Journal of Cellular Biochemistry 102:1036–1042 (2007)

Effect of Thermal Stress on Early and Late Passaged Mouse Lens Epithelial Cells

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Abstract Cataract is an age related disease of protein aggregation. It has been suggested that aging affects the cells ability to protect protein integrity. The protein integrity, which is essential for cellular homeostasis, is maintained by a complex system of refolding or degradation of damaged proteins. The heat shock proteins (hsps) are the major contributors in the maintenance of protein integrity. The heat shock transcription factor (HSF-1) is the master regulator of all hsp synthesis in response to stress. This investigation examined the role of HSF-1 in the regulation of hsp synthesis in early and late passaged αTN-4 cells. Data collected in this study revealed that the nucleotide sequence of HSF-1 mRNA obtained from early and late passaged αTN-4 cells were identical. When early and late passaged cell were exposed to thermal stress, their hsp expression were also similar. HSP-40 expression was detected after 2 h of heat stress, whereas HSP-70 and low molecular weight heat shock protein αβ crystallin showed significantly increased synthesis 18 h post heat stress. The late passaged αTN-4 cells ability to upregulate hsps in response to heat stress could be due to its high replicative activities. The data presented here suggests a relationship between the presence of functional HSF-1 and sustained proliferative activities of the late passaged αTN-4 cell. J. Cell. Biochem. 102: 1036–1042, 2007. © 2007 Wiley-Liss, Inc.

Key words: heat shock factor (HSF-1); HSP-70; HSP-40; αβ crystallin; heat stress; cataract; aging; protein aggregation; tissue cultured lens epithelial cells

Cataract is lens opacity that is considered to be an age-related disease of protein aggregation [Congdon et al., 2004; Ma et al., 2004]. Cherian-Shaw et al. [1999] reported that aging human lenses have significantly decreased chaperone activity. The normal aging process is similar to the diseases of aging and upregulation of HSF-1 can delay or retard aging [Hsu et al., 2003]. The lens provides us with a unique model system to examine aging effects on both the epithelial cells and differentiated fiber cells [Min et al., 2004]. The cellular homeostasis which is essential for the normal function of a cell, is maintained by protecting the integrity of both functional and structural proteins [Goldberg, 2003]. The molecular chaperones are the major participants in the maintenance of protein integrity and cellular homeostasis. Aging diminishes chaperone activity resulting in the development of many diseases including Alzheimer's and cataract [Dobson, 2003]. In both senile cataract and stress-induced cataract, epithelial cell homeostasis is initially compromised followed by eventual protein aggregation in the cortical or nuclear region of the lens [Bagchi et al., 2002; Goswami et al., 2003]. However it is not known how impaired homeostasis of epithelial cells leads to protein disorganization in either cortical or nuclear fiber cells. Most aged tissues and cells lose the ability to maintain their protein organization in response to oxidative, osmotic or other stressors [Gutsmann-Conrad et al., 1998; Singh et al., 1999; Hall et al., 2000; Sitte et al., 2000a]. This could be due to the inability of aged cells to increase the expression of heat shock proteins (hsps) [Liu et al., 1989; Lund et al., 2000]. Tissue cultured cells nearing the end of their proliferative cycle, proliferative senescence, also show significantly reduced response

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Received 15 February 2007; Accepted 20 February 2007
DOI 10.1002/jcb.21339

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to stressors relative to early passaged cells [Sitte et al., 2000a]. Liu et al. [1989] reported that the age related decline in the induction of HSP-70(1) is associated with the reduced activity of transcription factor, HSF-1. Lund et al. [2000] reported that older RBC's synthesize 87% less HSF-1 and 80% less HSP-70(1), compared to young RBC's. We showed that aging human lenses have considerable lower levels of HSF-1 and hsps, HSP-70(1) and HSP-40 [Bagchi et al., 2002]. Hsieh et al. [1998] demonstrated that the ability of tissue cultured human cells to respond to stress declines with age. Transgenic mouse overexpressing HSP-70(1) can protect animals from heart attack, stroke and other diseases associated with the oxidative stress [Angelids et al., 1991; Locke et al., 1996]. Zou et al. [2003] reported that the heart of transgenic mice overexpressing HSF-1 recovered from oxidative damage of reperfusion/ischemia. On the other hand, HSF-$1^{(-/-)}$ cells derived from mouse embryos showed increased susceptibility to heat induced apoptosis [Gutsmann-Conrad et al., 1999]. Thus HSF-1 activity is critical for maintaining cellular integrity after heat stress, HSF-$1^{(-/-)}$ mouse cells do not develop thermotolerence and undergoes heat induced cell death [Xiao et al., 1999; Zhang et al., 2002a].

