Environmental Mutagens & Carcinogens 25-2 : 51-59 (2005)

The Role of Heat Shock Protein 25 in Radiation Resistance

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(Received May 29, 2005 / Accepted June 18, 2005)

ABSTRACT **: Overexpression of HSP25 delayed cell growth, increased the level of $p21^{waf}$, reduced the levels of cyclin D1, cylcin A and cdc2, and induced radioresistance in L929 cells. We demonstrated that extracellular regulated kinase (ERK) and MAP kinase/ERK kinase (MEK) expressions as well as their activation (phospho-forms) were inhibited by *hsp25* overexpression. 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. In addition, HSP25 overexpression reduced reactive oxygen species (ROS) and increased expression of manganese superoxide dismutase (MnSOD) gene. Increased activation of NF-kB (IkB degradation) was also found in *hsp25*-overexpressed cells. Moreover, transfection of *hsp25* antisense gene abrogated all the HSP25-mediated phenomena. To further elucidate the exact relationship between MnSOD induction and NF-kB activation, dominant negative I-kBα (I-kBα-DN) construction was transfected to HSP25 overexpressed cells. I-kBα-DN inhibited HSP25 mediated MnSOD gene expression. In addition, HSP25 mediated radioresistance was blocked by I-kBα-DN transfection. Blockage of MnSOD with antisense oligonucleotides in HSP25 overexpressed cells, prevented apoptosis and returned the ERK1/2 activation to the control level. From the above results, we suggest for the first time that reduced oxidative damage by HSP25 was due to MnSOD-mediated downregulation of ERK1/2.**

Key words : **HSP25, radioresistance, ERK2, MnSOD, growth delay**

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 (Craig *et al.*, 1994) and that HSP27 protein participates in mediating physiological processes other than the stress response, including cellular differentiation and regulation of apoptosis (Kindas-Mugge and Trutinger, 1994). We earlier reported that overexpression of *hsp 25* 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 (Park *et al.*, 2000), thus leading us to 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 (Cho *et al.*, 2001).

It is generally accepted that the activation of the ERK cascade leads to cell proliferation (Grewal *et al.*, 1999). ERK1/2 pathway delivers a survival signal which counteracts proapoptotic effects elicited by JNK and the p38 activation (Pumiglia and Decker, 1997). However, investigations only recently have started to unravel in a number of cells that ERK mediates cell cycle arrest (York *et al.*, 1998), antiproliferation (Mohr *et al.*, 1998) as well as apoptotic (Murray *et al.*, 1998) and nonapoptotic death (Asada *et al.*, 2001). Requirement of ERK1/2 in mediating cisplatin-induced apoptosis of human cervical carcinoma HeLa cells and ovarian cell lines (Wang *et al.*, 2000) has also been demonstrated. Moreover, persistent activation of ERK1/2 contributes to glutamate-induced oxidative toxicity (Stanciu *et al.*, 2000).

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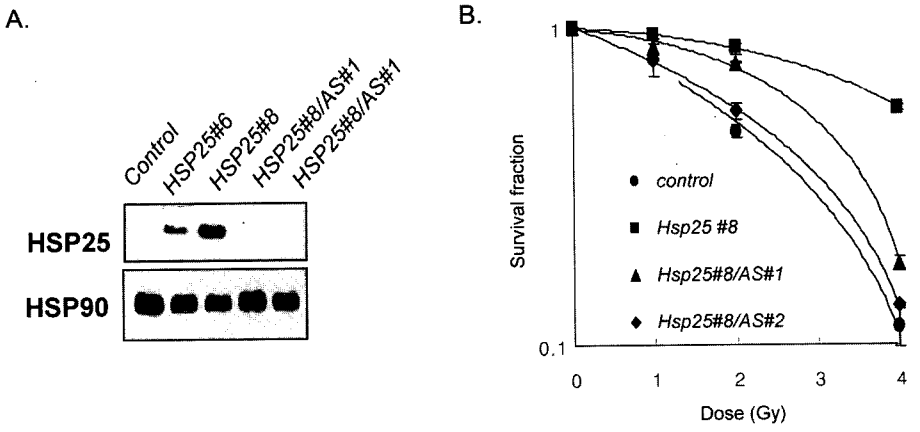

