PARTIAL CHARACTERIZATION OF A SECOND BASEMENT MEMBRANE COLLAGEN IN HUMAN PLACENTA

Evidence for the existence of two type IV collagen molecules

A. J. BAILEY, T. J. SIMS, V. C. DUANCE* and N. D. LIGHT*

Agricultural Research Council, Meat Research Institute, Langford, Bristol and **Department of Animal Husbandry, University of Bristol, Langford, England*

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1. Introduction

Basement membranes are extracellular structures occurring in many tissues including placenta, lung and vascular system, as well as the much thicker basement membranes of the kidney glomeruli and lens capsule.

The molecular composition of the collagen in basement membrane has recently been shown to be more complex than anticipated. Studies on lens capsule [1] indicated a molecule containing three identical α-chains (type IV) and these conclusions were recently supported by biosynthetic and pepsin digestion studies from this group [2]. On the other hand, it was concluded [3] that basement membranes were composed of many different collagenous peptides. More recently analysis of pepsin digests of placental membrane revealed, in addition to types I and III collagens, a new basement membrane-like collagen [4–7]. The molecular chain composition of this collagen has not yet been established. Although two non-identical chains have been characterized it is not yet clear whether they constitute one molecule, i.e. $(\alpha B)_2\alpha A$ [8] or two distinct molecules, i.e. $(\alpha A)_3$ and $(\alpha B)_3$ [9] in a 2:1 ratio. Antibodies raised against this collagen confirmed its identity as a basement membrane-associated collagen by immunofluorescent localization, the attached trophoblasts being clearly visible along the intensely stained membrane of the placental villi [10].

Recently we identified a second basement membrane-like collagen coprecipitating with type I from pepsin digests of human placenta [10]. We now report further characterization of this collagen demonstrating the presence of more than one α-chain possibly from different molecules and a similarity of its properties with the classical basement membranes of lens capsule and kidney glomeruli.

2. Materials and methods

2.1. *Pepsin digestion*

The human placenta was washed extensively in physiological saline for about 10 days at 4°C; suspended in 0.5 M acetic acid and digested with pepsin at a substrate:enzyme ratio of 10:1 for 24 h at 10°C. The solubilized collagens were then separated by the salt fractionation technique in [6,10]. The 2 M NaCl precipitate was subjected to the thermal gelation technique [11] to remove the contaminating type I and type III fibres.

Following short-term pepsin digestion (6 h at 10°C) of the washed placenta, the proportion of type I and III collagens was reduced and the three major components present in the original '2 M NaCl' precipitate were found to precipitate at 1.4 M and 1.8 M NaCl. These two precipitates were also subjected to the thermal gelation technique to remove contaminating type I and III collagen.

2.2. *Reduction and alkylation*

The lyophilized collagen (24 mg) was dissolved in 3 ml Tris buffer (pH 8.6), 0.3 ml EDTA solution (50 mg/ml) and made up to 12.0 ml with water. After

gassing with nitrogen for 30 min mercaptoethanol was added (0.1 ml), and the solution allowed to stand for 4 h at 22–25°C. Iodoacetic acid (0.268 g in 1 ml 1.0 N NaOH) 1 ml was added and the solution kept in the dark under nitrogen for 30 min. Another 0.1 ml of mercaptoethanol was added to inhibit further reaction and after 2 h the solution was dialysed against 0.05 M acetic acid in the dark [12].

A second more extensive reduction of the material in the native state was achieved by carrying out the reaction with 0.4 M dithiothreitol for 24 h and then alkylating with 0.1 M iodoacetamide for 24 h.

Reduction and alkylation of the collagen in the denatured state was achieved by the addition of urea to the initial collagen solution to give 8 M urea.

2.3. *Separation and cyanogen bromide analysis of isolated α chains*

The reduced and alkylated chains were separated on CM-cellulose, and each polypeptide chain analysed on SDS–acrylamide gels after cyanogen bromide digestion as in [13].

2.4. *Antibody production*

The preparation of antibodies in rabbits, the immunofluorescent staining, and the passive haemagglutination techniques were carried out as detailed in [6].

