Enhanced Induction of a 72 kDa Heat Shock Protein in Cultured Retroocular Fibroblasts

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Fibroblasts in the retroocular connective tissue appear to play a central role in the pathogenesis of Graves' ophthalmopathy (GO). We hypothesize that specific attributes, differentiating normal from GO retroocular fibroblasts, may render the latter more vulnerable to the ongoing immunopathological process in GO. We investigated whether GO fibroblasts differ from normal fibroblasts with respect to their sensitivity and capacity to express the inducible 72 kDa heat shock protein (HSP) in response to stressful environmental stimuli. Cultured retroocular fibroblasts derived from patients with GO and normal individuals were exposed to various changes in the culture medium that may simulate conditions in the affected retroocular space in vivo. HSP 72 reactivity was determined using sodium dodecyl sulfate polyacrylamide-gel electrophoresis of cellular extracts, followed by immunoblotting with a mouse monoclonal anti-HSP 72 antibody and quantitative scanning densitometry. In addition, indirect immunofluorescence was performed on parallel fibroblast monolayers. Following exposure to heat and acidic pH, deprivation from nutrients, and high monolayer density, GO fibroblasts expressed HSP 72 with significantly greater sensitivity and in significantly higher abundance than did normal fibroblasts. These results demonstrate that changes in the physiological environment induce HSP 72 expression in cultured fibroblasts. The enhanced sensitivity and capacity of GO retroocular fibroblasts to express the inducible HSP 72 in response to stressful stimuli may play a role in the autoimmune process affecting the retroocular space in GO. Invest Ophthalmol Vis Sci 33:466-470, 1992

Highly conserved heat shock proteins (HSPs) are involved in basic cellular functions such as protein refolding and degradation, and in transport processes across intracellular compartments.1-3 In addition, these proteins play a role in the binding and function of certain hormones and their receptors.4,5 An increase in HSP-synthesis in cells exposed to stressful environmental stimuli appears to be a universal phenomenon that is critical for the maintenance of cellular homeostasis.6 Such stimuli include elevated temperatures, reactive oxygen metabolites, tissue trauma caused by ischemia or reperfusion injury, inhibition of energy metabolism, cytokines, and infectious microorganisms.6,7 Recently, immunological functions have been suggested for HSPs, including intracellular processing, membrane anchoring, and surface presentation of antigens to the immune system.8-9 The expression of several HSPs, as well as antibodies against these HSPs, has been reported in a variety of infectious and autoimmune diseases.8 In addition, highly immunogenic HSPs, or molecules sharing epitope homologies with these proteins, are capable of eliciting specific T cell responses.10,11 Thus, the expression of certain HSPs, as well as their local abundance, may be important in site-directed autoimmune responses.

Increased numbers of monocytes/macrophages, mast cells, and lymphocytes, and the accumulation of extracellular matrix compounds such as glycosaminoglycans are prominent histologic features of the retroocular tissues involved in Graves' ophthalmopathy (GO).12,13 Fibroblasts in the retroocular connective tissue appear to play a central role in the pathogenesis of this autoimmune condition, in part by their capacity to synthesize glycosaminoglycans.14,15

We have demonstrated previously that retroocular fibroblasts differ from skin fibroblasts regarding their synthesis of glycosaminoglycans in response to triiodothyronine, glucocorticoids15 and interferon-gamma.16 In addition, we have reported that cultured retroocular fibroblasts from patients with GO express a 72 kDa HSP (HSP 72) under standard culture conditions, and that this expression persists for at least 8 cell passages. In contrast, retroocular fibroblasts from normal individuals express HSP 72 only after exposure to stressful stimuli, such as cytokines,7 reactive oxygen species,17 and prostaglandins.18

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We hypothesize that undefined specific attributes of Graves' retroocular fibroblasts, not present in normal retroocular fibroblasts, may render these cells more vulnerable to the ongoing immunopathologic process in GO. One possible feature particular to these fibroblasts could be their sensitivity to stressful environmental stimuli in terms of their capacity to express immunogenic molecules such as HSPs. Therefore, we investigated the influence of various culture conditions on retroocular fibroblasts HSP 72 expression in vitro, comparing cell monolayers derived from patients with GO with those of normal individuals.

**Materials and Methods**

**Cell Cultures**

Retroocular connective tissue was obtained from patients during orbital decompression surgery for severe GO (n = 3). Normal retroocular fibroblasts were obtained during ophthalmic surgery for unrelated conditions (n = 3). Prior to starting the experiments, cells were cultured in medium 199 containing fetal bovine serum (FBS) (10%, pH 7.25) as described previously.14 All cell strains were used between the 6th and 8th passages.

