Basement membrane heparan sulfate proteoglycan from the L2 rat yolk sac carcinoma

Mogens Fenger, Ulla Wewer* and Reidar Albrechtsen*

The Department of Clinical Chemistry, Rigshospitalet, Blegdamsvej 9 and **The University Institute of Pathological Anatomy, DK-2100 Copenhagen, Denmark*

Received 26 May 1984; revised version received 18 May 1984

Heparan sulfate proteoglycan from the L2 rat yolk sac carcinoma has been purified and partially characterized. The proteoglycan has an apparent M_r of 750000, 35% of which represents the core protein. The core protein seems to be homogeneous, whereas the heparan sulfate chains are heterogeneous with an M_r of about 50000–70000, with 30% of the glucosamine being *N*-sulfated. Antibodies raised against the core protein of the heparan sulfate proteoglycan reacted with basement membranes of various rat and human tissue.

Basement membrane *Proteoglycan* *Heparan sulfate* *Yolk sac carcinoma*

1. INTRODUCTION

Heparan sulfate proteoglycans (HS-PG) are integral components of the basement membrane as are laminin [1], type IV collagen [2], entactin [3], and nidogen [4]. Basement membrane HS-PG have in varying degrees been purified and characterized from the EHS-tumor [5], the PYS-2 [6] and the M1536-B3 [7] parietal endoderm cell lines. In addition, HS-PG have been isolated from authentic basement membrane of the rat kidney [8].

The L2 rat yolk sac carcinoma has been shown to produce laminin, type IV collagen and entactin [9], and it also seems to be a unique source of chondroitin sulfate proteoglycan [10].

Here we report on the purification procedure and partial characterization of a basement membrane HS-PG from the L2 rat yolk sac carcinoma.

2. MATERIALS AND METHODS

2.1. *Heparan sulfate proteoglycan from ascites*

Ascites fluid was collected from Lewis rats injected intraperitoneally with cells from the L2 yolk sac carcinoma cell line [9]. Aprotinin (NOVO A/S, Denmark) and phenylmethylsulfonyl fluoride (PMSF, Sigma, St. Louis, MO) were added to 500 KIE/ml and 1 mM final concentrations, respectively. The ascites fluid was cleared for cells and debris by centrifugation and dialyzed against several changes of water containing aprotinin and PMSF. NaCl was added to a final concentration of 0.35 M and the glycosaminoglycans (GAG) were precipitated with 10 ml 6% (w/v) cetyltrimethylammonium bromide (CTAB) per 150 ml ascites at room temperature overnight. The precipitate was collected by centrifugation, solubilized in a 5:1 mixture of 2 M NaCl and methanol, and the GAG was further precipitated by 3 vols of 96% ethanol. The GAG was finally solubilized in 0.1 or 0.5 M NaCl in phosphate buffer (pH 7.4), chromatographed on Sepharose CL-6B, and, after extensive digestion [11] by chondroitinase ABC (Seikagaku, Japan), on Sepharose CL-6B and 4B (Pharmacia, Uppsala). All chemicals used were of reagent grade or the best quality available.

2.2. *Antibodies to heparan sulfate proteoglycan*

Two rabbits were immunized with 100 μg of the purified HS-PG emulsified in Freund's complete adjuvants as in [12]. The reactivity of the antisera

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

to basement membranes was investigated on ethanol:acetic acid- or formalin-fixed paraffin-embedded tissues, using the unlabeled antibody peroxidase-antiperoxidase (PAP) technique [13].

2.3. *Other methods*

Uronic acid [14] and *N*-sulfate [15] were determined as described. Amino acid composition and hexosamine content were determined on an LKB amino acid analyzer. Iodination of the proteoglycans was performed by the Bolton-Hunter procedure [16]. Radiochemicals were purchased from New England Nuclear. The enzyme immunoassay, ELISA [12], was used to test for the presence of laminin, type IV collagen, entactin, and fibronectin.

3. RESULTS

3.1. *Isolation and identification of heparan sulfate proteoglycan*

HS-PG was isolated from ascites fluid and from extracts of solid tumor tissue of the L2 rat yolk sac carcinoma. The purification procedure included CTAB and ethanol precipitations, treatment with chondroitinase ABC, and gel filtration on Sepharose CL-6B and 4B (fig.1–3). The yield of HS-PG was approx. 5 mg/l ascites.

