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Retinal uptake of intravitreally injected Hsc/Hsp70 and its effect on susceptibility to light damage

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Purpose: To evaluate the uptake by the rat retina of an intravitreally injected mixture of the constitutive and inducible forms of the 70 kD heat shock protein (Hsc/Hsp70) and test its potential to protect photoreceptors from light damage.
Methods: Hsc/Hsp70 and actin (control protein) were labeled with fluorescein (referred to as fl-Hsc/Hsp70 and fl-actin). The labeled proteins were microinjected intravitreally into the normal or light damaged rat eye and each eye collected at three intervals after the injections. Retinal uptake of Hsc/Hsp70 or actin was studied in frozen sections using epifluorescence microscopy and in western blots of retinal homogenates using an anti-fluorescein antibody. Additionally, the cytoprotective effects of Hsc/Hsp70 were tested in rats that first were exposed to bright light (170 ft-c) for 24 h and then given an intravitreal injection of the protein immediately thereafter. Ten days later, photoreceptor damage was evaluated by measuring the area of the outer nuclear layer at fixed locations along the circumference of the retina.
Results: The fluorescein-labeled proteins were detected in the retina one h after administration and were retained there for more than 6 h. They were diffusely distributed, primarily in the nerve fiber layer, ganglion cell layer, and plexiform layers. Fl-Hsc/Hsp70 was also found in the outer nuclear layer (ONL) at 6 h after injection. At 24 h post-injection, the proteins were undetectable by epifluorescence microscopy of retinal sections, but could still be detected in western blots of retinal homogenates. The pattern of protein uptake was similar in light-damaged retinas. Ten days after light damage, the retinas in those eyes that received injections of Hsc/Hsp70 had greater ONL areas compared to either the light-damaged retinas of uninjected eyes or those that had received actin. The difference was statistically significant ($p<0.05$).
Conclusions: Intravitreally injected Hsc/Hsp70 is taken up by retinal cells and, when administered after an acute injury like light damage, increased the number of surviving photoreceptors.

In cells or tissues that are subjected to potentially lethal conditions, heat shock proteins (Hsps) accumulate intracellularly in great amounts and serve as molecular chaperones to effect cell recovery after stress by preventing the misfolding and aggregation of many kinds of cellular proteins. This is known as the heat shock response [1]. One of the most extensively studied sets of Hsps, the Hsp70 family, includes two functionally distinct isoforms, the constitutive form, Hsc70, and the stress-inducible form, Hsp70. The fundamental role of Hsc70 as a molecular chaperone includes the following: facilitation of protein folding and assembly during normal protein synthesis [2-5], removal of clathrin from coated vesicles [6], and translocation of certain newly synthesized proteins from the cytoplasm into the mitochondrion, the lysosome and other intracellular organelles [7-11]. Hsp70 appears to be more involved than Hsc70 with the prevention of misfolding and aggregation of cytoplasmic and nuclear proteins during abnormal, stressful conditions [2,12], since it is absent or at very low levels in normal cells, but accumulates to much higher levels following acute stress [2]. However, Hsp70 and Hsc70 have over 90% sequence identity and have been shown to function similarly in the stressed cell [13]. In fact, a recent report [14] indicates that overexpression of either form of the protein can inhibit apoptosis by interfering with the processing of procaspases 9 and 3, so the issue of whether they have distinct functions remains to be resolved. Nonetheless, there are a great number of reports documenting the protective effects of the Hsp70 family in numerous cells and tissues against a remarkably diverse variety of stressors and cytotoxic factors [15].

In the normal retina of the rat, Hsc70 immunoreactivity is abundant and is present in all layers except the outer segments (OS) [16,17]. Unlike most other tissues, immunoreactivity for Hsp70, the so-called stress-inducible form of the 70 kD Hsps, is also prominent in the normal retina, although its distribution is more restricted than that of Hsc70, being found only in the outer nuclear (ONL) and the inner segments (IS) [17]. This observation suggests that, in the retina, Hsc70 and Hsp70 have distinct functions, even under normal conditions [17]. Nonetheless, the retina still demonstrates a typical stress-inducible increase in Hsp70 in response to hyperthermia and heavy metal toxicity [18,19]. Following hyperthermic stress, Hsp70 mRNA was found to increase significantly in the ONL and IS and the protein increased in the OPL, inner plexiform layer (IPL), and retinal ganglion cell layer (RGCL) [18].

Previous work in this laboratory showed that the Hsp70 family could protect retinal cells against lethal damage. A brief period of hyperthermia in the whole animal was found to make

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retinal cells more resistant to later light damage [20]. A close correlation was observed between the timing of the rise and fall of retinal Hsps and the sensitivity of the photoreceptors to light damage [18]. Furthermore, preliminary tests suggested that an intravitreal injection of a mixture of Hsc70 and Hsp70 (purified from bovine brain) before light exposure inhibited the light-induced degeneration [21]. Another intriguing aspect of the retinal Hsc/Hsp70 response was observed in the Royal College of Surgeons (RCS) rat, in which a genetic defect leads to the degeneration of most of the photoreceptors within the first two months after birth. After the loss of the photoreceptors, the level of Hsc/Hsp70 in the remaining retinal cells was lower than that of a normal rat eye, which prompted the speculation that the photoreceptors contain a major portion of the Hsc/Hsp70 found in the normal retina [16].

This study documents the importance of the Hsps in the photoreceptor cell (PRC) response to lethal stress. Additionally, it supports the potential clinical use of Hsps for the treatment of eye injury and disease.

METHODS

Sources of materials and animals: Hsc/Hsp70 mixture (ratio of about 4:6 [22]) was purified from bovine skeletal muscle (provided by B. A. Margulis, Russian Academy of Science, St. Petersburg, Russia). This preparation was employed for three reasons. First, because it remains unresolved whether Hsc70 and Hsp70 have distinctive functions in the stressed cell (see Introduction), we felt that providing both proteins would increase the likelihood of a positive outcome. Second, the mixture mimics the situation found in the stressed cell, in which both Hsc70 and stress-induced Hsp70 co-mingle. Third, Margulis and co-workers had previously documented that their preparation had biological cytoprotective activities in cultured cells [22].

