STUDY OF GLOMERULAR BASEMENT MEMBRANE GLYCOPROTEINS RESISTANT TO HYDROLYSIS BY COLLAGENASE

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1. Introduction

We previously studied the soluble glycoproteins of murine and human glomerular basement membrane (GBM) hydrolyzed by clostridiopeptidase A (EC 3.4.24.3) [1–3]. These components were isolated from the supernatant obtained after digestion for 72 h and centrifugation at 5000 *g* for 15 min. The pellet (C. Col) represented the insoluble material resistant to hydrolysis by collagenase.

2. Materials and methods

2.1. *Pronase digestion of the insoluble material*

The pellet (C. Col) of human or rat GBM was suspended in 5 ml of 0.1 M Tris-HCl, 0.005 M calcium acetate buffer, pH 7.4, and then incubated with pronase (B grade Calbiochem, 45 000 PUK units/g). The mixture was stirred continuously. Experimental conditions were analogous to those described by Spiro [4]. Centrifugation (5000 *g* for 15 min) allowed the separation of the pronase digested material (S Pro) from the fraction which resisted digestion (C Pro). This latter fraction represented 8% (w/w) of the GBM proteins.

2.2. *Gel filtration of pronase digested fraction*

The solubilized fraction was loaded on to a Sephadex G-25 column (1.5 × 60 cm) equilibrated with a 0.1 M acetate buffer pH 5.0. The elution was carried out using 5 ml fractions and the same buffer. The glycoproteins were located by means of determination of absorbance at 280 and 260 nm as well the orcinol–sulfuric acid method [5].

2.3. *Chemical composition*

Amino acid composition was determined by a method adapted to the Technicon Autoanalyzer [6] after 24 h hydrolysis with 6 N HCl under vacuum in sealed tubes. The determination of the hexose content was carried out using the orcinol–sulfuric acid method adapted to the Technicon Analyzer [7].

The hexosamine content was determined using Neuhaus method [8] and the determination of fucose by Dische's method [9]. The sialic acid content was found using Warren's method [10]. Glucose content was determined with glucose oxidase [11].

2.4. *Reduction of cross-links of GBM*

The reduction by NaB^3H_4 (spec. act., 271 mCi/mmol) was carried out under the conditions suggested by Eyre and Glimcher [12]. Samples were diluted in 10 ml of Na^+PO_4 = 0.1 M, pH 7.4, and were treated by NaB^3H_4 (330 nM for 10 mg proteins) for 45 min. The reaction was stopped by addition of glacial acetic acid. After extensive dialysis and lyophilisation the samples were hydrolysed in 6 N hydrochloric acid under reduced pressure for 24 h: chromatographic analysis of radioactive reduced components was done with a Dowex 50 × 8 column (0.6 × 140 cm) and the Technicon Autoanalyzer [13]. Aliquots of radioactive effluent were analysed in an Intertechnique model SL 32 Scintillation counter. The identification of the cross-links was carried out by comparison with chromatographic diagrams of known samples (calf skin collagen).

