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Glycosylation of serum ribonuclease 1 indicates a major endothelial origin and reveals an increase in core fucosylation in pancreatic cancer

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Human pancreatic ribonuclease 1 (RNase 1) is a glycoprotein expressed mainly by the pancreas and also found in endothelial cells. The diagnosis of pancreatic cancer (PaC) remains difficult and therefore the search for sensitive and specific markers is required.

Previous studies showed that RNase 1 from human healthy pancreas contained only neutral glycans, whereas RNase 1 from PaC cell lines contained sialylated structures. To determine whether these glycan tumor cell-associated changes were also characteristic of serum RNase 1 and could be used as a marker of PaC, we have analyzed the glycosylation of serum RNase 1. The origin of serum RNase 1 was also investigated. Serum RNase 1 from two PaC patients and two controls was purified and the glycans analyzed by high-performance liquid chromatography (HPLC)-based sequencing and mass spectrometry. Although normal and tumor serum RNase 1 contained the same glycan structures, there was an increase of 40% in core fucosylation in the main sialylated biantennary glycans in the PaC serum RNase 1. This change in proportion would be indicative of a subset of tumor-associated glycoforms of RNase 1, which may provide a biomarker for PaC. Two-dimensional electrophoresis of the RNase 1 from several endothelial cell lines, EA.hy926, human umbilical vein endothelial cells (HUVEC), human mammary microvessel endothelial cells (HuMMEC), and human lung microvessel endothelial cells (HuLEC), showed basically the same pattern and was also very similar to that of serum RNase 1. RNase 1 from EA.hy926 was then purified and presented a glycosylation profile very similar to that from serum RNase 1, suggesting that endothelial cells are the main source of this enzyme.

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Introduction

Human ribonucleases have been widely studied in recent years because of their biological activity and possible therapeutic applications (Schein 1997). Our studies have focused on human pancreatic ribonuclease 1 (RNase 1), which is an enzyme that belongs to the RNase A superfamily and is the human counterpart of bovine RNase A (Sorrentino and Libonati 1997). RNase 1 has been isolated mainly from pancreas (Weickmann et al. 1981), which is the tissue with highest expression (Futami et al. 1997; Fernández-Salas et al. 2000), but it is also present in many different tissues and fluids such as serum, kidney, brain (Weickmann and Glitz 1982) and urine (Kurokawa et al. 1983). Recently, RNase 1 secretion by endothelial cells from different blood vessels including veins, arteries, and capillaries has been reported (Moenner et al. 1997; Landré et al. 2002).

In the 1970s, serum RNase 1 levels were proposed by many authors as a possible tumor marker of pancreatic cancer (PaC) (Reddi and Holland 1976; Maor and Mardiney 1978). However, further studies revealed that serum RNase 1 activity and concentration were neither sensitive nor specific enough for this purpose (Kurihara et al. 1984; Weickmann et al. 1984). In addition, increased levels of serum RNase 1 can be associated with age and different diseases such as pancreatitis and renal insufficiency (Weickmann et al. 1984).

The incidence of PaC in 2004 was estimated to be 58 700 cases in the European Union (the 12th incidence rank). Its mortality for the same year was estimated as 61 000 cases and it was sixth in the list of mortalities caused by cancer (Boyle and Ferlay 2005). PaC symptomatology is not clear and can be easily misinterpreted. Diagnosis is therefore difficult, and PaC is usually diagnosed late when it is already disseminated, making treatment difficult and contributing to the low survival rate (Saif 2006). The improvement of PaC diagnosis and, consequently, the implementation of more successful treatments require the identification of a sensitive and specific tumor marker.

One of the general features of tumor cells is the change in the glycosylation pattern of their cell surface glycoconjugates, which can also be reflected in the glycosylation of their secreted glycoproteins (Peracaula et al. 2003). Generally, *N*-glycans are more branched and more sialylated in tumors (Dennis et al. 1999; Gorelik et al. 2001). Lewis structures have been found to be overexpressed in carcinomas, and sialyl-Lewis^x (SLe^x) and sialyl-Lewis^a (SLe^a) have been

identified as tumor antigens (Dennis et al. 1999; Ørntoft and Vestergaard, 1999).

