Studies on the source and release of collagenase in thermally burned corneas of vitamin A–deficient and control rats

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In previous studies we found that a mild thermal burn of the vitamin A–deficient rat cornea caused collagenase release into the medium of corneas placed in culture media 72 hr after applying the burn. Collagenase was released only on day 1 of culture and was not released from identically burned corneas of control rats. We now demonstrate that in deficient corneas, this collagenase release on day 1 of culture increased gradually with increasing time between burn and sacrifice, reaching a maximum at 16 hr after burning and remaining high up to 72 hr. In control rats day-1 collagenase release also increased to a maximum at 16 hr after the burn but then declined to almost zero at 72 hr. Trypsin treatment of day-1 media from both control and deficient corneas, taken at 72 hr after the burn, showed an almost complete absence of latent (inhibited) collagenase. Histologic observations revealed a close correlation between the presence of infiltrating polymorphonuclear neutrophils (PMNs) and the ulcerative lesions seen in burned, deficient corneas. When PMN infiltration was blocked by application of a tissue adhesive, no ulceration occurred and collagenase activity in the day-1 media dropped to almost zero. If burned and unburned areas of deficient corneas were separated and cultured separately, the burned area (containing most of the PMNs) was found to have 10 times the collagenase activity of the unburned area. In the controls, PMNs and some collagenase activity was detectable only 16 hr after the burn. We concluded that in the burned, vitamin A–deficient cornea there is increased attraction of PMNs to the lesion, resulting in collagenase release by these and possibly other cells, and ultimately resulting in ulceration. (INVEST OPHTHALMOL VIS SCI 22:62-72, 1982.)

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...thelial defects, stromal edema, leukoma, and ulceration. Corneas from pair-fed control rats treated in the same way showed rapid re-epithelialization and no stromal lesions; collagenase release was minimal. Recovery from deficiency occurred within 24 to 48 hr after intragastric administration of vitamin A. We concluded that vitamin A deficiency is a necessary, but not sufficient, component of sterile stromal ulceration (keratomalacia) and that ulceration may be facilitated by some external insult such as a physical, chemical, or other trauma.

In this article we describe further investigations of collagenase release in vitamin A-deficient rat corneas. We attempted to determine whether in deficient corneas a control mechanism is lacking that normally prevents the release of a destructive enzyme. We also investigated the possible role of inflammatory cells in contributing to the collagenolytic system. In particular, we studied the following questions: (1) What is the time course of collagenase release after a thermal burn? (2) Is there a latent collagenase that can be activated by trypsin? (3) Is collagenase released when inflammatory cells are excluded from burned cornea? (4) What is the pattern of collagenase release when the burned and unburned segments of cornea are separated?

Materials and methods

**Corneal thermal burn.** Vitamin A-deficient and pair-fed control rats without clinically apparent eye lesions were obtained as described by Seng et al. In anesthetized animals, "mild" thermal burns were produced by brief (<1 sec) application of a thermal probe (Thermokeratophore; Frigitronics, Inc., Shelton, Conn.) of 2 mm diameter at 130°C to the central cornea. The burned area was about 5 mm², relative to a total corneal area of about 40 mm². After burning, corneas were irrigated with sterile saline and treated with topical erythromycin or bacitracin antibiotic. Animals were examined daily for the presence of ocular lesions, and topical antibiotic was reapplied. After appropriate intervals, animals were sacrificed by decapitation, the corneas were excised for morphologic study or for organ culture, and collagenase was assayed from the culture media, all as previously detailed.

**Activation of latent collagenase by trypsin.** Media harvests were prepared as described to give a crude collagenase solution, and trypsin was added to 20 μl of collagenase solution for a final trypsin concentration of 10 to 200 μg/ml. The mixture was then incubated at 37°C for 10 min. After incubation, a fivefold concentration of soybean trypsin inhibitor (100 to 1000 μg/ml) was added.

