

# Activation of Heat Shock Factor 1 by Hyperosmotic or Hypo-Osmotic Stress Is Drastically Attenuated in Normal Human Fibroblasts During Senescence

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We have previously reported that osmotic stress prominently induces the DNA binding activity of the heat shock transcription factor 1 (HSF1). In the present study, we examined the effects of medium osmolarity on both the activation of HSF1 and the programmed cell death in normal human fibroblasts during cellular senescence. The activation of HSF1 occurred rapidly in presenescent (early passage) IMR-90 cells when exposed to either hypo-osmotic or hyperosmotic stress. In contrast, the activation of HSF1 was significantly attenuated in senescent cells. Western blot analysis indicated that equal amounts of HSF1 were present as monomers in the cytoplasm of both presenescent and senescent cells in normal growth medium. Under either hypo-osmotic or hyperosmotic stress, trimerization and nuclear localization of HSF1 occurred in presenescent cells but not in senescent cells. More than 80% of HSF1 in senescent cells remained as monomers in the cytoplasm under osmotic stress, suggesting a defect in the signal transduction pathways that lead to HSF1 trimerization or a dysfunction in the HSF1 protein itself. Possible involvement of mitogen-activated protein kinase (MAPK) signal transduction pathways in the activation HSF1 was investigated by monitoring the activation of the three MAPKs, ERK1/2, JNK1/2, and p38, in cells exposed to hypo-osmotic or hyperosmotic stress. All three MAPKs were activated by hyperosmotic stress but not hypo-osmotic stress, suggesting that the MAPK signal transduction pathways may not be directly linked to the osmotic stress-induced activation of HSF1. In contrast to the rapid heat shock transcription factor (HSF) activation, apoptosis occurred only after long-term exposure to hypo-osmotic or hyperosmotic stress. Despite the prominent induction of HSF1 activation, the presenescent cells were more sensitive than the senescent cells to the osmotic stress-induced apoptosis. *J. Cell. Physiol.* 184:183–190, 2000.

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Normal diploid fibroblasts in tissue culture have a limited doubling potential (Hayflick and Moorhead, 1961). This feature, together with advantages of using tissue culture as an experimental model, make them a convenient system for studying the molecular mechanism of aging process and the effects of aging on stress response at cellular level (reviewed by Lee et al., 1996; Smith and Pereira-Smith, 1996; Campisi, 1999). Stress, either physical or chemical one, can induce distinct biochemical changes in living cells. Chief among the stress responses is the heat shock response, characterized by the biosynthesis of a group of highly conserved heat shock proteins (HSP) (reviewed by Nover and Scharf, 1991). In eukaryotic cells, heat shock protein (hsp) genes are under the control of heat

shock transcription factors (HSFs). Heat shock transcription factor 1 (HSF1), a member of the multigene heat shock factor family, is the key factor that mediates stress response. In higher eukaryotic cells, HSF1 is constitutively expressed and exists as an inactive monomer in the cytosol under nonstressed conditions

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(Morimoto, 1993, 1998; Wu, 1995). On heat shock or other stresses, HSF1 is converted from inactive monomers to active homotrimers. The activated HSF1 specifically recognizes heat shock response element (HSE) that consists of three or more contiguous inverted repeats of a 5-base pair sequence nGAAn located at the promoters of *hsp* genes (reviewed by Wu, 1995). Previous studies have shown that heat shock response exhibits an age-dependent attenuation in normal human diploid fibroblasts (Liu et al., 1989; Choi et al., 1990), suggesting that a progressive impairment of stress response occurs during aging even at cellular level.

Osmotic stress represents a prevalent environmental physical stress that is biologically relevant, not only for free-living organisms, but also for mammalian cells in vivo. For example, a large fluctuation of osmolarity, up to more than 1,000 mOsM, has been shown to exist in human renal medulla tissue (Garcia-Perez and Burg, 1991). Because hypo-osmotic stress and hyperosmotic stress represent two opposing physical forces, it is surprising to find that both of them are capable of inducing HSF1 activation (Huang et al., 1995; Caruccio et al., 1997). In the present study, we examined the effect of cellular aging on the osmotic stress-induced HSF1 activation in order to explore the mechanism and the physiologic significance of this unique osmotic stress response. Because heat shock and apoptosis appear to share some overlapping mechanism (Punyiczki and Fesus, 1998), we also examined whether osmotic stress may induce apoptosis and whether presenescent and senescent cells may respond to the osmotic stress differently. Mitogen-activated protein kinase (MAPK) signal transduction pathways have been implicated in many stress responses (for reviews, see Karin, 1998). To determine whether MAPK pathways may be directly linked to osmotic stress-induced HSF activation or apoptosis, we have also compared the three MAPK signal transduction pathways, ERK1/2, JNK1/2, and p38, in cells exposed to either hypo-osmotic or hyperosmotic stress. We found that aging process profoundly affected the osmotic stress-induced HSF1 activation. A possible mechanism that may cause the age-dependent attenuation of osmotic stress response is discussed.

