In Vitro Incorporation of Proline into Keratoconic Human Corneas

Uri Rehany* and Shmuel Shoshan†

Corneal buttons were obtained from four young adults with advanced keratoconus following perforating keratoplasty. The corneal buttons were incubated in organ culture media containing 3H-proline. Autoradiographs of corneal sections showed that the incorporation of the radioisotope was significantly higher in all layers of the keratoconic corneas than that found in the normal controls, indicating an increased protein biosynthesis in the former. It is suggested that in spite of the found increased synthetic activity, slow destruction and thinning of the cornea in keratoconus might occur as the result of inadequate compensation for tissue loss due to increased collagenolytic activity in the disease.


Keratoconus is a bilateral, dystropic, noninflammatory ectasia of the central part of the cornea, characterized by progressive thinning, destruction, and scarring. The onset of the disease usually coincides with adolescence. The cornea gradually becomes cone shaped with severe thinning of the apex. The visual acuity is decreased due to high myopic astigmatism and central scarring. In the advanced stages, corneal transplantation is required in most of the cases. The disease is usually limited to the cornea without other ocular or systemic manifestations. However, there are associations with other diseases, such as retinitis pigmentosa, Down’s syndrome, vernal conjunctivitis, atopic dermatitis, and also some connective tissue disorders such as Ehlers-Danlos syndrome. Usually, there is no recognizable genetic pattern, though it may be inherited as a recessive trait, more frequent among females. The etiology and pathogenesis of keratoconus are still an enigma. There are a variety of theories that include biophysical and atopic conditions, as well as hormonal, nutritional, and biochemical factors.1

Results obtained from histopathologic,2 ultrastructural,3-5 and enzymatic studies6-8 suggest that the slow destruction of the cornea might be due to an increase in collagenolytic activity in keratoconus. Electron microscopic studies also showed abundance of organized granular endoplasmic reticulum indicating involvement of keratocytes in active elaboration of proteins.9

It has been suggested that these findings indicated an attempt to repair the damaged collagenous connective tissue.

Biochemical studies of keratoconic corneas showed increased incorporation of proline and glucose in the structural glycoproteins rather than in the collagenous component.1 Moreover, no significant change was found in either the amount10 or distribution11 of collagen synthetized by human corneal stromal cultures from keratoconic corneas as compared with normal controls. Neither were significant differences found in the relative amount of the collagen types between normal and keratoconic corneas.12 The present autoradiographic study was initiated to correlate the biochemical and morphologic evidence for increased protein biosynthesis in keratoconic corneas.

Materials and Methods

Corneal buttons, diameter 7 mm from the center, were obtained by perforating keratoplasty from two female and two male patients, age 22-40 years, who suffered from advanced keratoconus with no clinically visible scars. They had no other ocular or systemic diseases. The corneal transplantations were performed for therapeutic reasons, and therefore no informed consent was required for further study of the excised tissue. Unaffected control corneas were obtained from four deceased individuals, age 27-52 years, 1-4 hr after death, who had no history of eye diseases. Corneal buttons from both groups were promptly placed in cold McCarey-Kaufman corneal bathing media (Warner Lambert Co.; Morris Plains, NJ) and cultured soon thereafter.

The corneal buttons were incubated separately for 24 hr at 37°C in 10 ml of organ culture media...
(Dulbecco's Modified Eagle's medium) supplemented with penicillin (250 μg/ml) streptomycin (10 μg/ml), ascorbic acid (50 μg/ml), and 250 μCi of tritiated proline (New England Nuclear, Boston, MA; NET-285 specific activity 5 Ci/mmol).

