Urol Res (1983) 11:163–166

Urological Research


# Glycosaminoglycans in Urothelial Carcinomas

H. Rübben, R. Friedrichs, H. W. Stuhlsatz and W. Lutzeyer

Departments of Urology (Head: Prof. Dr. W. Lutzeyer), Clinical Chemistry and Pathobiochemistry (Head: Prof. Dr. Dr. H. Greiling), Medical Faculty, Rheinisch-Westfälische Technische Hochschule Aachen, Federald Republic of Germany

Accepted: April 20, 1983

**Summary.** The glycosaminoglycan (GAG) content in human urothelial carcinomas was biochemically determined and compared to that of normal urothelium and bladder wall of the calf. The total GAG content was elevated in urothelial carcinomas, and the distribution pattern differed from that of normal urothelium and bladder wall. Whereas urothelial carcinomas contained heparan sulphate, dermatan sulphate, chondroitin 4-sulphate and chondroitin 6-sulphate, only heparan sulphate could be detected in the normal urothelium. The GAG determination was based on hexosamine analysis and thin layer chromatography after elution on Dowex 1 X 2 columns.

**Key words:** Urothelial bladder carcinoma, Calf urothelium, Calf bladder wall, Glycosaminoglycan content.

## Introduction

Proteoglycans are major constituents of the connective tissue stroma of most organs. They are complex macromolecules in which glycosaminoglycan (GAG) chains are covalently linked to a protein core. The chains consist of repeating disaccharide units, composed of a hexosamine (D-glucosamine or D-galactosamine) and an uronic acid.

An increased GAG synthesis and a change of the GAG distribution pattern compared to healthy mother tissue has been described in epithelial and mesenchymal tumours (melanoma, Wilms' tumor, breast carcinoma, leiomyoma) [1–4].

So far urothelial tumours have only been examined by histochemical methods; elevated GAG levels compared to normal urothelium have been discussed [5–7], but histochemical staining procedures are not specific, because not only GAG but also glycoproteins, polysaccharides and sialomucins are stained [7].

Beside a possible role of GAG in occurrence and growth pattern of carcinomas, GAG may protect against bacterial adhesion and stone formation. After removal of the urothelial surface coat with hydrochloric acid, bacterial attachment as well as crystalline aggregation to the injured epithelium are significantly increased. Intravesical instillation of heparin breaks off these effects [8, 9].

So far GAG have not been biochemically proven in the normal urothelium and surface coat as well as in urothelial tumours. The purpose of this study was to determine the GAG content and distribution pattern of the normal calf urothelium and human urothelial carcinomas.

## Experimental Procedures

### *Materials*

Immediately after surgical removal, one half of the tumour was carefully separated from muscle and connective tissue and stored in acetone. Five urothelial tumours without glandular or squamous cell metaplasia were analysed:

H. B., male, 47 yrs., 3.390 g dry weight of the tumor (d.wt.)

stage T1 NX M0, a moderately well differentiated urothelial carcinoma, recurrence of a urniary bladder carcinoma one year after open resection

K. D., male, 67 yrs., 5.200 g d.wt.

stage Ta N0 M0, a moderately well differentiated urothelial carcinoma, first tumour, open resection

T. H., male, 80 yrs., 0.800 g d.wt.

stage T2 NX M0, a poorly differentiated urothelial carcinoma, first tumour, open resection (1974 partial laryngectomy, because of a carcinoma of the larynx)

*Abbreviations:* ABC = chondrointin ABC lyase; AC = chondroitin AC lyase; CH4-S = chondroitin 4-sulfate; CH6-2 = chondroitin 6-sulfate; DS = dermatan sulfate; GAG = glycosaminoglycan (s); Gal-N = galactosamine; Glc-N = glucosamine; HS = heparan sulphate; KS = keratan sulphate; TLC = thin layer chromatography

**Table 1**

| bladder wall | Glc-N | Gal-N nmol g/d.wt. |
|---|---|---|
| 1.5 M NaCl | 860 | 570 |
| 3.0 M NaCl | 130 | 920 |
| calf urothelium | Glc-N | Gal-N nmol g/d.wt. |
| 1.5 M NaCl | 540 | – |
| 3.0 M NaCl | – | – |

M. L., male, 73 yrs., 4.410 g d.wt.

stage T3b NX M1, a poorly differentiated urothelial carcinoma, first tumour, open resection

M. M., female, 76 yrs., 1.310 g d.wt.

stage T2-3 N0 M0, a moderately well differentiated urothelial carcinoma, nephrectomy (renal pelvic carcinoma)

The glycocalyx and the urothelium of 15 calf urinary bladders were mechanically desquamated from the submucosa immediately after decapitation. The dried material consisted of 0.105 g of urothelium and 1.014 g of bladder wall (muscle layers and submucosa). The purity of the desquamated urothelium was shown by histological examination.