Fig. 1. Overexpression of hsp25 induced radioresistance.
A. Protein extracts of control vector, *hsp25*-transfected cells (clones *#6 and #8*), and *hsp25-antisense* (AS)-cotransfected cells (*hsp25#8/AS#1* and *hsp25#8/AS#2*) were prepared and assessed by western blot. *B.* Surviving fraction of vector- and *hsp25*-transfected cells (clone #8), and *hsp25-antisense* (AS)-transfected cells (*hsp25#8/AS#1* and *hsp25#8/AS#2*) were obtained by colony-forming assay after irradiation. Error bar indicates mean ± S.D. from three independent experiments.

Although it is still unclear how the MAP kinase pathway affects cellular survival or death, the results from earlier observations suggest that the MAP kinase cascade can be important for cellular response to reactive oxygen species (ROS). ROS act as mediators of cell death, and examples include the killing of cells by tumor necrosis factor (TNF) and chemotherapeutic drugs (Schreck *et al.*, 1991). ROS, generated either as by-products of normal cellular metabolism or under oxidative stress condition, leads to cell death and tissue damage when allowed to accumulate (Halliwell and Cross, 1994). To combat this, there exist various ROS enzymes, including multiple species of superoxide dismutase (SOD). MnSOD, a tetrameric enzyme located in the mitochondrial matrix, is the principal scavenger of superoxide in mitochondria (Fridovich, 1995). This evidence, together with a recent report of reduced survival and mitochondrial dysfunction in mice genetically deprived of MnSOD (Li *et al.*, 1995), support the notion that MnSOD acts mainly as a survival protein that is required to maintain mitochondrial integrity in cells exposed to adverse conditions. Although activation of the transcription factor NF-kB has been implicated in MnSOD induction, mechanisms involved in the induction of MnSOD expression under the conditions are poorly understood.

In this 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. In addition, we found that HSP25 facilitated MnSOD gene expression through NF-kB activation, which might be involved in HSP25-mediated radioresistance and HSP25-mediated increase of MnSOD level was involved in the attenuation of ERK1/2 activation and apoptosis.

Overexpression of HSP25 induced radioresistance.

To investigate the relationship between HSP25 and radioresistance, we first performed a clonogenic survival assay. As shown in Fig.1, *hsp25*-transfected cells (clone #8) showed increased clonogenicity against ionizing radiation-induced cytotoxicity. Moreover, co-transfection with *hsp25* antisense (#8/AS#1 and #8/AS#2) abrogated *hsp25*-mediated radioresistance (Fig.1B).

HSP25 overexpression downregulated ERK1/2 expression

Since we observed that overexpression of HSP25 induced cell growth delay (Park *et al.*, 2000; Cho *et al.*, 2001), we examined the upstream pathways of cell cycle regulation, particularly focusing on the MAP kinase pathway. As shown in Fig. 2A, both ERK1 and 2 expressions were dramatically decreased in the *hsp25*-transfected cells. Transient transfection of *hsp25* also decreased ERK1/2 expression (Fig. 2B). However, subsequent transfection with antisense *hsp25* cDNA recovered ERK1/2 expression to the level of control vector cells. These data suggested that HSP25 overexpression downregulated ERK1/2 expression. Other MAP kinases such