3. Results and discussion

Fig.1 indicates the elution pattern of the S Pro

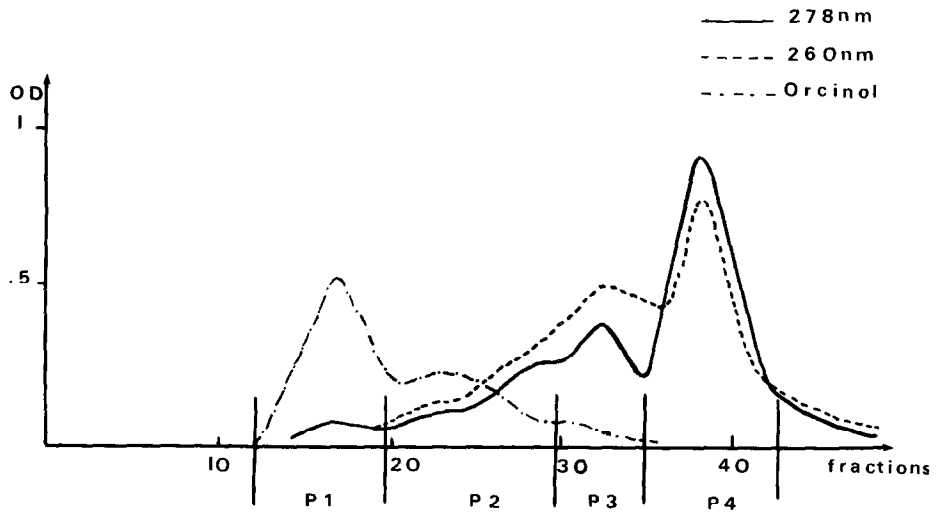

Fig.1. Molecular-sieve chromatography of pronase-soluble components of HGBM. Four fraction (P_1–P_4) were eluted. The composition of the major glycopeptide (P_1) was reported in table 1. Similar results were obtained with rat GBM.

fraction after gel filtration on Sephadex G-25. P_1 is the major glycopeptide.

In table 1 is shown the composition in amino acid and sugar of the fraction resistant to pronase digestion (C Pro), the fraction solubilized by this enzyme (S Pro) as well as that of the sub-fraction P_1.

In so far as concerns amino acid composition these fractions are characterized by their low content in hydroxylysine and a relatively high content in dicarboxylic amino acids (especially P_1) and in serine and threonine residues. The S Pro fraction stands out because of its relatively high content in glycine.

However, the differences are most striking in so far as concerns sugar content. The material having resisted both collagenase and pronase digestion (C Pro) contains glucose and hexosamine but contains no sialic acids. The solubilized material (S Pro) contains hexosamines, sialic acid but no glucose. The composition of the carbohydrate rich subfraction P_1 is presented in table 1. The sugar content of P_1 is similar to that of S Pro.

Table 2 shows the total d.p.m. per mg of each sample after radiochromatography on Dowex 50 × 8 and the relative d.p.m. of major radioactive peaks. It appears that the fixation of NaB^3H_4 is greater for the C Pro than for the S Pro fraction. According to the interpretation of chromatographic diagrams by Tanzer and Kefalides [14], it is apparent that the percentages of fixation of NaB^3H_4 corresponding to reduced aldehydes, to aldol condensation products, and to intermolecular cross-links vary depending upon the fraction studied. It should be noted in particular the high level of acidic and neutral components (fractions 32–92) in the products resistant to collagenase digestion (C Pro and S Pro) (fig.2).

These findings were interpreted using, as a starting point, the molecular model of GBM proposed by Kefalides [15,16].

The composition in carbohydrates and amino acids of the C Pro residue (the absence of hydroxylysine contrasting on one hand with the presence of glucose and on the other hand with the absence of sialic acid) is similar to the composition of epithelial basal membranes as reported by Johnson [17] and by Lee [18].

Being acquainted with the double origin (epithelio–endothelial) of GBM, one could imagine that the glycoproteins of high mol. wt. (1 000 000) might be of epithelial origin such as described by Kefalides [18] in the description of his molecular model. The absence of half-cystine residues in this fraction does not seem incompatible with such a hypothesis given two facts: first, the Kefalides hypothesis that these molecules may be linked by hydrogen bonds [15] and second,

Table 1
Amino acid composition of collagenase residue glycoproteins[a]

Fractions	C Pro H	C Pro R	S Pro H	S Pro R	S Pro P_1 H	S Pro P_1 R
Hyp	0	0	0	0	0	0
Asp	83.2	78.5	114.6	114.9	80.1	96.9
Thr	53.3	68.1	61.1	84.8	116.4	82.2
Ser	118.0	90.0	133.2	83.9	82.4	94.9
Glu	90.9	92.3	110.9	93.4	100.2	98.9
Pro	51.3	49.1	64.1	62.4	83.5	79.0
Gly	133.7	123.9	191.8	162.3	150.3	139.1
Ala	83.8	84.1	85.3	69.3	86.2	63.1
½ Cys	0	0	17.4	22.2	Traces	Traces
Val	52.9	57.9	39.2	41.3	42.9	34.9
Met	34.3	20.1	7.3	14.5	23.7	36.5
Ile	30.3	39.6	23.0	26.8	25.9	19.2
Leu	56.6	110.9	36.9	49.4	50.9	38.3
Tyr	13.6	25.5	22.4	18.1	15.0	12.8
Phe	49.8	35.2	18.7	24.2	16.5	17.1
Hyl	0	0	11.2	10.2	6.6	5.2
Lys	44.4	46.6	25.7	32.7	31.2	82.5
His	20.1	20.9	14.4	12.9	19.0	18.5
Arg	27.9	32.0	21.4	34.6	26.0	23.8

