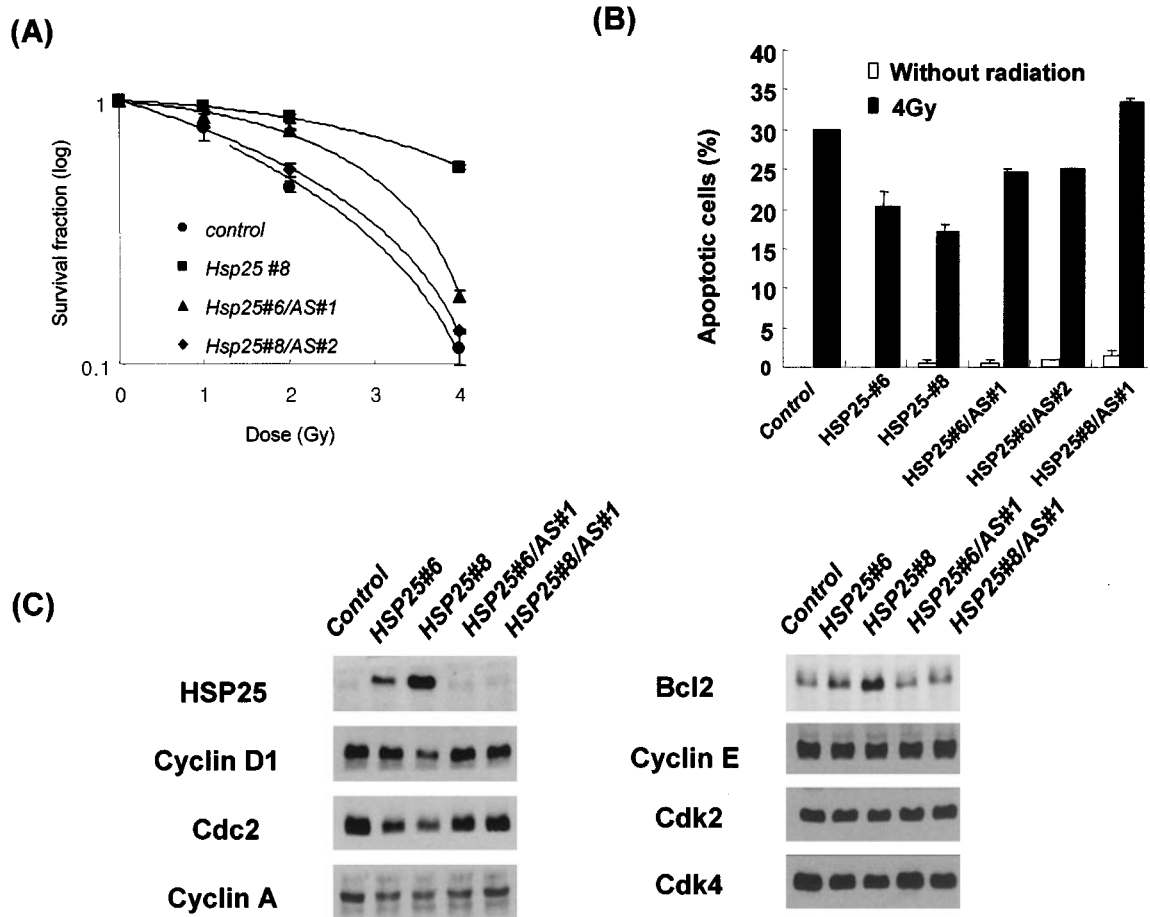
Figure 1 Overexpression of hsp25 induced radioresistance. (**A**) Surviving fraction of control vector-transfected cells (Control) and *hsp25* gene-transfected cells (HSP25#8) with or without subsequent *hsp25* cDNA antisense (AS) transfection (HSP25#6/AS#1 and HSP25#8/AS#1) were obtained by colony-forming assay after irradiation. (**B**) DNA fragmentation was measured by Hoechst 33258 staining 48 h after 4 Gy irradiation. Error bar indicates mean ± S.D. from three independent experiments. (**C**) Protein extracts were prepared, separated by SDS–PAGE, and analyzed by Western blot

showed an increased clonogenicity against ionizing radiation-induced cytotoxicity. In order to appreciate the mechanism of HSP25-induced radioresistance, we examined whether overexpression of *hsp25* inhibited radiation-induced apoptotic cell death, by assaying internucleosomal fragments of apoptotic cells at 48 h after irradiation. As seen in Figure 1B, γ-rays induced an intense fragmentation of DNA in vector control cells, while overexpression of *hsp25* (clones #6 and 8) drastically inhibited this apoptotic death. Moreover, subsequent transfection with antisense *hsp25* cDNA abrogated *hsp25*-induced radioresistance (Figure 1A,B). Furthermore, HSP25-mediated altered expressions of cyclin D1, -A, Cdc2, and Bcl-2 were also restored to the levels of control vector cells after transfection with antisense *hsp25* cDNA (Figure 1C).