RNase 1 has three potential N-glycosylation sites at Asn-34, Asn-76, and Asn-88. Taking into account the well-established finding that glycosylation is altered in tumor cells and could also be tissue-specific, we investigated whether the glycosylation of pancreatic RNase 1 could be altered in tumor situations and whether these changes could be used to identify the tumor state. Previous results from our group showed significant differences in glycan structures of RNase 1 from healthy pancreatic tissues and pancreatic adenocarcinoma cell lines (Peracaula et al. 2003). The main differences were a decrease in fucosylation and an increase in sialylation in the RNase 1 of tumor origin, resulting in the presence of SLe^x and SLe^a detected only in the RNase 1 secreted from the tumor cell lines.

With the aim of evaluating possible glycan modifications on serum RNase 1 that could be indicative of tumors, serum RNase 1 from two PaC patients' sera and two control donors' sera was purified and characterized by glycan sequencing. The glycosylation analysis of serum RNase 1 revealed that there is a 40% increase in glycoforms that are core fucosylated in the RNase 1 from the serum of pancreatic adenocarcinoma patients, and this could be of clinical value for PaC. In addition, we have also investigated whether the serum RNase 1 comes mainly from the pancreas or from other tissues which could also supply RNase 1 to the blood system. Thus, RNase 1 from several endothelial cell lines (EA.hy926, HUVEC, HuLEC, and HuMMEC) was compared by two-dimensional electrophoresis (2-DE) and a very similar pattern was observed among all the endothelial samples. It was further compared with the serum RNase 1, and the 2-DE pattern was again very similar, in contrast to the 2-DE pattern of healthy pancreas RNase 1 that showed a completely different profile. RNase 1 from all these endothelial cell lines and from serum contains sialic acid, since most of these 2-DE RNase 1 spots were digested to more basic ones after a treatment with neuraminidase. Owing to the high similarity of the 2-DE pattern among the different endothelial cell lines, RNase 1 was purified from the endothelial cell line EA.hy926, and its glycans were also characterized. The comparison of the glycan structures of RNase 1 from sera and endothelial cells suggested that the main source of serum RNase 1 is the endothelium rather than the pancreas.

Results

Serum RNase 1 purification

To analyze the glycan structures of serum RNase 1 of tumor origin and compare them with the structures expressed in sera from healthy donors, a purification procedure to obtain pure RNase 1 from serum was established. RNase 1 from two PaC patients' and two control donors' sera was purified using several chromatographic steps. The results for the purification of the RNase 1 of PaC B are shown in Figure 1 and are representative of the other sera.

First, a Cibacron Blue chromatography was performed since it is recommended for nucleic acids binding proteins. RNase 1 binds weakly to Cibacron Blue and can be released from the column with low salt concentration (Figure 1A). The second