The material obtained (indicated by bar in fig.3) was identified as HS-PG. Firstly, the *N*-sulfate content (fig.3) paralleled the uronic acid content. Secondly, a small aliquot was labelled with ^{125}I and chromatographed on Sepharose CL-6B. The

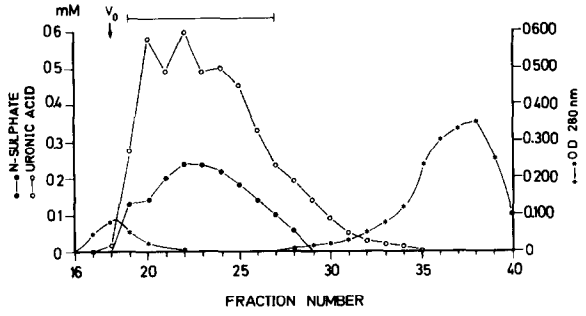

Fig.1. Gel filtration on a 2.6 × 96 cm column of Sepharose CL-6B in 0.1 M phosphate buffer (pH 7.4), 0.1 M NaCl of CTAB-precipitated material from L2 ascites fluid; 9.5-ml fractions were collected at room temperature at a flow rate of 25 ml/h. V_0 and V_T, void volume and total volume, respectively.

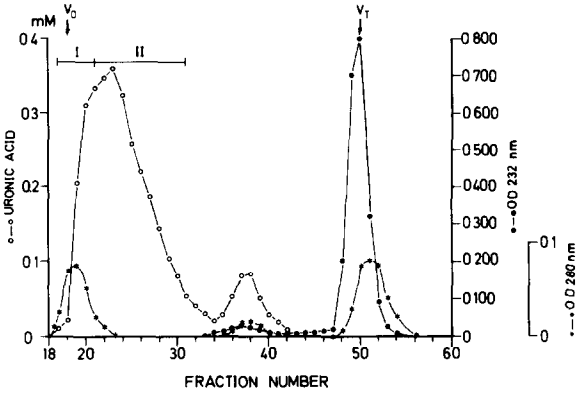

Fig.2. The pool indicated by the bar in fig.1 was rechromatographed after extensive digestion with chondroitinase ABC. Column and chromatographic conditions as in fig.1.

iodinated material eluted near the void volume was treated with 1 unit heparinase (Seikagaku) for 20 h at 37°C [17] and subsequently reapplied on the same Sepharose CL-6B column. As shown in fig.4, all iodinated protein was eluted near the total volume of the column, indicating that all the GAG was susceptible to heparinase. Thirdly, a ^{125}I aliquot was treated with 2 M NaOH at 37°C for 20 h and chromatographed on 1.1 × 96 cm AcA 34 (LKB). A symmetrical ^{125}I peak eluted with K_{av} = 0.32 (fig.5), indicating a homogeneous core protein and showing that the side chains probably were linked to the hydroxyl group of serine through xylose [18]. Furthermore, this elution profile corresponds to a molecular mass of approx. 200 kDa. Finally, no galactosamine could be detected (table 1). Laminin, type IV collagen, entac-

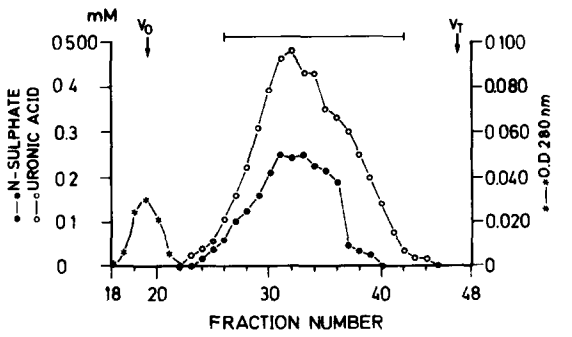

Fig.3. Gel filtration on a 2.6 × 96 cm column of Sepharose 4B of pool II from fig.2. Chromatographic conditions as in fig.1.

