The effect of thermal burns on the release of collagenase from corneas of vitamin A–deficient and control rats

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A mild trauma in the form of a thermal burn was applied to corneas of vitamin A–deficient rats and their pair-fed controls. The control corneas routinely showed rapid re-epithelialization without stromal changes. The corneas of deficient rats recovered more slowly, frequently exhibiting stromal edema, leukoma, and sometimes ulceration. Because collagenase is thought to initiate collagen destruction in corneal ulceration, the relationships among vitamin A status, severity of trauma, and collagenase levels were determined. Mild thermal burns were found to cause corneas from less severely deficient rats to ulcerate rarely but to release increased levels of collagenase, mainly on the first day of culture, as in the case of nonburned, severely deficient rats. Comparable burns of corneas of pair-fed control rats resulted in no ulceration and in very little collagenase release. Severe burns of either pair-fed control or normal rat corneas caused ulceration and collagenase release, but collagenase activity was maximal on the second and third days of culture. Differences in vitamin A status at time of burning gave rise to different patterns of collagenase. By following the development of the vitamin deficiency, it was determined that little active collagenase is released after mild burns of corneas in animals in the pre–weight plateau stage but that much more active enzyme is released when animals are in weight plateau or 5% weight loss stages. Studies of the effect of recovery from vitamin A deficiency on the response to mild thermal burn indicated that the longer the interval between feeding vitamin A and the burn, the lower the postburn level of collagenase in the day 1 medium. Thus it would appear that restitution of vitamin A status decreases the level of active collagenase after the mild thermal burn. The system developed here can be used to study the biochemical basis for ulceration in vitamin A deficiency, and the possibility exists that the ulceration characteristic of keratomalacia in people can be initiated by an environmental trauma.

Key words: cornea, vitamin A deficiency, vitamin A, rat, keratomalacia, collagenase, collagen, trauma, thermal burn, corneal ulceration, polymorphonuclear neutrophils
Keratomalacia is the rapid corneal ulceration that occurs in children who are severely deficient in vitamin A. It has been estimated that tens of thousands of new cases of this disorder occur each year in malnourished children. Although the exact sequence of events leading to tissue dissolution has not been elucidated, it would seem that as in cases of ulceration after chemical burns, thermal burns, or infection, the degradation of fibrillar corneal collagen is initiated by the enzyme collagenase. Indeed, Pirie et al. have recently described the release in culture of collagenolytic activity from vitamin A–deficient rat corneas.

It is unlikely that vitamin A deficiency alone causes corneal ulceration. Rather, it is our hypothesis that tissue changes specific to the deficiency can predispose the cornea to ulceration. This predisposition could occur either by the lowering of the threshold for the initiation of the ulcerative process, or by rendering ineffective the control mechanisms that normally prevent tissue destruction, or by both. In this view, vitamin A deficiency is a necessary but not a sufficient component of keratomalacia. The events leading to ulceration and destruction of the cornea would then have to be initiated by some external insult, be it physical or chemical or the result of infection.

In this communication, we compare vitamin A–deficient and pair-fed control rats in their response to mild thermal burn of the cornea. The differences observed, both clinically and biochemically, support the suggestion that the vitamin A–deficient cornea is at risk with respect to ulceration.

Materials and methods

Rats. Weanling Holtzmann albino rats were put on a vitamin A–deficient diet for 3 to 4 weeks and then paired by weight with normal control rats which were fed the same diet with a vitamin A supplement (200 μg of retinyl acetate per week). After they had been paired, the weights of the control and deficient rats were recorded. The amount of food eaten by the deficient rat on one day was given to its paired control rat on the next day.

For the vitamin A recovery experiments, rats were kept on a vitamin A–deficient diet until they developed deficiency symptoms (5% to 15% weight loss). Then retinyl acetate (150 μg in 0.2 ml of cottonseed oil) was given intraocularly to the deficient rats at different time intervals either before or after the thermal trauma.

