ISOLATION AND INITIAL CHARACTERIZATION OF HUMAN BASEMENT MEMBRANE COLLAGENS

Robert L. Trelstad and Karen R. Lawley

Experimental Pathology Laboratory, Department of Pathology, Shriners Burns Institute/Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114

Received April 13,1977

SUMMARY: Neutral solutions of pepsin extracted human collagens derived from glomeruli, kidney, aorta, lung, heart, bowel, spleen, skeletal muscle and skin were subjected to heat gelation at 37°C. Centrifugation of the gel provided two fractions: gelled pellet and non-gelled supernatant. Analysis of these two fractions by gel electrophoresis, molecular sieve and ion exchange chromatography, and amino acid and carbohydrate determinations indicated that the non-gelled supernatant contained a substantial enrichment of basement membrane like collagen. The initial characterization of lung derived basement membrane collagen indicated close similarities with those derived from glomeruli and whole kidney and differences with that obtained from the spleen.

INTRODUCTION: Basement membranes represent a class of cell surface materials which lie at the interface of the extracellular matrix and the external plasma membrane of the cell and whose composition include collagen like materials, glycoproteins and glycosaminoglycans (1,2,3).

The collagenous component from purified glomeruli and from lens capsules represents the prototype of basement membrane collagens. It was known to be a unique collagen even before the discovery of the heterogeneity of the collagen types found in the interstitium (4). Paucity of starting material and relative inaccessibility of tissues pure in basement membrane have made study of its composition difficult. For interstitial collagens, use of limited proteolytic digestion with pepsin followed by fractionation of the solubilized collagens by differential salt precipitation has expedited their study (4,5,6). Recent reports indicate that non-renal and non-ocular tissues bearing basement membranes contain a new molecular species of collagen with unusual solubility properties, whose composition is different from those found in the lens and glomeruli (1,7,8,16).

We report here the successful isolation of human basement membrane collagens

ISSN 0006-291X

from a number of tissues with partial characterization of those from lung, spleen and kidney.

MATERIALS AND METHODS: Human tissues were obtained at autopsy within 12 hours post mortem. Glomeruli were prepared from kidney slices and isolated from tubules by a graded sieving technique (9). The tissues studied were kidney and glomeruli, liver, lungs, spleen, bowel, skeletal muscle, cardiac muscle, aorta, skin, brain and whole blood.

Tissues were homogenized in 0.5M acetic acid adjusted to pH 2.0 to 2.5 with HCl and digested with pepsin (Worthington 2X crystallized) at 20-40 mg/gm wet weight for 3 days at 4°C. The pepsin digest was centrifuged and the extracted collagen solution neutralized with NaOH to pH 7.5 to inactivate pepsin and the solubilized collagens were then precipitated by the addition of NaCl to a final concentration of 20% w/v. The precipitated collagens were collected by centrifugation and the pellet resuspended in 0.4 ionic strength potassium phosphate buffer pH 7.6 and dialyzed against the same buffer. The neutral collagen solution was then placed in a 37° incubator for 4-16 hours to effect heat gelation. The heat gel was centrifuged at room temperature at 6,000 RPM for 30 minutes and the supernatant separated from the pelleted gel. The gel pellet was resolubilized in 0.1M acetic acid and resalted out by addition of NaCl to a final concentration of 10% w/v. The collagens in the heat gel supernatant were precipitated by the addition of NaCl to 20-30% w/v. The precipitate was collected following centrifugation and resolublized in either 0.4 ionic strength phosphate buffer or 0.1M acetic acid. The portion in phosphate buffer was reduced at 4°C for 16 hours by addition of mercaptoethanol at a concentration of 0.1% v/v. The reduced collagens were then dialyzed against 0.5M acetic acid, adjusted to pH 2.0-2.5 with HCl, which also contained 0.1% mercaptoethanol v/v. When equilibrated at acid pH, pepsin (20 mg/ml solution) was added and the collagens were digested at 4°C for 16 hours. Following repepsinization the solution was neutralized and the collagens salted out with NaCl at 20% w/v. For storage of both the heat gel precipitates and supernatants the solutions were dialyzed against 0.1M acetic acid and stored in solution.