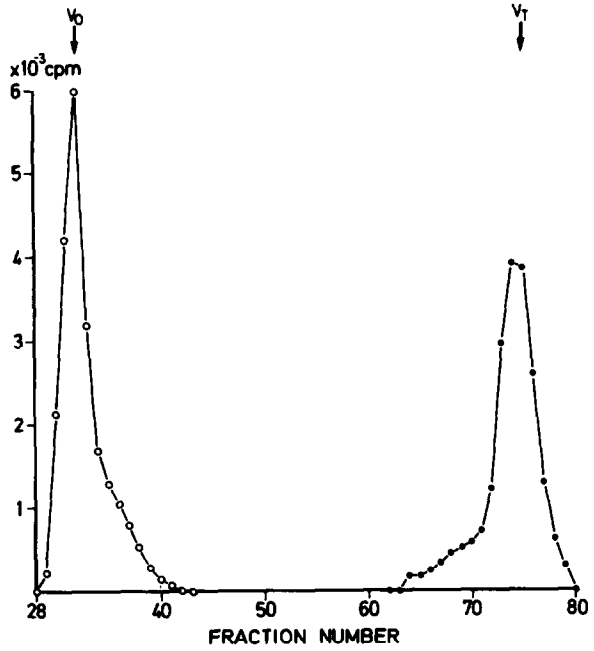

Fig.4. Chromatography on a 1.1 × 93 cm column of Sepharose CL-6B in 0.1 M phosphate buffer (pH 7.4), 0.5 M NaCl of ^{125}I-labelled material from fig.3 before (○—○) and after (●—●) digestion with heparinase. The column was eluted at room temperature with a flow rate of 15 ml/h, and 1.2 ml fractions collected. V_o and V_T, void volume and total volume, respectively.

tin, and fibronectin did not seem to contaminate the HS-PG as shown immunochemically by the ELISA assays, and by the amino acid analyses, which characteristically did not reveal any cysteine.

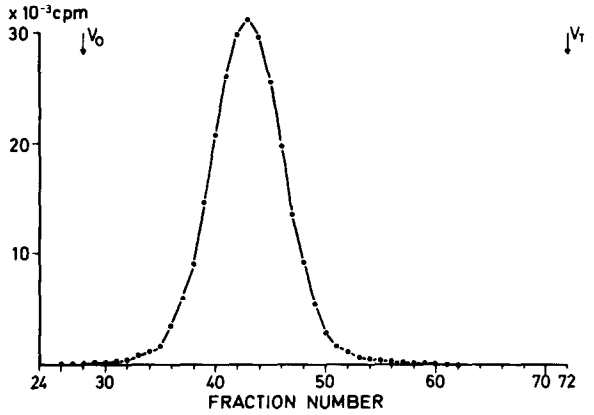

Fig.5. Chromatography on 1.1 × 96 cm column of AcA 34 of iodinated HS-PG treated with 2 M NaOH for 20 h at 37°C. Chromatographic conditions as in fig.4.

Table 1

Composition of the L2 HS-PG compared with the BM_1 HS-PG from the EHS-tumor [5] and with the L2 CS-PG [10]

	Residues/1000		
	L2 HS-PG	EHS HS-PG	L2 CS-PG
Aspartic acid	74	81	88
Threonine	42	68	34
Serine	101	84	183
Glutamic acid	173	154	116
Proline	36	81	61
Glycine	139	106	185
Alanine	75	70	68
Cysteine	Nil	?	Nil
Valine	79	63	72
Methionine	ND	13	10
Isoleucine	49	26	18
Leucine	66	81	20
Tyrosine	24	24	ND
Phenylalanine	24	32	43
Histidine	39	32	61
Lysine	37	29	22
Arginine	42	54	15
$GluNH_2$[a]	369	372	82
$GalNH_2$	Nil	24	2287
N-Sulfate	111		

[a] The number of hexosamine residues are expressed per 1000 amino acid residues

ND, not determined

Preliminary results indicate that a similar, if not identical, HS-PG was isolated from solid tumor tissue extracted with 4 M guanidine–HCl and 1% Triton X-100.

3.2. *Characterization of the isolated heparan sulfate proteoglycan*

The overall size of the HS-PG was roughly estimated to be 750 kDa based on its elution position (fig.1–3) on Sepharose CL-6B and 4B [5].

The core protein accounted for 35% of the HS-PG, as judged by the amino acid analyses. This corresponds to a molecular mass of 250 kDa, which is consistent with the elution profile on the AcA 34 column (fig.5), provided that the core protein attains a globular structure after alkali treat-

ment. However, the fact that it was eluted in the total volume of the Sepharose CL-6B (fig.4) suggests a lower molecular mass of the core protein. This discrepancy might reflect the presence in the heparinase preparation of proteolytic activity which could not be detected using, for example, iodinated albumin as a substrate (not shown).

To define further the size of the heparan sulfate chains (HS-chains), the HS-PG was treated with NaB^3H_4 (11 Ci/mmol) in 0.5 M NaOH at 37°C for 20 h. The liberated HS-chains were chromatographed on AcA 44 (LKB) and Sepharose 4B. The HS-chains show molecular size heterogeneity, as can be seen from fig.6. Most of the chains were eluted in the void volume of AcA 44, indicating a molecular mass above 50 kDa [19]. The HS-chains were included in the Sepharose 4B column and eluted in two main groups with K_{av} = 0.54 and 0.65, respectively. The exact molecular mass could not be determined because of a lack of suitable standards, but by calculating the specific activity of the labelled HS-chains (mol ^{3}H/mmol uronic acid), the molecular mass could be estimated at 50–70 kDa.