HSP25 overexpression downregulated ERK1/2 expression

Since we observed earlier that overexpression of HSP25 induced cell growth delay,[3] we examined the upstream

pathways of cell cycle regulation, particularly focusing on the MAP kinase pathway. As shown in Figure 2A,B, both ERK1 and 2 expressions and their kinase activities, with MBP or PHAS-1 as a substrate were dramatically decreased in the *hsp25*-transfected cells. When *hsp25* cDNA was overexpressed into RIF (Radiation Induced Fibrosarcoma) line, similar effects were obtained (Figure 1A). Transient transfection of *hsp25* also decreased ERK1/2 expression (Figure 2C). However, subsequent transfection with antisense *hsp25* cDNA recovered ERK1/2 expression to the level of control vector cells. Upstream protein expressions of ERK1/2 and MEK1/2 were also inhibited by *hsp25* overexpression and subsequent transfection with antisense *hsp25* cDNA restored these protein expressions. These data suggested that HSP25 overexpression downregulated ERK1/2 expression. In addition, when treated with 4 Gy radiation, ERK1/2 activation occurred in the control cells, while this activation was attenuated in the *hsp25*-transfected cells (Figure 2D). Other MAP kinases such as p38 and JNK expressions were not changed by *hsp25* overexpression (data not shown).
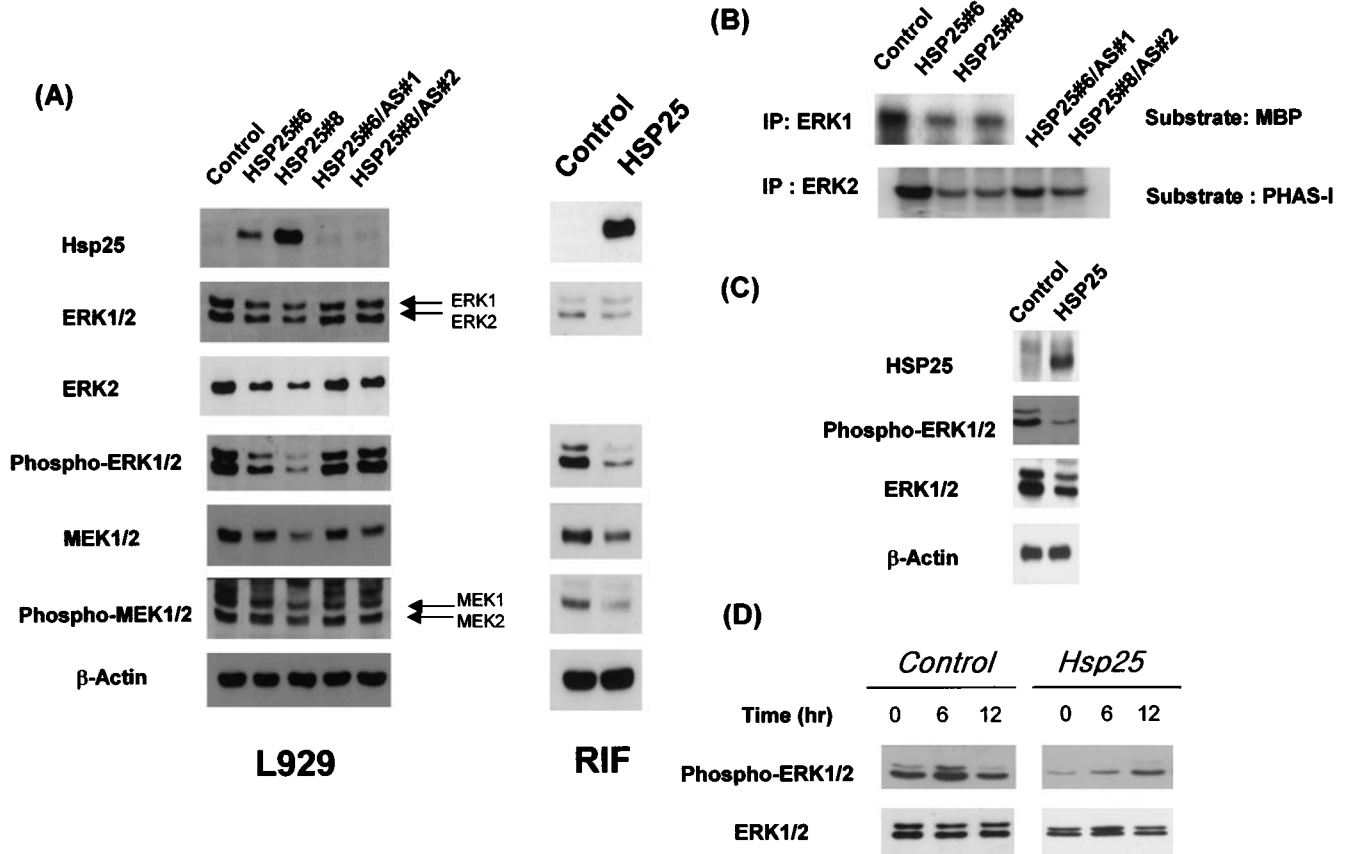
Figure 2 HSP25 overexpression downregulated ERK1/2 expression. (**A**) Protein extracts (60 μg) were prepared from control vector- (Control) and *hsp25*-transfected (HSP25#6 and HSP25#8) cells with or without subsequent *hsp25*-antisense (AS) transfection (HSP25#6/AS#1 and HSP25#8/AS#2), separated by SDS–PAGE, and analyzed by Western blotting. (**B**) ERK activity in cell lysates was measured by phosphorylation of phosphorylated heat and acid stable protein (PHAS-1), a substrate for ERK-2, and myelin based protein (MBP), a substrate for ERK-1. (**C**) Transient transfection of control vector and *hsp25* vector by lipofection into L929 cells was performed. Protein extracts (60 μg) were prepared, separated by SDS–PAGE, and analyzed by Western blotting. (**D**) Protein extracts (60 μg) were prepared from control vector- (Control) and *hsp25*-transfected (HSP25#8) cells which were harvested at 6 h after 4 Gy radiation, separated by SDS–PAGE, and analyzed by Western blotting