## MATERIALS AND METHODS

### Chemicals

All tissue culture supplies were purchased from Gibco BRL (Gaithersburg, MD). Ethylene glycol-bis(sulfosuccinimidyl succinate) (EGS), a homobifunctional NHS-ester cross-linker, was purchased from Pierce (Rockford, IL). [ $\gamma$ - $^{32}$ P]ATP (3,000 Ci/mmol) was obtained from ICN Chemicals, Radioisotope Division (Irvine, CA). All other chemicals were of standard reagent grade.

### Cell culture

IMR-90 human embryonic lung fibroblasts at low passage numbers were obtained from the Coriell Institute for Medical Research, Camden, New Jersey. Cells were maintained at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cell cultures were expanded through subcultivation to achieve a higher population doubling level (PDL) as previously described (Pang and Chen, 1993). Cells at PDL less than 25 are referred as presenescent or early

passage cells, cells at PDL more than 48 are referred as senescent cells. Cells grown to about 90% confluence were used for all the experiments. Hypo-osmotic stress and hyperosmotic stress was generated as previously described (Huang et al., 1995; Caruccio et al., 1997).

### HSE binding and electrophoretic mobility shift assay

The whole-cell extracts, nuclear extracts, and cytosolic extracts were prepared essentially as previously described (Huang et al., 1995; Caruccio et al., 1997). For nuclear and cytosolic extracts, cells were washed once with buffer B (10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.3 M sucrose, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF, 1  $\mu$ M pepstatin A, and 1  $\mu$ M leupeptin). The cell pellet was resuspended in buffer B and homogenized in a Dounce homogenizer with a type B pestle. Nuclei were then collected by a centrifugation at 3,300g for 15 min. The supernatant was saved as cytosolic extract. The nuclear pellet was suspended in buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 25% glycerol, 0.1 mM EGTA, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1  $\mu$ M pepstatin A, and 1  $\mu$ M leupeptin) for 30 min with gentle mixing. The suspension was centrifuged at 25,000g for 30 min and the supernatant was collected as nuclear extract. All extracts were dialyzed against buffer D (20 mM Hepes, 7.9, 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1  $\mu$ M pepstatin A, and 1  $\mu$ M leupeptin) and stored at -80°C. The protein concentration was determined using BCA Protein Assay Reagent (Pierce, Rockford, IL). For the binding reaction, cell extracts containing 20  $\mu$ g of proteins were mixed with 0.5  $\mu$ g of poly(dI-dC), 5  $\mu$ g bovine serum albumin (BSA), and approximately 250 pg of  $^{32}$ P-labeled oligonucleotides in a final volume of 20  $\mu$ l binding buffer containing 20 mM HEPES, pH 7.9, 150 mM KCl, 1 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 5% glycerol, and 1 mM DTT. After 30 min at room temperature, the binding reaction was analyzed by gel mobility shift assay in a 4% non-denaturing polyacrylamide gel at 4°C. The double-stranded synthetic consensus HSE, 5'-GCCTCGAAT-GTTCGCGAAGTTTCG-3', was synthesized in our laboratory using a Gene Assembler Plus DNA synthesizer (Pharmacia, Piscataway, NJ). The probe was labeled at the 5' end with [ $\gamma$ - $^{32}$ P] ATP using T4 polynucleotide kinase.

### Western blot analysis

Twenty micrograms of whole-cell extracts were incubated for 20 min at room temperature in the presence of one-tenth of EGS (6 mM in dimethyl sulfoxide). The reaction was quenched by an addition of glycine to 75 mM. The resultant mixtures were resolved on a 4%–10% gradient SDS-polyacrylamide gel, and transferred onto a piece of Immobilon-P transfer membrane (Millipore, Bedford, MA) using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad, Hercules, CA). The membrane was probed with a 1:5,000 dilution of polyclonal anti-HSF1 antibody, followed by an incubation with antirabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase. The antigen-antibody complex was detected by enhanced chemiluminescence (ECL; Amersham Pharmacia, Piscataway, NJ).