2.5. *Lectin labelling of collagen chains*

The α and β components of bovine type I collagen and the reduced and alkylated components of human placenta type IV collagen were separated in 7.5% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS [13]. Proteins were fixed in the gel with a solution containing 50% (v/v) methanol and 0.05% (v/v) glutaraldehyde prior to staining with ^{125}I-labelled phytohaemagglutinin (PHA) from *Phaseolus vulgaris* (red kidney beans) by the method in [14]. The lectin was labelled to spec. act. ~10^7 cpm/mg and 0.25 mg lectin was used in each incubation mixture. After lectin staining the gels were stained for protein with Coomassie blue and then dried prior to localization of the lectin-stained proteins by autoradiography.

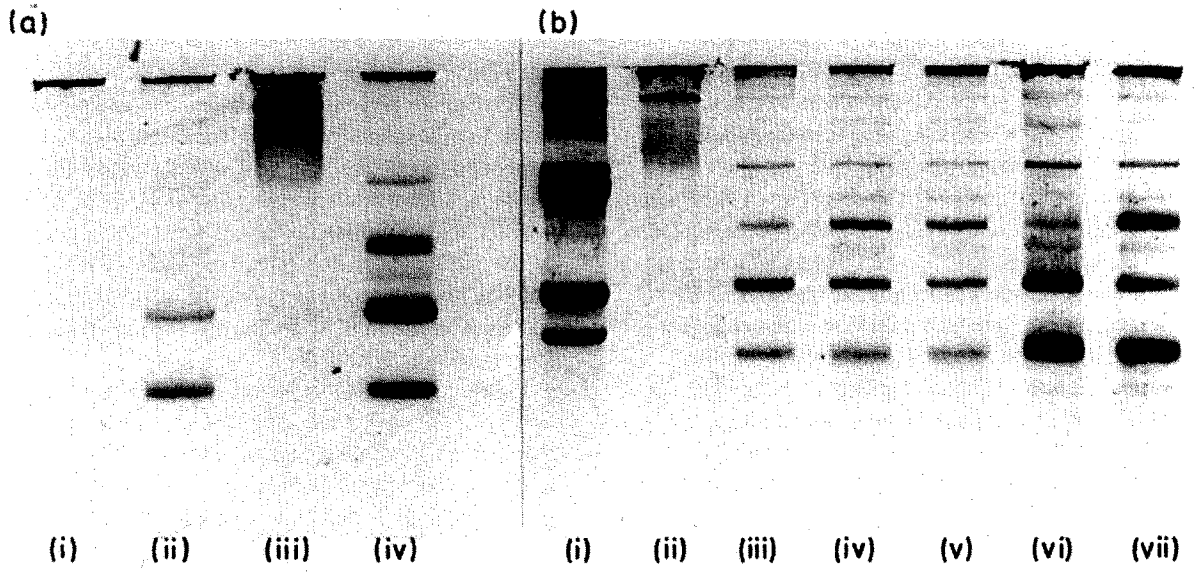


Fig.1(a). SDS–acrylamide gel electrophoresis pattern of the 1.4 M NaCl precipitate: (i) without mercaptoethanol; (ii) with mercaptoethanol and the 1.8 M NaCl precipitate; (iii) without mercaptoethanol; (iv) with mercaptoethanol, isolated from human placenta.

Fig.1(b). SDS–acrylamide gel patterns of 2 M NaCl precipitate isolated from human placenta; (i) type I collagen; (ii), (iii) 2 M NaCl precipitate without and with mercaptoethanol, respectively; (iv), (v) 2 M NaCl precipitate after rigorous reduction and alkylation without and with mercaptoethanol; (vi), (vii) 2 M NaCl precipitate after mild reduction and alkylation, without and with mercaptoethanol.

3. Results

SDS–acrylamide gel electrophoresis of the purified 2 M NaCl precipitate revealed three major bands with app. mol. wt $1.7–1.8 \times 10^5$, 1×10^5 and $7–8 \times 10^4$ all of which only appeared on the gels after pre-incubation with mercaptoethanol, indicating extensive disulphide bonding of initially high molecular weight components.

