PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 175, 336–341 (1984)

Heterogeneity in Keratoconus: Possible Biochemical Basis (41804)

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Abstract. Total protein and collagen content in normal and keratoconus corneas were determined. The protein content (expressed as a function of dry weight) in all keratoconus corneal samples was lower than that found in normal corneas. However, among the 11 keratoconus corneas examined, only 7 (group A) had the same hydroxyproline content (expressed as a function of dry weight) as normal corneas; 4 others (group B) showed significantly less. In tissue culture, four strains derived from keratoconus stroma (group I) produced total protein at the same rate as cells from normal controls. Four other strains (group II), however, had a decreased rate of protein synthesis. The amount of collagenous protein synthesized per microgram DNA by group I strains was similar to that found in normal cultures, whereas it was significantly reduced in group II cultures. We suggest that group I strains represent group A corneas. Group II strains, with a reduced level of both protein and collagen synthesis, may represent group B corneas. The defect in this group appears to be decreased total synthetic activity of corneal cells. The variation in our results suggests that keratoconus is a heterogeneous disease. The heterogeneity may explain the contradictory data that exist in the literature.

Keratoconus is a slowly progressive and potentially blinding corneal disease. The rapidity with which this disorder develops varies, as the central portion of the cornea gradually thins, scars, and becomes cone-shaped. Replacement of the corneal tissues by transplantation has been the current treatment of choice. Surgery is usually performed in the advanced stages of the disease when visual acuity is impaired.

The etiology of keratoconus is unknown. Nevertheless, it has been associated with a variety of inheritance patterns as well as many systemic diseases (1–4), including a heritable disorder of connective tissue (Ehler–Danlos syndromes, types II and IV). Extensive histopathologic and structural studies (5–8) conducted previously have shown fragmentation of the epithelial basement membrane, thickening of Bowman's layer, and the presence of electron-dense deposits in the stroma.

A number of investigations (9–14) have been directed toward elucidating the biochemical basis for keratoconus. However, data have been conflicting, and no clear mechanism of pathogenesis has been presented. Most recently, two independent studies (13, 14) have shown that keratoconus corneas, in organ culture, had higher levels of active collagenolytic activity than did normal corneas. It was suggested that abnormalities in collagen structure and/or collagenase activities may be a factor (or factors) in the development of keratoconus.

Although an increase in active collagenase activity implies a reduced collagen content in keratoconus corneas, varying results have been reported. While Buddecke and Wollensak (9) and Robert *et al.* (10) have shown a reduced collagen content in keratoconus corneas, Andreassen *et al.* (12) have not found such an abnormality. Because of these conflicts, we have reexamined the collagen content of keratoconus corneas and also have measured the total protein and collagen synthesized by cultures of keratoconus stromal cells. Our results support the notion that keratoconus is a heterogeneous group of diseases. The heterogeneity would account for the conflicting data presented previously.

Materials and Methods. Corneal buttons from 15- to 51-year-old patients with typical clinical symptoms of keratoconus were taken at the time of transplantation. Medical histories in terms of duration of disease, size and shape of the cone, degree of scarring, and the presence of other systemic diseases, were carefully recorded. Normal human corneas from individuals 5 to 68 years old were obtained

0037-9727/84 $1.50

from the Illinois Eye Bank. These corneas were used either for biochemical analysis or for establishing stromal cells in culture.

Biochemical analysis. Prior to analyses, the dry weights of keratoconus and normal human corneas were obtained by drying at 60°C for 8 hr. The tissues were dissolved in 2 ml of 1 *N* sodium hydroxide (NaOH) containing 1% sodium dodecyl sulfate (SDS) by heating at 90°C for 15 min and the protein content was determined using the Lowry method (15) with bovine serum albumin (BSA) as a standard.

The hydroxyproline content in the dried tissues was measured using one of two methods (16, 17). These differed in the hydrolysis procedure only, and comparable results were obtained. In one method (16), the tissue was hydrolyzed with 6 *N* hydrochloric acid (HCl) *in vacuo* for 24 hr at 110°C; after drying, the hydrolysate was dissolved in 1.5 ml of water and aliquots were taken for analysis. In the second method (17), the tissue was hydrolyzed with 4 *N* NaOH at 120°C for 10 min; the pH was adjusted to 6 with an equal volume of 1.4 *N* citric acid and samples were taken for analysis. The hydroxyproline content of all samples was determined using the chloramine-T oxidation procedure (16, 18).