Thermal burns. Rats without eye lesions were selected from animals having lost 5% to 15% weight after maintenance on the vitamin A–deficient diet. Animals were anesthetized by intraperitoneal injection of 0.5 ml of chloral hydrate solution per 100 gm body weight (3.6% solution for deficient; 7% for control rats). The eyes were then treated with 1 drop each of 0.5% proparacaine hydrochloride (E. R. Squibb, Burlington, Mass.). After application, the eyes were washed with saline and blotted dry. A thermal probe (Thermokeratophore; Frigitronics, Inc., Shelton, Conn.) of 2 mm diameter was used at 130°C to burn their corneas. An instant touch of the probe to the central cornea produced a "mild burn." The burn produced by applying the probe to the cornea twice at the same spot with increased pressure and 1 sec duration each time was defined as a "severe burn."

Clinical and histopathologic observations. Animals were examined at intervals by slit lamp. When ready, they were sacrificed, and their eyes were enucleated, placed in 10% neutral-buffered formalin, and prepared by standard techniques for paraffin-embedded sections. These were stained with hematoxylin and eosin, and serial sections were taken at four stepped intervals across the entire cornea. Representative sections were examined and photographed with a Zeiss microscope III.

Organ culture methods. Generally, unless otherwise stated, burned rats were sacrificed 3 days after burning. The reason for choosing this time interval was that Kenyon et al. had found ulceration of rabbit corneas, accompanied by collagenase release, to begin 3 days after a severe thermal burn. Corneas were cut into small pieces and cultured in Dulbecco’s modified Eagle’s Medium with NaHCO3 (0.2%), penicillin (110 U/ml), and streptomycin (100 μg/ml). Each culture dish containing six corneas and 1 ml of medium was placed in a sterile box and gassed with 5% CO2 + 95% O2 at 37°C. The medium was changed every day for 5 days, and the harvested medium was stored at −20°C. The sterility of cultures was confirmed by the absence of bacterial growth on nutrient agar plates subsequent to inoculation with media harvests.

Preparation of collagenase. The stored media were thawed and concentrated by ultrafiltration to 0.5 ml (filter PTGS, cutoff point 10^4 mol. wt.; Mil-
lipore Corp., Bedford, Mass.), for purposes of assay. Concentrated media were then dialyzed overnight against 0.025M Tris HCl buffer, pH 7.8 (4°C), containing 0.005M CaCl₂ and 0.1M NaCl, and then lyophilized. The lyophilized enzyme was then redissolved in 50 μl of double-distilled or deionized distilled water and was assayed.

**Characterization of collagenolytic activity.** Dialyzed harvests from thermally burned ulcerating rat corneas were incubated with 0.1% calf tail tendon collagen at 23°C. For the determination of cleavage products of collagen, reaction mixtures were electrophoresed in 6% or 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Collagenase activity was determined in a capillary "heat gel" system which employs undenatured (native) calf tail tendon collagen as substrate. This system discriminates active collagenase from other proteases that can degrade denatured collagen (gelatin). Collagenase activity was determined from the mean of three replicate assays incubated for 10 hr. Rates of collagen gel lysis were demonstrated to be proportional to enzyme concentration, over a range of approximately 0.05 to 0.25 mm of lysis per hour (unpublished data), as had been shown previously to hold for the enzyme from rabbit corneas. Data are expressed as millimeters of lysis per hour per cornea.

**Collagen solubilization.** The amount of hydroxyproline in the medium as a marker for solubilized collagen was determined by the method of Rojkind and Gonzales, after centrifugation of harvested media.

**Results**

**Clinical and histological observations**

*Nonburned animals.* In nonburned pair-fed control animals, the corneal epithelium remained intact and lustrous (Fig. 1, A) in contrast to vitamin A-deficient animals (weight loss 20%), which showed varying degrees of corneal xerosis, with punctate keratopathy evidenced by methylene blue staining of the irregular epithelium (Fig. 1, B). These deficient animals were never noted to show gross lesions such as stromal edema or ulceration. Severely deficient rats (>20% weight loss), on the other hand, frequently showed eye lesions such as stromal edema or leukoma and, rarely, ulceration.