Heat shock was induced by exposing the cell monolayers to elevated temperatures (43°C for 90 min). Cells were allowed to recover in fresh medium for 4 h after heat shock before harvesting in sodium dodecyl sulfate (SDS, 0.5%) using a rubber policeman. Protein concentrations were measured and cell lysates were subjected immediately to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or stored at -70°C until used.

**SDS-PAGE and Immunoblotting**

SDS-PAGE, immunoblotting, and quantification of HSP 72 immunoreactivity were performed as described previously.7 In brief, extracts of cultured fibroblasts were subjected to SDS-PAGE under reducing conditions using 4% polyacrylamide stacking gels and 10% polyacrylamide running gels. Following electrophoretic transfer onto nitrocellulose, immunoblotting was performed using a mouse anti-HSP 72 monoclonal antibody (StressGen Biotechnology Corp., Sidney, BC, Canada), biotinylated streptavidin-peroxidase complex (Amersham Corporation, Arlington Heights, IL), and a peroxidase substrate system with diaminobenzidine as the chromogen. HSP 72-immunoreactivity was quantitated using scanning densitometry, and the measured intensities were calculated as arbitrary units.

**Indirect Immunofluorescence**

Fibroblasts were plated directly on multichamber slides, grown to the appropriate degree of confluence, exposed to stress as indicated, and processed for indirect immunofluorescence as described previously.3 Briefly, viable monolayers were fixed in 100% methanol for cytoplasmic staining of HSP 72 using the mouse anti-HSP 72 monoclonal antibody (dilution 1:1000), biotinylated anti-mouse immunoglobulin (dilution 1:100), and fluorescein streptavidin (dilution 1:50). Air-dried and mounted slides were examined with a fluorescence Zeiss III photomicroscope equipped with epillumination (final magnification ×240). Parallel monolayers with the primary antibody replaced by unrelated monoclonal antibodies of the same isotype and with each step omitted in turn were examined to ensure specificity of staining and to exclude cross-reactivities between the antibodies and conjugates employed.

**Statistical Analysis**

Inter- and intra-assay variabilities for measurements of HSP 72 by SDS-PAGE and immunoblotting were determined and described previously.7 All data points represent the mean ± SD of samples derived from fibroblast monolayers of three different patients or normal individuals. Student's t-test for analysis of paired and unpaired data was used to assess the level of statistical significance.

**Results**

Exposure to elevated temperatures induced HSP 72 expression in normal retroocular fibroblasts and strongly enhanced HSP 72 expression in GO retroocular fibroblasts (Fig. 1). Minimal temperatures required to induce a significant increase in HSP 72 expression were 40°C for GO retroocular fibroblasts (P < 0.001) and 42°C for normal retroocular fibroblasts (P < 0.0001). Both patient and normal fibroblasts displayed their maximal level of HSP 72 expression at 43°–44°C. However, the abundance of HSP 72 ex-
expression at these maximally effective temperatures was significantly higher in GO retroocular fibroblasts than in normal controls ($P < 0.001$).

Figure 2 demonstrates the pattern of HSP 72 expression during 16 hours of recovery following heat stress at 43°C for 90 min. Significantly higher levels of HSP 72 expression were detected in retroocular fibroblasts from patients with GO at each time point ($P < 0.001$). HSP 72 expression was maximal at 4-6 h following heat stress in cells from patients with GO and normal individuals. However, while HSP 72 expression in normal cells returned to undetectable levels within 13 h, levels of HSP 72 expression in GO fibroblasts declined more slowly and remained elevated over baseline throughout the study period of 16 h.

Exposure of retroocular fibroblast monolayers to tissue culture medium of various pH values for 120 min induced changes in HSP 72 expression (Fig. 3). Exposure to a medium of acidic pH values induced a characteristic shift in cellular HSP 72 expression from the cytoplasm to the nuclear compartment, followed by redistribution during the recovery period (Fig. 4). Both patient and normal fibroblasts showed greater sensitivity to acidic than to alkaline pH alterations. A significantly higher abundance of HSP 72 expression was detected in GO fibroblasts compared with normal fibroblasts at all study points ($P < 0.001$). The minimal acidic pH change required to significantly increase HSP 72 expression over baseline (pH 7.25) was determined as $-0.25$ (pH 7.0) for GO fibroblasts ($P < 0.05$) and $-0.75$ (pH 6.5) for normal fibroblasts ($P < 0.05$). Exposure to a more acidic extracellular environment (pH 6.0) significantly enhanced HSP 72 expression in GO fibroblasts ($P < 0.05$) but did not significantly alter HSP 72 expression in normal fibroblasts. In patient fibroblasts, exposure to alkanalyzed culture medium (pH 9.0) resulted in a significant increase in HSP 72 expression ($P < 0.05$). No significant increase over background HSP 72 reactivity was detected in normal retroocular fibroblasts exposed to alkaline culture conditions.