All experiments were performed in duplicate. Two-tailed Student's *t* tests were used to determine the statistical significance of the data.

Cell cultures. The mid- to posterior portion of the stromal layer was taken from both keratoconus and normal human corneas as described (19); this entailed stripping off Descemet's membrane with the attached endothelium and then removing the superficial epithelial stromal layer. The explant was cultured by standard procedures (20) in Eagle's minimum essential medium (MEM) containing 10% fetal calf serum (FCS, GIBCO Laboratories). After 4–5 weeks when cultures were confluent, cells were trypsinized and subcultured.

Under phase-contrast microscopy, the cells derived from keratoconus and normal corneas looked alike. As shown previously (21), both types of cells had the long, thin, and spindle-shaped appearance of fibroblasts. The growth patterns and the growth rates of keratoconus and normal control cells in culture were also similar.

Specimens from which stromal cells were derived were used for tissue culture purposes only. No biochemical analysis was performed on any of them.

Protein and collagen synthesis in culture. Confluent cultures of corneal stromal cells in early passages (between passage numbers 1 and 3) were preincubated for 2 hr in MEM supplemented with 0.4% dialyzed FCS and 50 μg/ml of ascorbic acid. They were subsequently labeled either with 5 μCi/ml of [4,5-^{3}H]leucine (59.8 Ci/mmole) or with 5 μCi/ml of [2,3-^{3}H]proline (32.2 Ci/mmole) (both from New England Nuclear Corp.) for 2 hr. At the end of the labeling period, the medium was removed and the cell layer was washed three times with phosphate-buffered saline (PBS). Washes were combined with the medium. Cells were scraped in a total of 2.0 ml of cycloheximide (50 μg/ml), freeze–thawed three times, and disrupted by sonication for 20–30 sec on ice. The radioactive proteins present in both the medium and the cell layer were precipitated by cold 10% trichloroacetic acid (TCA) and 0.5% tannic acid. The precipitates were collected on glass fiber filters, washed with 5% TCA, dried, and counted in toluene-Liquifluor. Radioactivity incorporated into collagenous proteins was determined after digestion with the purified *Clostridial* collagenase (Advance Biofactures, Form III) according to the method of Peterkofsky and Diegelmann (22). The DNA content in the cell layer was measured as previously described (23).

Results. The corneas received from the eye bank and the operating room were in highly variable states of dehydration. Nonetheless, keratoconus corneas had a reduced percentage dry weight compared to normal ones ($P < 0.005$) (Table I). This difference persisted even when one very high normal value was omitted from the calculations (mean = 17.5; SD = 3.3; $P < 0.01$).

The amount of protein present in keratoconus corneal tissues was significantly ($P < 0.005$) lower than that found in normal corneas (Table I). All data were expressed as a function of dry weight rather than per cornea since only part of the cornea was usually received. Also, in some cases, corneas were cut in half to permit assays for both protein and hydroxyproline content.

TABLE I. PERCENTAGE DRY WEIGHT AND PROTEIN CONTENT OF KERATOCONUS AND NORMAL HUMAN CORNEAS

Sample	Age of donor (years)	Percentage dry weight	μg Protein/mg dry weight
Keratoconus	20	9	523
	23	8	—
	24	13	545
	25	20	694
	28	7	575
	29	9	618
	31	11	—
	31	10	—
	33	12	550
	33	12	580
	36	8	560
	36	12	643
	41	10	490
	43	9	693
	44	10	—
	45	7	500
	51	15	593
		10.7 ± 3.1	582 ± 63
Normal	5	20	750
	23	13	708
	29	13	—
	38	22	760
	41	20	—
	41	18	707
	42	15	—
	48	53	763
	49	20	740
	52	18	712
	53	15	756
	53	13	—
	56	20	692
	57	18	706
	61	23	713
	64	18	669
	68	14	723
		19.6 ± 8.9	723 ± 28

It should be noted that the values presented in Table I are not an exact quantitation of the protein content (24) of the cornea. Collagen, which constitutes a major portion of the protein in this tissue, is hypochromic when compared to BSA in the Lowry assay. We found that 100 μg of purified rat tail type I collagen was equivalent to only 60 μg of BSA.