The three major components consistently observed in all preparations after carboxymethylation have been designated 170 k, 100 k and 70 k. An additional band at 200 k was occasionally observed (fig.1). These figures do not strictly represent the molecular weight of the chains owing to the anomalous mobilities of collagen polypeptides on SDS–acrylamide gel electrophoresis.

The relative proportions of all these components depended on the extent of the pepsin treatment. Indeed, short-term pepsin treatment allowed the separation of two precipitates, one at 1.4 M and 1.8 M NaCl each containing different proportions of these components (fig.1a).

Analysis of the components following mild reduction and alkylation under non-denaturing conditions revealed that the 70 k in addition to the 100 k chain was present, the 170 k component only appearing after denaturation and subsequent mercaptoethanol treatment (fig.1b. vii). The material reduced and alkylated in the native state was digested with pepsin for a second time to demonstrate whether further pepsin digestion of the molecule could be achieved following cleavage of the disulphide bonds. Subsequent acrylamide gel analyses demonstrated that the pepsin had no further effect on these components. These results indicate that the 70 k polypeptide is not derived from either of the higher molecular weight components. Further support for this conclusion was achieved by the use of ^{125}I-labelled lectin specific for *N*-acetyl galactosamine and galactose when only the 70 k component was labelled (fig.2). Under more rigorous conditions all three chains were reduced and alkylated (fig.1b. iv, v) but again further pepsin treatment had no effect on the three components.

The supernatant obtained after heat gelation of the 2 M NaCl precipitate was denatured, reduced and alkylated and the components separated on CM-cellulose (fig.3a,b). Three peaks were obtained eluting prior to the known elution position of α 1 type I collagen, peak 1 corresponded to the 170 k component, peak 2 to the 100 k component and peak 3 to the 70 k component. The relative proportion of these chains recovered from the CMC did not correspond to those observed on the gels. All three chains possessed amino acid compositions characteristic of type IV collagen, i.e., low alanine and arginine

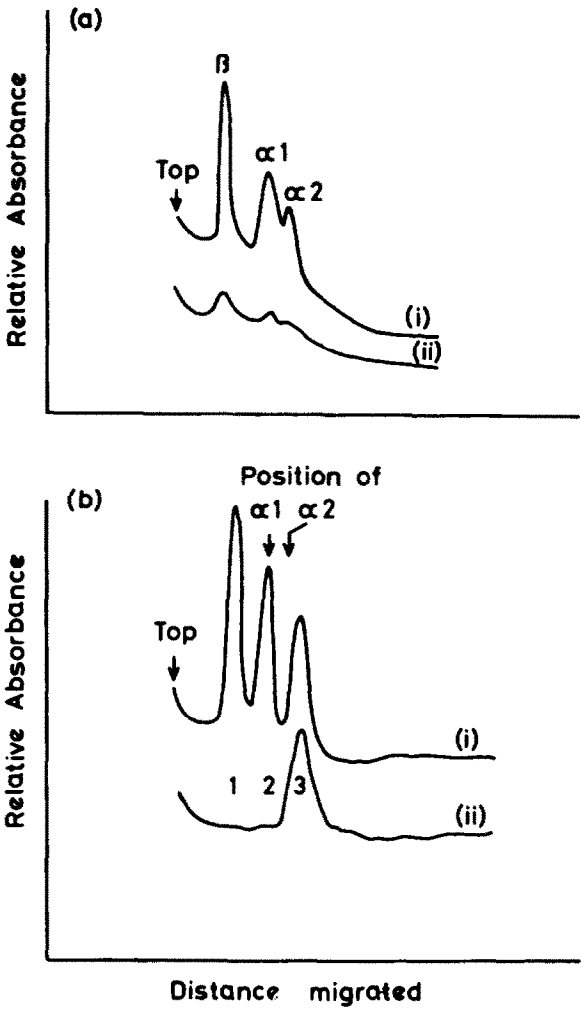
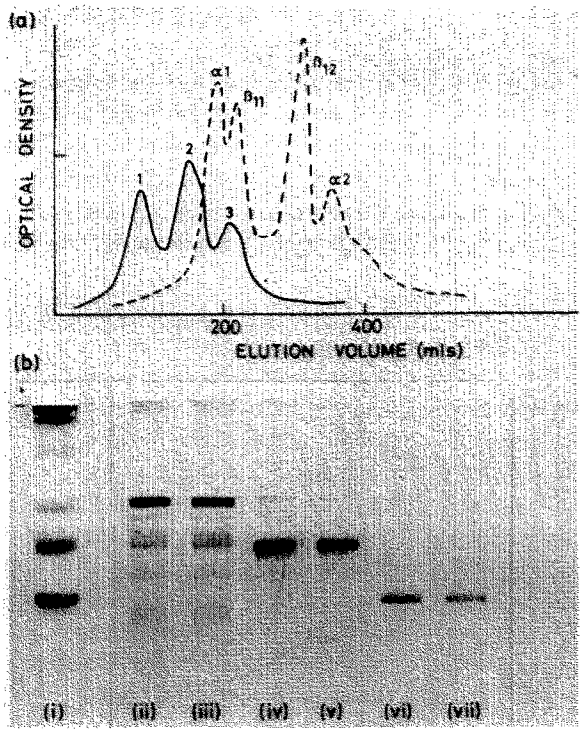


