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Rapid report

Pancreatic carcinoma is characterized by elevated content of hyaluronan and chondroitin sulfate with altered disaccharide composition

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Abstract

The amount and the types of glycosaminoglycans (GAGs) present in human pancreatic carcinoma were examined and compared with those in normal pancreas. Human pancreatic carcinoma contained increased levels (4-fold) of total GAGs. Particularly, this carcinoma is characterized by a 12-fold increase of hyaluronan (HA) and a 22-fold increase in chondroitin sulfate (CS) content. CS in pancreatic carcinoma exhibited an altered disaccharide composition which is associated with marked increase of non-sulfated and 6-sulfated disaccharides. Dermatan sulfate (DS) was also increased (1.5-fold) in carcinoma, whereas heparan sulfate (HS), the major GAG of normal pancreas, becomes the minor GAG in pancreatic carcinoma without significant changes in the content and in molecular size. In all cases, the galactosaminoglycans (GalGAGs, i.e. CS and DS) derived from pancreatic carcinomas were of lower molecular size compared to those from normal pancreas. The results in this study indicate, for the first time, that human pancreatic carcinoma is characterized by highly increased amounts of HA and of a structurally altered CS.

Keywords: Pancreas; Carcinoma; Glycosaminoglycan; Proteoglycan

Glycosaminoglycans (GAGs) are highly anionic macromolecules, which are the major components of cell surface and extracellular matrix [1]. The GAG molecules, because of their complex polyanionic nature, have been implicated in a number of biological processes and functions [2,3]. In recent years, GAGs contained in a wide variety of tumors have been studied and, in some instances, their chemical and structural characteristics were compared with the GAGs from normal tissues [4–12]. Pancreatic carcinoma, which is one of the common malignant tumors in human, is of unfavorable prognosis with the highest frequency of mortality and poor survival time. One of the most pathological characteristics of this carcinoma is its early invasion of the surrounding tissues and the metastasis to lymph nodes, liver, lung, peritoneum and bones [13]. Although GAGs have been characterized from a wide variety of normal and neoplastic tissues, very little is known about GAG content, composition and structural modifications in human pancreas and pancreatic carcinoma. In fact, information on these molecules in pancreatic tissues based mainly on immunohistochemical studies [14–17]. In the present study, the type of GAGs, their composition and fine chemical

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structure were examined in poorly differentiated pancreatic carcinoma and normal pancreas. The results indicated significant quantitative changes of GAGs in pancreatic carcinoma. Furthermore, significant structural modifications regarding the sulfation pattern and the molecular size of particular GAGs were also noted.

Sepharose CL-6B and DEAE-Sephacel were obtained from Pharmacia (Uppsala, Sweden). Papain twice crystallized, chondroitinases ABC and B (*Flavobacterium heparinum*) and GAGs standards were from Sigma (St. Louis, MO, USA). All other chemicals used were of the best commercially available grade. Uronic acid was determined by the Bitter–Muir modified carbazole reaction [18]. Analysis for glucosamine and galactosamine was performed on a Beckman amino acid analyzer [19]. Unsaturated disaccharides released following treatment of GAGs with chondroitinase ABC were determined by high-performance liquid chromatography (HPLC) [20]. Determination of glucuronic acid and iduronic acid was also performed by HPLC [21]. Pancreatic carcinomas were collected over a period of 30 months from 12 patients (7 males/5 females) aged 45–65 years who underwent pancreatectomy for pancreatic adenocarcinoma in the University Hospital of Patras. A specimen was taken from the center of the tumor (approximately 1 g) for pathological and biochemical examinations. Pathological examination of the neoplastic tissue showed poorly differentiated adenocarcinoma in 9 of the 12 cases. Normal human pancreas specimens were collected from five autopsies (3 males and 2 females) aged 45–65 years and were free from specific histopathological changes. All specimens were immediately stored at −70°C. Normal pancreas ($n=5$) and poorly differentiated pancreatic carcinomas ($n=9$) have been separately analyzed for their GAG content.