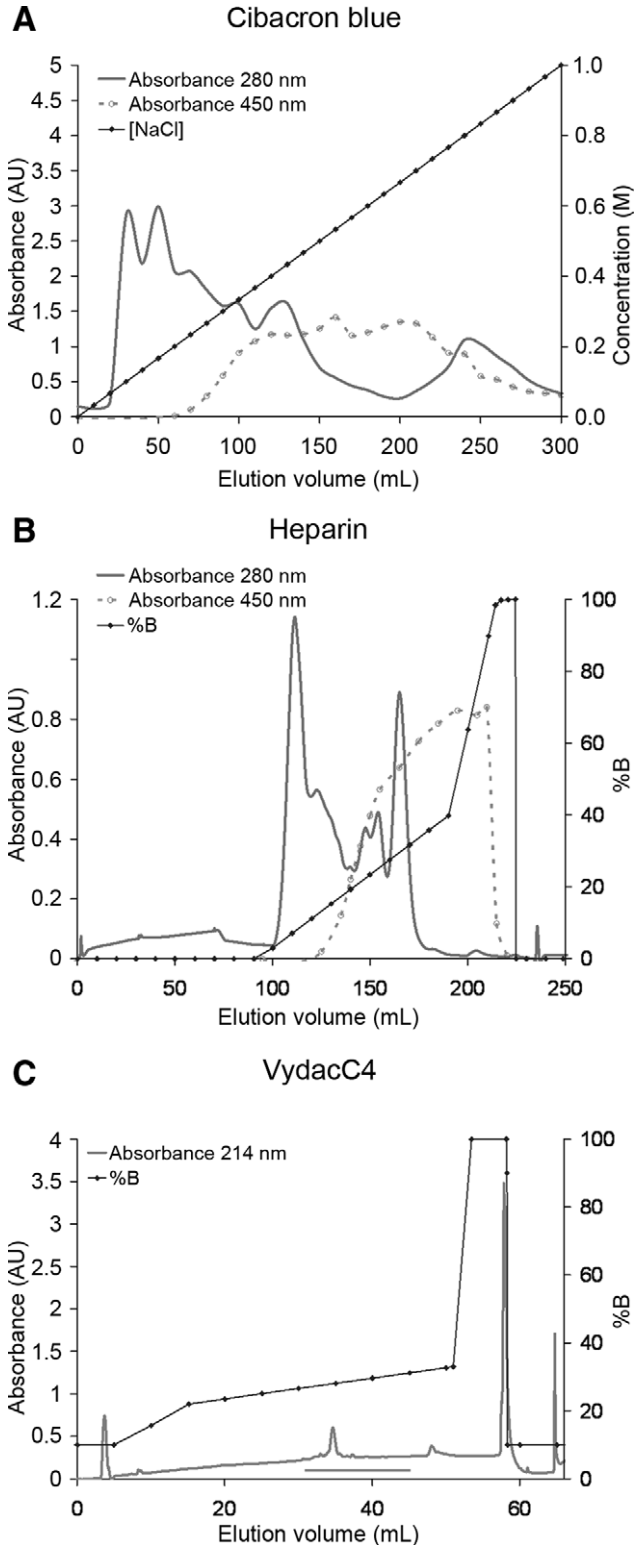


Fig. 1. Purification of serum RNase 1 from PaC B. (**A**) Cibacron Blue affinity chromatography: RNase 1 eluted with the saline gradient (25 mM Tris–HCl, 0.02–1 M NaCl, pH 7.5) and detected by sandwich ELISA (absorbance at 450 nm). (**B**) Heparin affinity chromatography: RNase 1 (absorbance at 450 nm) eluted between 20% and 60% of buffer B (25 mM sodium phosphate, 1.5 M NaCl, pH 6). (**C**) Reversed-phase chromatography: RNase 1 eluted between 27% and 31% of buffer B (0.1% trifluoroacetic acid in acetonitrile). Absorbance at 280 or 214 nm was indicative of the total protein content. The bar indicates the RNase 1-containing fractions.

chromatographic step was an affinity chromatography using heparin as adsorbent. Its polyanionic structure allows the purification of several proteins that interact with DNA or RNA and is thus recommended for RNase 1 purification since it interacts weakly with the heparin (Sorrentino et al. 1988) (Figure 1B). The last chromatographic step was a reversed phase chromatography (Figure 1C) in which RNase 1 elutes gradually from highly glycosylated fractions to the unglycosylated form as shown by zymography (Bravo et al. 1994) (Figure 2A). In sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), highly glycosylated ribonuclease fractions have a higher apparent molecular mass than the low glycosylated fractions, and the unglycosylated form migrates at 15 kDa. Earlier studies have shown that the more highly glycosylated RNase 1 fractions purified from pancreas correspond to the protein with three and two glycosylation sites occupied, whereas low glycosylated fractions correspond to the protein with only one glycosylation site occupied (Ribó et al. 1994). The different serum RNase 1 fractions shown in the zymography (Figure 2A) were pooled in four fractions according to their apparent molecular mass.

