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Heat Shock Proteins of Adult and Embryonic Human Ocular Lenses

M. Bagchi,* M. Katar, and H. Maisel

Department of Anatomy and Cell Biology, School of Medicine,Wayne State University, Detroit, Michigan

Abstract We investigated the presence and distribution of heat shock proteins, HSP-70 [Horwitz, J. 1992. Proc Natl Acad Sci 89:10449–10453], HSP-40, HSc-70, HSP-27, and αβ-crystallin in different regions of adult and fetal human lenses and in aging human lens epithelial cells. This study was undertaken because heat shock proteins may play an important role in the maintenance of the supramolecular organization of the lens proteins. Human adult and fetal lenses were dissected to separate the epithelium, superficial cortex, intermediate cortex, and nucleus. The water soluble and insoluble protein fractions were separated by SDS–PAGE, and transferred to nitrocellulose paper. Specific antibodies were used to identify the presence of heat shock proteins in distinct regions of the lens. HSP-70 [Horwitz, 1992], HSP-40, and HSc-70 immunoreactivity was mainly detected in the epithelium and superficial cortical fiber cells of the adult human lens. The small heat shock proteins, HSP-27 and αβ-crystallin were found in all regions of the lens. Fetal human lenses showed immunoreactivity to all heat shock proteins. An aging study revealed a decrease in heat shock protein levels, except for HSP-27. The presence of HSP-70 [Horwitz, 1992], HSP-40, and HSc-70 in the epithelium and superficial cortical fiber cells imply a regional cell specific function, whereas the decrease of heat shock protein with age could be responsible for the loss of optimal protein organization, and the eventual appearance of age-related cataract. J. Cell. Biochem. 84: 278–284, 2002.

Key words: heat shock proteins; adult and fetal human lens; heat shock factor; HSP-27; α-crystallin

A detailed knowledge of how proteins retain their native organization is essential for the understanding of lens transparency. The mammalian lens preserves its clarity over a long period of time, indicating that the supramolecular structure of its proteins is protected from disorganization. However, due to the limited turnover of the lens proteins, their conformational preservation presents a serious problem. Maintenance of native protein structure is performed by a group of molecules named heat shock proteins. Horwitz [1992] reported that alpha crystallin, a small heat shock protein (sHSP) protects lens proteins from irreversible denaturation. The sHSPs are able to bind to a variety of partially unfolded or non-native proteins, and protect them from further denaturation [Tumminia and Russel, 1994]. Additionally in co-operation with large heat shock proteins (HSP-70 and HSP-40) and ATP, sHSP bound partially unfolded proteins can be refolded to their native configuration [Ehrnsperger et al., 1998]. HSP-70 and HSP-40 are also needed for proper folding of newly synthesized polypeptides [Freeman et al., 1995; Pierpaoli et al., 1997]. The large oligomeric proteins α and β crystallins, present additional problems for stringent protein folding, and probably require the active participation of HSP-70 and HSP-40 [Jaenicke, 1996]. It can be argued that the presence of an increased amount of insoluble high molecular weight protein in the opaque lens is due to the absence or malfunction of heat shock proteins [Manski et al., 1979]. Osmotic or oxidative stress could induce protein misfolding or disassembly causing the disorganization of lens proteins and eventual opacity. Heat shock proteins and ATP could refold or reassemble unfolded proteins. If these corrective measures fail, denatured proteins are either targeted for digestion or cells enter apoptotic pathway. Both of these processes are facilitated by heat shock proteins [Heydari et al., 1993; Wagner and Margolis, 1995].

*Correspondence to: M. Bagchi, Ph.D., Department of Anatomy and Cell Biology, School of Medicine, Wayne State University, Detroit, Michigan 48201.
E-mail: mbagchi@med.wayne.edu

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The lens provides an excellent model for analyzing the role of heat shock proteins in the preservation of protein structure during various metabolic activities. The epithelial cell layer displays active protein synthesis and breakdown, whereas the nuclear region of the lens contains embryonic proteins with no turnover. Nevertheless, the structural integrity of all proteins is preserved in clear lenses.