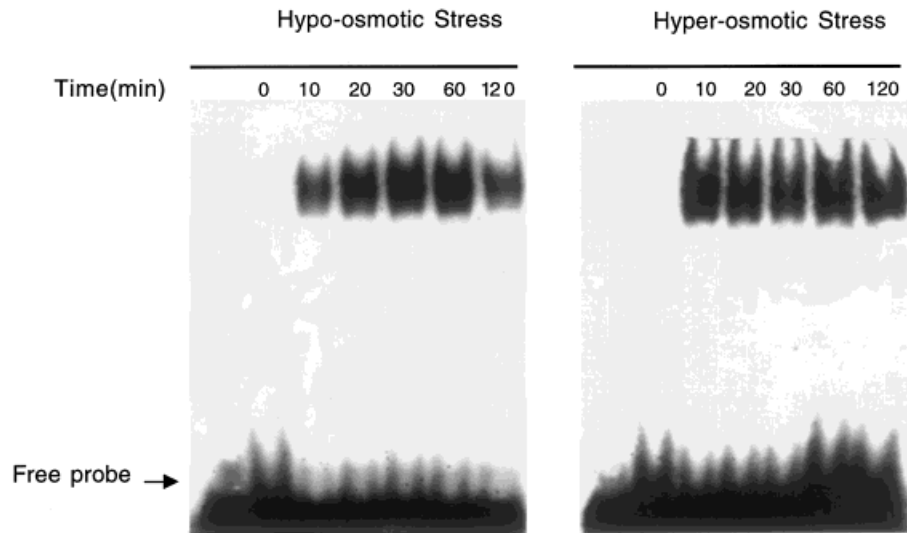


Fig. 1. Time course of heat shock transcription factor 1 (HSF1) activation in IMR-90 cells under hypo-osmotic and hyperosmotic stress. Confluent IMR-90 cells (PDL = 21) were incubated in a pre-warmed sorbitol solution (37°C) at 100 mOsM (hypo-osmotic stress) or

800 mOsM (hyperosmotic stress). Cells were harvested at indicated time, and whole-cell extracts were prepared for DNA binding and gel mobility shift assay on a 4% PAGE using the  $^{32}\text{P}$ -labeled heat shock cis-element (HSE) as the probe.

#### MAPK signal transduction pathway assay

HeLa cells at 90% confluence were incubated in a prewarmed sorbitol solution (37°C) at various concentrations for 30 min. Cells were then harvested and whole-cell extracts containing 30  $\mu\text{g}$  of proteins were analyzed by SDS-PAGE and Western blot analysis using polyclonal antisera against ERK1/2, JNK1/2, and p38 (Santa Cruz Biotechnology, Santa Cruz, CA) or using phosphospecific MAPK antibodies for ERK1/2, p38 (New England BioLabs, Beverly, MA) and JNK1/2 (Santa Cruz Biotechnology).

#### DNA fragmentation assay

Both attached and floating cells were collected and centrifuged at 2,000g for 5 min. The cell pellet was washed twice with ice-cold phosphate buffered saline and suspended in a lysis buffer (10 mM Tris, pH 7.5, 200 mM NaCl, 10 mM EDTA, 0.4% Triton X-100, and 0.1 mg/ml proteinase K) for 30 min at room temperature, followed by an incubation with RNase Cocktail containing RNase A and RNase T1 (Ambion, Austin, TX) for 30 min at 37°C. The genomic DNA was prepared by phenol/chloroform extraction and ethanol precipitation. The samples were analyzed on an agarose gel in the presence of 0.5  $\mu\text{g}/\text{ml}$  of ethidium bromide.

### RESULTS

#### Time course of the induction of HSF binding in normal human fibroblasts

We first determined the time courses of the effects of osmotic stress on the activation of HSF1 in young IMR-90 cells. Figure 1 shows that the activation of HSF induced by either hypo-osmotic or hyperosmotic stress in normal IMR-90 cells was rapid, reaching almost to the maximal level within 10 min after cells were exposed to hyperosmotic stress. Such rapid acti-

vation kinetics is similar to that commonly observed with heat shock (Wu, 1995), suggesting a similar, if not identical, activation mechanism may be operative in all these cases. These results are consistent with our previous finding that both hypo-osmotic and hyperosmotic stresses could induce the activation of HSF1 in mammalian cells with similar kinetics and comparable magnitude, despite the fact that they represent opposing physical forces. (Caruccio et al., 1997).

#### HSF1 activation in young and senescent human diploid fibroblasts

Next, we examined whether this osmotic stress-induced HSF activation response may exhibit an age-dependent change during cell senescence. Figure 2A shows that although the HSF1 activation occurred prominently in early passage cells after osmotic stress, this activation was substantially diminished in cells with higher PDL and became almost nondetectable in senescent cells. In order to ensure that the striking attenuation shown in Figure 2A is not an artifact, an internal control was included to show that equal amounts of cell extract were used for gel mobility shift assay (Fig. 2B). Moreover, we found that the Sp1 DNA binding activity did not exhibit any age-dependent attenuation with or without osmotic stress (data not shown). The degree of attenuation of HSF1 DNA-binding activity in senescent cells was consistently more pronounced under hypo-osmotic stress than hyperosmotic stress (Fig. 2A, lane 9 vs. lane 10). Thus, the osmotic stress-induced HSF1 activation exhibited a progressive decline as a function of the population doubling level of the cell culture. Similar age-dependent progressive decline in heat shock response has been reported for human diploid cells (Liu et al., 1989) and animals (Blake et al., 1991).