**Histopathologic examination.** For morphologic correlation of clinical and biochemical observations, both deficient and control animals were sacrificed at various intervals in all experiments. The globes or corneas were then dissected and immersed in formalin fixative, followed by routine paraffin embedding. Multiple sections were obtained at several levels throughout the central thermal lesions and unburned surrounding areas of each cornea, were stained with either hematoxylin-eosin or periodic acid-Schiff, and were examined and photographed with a Zeiss photomicroscope.

**Blockage of PMN infiltration.** The corneas were burned as usual, and the loosely adherent epithelium was gently debrided with a moist cotton-tipped applicator. Isobutyl 2-cyanoacrylate tissue adhesive (Braun, Melsungen, W. Germany) was applied to cover only the burned area. After air-drying the adhesive for 1 or 2 min, erythromycin ointment was applied. At intervals, animals were sacrificed, the tissue adhesive was carefully removed with jewelers' forceps, and the corneas...
Fig. 2. Time course of morphology after thermal burn. Top, At 6 hr after burn the vitamin A–deficient cornea (left) exhibited epithelial debris, acellular stroma, and acute inflammatory cells adherent to the endothelium. At the same time, the control cornea (right) showed a central epithelial defect, devitalized stromal cells, and no anterior chamber inflammatory response. Middle, At 16 hr after burn the deficient cornea (left) had absence of central epithelium and marked PMN infiltration of the stroma and anterior chamber, whereas the control cornea (right) showed a nearly acellular stroma. Bottom, At 48 hr after burn the deficient cornea (left) had a persistent epithelial defect, superficial stromal ulceration and severe stromal PMN infiltration in comparison to the control cornea (right), which featured continuous epithelium and intact stroma devoid of PMN or other cellular infiltration (All H & E; ×150 to 400).
were then dissected for histologic examination or for culture and collagenase assay.

Separation of central burned cornea ("button") from peripheral unburned cornea ("ring"). Rat corneas were burned as usual. Sixteen or 54 hr later, the animals were sacrificed and their corneas were dissected out and immersed in sterile culture media (Grand Island Biological Co., Grand Island, N.Y.). With the aid of a dissecting microscope, a 2.5 mm Elliott trephine was used to excise the central burned area (corneal button). These buttons were then cultured separately from the remaining peripheral unburned corneal rings. In one experiment buttons and rings were cut into halves. One-half button was cultured together with one-half ring, and the other half-button was cultured separately from the other half-ring for comparison.

Results

Time course of collagenase release after a thermal burn. In our previous work\(^1\) we compared the release of collagenase from thermally burned deficient and control corneas. When the animals were killed 72 hr after burning and the corneas were placed in organ culture, we found that on day 1 of culture, a much greater release of collagenase occurred from the deficient corneas than that from the control corneas. We subsequently wanted to determine the time course of this collagenase release: at what time after the burn does release of collagenase begin, rise to a maximum, and possibly decline in deficient and control animals? To that end, corneas of vitamin A-deficient and control rats were thermally burned in the usual manner, and groups of animals were sacrificed 0, 6, 16, 25, 40, 48, 54, and 72 hr later. The corneas were excised and cultured, and in each group the media were harvested for collagenase assay after day 1 of culture. At 6 hr after burning, the deficient corneas started to release substantial amounts of active collagenase into the culture medium. This activity peaked at 16 and 54 hr and remained elevated as long as 72 hr after the burn (Fig. 1). Control corneas released detectable but lesser amounts of active collagenase at 6 hr, reaching a maximum release at 16 hr of less than 50% of that from the deficient corneas and gradually decreasing to almost zero at 72 hr (Fig. 1). Media were also harvested from days 2 to 5 of culture, and the amount of collagenase release in both the deficient and control animals was found to be insignificant. In summary, both deficient and control corneas began to release active collagenase at an early time after the burn. This activity was sustained in deficient corneas, whereas in control corneas it rapidly decreased and disappeared.

