The development of enzyme activities in corneal connective tissue cells during the lag phase of wound repair

II. Formalin-resistant oxidase-like reaction

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A formalin-resistant oxidase-like reaction which is cyanide- and phenylhydrazine-sensitive, but azide insensitive, develops intense activity in corneal endothelial, epithelial, and connective tissue cells within 24 hours after injury to the rat cornea. This apparently new oxidase-like system cannot be detected in the normal cornea. It attains maximal activity between 24 and 48 hours after injury and disappears between the fourth and seventh postoperative days. No cytochrome oxidase activity could be demonstrated in either the normal or in the healing cornea.

We have shown that the so-called "lag phase" of wound repair is a period of intensive formation or activation of enzymes in corneal fibroblasts, endothelial and epithelial cells. Two enzymes, 5-nucleotidase and succinic dehydrogenase, which were not found in normal rat corneal fibrocytes, developed in these cells within 24 hours after injury and increased in activity during the first 7 postoperative days, the longest time period investigated. Cytochrome oxidase, in contrast, could not be detected at any of the time periods studied. However, the rapid development or activation of an apparently new, and as yet unidentified, oxidase-like system was found. This oxidase-like reaction first appeared (weakly) in the fibrocytes of 6 hour wounds, developed intensely between 12 and 24 hours after injury; and, in contrast to 5-nucleotidase and succinic dehydrogenase, disappeared between 4 and 7 days after wounding. It is the purpose of this report to describe the appearance and pattern of activity of this oxidase-like reaction in epithelial, endothelial, and connective tissue cells of healing corneal wounds.

Methods and materials

Wound material. Sprague-Dawley rats (200 to 300 grams) of either sex were used for all experiments. The animals were anesthetized briefly (1 to 2 minutes) with ether and wounds of 1 to 2 mm. in length were made in the center of the corneas with a sterile Swan needle knife. The animals were killed by decapitation at 6, 12, 24, 48, 96, and 168 hours after injury. The eyes were enucleated and either frozen and stored in liquid nitrogen or fixed in ice-cold Baker's calcium-formol for 24 hours and then stored in gum-arabic-sucrose solution until used.

Histochetnical methods. The cytochrome oxidase method of Burstone was used with N-phenyl-p-phenylenediamine as substrate and 1-hydroxy-2-naphthoic acid as coupler. Frozen sections were cut at 20μ in the cryostat. Three types of sections were used: (1) fresh-frozen, (2) fresh-frozen,
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sectioned, then fixed 1½ hours at room temperature in 10 per cent neutral formalin, and (3) corneas fixed for 24 hours in ice-cold Baker's calcium-formol. The sections were incubated for 1 to 4 hours at 37.5° C. All sections, including those which had been fixed in formalin before incubation, were routinely transferred to 1 per cent cobaltous acetate-10 per cent formalin for 1 hour after incubation. They were next rinsed through several changes of glass-distilled water and counterstained in Mayer's carmalum for 10 minutes. The carmalum was not more than 1 week old as it caused considerable fading of the reaction products as it aged. Following counterstaining, the sections were rinsed in glass-distilled water and mounted in either glychrogel or glycerine jelly.

In addition to formalin, the effect of the known cytochrome oxidase inhibitors, KCN and NaN₃, was tested. Phenylhydrazine, an inhibitor of some amine oxidases, was also used. As additional controls, cytochrome oxidase-rich tissues, liver and kidney (rat), were incubated in the same solutions and under the same conditions.

The corneas were sectioned coronally with the wound centered as much as possible. In such sections, the cells appear flat, spread out, and are considerably easier to identify than in sagittally cut sections. A minimum of 8 eyes were studied for each time period evaluated. Serial sections were cut with alternate sections used for the assay and for each chemical tested for possible inhibitory activity. With a thin cornea, such as that of the rat, only a few (about a dozen) sections are obtained before the anterior chamber is reached. It was necessary, therefore, to use different eyes for each inhibitor tested.