Glycan release and sequential oligosaccharide digestions of RNase 1 glycans from PaC patients' sera

Pooled glycosylated RNase 1 fractions from the PaC sera were electrophoresed in denaturing conditions and glycans were released from gel bands, corresponding to high, medium, and low glycosylated RNase 1 fractions, by in situ digestion with PNGase F (Figure 2B). Glycans were then fluorescently labeled with 2-aminobenzamide (2AB) and analyzed by normal-phase (NP)-HPLC in combination with exoglycosidase digestions and mass spectrometry (MS) analysis. In NP-HPLC, glycans were analyzed on the basis of their elution positions measured in glucose units (GU), using a dextran ladder to calibrate.

To determine whether the glycan pattern was dependent on the site occupancy of RNase 1, NP-HPLC was carried out with an aliquot of each of the different RNase 1 glycosylated fractions (high, medium, and low), before and after *Arthrobacter ureafaciens* sialidase (ABS) digestion (enzyme with broad specificity for α-linked sialic acids) (Figure 3). Each fraction showed a different glycan profile. The glycans of the higher molecular mass glycoforms had GUs from 6.5 to 13 and were found to contain both the glycans from the medium (GUs from 8 to 11) and low molecular mass glycoforms (GUs from 6.5 to 8.5) and some structures with higher GU (from 11 to 13) (Figure 3A). After ABS digestions, glycan structures obtained from the higher molecular mass glycoforms were again a combination of the glycans contained in the medium and low glycoforms, plus some structures with higher GUs (Figure 3B).

This pattern differs from those found in the RNase 1 glycans from healthy pancreas and from PaC cell lines. In both pancreas and pancreatic cell lines, RNase 1 glycans contained in low molecular mass glycoforms were present in all fractions and as the number of sites occupied increased, there was an increase in glycan structures of higher GU (Peracaula et al. 2003).

Glycans from high molecular mass glycoforms of RNase 1 from PaC A, which were representative of all the glycan structures present in each of the fractions of PaC A serum, were fully analyzed (Figure 4). Major glycans, A2G2S1, A2G2S2, FcA2G2S1, and FcA2G2S2, were biantennary, complex, sialylated structures (Figure 4A). Treatment with ABS (specific

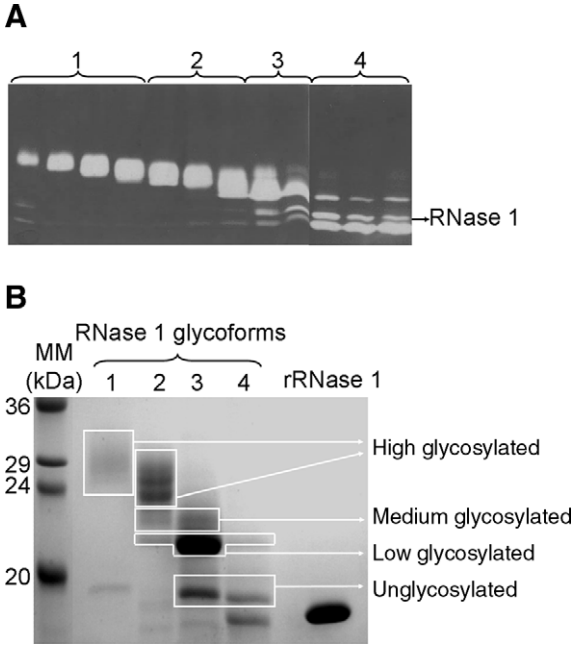


Fig. 2. Electrophoresis of RNase 1 pure fractions. (**A**) Zymography of the different RNase 1 fractions eluted from reversed-phase chromatography of RNase 1 from PaC B. RNase 1 fractions were pooled as indicated with numbers 1–4. The arrow indicates the unglycosylated RNase 1. (**B**) Coomassie Blue stained SDS–PAGE of the pooled fractions 1–4; 5 μg of recombinant unglycosylated RNase 1 (rRNase). MM, molecular weight markers. RNase 1 bands were excised from the gel (boxed) and separated as high, medium, low glycosylated, and unglycosylated RNase 1.