We believe that the aging lens, like other aged tissues, loses its ability to express optimal levels of hsps in response to stress leading to protein aggregation and disorganization and cataract. This investigation employed early and late passaged tissue cultured mouse lens epithelial cells (αTN-4) to determine whether repeatedly passaged cells (proliferative senescence), loose their ability to upregulate hsp synthesis in response to stress. We examined HSF-1 mRNA's of early and late passaged cells to determine the effects of passaging on HSF-1expression. Furthermore, we measured the expression of HSP-70(1), HSP-40 and αβ crystallins in early and late passaged cells in response to thermal stress. Results showed that early and late passaged (TN-4 cells have similar thermotolerence.

METHODS

Cell Culture and Heat Stress

Confluent αTN-4 cells [Yamada et al., 1990], obtained from Dr. P. Russel, National Eye Institute, were grown in DMEM, supplemented with 10% fetal bovine serum, in a humidified incubator at 37°C. The (TN-4 are lens epithelial cells obtained from transgenic mice. The mice were made transgenic by insertion of a hybrid gene comprising the murine (A promoter (−366 to +46) fused to the coding sequence of the SV-40 T antigen. The culture medium was maintained at 300 mmol and pH 7.2. Thermal stress on αTN-4 cells was produced by a 30 min exposure at 45°C. Some stressed cells were allowed to recuperate after heat exposure for 2 or18 h at 37°C. Total protein was isolated from heat stressed and control cells (without heat stress) and the protein concentration of each sample was determined by The Bradford method [Bidwell et al., 1993].

Western Blot

Equal amounts of protein from control and heated cells were separated by 10% SDS–PAGE. In all experiments duplicate gels were run. One gel was stained with Coomassie blue and the other gel was electrophoretically transferred to nitrocellulose paper. Specific heat shock proteins, HSP-70 and HSP-40 were identified by immunoblot analysis. All antibodies were obtained from Stressgen (Vancouver, Canada). Alkaline phosphatase conjugated secondary antibodies were utilized for color development.

HSF-1 DNA Extraction

Total RNA was isolated from early and late passaged αTN-4 cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The concentration of RNA was determined by absorption at 260 nM. Contaminating DNA was removed by incubation at 37°C for 30 min with amplication grade DNase (Life Technologies) in 10mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $Mgcl_2$ and 40 U or RNasin (5′3′inc. Boulder, CO). Total RNA was reverse transcribed. The cDNA equivalent to 100ng total RNA was subjected to PCR amplification. The following sets of primers was used for amplification, forward, 5′-atg gat ctg gcc gtg and ggc-3′, reverse—5′-gggg a cccc cta gg gac agt ggg gtc and ctt-3′. PCR commenced with a hot start at 94°C for 3 min and continued for 35 cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 57°C for 1 min, and extension at 72°C for 1 min. A final extension was conducted at 72°C for 5 min. and the PCR product was electrophoresed on a 1.5% agarose

gel. DNA bands were visualized with ethidium bromide staining. The appropriate DNA band, based on its molecular weight, was cut from the gel and purified using α1EX11 gel extraction kit (Quiagen, CA), sequenced at Wayne State University Nucleic acid core facility. The sequence was subjected to genebank search using Blast programme [Altschul et al., 1990].

RESULTS

The genebank search revealed that the amino acid sequence deduced from cDNA of αTN4 cells are homologous to mouse HSF-1, with alignment beginning at 400 amino acid of HSF-1 (Fig. 1). HSF1 mRNA from early and late passaged mouse lens cells is also identical. The protein profile and immunoblot results for early and late passaged cells exposed to heat for 30 min and than reincubated at 37°C for 2 h is shown in Figure 2. This figure also shows the data for control cells (no heat shock) kept at 37°C for duration of the experiment.