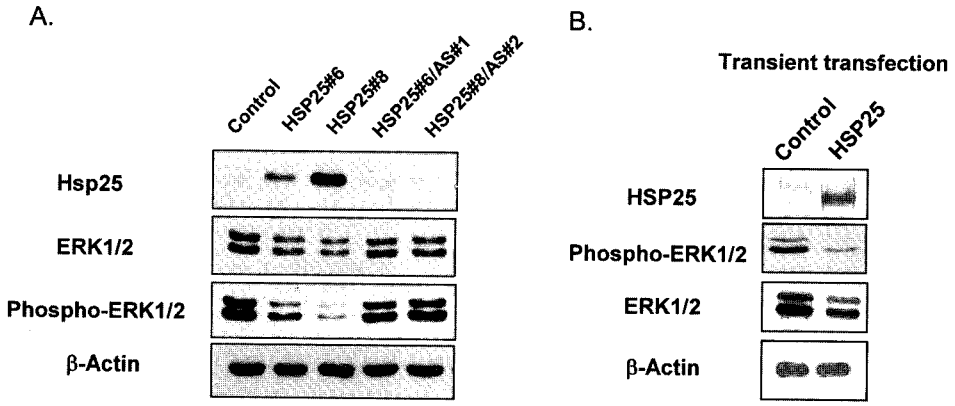

Fig. 2. HSP25 overexpression downregulated ERK1/2 expression.
A. Protein extracts (60 μg) were prepared from control vector-(Control) and *hsp25*-transfected (HSP25#6 and HSP25#8) cells with or without subsequent *hsp25*-antisense (AS) transfection (HSP25#6/AS#1 and HSP25#8/AS#2), separated by SDS-PAGE, and analyzed by western blotting. ***B.*** 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.

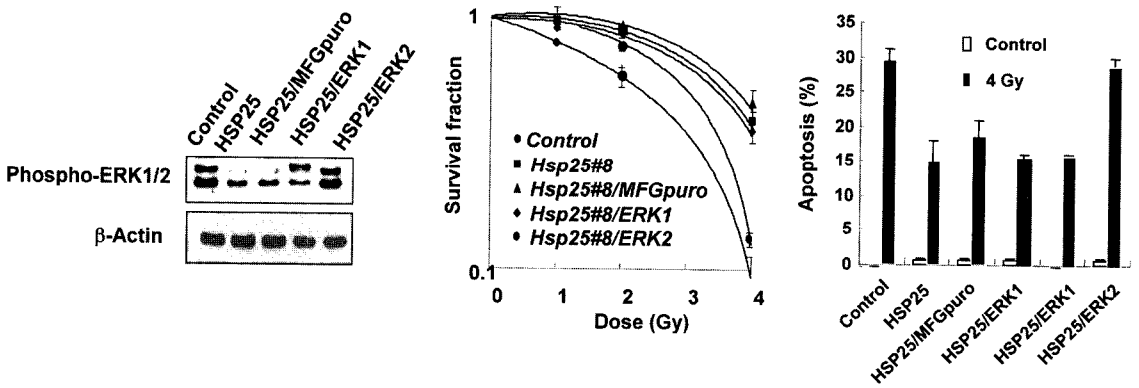

Fig. 3. Subsequent transfection of ERK-2 but not ERK-1 cDNA to hsp25-transfected cells restored altered HSP25-mediated cell cycle.
A. Control vector-(Control) and *hsp25*-transfected (HSP25) cells with or without subsequent transient transfection of vector control (HSP25#8/MFGpuro), ERK-1 (HSP25/ERK-1), or ERK-2 (HSP25/ERK-2) were harvested. Protein extracts (60 μg) 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 4Gy irradiation, as described in the Materials and Methods. Error bar indicates mean ± S.D. from three independent experiments.

as p38 and JNK expressions were not changed by *hsp25* overexpression (data not shown).

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 (Fig. 3A). When clonogenic survival and apoptosis assay were performed, ERK2 but not EKR1 transfected cells exhibited the inhibition of *hsp25*-mediated radioresistance (Fig. 3B and 3C), indicating that ERK2 inhibition was essential for *hsp25*-mediated radioresistance.