Half life of ERK1/2 Proteins was decreased by HSP25 overexpression

To determine whether the HSP25 overexpression affected *de novo* synthesis of ERK1/2 proteins, we assessed the protein expression in the presence of 10 μg/ml of cycloheximide (CHX), a potent inhibitor of protein synthesis. As seen in Figure 3A, the HSP25-overexpressed cells decreased steady-state levels of ERK1/2 protein compared with control cells. Northern blot analysis of the basal level of ERK2 mRNA detected no difference (Figure 3B), suggesting that ERK1/2 protein turnover was affected by HSP25 overexpression.

Inhibition of ERK1/2 activity by PD98059 induced radioresistance in L929 cells

To examine whether inhibition of ERK1/2 activity was responsible for radioresistance, PD98059, a MEK inhibitor, was treated. Irradiation activated ERK1/2 in the control vector transfected cells, while it did not in the HSP25 transfectant cells (Figure 4A). PD98059 induced radioresistance in both

cells, as evidenced by clonogenic survival assay or induction of apoptosis, and the effect was greater in the control vector cells (Figure 4B). These results suggested that inhibition of ERK1/2 activities were essential for radioresistance in L929 cells.

Inhibition of ERK2 but not of ERK1 expression was essential for the hsp25-mediated radioresistance

To test relationship between the level of ERK1/2 and *hsp25*-mediated radioresistance, ERK1 or ERK2 cDNA was transfected into the *hsp25* overexpressed cells and radioresistance was then examined. Using specific antibody for ERK1 or ERK2, increased level of ERK1 or ERK2 proteins as well as their activation (phospho-ERK1/2) were detected in the *hsp25*-transfected cells with no alteration of HSP25 expression (Figure 5A). When clonogenic survival and apoptosis assay were performed, ERK2 but not ERK1 transfected cells exhibited the inhibition of *hsp25*-mediated radioresistance (Figure 5B,C), indicating that ERK2 inhibition was essential for *hsp25*-mediated radioresistance.

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(B)
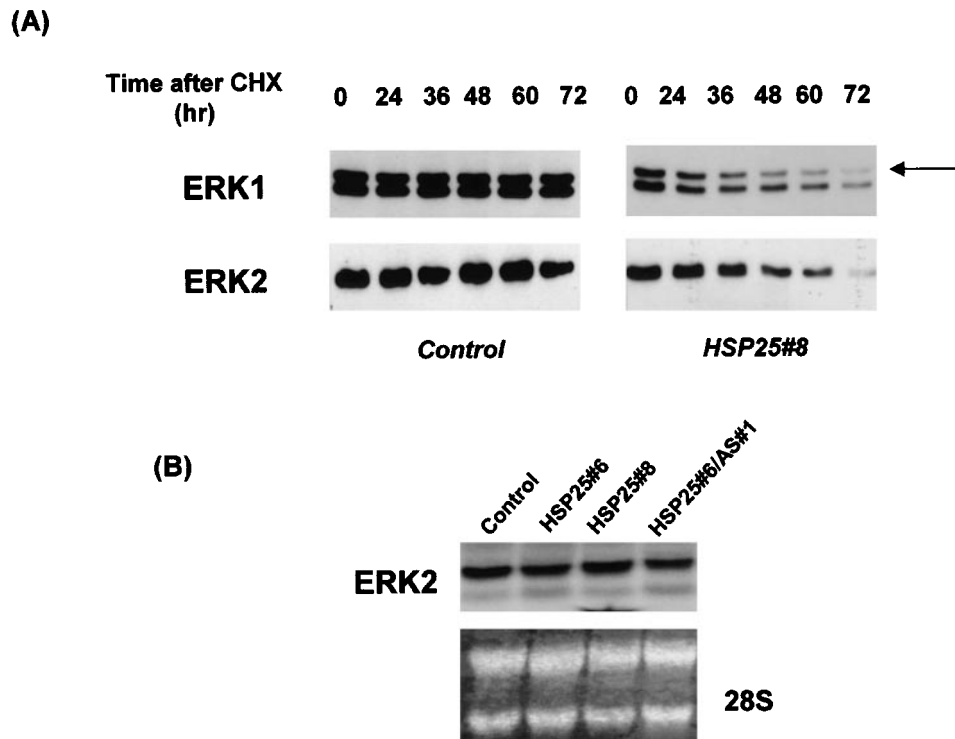
Figure 3 Half life of ERK1/2 proteins was decreased by HSP25 overexpression. (**A**) Cells were treated with cycloheximide (CHX, 10 μg/ml) for 24 h, and protein extracts (60 μg) were prepared at indicated times from control vector- (Control) and *hsp25*-transfected (HSP25#8) cells, separated by SDS–PAGE, and analyzed by Western blotting. (**B**) Northern blotting analysis of ERK in control vector- (Control) and *hsp25*-transfected (HSP25#6 and HSP25#8) cells with or without subsequent *hsp25*-antisense (AS) transfection (HSP25#6/AS#1)