This study determined the distribution of several heat shock proteins in different regions of adult and embryonic human lenses. The results revealed a distinct specificity of heat shock protein distribution in different regions of the human lens, dependent on the metabolic activity of these regions and loss of many heat shock proteins with aging.

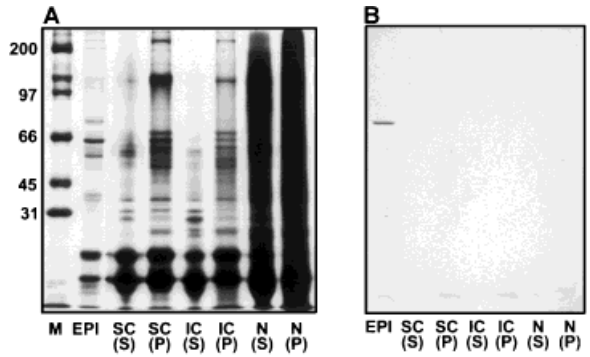

Fig. 1. SDS–PAGE profile of proteins isolated from a 52-year-old human male (**A**), and the corresponding immunoblot after reaction with polyclonal antibody to HSP-70 (**B**). Immunoblot. M, molecular weight markers; Epi, epithelium; SC, superficial cortex; S, soluble fraction; P, insoluble fraction; IC, intermediate cortex; N, nucleus.

MATERIALS AND METHODS

Isolation of Lens Proteins

Adult and fetal human lenses were obtained from the Oregon Lions Club and the Wayne State University School of Medicine Mortuary. The adult lenses were dissected to separate epithelium, superficial cortex, intermediate cortex, and nuclear fiber cells. The water soluble (WSF) and water insoluble fractions (WIF) of fiber cells were isolated as previously reported [Katar et al., 1993] and dissolved in 2% SDS containing buffer. The entire epithelium was dissolved in 2% SDS buffer. Intact fetal lenses were homogenized and the WSF and WIF fractions collected after centrifugation.

Western Blot Analysis

Lens proteins were separated by 10% SDS–PAGE, by Two-Dimensional gel electrophoresis [Bagchi et al., 2001a]. Some gels were stained with Coomassie blue and others were electrophoretically transferred to nitrocellulose paper [Atreya et al., 1989]. All antibodies and purified heat shock proteins were obtained from Stressgen (Victoria, BC, Canada). After exposure to the primary antibody, alkaline phosphatase labeled IgG (BioRad) was used as a secondary antibody, and color was developed using Bio-Rad reagents [Katar et al., 1993].

RESULTS

The SDS–PAGE protein profile of different regions of the fresh 52-year-old male human lens is shown in Figure 1A and the corresponding immunoblot using polyclonal antibody to HSP-70 in Figure 1B. HSP-70 [Horwitz, 1992] was detected in the epithelium. The WSF of the superficial cortical fiber cells showed only a faint band. HSP-70 was not detected in the deeper fibers of the lens. To further elucidate the presence of HSP-70 [Horwitz, 1992] in the WSF of the superficial cortical fibers, the protein was fractionated by two-dimensional gel electrophoresis. Figure 2A,B show the gel pattern and the immunoblot, respectively. Similar experiments with proteins obtained from other regions of the lens failed to reveal the presence of HSP-70 [Horwitz, 1992].

A distinctly different HSP-70 profile was present in the lens of a 53-year-old male obtained 24 h after death (Fig. 3A). HSP-70 was present in the epithelium, cortical, and nuclear fiber cells. The WIF contained a higher amount of HSP-70 [Horwitz, 1992] compared to the WSF. In addition several lower molecular weight reactions ($\approx$30 kDa) were evident in all regions of the lens. Adult chicken lens protein was used as a marker for HSP-70 [Horwitz, 1992] as it contains a significant amount of this protein [Horwitz, 1992; Bagchi et al., 2001b].

HSP-40 was detected in the epithelium and the superficial cortex WIF (Fig. 4). It is noteworthy that the epithelium and the WI fractions of the superficial and intermediate cortex also showed a cross-reaction with a protein of $\sim$80 kDa. This is likely due to the presence of HSP-40 dimers in these cells. Faint HSP-40 cross-reactive bands were also detected in the WSF of the intermediate cortex and both nuclear fractions.