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. Altered distribution

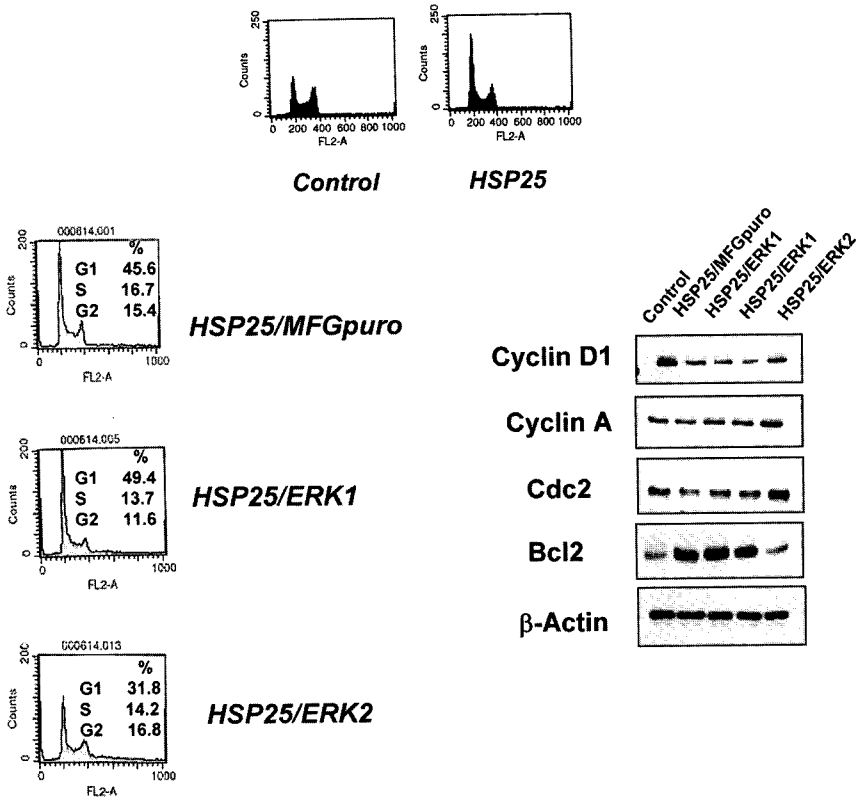

Fig. 4. HSP25-mediated growth delay was abolished by ERK-2 cDNA transfection.
A. Comparison of cell cycle distribution in asynchronized cells after transient transfection of ERK-1 or ERK-2 cDNA into *hsp25*-transfected cells ***B.*** After transfection of ERK-1 or ERK-2 cDNA to *hsp25*-transfected cells, protein extracts (60 μg) were separated by SDS-PAGE and analyzed by western blotting.

of cell cycle by HSP25 overexpression was also restored by ERK-2 transfection (Fig. 4A): 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 (Fig. 4B). In addition, increased induction of Bcl-2 by *HSP25* overexpression was also reduced to the control level.

Overexpression of HSP25 increased MnSOD gene expression as well as its enzyme activity

To understand the relationship between HSP25-mediated radioresistance and antioxidant enzyme activity, MnSOD gene expression was examined by northern analysis. HSP25 overexpressed cells (clones #6 and #8) showed increased MnSOD mRNA expression, while *hsp70* overexpressed cells did not (Fig. 5A). Also, increased enzyme activity was detected in *hsp25*-overexpressed cells (Fig. 5B). In addition, treatment of the cells with HSP25 antisense abolished these phenomena.