Particularly, known amounts of wet weight of tissues (normal and cancerous) were exhaustively digested with papain and the liberated GAG chains were precipitated by the addition of 5 vols. of ethanol. Consequently, GAG chains were further released from remaining peptide fragments by treatment with 0.05 M NaOH/1 M sodium borohydride at 45°C for 48 h under vacuum [22]. The solutions were brought to pH 5.0, with glacial acetic acid and the GAGs precipitated with 5 vols. of ethanol. The GAG content was estimated in each sample by measuring the uronic acid in the precipitates. No significant intrabatch differences were found in GAG contents between the various normal pancreas examined and between the various pancreatic carcinoma. Further analyses were performed on pooled normal pancreas and pancreatic carcinoma. Pooled GAGs were fractionated on a DEAE-Sephacel column as described elsewhere [23]. Characterization and quantitation of GAGs were performed following cellulose acetate electrophoresis [12], hexosamine analysis and treatment with specific chondro-/dermato-lyases. Disaccharide composition and sulfation pattern of galactosaminoglycans (GalGAGs) were estimated following digestion with chondroitinase ABC and HPLC analysis. Gel chromatographies and enzymic degradations were performed as described previously [12,24]. Digestion with chondroitinase B was performed in 0.05 M Tris–HCl, pH 8.0, containing 0.05% (w/v) bovine serum albumin, at 37°C for 24 h using 0.3 U enzyme per mg uronic acid. Statistical analysis was performed by the *t*-test using the microcall origin software (version 3.2). Data reported are the means ± S.D.

The results on the chemical composition (Table 1A) indicated that no significant intrabatch differences were found in the GAG contents, between the normal pancreas (320 ± 12 μg of uronic acid/g wet weight of tissue) and the pancreatic carcinomas (1615 ± 54 μg of uronic acid/g wet weight of tissue) examined. For these reasons, further analyses were performed on pooled normal pancreas and pancreatic carcinomas. The overall GAG content in pancreatic carcinoma was found significantly increased (4-fold, $P \leq 0.001$) as compared to normal pancreas. Pancreatic carcinoma is characterized by an 8.5-fold net increase in GalGAGs content and a 1.6-fold net increase in glucosaminoglycans (GlcGAGs) content as indicated by the amounts of galactosamine (GalN) and glucosamine (GlcN) (Table 1A). The molar ratios of GlcA/IdoA and GalN/IdoA revealed that total GAGs and GalGAGs in pancreatic carcinoma contained relatively lower proportions of iduronic acid in comparison to those of normal pancreas (Table 1A).

Anion-exchange chromatography on DEAE-Sephacel revealed the presence of three uronic acid containing populations in both normal and cancerous

tissues (Fig. 1A). The major population II of normal tissue (55% of the uronic acid-containing material) was eluted with 0.5 M NaCl. The remaining proportion of the recovered uronic acid-containing material was present in population I (9%) and population III (36%). In pancreatic carcinoma, however, population III was the major population, containing the 67% of total recovered uronic acid and eluted with 0.7 M NaCl. The remaining proportion of uronic acid was distributed in populations I (24%) and II (9%). Populations I, II and III were pooled, as indicated by bars, concentrated and taken for further analyses. Electrophoresis of all populations on cellulose acetate membranes (Fig. 1B) revealed that population I migrated with a mobility identical to that of standard hyaluronan (HA) and population II migrated as standard HS in both tissues. Population III gave two bands with the same electrophoretic mobility as that of standards of dermatan sulfate (DS) and chondroitin sulfate (CS). Chemical analyses of the populations I and II from both tissues showed that these populations contained only glucosamine confirming their HA and HS nature, respectively. The population III contained predominantly ($>98\%$) galactosamine and, therefore, appeared to be GalGAGs. Populations I, II and III, namely, HA, HS and GalGAGs, respectively, quantified in terms of uronic acid and by cellulose acetate electrophoresis [12]. It must be noted, that the quantitation of GAGs by uronic acid analysis was in very close agreement with that obtained by electrophoretic determination. The results on the composition of GAGs in normal and cancerous tissues are presented in Table 1B. Normal pancreas contained mainly HS, corresponding to 55% of total GAGs. The minor type of GAG in normal pancreas was the HA (9%). The GalGAGs were composed of DS (24%) and CS (12%). In pancreatic carcinoma the CS was (55%), showing a dramatic increase (22-fold) as compared to normal pancreas. HA content was also significantly increased 12-times, whereas DS content was increased only 1.5-times. In contrast, the amount of HS was almost unchanged.