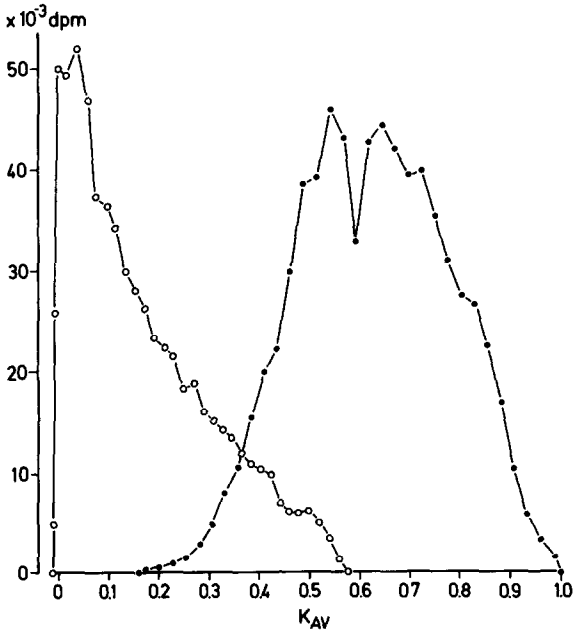

Fig.6. ^{3}H-Labelled HS-chains were chromatographed on a 1.1 × 96 cm column of AcA 44 (○—○), and on a 1.1 × 90 cm column of Sepharose 4B (●—●). Chromatographic conditions as in fig.4.

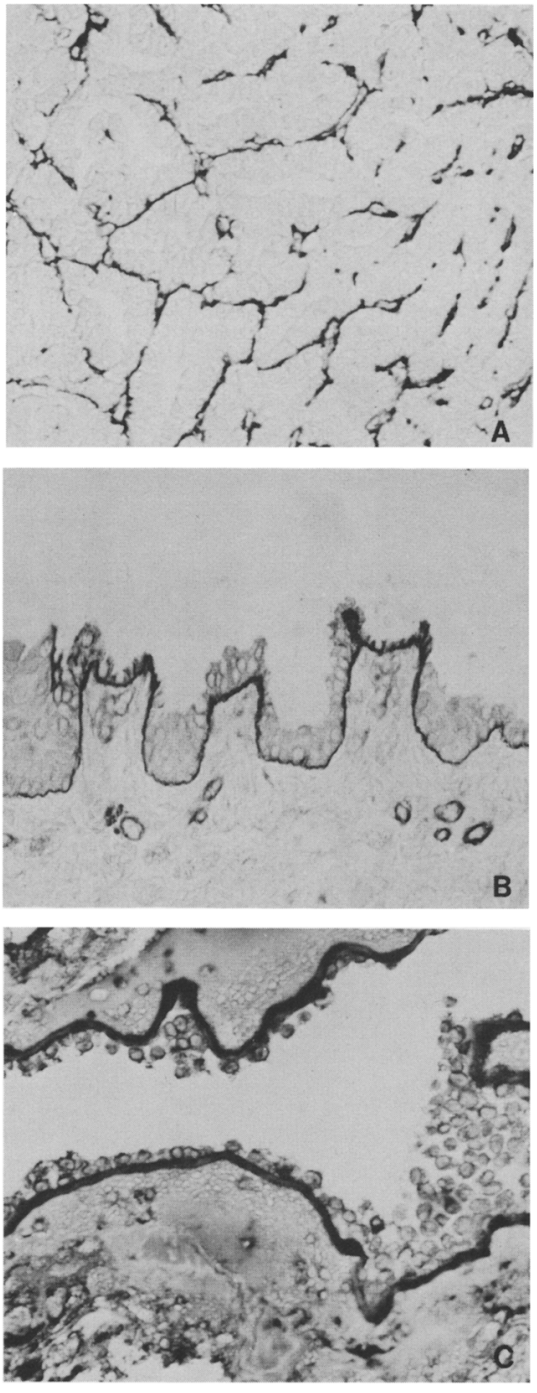

Fig.7. Immunostaining using antisera (dilution 1:100–200) raised against the isolated HS-PG. Note the intense staining reaction localized to basement membranes. (A) The L2 yolk sac carcinoma (× 260). (B) Mucosal epithelium of the normal rat tongue (× 260). (C) The Reichert's membrane of the parietal yolk sac endoderm of normal developing rat embryo (× 250).