Fig.2. Direct staining with ^{125}I-labelled PHA (lectin from *Phaseolus vulgaris*) of collagen polypeptide chains separated in SDS–polyacrylamide gels. (a) Type I collagen. (b) Type IV collagen. Each section shows: (i) a scan of the protein-stained gel (Coomassie blue) and below; (ii) a scan of the autoradiograph of the lectin-stained gel.



and a very high hydroxylysine: lysine ratio and significant amounts of 3-hydroxyproline were detected in the purified '2 M NaCl' precipitate (table 1). Preliminary characterization of the α-chains by cyanogen bromide digestion revealed some similarities between the 170 k and 100 k components, whilst the pattern of the 70 k component was completely different (fig.4).

Antibodies raised against the purified 2 M NaCl precipitate and tested by passive haemagglutination

Fig.3(a). Elution pattern of the type IV collagen (2 M NaCl precipitate) following reduction and alkylation in the denatured state on a CM-cellulose column: (———) type IV collagen; (- - - -) type I collagen.

Fig.3(b). SDS–acrylamide gel electrophoresis pattern illustrating the mobilities of components under the three peaks separated on the CM-cellulose column: (i) original sample type IV collagen (2 M NaCl precipitate); (ii), (iii) peak 1 with and without mercaptoethanol; (iv), (v) peak 2 with and without mercaptoethanol; (vi), (vii) peak 3 with and without mercaptoethanol.

Table 1
Amino acid composition type IV collagen α-chains isolated from human placenta

	Human placenta total type IV	CMC-chromatography		
		Peak 1	Peak 2	Peak 3
3-Hydroxyproline	7.0	n.d.	n.d.	n.d.
4-Hydroxyproline	121.7	156.0	134.5	57.9
Aspartic	55.8	37.0	53.2	59.5
Threonine	25.3	12.4	23.6	28.7
Serine	36.7	27.2	34.8	44.1
Glutamic	100.1	94.6	105.7	108.2
Proline	81.6	85.7	83.7	44.1
Glycine	290.9	337.0	322.6	339.5
Alanine	41.5	34.7	37.5	51.9
Cystine	5.6			
Valine	27.5	27.6	25.3	27.3
Methionine	9.9	8.6	3.1	9.6
Isoleucine	29.0	27.8	25.3	34.3
Leucine	49.8	51.0	45.0	54.6
Tyrosine	9.6	3.0	5.0	8.7
Phenylalanine	26.8	23.0	24.5	37.5
Hydroxylysine	36.3	50.9	38.0	33.0
Lysine	14.6	10.0	9.0	15.0
Histidine	5.1	Tr	2.7	4.3
Arginine	32.1	27.0	26.6	41.8