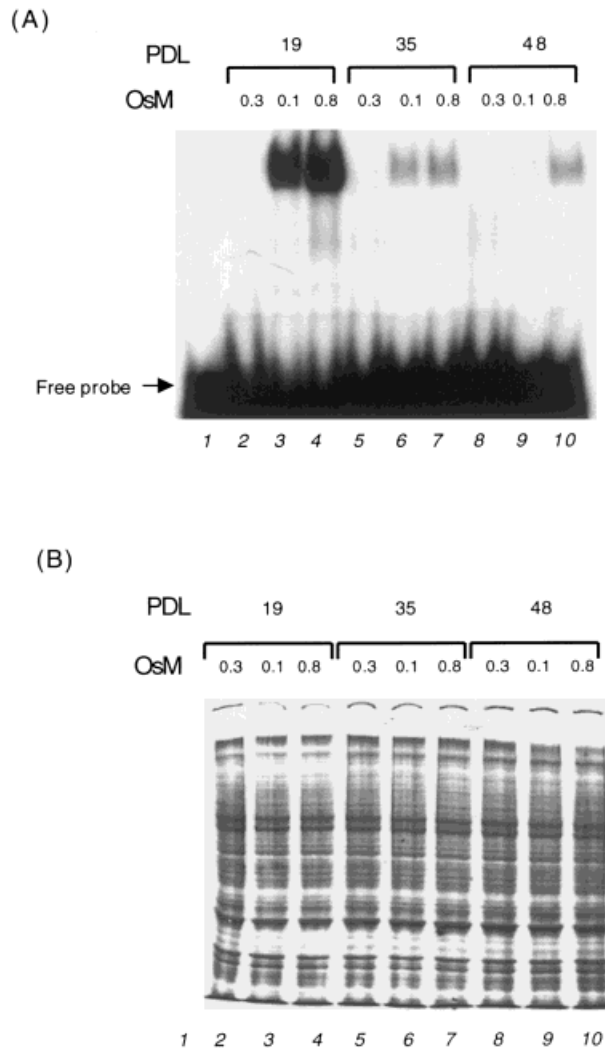


Fig. 2. A: Effect of hypo-osmotic and hyperosmotic stress on the heat shock transcription factor 1 (HSF1) activation in IMR-90 cells at different passage numbers. IMR-90 cells of three population doubling level (PDL), 19, 35, and 48 were grown to confluence. Cells were then incubated for 30 min in a sorbitol solution at 100, 300, and 800 mOsM. Whole-cell extracts were then prepared for HSF1 binding and gel mobility shift assay. B: Coomassie blue-stained SDS-polyacrylamide gel pattern of the same samples used for gel mobility shift assay. Each lane contained 25  $\mu$ g of protein.

### The activation of HSF1 in presenescent and senescent cells

The cause for the age-dependent attenuation of HSF1 activation in senescent cells could be due to any of the following reasons: a change in the subcellular localization of HSF1; a decrease in HSF1 protein levels, a defect in the signal transduction pathways; a dysfunction of HSF1 protein itself, probably due to some posttranslational modification; or any combination of the above. To determine the cause, we first examined the cellular localization of HSF1 DNA-binding activity in presenescent and senescent cells after osmotic stress. As expected, HSF1 DNA-binding activity was absent in either the nuclear or cytosolic fractions obtained from nonstressed cells (Fig. 3, lanes 2, 3, 8, and

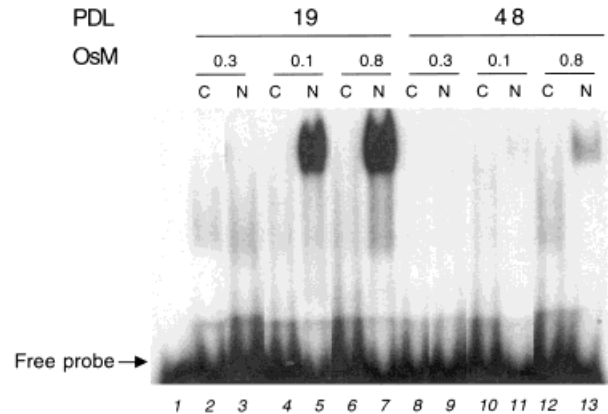


Fig. 3. Effects of medium osmolarity on heat shock transcription factor (HSF) activation in presenescent (population doubling level [PDL] = 19) and senescent (PDL = 48) IMR-90 cells. Confluent cells were incubated in a prewarmed sorbitol solution (37°C) at three different osmolarities for 30 min. Cells were harvested, and cytosolic (C) and nuclear (N) extracts were then prepared for DNA binding and gel mobility shift assay using the  $^{32}$ P-labeled heat shock cis-element (HSE) as the probe.