Carbohydrate composition of collagenase residue glycoproteins[b]

	C Pro H	C Pro R	S Pro H	S Pro R	S Pro P_1 H	S Pro P_1 R
Hexoses	24	24.7	17.6	11.8	22.1	23.2
Hexosamine	1.7	0.4	3.4	2.7	8.5	9.2
Sialic acid	0	0	4.4	4.5	3.1	6.7
Glucose	4.3	2.4	0	0	0	0
Fucose	0.9	0.7	nd	nd	2.4	1.8

[a]Results given as residues per thousand for amino acids.
[b]Carbohydrates, expressed as g for 100 g (dry weight).
nd = not determined.
H = human.
R = rat.

Table 2
Distribution of the radioactivity in reduced components of the human GBM

Samples	Total radioactivity[a] d.p.m./mg X 10^{-4}	Radioactivity of eluted fractions[b] 32–51	82–92[c]	95–107
HGBM	5.9 d.p.m./mg X 10^{-4}	6%	4.5%	14%
C Pro H	7.7 d.p.m./mg X 10^{-4}	10%	11 %	17%
S Pro P_1H	5.8 d.p.m./mg X 10^{-4}	4%	7 %	9%

Radioactivity expressed in d.p.m./mg X 10^{-4} is 20% higher in C Pro H than in S Pro P_1 H and native HGBM.
Results of ion exchange chromatography of acid hydrolyzates of reduced samples shows: an increased percentage of ^3H fixation in peaks corresponding to fractions 82–92 which are related to elution of *N*-ε hexosyl-hydroxylysines, *N*-ε hexosyl-lysines and aldol condensation products.

[a] Dry weight.
[b] Expressed as percentage of the total eluted radioactivity represented by each peak.
[c] Fractions 82–92 correspond to C, D, E components described by Tanzer and Kefalides [14].