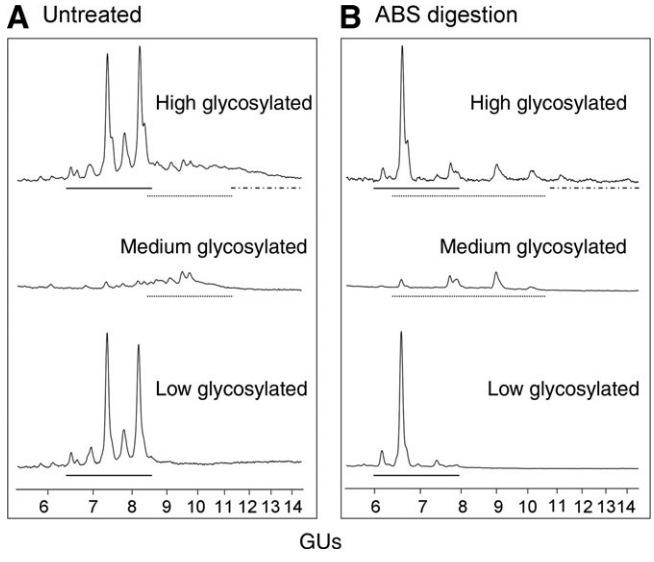


Fig. 3. NP-HPLC profiles of N-linked oligosaccharides obtained by in-gel digestion of the different glycosylated fractions of RNase 1 from PaC B. NP-HPLC profiles of (**A**) untreated oligosaccharides and (**B**) ABS-treated oligosaccharides. Structures present in the low glycosylated fraction are marked with a solid line, fractions present in the medium glycosylated fraction are marked with a dotted line, and fractions present only in the high glycosylated fraction are marked with a hyphen-dot line.