*Chemicals*

Papain (E.C. 3.4.22.2, crystalline suspension, 30 U/mg protein) was obtained from Sigma Chemical Comp., St. Louis, USA. Dowex 1 X 2 (200–400 mesh, $Cl^-$) was from Serva, Heidelberg, FRG. Chondroitin AC lyase (E.C. 4.2.2.5) and chondroitin ABC lyase (E.C. 4.2.2.4) were from Seikgaku Kogyo Comp., Tokyo, Japan. Cellulose strips (0.1 mm thin) and all other chemicals were from Merck, Darmstadt, FRG, Control samples of reference of heparan sulphate, dermatan sulphate, chondroitin 4-sulphate, chondroitin 6-sulphate and keratan sulphate (5 mg/ml) were kindly provided by one of the authors, H. W. S. [10, 11].

*Methods*

*Proteolysis.* After removal of acetone and drying at 65 °C, the dry weight was estimated and the material was triturated. Proteolysis was carried out to separate the GAG chains from the protein core. The samples were completely proteolysed by incubation at 65 °C for 30 h in a papain buffer with a separate addition of papain after 15 h (papain buffer: 0.5 mg of the enzyme and 88 mg cysteinium chloride were dissolved in 100 ml $H_2O$ containing 1.316 g sodium acetate and 0.186 g titriplex III, adjusted to pH 7.0). The ratio of dried material to papain buffer solution was 100 mg to 5 ml. The reaction was terminated by adjusting the proteolysate to pH 1.3 with 5.0 M HCl. After standing for 12 h at 4 °C, the precipitate was discarded after centrifugation. The supernatant was adjusted to pH 7.0 with 5.0 M NaOH [10, 11].

*Dowex 1 X 2-Chromatography.* Dowex 1 X 2 is an anionic exchanger. After papain digestion the solutions were at once applied to Dowex 1 X 2 columns (6 x 60 mm) which were equilibrated with 0.15 M NaCl. Elution was carried out with 20 ml each of 0.15 M NaCl, 0.5 M NaCl, 1.5 M NaCl and 3.0 M NaCl. Most of the sulphated GAG were eluted with 1.5 M and 3.0 M NaCl. Dermatan sulphate was usually sulphated to a higher degree than heparan sulphate and therefore fixed better at the anionic exchanger. Most of the dermatan sulphate was therefore found in the 3.0 M NaCl eluate [10, 11].

The four eluates are dialysed against water to remove the saline solution, evaporated to dryness and redissolved in 5 ml $H_2O$ [10, 11].

*Hexosamine Analysis.* Glucosamine and galactosamine of all four eluates (0,15 M NaCl, 0.5 M NaCl, 1.5 M NaCl and 3.0 M NaCl) were determined from 1 ml of the above solution in presence of the amino acids using an amino acid analyser (TSM, Technicon) after hydrolysis in 3 M HCl at 105 °C for 15 h [10, 11].

*Enzymatic Degradation.* Chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate could be degraded by the specific enzymes condroitin AC lyase and chondroitin ABC lyase to produce unsaturated disaccharides containing a reducing end [12]. The 1.5 M NaCl and the 3.0 M NaCl eluates were evaporated to dryness and redissolved in 60 µl $H_2O$. Two aliquots of 20 µl were subjected to chondroitin AC lyase and chondroitin ABC lyase digestion. The enzymatic digestion was carried out as described [13].

*Thin Layer Chromatography.* Ascending chromatography was carried out in six different concentrations of a calcium and barium acetate system (volume ratios calcium or barium acetate:$H_2O$ ethanol 5:3:12; 5:0:5; 5:1:4: 5:2:3; 5:3:2 and 5:4:1). In each concentration the migration distance was exactly 2 cm. The chromatoplates were stained with toluidin blue.