Overexpression of HSP25 activated NF-kB

The MnSOD gene expression is modulated by NF-kB (Darville and Eizirik, 2000) In order to delineate whether NF-kB activation, which involves proteolyic degradation of its inhibitor subunit (I-kB) followed by nuclear translocation (Baldwin, 1996) is necessary for HSP25 mediated events, I-kB degradation was examined. As shown in Fig. 6A, decreased expression of I-kB was detected in *hsp25*-overexpressed cells (clones #6 and #8), while antisense treatment reduced the degradation. These results, therefore, suggested that HSP25 overexpression affected NF-kB activation. In addition, 20 Gy radiation increased HSP25-mediated phenomena (Fig. 6B and 6C),

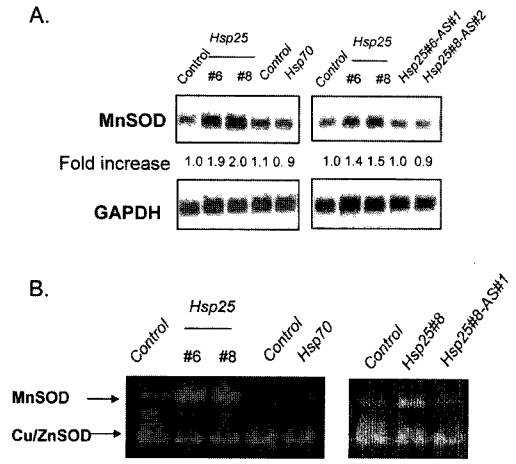

Fig. 5. Overexpression of HSP25 increased gene expression of antioxidant enzyme as well as their enzyme activity.
A. Total RNAs (30 μg per lane) from control vector and *hsp25*-transfected cells (clone #6 and #8), inducible *hsp70*-transfected cells, and *hsp25*-antisense (AS)-transfected cells (*hsp25#8/AS#1* and *hsp25#8/AS#2*) were prepared and analysed by formaldehyde/agarose gel electrophoresis, and transferred to a nylon membrane. *B.* The cells from control vector and *hsp25*-transfected cells (cone #8), inducible *hsp70*-transfected cells, and *hsp25*-antisense (AS)-transfected cells (*hsp25#8/AS#1*) were harvested, and 100 μg whole cell proteins were electrophoresed on SDS/12% polyacrylamide gel and analyzed for MnSOD protein.

Dominant negative I-kBα mutant transfection to HSP25 overexpressed cells inhibited MnSOD mRNA expression and radioresistance

To elucidate direct correlation between NF-kB activation and MnSOD expression, we made dominant negative I-kBα mutant vector (I-kBα-D/N). MFG retroviral vector (MFGpuro), control and HA-tagged I-kBα-D/N mutant vectors were transfected to HSP25 overexpressed cells. As shown in Fig. 7A, overexpressed HA protein was detected in I-kB-D/N transfected cells. HSP25-mediated overexpression of MnSOD mRNA was decreased in I-kBα-D/N transfected cells (Fig. 6B) and radioresistance induced by HSP25 was also abolished (Fig. 6C).

Treatment of MnSOD AS in HSP25 overexpressed cells recovered ERK1/2 phosphorylation and apoptosis to the level of control cells

To investigate interrelationship between MnSOD gene expression, ERK1/2 phosphorylation and apoptosis, the HSP25-overexpressed cells were treated with MnSOD antisense. As shown in Fig 8A, Northern blot analysis revealed that introduction of MnSOD antisense reduced the expression of MnSOD mRNA level, and DNA

