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COLLAGEN SYNTHESIS BY CULTURES OF STROMAL CELLS FROM NORMAL HUMAN AND KERATOCONUS CORNEAS*

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Summary

Keratoconus is a disease which thins and scars the central cornea. Confluent cultures of corneal stromal cells were derived from patients with keratoconus. The collagen synthesized by these cultures was compared to the collagen synthesized by age-matched normal human corneal stromal cultures. Although the amount of collagen and the types of collagen synthesized were similar, relative proportion of type I collagen and A, B chains produced was significantly altered in keratoconus cultures. The DEAE-cellulose chromatograms of procollagen in the medium fraction were different, not only between normal control and keratoconus cultures but also among keratoconus patients.

Introduction

Keratoconus is a disease which results in a gradual thinning and distortion of the central cornea. Although ultrastructural changes have been described (1-4), the pathogenesis of this condition remains unclear. It has been noted that keratoconus is sometimes seen in patients with hypermobility of the joints, an association suggesting a generalized heritable disorder of connective tissue (5) such as Ehlers-Danlos syndrome (6).

It is now well established that four genetically distinct types (I-IV) of collagen (7) and possibly additional types of collagen, such as A and B chains (8,9) exist in mammalian tissues and cell cultures. These collagens are distributed in a tissue-specific manner. In corneal stroma, the collagen

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is highly glycosylated and consists mainly of type I collagen (11, 12). Type III (12) and A, B-like chains (13) have also been identified in corneal stroma. The collagen in the cornea of patients with keratoconus has not been studied extensively, although abnormalities in the collagen cross-linking (14), the hydroxylation of lysine, and the glycosylation of hydroxylsine (15) have been suggested. It has also been reported that the total collagen is decreased and the amount of structural glycoprotein is relatively increased (15).

Cells grown in tissue culture have provided a good system for detailed biochemical analyses in relation to various diseases (16, 17). For this reason we have established cultures of corneal stromal cells from patients with keratoconus to examine the synthesis of collagen. Parallel experiments were performed with normal human corneal stromal cell cultures as controls.

Materials and Methods

Age-matched normal human corneas were obtained from the New England Eye Bank. Corneal buttons from patients with keratoconus were placed into McCarey-Kaufman medium (18) and sent to our laboratory.

The middle portion of the corneal stromal layer was explanted and cultured as described previously (19). After 3-4 weeks, when cultures were confluent, cells were trypsinized and passed. Normal control and keratoconus cells appeared similar under phase contrast microscopy. Collagen studies were performed between the 2nd and the 7th passages.

Four flasks (75 cm^2) of confluent corneal cultures were incubated for 24 hours with Eagles' minimum essential medium (Cat.# 12-125A, Microbiological Co.) containing 5 µCi/ml each of (^{3}H)glycine and (^{3}H)proline in the presence of β-aminopropionitrile and ascorbic acid (100 µg/ml). After labeling, the medium was removed from each flask and pooled. The cultures were washed six times with 8 ml of Dulbecco's phosphate buffered saline (1 x, pH 7.0, Cat.# 17-513A, Microbiological Co.), and then combined with the medium fraction. The cells were harvested in 0.5 M acetic acid and broken with a Polytron homogenizer as the cell layer fraction.

Aliquots of the cell layer and the medium fraction were dialyzed extensively against distilled water, lyophilized, and the amount of collagenous and non-collagenous proteins were determined with a purified bacterial collagenase (Advance Biofactures, Form IV) as described previously (20). All experiments were performed in duplicate.

The cell layer together with 5 mg of carrier type I collagen [prepared from lathyritic rat skin (21)] was extracted with 0.5 M acetic acid at 4°C overnight. After centrifugation at 15,000 x g for 40 minutes, the supernatant was dialyzed against 0.05 M sodium acetate buffer, pH 4.8, heated and placed on a column of carboxylmethyl (CM)-cellulose as previously described (22). Certain cell homogenates were suspended in

0.5 M acetic acid and treated with pepsin (1 mg/10 ml) for 16 hours at 4°C before analyses by CM-cellulose column chromatography.

The individual α chains were obtained from the CM-cellulose column, pooled, desalted by a Biorad P-2 column, and lyophilized for slab gel electrophoresis. Samples were treated with 1% sodium dodecyl sulfate (SDS) at 80°C for 5 min, and certain samples were reduced with 10 mM of dithiothreitol prior to layering onto 5% SDS-polyacrylamide slab gels (23). After the completion of electrophoresis, the gels were fixed and stained in Coomassie Blue. The radioactively labeled bands were detected by fluorography (24).