The antisera raised against the purified HS-PG were applied in immunohistochemical investigations. A moderate to intense immunostaining of the extracellular matrix of the rat yolk sac carcinoma was seen. A variety of normal rat tissues (salivary gland, tongue, tracheal tissue, intestine, kidney, skin) exhibited an intense staining reaction of the basement membranes (fig.7). Immunostaining of basement membranes of the rat embryo was likewise observed, with the Reichert's membrane being particularly positive (fig.7). In addition, cross-reactivity to basement membranes of human tissue was demonstrated.

4. DISCUSSION

A number of different populations of HS-PG have been identified, some of which are constituents of basement membranes [20]. Although the major proteoglycan produced by the L2 rat yolk sac carcinoma is chondroitin sulfate proteoglycan [10], this study shows that small amounts of HS-PG also are synthesized by this tumor.

The L2 HS-PG seems to share some characteristics (e.g., molecular mass, amino acid composition, and basement membrane localization) with the BM_1, which was purified from the EHS-tumor as in [5]; but further studies are needed to define, for instance, their immunological similarities.

Our immunostaining results demonstrate that antisera raised against the L2 HS-PG react distinctly with authentic basement membranes, in a manner similar to that described for antisera against BM_1 [21,22]. The fact that the antibodies also reacted with human tissue indicates the usefulness of such antisera in studies of various normal and pathological states.

ACKNOWLEDGEMENTS

Part of this work was supported by the Danish Cancer Society. We thank F. Hansen for performing the amino acid analyses; E. Engvall, A.E. Chung, and E. Ruoslahti for antisera to type IV collagen, entactin and fibronectin, respectively. Aa. Valsted, B. Valentin, and B. Lindholm gave excellent technical assistance.

REFERENCES

[1] Timpl, R., Rohde, H., Robey, P.G., Rennard, S.I., Foidart, J.-M. and Martin, G.R. (1979) J. Biol. Chem. 254, 9933–9937.
[2] Kefalides, N.A., Alper, R. and Clark, C.C. (1979) Int. Rev. Cytol. 61, 167–228.
[3] Carlin, B., Jaffe, R., Bender, B. and Chung, A.E. (1981) J. Biol. Chem. 256, 5209–5214.
[4] Timpl, R., Dziadek, M., Fujiwara, S., Nowack, H. and Wick, G. (1983) Eur. J. Biochem. 137, 455–465.
[5] Hassell, J.R., Robey, P.G., Barrach, H.-J., Wilczek, J., Rennard, S.I. and Martin, G.R. (1980) Proc. Natl. Acad. Sci. USA 77, 4494–4498.
[6] Oohira, A., Wight, T.N., McPherson, J. and Bornstein, P. (1982) J. Cell Biol. 92, 357–367.
[7] Lowe-Krentz, L.J. and Keller, J.M. (1983) Biochemistry 22, 4412–4419.
[8] Kanwar, Y.S., Hascall, V.C. and Farquhar, M.G. (1981) J. Cell Biol. 90, 527–532.
[9] Wewer, U. (1982) Dev. Biol. 93, 416–421.
[10] Oldberg, Å., Hayman, E.G. and Ruoslahti, E. (1981) J. Biol. Chem. 256, 10847–10852.
[11] Saito, H., Yamagata, T. and Suzuki, S. (1968) J. Biol. Chem. 243, 1536–1542.
[12] Engvall, E., Krusius, T., Wewer, U. and Ruoslahti, E. (1983) Arch. Biochem. Biophys. 222, 649–656.
[13] Albrechtsen, R., Nielsen, M., Wewer, U., Engvall, E. and Ruoslahti, E. (1981) Cancer Res. 41, 5076–5081.
[14] Blumenkrantz, N. and Asboe-Hansen, G. (1973) Anal. Biochem. 54, 484–489.
[15] Shively, J.E. and Conrad, H.E. (1976) Biochemistry 15, 3932–3942.
[16] Bolton, A.E. and Hunter, W.M. (1973) Biochem. J. 133, 529–539.
[17] Linker, A. and Hovingh, P. (1972) Methods Enzymol. 28, 902–911.
[18] Robinson, H.C., Horner, A.A., Höök, M., Ögren, S. and Lindahl, U. (1978) J. Biol. Chem. 253, 6687–6693.
[19] Johnson, E.A. and Mulloy, B. (1976) Carbohydr. Res. 51, 119–127.
[20] Hascall, V.C. and Kimura, J.H. (1982) Methods Enzymol. 82, 769–800.
[21] Laurie, G.W., Leblond, C.P. and Martin, G.R. (1983) Am. J. Anat. 167, 71–82.
[22] Foidart, J.B., Foidart, J.M., Hassell, J. and Mahieu, P. (1983) Renal Physiol. 6, 163–170.