9). After osmotic stress, almost all the HSF1 DNA-binding activity in early passage cells appeared in the nuclear fractions (Fig. 3, lanes 5 and 7). In contrast, HSF1 DNA-binding activity in senescent cells was absent in either the cytosolic or nuclear fractions under hypo-osmotic stress (Fig. 3, lanes 10 and 11). It was barely detectable, however, in the nuclear fraction under hyperosmotic stress (Fig. 3, lane 13 vs. lane 12). Thus, the HSF1 DNA-binding activity existed only in the nuclear fraction, even for the senescent cells (Fig. 3, lane 13).

We then compared the protein levels of HSF1 in presenescent and senescent cells either under nonstressed condition or under hyperosmotic stress. Under nonstressed condition, the HSF1 protein was detectable only in the cytosol in both presenescent and senescent cells (Fig. 4A, lanes 1 vs. 2 and lanes 3 vs. 4). Importantly, the protein levels of HSF1 in presenescent and in senescent cells were almost identical (Fig. 4A, lane 1 vs. lane 3), indicating that there was no age-associated decrease in HSF1 in senescent cells. Figure 4A also shows that after hyperosmotic stress, the nuclear translocation of HSF1 occurred prominently in presenescent IMR-90 cells (Fig. 4A, lane 6 vs. lane 5) but not in senescent cells (Fig. 4A, lane 7 vs. lane 8). Thus, although both presenescent and senescent cells contained the same amounts of HSF1 proteins, the HSF1 proteins in senescent cells appeared to be incapable of nuclear translocation under osmotic stress. Because trimerization of HSF1 leads to its accumulation within the nucleus (Morimoto, 1993), we examined whether the HSF1 monomers in senescent cells were capable of forming trimers under osmotic stress. To demonstrate trimer formation, we have taken advantage of the fact that once a trimer is formed, it can be covalently cross-linked by small cross-linker molecule and thus exhibits a retarded mobility on SDS-PAGE as compared to the monomer. Figure 4B shows that under nonstressed condition, HSF1 proteins in both presenescent and senescent cells could not