The SDS–PAGE protein profiles of heat shocked and control cells, both from early and late passaged appeared identical. However, immunoblots showed that levels of HSP-40 in heat stressed cells (early and late passaged) were considerably higher than in the control cells. Levels of inducible HSP-70(1) did not display any difference between control and stressed cells. Experiments were also performed on cells that were allowed to recuperate for 18 h after heat stress. Figure 3 shows that the protein profiles of all cell groups were similar. However, levels of HSP-70(1), HSP-40 and αβ crystallin were markedly higher in the heat stressed cells. We also examined the effect of severe heat stress on the early and late passaged cells. In these experiments tissue cultured αTN-4 cells were exposed to DMEM at 50°C for 20 min. After 20 min of heat exposure cells were incubated at 37°C for another 2 h, then the total protein of control and heated cells were separated by SDS–PAGE. Figure 4 shows that the protein profiles both heated and control early passaged cells were similar. However, the profile of late passaged cells exposed to heat stress at 50°C were significantly different, and displayed a distinct band around 70 kDa, which is not present in either control late passaged or heated early passaged cells. At present, identity of this protein is not known. Western blot analysis revealed that the 70 kDa protein is not a member of the heat shock protein family. Figure 5 shows the morphology of early and late passaged heat stressed cells after 18 h of recuperation in 37°C medium. Both groups of cells look similar and show no sign of dead or dying cells.

DISCUSSION

Aged tissues, or late passaged tissue cultured cells, have diminished stress tolerance [Gutsmann-Conrad et al., 1999; Hall et al., 2000; Sitte et al., 2000b; Ruotolo et al., 2003; Soti and Csermely, 2003]. The loss of stress tolerance could be due to decreased levels of heat shock proteins as a result of the inability of HSF-1' to upregulate hsps in response to stress. Thus, either decreased levels or diminished activity of HSF-1 can prevent overexpression of hsp in response to stress [Malhotra et al., 2002; Zhang et al., 2002b]. We determined HSF-1mRNA levels of both early and late passaged αTN-4 cells. The results showed that HSF-1 mRNA isolated from early and late passaged (TN-4 cells were identical. This finding can be explained if late passaged (TN-4 cells had not yet reached proliferative senescence.

It is also possible that the functional activity of HSF-1 mRNA is different even though the sequences are similar. To test this hypothesis,

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400
SHGFSVDTSALLDIQELLSPQEPPRPIEAENSNPDSGKQLVHYTAQPLFLL Mouse HSF-1
-------------------------------------------------- αTN4-cDNA

                                  483
DPDAVDTGSSELPVLFELGESSYFSEGDDYTD    Mouse HSF-1
--------------------------------    αTN4-cDNA
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Fig. 1. The amino acid sequence of cDNA obtained from αTN4 cells is homologous to mouse HSF-1.Homology between the amino acid sequence from αTN4cDNA to mouse HSF-1 protein is indicated by dashed lines. The number above denotes the HSF-1 amino acid that aligns with the amino acid deduced from the αTN4cDNA.

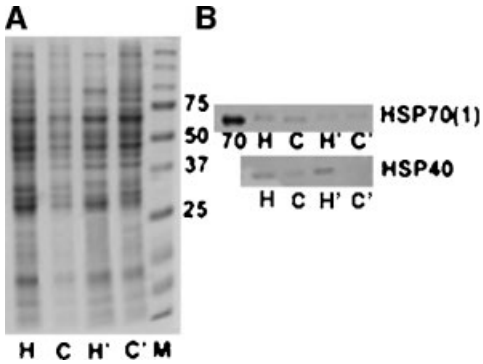

Fig. 2. SDS–PAGE protein profile of control and heat shocked αTN-4 cells. All heat stressed cell were exposed to 45°C thermal stress for 30 min. Heat stressed cells were then transferred to 37°C medium and allowed to recuperate for 2 h. Total protein was extracted from all cell groups, and immunobloted with antibodies to HSP-70(1) and HSP-40. **A**: SDS–PAGE profile; (**B**) immunoblot; H, heated (late passaged); H′, heated (early passaged); C, control (late passaged); C′, control (early passaged); 70, HSP-70(1) protein; M, molecular weight markers.