For histologic study, 42 eyes were examined, representing deficient and control animals at 6, 16, 24, and 48 hr after burning (Fig. 2). In deficient animals there was no evidence of epithelial regeneration at 6 hr, and the central stroma appeared devoid of viable keratocytes or inflammatory cells. A substantial inflammatory reaction developed in the anterior chamber and numerous polymorphonuclear leukocytes (PMNs) adhered to the corneal endothelium. Control corneas

Fig. 3. Trypsin activation of collagenase inhibited by serum. Collagenase was obtained from day 1 culture of six mildly burned vitamin A-deficient rat corneas. Two microliters of rat serum (diluted 1:10) was added to the crude collagenase and incubated for 30 min at room temperature before trypsin was added. The trypsin titration was done by the addition of 10 μl of trypsin (concentration indicated on the graph) into 20 μl of crude collagenase solution and incubation at 37°C for 10 min. Any excess trypsin activity would then be stopped by 10 μl of five times excess of soybean trypsin inhibitor. The collagenase activity was determined by the standard assay. Dashed line, Active collagenase activity before inhibition by rat serum.
Fig. 4. Activation of latent collagenase by trypsin on day 1 of rat corneal cultures begun 72 hr after mild thermal burn. The difference between points is insignificant (p > 0.05). The points are the means of four to five experiments with a total of four to five culture dishes (four corneas/dish). Bars represent the S.E.M. The experiment was done as described in the legend to Fig. 3.

Fig. 5. Exclusion of inflammatory cells from burned cornea. In the central burned cornea of a deficient animal (with tissue adhesive applied immediately after burning) this specimen, taken 3 days later, showed that epithelium was absent and that the stroma remained entirely intact and completely acellular (compare with Fig. 2). (H & E; ×150).

at this time appeared essentially identical except that no inflammatory cells were discernible within the stroma or anterior chamber. At 16 hr deficient eyes displayed marked stromal and anterior chamber inflammatory response, whereas control eyes showed epithelial regeneration, acellular stromas, and minimal anterior chamber inflammation. By

48 hr all deficient eyes showed evidence of corneal epithelial defects, stromal ulceration, and massive PMN infiltration of the stroma and anterior chamber. In contrast, control corneas had completely re-epithelialized and had no ulceration or stromal inflammation and no anterior chamber reaction. These observations suggest that in the deficient eye there is marked inflammation of both cornea and anterior chamber with continuing and increasing recruitment of PMNs, whereas in nondeficient control eyes there is only brief and minimal inflammatory response.

Trypsin activation of latent collagenase.

The preceding experiments raised the question of how the released collagenase in the control corneas was inhibited, degraded, or somehow eliminated, whereas in deficient corneas it continued to be active or to be produced. Although the histologic observations suggested that the ongoing infiltration of acute inflammatory cells might provide a continuing supply of collagenolytic enzymes, it seemed important to determine the exis-
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tence of an inhibited or latent (trypsinactivatable) enzyme in the control corneas.

A trypsin titration curve was first established for activation of released collagenase that had been inhibited by treatment with rat serum (Fig. 3). The collagenase was inhibited 50% by the amount of serum used (0.2 μl); 25 μg/ml of trypsin could completely recover the active collagenase, and 200 μg/ml of trypsin increased the collagenase activity to 1.5-fold in a 10 min incubation, thereby demonstrating the presence of activatable latent collagenase.

To study latent collagenase, rats were sacrificed either 16 hr or 72 hr after burning. The corneas were immediately placed in culture, and the day 1 media were then titrated with the same concentration range of trypsin (10 to 200 μg/ml) previously determined to activate the collagenase. In both deficient and control corneas no significant amount of latent collagenase was detected at 16 or 72 hr after burning (Fig. 4). Thus, for controls, the collagenase at 16 hr was fully active (data not shown) and the lack of collagenase release at 72 hr was not caused by inhibition of the enzyme. For deficient corneas the elevated level of collagenase at 72 hr was also in the fully active form.