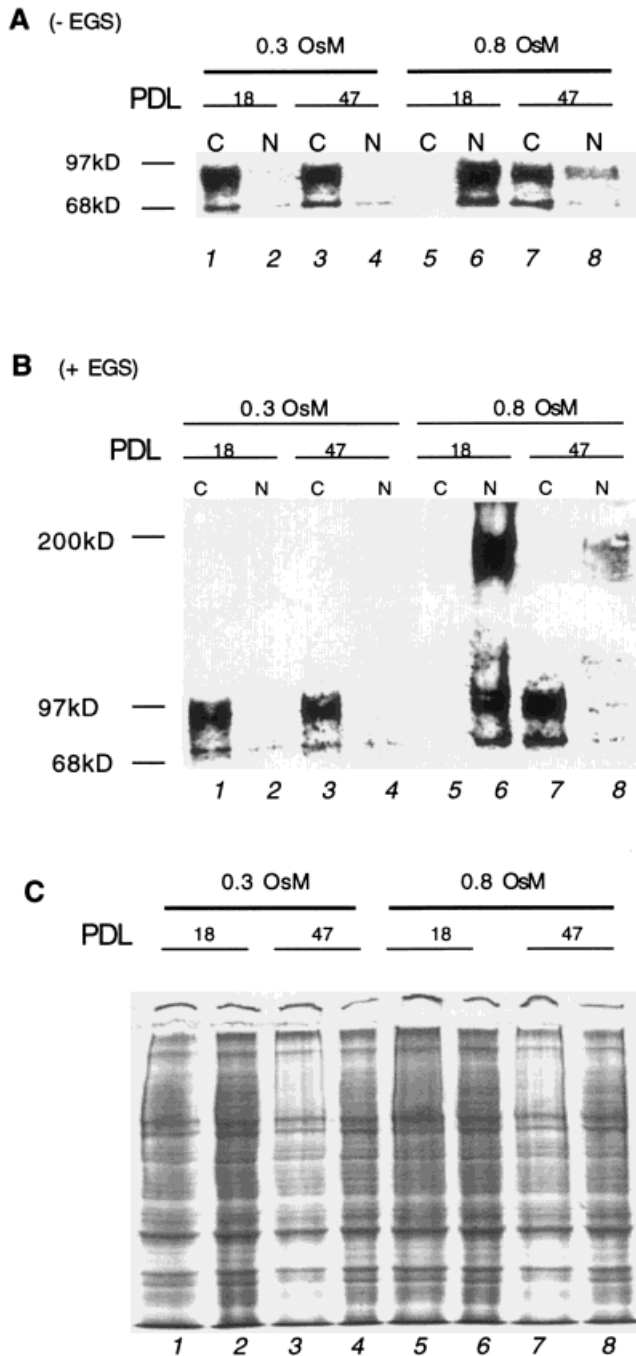


Fig. 4. Western blot analysis of the size of heat shock transcription factor 1 (HSF1) in presenescent (population doubling level [PDL] = 18) and senescent (PDL = 47) cells during hyperosmotic stress. IMR-90 cells were incubated in Dulbecco's medium at 315 or 800 mOsM at 37°C for 30 min. Cytosolic and nuclear extracts were prepared from these cells as described in Materials and Methods. The extracts were then incubated in the absence (-EGS; **A**) or in the presence (+EGS; **B**) of cross-linker ethylene glycol-bis(sulfosuccinimidyl succinate) (EGS) for 20 min at room temperature. The reaction mixture was then subjected to a 4%–10% gradient SDS-PAGE and Western blot analysis using polyclonal anti-human HSF1 antibody at 1:5,000 dilution. Coomassie blue-stained SDS-polyacrylamide gel pattern of the same samples used for Western blot analysis was shown in (**C**). Each lane contained 25  $\mu$ g of protein.

be cross-linked by EGS, indicating that they remained as monomers in the cytosol (Fig. 4B, lanes 1 and 3). Under osmotic stress, the HSF1 proteins present in the nuclear fraction of presenescent cells were mostly cross-linked by EGS, indicating trimer formation (Fig. 4B, lane 6). However, the majority of HSF1 polypeptides (> 80%) in the osmotic stress-treated senescent cells remained as monomers in the cytosol and could not be cross-linked by EGS (Fig. 4B, lane 7). Taken together, the data suggested that the steps involved in HSF1 trimerization and nuclear translocation were compromised in senescent cells during osmotic stress.

#### MAPK signal transduction pathway in osmotic stress response

MAPK signal transduction pathways play an important role in mediating biologic signals generated by hormones, cytokines, growth factors, or stresses to the nucleus via a cascade of phosphorylation reactions that regulate the activity of various transcription factors (Karin, 1998). In yeast, a two-component osmosensing system, Sln1p and Ssk1p, is known to regulate the MAPK cascade (Maeda et al., 1994). In mammalian cells, it has been shown that HSF1 can serve as an *in vitro* substrate for kinases of all three MAPK families (Kim et al., 1997). It is therefore possible that MAPK cascade may be involved in osmotic stress-induced HSF1 activation. Because HSF1 activation can be induced by either hypo-osmotic or hyperosmotic stress, we reasoned that if the MAPK signal transduction pathways are involved in HSF1 activation, they should respond to hypo-osmotic and hyperosmotic stress equally well. The effects of medium osmolarity on the activation of MAPK pathways was monitored by the phosphorylation of the each MAPK using phosphospecific antibodies that only recognize the phosphorylated form of MAPKs. Figure 5 shows that whereas hyperosmotic stress (> 0.5 OsM) activated ERK1/2, JNK 1/2, and p38 kinase (Fig. 5, lanes 6–11), hypo-osmotic stress (0.1–0.2 OsM) had little or no effect on all these three kinases (Fig. 5, lanes 2 and 3). The control experiments indicated that MAPK activation was not detected in the cells maintained in Dulbecco's medium (Fig. 5, lane 1) and that anisomycin, an agonist for the MAPK cascade (Zinck et al., 1995), activated all three kinases (Fig. 5, lane 11). In all cases, the protein levels of these three kinases remained unchanged under either hypo-osmotic or hyperosmotic stress. Because the activation of MAPKs responded to hypo-osmotic and hyperosmotic stress in a different manner, it seems unlikely that MAPK cascade can be involved in the osmotic stress-induced activation of HSF1.