These results demonstrated that pancreatic carcinoma exhibits a statistically significant increase in GalGAGs content, which is mainly due to the increase of CS content. The increase in GlcGAGs content in pancreatic carcinoma is due to the increase of HA. Previous studies have shown that the amounts and composition of GAGs undergo dramatic changes in various neoplastic tissues [8,9,12]. CS content markedly affected in a variety of neoplasms of both epithelial and mesenchymal origin [6,7,25] and increased 12-, 9- and 24-fold in human colon carcinoma [8], rectum carcinoma [12] and hepatocellular carcinoma [9], respectively. In the present study, the 22-fold increase of CS in pancreatic carcinoma is of the same magnitude to that of hepatocellular carcinoma. The characteristic increase of HA in pancreatic carcinoma may possibly be related to the degree of malignancy of this carcinoma since most malignant solid tumors contain and/or produce increased

Table 1
(A) Chemical composition and (B) GAG distribution, of isolated GAGs from normal pancreas ($n=5$) and pancreatic carcinomas ($n=9$)[a]

	Normal pancreas	pancreatic carcinoma	Net increase (*x*-fold)
(A) Chemical composition			
Uronic acid	320 ± 12	1615 ± 54	4.0
Galactosamine	125 ± 5	1190 ± 43	8.5
Glucosamine	225 ± 9	586 ± 21	1.6
GlcA/IdoA[b]	3.0	10.0	
GalN/IdoA[b]	1.5	8.2	
(B) GAG composition			
Hyaluronan	29 ± 3 (9)	388 ± 32 (24)	12.0
Heparan sulfate	176 ± 16 (55)	145 ± 12 (9)	–
Dermatan sulfate	77 ± 6 (24)	194 ± 16 (12)	1.5
Chondroitin sulfate	38 ± 3 (12)	888 ± 72 (55)	22.0

[a]The results are expressed as μg/g wet weight of tissue. Values are the mean ± S.D. Percent values are given in parentheses.
[b]Molar ratios of glucuronic acid (GlcA) to iduronic acid (IdoA) and *N*-acetyl-galactosamine (GalN) to iduronic acid (IdoA).

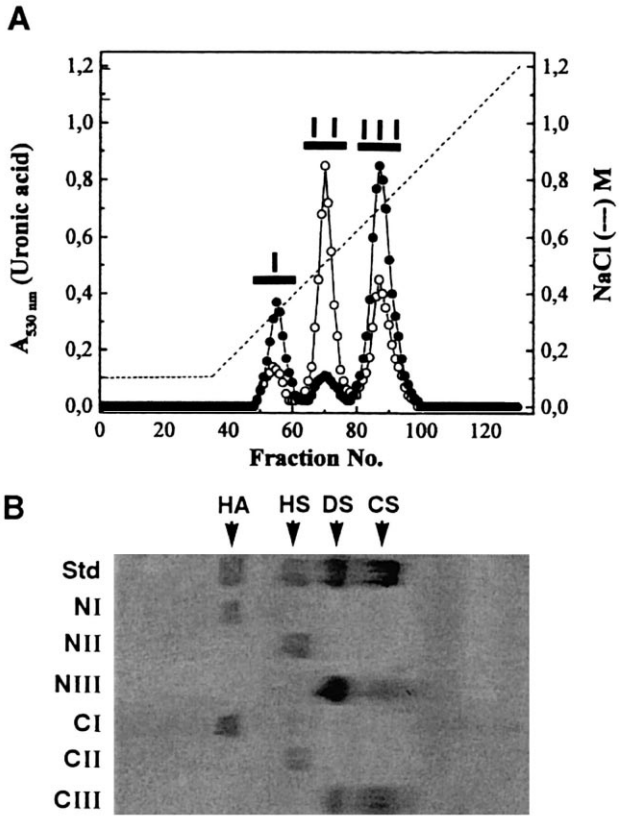

Fig. 1. (A) Fractionation of GAGs (3 mg of uronic acid) from normal pancreas (○) and pancreatic carcinoma (●) by anion-exchange chromatography on DEAE-Sephacel column (15×1.6 cm i.d.). The column was eluted stepwise with 3 vols. of 0.1 M NaCl and 10 vols. of a NaCl linear gradient ranging from 0.1 to 1.2 M NaCl. Fractions of 3.1 ml were collected and analyzed for uronic acid. (B) Electrophoresis of fractionated populations from normal pancreas (N) and pancreatic carcinoma (C), on cellulose acetate membranes using 0.1 M pyridine–0.1 M formic acid, pH 3.1. Std, standards of HA, HS, DS and CS.

