Heat Shock Protein Expression in Human Conjunctiva

Alejandro Berra, James E. Dutt, Mahnaz Nouri, and C. Stephen Foster

Purpose. To examine the distribution of human heat shock proteins (HSPs) HSP90, inducible HSP70 (iHSP70), constitutive and inducible HSP70 (cHSP70), HSP65, and human HSP27 in conjunctival biopsy specimens of ocular cicatricial pemphigoid (OCP), atopic keratoconjunctivitis, and healthy persons with cataract.

Methods. Using an immunoperoxidase technique, conjunctival biopsy specimens from ten patients with ocular cicatricial pemphigoid, ten patients with atopic keratoconjunctivitis and ten healthy persons undergoing cataract surgery were analyzed with a panel of monoclonal antibodies directed against human HSPs.

Results. Large amounts of HSP90 and HSP27, and lesser amounts of cHSP70, iHSP70, and HSP65 were present in atopic keratoconjunctivitis and normal epithelium; less of these proteins were seen in OCP conjunctival epithelium. In atopic keratoconjunctivitis and normal tissue the substantia propria contained a few HSP-positive cells, and the vascular endothelium was consistently negative for all of the HSPs. In sharp contrast, OCP stroma contained large numbers of cells staining for HSP27, HSP90, and iHSP70, and the vasculature was strongly positive, particularly for HSP90, cHSP70, and HSP27.

Conclusion. These results indicate that normal and atopic keratoconjunctivitis epithelia express HSP90 and HSP27 and some form of HSP65 and HSP70. The differences between normal, atopic keratoconjunctivitis, and OCP stromal staining suggest an upregulated expression of HSP90, cHSP70, and HSP27 at the site of inflammation in OCP, the stroma, from cytokine release. The striking presence of HSP in the conjunctival vascular endothelium from OCP patients suggests a previously unappreciated role of the vasculature in OCP.


Cicatricial pemphigoid is a systemic autoimmune disease that affects the eyes in approximately 70% of cases. It causes chronic cicatrizing conjunctivitis with subepithelial fibrosis, symblepharon formation, fornix foreshortening, and meibomian duct obstruction. Untreated, this progressive disease inexorably leads to xerosis, corneal opacification, profound visual loss, and, in some cases, loss of the eye.1

We previously reported on the inflammatory cell types invading the substantia propria of conjunctiva from 130 patients with active cicatricial pemphigoid.1 We used monoclonal antibodies to characterize the inflammatory mononuclear cell phenotypes and2 found that a significant number of conjunctival specimens exhibited perivasculitis and vasculopathy.1,2 We also found that the conjunctiva from ocular cicatricial pemphigoid (OCP) patients contained a proportionately increased number of T cell receptor (TCR) gamma/delta expressing cells.3 Specific antigens that gamma/delta T cells recognize, such as heat shock proteins (HSP), have been identified,4-8 but presence of HSP in conjunctiva has not been reported.

The HSPs are highly conserved proteins found in all organisms. Increased synthesis of these proteins occurs in response to many environmental stresses, including inflammation, fever, irradiation, viral infection, malignant transformation, exposure to oxidizing agents, heavy metal ions, ethanol, and anoxia.10 HSPs in the epithelia may be important in surveillance of epithelial cell integrity and may represent a first line of defense against transformation of epithelial cells induced by stress agents. In this study we examined the expression of HSPs in an epithelial tissue not previously investigated, the human conjunctiva, an integral and very active participant in the immunologic system of the eye.

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This study was undertaken to compare the expression and distribution of several HSPs in normal, atopic keratoconjunctivitis (AKC), and OCP conjunctiva.

**METHODS**

Biopsy specimens were obtained from the affected conjunctiva of 10 patients with cicatricial pemphigoid (4 men and 6 women), ten patients with atopic keratoconjunctivitis (5 men and 5 women) and from ten healthy persons with cataract but without evidence of other ocular disease (3 men and 7 women), who were undergoing cataract surgery. Informed consent was obtained from each patient before biopsy specimens were taken. In addition, the tenets of the Declaration of Helsinki were followed, and institutional human experimentation committee approval was granted. The mean age of the OCP patients was 72 years (range, 50 to 80 years). The mean age of the AKC patients was 64 years (range, 45 to 72 years). The mean age of the control group was 75 years (range, 50 to 89 years).