#### Osmotic stress and apoptosis

A variety of cellular stresses can initiate apoptosis, and some recent work shows that the underlying mechanism may involve stress-activated MAPK pathways (Kia et al., 1995; Cosulich and Clarke, 1996; Punyiczki and Fesus, 1998). Although both hypo-osmotic and hyperosmotic stress can induce the activation of HSF1 (Caruccio et al., 1997), we wonder whether they both also could cause apoptosis. If they do, it will be of interest to see whether the osmotic stress-induced apoptosis shows any age dependency, as in the case of HSF1 activation. Figure 6A shows that after an expo-

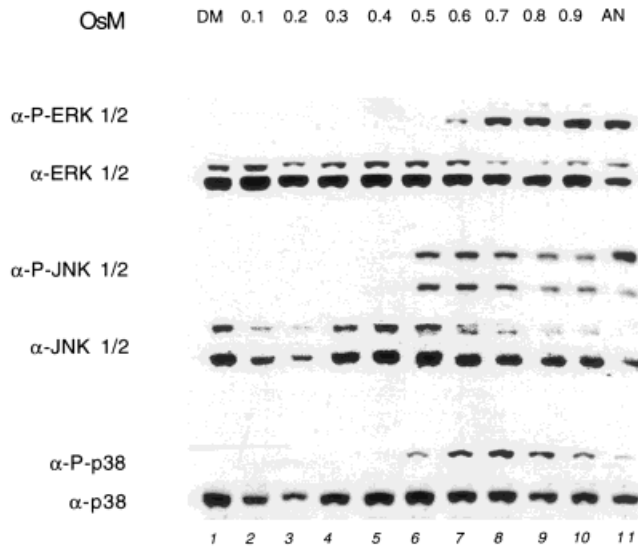


Fig. 5. Mitogen-activated protein kinase (MAPK) signal transduction pathways. The 90% confluent culture of HeLa cells were incubated for 30 min in a sorbitol solution (37°C) of various concentrations (0.1 Osm to 0.9 Osm) as indicated. Cells maintained in serum-containing Dulbecco's medium with (AN) or without (DM) anisomycin (1  $\mu$ g/ml) were used as positive and negative control, respectively. Cells were harvested and whole-cell extracts were prepared for SDS-PAGE and Western blot analysis using specific MAPK antibodies for ERK1/2, JNK1/2, and p38, and phosphospecific MAPK antibodies for ERK1/2, JNK1/2, and p38.

sure for 4 h to hyperosmotic stress, an extensive DNA fragmentation occurred in HeLa cells, indicating that osmotic stress can induce apoptosis. However, when both presenescent and senescent cells were exposed to the hyperosmotic stress for 4 h, no appreciable DNA fragmentation was observed (Fig. 6B). Further exposure of presenescent cells to hyperosmotic stress for up to 8 h resulted in an extensive DNA fragmentation (Fig. 6C, lane 2 vs. lane 1). Interestingly, senescent cells were still resistant to apoptosis under hyperosmotic stress (Fig. 6C, lane 4 vs. lane 3). Similarly, we found that hypo-osmotic stress induced apoptosis in presenescent cells but not in senescent cells (data not shown). These results indicated that despite the attenuated HSF1 activation, senescent cells were more resistant than presenescent cells in response to the osmotic stress-induced apoptosis.

## DISCUSSION

Maintenance of the optimal chemical potentials of metabolites is of vital importance for cell survival. It is therefore not surprising that living organisms have developed strategies to cope with osmotic stress (reviewed by Burg et al., 1997; Lang et al., 1998). One strategy that a living organism may use is to alter the expression of certain genes whose products may have critical regulatory or protective roles (Lang et al., 1998). In light of the role of HSPs as molecular chaperons in heat shock response, it is tempting to speculate that heat shock response also occurs in living organism during osmotic stress. Indeed, it has been shown that the HSF1 DNA-binding activity is prominently activated when mammalian cells are main-

tained in medium of hypo-osmolality or hyperosmolality (Huang et al., 1995; Caruccio et al., 1997; also Fig. 1). Because hypo-osmotic and hyperosmotic stress generally induce different sets of biological responses, including gene expressions (e.g., Lang et al., 1998), the observation that HSF1 activation occurs under both conditions (Fig. 1) is unique. The osmotic stress-induced HSF1 activation has also been shown to be uncoupled from *hsp* gene expression (Huang et al., 1995; Caruccio et al., 1997; Alfieri et al., 1996). Such uncoupling of HSF1-DNA-binding from *hsp* gene expression is not unusual and has been demonstrated in mammalian cells subjected to oxidative stress and salicylate treatment (Bruce et al., 1993; Jurivich et al., 1995). It is therefore possible that HSF1 may have biologic function other than regulating *hsp* gene expression. Recently, HSF1, but not HSPs, has been shown to be required for oogenesis and larval development of *Drosophila* (Jedlicka et al., 1997). Activated HSF1 has also been found in heat-shocked mitotic cells that are devoid of transcription (Jolly et al., 1999), suggesting a functional role of HSF1 on nuclear organization. Thus, our finding that osmotic stress-induced HSF1 activation was attenuated in normal human cells during cell senescence (Fig. 2) may suggest that HSF1 activation can be independently important for mammalian cells in response to hypo-osmotic or hyperosmotic stress.