The collagens were analyzed for amino acid and carbohydrate composition, capacity to form SLS (segment-long-spacing) crystallites, electrophoretic mobility on acid-urea and SDS polyacrylamide gels and chromatographic behavior on molecular sieve columns (6% Agarose equilibrated with 5.0M guanidine-HCl, 0.05M Tris-HCl pH 7.5, 0.001M dithiothreitol or 8% Agarose equilibrated with 1.0M $CaCl_2$, 0.05M Tris HCl pH 7.5) and on CM-cellulose cation exchange columns all as previously described (10-15).

RESULTS: The effectiveness of heat gelation as a fractionation method is readily assayed by amino acid analysis of the heat gel supernatants and precipitates. Differences between the two fractions can be seen by comparing the ratio of hydroxylysine to 4-hydroxyproline as shown in Table I. The hydroxylysine/4-hydroxyproline ratio generally is greater than 0.35 for the heat gel supernatant. In addition, the heat gel supernatant generally has a 3-hydroxyproline/4-hydroxyproline ratio of greater than 0.04 and as high as 0.10. Conversely the heat gel precipitate shows values more consistent with those found in interstitial collagen Types I, II and III.

Table I

Ratio of hydroxylysine/4-hydroxyproline in native collagens after heat gelation fractionation

	Kidney	Spleen	Aorta	Bowel	Lung	Heart	Skin	Liver
Supernatant	0.40	0.39	0.39	0.35	0.38	0.38	0.25	0.33
Precipitate	0.14	0.15	0.06	0.07	0.11	0.13	0.08	0.08

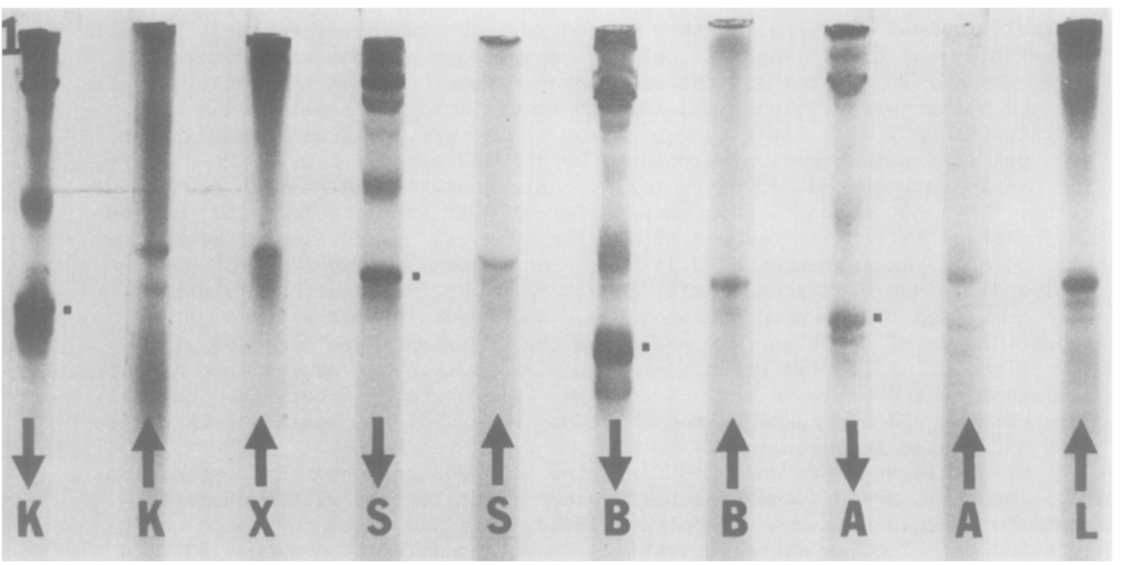

Figure 1: Acid-urea polyacrylamide electrophoretogram of heat gelled collagens from kidney (K), spleen (S), bowel (B), aorta (A), and lung (L). ↑ indicates heat gel supernatant, ↓ heat gel precipitate. X is the peak 1 material from the Agarose column (Figure 3). The gels were run at separate times, but those from the same tissues simultaneously. The position of α1 (I-III) is indicated by a closed square.