To exclude the possibility of an inhibitor that was not responsive to trypsin being present in control cultures, media from deficient corneas containing a known amount of collagenase were added to media from control corneas (incubated 72 hr after burning). No inhibition of the collagenase by control media was discernible either at the beginning or at the end of the culturing period.

Exclusion of inflammatory cells from burned cornea. Histologic observations from the collagenase release experiment suggested that the enzyme might be derived from infiltrating PMNs, a hypothesis first advanced by Pirie et al. to explain collagenolytic activity in unburned, vitamin A-deficient corneas. To test this possibility we tried to block PMN infiltration and then to determine collagenase release. Kenyon et al. demonstrated that a tissue adhesive, isobutyl 2-cyanoacrylate, can inhibit infiltration of PMNs into alkali-

burned rabbit corneas and prevent ulceration. We applied this adhesive immediately after a mild thermal burn to deficient and control corneas and either placed them in culture or prepared them for histologic study at 16 or 72 hr after burning.

Both deficient and control eyes retained the adhesive during the entire 72 hr experiment, and there was no clinical suggestion that the glue caused increased or decreased inflammation or tendency for infection. Indeed, in all eyes no corneal ulceration was apparent after removing the adhesive at the time of sacrifice. Histologic observations of the central corneas of both deficient and control animals were identical; neither showed evidence of epithelial recovery, stromal ulceration, or stromal infiltration by inflammatory cells or keratocytes (Fig. 5). The peripheral corneas beyond the burned areas, however, demonstrated considerable PMN infiltration in deficient animals but insignificant infiltration in controls.

Collagenase activity was determined in day 1 culture media of corneas with and without tissue adhesive. When animals were killed and corneas were placed in culture 16 hr after a mild thermal burn, the adhesive completely prevented the appearance of collagenase,
Fig. 8. Separation of centrally burned cornea button from peripheral unburned cornea ring. 

Top, In the deficient cornea, 16 hr after burning, the central button (left) demonstrated absence of epithelium, extensive stromal PMN infiltration, and numerous PMNs adherent to the endothelium. The peripheral ring (right) maintained an intact epithelium and had substantial PMN infiltration. Middle, In the deficient cornea, 54 hr after burning, the central button (left) exhibited irregular epithelial regeneration and marked intrastromal PMNs, whereas the unburned ring (right) showed minimal PMN response. Bottom, In the control cornea, 16 hr after burning, the central button (left) had a regenerated epithelium and an acellular stroma with no anterior chamber reaction. The peripheral ring (right) at 54 hr after burning exhibited normal epithelial morphology and insignificant stromal inflammatory response. (All H & E; x150 to 400).
both in deficient (Fig. 6, A) and control corneas (Fig. 6, B). When deficient corneas were placed in culture 72 hr after burning, application of adhesive had caused a fourfold to fivefold decrease in collagenase release (Fig. 7, A). Control corneas, of course, showed no collagenase at that time, whether treated with adhesive or not (Fig. 7, B). These results show that when re-epithelialization and PMN infiltration are blocked, collagenase activity decreases significantly.

Separation of central burned cornea from peripheral unburned cornea. In our previous observations of whole corneas after a thermal burn, the central burned area frequently contained most of the PMNs. To obtain further evidence for a correlation between PMN infiltration and collagenase release, we separated the central burned "button" (2.5 mm diameter) from the peripheral unburned "ring" for histologic and biochemical examination.

Histologic studies were performed on buttons and rings from a total of 17 eyes (Fig. 8). At 16 hr after burning, buttons from deficient corneas showed an absence of epithelium and the presence of substantial PMNs within the stroma and adherent to the endothelium. By 54 hr, although some epithelial recovery had occurred, inflammatory cell infiltration had increased. At both 16 and 54 hr the rings of deficient corneas showed moderate numbers of intrastromal PMNs. In contrast, control eyes taken into culture 16 and 54 hr after the burn exhibited epithelium that completely resurfaced the central button, and an acellular stroma; peripheral rings showed little or no inflammatory response.

