Effect of lysosomes on cultured rabbit corneal endothelial cells*

**Dharmendra V. Arya, Jean Mannagh, and A. Ray Irvine, Jr.**

Lysosomal preparations induced marked changes in cultured corneal endothelial cells, firstly causing the epithelial-like pattern of control growth state to become multilayered and mesothelial in appearance and behavior, and secondly causing cell detachment and death. These culture changes were reversible with removal of lysosomes and restoration of fresh growth media. It appears that the changes observed were due to cathepsin, since protease with catheptic activity similar to that of lysosomal preparations produced a similar effect, whereas the enzymes B-glucuronidase, aryl sulfatase, and acid phosphatase at concentrations found in the lysosomal preparations did not affect the cells. Trasylol, a mucoprotein protease inhibitor, reduced cell damage, thus suggesting the involvement of a protease or cathepsin in the reaction. A parallel between cathepsin concentration and the extent of cellular detachment was observed.

**Key words:** corneal endothelium, tissue culture, lysosomes, cathepsin, cellular detachment.

Clinical observations and studies of routine surgical specimens strongly suggest that pathologic states giving rise to leukocytic exudates in the anterior chamber are accompanied by marked changes in the corneal endothelium. Patients with fulminating endophthalmitis may rapidly develop corneal edema and opacification. Microscopically, eyes so afflicted may show endothelial cell necrosis with partial or complete destruction of this layer.1 Irvine, Mannagh, and Yuhasz2 using cultured rabbit corneal endothelial cells demonstrated that exposure to leukocytic exudates resulted in a dramatic change in culture characteristics.

Although De Duve,3 who originally isolated lysosomes from rat liver, suggested that lysosomal components could play a role in the development of tissue damage, however, direct tests of the capacity of lysosomes to induce tissue injury were not undertaken until Cohen and Hirsch4 showed that the specific cytoplasmic granules of rabbit peritoneal polymorphonuclear (PMN) leukocytes were lysosomal in nature. Lyed leukocyte granules have been shown to cause various tissue reactions such as increase in vascular permeability, emigration...
of leukocytes, disruption of mast cells, hemorrhage, and cell injury. The present investigation was undertaken to study the effect of lysosomes on corneal endothelial cells in tissue culture.

Materials and methods

Isolation of lysosomes. Rabbit peritoneal PMN leukocyte lysosomes were isolated by differential centrifugation according to Cohen and Hirsch. Lyosomes from liver and kidney were prepared by the method of Sawant and co-workers. Isolated lysosomes were suspended in Hank's balanced salt solution (Hank's BSS), ruptured by 15 rapid freezings and thawings, centrifuged by 2,000 x g for ten minutes, and the supernate collected.

Assay methods. Lysosomal B-glucuronidase was estimated according to Allison and Sandelin using phenolphthalein-B-glucuronide as the substrate (Sigma Chemical Co., St. Louis, Mo.). For acid phosphatase, sodium-B-glycerophosphate (Sigma Chemical Co., St. Louis, Mo.) was used as the substrate, and released inorganic phosphate quantitated by the method of Lowry and Lopez. The procedure of Roy, using p-nitrocatechol sulfate (Sigma Chemical Co., St. Louis, Mo.) as the substrate, was used for aryl sulfatase. To determine the catheptic activity, hemoglobin (sheep red blood cells, type III) was used, and released tyrosine estimated according to Anson. Lysosomal proteins were estimated by the method of Lowry and co-workers.

Tissue culture. Two types of rabbit corneal endothelial cell preparations were used, primary cell cultures and a cell line. In both, the corneal endothelial cells were removed enzymatically and established in Rose chambers. In primary cultures, the endothelial cells from one rabbit eye were seeded into a Rose chamber. The cells from the fellow eye were used as a control. Primary cultures were used when they became a monolayer (eight to ten days). A rabbit corneal endothelial cell line in the fifty-fourth passage was seeded with 100,000 cells per milliliter. It was treated when it became a monolayer (48 hours).