## Results

The results of the hexosamine analysis of the 1.5 M NaCl and the 3.0 M NaCl eluates are shown in Fig. 2 and 3. The hexosamine content extended from 230 nmol/g d.wt. to 3.05 µmol/g d.wt. in the 1.5 M NaCl eluate and from 50 nmol/g d.wt. to 2.82 µmol/g d.wt. in the 3.0 M NaCl eluate. All tumours showed different hexosamine contents as well as different glucosamine/galactosamine ratios. The most advanced urothelial carcinoma (T3b, G3) exhibited the highest hexosamine levels.

The difference between human urothelial carcinomas and normal calf urothelium were evident. Only glucosamine was found in the 1.5 M eluate of the normal calf urothelium and in the 3.0 M NaCl eluate no GAG could be detected. In the bladder wall of the calf glucosamine and galactosamine were found (Table 1).

The hexosamine content in the 0.15 M NaCl and 0.5 M NaCl eluates mainly belonged to glycoproteins which also contained glucosamine and galactosamine as constituents. In contrast to the GAG glycoproteins were not fixed at the anionic exchanger. Hexosamine levels up to 11.2 µmol/g d.wt. were measured (not depicted). The two eluates were not considered further for thin layer chromatography or enzymatic degradation.

Heparan sulphate, dermatan sulphate, chondroitin 4-sulphate and chondroitin 6-sulphate were found in human urothelial tumours (Fig. 4 and 5), whereas only heparan sulphate was detected in normal calf urothelium (Fig. 1).

| calf | urothelium | | bladder wall | |
|---|---|---|---|---|
| 1,5 MNa Cl | Ca Ac | Ba Ac | Ca Ac | Ba Ac |
| DS | – | – | + | (+) |
| HS | (+) | – | + | – |
| CH-4-S | – | – | + | – |
| CH-6-S | – | – | – | – |
| KS | – | – | – | – |

**Fig. 1.** Qualitative GAG identification of normal calf urothelium and bladder wall with TLC of the 1.5 M NaCl Dowex eluates in the barium and calcium acetate system. *Legend*: + = clearly identified; (+) = questionably identified; – = not identified

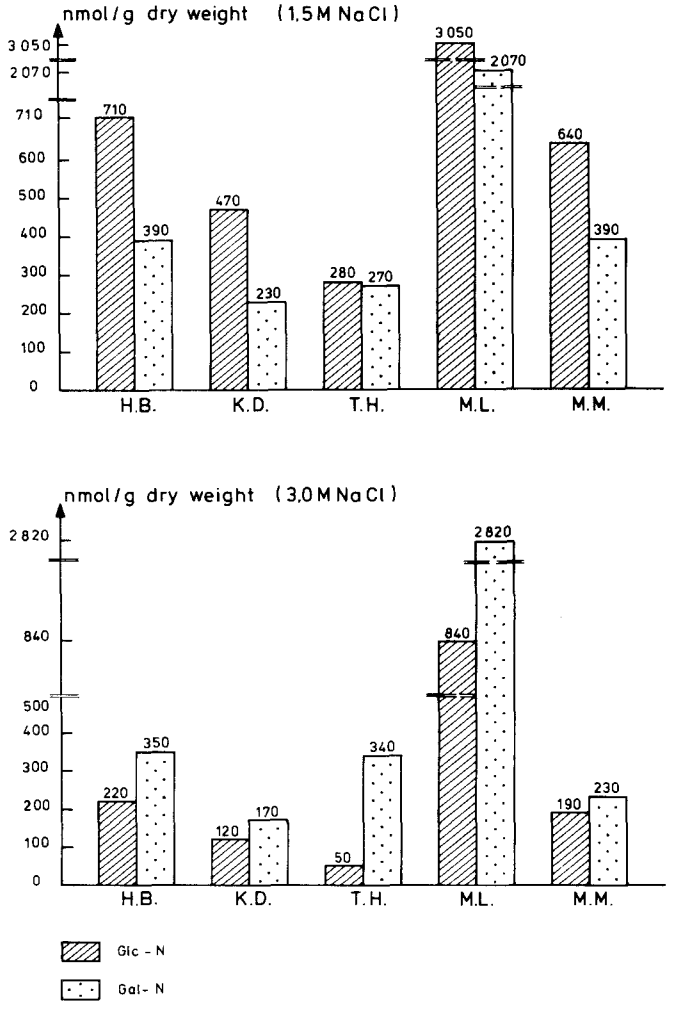


**Fig. 2.** Quantitative hexosamine analysis of urothelial carcinomas in the 1.5 M and 3.0 M NaCl Dowex eluates

**Fig. 5.** Qualitative GAG identification of urothelial tumors with ▶ TLC of the 3.0 M NaCl Dowex eluates in the barium and calcium acetate system. *Legend*: + = clearly identified; (+) = questionably identified; – = not identified