Central buttons were trephined at 16 or 54 hr after burning and were cultured for 1 day separately from peripheral rings. At 16 hr after burning (Fig. 9), the central buttons (containing most of the PMNs) showed 10 times more collagenase activity (per unit surface area, see figure legend) than that in the peripheral rings. In control corneas, only the unburned area, which contained some PMNs, showed a small amount of collagenase activity (data not shown).

At 54 hr after burning, the buttons, which were heavily infiltrated by PMNs, again had 10 times the activity of the rings (Fig. 9). In the controls, collagenase activity was insignificantly small in both buttons and rings (data not shown).

These findings further confirm the close correlation between the presence of PMNs and the release of active collagenase. However, we noted that the combined total activity of button and ring was never more than 10% to 20% of the activity routinely found in day 1 medium of whole deficient cornea after a mild burn. When we incubated one-half button with one-half ring, we detected no increase in activity over that found when they were incubated separately (data not shown).

Discussion

The results of our previous work suggested that corneas from vitamin A-deficient rats might have a greater risk of ulceration.
as a result of environmental trauma. This was inferred from the different response of deficient and pair-fed control rat corneas to the same mild thermal burn. The burns caused no corneal lesions in pair-fed control rats; little or no collagenase release was detected from these corneas in organ culture. On the other hand, deficient rats showed severe corneal ulceration and a high level of collagenase. The collagenase was detected mainly in media harvested on the first day of culture.

We then faced the question: is collagenase activity caused by (1) a process peculiar to deficient corneas, which is lacking in controls or (2) similar collagenase release in deficient and control corneas, followed by inhibition of the enzyme(s) in the control corneas? To distinguish between these possibilities we carried out a time-course study of collagenase release. Our data showed that corneas of pair-fed control rats released significant amounts of collagenase during the early hours after trauma, and the activity then declined to almost zero. In deficient rats the activity continued at a high level. We therefore concluded that the release of collagenase in deficient and control corneas is very similar in the early hours after the trauma. The enzyme disappears in the control corneas at later times, whereas it continues to be made in deficient corneas. This result, then, favored hypothesis 2 above.

Our next task was to decide whether the decline of collagenase activity in the controls was indeed caused by an inhibitor that is absent in deficient corneas but present in controls. The trypsin activation experiment failed to demonstrate an inhibitor; at 72 hr after the burn there was only an insignificant amount of latent collagenase evident in both deficient and pair-fed control corneas. Thus inhibition alone is clearly not the reason why control corneas show little collagenase and fail to ulcerate after the burn. The lack of collagenase inhibitor in the control corneas was further proved by an experiment in which the same amount of collagenase was recovered after the addition of control media to the deficient media. This proved that no unstable inhibitors were present in the control media that were not released by trypsin.

Weimar suggested that injured corneal epithelium released a substance after linear wounds that was chemotactic for PMNs. Pandolfi and Astrup reported that injured corneal epithelium released plasminogen activator. Recently Berman et al. showed that plasminogen activator is secreted by ulcerating corneas. This activator stimulates plasminogen to form plasmin, which then cleaves C (a component of complement) to C, a chemotactic substance for PMNs. The presence of PMNs as the major inflammatory cells in injured cornea has been reported by several laboratories, and these PMNs have been suggested to be a major source of collagenase in wounded cornea. In 1975 Pirie et al. reported that PMNs are overwhelmingly dominant over other types of inflammatory cells in the ulcerating corneas of severely vitamin A-deficient rats. To further strengthen the hypothesis, Rowsey et al. found that intracorneal injection of rabbit PMN lysosomal fraction can induce morphologically detectable lysis of the stroma.

Therefore we developed the hypothesis that mild trauma to the corneal epithelium induces PMN infiltration in control and deficient rats. In the deficient cornea, however, this inflammatory response is accelerated and continues to attract PMNs (possibly in relation to the persistence of epithelial defects), whereas the control cornea heals rapidly and PMNs cease to infiltrate. This hypothesis would also explain the fact that the major collagenase activity is found mainly in media harvested on the first day of culture; existing infiltrated inflammatory cells would release the enzyme in culture but would not themselves survive, proliferate, or continue to produce enzyme, as might epithelial and stromal cells.