Electrophoretic mobility in 7.5% polyacrylamide gels at acid pH indicates a clear difference between the two heat gel fractions (Figure 1). Several components are present in the acid-urea gels of materials which had not been reduced and repepsinized in solution: a major component remaining at the gel top, and components migrating between standard β and α chains (Figure 1). Staining the gels with periodic acid-Schiff revealed prominent staining of

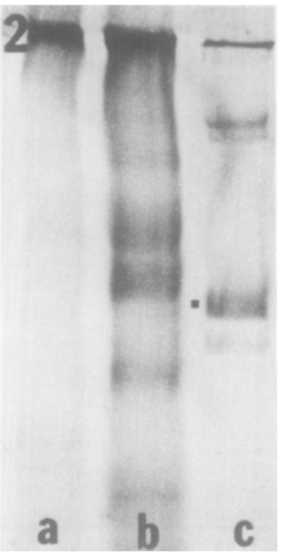

Figure 2: SDS-polyacrylamide electrophoretogram of heat gelled kidney collagens showing effects of native reduction - repepsinization. a) Heat gel supernatant b) Heat gel supernatant after native reduction - repepsinization c) Heat gel precipitate. All samples were reduced after denaturation. The position of α1 (I-III) is indicated by a closed square.

all bands, particularly the faster migrating materials present above the α chains. Electrophoresis of the material on SDS gels without reduction and repepsinization was usually unsuccessful although faint amounts of materials could on occasion be seen in the gels. Following reduction and repepsinization, however, several prominent components were present in 5.0% gels with mobilities both greater and lesser than standard chains (Figure 2). Owing to the anomalous behavior of collagens and/or glycoproteins in SDS gels, the molecular weights of these materials will require further confirmation by other methods.

Molecular sieve chromatography on Agarose of heat gel supernatant collagens obtained from the kidney and not subjected to reduction-repepsinization revealed at least four components. On 6% Agarose (Bio-Gel A-5M), equilibrated in 5M guanidine HCl, pH 7.5, material eluted at the void volume of the column, at the expected elution position of a gamma chain followed by material at

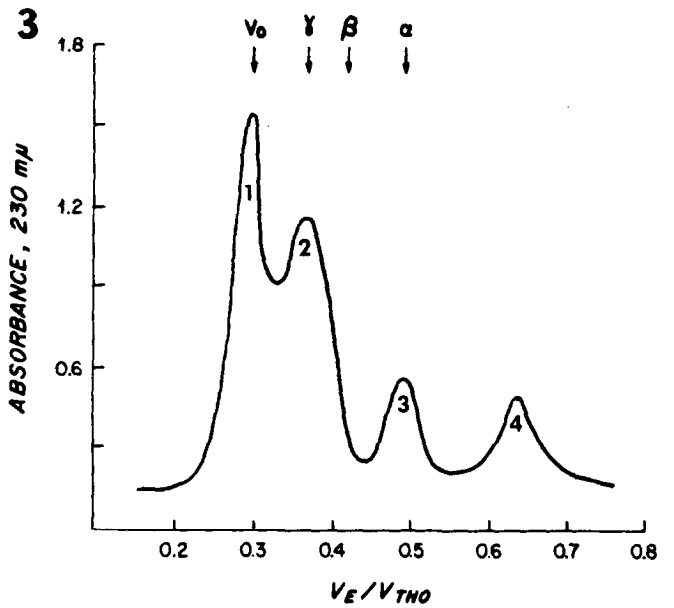

Figure 3: Chromatogram of the heat gel supernatant of kidney collagen on Agarose A5M equilibrated with 5.0M guanidine - HCl, pH 7.5. The elution position of standard γ, β and α chains are noted. The amino acid composition of each of these peaks is given in Table II.