The monolayers were washed with Hank's BSS before exposure to lysosomes or the enzymes. All dilutions of lysosomes or enzymes were made in Hank's BSS. Hank's BSS was used as media in the controls. Resulting changes were recorded by time-lapse cinemicrography (five frames per minute) and serial or repeated still-phase photomicrography. The number of cells detached from the glass surface was calculated by counting the cells in ten fields at random at each time interval.

Results

As observed by time-lapse cinemicrography, the cells in culture media and in
Fig. 2. Rabbit corneal endothelial cell line 17 hours after exposure to lysed rabbit peritoneal polymorphonuclear leukocyte lysosomes containing 0.723 µg tyrosine per minute per milliliter of catheptic activity. The cells were round, short, and refractile, and most had left their attachment to the glass. (Magnification ×400; phase contrast.)

Hank's BSS appeared as an epithelial-like sheet remaining in a monolayer and exhibiting contact inhibition as evidenced by minimal motility and membrane activity (zeiosis) (Fig. 1). The cultures exposed to lysosomes (250 µg of lysosomal protein per milliliter) became mesothelial in appearance and behavior, becoming multilayered, losing contact inhibition and showing increased motility and zeiosis. Long fimbriated processes rapidly extended and retracted and pinocytosis increased. Some of the cells became rounded and came off the glass surface (Fig. 2). This cell detachment from the glass surface has been taken as one criterion of cell damage. At any time after exposure to the lysosomes, those cells remaining attached to the glass would multiply if replenished with fresh media, whereas only a portion of those cells that came off the glass would attach and multiply if seeded in fresh media. Of the detached cells left in contact with the lysosomes (250 µg of lysosomal protein per milliliter) for 24 hours, only about 50 percent would grow when seeded in fresh media.

A particularly significant observation were the changes in tissue culture characteristics occurring 48 to 72 hours following removal of lysosomes and replacement of the control growth media. Under such conditions, the cells would revert to the epithelial-like pattern of the control state. They would become monolayered in an epithelial-like pattern, zeiosis would decrease and the cells would exhibit contact inhibition.

The enzymes B-glucuronidase, aryl sulfatase, and acid phosphatase at concentrations found in the lysosomal preparations Table I, did not detach the cells, whereas protease (Sigma Chemical Company, St. Louis, Mo.) with catheptic activity (0.734 µg of tyrosine per minute per milliliter) similar to that of lysosomal preparation (0.723 µg of tyrosine per minute per milliliter, and 250 µg of protein per milliliter)
produced similar cell detachment (Fig. 3). The per cent detachment caused by varied concentrations of cathepsin is given in Table II. There seems to be a parallel between the catheptic activity and the extent of cellular detachment. Similar results were obtained with the cell line and the primary endothelial cell cultures. Lysosomes from rabbit liver, rabbit kidney, and rat liver with similar catheptic activity exerted a similar effect. Intact lysosomes with 0.723 µg of tyrosine per minute per milliliter catheptic activity damaged about 10 per cent of the cells in 18 hours, whereas lyzed lysosomes with equivalent catheptic activity damaged about 95 per cent of the cells during the same period.

Ten Kallikrein inhibitor units per milliliter of trasylal, an antiprotease, protected the cells from the cell-detaching effect of lysosomal preparation with 0.723 µg of tyrosine per minute per milliliter of cathepsin concentration.

Hydrocortisone acetate (Upjohn Company, Kalamazoo, Mich.) and vitamin A palmitate (E. R. Squibb and Sons, Indianapolis, Ind.) were used with whole lysosomal preparations (catheptic activity—0.723 µg of tyrosine per minute per milliliter) to study their influence on the cell-damaging effect of lysosomes on corneal endothelial cells. Hydrocortisone acetate at a concentration of 10 µg per milliliter reduced cell damage, whereas one unit per milliliter of vitamin A palmitate accelerated cell destruction.