The biopsy specimens were obtained under identical conditions: subconjunctival 2% lidocaine was injected immediately before biopsy. The 4 x 4 mm specimens were harvested from superior bulbar conjunctiva adjacent to the limbus. Each specimen was bisected for light microscopy and immunohistochemical analysis.

Specimens for light microscopy were immersed in Karnovsky’s fixative (1% paraformaldehyde, 1.25% glutaraldehyde, 0.13% sucrose, and 25 mM sodium phosphate in 150 mM sodium cacodylate buffer, pH 7.2) for 24 hours, dehydrated using a graded series of ethanols, embedded in glycol methacrylate (LKB Producter, AB Bromma, Sweden), and sectioned at 2 μm on a Sorval (Newtown, CT) JB-4 microtome. Sections were stained with alkaline Giemsa, periodic acid-Schiff reaction, and hematoxylin and eosin.

Specimens for direct immunofluorescence and immunoperoxidase staining were snap frozen in liquid nitrogen and embedded in Tissue-Tek OCT compound (Ames Company, Division Miles Laboratory, Elkhart, IN). Four-micron sections were then cut in an IEC (Needham Heights, MA) microtome cryostat, mounted on gelatin-coated slides and stored at -70°C. Direct immunofluorescence staining was performed using fluorescein- or rhodamine-labeled goat immunoglobulin G directed against each of the human immunoglobulins (IgG, IgA, IgM, IgD, IgE), complement components C3 and C4, fibrin, and albumin (Organon Teknika-Cappel, Durham, NC). Tissue was examined by a masked observer with a Zeiss (Oberkochen, Germany) Photomic III fluorescence microscope as described by Foster. For immunoperoxidase procedures, all the sections were stained simultaneously. Cryostat sections were air dried and fixed in acetone. Sections were then incubated for 20 minutes with normal mouse serum (HSP 90) or with normal goat serum (HSP 70, cHSP 70, HSP 65, and HSP 27). The sections were then incubated for 45 minutes with the panel of primary monoclonal antibodies listed in Table 1. After a phosphate-buffered saline rinse and a 30-minute block for endogenous peroxidase using 0.3% H2O2 in phosphate-buffered saline, all sections were incubated for 45 minutes with a 1:500 dilution of Biotin-SP-AffiniPure goat anti-mouse IgG(H + L) or Biotin-SP-affiniPure mouse anti-rat IgG(H + L) (Jackson ImmunoResearch, West Grove, PA). After a final incubation with a 1:1000 dilution of peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratory), the reactions at sites of antibody binding were developed in peroxidase substrate containing 3-amino-9-ethylcarbazole and hydrogen peroxide in 0.1 M acetate buffer. The specimens were then fixed in formalin, counterstained with Gill’s Number 3 hematoxylin, and coverslipped with Vinol 205 (Air Products and Chemical Allentown, PA).

Experimental controls were tissue sections incubated without the primary antibodies, secondary antibodies, or streptavidin. Positive cells were counted in three representative high-power fields (X450) from each of the ten cicatrical pemphigoid, ten atopic keratoconjunctivitis, and ten normal specimens with a 10 x 10-mm ocular grid on an American Optical Microstar 110 microscope (Buffalo, NY). A 2 x 10 mm grid was used for epithelial counts. All counts were performed in a masked fashion. The mean value of the number of positive cells/mm² and standard errors of the mean were calculated for each HSP in cicatrical pemphigoid.