The medium fraction was dialyzed against solutions of 0.15 M NaCl, 0.05 M Tris and 0.02 M EDTA, pH 7.4, containing the protease inhibitors phenylmethyl sulfonyl fluoride (10 mM) and p-chloromercuribenzoate (1 mM). Samples were prepared and chromatographed on DEAE-cellulose columns as previously described (25) to separate collagen, procollagen Type I, and procollagen type III.

Results and Discussion

Collagen synthesis by confluent cultures of keratoconus and normal human stroma is shown in Table I. The amount and percentage of collagen synthesized by these cultures were similar. More labelled collagenous products were found in the medium fraction than in the cell layer.

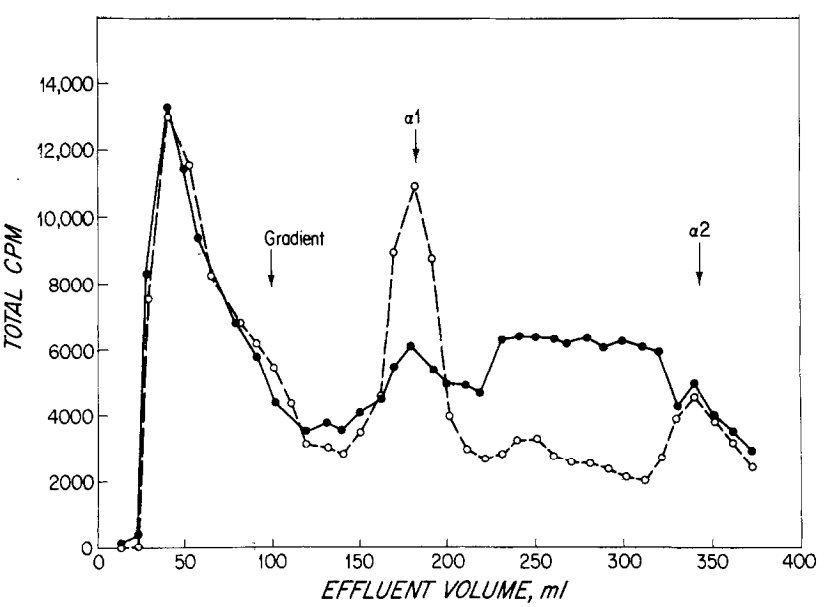
The labeled protein from the cell layer of keratoconus and normal control cultures was extracted and chromatographed on CM-cellulose together with carrier rat skin type I collagen. The carrier standard had the typical $\alpha_1(I)$, and α_2 peaks with β_{12} component preceeding the α_2 peak. The elution patterns of cell layer samples are shown in Fig. 1, representing typical profiles from four normal samples and five keratoconus samples. The radioactivity profile of the labeled normal control samples (open circles) had an $\alpha_1(I)$ peak, and an α_2 peak. Similar elution patterns were found in all cultures from normal corneas, with the ratio of $\alpha_1(I)$ to α_2 varying slightly between 2.0 and 2.3. The labeled material appearing between the $\alpha_1(I)$ and the α_2 peaks was collagenase sensitive demonstrating that it was collagen.

The CM-cellulose chromatogram of samples from keratoconus cultures (Fig. 1, closed circles) showed marked differences in the proportions of various peaks compared to normal control samples. There was no discrete α_1 and α_2 peaks. The radioactivity in the region between $\alpha_1(I)$

Table I
Collagen synthesis by cultures of normal human and keratoconus stromal cells

Cell Type	Age of Donor (years)	(^{3}H)proline and (^{3}H)glycine incorporated into collagenous proteins(cpm/mg of dry weight)			(^{3}H)collagenous proteins as % of total (^{3}H)proteins	
		Cell	Medium	Cell + Medium	Cell	Medium
Normal human	17	3164	12617	15718	2.9	8.9
	26	2255	8901	11156	2.0	11.0
	40	1177	13425	14602	2.4	16.9
	51	2130	7954	10084	3.0	19.6
Keratoconus	23	3234	22937	26171	2.6	18.5
	31	2556	13677	16233	3.0	17.7
	44	2745	11040	13785	2.3	18.9
	48	6530	8465	14995	3.8	9.2
	68	3079	12347	15426	3.0	9.8