Table I. Enzyme activities in the rabbit peritoneal polymorphonuclear leukocyte lysosomes, expressed as product formed per milligram of lysosomal protein

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Product formed/min./mg. of protein</th>
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<tbody>
<tr>
<td>B-glucuronidase</td>
<td>0.744 µg Phenolphthalein</td>
</tr>
<tr>
<td>Aryl sulfatase</td>
<td>0.504 µg p-Nitrocatechol</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>1.555 µg Inorganic phosphate (Pi)</td>
</tr>
<tr>
<td>Cathepsin</td>
<td>2.895 µg Tyrosine</td>
</tr>
</tbody>
</table>

Fig. 3. Rabbit corneal endothelial cell line 17 hours after treatment with protease containing 0.734 µg tyrosine per minute per milliliter of catheptic activity. Most of the cells had detached; those remaining were round and refractile. (Magnification x400; phase contrast.)
Table II. Number of corneal endothelial cells detached from glass surface after exposure to lysed rabbit peritoneal polymorphonuclear leukocyte lysosomes, in Rose chambers

<table>
<thead>
<tr>
<th>Cathepsin (μg tyrosine/min./ml.)</th>
<th>Lysosomal protein (μg/ml.)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>6</th>
<th>10</th>
<th>18</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.895</td>
<td>1,000</td>
<td>3.5*</td>
<td>10</td>
<td>23</td>
<td>55</td>
<td>92.5</td>
<td>99.9</td>
<td>100</td>
</tr>
<tr>
<td>0.723</td>
<td>250</td>
<td>0</td>
<td>3</td>
<td>15</td>
<td>30</td>
<td>57.5</td>
<td>95</td>
<td>99.9</td>
</tr>
<tr>
<td>0.3615</td>
<td>125</td>
<td>0</td>
<td>1.5</td>
<td>5</td>
<td>10</td>
<td>13.5</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>0.0723</td>
<td>25</td>
<td>0</td>
<td>1</td>
<td>1.5</td>
<td>5</td>
<td>5.5</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>0.00723</td>
<td>2.5</td>
<td>0</td>
<td>3</td>
<td>5</td>
<td>6.5</td>
<td>3</td>
<td>5</td>
<td>6.5</td>
</tr>
<tr>
<td>Hank’s BSS</td>
<td>0</td>
<td></td>
<td></td>
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<td></td>
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</table>

*Expressed as per cent cells detached.

Discussion

Isolated lysosomes caused both cellular detachment and digestion of detached rabbit corneal endothelial cells in vitro. Vitamin A, a lysosomal membrane labilizer, when used with intact lysosomes, accelerated cell detachment; whereas cortisone, a lysosomal membrane stabilizer, reduced the cellular detachment, thus emphasizing the importance of environment on lysosomal integrity. The cellular detachment was inhibited by trasyal, a mucoprotein protease inhibitor, indicating the involvement of lysosomal protease(s) or cathepsin(s) in the reaction.

It has been suggested by many investigators that activation or release of proteases into the tissue constitutes a significant pathogenic mechanism in tissue injury and inflammation. Evidence for the involvement of protease in tissue injury was provided by Halpern who found that proteolytic enzyme inhibitors blocked the Shwartzman-like lesions produced by lysed PMN leukocyte lysosomes, thus suggesting the importance of lysosomal acid protease in the reaction. The capillary permeability induced by Burke and co-workers could also be inhibited by trasyal indicating that lysosomal proteases were the inciting material.

Our results of an in vitro study also seem to indicate that cell damage is caused mainly by the lysosomal proteases, since protease with catheptic activity similar to that of lysosomal preparations caused a similar effect, and particularly since protease inhibitor caused inhibition of cell damage. Although the lysosomal preparations used in these experiments were not highly purified, there seems to be a parallel between the cellular detachment and cathepsin concentration.

From the experimental point of view, cells and tissue in culture differ in many ways from those in the body. Every cell removed from the body, either in isolation or as a part of a tissue or an organ, is inevitably altered. Nevertheless, the information obtained by tissue culture has been valuable to the understanding of the normal working of the cells or tissue in question or to the understanding of cellular behavior in general.

It was of interest that the morphologic changes induced in the cultured cells by sublethal concentrations of lysosomal cathepsins were similar to those previously reported after exposure of cultured endothelial cells to PMN leukocytes and crude PMN leukocyte lysosomal preparations. The cells reverted to the epithelial-like pattern of the control culture after cathepsin was washed from the culture and replaced by fresh culture media. This emphasizes that a change in cultured cells may, at least in these, be due to environment rather than to permanent metaplasia.

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REFERENCES