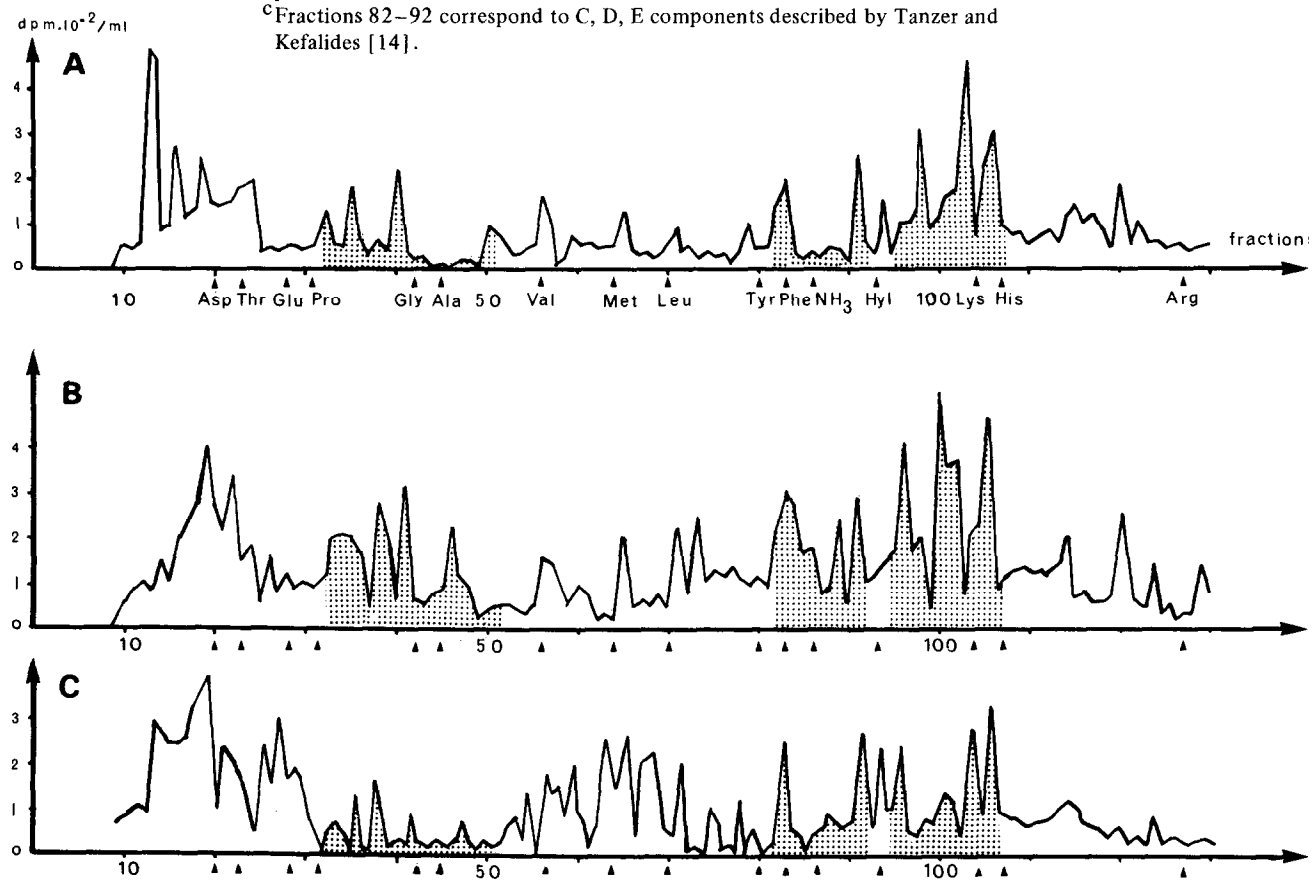

Fig.2. Elution patterns of ion exchange chromatography of acid hydrolysates of (A), human GBM; (B), C Pro; and (C), S Pro. The diagram of radioactive components was made in regard to amino acid elution.

the existence of a large number of intermolecular cross-links in this fraction.

Also it was found that it was impossible to solubilize this material either by detergent, (sodium dodecylsulfate (conc., 1%)) or denaturing agents (8 M, urea), or by reducing agents (mercaptoethanol, 0.75 M), a finding already reported by Johnson [17]. The origin of the S Pro fraction should be discussed in function of seemingly contradictory findings such as its glycine content (and thus the presence of material rich in collagen), its very low hydroxylysine content, and the absence of glucose.

It has previously been described [19,20] that after digestion of collagen by collagenase, a core remains after dialysis. This core represents 10% of the collagenous material and is composed of peptides coming from terminal chain regions and from polar regions of the collagen molecule.

In addition, Kefalides [16] described a low mol. wt. glycoprotein (glycoprotein extension) possessing a heterosaccharidic sugar part and linked to the ends of the collagen α_1 chains by disulfide bridges.

If we admit that the action of the collagenase on the basement membrane is the same as that observed for interstitial collagen, we may imagine that the fraction S Pro could contain glycopeptides having an α_1 collagen-like C-terminal end without disaccharidic glycans and being linked to the glycoprotein extension described elsewhere. These glycopeptides would be liberated after the proteolytic action of pronase on the high mol. wt. starting material. The fraction P_1 would be made up of S Pro glycopeptides possessing a short collagen peptidic residue.

The low Hyl content and the differences in the Lys content of this fraction between man (3.12%) and rat (8.25%) could be explained by participation in varying proportions (depending upon the maturity of the collagen or upon the species) of these amino acids in intermolecular cross-links of the type described by Zimmermann [21].

Another explanation of the presence of glucose in the C Pro fraction despite the absence of hydroxylysine could be the existence of aldol-type links in the native state or its appearance during fractionation (ultrasonic effects) between the disaccharidic glycans of collagen elements and lysine residues of the structural glycoproteins. In support of this hypothesis is the high content of lysine after acid hydrolysis, the high level of radioactivity of fractions corresponding to N-ϵ-hexosyl-Hyl, and to the aldol condensation products as well as the high neutral hexose content found in this fraction.

In addition such a hypothesis could allow us to explain the difficulties that Hudson and Spiro [22], Lehotay [23] and Bardos et al. [2] had in order to obtain BM large glycopeptides possessing only one kind of glycan.

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