At the end of the incubation time, the corneas were removed from the medium, rinsed with normal saline several times, and fixed in 4% neutral buffered formalin solution, followed by paraffin embedding and sectioning for autoradiography. All the sections were 6 μm thick, and they were taken from the central part. Some sections were stained with hematoxylin and eosin only. Autoradiography was performed by dipping the sections in Ilford K-5 nuclear emulsion, thus obtaining an even thickness of the film formed after the emulsion dried. The optimal exposure time was found to be 6–12 hr in the cold. For quantitation of the incorporated radioisotope, the number of silver grains were counted over 10 groups of 100 consecutive cells within the central corneal epithelium, stroma and endothelium in both the keratoconic and control sections. Comparisons of the results were made between the corresponding layers by relating the four keratoconic corneas to the four controls. The results were expressed as mean values ± standard deviations, and statistical significance was assessed following the Mann-Whitney U-test.

**Results**

Figures 1 and 2 show the autoradiographs of the different corneal layers both in bright- and dark-field illumination. One can see clearly that all the layers

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**Fig. 1. A**, Photomicrograph of a dark-field illuminated corneal epithelium (×500). Normal cornea. Note the thinning and the radioisotopic density of the keratoconic epithelium. Insets, higher magnification (×1,250) with bright illumination, at which silver grains were counted. Note the conspicuously increased number of silver grains over the epithelial cells of the keratoconic cornea.

**Fig. 1. B**, Keratoconic cornea. Inset, see inset legend of Figure 1A for description.
of keratoconic corneas show conspicuously increased density of silver grains, which at higher magnification and bright light illumination, could be counted fairly accurately.

Figure 3 shows the results obtained from counting the silver grains over 100 consecutive cells of the different corneal layers following development of the autoradiographs. From the obtained figures (mean values ± standard deviation), it is evident that in all layers of keratoconic corneas there is a significantly higher incorporation of the radioisotope than in the controls ($P < 0.015$).

**Discussion**

In this study, a significantly increased number of silver grains was shown to be deposited in all layers of keratoconic corneas following the administration of $^3$H-proline. We used radioparine because it constitutes, together with its hydroxylated form, over 25% of all amino acids of collagen. The latter is the major protein of the cornea, comprising 70–80% of its dry weight. Our results are in agreement with earlier morphologic and biochemical studies on keratoconus in which apparent augmentation of pro-
tein biosynthetic activity has been suggested. Thus, it has been shown that the specific activity of hydroxyproline, which was isolated from the soluble collagen fraction of the cornea, was higher in keratoconic corneas than in the normal controls, while that of the polymeric insoluble collagen was lower in keratoconic corneas than in the controls. The results were interpreted as indicating a higher rate of synthesis of collagen, followed by a lower rate of its polymerization and subsequent lesser contribution to the stroma. The latter has been suggested to be due to an increased catabolism of the newly synthesized collagen. This interpretation was supported further by results obtained from studies in which increased collagenolytic activity has been demonstrated directly in keratoconic corneas.

The fact, however, that the collagenase in the keratoconic tissue, unlike that in the control, was found to be in the activated form, suggests a possible defect in the level of regulation of the collagenase activation as a pathogenic factor in keratoconus. The turnover rate of the connective matrix macromolecules is the result of a dynamic state determined by the rates of their synthesis and degradation. The increased catabolism, as seen in many connective tissue diseases, is a disturbance in this normal equilibrium due to enhancement of proteolytic enzymatic activity. All layers of the cornea are capable of synthesizing the different macromolecules, and the relative rate of synthesis is adapted to the local needs as determined by the micromorphology of the tissue. Faulty regulation of the relative rate of synthesis and/or degradation of the matrix macromolecules may be the pathogenetic basis of some of the corneal dystrophies and also of keratoconus.

The herewith reported conspicuous increase in the incorporation of radioproline supports earlier electronmicroscopic data, which showed abundance of organized rough endoplasmic reticulum in keratoconic keratocytes, thus indicating increased protein synthetic activity. However, we suggest a possibility that the increased biosynthetic activity in keratoconus may turn out to be inadequate to compensate for the tissue loss in the disease, when concomitant with the demonstrated increased collagenolytic activity, thus resulting in the slow destruction and thinning of the cornea.

Key words: cornea, keratoconus, autoradiography, connective tissue, collagenolytic activity

References