HSP25 mediated growth delay was abolished by ERK2 transfection

Since HSP25 overexpression resulted in growth delay and this growth delay was partially responsible for the HSP25-mediated radioresistance, the growth curve was examined after ERK-2 transfection. As shown in Figure 6A, growth delay by HSP25 overexpression was restored to the level of control cells after transfection with ERK-2 cDNA, however, no change was detected when ERK-1 cDNA was transfected. To examine whether the restoration of cell growth by ERK-2 cDNA transfection was due to alteration of cell cycle, cell cycle was analyzed by flow cytometry. For this study, 400 μM mimosine, known to reversibly block cells at the G1/S boundary, was employed. Late G1 arrest was induced in both control and *hsp25*-transfected cells by 20 h of drug treatment. A 10 h after washing mimosine off, little more than half of the HSP25 plus ERK-2 transfected cells had already passed G2/M phase, but HSP25 alone and HSP25 plus ERK-1 transfected cells did not yet pass (Figure 6B). These data further indicated that the alteration of cell cycle by HSP25 was restored to control cell level by subsequent transfection with ERK-2 cDNA. Altered distribution of cell cycle by HSP25 overexpression was also restored by ERK-2 transfection (Figure 6C): Increased G1 peak by HSP25 overexpression was also diminished by ERK-2 cDNA transfection. A question

of whether the restoration of cell growth induced by ERK-2 cDNA transfection was due to restored expression of cell cycle related proteins was analyzed by Western blot and the result indicated that reduced basal levels of Cdc2, cyclin D1 and cyclin A proteins by HSP25 overexpression was restored by ERK-2 cDNA transfection, but not ERK-1 cDNA transfection (Figure 6D). In addition, increased induction of Bcl-2 by *HSP25* overexpression was also reduced to the control level.

Discussion

It is generally accepted that the activation of the ERK cascade leads to mitogenic effect.[7,8] We earlier observed that HSP25-mediated radioresistance was correlated with growth inhibition and increased induction of Bcl-2 protein.[3] Subsequent transfection with antisense *hsp25* cDNA abrogated *hsp25*-induced radioresistance. Simultaneously, HSP25-mediated altered expressions of cyclin D1, -A, Cdc2, and Bcl-2 were also restored to the levels of control vector cells by transfection with antisense *hsp25* cDNA (Figure 1), suggesting that HSP25 induced radioresistance through cell cycle regulation and Bcl-2 induction. We also observed decreased expressions of ERK-1 and -2 along with inactivation of ERK1/2 (decrease in the level of phospho-ERK/2 proteins) in the *hsp25*-overexpressed cells with no alteration of other MAP kinase expressions such as

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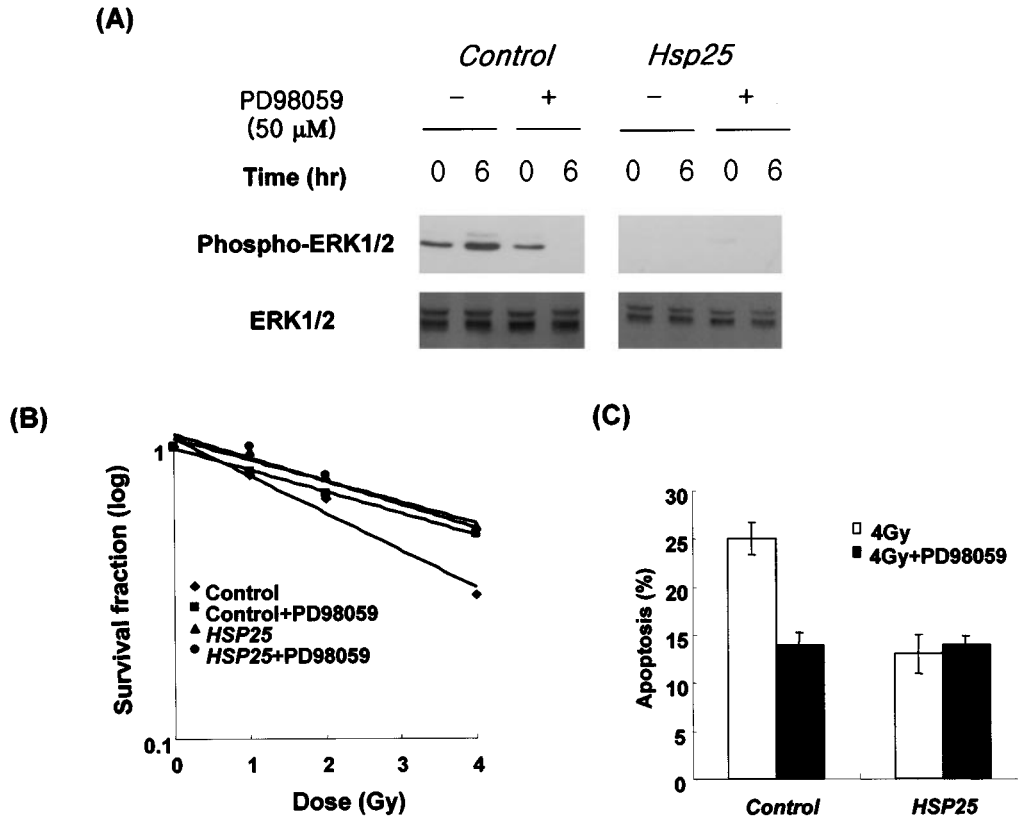
Figure 4 Inhibition of ERK1/2 activity by treatment with PD98059 induced radioresistance. Control vector-(Control) or *hsp25*-transfected (HSP25) cells were pretreated with 50 μM PD98059 for 2 h, followed by irradiation with 4 Gy. (**A**) At indicated times, protein extracts (60 μg) were separated by SDS–PAGE, and analyzed by Western blotting. (**B**) Relative viability was obtained by colony-forming assay. (**C**) DNA fragmentation was measured by Hoechst 33258 staining 48 h after irradiation. Error bar indicates mean $\pm$ S.D. from three independent experiments