Results

This series was originally planned as an evaluation of the possible development of cytochrome oxidase activity in the healing wounds, as this enzyme is known to be absent in the normal cornea. Corneas fixed in cold (2 to 4° C.) formalin for 24 hours were just as active as fresh-frozen sections and the pattern of distribution of the activity was identical. It appeared, therefore, that the reaction might represent an unknown oxidase of some type. The distribution of the reaction product was entirely different from that of the M-Nadi reaction which is also formalin resistant. In addition, the M-Nadi reaction differs from the oxidase-like reaction observed in the healing cornea in that the oxidase-like reaction is inhibited by KCN and the M-Nadi reaction is not. It did not appear to be a peroxidase as it was not inhibited by NaN₃.

Further details on the pattern of action of various inhibitors on this unknown formalin-resistant oxidase-like reaction will be presented below following the description of the results obtained in the wounded eyes.

Normal. The cells of all layers of the cornea were negative (Figs. 1, 8, 10). The occasional polymorphonuclear leukocytes found were negative.

Six-hour wounds. There were numerous weakly positive granules in most of the stromal cells throughout the sections. The activity was greatest, however, in stromal cells at the wound edge. The polymorphonuclear leukocytes also were weakly positive. Those at the wound edge were more positive than those away from the wound edge. The epithelium developed a distinct activity which was greatest at the wound edge. The endothelium also had some activity. In general, a definite, but very weak, activity had developed in cells of all types by 6 hours after injury.

Twelve-hour wounds. The activity in the stromal fibrocytes was distinctly increased over that found at 6 hours and appeared to be about equal in those cells found at the wound edge and in those away from the wound (Fig. 2). The invading polymorphonuclear leukocytes were now more active than the stromal cells. Both the endothelium and the epithelium were more active than at 6 hours, and the activity in both increased near the wound area.

Twenty-four hour wounds. The activity found at 24 hours after injury was vastly increased over that found in 6 and 12 hour wounds (Compare Figs. 2 and 3). The results were comparable with those found at 24 hours with 5-nucleotidase. Corneas in which the inflammatory reaction was well developed had an intensely positive reaction in the stromal cells and an even greater reaction in the polymorphonuclear leukocytes. The stromal cells (or fibro-

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*Weimar and Haraguchi: Unpublished observations.
blasts) at the wound edge were about even in the intensity of their reaction with the stromal cells away from the wound.

When the inflammatory reaction was weak at 24 hours, the activity in the stromal cells and in the polymorphonuclear leukocytes, although strong, was much weaker than that found in corneas with a well-developed inflammatory reaction. The activity in the fibroblasts and in the polymorphonuclear leukocytes at the wound edge was distinctly greater than that found in both types of cells away from the wound edge.

The activity in the endothelium and epithelium was also quite intense at this time period (Figs. 9, 11), particularly in those eyes with well-developed inflammatory reactions. It was much weaker in those with less inflammatory reaction.

**Forty-eight-hour wounds.** The inflammatory reaction was now more uniformly developed and the enzyme activities were fairly uniform from cornea to cornea. The fibroblasts at the wound edge as well as the stromal cells away from the wound were very rich in activity (Fig. 4). The reaction in the white blood cells of all types, however, was much greater than that of either the fibrocytes or fibroblasts. In fact, the activity in the white blood cells of all types was so intense that it was necessary to shorten the incubation periods at both 24 and 48 hours from 4 to 3 hours in order to identify the cell types involved. When the shorter incubation period was used, the fibroblasts at the wound edge were again found to be more active than the fibrocytes away from the wound. The endothelium and epithelium were at the peak of their activity at this time period.

The over-all activity of the stromal cells was more uniform at 48 hours than at 24 hours, but the richest sections at 24 hours were generally more intense than those at 48 hours. Frequently, wounds were found at both 24 and 48 hours which had a much greater activity in the connective tissue near the epithelium than in the areas away from the epithelium (Fig. 5). With suitable counterstaining and magnification, however, the richness of such areas adjacent to the epithelium was found to be due to white blood cells of all types (Fig. 6). The weak areas away from the epithelium were found to have very few white blood cells present (Fig. 7). The stromal cells in the area with numerous white blood cells present were much more active than the stromal cells

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**Fig. 1.** Formalin-resistant oxidase-like reaction. Negative normal corneal fibrocyte. (Four-hour incubation. Mayer's carmalum stain. x1,000.)