To infer that PMNs were the source of the enzyme activity, we first blocked their infiltration with tissue adhesive after burning and then assayed for collagenase. Simultaneous histologic and biochemical studies showed
that the tissue adhesive successfully blocked PMN infiltration and significantly decreased collagenase activity. This covariation between PMN invasion and collagenase release strongly suggests an integral relationship between the leukocytes and the enzyme.

The experiment in which the burned center ("button") and unburned surround ("ring") of cornea were separated and incubated separately further supported the hypothesis that PMN appearance is closely related to collagenase release; the central button contained the majority of PMNs and had 10 times the collagenase activity of the peripheral ring (per square millimeter).

PMNs could contribute to collagenase activity in several different ways. The first is to produce the enzyme directly. Although Lazarus et al. 14 and Robertson et al. 15 reported extracting collagenase directly from PMN granules, it is known that the collagenase content of individual PMNs is very small. However, the situation in vivo is likely to be quite different, since the ongoing infiltration of innumerable PMNs might lead to significant aggregate accumulation of collagenase activity. We attempted to extract collagenase directly from burned, deficient corneas by the method of Leonard and Maddison, 16 but we were unable to detect activity presumably because of the very small amount present, given the poor yield of the extraction procedure. 17

PMNs may also contribute to collagenase activity by releasing hydrolases and proteases during the phagocytic process. Werb and Aaggeler 18 reported that proteases can induce fibroblasts to secrete collagenase. Thus it is possible that PMNs could induce collagenase production of the stromal fibroblast keratocytes. In fact the PMN-containing central "button," incubated by itself, was found to produce less collagenase than the whole cornea. The sum of button and ring collagenase activity had five to 10 times less collagenase activity than the equivalent whole cornea. This implies that separation of PMNs from the surrounding fibroblasts results in a severe decrease in release of the enzyme from the cornea. Full collagenase release occurs only when PMNs and fibroblasts are together, suggesting that mediator(s) between the infiltrating PMNs and the surrounding epithelial and stromal cells might be necessary for the production or release of enzyme. Kenyon et al. 4 observed that in the alkali-burned rabbit cornea, PMNs actively degranulated and were phagocytic; this could release proteases, hydrolases, plasminogen activators, and other inflammatory mediators. 10 It is possible that one of these could be the activator or stimulator of collagenase, as observed by Werb and Aaggeler. 18 On the other hand, PMNs observed in the unburned area were quiescent. 4 These PMNs did not actively release these enzymes, although they could be passively released by bursting during culture. It is important to note that the putative mediator cannot be transferred from PMNs to keratocytes through the culture media: when one-half button was incubated with one-half ring, no more activity was found than when they were incubated separately.

From Fig. 9 we observed that in the burned area, detectable collagenase activity was found only on day 1 of culture. Yet in the unburned area, collagenase activity, though very small, was detectable on days 2 to 5 in addition to day 1. Histologic examination showed that under the burned area there were PMNs but no fibroblasts; under the unburned area there were fibroblasts and few PMNs. The collagenase activity detected from days 2 to 5 in the unburned area could therefore come from those fibroblasts. It is interesting that in human ulcerating corneas, Gordon et al. 19 recently used immunofluorescence to show that collagenase was localized in the stroma, leading to the hypothesis that stromal fibroblasts are a source of corneal collagenase.

It is undoubtedly the case that corneal ulceration in vivo involves the interaction of multiple cell types to provide the chemotactic factors, mediators, activators, and proteases that result in stromal matrix degradation. Our studies in the vitamin A-deficient rat, however, favor a model in which ocular
surface trauma and delayed epithelial recovery precipitate an enhanced inflammatory response whereby PMNs are the most probable effectors of stromal destruction.

REFERENCES