The total amounts of HSF1 in presenescent and senescent cells were comparable either before or after osmotic stress (Figs. 3 and 4). However, on osmotic stress, HSF1 in presenescent cells formed trimers and became nuclear localized, whereas HSF1 in senescent cells remained in the cytoplasm as inactive monomers (Fig. 4). Our results provided direct evidence that rather than the decrease in protein amounts, the age-dependent suppression of HSF1 DNA-binding activity was due to the inability of the HSF1 in senescent cells to form trimer and to move into the nucleus. It has recently been shown that mutation of the a putative nuclear localization signal (NLS) in *Drosophila* HSF1 prevents nuclear translocation but not trimerization of HSF1 during heat shock (Orosz et al., 1996), suggesting that HSF1 trimerization may precede nuclear localization. If this is the case, our data would suggest that in senescent cells either HSF1 itself is incapable of forming trimer or the signal transduction pathway that lead to HSF1 activation is defect.

MAPKs form the central elements of signal transduction pathways that lead to and activate transcription factors in the nucleus and other effectors throughout the cell (Ip and Davis, 1998). HSF1 undergoes hyperphosphorylation on heat shock or other stresses (Larson et al., 1988; Cotto et al., 1996) and appears to be a good substrate of MAPKs (Kim et al., 1997). However, the finding that all three MAPKs, ERK1/2, JNK1/2, and p38, were activated only by hyperosmotic stress and not by hypo-osmotic stress (Fig. 5) makes it difficult to envision that these three kinases can be involved in the HSF1 activation induced by both kinds of osmotic stress. Alternatively, it is possible that although hypo-osmotic osmotic stress and hyperosmotic stress engage different signal transduction pathway, these different pathways could independently lead to HSF1 activation. If this is the case, MAPK pathways may still be involved in hyperosmotic stress-induced

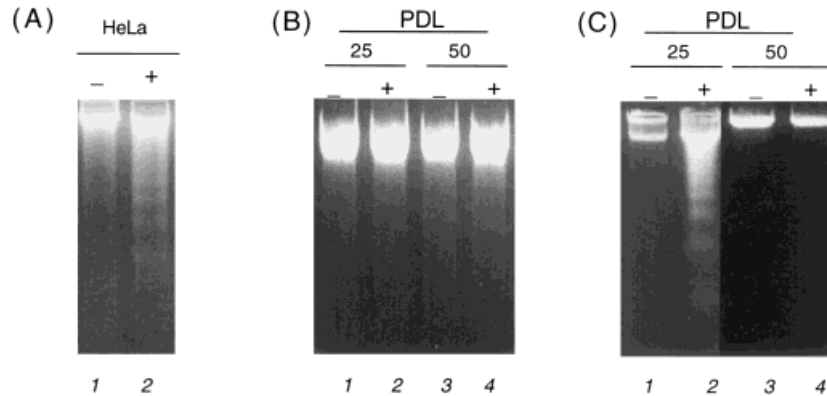


Fig. 6. Effects of osmotic stress on DNA fragmentation. Confluent HeLa cells (A) and IMR-90 (presenescent and senescent) cells (B and C) were incubated in fresh Dulbecco's medium in the absence (-) or in

the presence (+) of 0.7 M sorbitol for 4 h (A and B) and 8 h (C). Cells were harvested for DNA fragmentation analysis as described in Materials and Methods.

HSF1 activation and thus be relevant to senescence. Further studies are needed to identify the pathways that cause HSF1 activation and to the underlying mechanism that causes dysfunction of HSF1 in senescent cells.

We have previously speculated that the activated HSF1 may have a role in stabilizing chromosomal DNA during osmotic stress (Caruccio et al., 1997). Similar notion that HSF1 may have a structural role in protecting hypersensitive or fragile sites of the genome has also been advanced recently by Jolly et al. (1997). If the activated HSF1 does have a protective role of genome, one would expect that presenescent cells will be more resistant than senescent cells to the osmotic stress-induced apoptosis. This apparently is not the case. Instead, we found that senescent cells were much more resistant than presenescent cells to the osmotic stress-induced DNA fragmentation (Fig. 6). It is interesting to note that the propensity of presenescent cells to undergo programmed cell death has been previously noted for human fibroblasts (Wang, 1995) and for T lymphocytes in mice (Spaulding et al. 1997). In light of these studies, it is possible that the resistance to apoptosis by senescent cells under osmotic stress may be an independent phenomenon, unrelated to HSF1 activation. Alternatively, it is also possible that the HSF1 activation, uncoupled from HSP transcription, could mark presenescent cells for programmed cell death. Thus, despite the recent finding that HSF1 activation can be correlated with apoptosis (Schett et al., 1999), the precise role of HSF1 activation in the osmotic stress-induced apoptosis remains to be determined.

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