n.d., not determined

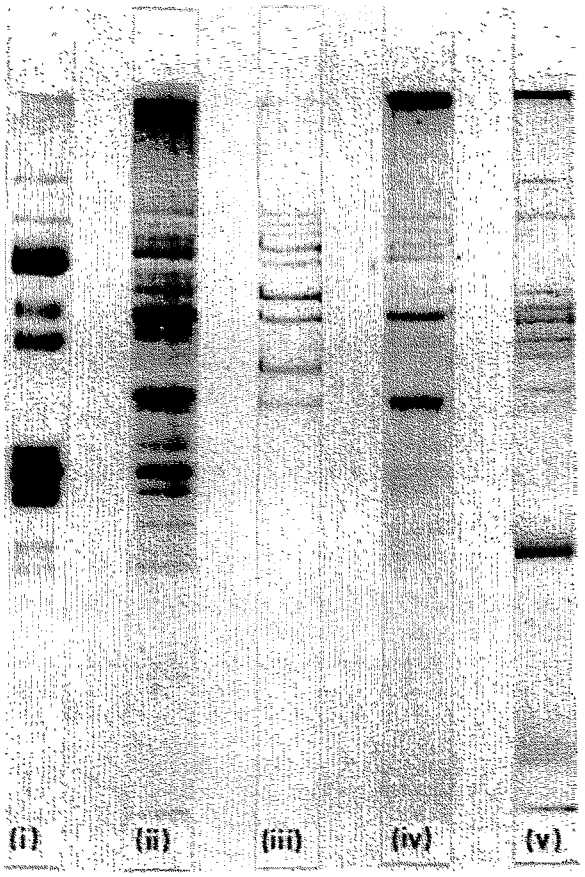


Fig.4. SDS–acrylamide gel pattern of the cyanogen bromide digests of the components separated on the CM-cellulose column. Cyanogen bromide peptides of: (i) neutral salt-soluble type I collagen; (ii) reduced and alkylated placenta type IV; (iii) CM-cellulose peak 1; (iv) CM-cellulose peak 2; (v) CM-cellulose peak 3.

did not crossreact with the type AB collagen obtained from placenta. The antibodies did, however, crossreact with the classical basement membrane collagens from lens capsule and glomeruli. More importantly immunofluorescent staining of placenta produced an intense staining of the basement membrane of the placental villi confirming the identity of this collagen as a basement membrane collagen.

4. Discussion

The collagenous fraction isolated from human placental villi following pepsin digestion and salt fractionation has certain characteristics typical of the type IV collagens of the classical basement membranes from lens capsule and glomeruli, i.e., amino acid composition and the ability of antibodies raised against it to specifically stain basement membrane. Detailed acrylamide gel analyses of this collagen, however, revealed the presence of three distinct α-chains.

The lectin binding studies and the differences in cyanogen bromide patterns clearly indicate that the 70 k component is not derived from either the 100 k or 170 k component. Furthermore, the difference in sensitivity in the production of the 170 k and 70 k chains with mercaptoethanol and dithiothreitol from the native high molecular weight components and the different proportions of these components in the 1.4 M and 1.8 M NaCl precipitates, strongly suggests that the two chains are derived from different molecules.

The cyanogen bromide patterns suggest that the 100 k could be a fragment of the 170 k component. However, rigorous reduction and alkylation followed by a second pepsin digestion failed to convert the 170 k to the 100 k component. Confirmation of their identity or otherwise requires further detailed analysis. However, the presence of two or even three different α-chains whether from one or more molecules conflicts with the model proposed [2] for lens capsule basement membrane of a single molecule comprised of three identical chains. This latter model is supported by biosynthetic studies on glomeruli in which a single 140 k component has been obtained [17]. However, our own studies on the pepsin digests of classical basement membrane from lens capsule and glomeruli [10] revealed almost identical electrophoretic and chromatographic properties to those of the placenta type IV, again indicating the existence of two or three different α-chains. A single 100 k and a 70 k component has been reported [7] in placental extracts employing a second pepsin digest after reduction and alkylation and these may be related to two of the three chains reported here. A high molecular weight component (135 k) which is readily degraded by pepsin has been isolated [16] from murine tumours. The relationship of the tumour basement membrane collagen to other basement membranes is not at present clear.

From the results reported here, it would appear

that the collagen of basement membrane is more complex than previously proposed, there being a mixture of different 'type IV' molecules, the proportion of each depending on the function of the particular membrane.

Acknowledgements

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