The qualitative GAG determination was based on thin layer chromatography in a calcium and barium acetate system, in which the GAG types were precipitated in different concentrations of ethanol. In the calcium acetate system all GAG migrated. The dermatan standard was precipitated as the first GAG, keratan sulphate as the last one. The non-digested portion of the eluate contains heparan sulphate, dermatan sulphate, chondroitin 4-sulphate and chondroitin 6-sulphate. Chondroitin 4-sulphate and chondroitin 6-sulphate were missing in the portion

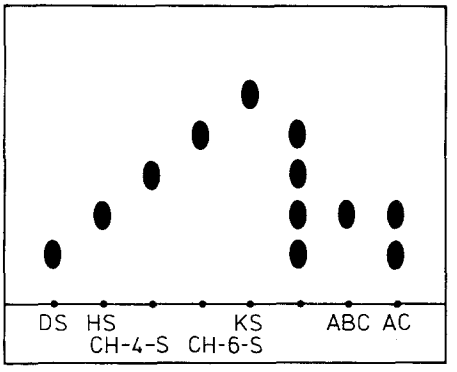


**Fig. 3.** The 1.5 M NaCl Dowex eluate of the tumor M. M. subjected to TLC in the calcium acetate system

| tumors | H.B. | | K.D. | | T.H. | | M.L. | | M.M. | |
|---|---|---|---|---|---|---|---|---|---|---|
| 1,5 M Na Cl | Ca Ac | Ba Ac | Ca Ac | Ba Ac | Ca Ac | Ba Ac | Ca Ac | Ba Ac | Ca Ac | Ba Ac |
| DS | + | + | – | (+) | + | (+) | – | – | + | (+) |
| HS | + | + | (+) | (+) | – | + | (+) | (+) | + | + |
| CH-4-S | – | – | – | – | + | – | – | – | + | – |
| CH-6-S | (+) | – | – | – | + | – | – | – | + | – |
| KS | – | – | – | – | – | – | – | – | – | – |

**Fig. 4.** Qualitative GAG identification of urothelial tumors with TLC of the 1.5 M NaCl Dowex eluates in the barium and calcium acetate system. *Legend*: + = clearly identified; (+) = questionably identified; – = not identified

| tumors | H.B. | | K.D. | | T.H. | | M.L. | | M.M. | |
|---|---|---|---|---|---|---|---|---|---|---|
| 3,0 M Na Cl | Ca Ac | Ba Ac | Ca Ac | Ba Ac | Ca Ac | Ba Ac | Ca Ac | Ba Ac | Ca Ac | Ba Ac |
| DS | + | + | + | + | + | + | + | + | + | + |
| HS | (+) | – | (+) | (+) | – | + | (+) | (+) | + | + |
| CH-4-S | – | – | + | – | – | – | + | – | – | – |
| CH-6-S | + | – | + | – | + | – | + | + | – | – |
| KS | – | – | – | – | – | – | – | – | – | – |

digested with chondroitin AC lyase; in addition to these two GAG types dermatan sulphate was also missing in the portion digested with condroitin ABC lyase (Fig. 3). In the barium acetate system dermatan and heparan sulphate did not leave the start, the keratan sulfate standard migrated best and chondroitin 4-sulphate and keratan sulphate could be separated (not depicted). Keratan sulphate could be excluded in any of the samples analysed.

## Discussion

A first quantitative study of the GAG content in urothelial carcinomas is described. GAG can be only presumed from the histochemical results published so far [5–7]. The GAG content in human urothelial carcinomas is elevated compared to normal calf urothelium. In normal calf urothelium we find heparan sulphate in a concentration of 540 nmol/g d.wt. In urothelial carcinomas however dermatan sulphate, chondroitin 4-sulphate and chondroitin 6-sulphate are also found. The most advanced carcinoma exhibits the highest GAG level.