**Fig. 2.** Formalin-resistant oxidase-like reaction. Twelve-hour wound. Development of activity in fibrocyte at the wound edge. (Four-hour incubation. Mayer's carmalum stain. x1,000.)

**Fig. 3.** Formalin-resistant oxidase-like reaction. Twenty-four-hour wound. Center intensely positive developing fibroblast at the wound edge, with two positive polymorphonuclear leukocytes just to the right. (Four-hour incubation. Mayer's carmalum stain. x1,000.)

**Fig. 4.** Formalin-resistant oxidase-like reaction. Forty-eight-hour wound. Positive spindle-shaped fibroblasts at the wound edge. (Four-hour incubation. Mayer's carmalum stain. x1,000.)

**Fig. 5.** Formalin-resistant oxidase-like reaction. Twenty-four-hour wound. The intensely reactive area to the left is the side toward the epithelium. The nearly black areas represent leukocytes. The area to the right has no leukocytes present. (Four-hour incubation. Mayer's carmalum stain. x100.)

**Fig. 6.** Formalin-resistant oxidase-like reaction. Twenty-four-hour wound. A higher magnification of the leukocyte-rich area in Fig. 5. There is intense activity in both the leukocytes and the fibrocytes. (Four-hour incubation. Mayer's carmalum stain. x400.)

**Fig. 7.** Formalin-resistant oxidase-like reaction. Twenty-four-hour wound. A higher magnification of the leukocyte-free area in Fig. 5. Distinct activity is present in the fibrocytes, but it is definitely weaker than that seen in the leukocyte-rich area, Fig. 6. (Four-hour incubation. Mayer's carmalum stain. x400.)
in the area with fewer white blood cells. (Compare Figs. 6 and 7.)

A further comparison of Figs. 5, 6, and 7, especially Fig. 5, clearly shows the difficulty of determining the identities of the cells possessing histochemical activity without suitable counterstaining and high magnification.

**Ninety-six-hour wounds.** There was a definite decrease in activity at this time. The fibroblasts at the wound edge were still very active but, as a rule, the stromal cells away from the wound had greatly decreased in activity from that found at 24 and 48 hours after injury. The activity in the white blood cells, although greater than that of the stromal cells, had also diminished considerably from that seen at 24 and 48 hours. The activity in both the endothelium and epithelium resembled that of 12 hour wounds in that it was very weak to absent away from the wound but definitely enhanced in the wound area.

**Seven-day wounds.** Only slight activity was found at this time in the fibroblasts at the wound edge and many were totally negative. Stromal cells away from the wound edge were negative. The epithelium and endothelium also were negative.

**Effect of various inhibitors on the formalin-resistant oxidase-like reaction.** A series of inhibitors were selected to attempt to
distinguish between the activity in the fresh-frozen and formalin-fixed tissues. Although, of course, the full activity remaining after 24 hours' fixation in formalin eliminated cytochrome oxidase as the oxidase-like system present in those sections, it seemed that it might be possible to find an inhibitor which would eliminate the activity in the formalin-fixed sections and only partially decrease that in the fresh-frozen sections. The activity remaining in such fresh-frozen sections would be due presumably to cytochrome oxidase.

The inhibitors selected were KCN and NaN₃, known inhibitors of cytochrome oxidase, and phenylhydrazine, an inhibitor of some amine oxidases. Wounds of 48 hours' duration were used to evaluate the inhibitors because the oxidase-like activity in such wounds was the most uniform at this time period in all three layers of the cornea as well as in the invading white blood cells of all types. The results are summarized in Table I.

Identical results were obtained with all the inhibitors tested for both fresh-frozen and formalin-fixed sections of the cornea: (1) Formalin, as indicated, did not alter the pattern of activity in the corneal wounds but totally inhibited the oxidase activity of the liver and kidney; (2) azide did not inhibit the corneal oxidase-like reaction but totally suppressed the oxidase activity of the liver and kidney; and (3) cyanide inhibited the oxidase-like activity in the cornea and in the control sections of liver and kidney. The failure of either sodium azide or the formalin fixation to inhibit the reaction found in the wounded corneal tissues eliminates cytochrome oxidase as the source of the activity found.

Further tests on formalin-fixed sections of the cornea with phenylhydrazine indicated that the formalin-resistant oxidase-like reaction was very sensitive to this reagent, being inhibited by 0.001M.

