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THE PRESENCE OF EC COLLAGEN AND TYPE IV COLLAGEN IN BOVINE DESCEMET'S MEMBRANES

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SUMMARY: When bovine Descemet's membranes (DMs) were characterized after limited pepsinization the major component in DM was found to be endothelial cell (EC) collagen. Phenol extractions of the undigested pepsin residue recovered only type IV collagen. This study provides evidence that EC collagen may be produced by corneal endothelial cells *in vivo*.

Descemet's membrane, the specialized basement membrane produced by corneal endothelial cells, contains a collagenous and noncollagenous component. Discrepancies exist not only as to the collagen types within Descemet's membrane (DM) but also in the collagenous synthetic activity of corneal endothelial cells *in vitro*. These reported inconsistencies of collagen types may be attributable to DM insolubility, species differences or variations in analytical techniques. While bovine DM contains type IV (1) and possibly type I and V collagens (2), cultured bovine corneal endothelial cells produce predominately type III collagen with small amounts of I, IV, and V collagens (3,4). Rabbit DM reportedly contains only type IV collagen (5,6). *In vitro* rabbit corneal endothelial cells are phenotypically stable and synthesize type IV collagen, but not types I, III, and V (5,7-9). In addition, these cells, along with vascular endothelial cells and astrocytoma cells, produce *in vitro* a novel class of collagen molecules, EC collagen (10-13). Since EC collagen is not described previously in any *in vivo* tissue, it was not clear if its *in vitro* synthesis was a tissue culture-induced artifact.

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The present study demonstrates that the soluble (pepsin-extractable) and the insoluble (phenol-extractable) fractions of bovine DM contain EC and type IV collagens, respectively.

MATERIALS AND METHODS

Fresh bovine eyes were obtained from a local slaughterhouse and kept on ice prior to dissection. Isolated DM (1) were purified by sequential detergent, DNase treatment (14) followed by two pepsin extractions (0.5 mg/ml in 0.5M acetic acid) at 4°C for 8 and 24 hours. Pepsin supernatants were then subjected to differential salt precipitations (1) and the final supernatants (FS) containing the pepsin-extractable bovine DM collagen were lyophilized for further characterization. The indigestible DM residues after pepsin digestions were washed thoroughly in ice water, lyophilized and extracted with freshly prepared phenol:acetic acid:water (1:1:1, w/v/v/, pH 1.25, 8 mg/ml) for 7 days at 60°C (15). Supernatants were extracted with cold ether (3x), and the aqueous layers lyophilized for further analyses. Aliquots of pepsin-FS and phenol extract (PE) were lyophilized and hydrolyzed in 6N HCl at 110°C for 24 hours and applied to a Durham D500 analyzer for amino acid analysis. Pepsin-FS and PE samples were labelled with ^{125}I-Bolton-Hunter reagent (16) and fractionated by sodium dodecyl sulfate polyacrylamide gel electrophorsis (SDS-PAGE) (17). Bands of interest were cut out and treated with proteinase K for 2-dimensional peptide mapping (18). Type IV and V collagen standards were isolated from human placenta (19). EC collagen standards were isolated from culture medium of rabbit corneal endothelial cells (9) as described by Sage and coworkers (12).

RESULTS AND DISCUSSION

Due to the highly crosslinked nature of DM, less than 50% of its collagen could be released by limited pepsinization, thereby, making it difficult to confidently characterize collagen types present in the DM. Sequential pepsin and phenol treatments were used to extract the collagens in their entirety from DM. Phenol extraction (PE) is an established method to isolate insoluble collagens (15,20) and in the present study is applied successfully to extract all of the pepsin-resistant, insoluble DM collagens. Using the combined technique of pepsin and phenol extraction, three different DM collagen fractions were analyzed: 1) 8 hour pepsin-extractable final supernatant collagen (FS), 2) 24 hour pepsin-extractable supernatant and 3) phenol extractable collagen (PE).