By light microscopy, nondeficient controls demonstrated entirely unremarkable morphology (Fig. 2, A), and deficient animals (5% to 15% weight loss) showed only minimal superficial epithelial keratinization with no stromal abnormality (Fig. 2, B). Severe vi-
Fig. 2. For legend, see facing page.
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Vitamin A deficiency, however, resulted in marked epithelial surface keratinization, granulosa cell layer formation, stromal infiltration by inflammatory cells, and peripheral stromal neovascularization (Fig. 2, C and D).

**Thermally burned animals.** In response to mild thermal burns, nondeficient controls showed rapid epithelial recovery, did not show significant stromal opacification, and never ulcerated. After the burn, deficient animals (5% to 15% weight loss) often exhibited stromal edema and leukemia but rarely ulceration. In contrast, with severe deficiency a mild burn resulted in rapid stromal ulceration and frequent perforation (Fig. 1, C). Severe thermal burns resulted in stromal ulceration in both normal and vitamin A–deficient animals. Histologically, no inflammatory response was noted in corneas of mildly burned, nondeficient controls (Fig. 2, E). In contrast, deficient animals 3 days after mild thermal burn usually showed intact epithelium and nonulcerating stroma with moderate acute inflammatory infiltrate (Fig. 2, F).

In the severely deficient animal, loosely adherent epithelium often terminated at margins of ulcerating stromas that were heavily infiltrated by acute inflammatory cells (Fig. 2, G and H).

**Characterization and assay of collagenolytic activity.** Electrophoresis of the reaction products of collagen with media harvests from both mildly burned deficient and severely burned normal corneas demonstrated that collagen had been cleaved intrahelically into approximately ¾-length and ¼-length products (Fig. 3). As described by Pirie et al., the initial cleavage products appear to have been degraded further, presumably by other proteases in the media.

Control corneas given mild burns showed no lesions 3 days after burning. Upon culturing of these corneas, low levels of collagenase were detected on days 2 and 3 (Fig. 4, A). Much more collagenase was detected in cultures of corneas from vitamin A–deficient rats (weight loss 10.6% ± 0.65 S.E.M.) after the mild thermal burn. The active collagenase level was greatest on day 1 of culture (Fig. 4, A). When nonburned corneas were cultured from control (pair-fed) and from vitamin A–deficient rats without eye lesions (weight loss 20.7% ± 5.4 S.E.M.), no detectable collagenase was released within the limits of sensitivity of the assay, even after 5 days of culture.

Nonburned corneas of severely deficient rats (weight loss > 20%; stromal edema and leukemia in corneas) released considerable active collagenase into the medium when placed in culture. Collagenase levels were found to be highest on the first day of culture.

**Fig. 2.** A, Pair-fed control animal exhibits normal corneal cytoarchitecture with respect to uniform, five-layered, nonkeratinizing epithelium, avascular stroma devoid of inflammatory cells, and unremarkable Descemet’s membrane and endothelium. (×100.) B, Vitamin A–deficient animal (10% weight loss) shows only minimal keratinization of superficial epithelial cells. (×250.) C, Severe vitamin A deficiency (20% weight loss) results in marked epithelial surface keratinization, granulosa cell layer formation, stromal inflammatory infiltration, and stromal neovascularization (peripherally). (×250.) D, Severely deficient animals show infiltration of central stroma by acute inflammatory cells, predominantly PMNs. (×400.) E, Pair-fed control animal 3 days after mild thermal burn has re-established intact corneal epithelial layer. The stroma is intact but acellular, since neither keratocytes nor inflammatory cells have repopulated the central stroma. (×250.) F, Deficient animal (10% weight loss) 3 days after mild thermal burn shows intact epithelium and nonulcerating stroma which is, however, moderately infiltrated by acute inflammatory cells. (×150.) G, Severely deficient animal 3 days after mild thermal burn demonstrates extensive central epithelial defect (left) and nonadherent marginal epithelium (right), with ulcerating stroma heavily infiltrated by acute inflammatory cells. (×100.) H, Severely deficient animal 3 days after mild thermal burn exhibits stromal ulcer (left) with epithelium (right) terminating abruptly at margin of stromal defect. The stromal lamellae appear diffusely disorganized and fragmented, and they are infiltrated by PMNs. (×250.)
Fig. 3. Cleavage of collagen by media concentrates. Gel electrophoresis of the denatured products of hydrolysis of collagen in solution. Concentrated culture media were incubated with calf tail tendon collagen in solution at 23° for times indicated below and then acidified to pH 3.5 by acetic acid. Incubation has resulted in degradation of the β-dimers (β11, β12) to ¼-length dimers (β11, β12); of single α-chains (α1, α2) to ¼-length α-chains (α1, α2). Left panel, Media from mildly burned vitamin A-deficient rat corneas. (1) Calf tail tendon collagen incubated 72 hr in buffer; (2) combined day 1 to day 4 culture concentrate; (3) calf tail collagen and 20 μl of day 1 culture concentrate incubated 17 hr; (4) calf tail collagen and 150 μl of day 4 culture concentrate incubated 17 hr. The one-fourth fragments are not resolved by the 6% gel used and migrate with the buffer front.