c-Jun NH$_2$-terminal kinases (JNK, also called SAPK) and the p38. Measurement of kinase activity of ERK-1 and -2 also confirmed our observations (Figure 2). In addition, radiation-induced ERK1/2 activation was also inhibited by HSP25 overexpression (Figure 4). These results strongly indicated that the downregulation of ERK1/2, in particular ERK2 gene expression might relate to the HSP25-mediated radioresistance.

In an attempt to elucidate the mechanism of ERK1/2 protein regulation by HSP25 overexpression, we measured steady-state levels of ERK1/2 proteins by treatment with CHX. The steady-state levels of ERK1/2 proteins were rapidly decreased in the HSP25 overexpressed cells, indicating that HSP25 altered the half-life of ERK1/2 proteins without altering mRNA levels of these genes (Figure 3). We cannot offer any explanation on how HSP25 regulates the half-life of ERK1/2 protein. There exists a possibility that protein degradation system might have been altered by HSP25 overexpression, because similar observation on the half-life of cyclin D1 protein which was downregulated by *HSP25* overexpression (data not shown). The other possibility is a reactive oxygen species (ROS) system: Our preliminary data revealed that HSP25-induced inhibition of ERK1/2 expressions might have been mediated through Ras-Raf signal transduction pathway, but not epidermal growth factor receptor (EGFR) or protein kinase C (PKC)

pathways (unpublished data). NF-κB mediated increase in manganese superoxide (MnSOD) expression, which is free radical scavenging enzyme that defends cells from oxidant stress by distorting superoxide anion (O$_2$$^-$) and reduces ROS, was also observed in the HSP25 overexpressed cells (data not shown). Therefore, it is quite possible that ROS in the HSP25-overexpressed cells resulted in HSP25-mediated ERK1/2 downregulation. Since Ras is critical for ERK1/2 activation by ROS,[13,14] reduction of ROS level may be responsible for the downregulation of ERK1/2. Recently, it has been shown that ROS stimulate intracellular signal events such as c-Src, Ras, and ERK1/2.[15,16] and Guyton *et al.*[17] also showed that H$_2$O$_2$-stimulated ROS activated ERK2 in PC12 cells.