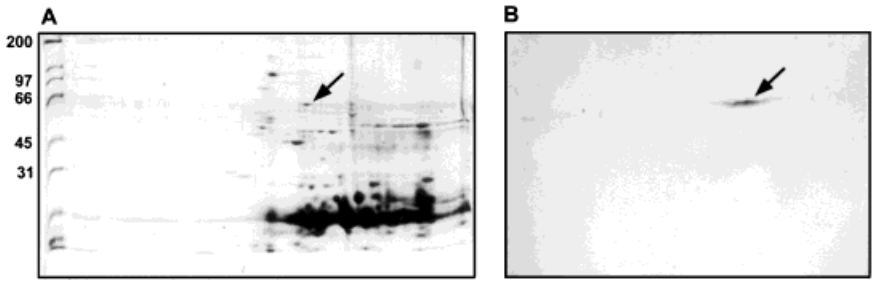

Fig. 2. Two-dimensional gel electrophoresis of proteins obtained from soluble fraction of human lens superficial cortical fiber cells, and stained with Coomassie blue (**A**). Duplicate set of two-dimensional SDS–PAGE separated proteins were blotted onto nitrocellulose paper and immunostained with polyclonal anti-HSP0-70 [Horwitz, 1992] antibodies (**B**).

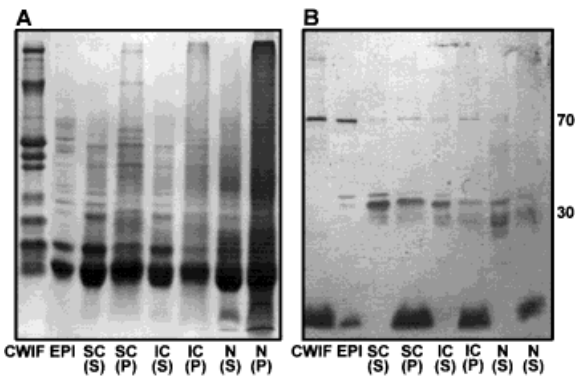

Fig. 3. Immunoblot of proteins obtained from a 53-year-old human male (24 h after death). Protocol used was similar to Figure 1. Immunostained with polyclonal anti-HSP-70 [Horwitz, 1992]. CWIF, chicken cortical WIF.

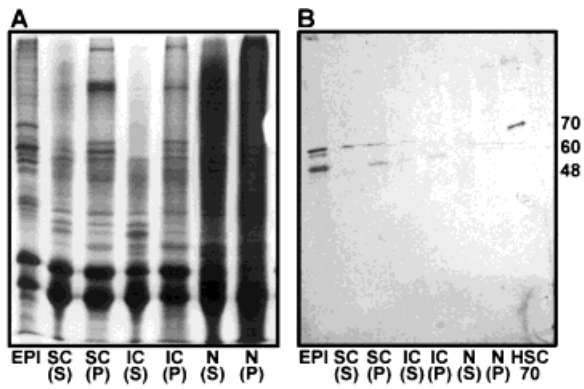

Fig. 5. Immunoblot of proteins obtained from a 52-year-old human lens. Monoclonal HSc-70 antibodies was used for immunostaining. HSc-70, purified HSc-70 protein.

Figure 5 shows the cross-reaction between HSc-70 monoclonal antibodies and proteins from discrete areas of the human lens. It is interesting to note that even though there were cross-reactions in the different lens fractions, the molecular weight of all these reactive proteins was significantly less than that of the purified HSc-70 protein. The epithelium showed three prominent bands, whereas the cortical regions reacted mainly with a protein of ~60 kDa. There were no reactions with nuclear protein. It is evident from Figure 5B that HSc-70 cross-reactive protein decreases significantly in the superficial and intermediate cortical region.