Right panel, Media from severely thermally burned ulcerating rat corneas incubated for 4 days (3). Fragments with sizes between α1 and α2 as well as fragments smaller than α1 are produced. Cleavage has also formed the ¼-length fragment α2. The α2 fragment is nearly completely missing, indicating its continued degradation to small-molecular-weight fragments. Shown for comparison are β and α components of undegraded collagen (1) and ¼- and ½-length collagen reaction products from incubation with rabbit corneal fibroblast collagenase (2) used as markers. Acrylamide concentration, 10%. Notation is same as left panel except that β and β' not resolved into β11 and β12, nor α into α1, α2, ¼-length α1, α2, ¼-length α2.

(Fig. 4, B). Thus less severely vitamin A-deficient rats without eye lesions (Fig. 4, A) showed a pattern of collagenase after burning closely similar to that of nonburned severely deficient corneas (Fig. 4, B).

Severe thermal burns of corneas from control animals caused ulceration in almost all animals and the presence of active collagenase in the culture media. As opposed to the deficient corneas, active collagenase was maximal on day 2 of culture and continued through day 3 (Fig. 4, A).

To study corneal collagen solubilization as an index of actual stromal destruction, determinations were made of hydroxyproline-containing fragments in daily harvest of media. Inspection of hydroxyproline profiles (Fig. 5, A) shows that more collagen was solubilized on day 1 in culture of burned deficient corneas than on day 1 in cultures of burned or nonburned pair-fed control corneas. Thereafter, daily solubilization of corneal collagen appeared comparable in culture of burned deficient and pair-fed controls. Nonburned pair-fed controls showed much less collagen solubilization. Despite the fact that collagenase showed a peak at day 1 in culture of burned deficient corneas, the peak of collagen solubilization by such corneas was on day 2 of culture. When the first harvest
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Fig. 4. Collagenase activities in cultures of corneas. Culture media were harvested daily for 5 days and assayed for collagenase. A, -•- - Mildly burned vitamin A-deficient corneas. Each point is the mean of five separate experiments, with a total of 17 culture dishes (five to six corneas per dish). Bars represent S.E.M. -Δ-, Severely burned, pair-fed control corneas. Each point is the mean of two experiments, with a total of eight culture dishes, (five to six corneas per dish). Bars represent the range. •, Mildly burned, pair-fed control corneas. Numbers of corneas same as for severely burned control corneas. B, Nonburned, severely vitamin A-deficient corneas. Each point represents the mean of three experiments; bars give S.E.M. Culture dishes (five to six corneas per dish) used in each experiment were five, four, and two.

was made after 2 days of culture, the initial hydroxyproline levels were much higher than in cultures of burned and nonburned pair-fed controls (Fig. 5, B). Thereafter, daily solubilization of collagen was comparable in all three types of cultures. These data are interpreted to mean that there is a lag in collagen degradation after introduction of the corneas to culture, so that even though collagenase levels are higher initially in burned deficient cultures, solubilized collagen does not reflect the initial difference in collagenase levels.