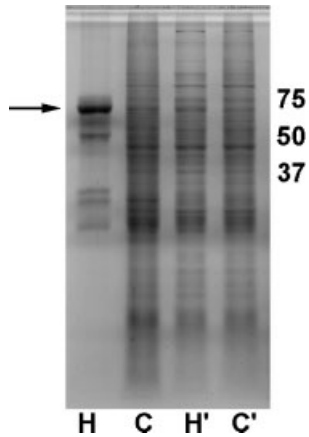

Fig. 4. SDS–PAGE protein profile of control and heated αTN-4 cells. Experimental cells were exposed to 50°C for 20 min than transferred to 37°C and allowed to recuperate for 2 h. Total protein was extracted from all cell groups. The arrow points to a distinct 70 kDa protein band.

semi confluent early and late passaged cells were exposed to heat for 30 min and then allowed to recuperate for 2 h. When levels of hsps were examined in these two groups of cells, HSP-70(1) synthesis was minimal, but both early and late passaged cells showed a marked increase of HSP-40. This suggests that HSP-40, a foldase chaperone, is upregulated immediately after exposure to heat, whereas HSP-70(1) synthesis required several hours of recuperation. Whether early HSP-40 synthesis plays a role in the subsequent upregulation of HSP-70(1) is not known. However, both HSP-70(1)

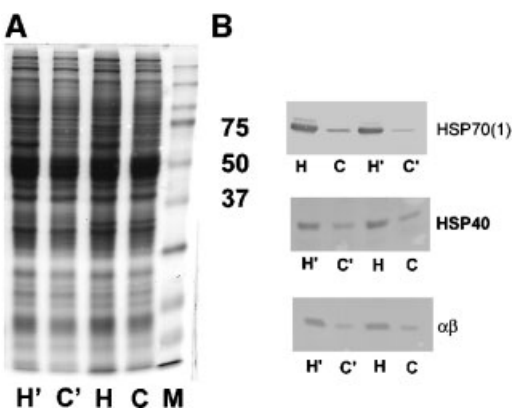

Fig. 3. Immunoblot of proteins isolated from early and late passaged cells exposed to heat stress. All heated cells were allowed to recuperate for 18 h at 37°C. **A**: SDS–PAGE profile; (**B**) immunoblot.

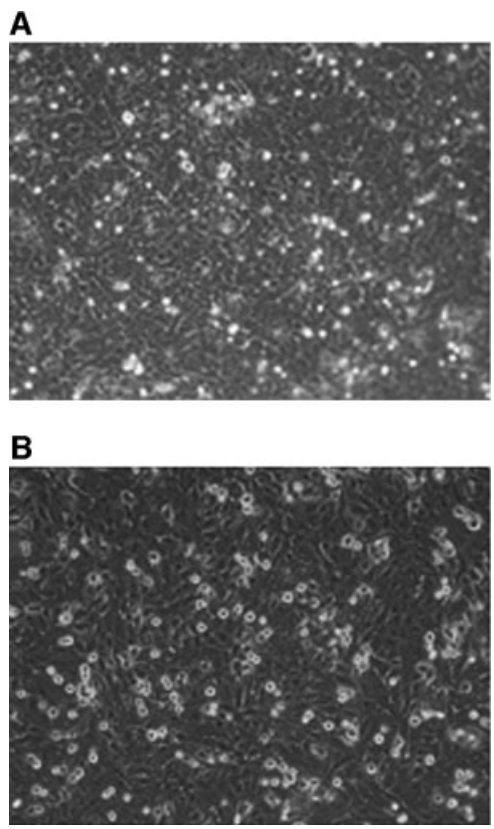

Fig. 5. Morphology of αTN-4 cells, heat stressed for 30 min at 45°C and allowed to recuperate at 37°C for 18 h. **A**: early passaged αTN-4 cells; (**B**) late passaged αTN-4 cells.

and HSP-40 work in tandem to protect proteins from degradation and are able to refold partially damaged protein [Liberek et al., 1995].