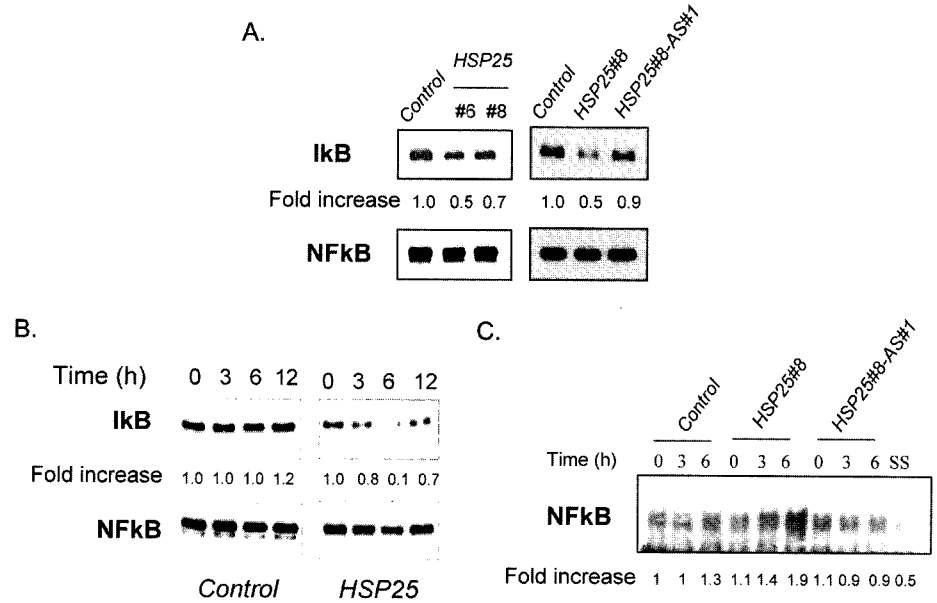

Fig. 6. Overexpression of HSP25 activated NF-kB.
A. Protein extracts of control vector and hsp25-transfected cells (clones #6 and #8), and hsp25-antisense (AS)-transfected cells (hsp25#8/AS#1) were prepared and assessed by western blot. ***B.*** Protein extracts of control vector and hsp25-transfected cells (clone #8) after 20 Gy of g-rays were prepared and assessed by western blot. ***C.*** After 20 Gy radiation, the nuclear and cytosol proteins were extracted, incubated with [^{32}P]-oligonucleotide containing NF-kB consensus sequence, and then electophoresed on 6% nondenaturing polyacylamide gel. Autoradiographs were obtained and radioactive signals were quantified using a computing densitometer.

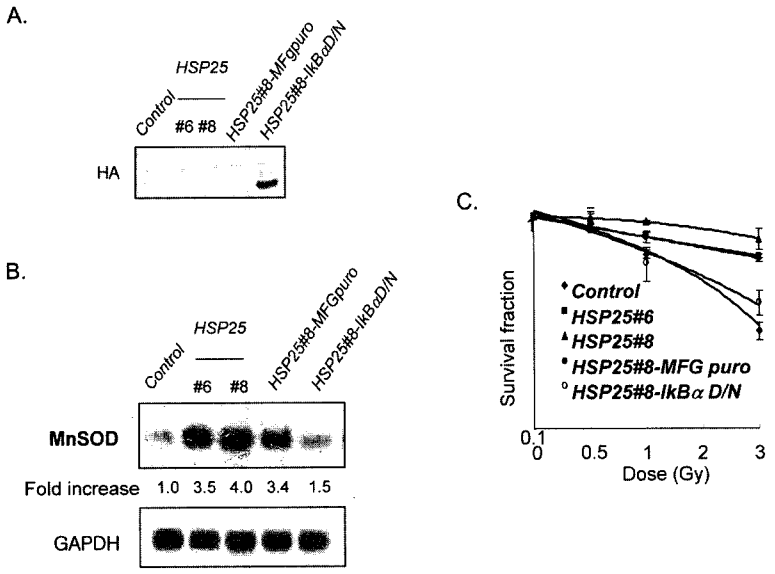

Fig. 7. Transfection of I-kB dominant negative mutant to HSP25 overexpressed cells reduced MnSOD mRNA expression and radioresistance.
A. Protein extracts of control vector and *hsp25*-transfected cells (clones #6 and #8), MFGpuro control vector transfected *hsp25* cells (*HSP25#8-MFGpuro*) were prepared and assessed by western blot with HA-antibody. ***B.*** Total RNAs (30 μg per lane) from control vector and *hsp25*-transfected cells (clone #8), MFGpuro control vector transfected *hsp25* cells (*HSP25#8-MFGpuro*), were prepared, analysed by formaldehyde/agarose gel electrophoresis, and transferred to a nylon membrane as described in Materials and Methods. ***C.*** Surviving fraction of control vector and *hsp25*-transfected cells (clone #8), MFGpuro control vector transfected *hsp25* cells (*HSP25#8-MFGpuro*) were obtained by colony-forming assay after irradiation. Error bar indicates mean ± S.D. from three independent experiments.