When the hydroxyproline content was expressed as a function of dry weight, seven keratoconus corneas (group A) had the same values as normal corneas (Table II). Four other keratoconus corneas (group B) showed a significant reduction ($P < 0.001$) of hydroxyproline content.

There was no correlation (correlation coefficient < 0.1) between either protein or hydroxyproline content and donor age.

The synthesis of protein and collagen by confluent cultures of keratoconus and normal control cells is presented in Table III. Based on the incorporation of [^{3}H]leucine into TCA-precipitable material, four keratoconus strains (group I) were found to produce total protein at the same rate as cells from normal controls. Four others (group II) showed a decreased rate of protein synthesis. When collagen synthesis was expressed as a percentage of total protein synthesis, group I keratoconus cultures had a value similar to that found in normal cultures. Group II keratoconus cultures had a higher ($P < 0.05$) percentage collagen synthesis. However, because of the decrease in total protein synthesis, these latter cultures produced

TABLE II. HYDROXYPROLINE CONTENT OF KERATOCONUS AND NORMAL HUMAN CORNEAS

Sample	Age of donor (years)	μg Hydroxyproline/ mg dry weight
Keratoconus		
A	20	93
	23	100
	24	98
	31	98
	33	106
	36	95
	44	98
		97 ± 3
B	29	81
	31	42
	41	55
	51	71
		62 ± 17
Normal	29	91
	38	110
	41	92
	42	102
	49	83
	52	89
	53	117
	53	110
	57	96
	61	92
	68	89
		97 ± 10

TABLE III. PROTEIN AND COLLAGEN SYNTHESIS BY CULTURES OF KERATOCONUS AND NORMAL HUMAN STROMAL CELLS

Sample	Age of donor (years)	[^{3}H]Leucine incorporated (cpm/μg DNA)	[^{3}H]Proline-labeled collagenous protein (cpm/μg DNA)	Percentage collagen synthesis[a]
Keratoconus				
Group I	28	855	108	2.8
	30	728	127	2.3
	33	1183	160	2.5
	40	1143	176	2.8
		977 ± 192	143 ± 31[b,c]	2.6 ± 0.2[b,e]
Group II	15	437	69	3.2
	29	479	40	2.9
	34	355	88	3.2
	67	326	46	2.8
		399 ± 61[d]	61 ± 22[d]	3.0 ± 0.2[d]
Normal	5	1011	100	2.4
	12	1120	94	1.9
	21	997	139	2.8
	43	1142	55	1.4
	53	740	86	2.8
		1002 ± 143	95 ± 30	2.2 ± 0.6

[a] Percentage collagen synthesis was determined from the proline-labeling experiments by the following formula: Collagenous cpm/collagenous cpm + 5.4 (total cpm − collagenous cpm).
[b] Compared to group II $P < 0.05$.
[c] Compared to normal $P < 0.1$.
[d] Compared to normal $P < 0.05$.
[e] Compared to normal N.S.

less collagenous protein per microgram DNA than did the strains derived from normal cells ($P < 0.05$).

Discussion. The present study demonstrates that keratoconus corneas contain less protein per milligram dry weight compared to normal controls. On the basis of the hydroxyproline data expressed as a function of dry weight, the 11 keratoconus corneas examined clearly can be separated into two categories (Table II): those that have the same content as the normal corneas (A) and those that have approximately two-thirds the hydroxyproline content (B). The hydroxyproline values in normal and group A keratoconus corneas are the same as reported previously (12).

Cultured stromal strains derived from keratoconus corneas also can be divided into two groups (Table III). One group (I) produces protein at a normal rate while the other (II) shows a reduced rate. The existence of these two groups of keratoconus strains suggests that the defect(s) which cause(s) the biochemical differences among keratoconus corneas continue(s) to be expressed in culture.