When isolated bands from the 8 hour pepsin-FS and PE were compared by 2-dimensional peptide mapping (a very sensitive,

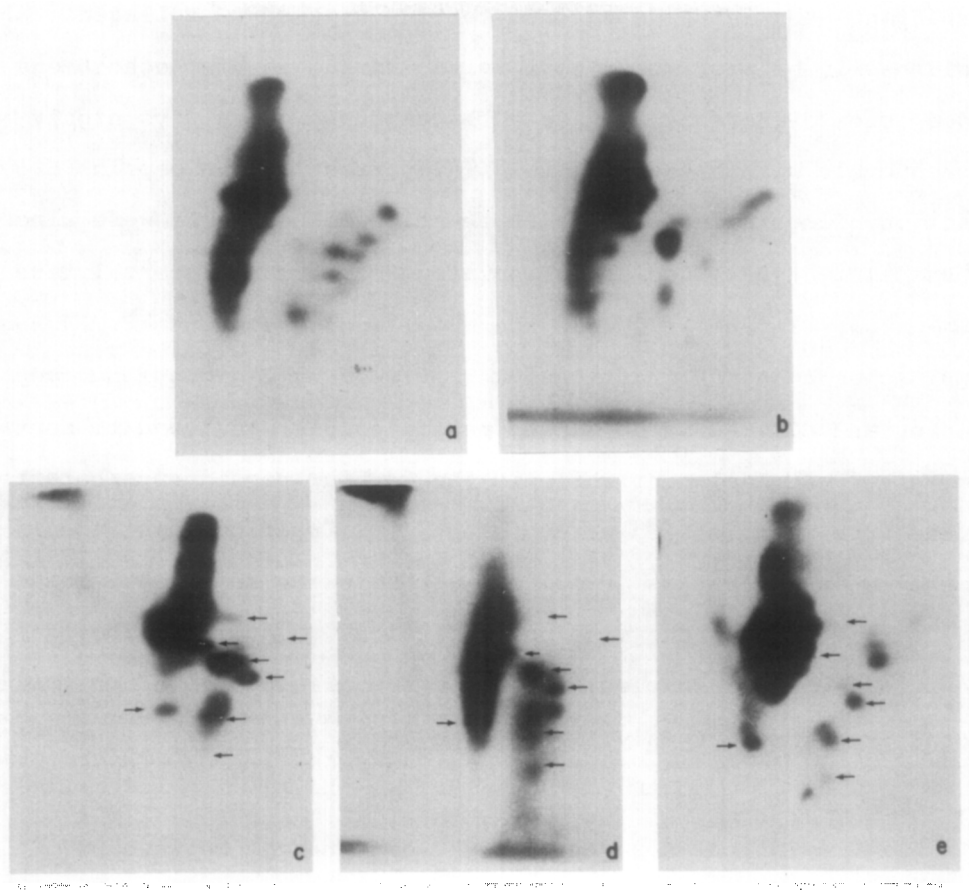

Figure 1. ^{125}I peptide maps of DM-collagens and collagen standards. Collagens were radioiodinated, resolved on SDS-PAGE, bands of interest were cut out, digested with proteinase K and mapped on cellulose plates by 2-dimensional electrophoresis and chromatography.

a) α_1(IV) collagen standard isolated from human placenta
b) 7 day phenol extract from bovine DM
c) EC collagen (50K) isolated from bovine DM final supernatant (FS)
d) EC collagen standard (50K) from rabbit endothelial cell culture medium
e) EC collagen (50K) isolated from human DM

reproducible method of protein identification) the differences in fingerprints were significant. While maps of pepsin-FS collagens were unique when compared to interstitial, type IV and type V collagens (data not shown), they corresponded favorable to 2-dimensional maps of EC collagen isolated from rabbit corneal endothelial cell culture medium and human DM (Figure 1c-e). Minor

variations seen in these various peptide maps of EC collagen could easily be attributed to in vitro versus in vivo conditions and species differences, since collagen maps were always highly reproducible within each specie. In contrast to the pepsin-FS fraction, fingerprints of the PE fraction of bovine DM were almost identical to type IV collagen isolated from human placental tissues (Figure 1). In summary, when the 3 collagen fractions (8, 24 hour pepsin digest and PE fractions) were viewed by 2-dimensional peptide mapping and SDS-PAGE, the 8 hour pepsin sample was found to contain exclusively EC collagen, while in the 24 hour pepsin supernatant EC and type IV molecules were present. PE fractions contained almost exclusively type IV collagen. While the major analyses were carried out on the more pure fractions, 8 hour pepsin-FS and phenol extract samples, analyses were also completed on the second fraction, but only EC and type IV collagens were found.