Phenylhydrazine was found to cause some color changes in the substrate mixture containing N-phenyl-p-phenylenediamine. In order to eliminate the possibility that the action of phenylhydrazine was on N-phenyl-p-phenylenediamine rather than against the oxidase, corneal sections were preincubated for 15, 30, 60, and 120 minutes in 0.001M and 0.005M phenylhydrazine, rinsed through two changes of fresh incubation medium minus the substrate, and then incubated for 3 hours in the usual substrate (with no phenylhydrazine present).

Preincubation in either 0.001M or 0.005M phenylhydrazine for 1 or 2 hours eliminated all but the faintest suggestion of activity. Preincubation in either 0.001M and 0.005M phenylhydrazine for 30 minutes eliminated most of the activity in the white blood cells, but there was definitely a little activity remaining in the fibroblasts at the wound edge. (In the control sections, the white blood cells had the most activity.)

Table I. Influence of various inhibitors on the oxidase-like activity* of fresh-frozen and formalin-fixed corneas†

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Final concentration</th>
<th>Corneas</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fresh-frozen</td>
<td>Formalin-fixed</td>
<td>Fresh-frozen</td>
</tr>
<tr>
<td>KCN</td>
<td>0.002M</td>
<td>—§</td>
<td>+++</td>
<td>—</td>
</tr>
<tr>
<td>NaN₃</td>
<td>0.002M</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Phenylhydrazine</td>
<td>0.005M</td>
<td>—</td>
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</tbody>
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*Burstone method for cytochrome oxidase.³
†Forty-eight hour wounds.
‡Tested both on sections fixed 1½ hours at room temperature and corneas fixed 24 hours in ice-cold Baker's calcium-formal.
§No activity.
‖Activity.
In the kidney, very distinct zones of complete inhibition and no inhibition were found with phenylhydrazine.
The epithelium and endothelium were negative. When the sections were preincubated in phenylhydrazine (0.001M and 0.005M) for 15 minutes, the activity was reduced to that of 6 or 12 hour wounds. Again, the reduction of activity in the white blood cells appeared to be greater than that of the fibrocytes and fibroblasts. Phenylhydrazine, under the same conditions, eliminated all oxidase activity in the liver, but a distinct pattern of positive and negative areas was found in the kidney.

Since the formalin-resistant oxidase-like activity was essentially negative at 7 days after injury, it was thought it might now be possible to detect cytochrome oxidase at this time period by using fresh-frozen sections. Alternate sections from 7 day wounds were incubated as fresh-frozen sections for cytochrome oxidase or fixed in formalin and then tested for formalin-resistant oxidase-like activity. However, both the formalin-fixed and the fresh-frozen preparations were practically negative and again had identical patterns of distribution of the faint activity that was found. Thus, no evidence for the appearance of cytochrome oxidase at any of the postoperative time periods studied could be obtained. The oxidase-like activity found in the healing corneal tissues differs from cytochrome oxidase in that it is not inhibited by azide or formalin under conditions which inhibited known preparations of cytochrome oxidase.

Discussion

The results confirm the previous finding of intensely altered biochemical activities of corneal fibrocytes, as well as endothelial and epithelial cells, within 24 hours after injury. Although this apparently new oxidase-like activity develops in the wounded corneal tissues at approximately the same time as 5-nucleotidase and succinic dehydrogenase, it attains maximal activity between 24 and 48 hours after injury and then disappears between 4 and 7 days after injury in contrast to 5-nucleotidase and succinic dehydrogenase which continue to develop throughout this postoperative period.

It is not known if the appearance of this oxidase-like activity is a general phenomenon of wound healing or if it is a system peculiar to the cornea. The system is now being investigated in healing skin wounds.

It has not been possible with histochemical methods to identify this apparently new oxidase-like system which develops in the corneal cells between 6 and 24 hours after injury. The reaction has not been found in normal rat liver or kidney or in normal rabbit corneas. However, following injury to the rabbit cornea, the oxidase-like reaction develops in the rabbit corneal cells and white blood cells in a pattern similar to that found in the wounded rat corneas. The nature of this oxidase-like activity is now being investigated in this laboratory with biochemical methods.

REFERENCES