As shown in Figure 4, MEK inhibitor PD98059, which consequently inhibited ERK1/2 activation, induced radioresistance in L929 cells. In particular, reduction of ERK2 level and inhibition of its activation might have been responsible for HSP25-mediated radioresistance (Figure 4). These observations were somewhat surprising, because ERK pathway is known to be critical in the control of cellular growth and cell survival responses to mitogenic signals in many different cell systems, including those with tyrosine kinase, G protein-coupled and cytokine receptors.[18,19] Many studies support the general view that activation of the ERK pathway delivers a survival signal which counteracts proapoptotic effects
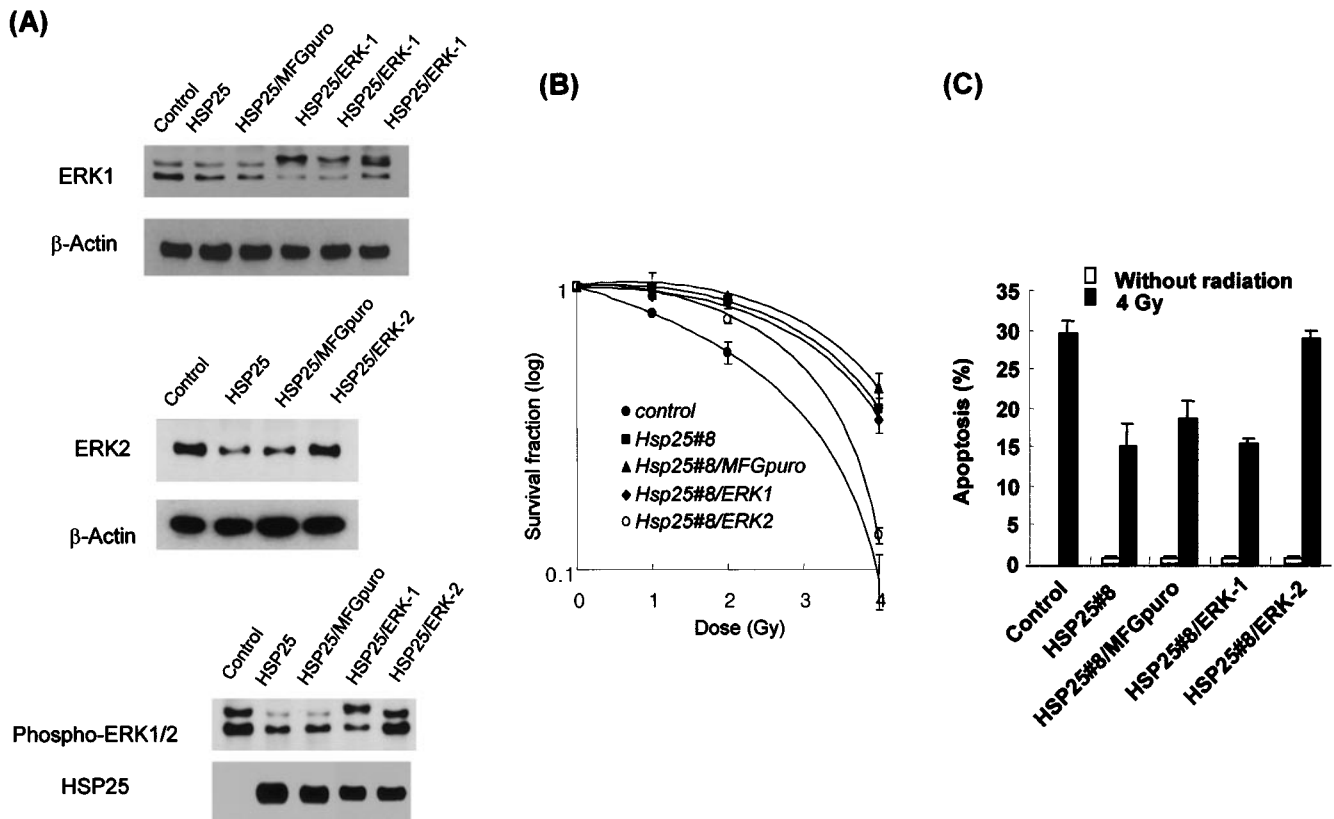
Figure 5 Subsequent transfection of ERK-2 but not ERK-1 cDNA to hsp25-transfected cells restored altered HSP25-mediated cell cycle. (**A**) Control vector- (Control) and hsp25-transfected (HSP25) cells with or without subsequent transient transfection of vector control (HSP25#8/MFGpuro), ERK-1 (HSP25/ERK-1), or ERK-2 (HSP25/ERK-2) were harvested. Protein extracts ($60\,\mu g$) were separated by SDS–PAGE and analyzed by Western blotting. (**B**) Surviving fraction was obtained by colony-forming assay after irradiation. (**C**) DNA fragmentation was measured by Hoechst 33258 staining 48 h after 4 Gy irradiation, as described in the Materials and Methods. Error bar indicates mean $\pm$ S.D. from three independent experiments

elicited by JNK and the p38 activation.[20] However, requirement of ERK in mediating cisplatin-induced apoptosis of human cervical carcinoma HeLa cells and ovarian cell lines[21,22] has also been demonstrated. Moreover, persistent activation of ERK1/2 contributes to glutamate-induced oxidative toxicity.[23] In the present study, we also provided evidence that ERK-2 cDNA transfection which affected cell growth, Bcl-2 induction, and finally radioresistance induction (Figures 5 and 6) abolished radiation-induced cell death in the HSP25 overexpressing cells.

In this paper, we described possible involvement of ERK2 in the development of radioresistance in the HSP25-overexpressed L929 cells. Our proposed model shown in Figure 7 might provide important insight in understanding how HSP25 induces radioresistance. This model would also provide a guideline to further in-depth study on the mechanism of radioresistance induction by HSP25.

Materials and Methods

Cell culture

Murine L929 cells were cultured in Dulbecco's minimal essential medium (DMEM) (GIBCO, Gaithersburg, MD, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS, GIBCO) and antibiotics at 37°C in a humidified incubator with a mixture of 95% air and 5% CO_2. L929 transformant clones were obtained from stable transfection with phsp6 (containing the complete genomic sequence for murine hsp25) and pBC vector (Stratagene, La Jolla, CA, USA).[3,24] Hsp25 transfectants were subsequently transfected with plasmids containing antisense hsp25 cDNA which was kindly provided by Dr Davidson,[25] and stable transfectants were selected with 6 μg/ml puromycin for approximately 2 weeks, followed by continued growth in the presence of 4 μg/ml puromycin. Clones with downregulated HSP25 protein detected by Western blotting, were selected. Plasmid pcDNA3 with ERK1 or -2[26] was transiently transfected to HSP25 overexpressed cells using lipofection.