Each flask of confluent cultures was incubated with 3 ml of Eagle's minimum essential medium containing 5 µCi/ml each of (^{3}H)proline and (^{3}H)glycine for 24 hours in the presence of β-aminopropionitrile and ascorbic acid. The amount and percentage of (^{3}H)collagenous proteins were determined by collagenase assays as described in Material and Methods.



and α_2 chains was increased to 40-50% of the total in all keratoconus cultures as opposed to 20-25% in normal control cultures. This material could be procollagen (26), type III collagen (16), and/or A, B chains (8) based on its elution from CM-cellulose columns.

To further identify this collagenous material, another set of keratoconus and normal control samples were treated with pepsin to remove the procollagen extension peptides. When examined by CM-cellulose column chromatography, these samples still contained similar proportions of collagenous proteins eluting between the α_1 and α_2 chains. Analysis of these proteins by SDS-gel electrophoresis resulted in two radioactive bands migrating slower than the α_1 band in the A, B chain region (8). No radioactive α_1 band was found even after reduction with dithiothreitol indicating the absence of type III collagen. The data, therefore, is consistent with the idenfication of A, B-like chains in keratoconus and normal control cultures. This differs from a previous report (27) which showed that type I collagen was the only collagenous product in normal human corneal stromal cultures.

Procollagens in the medium fraction of corneal cultures were analyzed by DEAE-cellulose column chromatography. Fig. 2 shows the elution patterns of radioactive procollagens from the most severely affected keratoconus cultures (closed circles) and the representative normal control cultures (open circles). The product appearing at peak I was identified by SDS-gel electrophoresis as type I procollagen. The component at peak II, which was completely collagenase digestable, represented approximately 6-15% of the radioactivity in four normal

Figure 1. CM-cellulose chromatogram of labeled collagen isolated from the cell layer of keratoconus (●---●) and normal control cultures (o---o).

The column (1.5 x 10 cm) was equilibrated with 0.05 M sodium acetate buffer pH 4.8 and elution was performed with a linear gradient from 0 to 0.1 M NaCl over a total volume of 400 ml. Fractions of 5 ml were collected and assayed for radioactivity. Carrier collagen was type I collagen isolated from lathyritic rat skin.

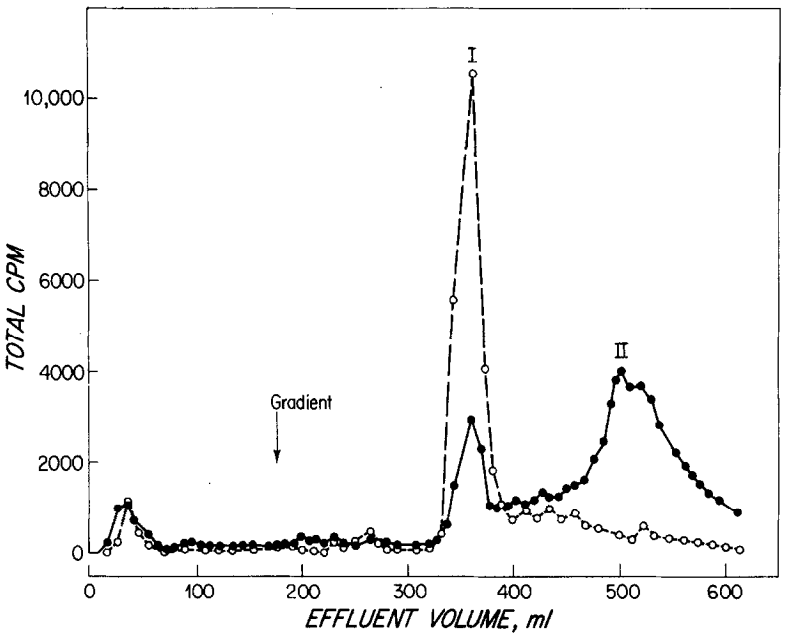


Figure 2. DEAE-cellulose chromatogram of the procollagen purified from the medium fraction of keratoconus (●---●) and normal control cultures (o---o).

Column was equilibrated with 0.05 M Tris pH 8.0, 2 M urea. Chromatography was performed at 4°C with an elution volume of 600 ml and the gradient from 0 to 0.2 M NaCl. Fractions of 11 ml were collected and assayed for radioactivity.

control cultures, but was increased in five keratoconus cultures to approximately 30-70% of the total procollagen depending on the patient analyzed.