amounts of HA. The increase of HA may correlate with poor differentiation and decreased survival rates in some human carcinomas [2]. The accumulation of GAGs, in particular, of HA and CS, in the extracellular matrix surrounding the tumor could play an important role for the remodeling and reconstruction of an environment which supports cellular proliferation and invasion via a hydrated GAG-rich pericellular matrix. On the other hand, the relative small proportion of DS (as percentage of total net increase of GalGAGs) could result in an altered extracellular matrix since L-iduronic acid residues of DS [26] are directly involved in the orderly arrangement of collagen fibrils [27]. The resulting less organized extracellular matrix may favor the infiltration of malignant cells [7,28]. HS, as reported previously, is the minor type of GAGs in pancreatic carcinoma. In a previous study [15], it has been also reported that a type of heparan sulfate proteoglycan (HSPG), in non-carcinomatous parenchyma of the pancreas is higher than that in pancreatic adenocarcinoma and the HSPG content of adenocarcinomatous parenchyma is closely correlated with its degree of differentiation and the clinical prognosis.

Electrophoresis of isolated GalGAGs from normal pancreas (Fig. 2A, inset) and pancreatic carcinoma (Fig. 2B, inset) on cellulose acetate membranes, before digestion with chondroitinase B gave two bands belonging to DS and CS. Electrophoresis of isolated GalGAGs after digestion with chondroitinase B gave only the CS band, revealing the degradation of DS band. Chromatography of isolated GalGAGs from normal pancreas on Sepharose CL-6B column resulted in a major peak with a partition coefficient (K_{av}) 0.5 (corresponding to M_r 33-kDa) and a broad incompletely resolved slowly eluting peak with a K_{av} 0.65 (corresponding to M_r 19-kDa) (Fig. 2A). Digestion of GalGAGs with chondroitinase B, a lyase which cleaves β(1–4)-galactosaminyl-iduroronic acid bonds, and subsequent chromatography on the same column showed that the peak of K_{av} 0.5 'disappeared' and the degradation's products (about 70% of total uronic acid) were eluted to the V_t of the column. The GalGAG chains in 'disappeared' peak were characterized as DS chains. The GalGAG chains in the chondroitinase B resistant peak of K_{av} 0.65 were characterized as CS chains. The GalGAG chains derived from the pancreatic carcinoma were chromatographed on Sepharose CL-6B (Fig. 2B) and eluted as two incompletely resolved but distinct peaks with K_{av} values of 0.58 (M_r 24 kDa) and 0.69 (M_r 17 kDa). Chromatography of pancreatic carcinoma-derived GalGAGs after digestion with chondroitinase B on the same column revealed that the peak of K_{av} 0.58 had disappeared and the products of degradation (18% of uronic acid) were eluted to the V_t of the column. The disappeared peak contained DS chains and the chondroitinase B-resistant material which eluted with K_{av} 0.69 represented the CS chains. These results indicated that the CS and DS chains presented in pancreatic carcinoma are of lower molecular size as compared with those derived