Amino acid analyses verified the differences between DM pepsin-FS and PE fractions (Table I). Analyses of the PE exhibited a rather characteristic pattern of type IV collagen (Table I), with low glutamic acid, valine, arginine, and elevated isoleucine and leucine values. The most outstanding feature of the pepsin-FS fraction was the very low glycine content suggesting nonhelical portions within the molecule. In comparison to other basement membrane collagens, EC collagen had decreased amounts of hydroxyproline and hydroxylysine residues (Table I). This lower extent of hydroxylation had been reported for in vitro EC collagen (11) and was suggested to represent 1) a lower degree of hydroxylation of the imino acids or 2) underhydroxylation due to tissue culture conditions. Since our system is in vivo the latter theory can be excluded. Pepsin-FS had a hydroxyproline/proline ratio of 0.93, which was in close

Table I

Amino Acid	Bovine Descemet's Membrane Phenol extract 7 days	Sheep Descemet's Membrane* I(IV)	Human Placenta 100K**	Bovine Descemet's Membrane Pepsin Final supernatant
4-Hypro	110.0	165.0	113.5	88.0
Asp	32.0	30.0	46.0	60.0
Thre	23.0	18.0	22.0	38.0
Ser	27.0	25.0	45.0	54.0
Glu	88.0	78.0	82.0	93.0
Pro	101.0	90.0	83.0	95.0
Gly	315.0	320.0	317.0	255.0
Ala	37.0	32.0	34.0	46.0
1/2 Cys	-	8.0	-	-
Val	34.0	25.0	33.0	46.0
Met	14.0	9.5	11.0	4.0
Ileu	31.0	24.0	35.0	40.0
Leu	65.0	52.0	56.0	68.0
Tyr	12.0	3.0	3.9	14.0
Phe	20.0	22.0	23.0	24.0
His	9.5	7.8	6.3	12.0
Hylys	29.0	43.0	58.0	16.0
Lys	18.0	15.2	7.7	20.0
Arg	32.0	30.0	21.0	26.0

*Kefalides, N.A. (1971) Biochem. Biophys. Res. Comm. 45, 226-234.

**Sage, H., Woodbury, R.G. and Bornstein, P. (1979) The Journal of Biological Chemistry 254, 9893-9900.

agreement with hydroxyproline/proline ratios of EC collagens-50K and 60K peptides reported by other investigators (Table II).

EC collagen is a recently described molecule and its position in the collagen family is not understood at this time. It is extremely pepsin sensitive (10,12,21) as reflected by the fact that 80% of the pepsin-FS fraction was recovered as the low molecular weight 50K peptide. Fractionation of pepsin-FS

TABLE II

COMPARATIVE HYDROXYPROLINE/PROLINE RATIOS FOR EC COLLAGEN

Protein Sample	Total Hydroxyproline/Proline
a) EC p-FS	0.93
b) EC (60K)	0.89
c) EC 2 (50K)	1.05

a) EC collagen of bovine DM (80% represented by 50K peptides) isolated from pepsin final supernatant

b) EC collagen of rabbit endothelial cell culture medium; Benya, P.D. (1980) Renal Physiol. Basel, 3, 30-35.

c) EC collagen of bovine aortic endothelial cell culture medium, pepsin-resistant fragment of EC 2 (50K); Sage, H., Pritzl, P. and Bornstein, P. (1980) Biochem. 19, 5747-5755.

by SDS-PAGE showed bands of 300K, 200K, 100K, and 50K peptides which were resistant to further pepsin or B-mercaptoethanol treatment (data not shown). However, each of the fractions were sensitive to bacterial collagenase (data not shown). Our data suggest that the basic unit of the EC collagen is a 50K molecule which is capable of aggregation into higher molecular weight forms. This concept of the EC molecule is in complete agreement with the hypothetical model of EC collagen proposed elsewhere (10).

In vitro studies on bovine corneal endothelial cells reported type III collagen as being the major component deposited in the extracellular matrix and secreted into the media (3,4). However, these results could not be supported by the present study on collagen components of bovine DM in vivo. In contrast, our data is in agreement with reports on cultured rabbit endothelial cells (7,10,11), therefore, strongly suggesting the production of EC collagen by corneal endothelial cells in vivo.

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