Variation found in the DEAE-cellulose chromatograms among patients with keratoconus suggests that this corneal disease is heterogeneous. This has also occurred in studies with skin fibroblasts from patients with osteogenesis imperfecta (16). In some of the keratoconus corneal buttons, a scarred area was observed. It is not clear at present whether the defects in the collagen synthesis are due to the diseased cells themselves or are associated with the process of repair.

We have also demonstrated abnormalities in the metabolism of glycosaminoglycans produced by these same keratoconus cultures. The synthesis of cell layer-related heparan sulfate is significantly reduced (28). This suggests that keratoconus may be a disease of matrix which shows modifications in both collagen and glycosaminoglycan syntheses.

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References

1. Teng, C.C. (1963) Amer. J. Ophthalmol. 55, 18-47.
2. Patta, CL., Loyon, L., and Roucher, F. (1970) Arch. Ophthalmol. (Paris) 30, 403-417.
3. Kim, J.O., and Hassard, D.T.R. (1972) Canad. J. Ophthalmol. 7, 176-180.
4. Iwamoto, T., and Devoe, A.G. (1975) Arch. Ophthalmol. (Paris) 35, 65-72.
5. Robertson, I. (1974) Aust. J. Ophthalmol. 2, 144-147.
6. Uitto, J., and Prockop, D.J. (1974) Molecular Pathology, pp. 670-688, Thomas, Springfield, Illinois.
7. Miller, E.J. (1976) Molecular and Cellular Biochemistry 13, 165-192.
8. Burgeson, R.E., El Adli, F.A., Kaitila, I.I., and Hollister, D.W. (1976) Proc. Natl. Acad. Sci. USA 73, 2579-2583.
9. Chung, E., Rhodes, R.K., and Miller, E.J. (1976) Biochem. Biophys. Res. Comm. 71, 1167-1169.
10. Katzman, R.L., Kang, A.H., and Dixit, S.N. (1974) Biochim. Biophys. Acta. 336, 367-369.
11. Freeman, I.L. (1978) Invest. Ophthalmol. and Vis. Sci. 17, 171-177.
12. Schmut, O. (1977) Exp. Eye Res. 25. 505-509.
13. Hong, B.S., Cannon, D.J. and Davison, P.F. (1978) Fed. Proc. 37, 1528.
14. Cannon, J. and Foster, C.S. (1978) Invest. Ophthalmol. and Vis. Sci. 17, 63-65.
15. Robert, L., Schillinger, G., Moczar, M., Junqua, S., and Moczar, E. (1970) Arch. Ophthalmol. (Paris) 30, 590-607.
16. Penttinen, R.P., Lichtenstein, J.R., Martin, G.R., and McKusick, V.A. (1975) Proc. Natl. Acad. Sci. USA 72, 586-589.
17. Dorfman, A., and Matalon, R. (1976) Proc. Natl. Acad. Sci. USA 73, 630-637.
18. McCarey, B.E., and Kaufman, H.E. (1974) Invest. Ophthalmol. 13, 165-173.
19. Baum, J.L., Niedra, R., Davis, C., and Yue, B.Y.J.T. Arch. Ophthalmol. (in press).
20. Peterkofsky, B., and Diegelmann, R. (1971) Biochemistry 10, 988-994.
21. Piez, K.A. (1967) Treatise on collagen, Vol. 1, Chemistry of Collagen, pp. 201-248, Academic Press, New York.
22. Miller, E.J., Martin, G.R., Piez, K.A., and Powers, M.J. (1967) J. Biol. Chem. 242, 5481-5489.
23. Laemmli, U.K. (1970) Nature 227, 680-685.
24. Laskey, R.A., and Mills, A.D. (1975) Eur. J. Biochem. 56, 335-341.

25. Smith, B.D., Byers, P.H., and Martin, G.R. (1972) Proc. Natl. Acad. Sci. USA 69, 3260-3262.
26. Moro, L., and Smith, B.D. (1977) Arch. Biochem. Biophys. 182, 33-41.
27. Stoesser, T.R., Church, R.L., and Brown, S.I. (1978) Invest. Opthalmol. and Vis. Sci. 17, 264-271.
28. Yue, B.Y.J.T., Baum, J.L., and Silbert, J.E. Manuscript submitted for publication.