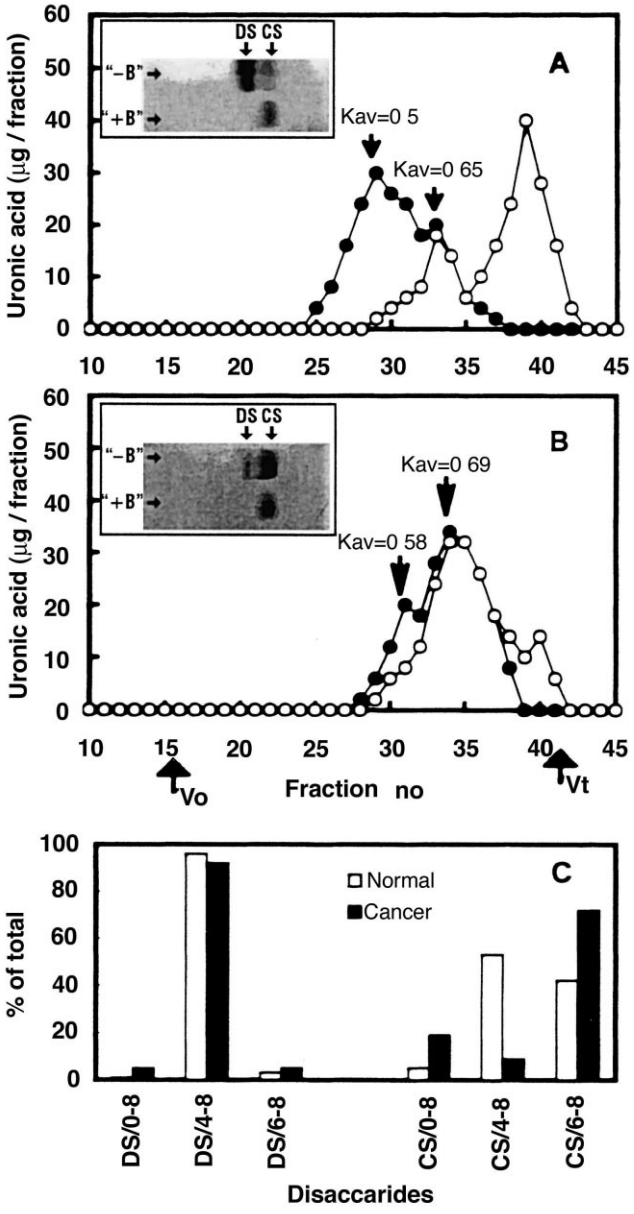

Fig. 2. Gel chromatography of GalGAGs isolated from normal pancreas (A) and pancreatic carcinoma (B) on Sepharose CL-6B (110×0.6 cm i.d.), before (●) and after (○) digestion with chondroitinase B. The column was eluted with 0.5 M sodium acetate buffer, pH 7.0. Fractions of 0.9 ml were collected and analyzed for uronic acid. Insets show the electrophoretic profiles of GalGAGs isolated from both tissues before and after digestion with chondroitinase B. Electrophoreses were performed on cellulose acetate membranes as mentioned above. The disaccharide composition of CS and DS isolated from both tissues (C) was obtained by HPLC analyses.
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from normal pancreas. In contrast, the HS chains from both tissues, normal and cancerous, showed identical chromatographic profiles having the same M_r 18-kDa (figure not shown).

The products of digestion derived by chondroitinase B (belonging to DS chains), which eluted to the V_t of the column, and the chondroitinase B-resistant materials (belonging to CS chains) were pooled, concentrated and digested with chondroitinase ABC. The obtained unsaturated disaccharides were analyzed by HPLC. The results on disaccharide composition in DS and CS chains are presented in Fig. 2C. In normal pancreas, the predominant disaccharide units in both DS and CS chains are the 4-sulfated disaccharides (95% and 58% of total disaccharides, respectively). In pancreatic carcinoma the sulfation pattern of DS was almost unaffected, but the sulfation pattern of CS was significantly altered. The results demonstrate that the CS chains in pancreatic carcinoma were composed predominantly of 6-sulfated disaccharides (72%), whereas the proportion of 4-sulfated disaccharides (9%) was significantly decreased. Furthermore, the proportion of non-sulfated disaccharides (19%) of CS in pancreatic carcinoma was markedly increased in comparison with that of normal pancreas (5%) (Fig. 2C).

The coexistence of non-sulfated with 6-sulfated disaccharides in this extent, suggests that in pancreatic carcinoma, the predominant type of GAG is an undersulfated CS, a more primitive species found in abundance in developing organs and in the stroma of various epithelial tumors [29]. It is noteworthy that similar tumor-associated changes in disaccharide composition and sulfation pattern are also observed in numerous carcinomas [9,11,12] with morphogenetic features similar to pancreatic carcinoma. Alterations in sulfation pattern of CS may well reflect modified biological roles of this molecule between normal and neoplastic tissues, since sulfation is a non-random and biosynthetically regulated process [30]. The lower molecular sizes and the more heterogeneity of the GalGAG chains of pancreatic carcinoma than those from normal tissue must be due, at least partly, to the presence of variable, but significantly increased proportions of non-sulfated disaccharides in GalGAG chains. Furthermore, the quantitative data revealed that the CS chains, that are

accumulated in the extracellular matrix of the pancreatic carcinoma are relatively smaller, but are much more numerous than their normal counterparts. The nature of proteoglycan (PG) involved in the accumulation of CS is unknown, but according to previous studies [11,29], the presence of versican and decorin is a likely candidate. The speculation that these PGs provide the core protein for CS chains remains to be studied.

In conclusion, the present study indicated that pancreatic carcinoma in comparison to the normal pancreas, contained much higher amounts of HA and CS chains which exhibited altered sulfation pattern and reduced molecular size. The present data provide further support to the concept that the high levels of HA correlate with poor differentiation and decreased survival rates in some human carcinomas, and HA is one of the key molecules implicated in the invasive growth of carcinomas. Furthermore, our results in pancreatic carcinoma support the concept that the CS accumulation may reflect a more general phenomenon associated with tumor growth and progression, since CS is markedly elevated in a variety of mesenchymal and epithelial neoplasms.

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