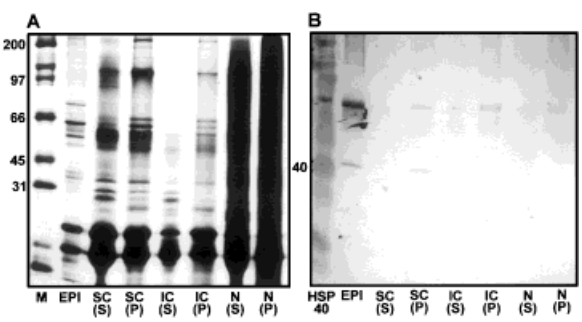

Fig. 4. Immunoblot of proteins obtained from a 52-year-old human male. Protocol used as in Figure 1. Polyclonal anti-HSP-40 antibodies were used for immunostaining. HSP-40, purified HSP-40, used as control.

The presence of the small heat shock proteins HSP-27 and αβ-crystallins was also determined. Figure 6B shows that HSP-27 was detected in all regions of the lens with trace amounts in the nuclear WIF. αβ-crystallin was present in all regions of the human lens (Fig. 6C).

The presence of heat shock protein in human lenses of different ages was also determined. Total proteins were extracted from the epithelial cells of 52-, 59-, 68-, 70-, 72-, and 76-year-old clear human lenses (Fig. 7A). Both HSP-70 [Horwitz, 1992] and HSP-40 were present in 52–72-year-old human lenses (Fig. 7C). Even though the 76-year-old human lens was transparent, it is possible that there was some protein breakdown since a clear protein banding

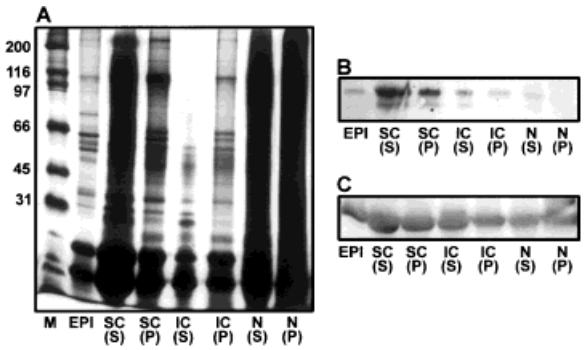

Fig. 6. Immunoblot of proteins obtained from a 52-year-old human lens. Polyclonal anti-HSP-27 and anti-αβ crystallin antibodies were used for immunostaining. **B**, immunostaining with HSP-27 AB; **C**, immunostaining with αβ crystallin AB.

pattern was not evident, and HSP-70 [Horwitz, 1992] and HSP-40 were not detected.

When antibodies to HSc-70, HSP-27, and NM-60 were used, it was found that HSc-70 (Fig. 8B) cross-reacted with lens proteins of 66 kDa or less and this decreased with age so that at 72 years, only a trace amount of HSc-70 cross-reaction was visible. It is interesting to note that HSP-27 increased markedly in older human lenses (Fig. 8C). The NM-60 heat shock transcription factor protein was present in the 52- and 59-year-old human lens epithelium, trace amounts in the 68-year lens, but was not detected in 70- and 72-year-old human lens epithelium.

The presence of HSP-70 [Horwitz, 1992] in human embryonic lenses was also determined. The protein profiles of 20-, 22-, and 27-week-old embryonic human lenses look similar (Fig. 9A)

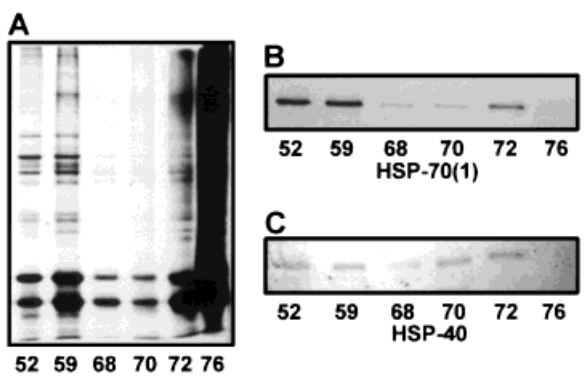

Fig. 7. Immunoblot analysis of proteins obtained from epithelial cells of 52–76-year-old human lenses. Polyclonal anti-HSP-70 [Horwitz, 1992] and anti-HSP-40 antibodies were used. **B**, immunoblot with HSP-70 [Horwitz, 1992] AB; **C**, immunoblot with HSP-40 AB; 52, 59, 68, 70, 72, 76, ages of the human lens.