**TABLE 1. Monoclonal Antibodies Used to Characterize Specimens**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Vendor</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-90kD</td>
<td>1:200</td>
<td>StressGen</td>
<td>Human HSP90&lt;sup&gt;21,22&lt;/sup&gt;</td>
</tr>
<tr>
<td>Anti-72/73kD</td>
<td>1:500</td>
<td>StressGen</td>
<td>HSP72/73 (inducible and constitutive form of HSP70)&lt;sup&gt;23,24&lt;/sup&gt;</td>
</tr>
<tr>
<td>Anti-72kD</td>
<td>1:500</td>
<td>StressGen</td>
<td>HSP72 (inducible HSP70)&lt;sup&gt;25,26&lt;/sup&gt;</td>
</tr>
<tr>
<td>Anti-65</td>
<td>1:30</td>
<td>UNDP/World Bank/WHO special program for research and training in tropical disease</td>
<td>HSP65&lt;sup&gt;27&lt;/sup&gt;</td>
</tr>
<tr>
<td>Anti-27kD</td>
<td>1:200</td>
<td>StressGen</td>
<td>Human HSP27&lt;sup&gt;28,29&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>Downloaded From: http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933179/ on 05/31/2017</sup>
TABLE 2. Conjunctiva of Cicatricial Pemphigoid Patients: Direct Immunofluorescence Staining

<table>
<thead>
<tr>
<th>Patient</th>
<th>Epithelial BMZ</th>
<th>Epithelial Cells</th>
<th>Cells in Substantia Propria</th>
<th>Vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IgA, IgG, IgM, IgE, C4</td>
<td>—</td>
<td>IgA, C3</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>IgA, IgG, IgD</td>
<td>IgA, IgM, IgE</td>
<td>IgA, IgG</td>
<td>IgD</td>
</tr>
<tr>
<td>3</td>
<td>IgG, C5</td>
<td>IgA, C4</td>
<td>IgA, IgG, C3</td>
<td>C3</td>
</tr>
<tr>
<td>4</td>
<td>IgA, IgG</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>IgA, IgG</td>
<td>IgA, IgG</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>IgA, IgG</td>
<td>IgA, IgG, IgM, IgD</td>
<td>IgA, IgG</td>
<td>C3, C4</td>
</tr>
<tr>
<td>7</td>
<td>IgA, IgG</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>IgA</td>
<td>IgA, IgG</td>
<td>IgA, C3</td>
<td>IgM, C3</td>
</tr>
<tr>
<td>9</td>
<td>IgG, C4, C3</td>
<td>IgA, IgG, IgC4</td>
<td>—</td>
<td>C3</td>
</tr>
<tr>
<td>10</td>
<td>IgA, IgG</td>
<td>IgA, IgG, C4</td>
<td>IgA</td>
<td>IgM</td>
</tr>
</tbody>
</table>

TABLE 3. HSP Expression in Conjunctival Epithelium

<table>
<thead>
<tr>
<th>HSP90</th>
<th>OCP</th>
<th>AKC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>92.8 ± 9.2</td>
<td>21.1 ± 10.0*</td>
</tr>
<tr>
<td></td>
<td>15.6 ± 8.2</td>
<td>10.4 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>25.9 ± 9.3</td>
<td>24.5 ± 12.4</td>
</tr>
<tr>
<td></td>
<td>14.3 ± 6.2</td>
<td>9.2 ± 7.0</td>
</tr>
<tr>
<td></td>
<td>92.4 ± 16.3</td>
<td>34.8 ± 16.1</td>
</tr>
</tbody>
</table>

OCP = Ocular cicatricial pemphigoid; AKC = atopic keratoconjunctivitis.  
| * P < 0.05 (versus normal and AKC conjunctival epithelium). |

mean value of numbers of cells staining positive count/mm² ± SEM.

The means of the numbers of HSP expressing cells in normal, AKC, and OCP conjunctiva identified by monoclonal antibodies appear in Tables 3, 4, and 5.

Normal and AKC epithelium showed more numbers of cells than OCP epithelium that stained for HSP90 (92.8 and 87.4 versus 21.1) and HSP27 (92.4 and 89.6 versus 34.8). The intensity of staining for HSP90 and HSP27 was greater for normal and AKC conjunctiva than for cicatricial pemphigoid epithelium (Figs. 1, 2). The differences in positive cells between normal and AKC versus OCP conjunctiva were highly significant for HSP90 (P < 0.05). There were no significant differences in the numbers of cells expressing cHSP70, iHSP70, and HSP65 among normal, AKC, and OCP specimens.