Subconfluent degrees of cell density did not alter HSP 72 expression in normal or patient retroocular fibroblasts. However, in contrast to normal fibroblasts, HSP 72 expression in patient fibroblasts were significantly increased at 100% confluency ($P < 0.05$) and in the “crowded state” (over 100% confluency; $P < 0.001$) (Fig. 5). In “crowded” monolayers from patients with GO, deprivation from nutrient supply (starvation by exposure to nonrenewed medium 199 containing 1% FBS for 10 days) enhanced HSP 72 expression three-fold over controls fed every three days with fresh medium 199 containing 10% FBS ($P < 0.001$). Starvation of “crowded” monolayers from normal individuals also significantly increased the abundance of HSP 72 expression over fed controls ($P < 0.001$). However, this increase was of significantly smaller magnitude than that observed in GO patient fibroblasts ($P < 0.001$).

**Discussion**

Proptosis, impairment of extraocular muscle function, and optic nerve compression are clinical hallmarks of GO. These conditions are consequences of the increased volume and pressure that arise from expanding retroocular structures within the limited space of the bony orbit. This increase in tissue volume is largely a result of the accumulation of glycosaminoglycans, hydrophilic macromolecules produced by fibroblasts within the orbit. The inflammatory and edematous changes commonly observed in the eyes and periorbital regions can be attributed to venous congestion, inflammatory cellular infiltrates, and the release of inflammatory and immune mediators within the orbit.
We reported previously that retroocular fibroblasts from patients with GO, unlike normal fibroblasts, express the inducible HSP 72 under standard culture conditions. The current study examines the modulation of HSP 72 expression by certain modifications in the extracellular milieu of cultured retroocular fibroblasts, in analogy to pathophysiological conditions likely present with the orbit in vivo. We compared HSP 72 expression in fibroblasts from patients with GO with that in normal retroocular fibroblasts to determine whether patients’ fibroblasts are more susceptible to environmental stress in terms of HSP 72 expression.

Our results demonstrate that significant differences exist in the sensitivity and magnitude of stress-induced HSP 72 expression between cultured normal and GO fibroblasts. Furthermore, this increased sensitivity and magnitude of HSP 72 expression is observed in GO fibroblasts that have been subcultured 6–8 times. We reported previously that baseline HSP 72 expression is present only in fibroblasts from patients with GO and that this expression persists for up to 8 cell passages. These observations argue against a “carry-over” phenomenon, in which stressful stimuli might be transferred into primary cultures, because HSP 72 expression is demonstrated in new generations of GO fibroblasts cultured under standard conditions. Therefore, apparently some factor in GO is responsible for the initial stimulation of HSP 72 expression in vivo and that the effect of this factor persists even in its absence in vitro. The effect is manifest in GO fibroblasts as baseline HSP 72 reactivity and as increased sensitivity of HSP 72 expression in response to subsequent stressful stimuli. Thus, a chronic or pri-
Fig. 5. HSP 72 expression in cultured normal and GO orbital fibroblasts (OF) at various states of cell confluency and nutrient supply. "Crowded" cell monolayers were over 100% confluent. "Starved" cells were exposed to nonrenewed medium 199 containing 1% FBS for 10 days.

mary alteration in cellular homeostasis or function, initially induced in vivo, may be responsible for our observations in vitro.

In conclusion, our results demonstrate an increased sensitivity and magnitude of HSP 72 induction in Graves’ retroocular fibroblast cultures, compared with cultures of normal retroocular fibroblasts, when exposed to various stressful environmental stimuli of potential biological relevance in the pathogenesis of GO. Chronic stimulation of expression of the inducible 72 kDa HSP in vivo may actively contribute to the local autoimmune process in GO.

Key words: Graves’ ophthalmopathy, heat shock protein, stress response, retroocular fibroblasts, autoimmunity

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References