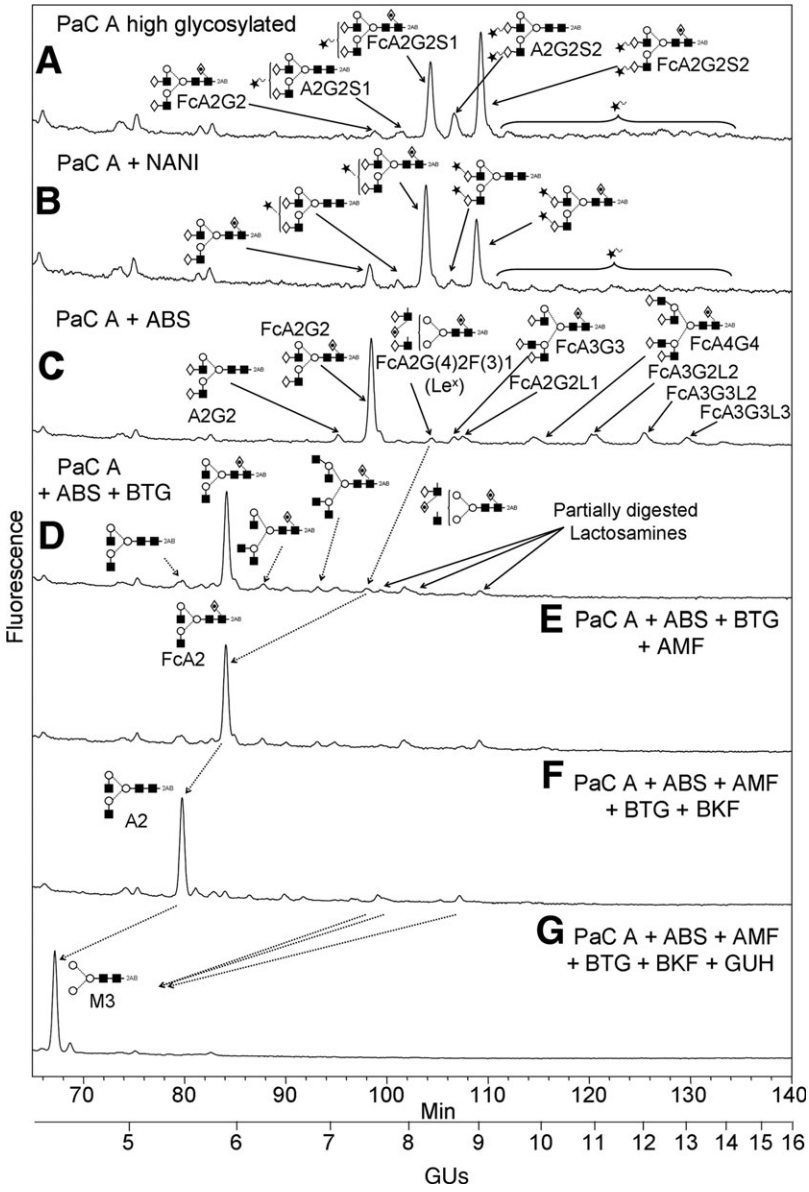


Fig. 4. Sequential exoglycosidase digestions of N-linked oligosaccharides obtained by in-gel digestion with PNGase F of the high glycosylated RNase 1 from PaC A. One aliquot was analyzed directly by NP-HPLC (**A**) and the remaining were treated with arrays of exoglycosidase enzymes prior to NP-HPLC analysis as indicated in each panel [(**B**)–(**G**)]. Dotted arrows indicate the shifts of the glycans digested by the subsequent enzyme array. A1-4 indicates the number of antennae linked to the trimannosyl core; G1-4 indicates the number of terminal galactose residues in the structure; G(4), galactose linked 1-4 to GlcNAc; Fc, fucose linked α1-6 to core GlcNAc; F(3), outer-arm fucose linked 1-3 to GlcNAc; M, mannose; S, sialic acid; and L, Lactosamine extension with the linkage Galβ1-4GlcNAc. Symbol representation of glycans for this and later figures: GlcNAc, filled square (■); mannose, open circle (○); galactose, open diamond (◇); fucose, open diamond with a dot inside (◈); sialic acid, filled star (★); beta linkage, solid line; alpha linkage, dotted line; unknown linkage, ∼; the linkage itself is indicated by the angle linking adjacent residues, thus 1-4-linkage, horizontal line (-); 1-3-linkage, angled line (/); 1-6-linkage, angled line (\); 1-2-linkage, vertical line (|).

enzyme for sialic acid linked α2-3,6) and *Streptococcus pneumoniae* neuraminidase (NANI; specific enzyme for sialic acid linked α2-3) digested these major glycans and the rest of the structures to glycans with lower GU, indicating that these glycans were all sialylated and presented both α2-3 and α2-6-linked sialic acid (Figure 4B and C). After complete sialic acid digestion, tri- and tetra-antennary glycans were detected in addition to the major biantennary structures.

The presence and linkages of galactoses and outer arm fucoses in these structures were also determined. There was no difference between the digestion with bovine testes β-galactosidase (BTG; specific activity for β1-3/4 > 6-linked galactose) and *Streptococcus pneumoniae* galactosidase (SPG; specific for galactose linked β1-4), indicating that all galactoses were linked β1-4 (data not shown). Digestion with almond meal α-fucosidase (AMF; specific activity for α1-3 and α1-4-linked fucose) showed a biantennary structure with an outer arm Fuc linked α1-3 forming the antigen Lewis^x (Le^x) (Figure 4D and E), which would have come from a structure containing the antigen SLe^x. *Xanthomonas manihotis* α1-2 fucosidase (XMF; specific activity for α1-2 fucose) did not show any outer arm fucose linked α1-2

(data not shown). The presence of poly-*N*-acetyllactosamines was demonstrated by the combination of ABS, BTG, bovine kidney fucosidase (BKF), and glucosaminidase (GuH; broad activity for *N*-acetylglucosamine), which digested the glycans to the trimannosyl chitobiose core (M3) (Figure 4F and G). No hybrid or high mannose structures were detected.

RNase 1 from the two PaC serum samples (PaC A and PaC B) presented the same general NP-HPLC profiles and glycan structures (Table I). More complex *N*-glycans with poly-*N*-acetyllactosamine extensions were found in PaC A after the glycans were treated with ABS (Figure 4C).