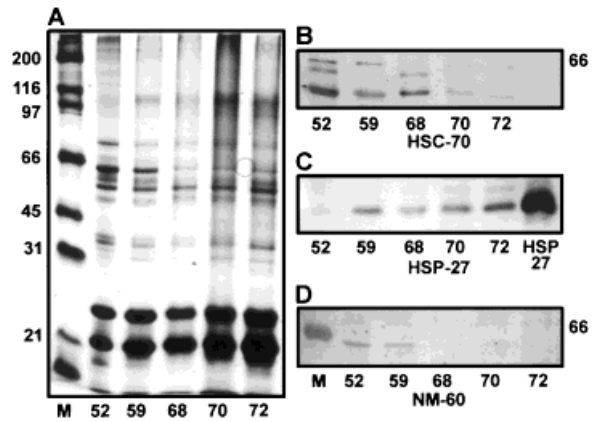

Fig. 8. Immunoblot of epithelial proteins obtained from aging human lenses. Polyclonal anti-HSc-70, anti-HSP-27 and anti-NM-60 antibodies were used. **B**, immunoblot with HSc-70 AB; **C**, immunoblot with HSP-27 AB; **D**, immunoblot with NM-60 AB; M, molecular weight markers; 66, denotes molecular weight.

and also show cross-reaction with HSP-70 [Horwitz, 1992]. Fetal lenses were obtained after a minimum of 24 h of death and so HSP-70 [Horwitz, 1992] cross-reaction with lower molecular weight protein could be due to partial proteolysis of lens protein. Figure 10 shows the presence of HSc-70 in the fetal human lenses. Fetal liver protein and purified HSc-70 protein were used as controls.

HSP-40 was also detected in the 16-week-old fetal human lenses (Fig. 11).

DISCUSSION

The results of these studies revealed that the inducible heat shock proteins HSP-70 and

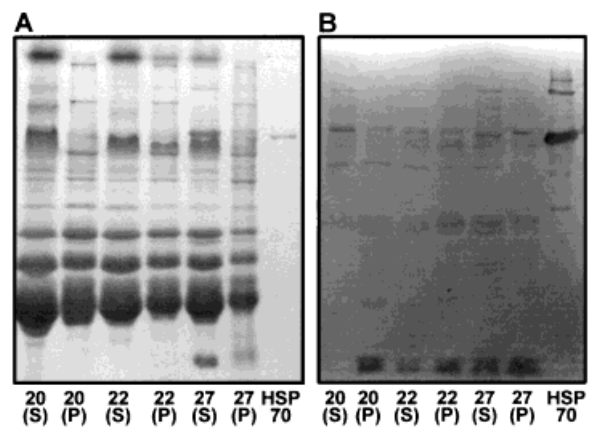

Fig. 9. Immunoblot of proteins obtained from human fetal lenses. Polyclonal anti-HSP-70 [Horwitz, 1992] antibodies were used for immunostaining. **A**, stained gel; **B**, immunoblot; S, soluble protein; P, insoluble protein; HSP-70, purified HSP-70 protein; 20-, 22-, 27-week old fetal lens.

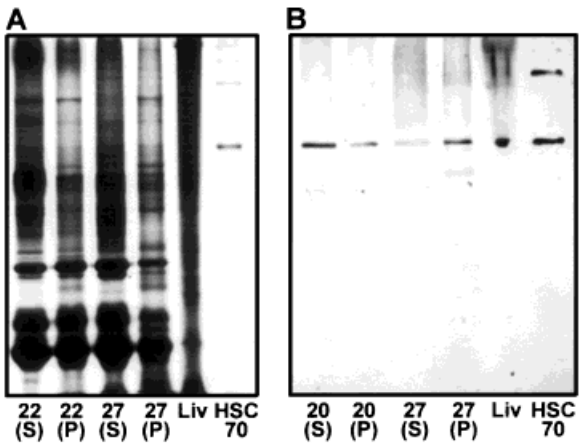

Fig. 10. Immunoblot analysis of proteins obtained from fetal human lenses. Polyclonal anti-HSP 40 antibody was used for immunostaining. LIV, protein obtained from fetal human liver (22-weeks-old); HSP-40, purified HSP-40.