When heat stressed cells, from both early and late passaged cells, were allowed to recuperate for 18 h, all hsps including αβ crystallin showed a significant increase. Thus thermal stress not only upregulated foldase chaperones HSP-70 and HSP-40, but also the small heat shock protein αβ crystallin. The significance of the upregulation of αβ crystallin is not clear, as this holdase chaperone, is needed at the time of thermal shock to protect cellular protein from degradation. It could be argued that thermal stress affects the HSF-1 transcription factor, and since it is the master promoter of all heat shock proteins, it also upregulated synthesis of αβ crystallins [Zhang et al., 2002b; Soti and Csermely, 2003; Sun and MacRae, 2005]. Since αβ crystallin is involved in the stabilization and regulation of the cytoskeleton [Fujita et al., 2004] its upregulation could be indicative of its role in reestablishing cellular morphology in the heat stressed cells. Our results showed that, when early and late passaged cells were exposed to 45°C, both cell groups were able to recuperate and upregulate hsps in the same fashion. The protein profiles of all cell groups were similar, suggesting that by 18 h of recuperation all heat stressed proteins were either refolded, completely degraded or were not damaged.

Experiments were also performed to examine whether early and late passaged lens epithelial cells exposed to extreme heat have similar thermotolerence. Both cell groups were exposed to 50°C for 20 min and were allowed to recuperate for 2 and 18 h. Cells examined at 2 h of recuperation showed normal morphology, but were all dead at 18 h. SDS–PAGE analysis of the total proteins of heat stressed cells after 2 h of recuperation showed significant alterations, probably due to protein degradation. A prominent band was visible at 70 kDa. However, the protein profiles of control late passaged, and control and heated early passaged cells looked similar. These data suggest that late passaged cells are more susceptible to extreme heat stress. Molecular events associated with protein degradation of late passaged cells exposed to extreme heat stress are not known. Immunostaining showed that the prominent 70 kDa proteins is not HSP-70(1). Effects of heat on cell phenotype were also examined. Altered cell structure could indicate possible onset of apoptosis. Figure 5 depicts morphology of heat stressed cells after 18 h of recuperation. These cells look similar to control and are able to divide normally, if maintained in tissue culture. This data suggest that both early and late passaged lens epithelial cells were able to endure 45°C heat stress for 30 min, indicating the presence of functional HSF-1 and ability of these cells to upregulate hsp synthesis in response to heat stress and maintain protein integrity and cellular phenotype.

Gutsmann-Conrad et al. [1999] reported that HSF-1 levels of old rat splenocyte and hepatocytes were similar to that of younger rats, however, the ability of old rats to express hsp decreased significantly indicating decreased functional activity of HSF-1 in the older cells. Heydari et al. [1993] reported that the age related decline in HSP-70 expression was caused at the transcriptional level, by decreased binding of HSF-1 to the heat shock element of HSP-70. The down regulation of hsp synthesis could also be modulated by alterations in the nuclear matrix organization initiated by changes in the aging Lamin-C structure [Stein, 1993; Bagchi et al., 2007]. The lens epithelial cells (αTN-4) used in these experiments were immortalized with introduction of α-crystallin promoter fused with T-antigen, thus these cells do not display proliferative senescence and an age related decrease of HSF-1 activities. Recent studies showed that hsps are overexpressed in prostate cancer (PC) cells [Wang et al., 2004]. A dominant negative construct of HSF-1 introduced in the PC cells depleted cellular hsp levels and affected the cell cycle regulation of the malignant cells. Thus, HSF-1 could exert regulatory effects on cell proliferation, and depressed HSF-1 activity could be related to the development of the proliferative senescence. Xiao et al. [1999] reported that normal HSF-1 expression is required for postnatal growth, as both male and female HSF-1 negative mice were significantly smaller than wild type. Thus suggesting that the presence of functionally active HSF-1 are essential for sustained cell division.

In conclusion, data obtained from this investigation revealed that both early and late passaged αTN-4 cells exposed to thermal stress upregulated hsp. HSP-40 synthesis began within hours of heat stress, however upregulation of HSP-70 and αβ crystallin synthesis required 18 h post stress. The ability

of late passaged αTN-4 cells to upregulate hsps could be due to sustained proliferation of these tissue-cultured cells. We are investigating this possibility.

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