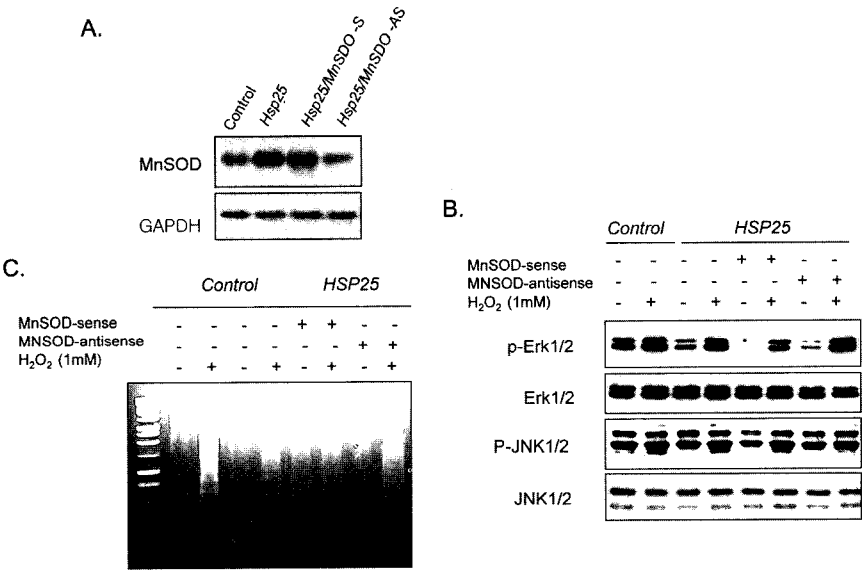

Fig. 8. Treatment of HSP25 overexpressed cells with antisense MnSOD.
A. Synthetic phosphrothioates sense or antisense of MnSOD were transfected to HSP25 overexpressed cells (HSP25#8), and total RNAs (30 μg per lane) were prepared and analysed by formaldehyde/agarose gel electrophoresis and transferred to a nylon membrane. ***B.*** Cells were treated with 1mM H_2O_2 and lysed with lysis buffer, followed by incubation with RNase and finally with proteinase K. DNA was separated by electrophoresis in 1.5% agarose gels and stained by ethidium bromide. ***C.*** Protein extracts (60 μg) were prepared, separated by SDS-PAGE, and analyzed by western blotting.

laddering data also revealed that MnSOD antisense increased H_2O_2-mediated ERK1/2 activation and apoptosis (Fig. 8B), and tyrosine kinase phosphorylation to the control levels (Fig. 8C).

Discussion

We observed that HSP25-mediated radioresistance was correlated with growth inhibition and increased induction of Bcl-2 protein (Park *et al.*, 2000; Cho *et al.*, 2001). 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, 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 c-Jun NH_2-terminal kinases (JNK, also called SAPK) and the p38. These results strongly indicated that the downregulation of ERK1/2 in 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 cycloheximide. 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 (Cho *et al.*, 2002). NF-kB 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 (Aikawa *et al.*, 1997; Irani *et al.*, 1997), 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 (Devary *et al.*, 1992; Baas and Berk, 1995), and Guyton *et. al.* (1996), also showed that H_2O_2-stimulated ROS activated ERK2 in PC12 cells.