Irradiation

Cells were exposed to γ-rays with ^{137}Cs γ-ray source (Atomic Energy of Canada, Ltd., Ontario, Canada) with dose rates of 3.81 Gy/min.

Colony-forming assay

Clonogenicity was compared, as described previously,[3,27] by using a colony-forming assay. Cells were seeded into 6 cm Petri dishes at densities to produce approximately 500 colonies per dish in control, and were incubated for 7–14 days. Colonies were fixed (75%

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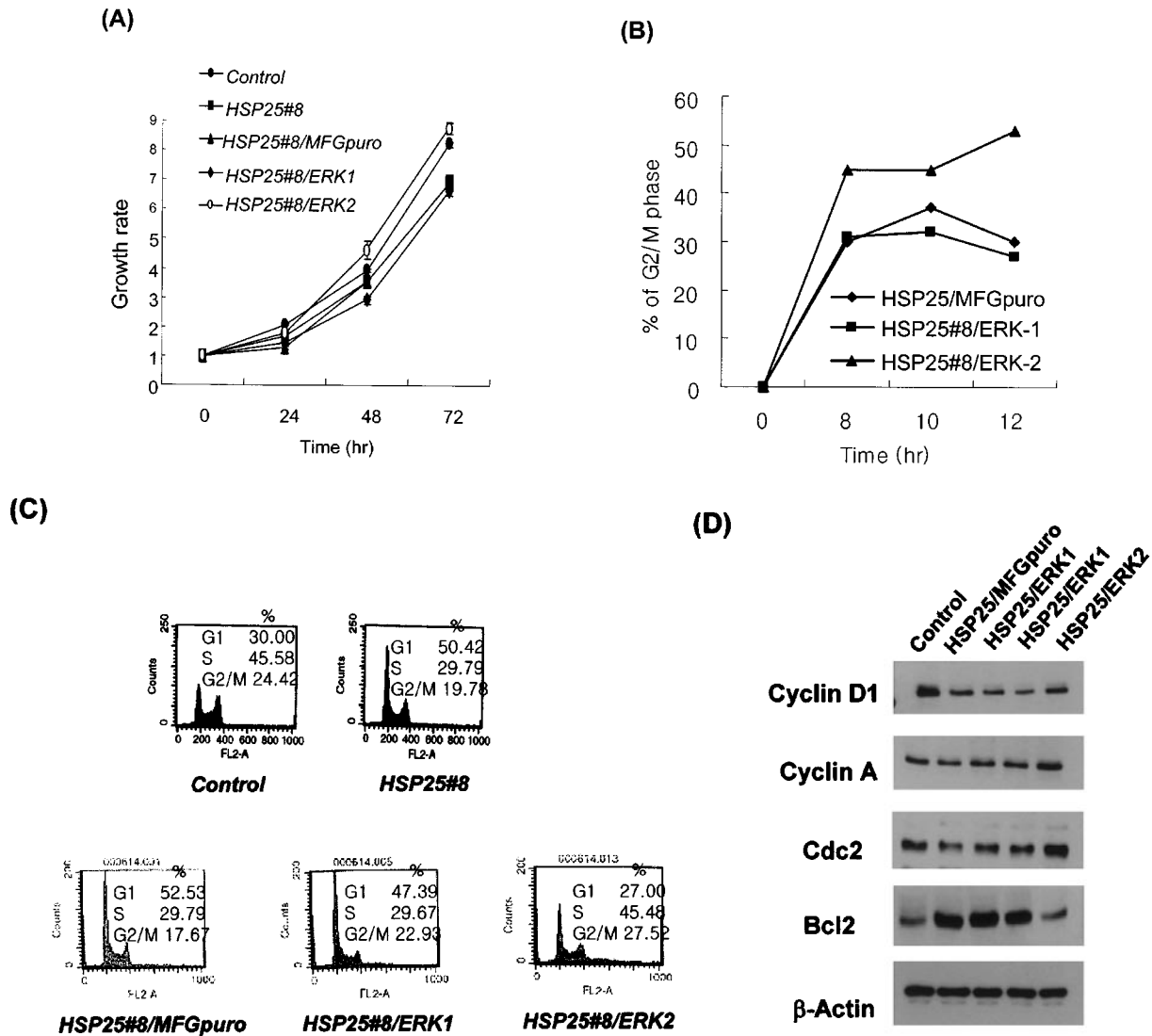
Figure 6 HSP25-mediated growth delay was abolished by ERK-2 cDNA transfection. After transient transfection of ERK-1 or ERK-2 cDNA into *hsp25*-transfected cells (HSP25#8/ERK1 or HSP25#8/ERK2), cell growth rate was measured at indicated times by counting cell number. (**A**) Relative growth rates of these cells were compared. (**B**) Cells were preincubated for 20 h with 400 μM mimosine for synchronization, washed, and incubated for various times before cell cycle analysis. (**C**) Comparison of cell cycle distribution in asynchronized cells after transient transfection of ERK-1 or ERK-2 cDNA into *hsp25*-transfected cells. (**D**) After transfection of ERK-1 or ERK-2 cDNA to *hsp25*-transfected cells, protein extracts (60 μg) were separated by SDS–PAGE and analyzed by Western blotting
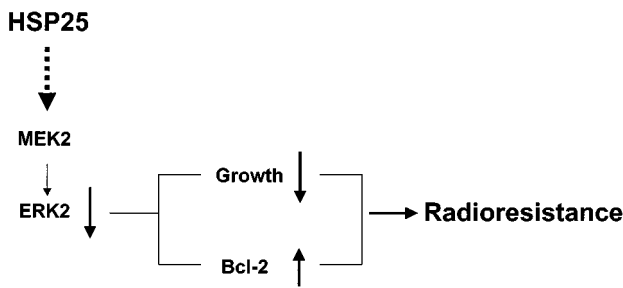
Figure 7 Proposed model of HSP25 mediated radioresistance. In the proposed model, HSP25 downregulates ERK-2 expressions and affects cell growth and Bcl-2 induction, resulting in induction of radioresistance