Substantia Propria

Histologic changes most notable in the stroma of cicatricial pemphigoid specimens included the presence of dramatically increased numbers of inflammatory cells (plasma cells and lymphocytes being especially abundant) and increased numbers of mast cells.

The substantia propria of AKC and normal conjunctiva contained few cells with light staining for HSP90, cHSP70, iHSP70, and HSP65 among normal, AKC, and OCP specimens.

TABLE 4. HSP Expression in Conjunctival Substantia Propria

<table>
<thead>
<tr>
<th>HSP90</th>
<th>OCP</th>
<th>AKC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2.4 ± 1.7</td>
<td>62.4 ± 12.3*</td>
</tr>
<tr>
<td>cHSP70</td>
<td>26.0 ± 1.0</td>
<td>7.8 ± 4.1</td>
</tr>
<tr>
<td>iHSP70</td>
<td>3.5 ± 1.2</td>
<td>3.8 ± 1.7</td>
</tr>
<tr>
<td>HSP65</td>
<td>3.5 ± 0.5</td>
<td>3.5 ± 1.0</td>
</tr>
<tr>
<td>HSP27</td>
<td>6.1 ± 1.9</td>
<td>49.4 ± 0.5*</td>
</tr>
</tbody>
</table>

OCP = Ocular cicatricial pemphigoid; AKC = atopic keratoconjunctivitis.  
* P < 0.05 (versus normal and AKC conjunctival substantia propria).  
Mean value of numbers of cells staining positive count/mm² ± SEM.
were found for HSP90 (2.4 and 9.2 versus 62.4) and for HSP27 (6.1 and 10.1 versus 49.4; Table 4, Figs. 3, 4). The number of cells staining for cHSP70, iHSP70, and HSP65 was not significantly different for normal, AKC, and pemphigoid specimens.

**Vessels**

The most striking histologic finding in the conjunctival vessels of cicatricial pemphigoid patients was vasculopathy with perivascular inflammatory cell infiltration (4 patients) and large numbers of mast cells around vessels (4 patients).

Direct immunofluorescence staining showed deposits of immunoglobulins IgM (3 patients), IgD (1 patient), and C3 (4 patients).

Dramatic differences in the expression of HSP were found in cicatricial pemphigoid vasculature compared to normal controls and AKC specimens (Table 5, Figs. 3, 4). A large number of cicatricial pemphigoid vessels showed intense staining for HSP90 (62.4 ± 7.0). Some OCP vessels expressed moderate staining for HSP27 (18.2 ± 4.1); none of the ten normal and ten AKC specimens showed vascular expression of any HSP.

Significantly more cells of the vascular endothelium (P < 0.05) stained for HSP90 and HSP27 in the tissue of OCP patients than in normal and AKC conjunctiva.

**DISCUSSION**

Normal and AKC epithelium showed large numbers of cells that stained for HSP90 and HSP27; the differ-
TABLE 5. HSP Expression in Conjunctival Vessels

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>OCP</th>
<th>AKC</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP90</td>
<td>0</td>
<td>62.4 ± 7.0*</td>
<td>0</td>
</tr>
<tr>
<td>cHSP70</td>
<td>0</td>
<td>1.8 ± 1.2</td>
<td>0</td>
</tr>
<tr>
<td>iHSP70</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HS65</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HSP27</td>
<td>0</td>
<td>18.2 ± 4.1*</td>
<td>0</td>
</tr>
</tbody>
</table>

OCP = Ocular cicatricial pemphigoid; AKC = atopic keratoconjunctivitis.
* P < 0.05 (versus normal and AKC conjunctival vessels).

Mean value of numbers of cells staining positive count/mm² ± SEM.

ences in comparison to OCP epithelium were highly significant for HSP90 (P < 0.05). The intensity of staining for HSP90 and HSP27 was greater for normal and AKC conjunctiva than for cicatricial pemphigoid epithelial specimens. Fewer cells were noted that stained for cHSP70, iHSP70, and HS65 in either normal, AKC, or OCP epithelium. There were no significant differences in the expression of cHSP70, iHSP70, or HS65 between normal, AKC, and OCP specimens. We hypothesize that normal and AKC conjunctival epithelium expresses HSPs in response to environmental stress. Why OCP conjunctival epithelium expresses reduced amounts of HSPs is unclear, as is the possible significance of this reduced expression in the pathogenesis of the disease.