MALDI, ESI, and MS-ESI-LC analyses of RNase 1 glycans from PaC patient serum

Matrix-assisted laser desorption/ionization (MALDI) and negative ion nanospray MS/MS (Harvey 2005) analysis of the pool of high, medium, and low glycosylated fractions from serum sample PaC B was carried out after ABS treatment (Table I). Structures were consistent with the HPLC data, being mainly fucosylated bi-, tri-, and tetra-antennary complex glycans with poly-*N*-acetyllactosamine extensions. Tri-antennary glycans were mainly branched on the 3-antenna but traces of 6-branched compounds were also detected. All fucosylated glycans contained a fucose residue at the 6-position of the reducing-terminal GlcNAc (Harvey 2005), and three compounds were found with additional fucosylation in one of the antennae, corresponding to glycans containing the Le^x antigen. The second fucose in the difucosylated structure appears to be mainly on the 3-antenna as determined by electrospray (ESI)-MS/MS but there could also be a small amount on the 6-antenna. MS/MS spectra of the other difucosylated compounds were too weak to determine the location of the antenna-linked fucose.

The major ion masses found using ESI-LC/MS (PaC B-pooled fractions) were 966.8, 1039.9, 1112.4, and 1185.4, with compositions $Hex_5HexNAc_4Neu5Ac_1$, $Hex_5HexNAc_4Fuc_1Neu5Ac_1$, $Hex_5HexNAc_4Neu5Ac_2$, and $Hex_5HexNAc_4Fuc_1Neu5Ac_2$ corresponding to the main sialylated biantennary structures described earlier (Table I).

A summary of the glycan structures of PaC B before and after ABS digestion is shown in Table I.

Sequential oligosaccharide digestions of RNase 1 glycans from control donor serum

Serum samples from two healthy donors were also analyzed (Control A and B). The amount of RNase 1 in control sera was lower than that from the cancer sera. Therefore, all the purified glycosylated RNase 1 fractions were pooled for the glycan analysis of each control. RNase 1 oligosaccharides from these control samples were released from band gels by in situ digestion with PNGase F and fluorescently labeled with 2AB, as described earlier.

The glycan profiles of these controls, obtained before and after ABS and BTG digestions (Figure 5A and C), show the presence of the same glycan structures as in the RNase 1 from cancer patients' sera but with a significantly lower proportion (40%) of core fucosylated structures in comparison with glycans from both high and low glycosylated RNase 1 fractions from PaC sera. Sequential oligosaccharide digestions confirmed the presence of sialylated complex glycans, with A2G2S1 and A2G2S2 with and without core fucosylation as the predominant structures. The analysis after the digestion with ABS + BTG shows the poly-*N*-acetyllactosamine structure with the lowest GU that was present in the glycans from RNase 1 from cancer patients' sera. Larger poly-*N*-acetyllactosamines were not detectable. The structure containing the Le^x antigen and FcA3 and FcA4 structures were also detected, as for the RNase 1 glycans from PaC patient sera (Figure 5C).

2-DE of partially purified RNase 1 from healthy pancreas, serum samples, and endothelial cell lines conditioned media

HUVEC, HuMMEC, and HuLEC were cultured in Dulbecco's Modified Eagle Media (DMEM), 20–30% fetal bovine serum (FBS) (Moenner et al. 1997), and the conditioned media was enriched in RNase 1 by two affinity chromatographies to Cibacron Blue and heparin, as described for serum samples.

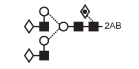
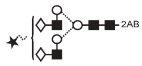
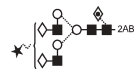
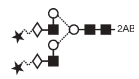
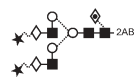
Partially purified RNase 1 from EA.hy926-conditioned media and from the serum sample PaC A was obtained also after Cibacron Blue and heparin affinity chromatographies and used for the 2-DE.

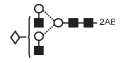
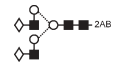
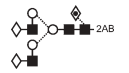
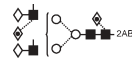
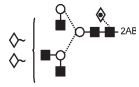
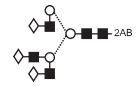
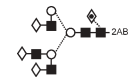
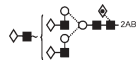
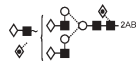
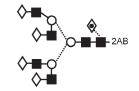
RNase 1 from healthy pancreas was obtained by acid extraction and acetone precipitation as described in Peracaula et al. (2003).