methanol and 25% acetic acid) and stained with 0.4% trypan blue. The number of colonies consisting of 50 or more cells was scored.

Detection of apoptosis

Cells were plated on glass slides and irradiated. After 24 or 48 h, cells were fixed in 70% ethanol, washed with PBS, and were incubated with 1 μg/ml bisbenzimide trihydrochloride in PBS (Hoechst No. 33258) for 30 min at room temperature in the dark. Specimens were viewed by fluorescence microscopy using Olympus BX-40 microscope. For each determination, 200 cells at least were scored. Apoptosis was characterized by chromatin condensation and fragmentation, giving rise to 'apoptotic bodies'.

G1-phase synchronization

Mimosine, a plant amino acid which reversibly blocks cells at the G1/S boundary by inhibiting initiation of DNA replication and accumulating p21^{WAF}, was used to obtain populations with a greater degree of synchrony in early S phase. Cells were plated 1 day prior to a 20 h exposure to 400 μM mimosine. Following treatment, medium was changed to drug-free medium, and cells were harvested thereafter at various time intervals for cell cycle distribution studies.

Cell cycle analysis

For cell cycle analysis, cells were fixed in 80% ethanol at 4°C for at least 18 h. The fixed cells were then washed once with PBS-EDTA and resuspended in 1 ml of PBS. After addition of 10 μl propidium iodide (5 mg/ml) and 10 μl RNase (10 mg/ml), the samples were incubated for 30 min at 37°C and analyzed by FACScan flow cytometer (Becton Dickinson, Frankin 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 incubated with a 1 : 1000 dilution of antibodies against cell cycle-related proteins; the mouse monoclonal anti-cyclin B1, anti-cdc2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), the rabbit polyclonal anti-cdk2, -cdk4, -cyclin D1, -Bcl-2 (Santa Cruz Biotechnology), and -cyclin E antibodies (Upstate Biotechnology, Inc., Lake Placid, NY, USA) were used. For the detection of HSP25, blots were incubated with a 1 : 1000 dilution of goat polyclonal anti-HSP25 antibody (Santa Cruz Biotechnology). Anti-phospho ERK1/2 or anti-ERK1/2 (New England Biolabs Inc.), anti-phospho MEK-1/2 or anti-MEK1/2 (New England Biolabs Inc.) antibodies were also used. 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.).

ERK kinase assay

ERK activities in cell lysates were measured by phosphorylation of phosphorylated heat and acid stable protein (PHAS-1), a substrate for ERK2,[28] and myelin based protein (MBP), a substrate for ERK1.[29] Briefly, subconfluent cell monolayers on 100 mm dishes were washed twice with PBS, and scraped with 800 μl of lysis buffer [120 mM NaCl, 40 mM Tris (pH 8.0), 0.1% NP40]. ERK1 or ERK2 were immunoprecipitated by incubating 200 μl of lysate with 2 μg of antibody (Santa Cruz Biotechnology, Inc.) for 2 h, and then by adding 20 μl of protein A agarose (Santa Cruz Biotechnology). After an overnight incubation at 4°C with end-over and washed three times with lysis buffer and once with 250 mM HEPES (pH 7.4), 10 mM MgCl$_2$, and 200 μM Na$_2$VO$_4$. The ERK pellets were resuspended in lysis buffer containing 120 μg of PHAS-1 or MBP substrate along with 3–5 μCi of [γ^{32}P] ATP in a final volume of 180 μl. Kinase reaction took place for 30 min at room temperature and were stopped by adding 4 × SDS–PAGE reducing sample buffer and boiling for 10 min. ERK1-MBP or ERK2-PHAS-1 samples were resolved on SDS polyacrylamide gels, dried, and autoradiographed.

Measurement of half-life of ERK1/2

To determine the half-life of ERK1/2 protein, cells were grown as described above and treated with 10 μg/ml of cycloheximide (CHX) in culture media for 24 h. After indicated times, proteins were extracted, resolved by polyacrylamide gel electrophoresis and analyzed by Western blotting as described earlier.

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