The control protein, bovine skeletal muscle actin, was purchased from Sigma (St. Louis, MO; catalog number A3653). It was selected for this purpose, rather than the typically used bovine serum albumin (BSA) because it has more in common with the Hsps than BSA. It is a cytoplasmic protein, not known to be secreted and the tertiary structure of its ATP-binding domain is very similar to that of Hsp70 [23]. Fluorescent labeling of both actin and Hsc/Hsp70 was accomplished using 5-(and 6-)-carboxyfluorescein, succinimidyl proprionatefrom Molecular Probes (Eugene, OR; catalog number C1311).

Normal male albino Sprague-Dawley rats were purchased from Zivic Laboratories, Inc. (Pittsburgh, PA) at about 50 days of age (about 250 g) and were housed for at least one week prior to use. All procedures concerning animals in this study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Labeling purified Hsc/Hsp70 and actin with fluorescein: Hsc/Hsp70 and the control protein, actin, were labeled with fluorescein succinimidyl propionate according to standard procedures recommended by Molecular Probes. This form of fluorescein binds covalently to lysine residues of the protein under the basic aqueous conditions under which the labeling reaction was conducted. The labeled proteins were separated from free fluorescein by repeated filtration of the protein solution through a Millipore (Bedford, MA) filter centrifuge tube with a 10 kD pore size. The filtration was repeated three times, which allowed the reaction solution to be replaced with a physiologically compatible solution, sterile buffered saline. The success of labeling was verified by running a portion of each protein sample on a western blot and probing it with an antifluorescein monoclonal antibody (see western blot method below).

Light damage: Animals were kept in 12 h cyclic light (2~6 ft-candles) for at least one week before treatment. They were then exposed to 175 ft-c of illumination from standard Cool-White™ fluorescent tubes for 24 h at room temperature, beginning at about 9:00 AM (without prior dark-adaptation). Immediately after light exposure, the animals were given intravitreal injections of test or control solutions (2 µl, see below) and then kept in cyclic light for the remaining time.

Intravitreal injection: Animals were injected intramuscularly with a mixture of 10 mg/kg xylazine and 50 mg/kg ketamine to induce anesthesia. A single injection of 2 µl of protein solution in buffered saline into the vitreous cavity was made in the following manner. A standard sterile, disposable, 30 gauge hypodermic needle was broken from its Luer-lock hub and the end inserted into a length of polyethylene tubing (Intramedic PE10 or the equivalent). The other end of the tubing was connected to a 25 µl Hamilton syringe held in a Hamilton repeating dispenser device (PB600-10), which allowed 0.5 µl to be injected with each push of the dispenser button. The syringe and a portion of the tubing volume were back-loaded with deionized, Millipore-filtered water and then the protein solution to be injected was drawn up through the hypodermic needle into the tubing, separated from the water by a small air bubble. The volume of protein solution drawn up was sufficient to make injections into several eyes, but the hypodermic needle was replaced with a new one for each injection.

To study the retinal uptake of the protein, groups of normal rats were given intravitreal injections of 10 µg of fl-Hsc/Hsp70 in one eye and 10 µg of fl-actin (control protein) in the other eye. The effect of Hsp70 on the light-induced photoreceptor cell death was studied by similar injections immediately after the light exposure of 10 µg of each of the two proteins, but without prior labeling with fluorescein. After the injections, the animals were kept in a 30 °C chamber to prevent them from becoming hypothermic until the anesthetic wore off. The rats were then maintained at room temperature and 2~6 ft-c of illuminance during the remaining time.

Western blot: Immediately after euthanizing each rat using CO_2 inhalation, the retinas were extruded and homogenized in sample solution (8 M urea, 2% SDS). The protein concentration of the samples was determined by protein assay (BioRad Coomassie blue dye binding assay, Hercules, CA). 100 ng protein were loaded in each lane of a 1.5 mm, 10% SDS-polyacrylamide gel (Pharmacia Hoefer minigel apparatus, Amersham Pharmacia Biotech, Inc., Piscataway, NJ) for electrophoresis followed by western blotting. The blots were incubated with a mouse monoclonal primary antibody against

fluorescein (Molecular Probes; catalog number A-6421) for one h, then probed with secondary antibody, anti-mouse IgG conjugated to HRP. The antigen-antibody complexes were detected by diaminobenzidine (DAB) or Vector VIP substrate kit for peroxidase (Vector Laboratories Inc., Burlingame, CA).

Histology: At 1, 6, or 24 h after injection, the eyes were collected and fixed by immersion in 4% paraformaldehyde overnight. Following removal of cornea and lens, the whole eye cups were embedded in OTC Compound (Ted Pella, Inc., Redding, CA). Sections were cut on a Leica CM3000 freezing microtome (Leica, Deerfield, IL) at 12 µm. Retinal sections through the optic nerve in the superior-inferior plane were collected. The sections were mounted on ProbeOn Plus microscope slides (Fisher Scientific, Pittsburgh, PA) and covered with Immu-Mount (Shandon Corp, Pittsburgh, PA). Several normal, uninjected eyes also were collected to serve as controls. This same procedure was to prepare sections to evaluate the uptake of the fl-Hsc/Hsp70 in the retina immediately after light damage. Sections were examined by epifluorescence microscopy and photographed.