No significant differences in medical histories and severity of clinical symptoms could be identified between either groups A and B or groups I and II. Therefore, the variation in collagen content of keratoconus corneas and the synthetic activity of keratoconus stromal cells may not be due to differences in disease progression. Rather, the variation in results suggests that this disease may be comprised of a heterogeneous group of defects that manifest similar clinical symptoms but have varied pathogeneses. Such heterogeneity has been observed in other connective tissue diseases such as the Ehler–Danlos syndromes and osteogenesis imperfecta, which were originally thought to be homogeneous defects (3).

Our results further indicate the importance of normalizing the data to dry weight. For example, a report (13) showing a reduced collagen content in keratoconus corneas had data expressed as hydroxyproline content per cor-

nea. It is expected that keratoconus tissue would contain less collagen than normal corneas of the same diameter because of the thinning in the central portion. If we normalize the data of Kao *et al.* (13) to dry weight, then the normal value of collagen is found, and those corneas would be equivalent to our group A.

In order to determine the biochemical defect(s) associated with keratoconus, we have constructed a table (Table IV) that presents the calculated protein, collagen, and nonprotein contents for corneas in which both protein and hydroxyproline contents were measured. Data from Tables I and II were used. In addition, it was necessary to take into account the reduced chromogenicity of collagen compared to BSA in the Lowry assay, and the hydroxyproline level in corneal collagen (1.05 μmol/mg or ~14% w/w) (25). Because of these corrections, the values for total protein per milligram dry weight are higher than those presented in Table I.

The results shown in Table IV indicate that keratoconus corneas in both groups A and B contain an elevated level of nonproteinaceous compound(s). The mechanism for this alteration is not yet clear.

Table IV, in addition, suggests that the segregation of keratoconus corneas into groups A and B may depend on the presence of other biochemical defects. Group A corneas have a normal collagen content. In culture, keratoconus stromal strains in group I show a normal percent collagen synthesis. Based on these observations, we suggest that the group I keratoconus strains were derived from specimens that would have been categorized into group A, had they been analyzed biochemically.

By extension, group II stromal cells, which show reduced protein synthesis, are presumed to represent group B corneas. The decrease in

TABLE IV. PROTEIN, COLLAGEN, AND NONPROTEIN CONTENTS IN KERATOCONUS AND NORMAL HUMAN CORNEAS

Sample	μg Noncollagenous protein per mg dry weight[a]	μg Collagenous protein per mg dry weight	μg Nonprotein per mg dry weight
Keratoconus			
A	125	664	211
	125	700	175
	126	757	117
	153	679	168
	132 ± 14[b,c]	700 ± 41	168 ± 39[b]
B	271	578	151
	254	393	353
	289	507	204
	271 ± 18[d]	493 ± 93[e]	236 ± 105[b]
Normal	288	787	<0
	384	593	23
	330	636	34
	254	836	<0
	294	686	20
	319	657	24
	341	636	23
	316 ± 42	690 ± 89	18 ± 13

[a] The amounts of noncollagenous protein, collagenous protein, and nonprotein were calculated from data in Tables I and II by the following formulas: μg collagenous protein/mg dry weight (C) = (μg hydroxyproline/mg dry weight) ÷ 0.14; μg noncollagenous protein/mg dry weight (NC) = (μg total protein/mg dry weight) − C × 0.60; μg nonprotein/mg dry weight = 1000 − C − NC.

[b] Compared to normal $P < 0.005$.

[c] Compared to group B $P < 0.001$.

[d] Compared to normal $P < 0.1$.

[e] Compared to normal $P < 0.01$.

both collagenous and noncollagenous protein in this group (Table IV) can then be explained by a decrease in the total synthetic activity of corneal cells. The basis for this alteration is currently under study.

In conclusion, the present study clearly shows on the basis of biochemical analyses and synthesis by cultured stromal cells that keratoconus is a heterogeneous group of diseases. The heterogeneity in keratoconus may explain the contradictory findings in the literature (9, 10, 12), while it emphasizes the importance of studying corneal buttons individually.

We thank Ms. Judy Elvart for her technical assistance. This work was supported by Grants 3890, PHS EY 1792, and EY 7038 from the National Eye Institute, Bethesda, Md., and by an unrestricted award from Research to Prevent Blindness, New York City.

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Received September 2, 1983. P.S.E.B.M. 1984, Vol. 175.
Accepted November 18, 1983.