positions of an α chain (100,000 daltons) and a smaller peptide (Figure 3). This pattern was not altered by prior treatment of the denatured protein at pH 8 with reducing agents with or without subsequent repepsinization. Similar chromatographic profiles were obtained on 8% Agarose (Bio-Gel A-1.5M) equilibrated with 1M $CaCl_2$, pH 7.5, but often this solvent caused the material to precipitate even after heating to 100°C for 15 minutes. The large molecular weight materials eluting in the void volume and the γ fraction from the 6% Agarose column (Figure 3, peaks 1 and 2) revealed on electrophoresis in acid-urea gels a pattern similar to that of unfractionated material, (Figure 1) suggesting the presence of persistent aggregates under the chromatographic conditions. The material eluting from the Agarose column in the region slightly before standard α chains (Figure 3, peak 3) electrophoresed as a distinct band between the standard β and α chains (not shown). The fourth

Table II
Amino Acid Composition in Residues/1000

	NATIVE				DENATURED			
	Kidney		Lung	Spleen	Agarose Peaks (Fig. 3)			
	Gel	Non-gel	Non-gel	Non-gel	1	2	3	4
3-OHPRO	5	9	2	7	11	12	8	6
4-OHPRO	113	97	107	69	129	120	119	123
ASP	50	54	55	55	52	52	52	57
THR	18	25	26	31	22	27	22	26
SER	38	36	41	43	32	32	48	46
GLU	71	85	90	83	96	94	85	92
PRO	90	62	84	54	68	68	62	70
GLY	305	326	302	356	331	345	328	329
ALA	98	41	38	41	35	31	32	31
1/2 CYS	2	8	5	11	2	4	3	5
VAL	28	29	27	32	19	26	27	22
MET	11	13	14	12	13	13	15	11
ILEU	17	24	24	23	20	18	28	22
LEU	32	55	49	54	50	48	51	48
TYR	1	13	15	21	9	8	8	8
PHE	15	27	25	28	24	21	20	18
OHLYS	16	39	41	27	48	43	49	47
LYS	27	16	15	17	2	1	9	1
HIS	8	10	7	10	6	6	6	5
ARG	55	31	33	26	31	31	28	33

peak from the Agarose column (Figure 3) was not successfully detected after concentration and electrophoresis in the acid-urea gel system.

Chromatography of denatured kidney heat gel supernatants on carboxymethylcellulose demonstrated material eluting at the beginning of the chromatogram despite the low ionic strength of the starting buffer (0.02M sodium acetate, pH 4.8) followed by a peak at a buffer concentration of 0.05-0.06M sodium acetate with lesser amounts of material present later in the chromatogram. Reduction and pepsinization did not substantially alter the chromatographic pattern.

Amino acid analyses of the native materials and fractions from the Agarose column are given in Table II. These selected analyses from kidney, lung and spleen show clearcut differences between the heat gel supernatant and the heat gel precipitate. Surprisingly few differences were found in composition among the four components separated on the Agarose column. The values

obtained from the heat gel supernatant fraction of the isolated glomeruli were essentially identical to those for the heat gel supernatant from the whole kidney.

Amino acid analysis of alkaline hydrolysates of kidney heat gel supernatants (2N NaOH for 24 hours at 110°) indicated that over 90% of the hydroxylysine residues were glycosylated. Analyses of hydrolysates of the same material for 4 hours in 6N HCl at 110° on a short column of the amino acid analyzer demonstrated the presence of glucosamine.

The native state of the heat gel supernatant collagens was indicated by: 1) its resistance to peptic digestion, both initially in the tissue and following reduction and repepsinization and its susceptibility to peptic digestion after gentle (50°C for 10 minutes) heat denaturation 2) its capacity to precipitate at both low and high ionic strengths, conditions typical for native collagen and 3) its capacity to form SLS crystallites.

The heat gel supernatant collagens did not form SLS crystallites unless the sample was subjected to reduction and repepsinization and those which then formed were different from crystallites of Types I, II and III.

DISCUSSION: Heat gelation is a purification method currently in use in the isolation of several different polymerizing macromolecules including actin and tubulin (18,19). The heat gelation reaction of collagen has been studied extensively in respect to the problem of collagen fibrillogenesis (20), but has not been used as a principal purification method.

The lens capsule and glomeruli are prototypes of basement membrane rich structures and identification of material as a basement membrane collagen at present requires comparison with these materials (1,16). The recently isolated collagens from the placenta and internal organs by Burgeson et al. (7) and Chung et al. (8) although possibly basement membrane in origin are different from those reported by other workers (1,16,17) and the data presented here. It is quite possible, however, that the basement membrane collagens from

different tissues represent a heterogeneous class of molecules similar to what has now been found for the interstitial collagens (4).