Collagenase during development and recovery from vitamin A deficiency. The response to mild burns is correlated with the extent of progressive vitamin A deficiency (Fig. 6, A). At the stage when there was still sufficient vitamin available to support growth (preplateau) a small amount of collagenase was detected on days 2 to 5 of culture. When growth ceased (plateau stage), presumably after a decline in tissue vitamin A, the mild burn caused the appearance of collagenase activity on days 1 to 2 of culture. The amount of collagenase detected increased with advancing vitamin A deficiency. Conversely, upon intragastric intubation of one dose of retinyl acetate (150 μg), complete recovery occurred from the effects of deficiency on the cornea, provided the vitamin was given 48 hr before the mild burn; a very low level of collagenase was detectable on day 1 of culture, and a small amount was detected on days 2 to 5 (Fig. 6, B). If the vitamin was given 24 hr before the burn, some collagenase was detected on day 1 of culture, and more was detected if the vitamin was given immediately after or 24 hr after burning.

Discussion

The results of the current studies suggest that corneas from vitamin A-deficient rats...
Fig. 5. Release of hydroxyproline (OH-Pro) (micrograms per cornea) into the media of cultures of rat corneas. ——, Mildly burned, vitamin A-deficient corneas. Each point is the mean of three separate experiments, with a total of three culture dishes (four to six corneas per dish). Bars represent S.E.M. ---, Mildly burned, pair-fed control corneas. Numbers of corneas same as for deficient ——, Nonburned, pair-fed control corneas. A, Culture media harvested daily and assayed for hydroxyproline.6 B, Culture media initially harvested 2 days after culturing, then daily, and assayed for hydroxyproline.

are at increased risk of ulceration as a result of trauma. This was demonstrated by the differential clinical and biochemical responses to the same mild burn by the deficient and the control corneas. The burns caused no corneal lesions in pair-fed controls, and little or no collagenase was detected in media from such corneas. Control corneas could be caused to ulcerate but only by severe burns, and the pattern of release of collagenase differed from that of the burned vitamin A-deficient corneas. We were also able to confirm the observation of Pirie et al.¹ that nonburned corneas from severely vitamin A-deficient rats (>20% weight loss; gross eye lesions) released active collagenase into the medium. The collagenase was detected mainly on the first day of culture.

The difference in patterns of collagenase activity (Fig. 4), mainly on day 1 for the deficient (burned and nonburned) and days 2 and 3 for the burned pair-fed controls, is of significance in that it might bear upon the mechanism of ulceration in the vitamin A-deficient cornea. The recovery experiment in particular (Fig. 6, B) shows that on days 2 to 5 the levels of detectable collagenase present in the media are almost identical for corneas from animals whose day 1 collagenase values do differ as a function of vitamin A status. Moreover, collagenase values for days 2 to 5 are comparable, whether vitamin A was or was not restored to the deficient animals. It is clear, then, that the day 1 media reflect the vitamin A status. Collagenase levels on subsequent days of culture are thought to reflect the results of the tissue injury involved in the scissoring and mincing procedures used to prepare corneas for culture.

The hydroxyproline data (Fig. 5) are also in support of the interpretation that day 1 media reflect the vitamin A status. They suggest that the greatest daily solubilization of corneal collagen in vitro in culture of burned vitamin A-deficient corneas occurs during the early part of the culture period. Thereafter collagen solubilization occurs at slower rates in all types of cultures. It is to be noted that although pair-fed controls do not show persistent corneal pathology after thermal burns in vivo, they do demonstrate solubilization of corneal collagen in organ culture somewhat above that of nonburned controls (Fig. 5). If one considers the hydroxyproline profile (Fig. 5, B) in nonburned controls as reflecting merely the tissue mincing and culturing and subtracts this from the burned control profile, it becomes clear that nearly all the hydroxyproline in the burned control is due to tissue preparation. Despite the fact that there is a relationship between collagenase, vitamin A status, and hydroxyproline release, our results show that hydroxyproline solubilization in culture is not correlated with collagenase levels and most likely reflects the activity of other proteases as well.