Evaluation of retinal light damage: To study the effect of Hsp70 on the extent of PRC death in the light-damaged retina, rats were divided into three treatment groups. One group of animals was exposed to 175 ft-c of illuminance for 24 h as described earlier. Immediately after the light exposure, one eye received an injection of 10 µg Hsc/Hsp70 (~140 pmol) and, in the other eye, an equal amount (by weight) of actin (~230 pmol). The animals were returned to regular cyclic light for 10 days afterwards, to allow the destroyed photoreceptors to be phagocytosed and cleared from the retina. The second group served as a positive light damage control, being subjected to identical light exposure, but given no intravitreal injections, and then kept in regular cyclic light for ten days. The third group were age-matched normal rats kept in normal, cyclic light for the duration of the experiment.

Sections of the eyes prepared after methacarn fixation and paraffin-embedding were stained with hematoxylin and eosin (H and E) for light microscopy. Digital images of the retinas were collected at six equally spaced points along the retina from inferior to superior. The program, NIH Image (Version 1.60, a version of the public domain software by the same name, prepared by Scion Corp., Frederick, Maryland), was used to calculate the area of the ONL in a 150 µm wide rectangle, an index of the survival of photoreceptor cells used previously [24]. Five eyes were evaluated for each treatment. The data were analyzed by repeated measures ANOVA followed by the Newman-Keuls test for individual differences using the statistics software, GB-Stat (Version 5.4.3; Dynamic Microsystems, Inc., MD). This method permitted the comparisons of the patterns of variation in ONL thicknesses throughout the midsagittal planes of the retinas of each treatment group.

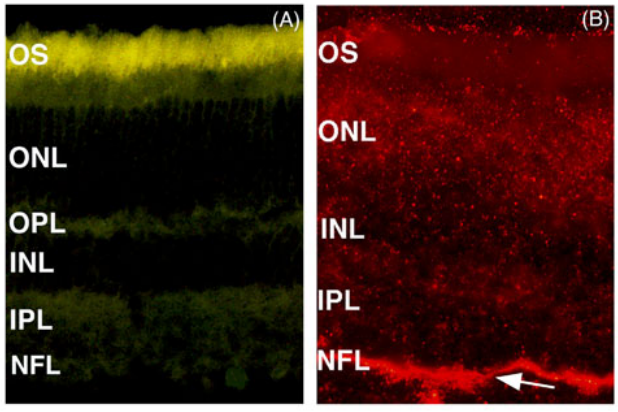

Figure 1. Epifluorescence micrographs of rat retinal sections from control and rhodamine bead-injected eyes. **A**: Untreated, normal retinal section showing autofluorescence, which appeared as yellow to greenish yellow and was especially bright in the OS. **B**: Retinal section from an eye taken six h after an intravitreal injection of 10 µg of rhodamine beads. The arrow indicates the high uptake of fluorescent beads in the NFL. Lower levels of diffuse fluorescence can be seen throughout the other retinal layers. In many places, clusters of beads can be seen as punctate fluorescence. 100x magnification. n=4.

RESULTS

Retinal distribution of Hsc/Hsp70 following intravitreal injection: Control retinal sections were examined first to check for autofluorescence and to confirm that the intravitreally injected solution could diffuse into the retina. Figure 1A shows

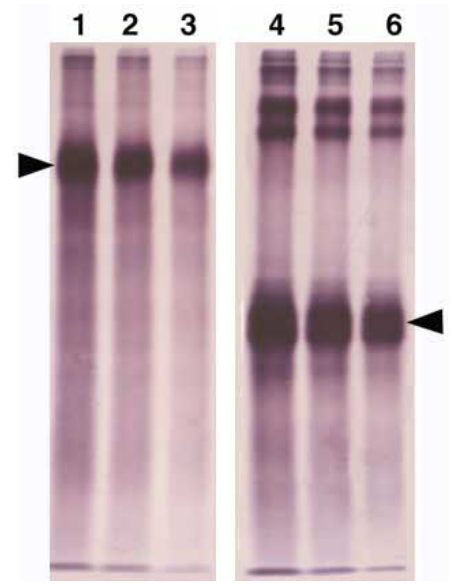

Figure 2. Western blot analysis of fluorescein-labeled Hsc/Hsp70 and actin. Lanes 1-3 are samples of 100, 50, and 25 ng fl-Hsc/Hsp70, respectively. The left arrowhead indicates intact fl-Hsc/Hsp70. This blot did not resolve as separate bands the Hsc70 and Hsp70 present in this preparation because of the relatively small difference in molecular weights. The relative purity of this preparation was confirmed by the fact that even in the lane with 100 ng fl-Hsc/Hsp70, only traces of other proteins of higher or lower molecular weights were detected. Lanes 4-6 are samples 100, 50, and 25 ng fl-actin. This preparation did contain significant amounts of some higher molecular weight proteins, but these were not considered to be of concern, since it was used as a negative control.

the pattern of autofluorescence observed in the untreated retina viewed using a long pass barrier filter for fluorescein. Autofluorescence was minimal in all layers except for the outer segments (OS). Although the yellow-green color of this autofluorescence was different from the bright green signal of fluorescein, it precluded unambiguous confirmation of uptake of fluorescently labeled material into the OS layer, as presented below (see Figure 3). Figure 1B illustrates the distribution of a suspension of 50 nm rhodamine-labeled beads one h after an intravitreal injection. As expected, the nerve fiber layer (NFL) contained the greatest fluorescence because it is in direct contact with the vitreal space into which the injection was made. Fluorescent beads were also seen scattered throughout the rest of the retina, thus confirming that the injected material was able to diffuse into the retina relatively rapidly.

Prior to intravitreal injection of fl-Hsc/Hsp70 or fl-actin, the integrity of the labeled proteins was checked by SDS-PAGE followed by western blotting and detection of the proteins using an antifluorescein antibody. As shown in Figure 2, both proteins remained intact after going through the labeling reaction and subsequent isolation by filtration through filters with a 10 kD pore size. This analysis also revealed the skeletal muscle actin preparation used as a control protein did include some higher molecular weight proteins. No attempt was made to identify those other proteins.