The basement membrane like collagens reported here are a group of molecules of similar composition, but dissimilar size as shown by both Agarose chromatography and SDS gel electrophoresis. On the SDS gels there are two major components in the kidney heat gel supernatant with mobilities between α1(I-III) chains and β components which resemble the pattern of A and B chains reported by Burgeson et al. (7), two faster moving materials with apparent lower molecular weights than α chains and a fraction which remained at the gel top. The composition of the kidney starting materials and the fractions from the Agarose columns reported here, however, do not resemble the A and B chains, but rather the collagens previously described from glomeruli and the murine tumor (1,16,17). This diversity of collagen composition and size may reflect true differences in the molecules and/or limited proteolysis both in situ prior to isolation, and during extraction and purification (2,8,21). Biosynthetic studies directed at each of these tissues should help clarify these questions (22,23).

Acknowledgements: Reduction-repepsinization was suggested by discussions with Drs. Dehm, Clark, Timpl and Martin at a Symposium on the Biology and Chemistry of Basement Membranes, Philadelphia, 1976 organized by Dr. Kefalides. Supported by the Shriners Burns Institute and Grants from the NIH (HL 18714 and AM 18729).

References

1. Kefalides, N.A. (1975), J. Invest. Dermatol. 65:85-92.
2. Hudson, B.G., and Spiro, R.G. (1972), J. Biol. Chem. 247:4229-4238.
3. Trelstad, R.L., Hayashi, K., and Toole, B.P. (1974), J. Cell. Biol. 62:815-830.
4. Miller, E.J. and Matukas, V.J. (1974), Fed. Proc. 33:1197-1204.
5. Trelstad, R.L., Catanese, V.M. and Rubin, D.F. (1976), Analyt. Biochem. 71:114-118.
6. Epstein, E.H. (1974), J. Biol. Chem. 249:3225-3231.
7. Burgeson, R.E., El Adli, F.A., Kaitila, I.I. and Hollister, D.W. (1976), Proc. Natl. Acad. Sci. USA 73:2579-2583.
8. Chung, E., Rhodes, R.K. and Miller, E.J. (1976), Biochem. Biophys. Res. Commun. 71:1167-1174.
9. Misra, R.P. (1972), Amer. J. Clin. Path. 58:135-139.
10. Trelstad, R.L. and Lawley, K.R. (1976), Analyt. Biochem. 70:287-289.
11. Ortec Application Note AN 32A (1973) p. 39.
12. Stark, M. and Kühn, K. (1968), European J. Biochem. 6:534-541.

13. Piez, K.A. (1968), Analyt. Biochem. 26:305-312.
14. Piez, K.A., Eigner, E.A. and Lewis, M.S. (1963), Biochemistry 2:58-66.
15. Bruns, R.R. and Gross, J. (1973), Biochemistry 12:808-815.
16. Daniels, J.R. and Chu, G.H. (1975), J. Biol. Chem. 250:3531-3537.
17. Orkin, R.W., Gehron, P., McGoodwin, E.B., Martin, G.R., Valentine, T. and Swarm, R. (1977), J. Exp. Med. 145:204-220.
18. Adelstein, R.S. and Kuehl, W.M. (1970), Biochemistry 9:1355-1364.
19. Shelanski, M.L., Gaskin, F. and Cantor, C.R. (1973), Proc. Natl. Acad. Sci. USA 70:765-768.
20. Trelstad, R.L., Hayashi, K. and Gross, J. (1976), Proc. Natl. Acad. Sci. USA 73:4027-4031.
21. Freytag, J.W., Ohno, M., and Hudson, B.G. (1976), Biochem. Biophys. Res. Commun. 72:796-803.
22. Williams, I.F., Harwood, R. and Grant, M.E. (1976), Biochem. Biophys. Res. Commun. 70:200-206.
23. Minor, R.R., Clark, C.C., Strause, E.L., Koszalka, T.R., Brent, R.L. and Kefalides, N.A. (1976), J. Biol. Chem. 251:1789-1794.