HSP-40 are mainly present in the epithelial and superficial cortical fibers of adult human lenses in regions with active protein synthesis [Rothstein and Bagchi, 1969; Bagchi and Gordon, 1978]. Human lenses obtained 24 h after death showed a somewhat different protein pattern and cross-reaction with HSP-70 antibody. It is evident from Figure 3B that all regions of the lens, except the nuclear WIF, contained HSP-70. The presence of low molecular weight cross-reactions could be due to proteolytic cleavage of HSP-70. The absence of such reactions in freshly isolated human lenses could be due to the masking of the HSP-70 antibody binding epitopes by cellular proteins. Both bovine and calf lenses showed similar lower molecular weight cross-reactions with HSP-70 as there is always a few hours delay in obtaining lenses from the slaughter house (personal observation), whereas freshly sacrificed chicken lenses do not show any HSP-70 breakdown products [Bagchi et al., 2001b]. The data presented suggest that HSP-70 not only participates in protein refolding, but also binds with it to prevent further denaturation. This is especially important in the lens where most of the proteins have a low turnover rate. The lens is one of the few ocular tissues to show the presence of HSP-70 in unstressed conditions [Dean et al., 1999].

HSP-40, a Dnaj like protein, which participates in the refolding of denaturated protein in association with HSP-70 and ATP, is also present in epithelium and superficial cortical fiber cells [Silver and Way, 1993; Liberek et al., 1995]. It is evident from Figure 4B that HSP-40 is present in lens cells both as monomers and dimers. The HSP-40 dimer is detectable in all areas of the lens, whereas monomers were noted only in the epithelium and the WIF of the superficial cortical fibers. The presence of HSP-40 dimers is intriguing. It is possible that dimerization of HSP-40 is an inherent characteristic of this protein as purified HSP-40 also showed distinct dimerization.

The constitutive heat shock protein, HSc-70, is not stress inducible; it is present in most ocular tissues [Freeman and Morimotto, 1996; Morales et al., 1998] and plays an important role in stress induced expression of heat shock proteins [Silver and Way, 1993]. Figure 5B shows that all regions of the adult human lens contain the truncated form of HSc-70, most abundantly in the epithelium. The presence of

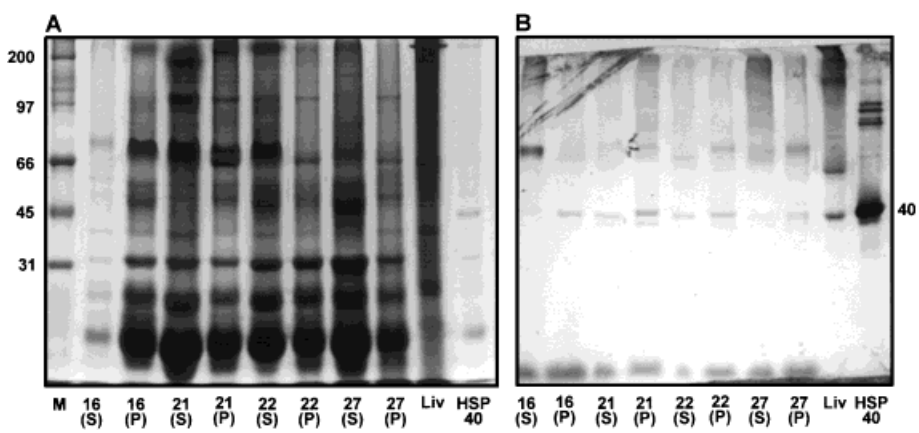

Fig. 11. Immunoblot of protein obtained from fetal human lenses. Monoclonal anti-HSc-70 ABs were used for immunostaining. HSc-70, purified HSc-70.

truncated HSc-70 in adult human lenses could be due to proteolysis since fetal HSc-70, as shown in Figure 10, has the same molecular weight as the purified HSc-70.