Next, the uptake of fl-Hsc/Hsp70 or fl-actin by the retina was studied at 1 h, 6 h, and 24 h after intravitreal injection. One h after injection, fl-Hsc/Hps70 was observed diffusely throughout the layers of the retina, especially in the NFL and plexiform layers (Figure 3A). Additionally, some of the pro-

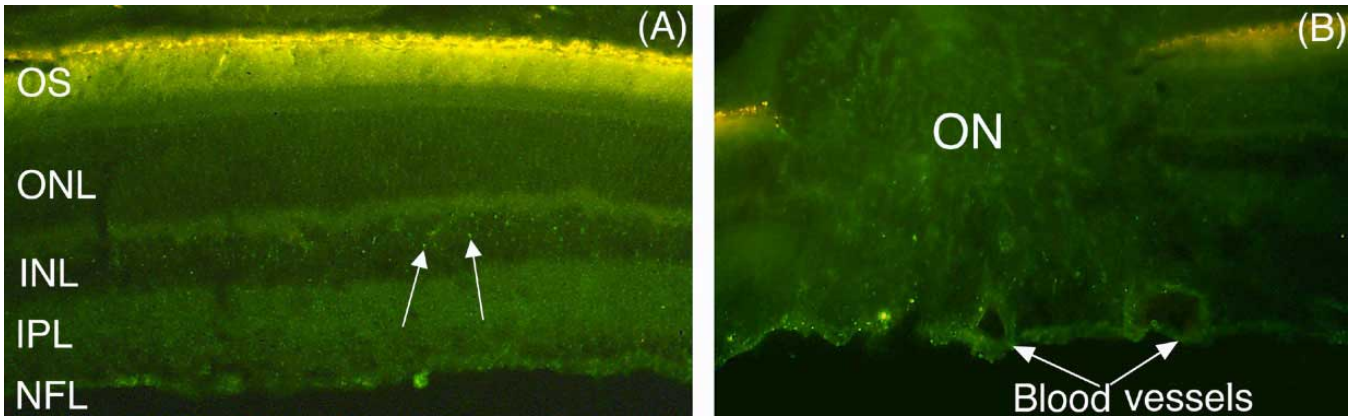

Figure 3. Epifluorescence micrographs of different regions of retinal sections from two eyes 1 h after intravitreal injection of fl-Hsc/Hsp70. **A**: Fl-Hsc/Hsp70 is apparent in all layers of the retina, but appears to be more prominent in neuropil layers, NFL, IPL, and OPL. In the INL, the arrows indicate vesicular accumulations of the protein. The OS is autofluorescent (compare to Figure 1A), so it was not possible to determine if fl-Hsc/Hsp70 was present there. **B**: In a section from another eye showing the intraretinal part of the optic nerve, fl-Hsc/Hsp70 can be seen in the walls of two blood vessels, as well as in the neuropil of the optic nerve. 50x magnification. n=13.

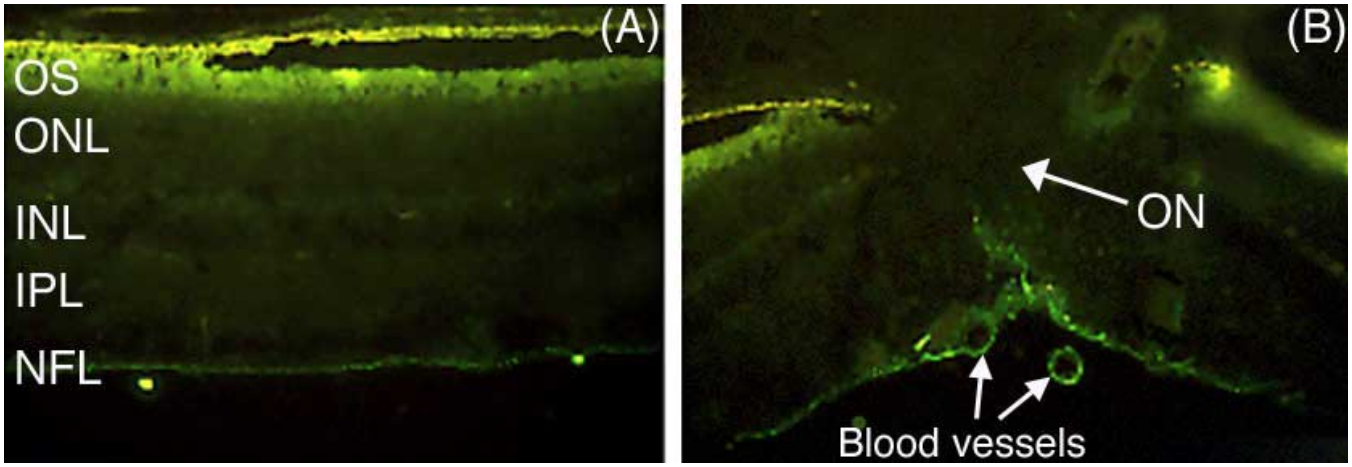

Figure 4. Epifluorescence micrographs of different regions of retinal sections from two eyes 1 h after intravitreal injection of fl-actin. **A**: Most of the fl-actin is seen in the NFL, with little fluorescence detectable in other retinal layers. The bright signal in the OS is attributed to autofluorescence, as explained in Figure 1A and Figure 3A. **B**: Cells comprising the walls of blood vessels near the intraretinal portion of the optic nerve were found to take up fl-actin in a manner similar to that seen for fl-Hsc/Hsp70 injected eyes (compare to Figure 3B). 50x magnification. n=9.

tein was concentrated in vesicular structures (Figure 3A, arrows). The fl-Hsc/Hsp70 was also found in the walls of blood vessels, as exemplified in region of the optic nerve shown in Figure 3B. In comparison, fl-actin was not as diffusely distributed through the retinal layers at one h post-injection, but was more confined to the inner retina layers, mainly the NFL, as shown in Figure 4A. Neither was it found in vesicular accumulations. However, it too, was found within the walls of blood vessels near the optic nerve (Figure 4B).