The present studies do not provide an answer to the question why nonburned severely vitamin A-deficient corneas some-
Fig. 6. A, Collagenase activities in cultures of corneas from mildly burned pre-weight plateau, plateau, and vitamin A-deficient rats. Culture media were harvested daily for 5 days and assayed for collagenase. ---Ø---, Preplateau rats; ---Δ---, plateau rats; ---○---, deficient rats (5% to 10% weight loss, no eye lesions). Each experiment was done with the combined media from two culture dishes (six corneas per dish). B, Collagenase activities as a function of recovery from vitamin A deficiency. Corneas were removed from mildly burned deficient rats (5% to 15% weight loss, no eye lesions) at various times after vitamin A administration and cultured for 5 days with daily change of medium. Collagenase activities were determined. ---●---, Vitamin A supplementation 48 hr before burning (exp. 1); ---□---, vitamin A supplementation 24 hr before burning (exp. 2); ---○---, vitamin A supplementation 24 hr after burning (exp. 3); ---Δ---, vitamin A supplementation immediately upon burning (exp. 4); ---A---, no vitamin A supplementation (exp. 5). The numbers of culture dishes used for each experiment were two, two, three, three, two, respectively, with four to six corneas per dish. For experiments 1, 2, and 5, the points represent means of two separate experiments, and the bars show the range; for experiments 3 and 4, points represent the means of three separate experiments, with bars representing S.E.M. Combined collagenase values for experiments 1 and 2 (day 1) are statistically significantly different from combined values of experiments 3, 4, and 5 (day 1) (p < 0.01).

Times ulcerated and why thermal burns of less severely vitamin A-deficient corneas sometimes also caused ulceration. In keeping with the hypothesis that trauma of some sort is necessary to initiate the events leading to ulceration, it is possible that some severely vitamin A-deficient animals have suffered trauma. This may have caused the release of destructive mediators which are successfully regulated in well-nourished animals. Ulceration in the present studies is correlated with the presence in the ulcer region of polymorphonuclear neutrophils (PMNs). However, their exact roles are not understood. PMNs have been implicated in ulceration from diverse causes. Indeed, in a study of ulcerations in vitamin A-deficient rat corneas, Pirie et al. observed that higher levels of collagenolytic activity were correlated with the appearance of PMNs in the vitamin A-deficient corneas. Possibly the observed inability of the burned corneal epithelium to heal in vitamin A-deficient animals results from the continuous infiltration of the corneal stroma by PMNs. A role of persistent epithelial defects in attracting PMNs into the cor-
nea has been discussed elsewhere. PMNs do contain hydrolases that can attack collagen; and it would seem that PMN-derived elastase and cathepsin, in addition to collagenase, might all contribute to the collagen destruction of corneal ulceration. In this regard, hydrolase-containing vesicles might be more labile in vitamin A deficiency, so that hydrolases more readily reach the extracellular matrix. Some PMN hydrolases, so released, could then facilitate PMN infiltration of the stroma.

Although PMNs appear to participate in corneal ulceration, roles for other cell types are not ruled out. Indeed, rabbit corneal fibroblasts in cell culture produce a collagenase with the same chromatographic and activation properties as that obtained from organ cultures of corneas that are ulcerating after alkali burns.

We have shown in this report that a mild thermal trauma inflicted on corneas, even at the earliest stage of deficiency (Fig. 6,A), caused corneal lesions and release of active collagenase whereas a trauma of the same magnitude to control corneas caused no lesions and resulted in little detectable collagenase release. Thus trauma can initiate biochemical events in early stages of vitamin A deficiency that may ultimately lead to stromal ulceration. The possibility exists, then, that the very erratic and usually unilateral appearance of ulceration characteristic of keratomalacia, both in rat and man, may also be initiated by an environmental trauma.

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