Chondroitin 4-sulphate and chondroitin 6-sulphate are found in high concentrations in embryonic and neonatal tissues. In healthy adult tissues the content of these two GAG types significantly declines, or they completely disappear. Because of a high content in carcinomas chondroitin 4-sulphate and chondroitin 6-sulphate are discussed as stimulants of tumour growth [4, 14].

Our results give a first indication that GAG may be involved in the biological behaviour of urinary bladder carcinomas. Although the elevated GAG content in human urothelial carcinomas compared to normal calf urothelium is proven, the GAG content and GAG distribution pattern cannot yet be correlated to the depth of infiltration or grade of differentiation.

A study of urinary GAG excretion in individuals with bladder cancer detected an elevated excretion in 53% of the cancer cases, the highest levels found among individuals with metastatic disease. Therefore GAG are discussed as markers of bladder cancer [15].

Further studies to characterise the GAG production of cultured urothelial cells are carried out in our laboratory.

These studies are necessary because the elevated urinary GAG excretion may reflect disturbances of the turnover in connective tissues. Cell culture analysis may also rule out a possible falsification of our results by the GAG content of the carcinomas' vasculature and connective tissue.

## References


1. Bhavanandan, VP (1982) Glycosaminoglycans of cultured human fetal uveal melanocytes and comparison with those produced by cultured human melanoma cells. Biochemistry 20:5595
2. Hopwood JJ, Dorfman A (1978) Glycosaminoglycan synthesis by Wilms' tumor, Pediatr Res 12:52
3. Chandrasekaran EV, Davidson EA (1979) Glycosaminoglycans of normal and malignant cultured human mammary cells. Cancer Res 39:870
4. Dietrich CP, Sampaio LO, Toledo OMS, Cassaro CMF (1977) Cell recognition and adhesiveness: A possible role for the sulfated mucopolysaccharides. Biochem Biophys Res Commun 75:329
5. Stiller D (1964) Histochemische Untersuchungen über Sekretionserscheinungen in Papillomen und Karzinomen der Harnblase. Beitr Pathol Anat 129:377
6. Hukill PB, Vidone RA (1965) Histochemistry of mucus and other polysaccharides in tumors I. Carcinoma of the bladder. Lab Invest 14:1624
7. Monis B, Dorfman HD (1967) Some histochemical observations in transitional epithelium of man. J Histochem Cytochem 15:475
8. Gill WB, Jones KW, Ruggiero KJ (1982) Protective effects of heparin and other sulfated glycosaminoglycans on crystal adhesion to injured urothelium. J Urol 127:152
9. Parsons CL (1982) Prevention of urinary tract infection by the exogenous glycosaminoglycans sodium pentosanpolysulfate. J Urol 127:167
10. Stuhlsatz HW, Hirtzel F, Keller R, Cosma S, Greiling H (1981) Studies on the polydispersity and heterogenicity of proteokeratan sulfate from calf and porcine cornea. Hoppe Seylers Z Physiol Chem 362:841
11. Stein T, Keller R, Stuhlsatz HW, Greiling H, Ohst E, Müller E, Scharf HD (1982) Structure of the linkage-region between polysaccharide chain and core protein in bovine corneal proteokeratan sulfate. Hoppe Seylers Z Physiol Chem 363: 825
12. Suzuki S, Saito H, Yamagata T, Anno K, Seno N, Kawai Y, Furuhashi T (1968) Formation of three types of disulfated disaccharides from chondroitin sulfates by chondroitinase digestion. J Biol Chem 243:1543
13. Greiling H, Eberhard A (1974) Chondroitin-4-sulfat, Chondroitin-6-sulfat and Dermatansulfat. In: Bergmeyer HU (ed) Methoden der enzymatischen Analyse. 3rd ed, p 1210
14. Sampaio LO, Dietrich CP, Filho OG (1977) Changes in sulfated mucopolysaccharide composition of mammalian tissues during growth and in cancer tissues. Biochim Biophys Acta 498:123
15. Hennessey PT, Hurst RE, Hemstreet III GP, Cutter G (1981) Urinary glycosaminoglycan excretion as a biochemical marker in patients with bladder carcinoma. Cancer Res 41:3968



Dr. H. Rübben
Department of Urology
Medical Faculty
RWTH Aachen
D-5100 Aachen