At six h post-injection, the level and distribution of fl-Hsc/Hsp70 and fl-actin in the retina was more widespread than at one h. Both proteins were detected diffusely throughout the retina (Figure 5A and Figure 6), being especially prominent in the plexiform layers (IPL and OPL) and the NFL. In the fl-Hsc/Hsp70-treated eye, even the inner and outer segments (OS) appeared to contain the protein, based on the green rather than yellow fluorescence observed there (Figure 5A). However, this distribution pattern was not present throughout the retina, as illustrated in Figure 5B, which shows the optic nerve region of the same retina as in Figure 5A. Here the fl-Hsc/Hsp70 is seen mainly as globular accumulations in perivascular cells surrounding the major ophthalmic blood vessel, while the retina and optic nerve contain little fl-Hsc/Hsp70 (Figure 5B). Three other features of the distributions of both fluorescent proteins were also notable. First, at this time interval, as well as at one h (Figure 3 and Figure 4), neither fluorescent protein was detectable in the nuclear layers of the retina. Second, punctate accumulations of fl-Hsc/Hsp70 and fl-actin were seen throughout the retina (arrowheads in Figure 5A and arrows in Figure 6). Third, fluorescein-labeled proteins were frequently observed to be concentrated in the walls of the retinal capillaries and larger blood vessels, as noted previously in the one-h post-injection retinal specimens (Figure 3 and Figure 4) and shown in Figure 5A, Figure 5B, and Figure 6.

At 24 h post injection, little of either of the fluorescein-labeled proteins were detectable in all of the retinal sections examined (n=10 for each protein; images not shown). The levels of exogenous proteins probably had been reduced by diffusion throughout the retina, as well as by turnover or by dispersal into the blood stream by this time.

Distribution of exogenous proteins following intravitreal injection in the light-damaged retina: Ten µg of fl-Hsc/Hsp70 or fl-actin were injected intravitreally into the eyes of rats immediately after the 24 h light exposure period. The patterns of uptake of the proteins in the light-damaged retinas 1 h, 6 h, and 24 h post-injection were similar to those seen in the normal retinas. At 1 h, the injected fl-Hsc/Hsp70 was detected in the NFL, INL, IPL, and RGCL, as well as in the walls of blood

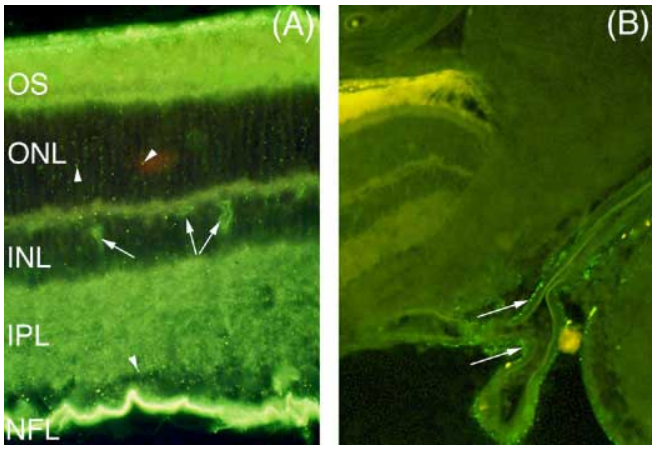

Figure 5. Epifluorescence micrographs of two regions of the retina from one eye at 6 h after an intravitreal injection of fl-Hsc/Hsp70. **A**: This image of the retina about midway between the optic nerve and ora serrata shows the higher level of uptake of fl-Hsc/Hsp70 seen at six h post-injection compared to one h (Figure 3). The pattern of uptake, however, is similar to that seen at one h, with the brightest fluorescence seen in the neuropil layers of the retina, the NFL, IPL, and outer plexiform layer (not labeled, but seen as a thin layer of fluorescence above the INL). Relatively little fluorescence was observed in the nuclear layers, the ganglion cell layer (the thin, dark band just above the NFL), INL, and ONL. In this case, the NFL fluorescence was extremely bright, and the OS (including the inner segment layer just below) was clearly green in color, rather than the yellow typical of autofluorescence, suggesting that fl-Hsc/Hsp70 was present in high levels there as well. Vesicular accumulations of fl-Hsc/Hsp70 are seen in all retinal layers (arrowheads indicate examples) and uptake was also obvious in capillary walls (arrows). **B**: In the optic nerve (ON) region of the same retina as in Panel **A**, there was less fl-Hsc/Hsp70 present, except in perivascular elements seen around the major ophthalmic blood vessel. Note also that the outer segment layer seen in the upper left here shows the yellow color of autofluorescence, in contrast to the green fluorescence in the OS in Panel **A**. 50x magnification. n=11.

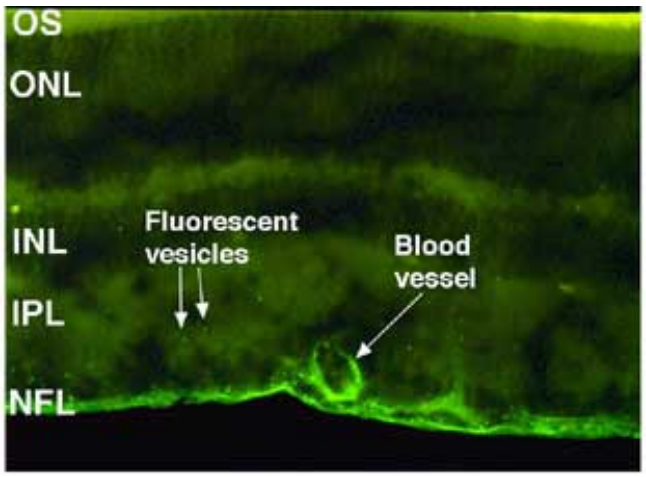

Figure 6. Epifluorescence micrograph of one region of the retina from an eye at 6 h after an intravitreal injection of fl-actin. This image taken from the mid-retina shows that the general pattern of uptake of fl-actin was similar to that of fl-Hsc/Hsp70 at six h, including the presence of vesicular accumulations of the protein and the greater uptake into perivascular or vascular cells. The lower intensity of fl-actin fluorescence compared to fl-Hsc/Hsp70 cannot be ascribed to uptake differences, as the specific activity of fluorescein labeling of the two proteins may not have been equivalent. Most of the IS and OS layers were not included in the image because the lower intensity of fl-actin fluorescence in these retinas made it difficult to distinguish the fluorescence there from background autofluorescence. 50x magnification. n=11.