Jakob et al. [1999] proposed that there are two classes of heat shock protein. Holdases, are ATP-independent small HSPs (sHSP), which stably bind folding intermediate or partially denatured proteins and suppress otherwise lethal aggregation. "Foldases," such as HSP-70 and HSP-40, subsequently interact with Holdase bound intermediates in the presence of ATP, and convert them to their native state [Freeman and Morimotto, 1996].

Thus, we examined the sHSPs (Holdase) Hsp-27 and αβ-crystallin of adult human lenses [Inaguma et al., 1995]. HSP-27 is present in most of the regions of the lens and appears to be in higher concentration in the WSF of the superficial cortical fiber cells, αβ-crystallin is found in all areas of the lens including the nuclear WIF. It is evident that αβ-crystallin through its extensive distribution is probably the most important "Holdase" heat shock protein of the lens [Frederikase et al., 1994].

It has been reported that aging causes decreased heat shock protein activity, inducing protein denaturation and eventual apoptosis of eukaryotic cells [Heydari et al., 1993]. The human lens provides an excellent model system to study the effect of aging on heat shock proteins, as disorganization of its constituent protein induces opacity, an excellent morphological marker.

We examined the heat shock protein of clear lenses obtained from 52–76-year-old humans, and focused our study on the epithelial heat shock proteins. Figure 7 clearly shows that both inducible HSP-70 and HSP-40 are present in the epithelial cells of 52–72-year-old human lenses. Even though the lens of the 76-year-old human was clear, HSP-70 and HSP-40 were not detected. We used excessive amounts of 76-year-old human lens epithelial cell protein to confirm the absence of both HSP-70 and HSP-40. Constitutive HSc-70 is also present in the epithelial cells of all human lenses tested, but decreased with age (Fig. 8B). Small heat shock protein HSP-27 increased significantly with age, possibly to protect partially denatured protein from further denaturation or non-specific aggregation (Fig. 8C). It has been reported that in COS cells, HSP-27 can induce increased levels of glutathione, which protects cellular protein from oxidative and hypertensive stress [Singh et al., 1999]. Figure 8D shows that NM-60, a nuclear matrix protein, with extensive similarity to the heat shock transcription factor, decreased with age and was not detected in the epithelium of 72-year-old human lenses. A decrease in synthesis of NM 60, a regulatory protein of HSP synthesis, could induce a decline of total HSPs, disorganization of native protein, and eventual cataract formation [Bagchi et al., 2001a].

Fetal lenses show extensive protein synthesis and is therefore advantageous for the detection of heat shock proteins. Inducible HSP-70, constitutive HSc-70 and HSP-40 were detected in these lenses. It is interesting to note that fetal HSc-70 maintained its molecular weight, similar to universal HSc-70 (Fig. 10). Thus it can be proposed that after birth or due to aging HSc-70 shows post-translational modification, or that the adult lens HSc-70 is a new gene product.

The inducible HSP-70, is also present in both soluble and insoluble fractions of the fetal lens. The lower molecular weight cross-reactions could be due to breakdown products of HSP-70, since the lenses were collected at least 24 h after death. HSP-40 is also present in the fetal lens and shows a higher amount in the insoluble fraction.

In this investigation, we examined human lenses spanning from fetal life to 76 years after birth and detected both small and large heat shock proteins. The metabolically active anterior epithelial cells of the adult lens contain all heat shock proteins whereas the metabolically inactive nucleus contains only the sHSPs αβ-crystallin. The aging study showed that the large heat shock proteins, HSc-70, HSP-70 and HSP-40, decline with age. This decline could result in changes in the supra-molecular organization of the lens proteins and resultant opacity [Cherian and Abraham, 1995; Takemoto, 1996].

It is also interesting to note that unlike most ocular tissues, heat inducible HSP-70 is present in normal unstressed lenses. Thus it can be suggested that the normal microenvironment of the lens is stressful, inducing the continuous expression of HSP-70. In this regard, the retina, another ocular tissue which is always at a risk of hypoxia, displays the presence of HSP-70 in its inner segment of photoreceptors [Dean et al., 1999].

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