Many studies support the general view that activation of the ERK pathway delivers a survival signal which counteracts proapoptotic effects elicited by JNK and the p38 activation (Hayakawa *et al.*, 1999) However, requirement of ERK in mediating cisplatin-induced apoptosis of human cervical carcinoma HeLa cells and ovarian cell lines (Wang *et al.*, 2000; Persons *et al.*, 1999) has also been demonstrated. Moreover, persistent activation of ERK1/2 contributes to glutamate-induced oxidative toxicity (Stanciu *et al.*, 2000). We also provided evidence that ERK-2 cDNA transfection which affected cell growth, Bcl-2 induction, and finally radioresistance induction, abolished radiation-induced cell death in the HSP25 overexpressing cells (Park *et al.*, 2000).

MnSOD, which converts superoxide radical to hydrogen peroxide and molecular oxygen within mitochondrial matrix, and cytosolic glutathione peroxidase (GPx), which converts hydrogen peroxide into water, are primary antioxidant enzymes whose expressions are essential for the survival and development of cellular resistance to oxygen-mediated cytotoxicity. In the present study, we showed that HSP25 overexpression resulted in a coordinate increase in MnSOD mRNA and enzymatic activity.

Numerous studies have demonstrated that NF-kB is involved in the induction of various genes and that activation of NF-kB by these stimuli is a consequence of proteolytic degradation of its inhibitor subunit (I-kB) immediately followed by nuclear translocation. Activation of NF-kB can induce MnSOD, suggesting that NF-kB is intimately involved in the regulation of MnSOD expression (Baeuerle and Baltimore, 1989). HSP25-overexpressed cells showed increased degradation of I-kB and increased nuclear binding activity of NF-kB after radiation. Moreover, in our dominant negative I-kBα mutant vector experiments also suggested that HSP25-mediated NF-kB activation is responsible for MnSOD gene expression (Yi *et al.*, 2002). MnSOD is uniquely induced in response to oxidative stress condition, underscoring the importance of protecting the energy-producing machinery within the mitochondria from oxidative damage. Treatment of the HSP25 overexpressing cells with MnSOD antisense restored the induction of apoptosis and expressions of ERK1/2 to the levels of the control cells, without altering JNK1/2 phosphorylation. When MnSOD gene was overexpressed in L929 cells, similar phenomena such as reduced expression of ERK1/2 phosphorylation and

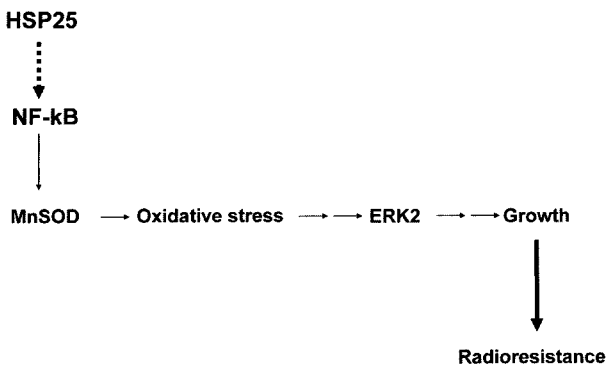

Fig. 9. Proposed model of HSP25 mediated radioresistance

tyrosine kinase phosphorylation, which were observed in the HSP25 overexpressing cells by H_2O_2 treatment (data not shown), occurred, suggesting that reduced oxidative damage by HSP25 overexpression was due to increased expression of MnSOD. We could not offer any explanation on how MnSOD downregulated ERK1/2 signaling, however, H_2O_2-induced superoxide anion might be a pivotal molecule in ROS-mediated ERK1/2 activation and apoptosis, and HSP25-mediated MnSOD inhibited superoxide anion production, which consequently resulted in resistance to oxidative stress.

In this paper, we described possible involvement of ERK2 and MnSOD in the development of radioresistance in the HSP25-overexpressed L929 cells. Our proposed model shown in Fig. 9 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.

Acknowledgment

This work was supported by the Korean Institute of Science & Technology Evaluation and Planning (KISTEP) and by the Ministry of Science and Technology (MOST), through the National Nuclear Technology Program.

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