vessels in the vicinity the optic nerve (not shown). By 6 h post-injection, both fl-Hsc/Hsp70 and fl-actin were more diffusely distributed throughout the retina and a punctate distribution of fluorescent proteins was found (not shown).

Confirmation that the fluorescent signal in the retina was from intact, labeled protein: In order to verify that the fluorescein signal viewed in retinal sections corresponded to intact Hsc/Hsp70 or actin, retinal homogenates from eyes injected with the proteins were analyzed on western blots probed with anti-fluorescein antibody. These analyses showed that the fluorescent material in the retina was primarily intact Hsc/Hsp70 (Figure 7) or actin (not shown) in both normal and light damaged retinas, even 24 h after the intravitreal injection. Additionally, there was a trend toward decreases in the amounts of fluorescein labeled protein between one h post-injection and the six and twenty-four h after injection, but we did not attempt to quantify that change. The fact that the blot indicates that there was still a considerable proportion of the injected fl-Hsc/Hps70 present 24 h later, whereas by fluorescence microscopy, little fluorescent protein was detected is a consequence of the greater sensitivity of the western blot method. The samples on the blot reflect the protein from the entire retina and not just what was present in one 12 µm section as viewed by microscopy.

Intravitreally injected Hsc/Hsp70 protects the retina from light damage: A preliminary histological study showed that an illuminance of 175 ft-c for 24 h caused about 50% loss of photoreceptor cells by ten days after the light exposure (data not shown). Therefore, this level of light exposure was deemed suitable to test for the protective effect of Hsc/Hsp70. The area occupied by the ONL in a midsagittal section of the eye, which is proportional to the number of surviving photoreceptor cells, was compared in Hsc/Hsp70-treated and control animals. We found that those eyes treated with Hsc/Hsp70 had more photoreceptor nuclei left ten days after light exposure than the actin-injected or untreated light-damaged eyes (Figure 8). Evaluation of the data by ANOVA confirmed a significant interaction between light-damage and injection of Hsc/Hsp70, with an overall $F=3.32$ and $p<0.017$ ($df=5$). Post-hoc analysis of individual differences in each section of the retina (Newman-Keuls) showed that the middle superior region of the retina to be significantly protected by Hsc/Hsp70 treatment. In the case of the actin-injected eyes, the changes in ONL area compared to uninjected light-damaged eyes did not achieve statistical significance. Even though, in the actin-treated eyes, the ONL areas of sectors 4 and 5 were greater than the corresponding sectors of the light-damaged-only group, those differences were negated by the overlap in ONL

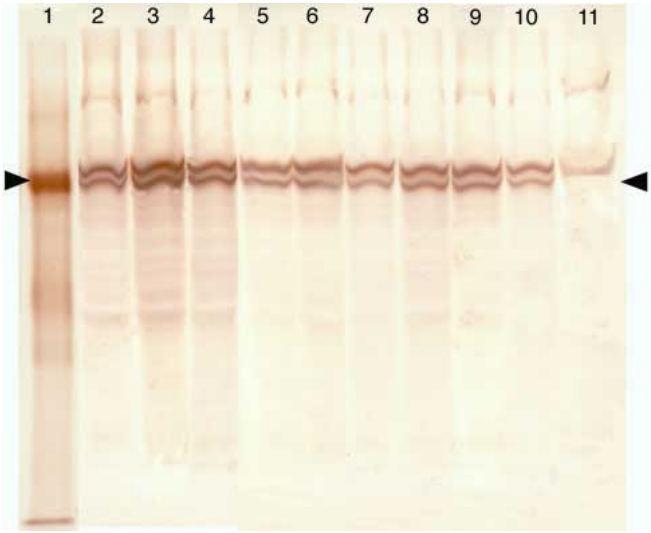

Figure 7. Western blot analysis of fl-Hsc/Hsp70 in normal and light-damaged retinas. Lane 1 is 20 ng of fl-Hsc/Hsp70 from the same preparation used for the intravitreal injections and the arrowhead indicates the prominent immunoreactive band at 70 kD, with very little fluorescein immunoreactivity at other molecular weights. The succeeding lanes are samples of light-damaged and normal retinas collected at increasing intervals after intravitreal injections of fl-Hsc/Hps70, as follows: 2 and 3, replicates of light damaged retinas one h post-injection; 4, normal retina one h post-injection; 5 and 6, light-damaged retinas six h post-injection; 7, normal retina six h post-injection; 8 and 9, light-damaged retinas 24 h post-injection; 10, normal retina 24 h post-injection; 11, normal retina from uninjected eye to show the nonspecific stained bands. The right arrowhead indicates the position of intact fl-Hsc/Hsp70 in the retinal samples, which lined up with the sample of purified fl-Hsc/Hsp70 shown in lane 1. The amount of each retinal sample loaded on the gel was 2.5% of the total retinal homogenate and each eye had received an injection of 10 µg fl-Hsc/Hsp70.