An examination of the substantia propria and vasculature revealed dramatic differences in HSP expression between normal, AKC, and OCP conjunctiva. The substantia propria of normal conjunctiva contained a few cells with light staining for HSP90, iHSP70, HS65, and HSP27. In sharp contrast, more cells were found for HSP90 and HSP27 in OCP stroma (P < 0.05). The number of cells staining for cHSP70, iHSP70, and HS65 was not significantly different for normal, AKC, and pemphigoid specimens. An important and exciting result was the striking difference in the expression of HSPs in cicatricial pemphigoid vasculature compared to normal controls and AKC specimens (P < 0.05; Table 5). A large number of cicatricial pemphigoid vessels showed intense staining for HSP90; some vessels expressed moderate staining for HSP27. Normal and AKC conjunctival vasculature revealed no HSP expression.

The disproportionately increased number of HSP90 and HSP27 expressing cells in OCP stroma and vessels represent a finding not noted in our patients with other forms of chronic conjunctivitis, such as atopic keratoconjunctivitis. The significance of this finding is currently unclear; but the vascular findings, coupled with Foster’s earlier observations of perivascularitis and vasculopathy in substantial numbers of OCP conjunctival specimens, suggests that abnormalities of blood vessels exist in tissues affected by pemphigoid.

OCP and AKC are disorders that have different underlying immunologic mechanisms. AKC is a chronic manifestation of several ocular surface disorders in the context of atopic dermatitis caused by Type I and probably Type IV hypersensitivity. Conversely, OCP is a systemic autoimmune disorder.

The relationship between HSP expression, gamma/delta T cells, and autoimmunity is not altogether clear. Recent data suggest that TCR γ/δ expressing lymphocytes have a role in autoimmune disease and may in fact recognize ligands by a similar mechanism to that of TCR α/β expressing lymphocytes. Alternatively, non-MHC-restricted recognition of tumor targets and stress cells by some TCR γ/δ cells has suggested to some investigators that these TCRs might recognize cell surface molecules not encoded by the MHC. Both HSPs and gamma/delta T cells have been identified in various human autoimmune disorders, such as in the synovial fluid of rheumatoid arthritis patients, brain tissue of multiple sclerosis patients, and possible autoimmune chronic gastritis of the antrum. Previously, we found that OCP patients contain an elevated number of T cells expressing the gamma/delta TCR, and now our observations show an increased number of HSP expressing cells in the same patient population.

Specific epitopes that γ/δ T cells recognize, such as HSPs, have been identified. Although some α/β T cells are also capable of recognizing HSPs, the γ/δ T cells generally recognize this type of antigen. The HSPs are highly conserved, not only among bacteria but also in eukaryotes, so that some γ/δ T cells might respond to epitopes common to both bacterial and eukaryotic antigens. Because HSPs may be produced at sites of inflammation, γ/δ T cells initially induced by bacterial antigens could conceivably mediate autoimmune disease by responding to autologous tissue, which is expressing HSPs because of inflammatory stress. Such recognition of HSPs would enable gamma/delta T cells to perhaps eliminate those cells that exhibit signs of stress, but it is unknown what mechanisms might trigger the autoimmune disease.

Immunohistopathologic studies have clearly demonstrated complement and immunoglobulin deposition in the epithelial basement membrane of OCP conjunctiva. This is the classic hallmark of the disease, although the particular OCP autoantigen has not yet been identified. The striking presence of HSPs in the conjunctival vascular endothelium of OCP patients suggests a previously unappreciated role of the vasculature in cicatricial pemphigoid. Additionally, the increased number of both HSP-expressing cells and gamma/delta T cells in OCP conjunctiva suggests a...
relationship between these two cell populations in the
tissue of patients with another systemic autoimmune
disease, but with primarily an ocular manifestation.

Key Words
heat shock protein, γδ T cell, ocular cicatricial pemphigoid,
autoimmunity, conjunctiva

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fluid-derived Yersinia-reactive T cells responding to
human 65 kDa heat shock protein and heat-stressed
21:2139-2143.