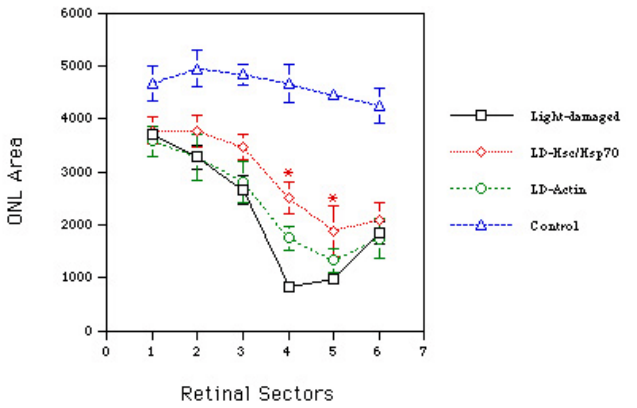

Figure 8. Inhibition of light-induced photoreceptor cell death in the eyes injected with Hsc/Hsp70 immediately after light exposure. The graph illustrates the area ($\mu^2 \pm$SEM) occupied by the ONL at each of six sectors from the inferior (1-3) and superior (4-6) retinas of four treatment groups, those maintained under normal illumination (blue, control), those which received intravitreal Hsc/Hsp70 after light damage (LD-Hsc/Hsp70, red), those which received intravitreal actin after light damage (LD-Actin, green), and those which were untreated after light damage (Light-damaged, black). Statistical comparisons (ANOVA) of the Hsc/Hsp70 and actin treatment groups with the untreated light-damaged group documented that only the Hsc/Hsp70 group had significantly more photoreceptors, with the greatest effect being in the superior retinal sectors which are most vulnerable to light damage (asterisks, $p<0.05$, sectors 4 and 5). $n=6$ per group.

areas in the other retinal sectors of the two groups.

Micrographs of retinas from each experimental group are shown in Figure 9. Both the whole retinal thickness and the thickness of ONL in the light-damaged-only group showed the greatest decline. Cystic structures were noted in those retinas, which are an indication of the severity of damage to the retina caused by the 24 h light exposure. The LD-Hsc/Hsp70 treated retinas clearly had more nuclei in the ONL, reflecting the cytoprotective effects of the Hsc/Hsp70. Little or no effect was observed in eyes treated with the control protein, actin.

DISCUSSION

We observed inter-animal differences in the pattern of distribution and intensity (relative amount) of the injected fluorescein-labeled proteins and rhodamine-labeled beads in the retina. The fluorescent material was associated unevenly with the NFL and other layers of the retina. We suspect that this uneven distribution was a consequence of the injection method and the viscous property of the vitreous, even though the injections were done as uniformly as possible. An example of this variation can be seen in Figure 5A and Figure 5B, which shows two regions of the retina from the same eye 6 h after injection with fl-Hsc/Hsp70.

Even though there was variation in distribution of injected protein, we were able to discern trends in our study of the time course of uptake of the fluorescently labeled proteins. Both fl-Hsc/Hsp70 and fl-actin were taken up by the inner retinal layers within one h after injection and then became more diffusely distributed in other retina layers at later times (Figure 3 and Figure 6). The diffuse uptake suggests that the fluorescein-labeled proteins were internalized by the cells. Such uptake of exogenously presented Hsc/Hsp70 has also been reported to occur within three h in a monocyte cell line [22]. The punctate fluorescence observed in several retinas suggests that endocytosis into vesicle-like structures (Figure 5A) accounted for at least some of the uptake. This interpretation is consistent with the observation of initial patching and capping of cell surface-bound Hsc/Hsp70 reported by Guzhova et al. [22]. However, that report suggests that Hsc/Hsp70 can enter a cell by other means, becoming distributed throughout the cytoplasm. The possibility of uptake of Hsc/Hsp70 by unconventional means is supported by the observations of Alder et al. [25] and a recent report by Arispe and De Maio [26], which both showed that Hsc/Hsp70 can insert into artificial lipid bilayers. Further work using higher resolution microscopy will be required to show definitively if the intravitreally administered Hsc/Hsp70 does become intracellularly distributed in the retina.

The pattern and time course of fl-Hsc/Hsp70 and fl-actin uptake in the retina seen here was very similar to the uptake of intravitreally injected biotinylated basic fibroblast growth factor (bFGF) in the rabbit, described by Lewis et al. [27]. They also found concentrated uptake at the retinal-vitreal interface (inner limiting membrane and nerve fiber layer) within two h of the injection of protein and, by six h, saw the protein become distributed in the inner retina. Of particular interest to us was the fact that they also saw a punctate distribution of bFGF and uptake of the protein in basal lamina and endothelial cells of retinal blood vessels. They interpreted the punctate distribution of bFGF as binding within synaptic terminals.

No difference was discernible by epifluorescence microscopy in the retinal uptake and distribution of injected fl-Hsc/Hsp70 and fl-actin in the normal retina. This observation suggests that there is not a specialized pathway or receptor-mediated mechanism for the interaction and uptake of Hsc/Hsp70 by retinal cells, although Hsp70 receptors recently have been found to exist in monocytes and related antigen-presenting cells [28,29].

The actin-treated, light-damaged eyes showed a trend toward increased PRC survival that did not achieve statistical significance. It is possible that this trend was a reflection of a weaker cytoprotective activity elicited by the injection itself. Such an event was found to occur in the light-damaged rat retina in response to injections of fibroblast growth factor (FGF) into the subretinal space [30]. Although the protective effect elicited by the needle penetration was significant in that case, it was still less than that seen when FGF was injected. The reason why the needle penetration elicited a significant protective effect in that study and not in this one may be a

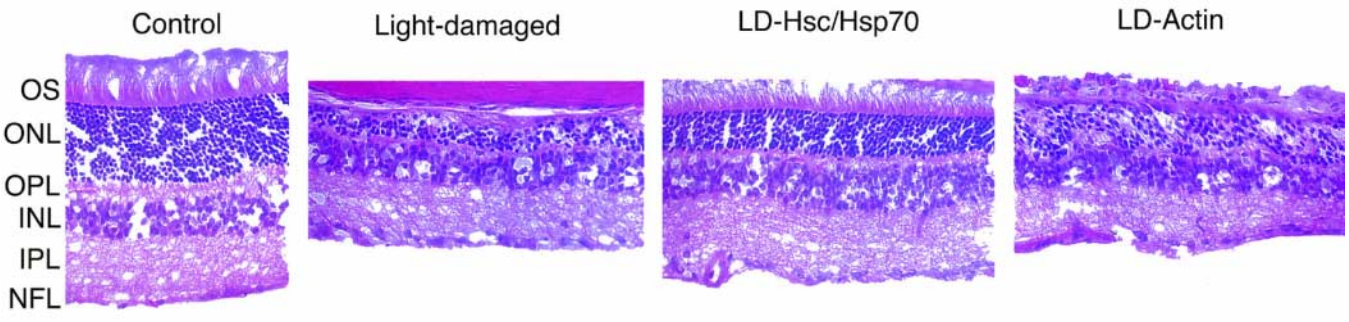

Figure 9. Light micrographs of examples of retinal sectors. Light micrographs of examples of retinal sectors from one rat in each of the treatment groups analyzed in Figure 8. Note that the number and relative structural organization of the ONL and OS layers in the LD-Hsc/Hsp70 example is more similar to the Control than the Light-damaged only and the LD-Actin examples. In fact, neither of the latter two show recognizable outer segments (OS), whereas the Hsc/Hsp70-treated retina does. 50x magnification.

consequence of the difference in location of the injections in the two studies, subretinal versus intravitreal.

Despite the absence of a uniform uptake pattern, intravitreally injected Hsc/Hsp70 was found to protect retinal photoreceptors from light-induced cell death (Figure 8 and Figure 9). This inhibition of light damage suggests that the protein retained its chaperoning and cytoprotective activity, even when supplied to cells from the extracellular space. The observation of cytoprotective activity by exogenously administered Hsc/Hsp70 has now been made in a number of different systems using a variety of stressors. These include nutrient deprivation in cultured smooth muscle cells [31], trophic factor withdrawal in cultured motor neurons [32], axotomy of the sciatic nerve [33], tumor necrosis factor-α(TNF-α) induced apoptosis of cultured premonocyte cells [22], and both hyperthermic stress and treatment with staurosporine in cultured neuroblastoma cells (unpublished observations). Despite the differences in the types of cells and stressors in these studies, they are united in one important characteristic: the predominant manner of cell death observed in each was apoptosis. In the case of the rat retina, two reports have shown that exposure to green or blue light for three to nine h caused significant photoreceptor apoptosis within the 24 h following light exposure [34-36]. Recent reports have indicated that endogenously produced Hsp70 can interrupt the sequence of events in apoptotic cell death [37,38]. Both Hsc70 and Hsp70 inhibited the processing of procaspases 9 and 3 and Hsp70 also inhibited the release of cytochrome C from mitochondria [14]. Thus, it seems that both of these Hsps can prevent apoptotic cell death, though their activities in this regard may not be identical. The observations described here suggest that the administration of Hsc/Hsp70 immediately after the light exposure prevents many of the light-damaged PRCs from initiating the apoptotic cascade that otherwise occurs in the first 24 h after the light exposure. They also imply that exogenously supplied Hsc/Hsp70 can function in a similar manner to that synthesized within a cell, although additional studies will be required to test this hypothesis directly.

The potential for Hsps to protect the retina was first shown over ten years ago by this laboratory [20]. In that study, the levels of endogenous Hsps were stimulated by whole body hyperthermia prior to the exposure to damaging light. Nearly full protection was observed when the interval between hyperthermia and light exposure was 18 h and that correlated with the time of the maximum increase in retinal Hsp70 content [18]. In the present study, there was a loss of about 37% of the PRCs in the Hsc/Hsp70 treated eyes. There are several reasons that could account for this difference, with two considered most likely. First, in the earlier study, the Hsps were elevated prior to the light damage, whereas in this investigation, the Hsps were injected after exposure the light. Thus, in the first study, the cells were protected before being exposed to the damaging treatment, whereas in the present study, there were likely some PRCs that had been damaged beyond the point of rescue during the 24 h of light exposure prior to the Hsc/Hsp70 administration. Second, hyperthermia certainly stimulated additional changes in the rats beyond those produced by an intravitreal injection of Hsc/Hsp70. Those additional changes included increases in two higher molecular weight Hsps in addition to Hsp70, as well as eliciting other hormonal and physiological alterations in the rats that may have contributed to PRC protection. Furthermore, we have yet to determine how the hyperthermia-induced increase in Hsp compares quantitatively to that achieved by intravitreal injection. It is possible that different timing or doses of Hsc/Hsp70 and other Hsps will produce a similar level of protection as that seen with hyperthermia.

The key observation in this report is that administration of Hsc/Hsp70 after PRC damage was effective in reducing PRC death. Since the damage was elicited during a 24 h period of light exposure, the result suggests that the interval between damage and treatment with Hsc/Hsp70 could range from min to h and still halt the progression of damaged cells to apoptosis. This post-injury protective effect means that Hsc/Hsp70 administration has promise clinically in the treatment of traumatic retinal injuries, such as retinal detachment, tears, or stroke. Furthermore, there are some chronic diseases, such as glaucoma and diabetic retinopathy, in which there is protracted retinal cell death by apoptosis, even after the primary problem, elevated intraocular pressure or blood glucose concentration, is ameliorated [39,40]. In those cases as well, it is possible that appropriately timed administration of Hsc/Hsp70 could delay the progressive loss of retinal cells. The present results warrant further study of the potential therapeutic application of Hsps in these and other retinal conditions.

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