After 2-DE electrophoresis, gels were transferred to polyvinylidene difluoride (PVDF) membranes, and RNase 1 was detected using specific antibodies. The 2-DE patterns obtained (Figure 6) for serum samples and endothelial cell line media were highly similar and different from the pattern obtained for healthy pancreas RNase 1. The different spots could be pooled in seven groups according to their pIs: 7.15 ± 0.04 (group a), 7.48 ± 0.05 (group b), 7.71 ± 0.04 (group c), 7.98 ± 0.05 (group d), 8.20 ± 0.07 (group e), 8.37 ± 0.03 (group f), and 8.44 ± 0.01 (group g). Although serum and endothelial cell line samples presented a similar pattern, in the case of endothelial cell lines, the higher Mw and low pI forms appear in higher proportion than in serum samples. The last group of spots (group g), which appear to be neutral forms, were not detected in endothelial cell line media RNase 1, whereas it is the only group present in healthy pancreas RNase 1 ($pI = 8.43$). Digestion of endothelial cell line RNase 1 with ABS shows that, in all cases, all the spots (from a to f) contain sialic acid as they all move to the neutral forms at pI 8.44 (Figure 6). Digestion with PNGase F moves all the spots to pI 8.42 with an Mw of 15 kDa, which indicates that all of them contain N-linked oligosaccharides (data not shown). Thus, RNase 1 glycoforms with pIs closer to 8.2–8.4 (groups e and f) represent RNase 1 glycoforms with lower sialic acid content. The higher the sialic acid content, the lower the pI becomes. Taking into account that the higher molecular mass spots correspond to the RNase 1 with more glycosylation sites occupied, the spots with lower pI and higher molecular mass (groups a and b) could be attributed to the heavily sialylated RNase glycoforms with all the N-glycosylation sites occupied.

The high similarity among the 2-DE patterns of all the endothelial cell lines suggests that they contain the same RNase 1 forms, including the sialylated N-linked glycoforms. Thus, EA.hy926 can be representative of the endothelial cell lines and can be used as a source for purifying the RNase 1 to characterize in detail the RNase 1 glycan structures.

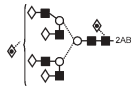
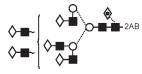
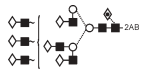
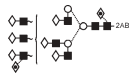
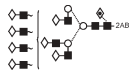
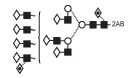
Table I. Glycans from RNase 1 from PaC B

GU	Structure[a]	ESI MS m/z			Composition[b]				Structure
		Found	Calculated	Ions	H	N	dH	S	
Undigested									
7.53	FcA2G2	ND	ND		ND	ND	ND	ND	
7.96	A2G2S1	966.8	966.8	$[M+2H]^{2+}$	5	4	0	1	
8.39	FcA2G2S1	1039.9 1050.9 1061.9 1072.8	1039.9 1050.9 1061.9 1072.9	$[M+2H]^{2+}$ $[M+H+Na]^{2+}$ $[M+2Na]^{2+}$ Na salt$[M+2Na]^{2+}$	5	4	1	1	
8.82	A2G2S2	1112.4	1112.4	$[M+2H]^{2+}$	5	4	0	2	
9.23	FcA2G2S2	1185.4 1196.4 1207.4 1218.4 1229.4	1185.4 1196.4 1207.4 1218.4 1229.4	$[M+2H]^{2+}$ $[M+H+Na]^{2+}$ $[M+2Na]^{2+}$ Na salt $[M+2Na]^{2+}$ Na_2 salt $[M+2Na]^{2+}$	5	4	1	2	

GU	Structure[a]	MALDI m/z ([M + Na]+)		ESI m/z ([M + H2PO4]−)		Composition[b]				Structure
		Found	Calculated	Found	Calculated	H	N	dH	S	
Digested after ABS treatment										
6.33	A2G1	1647.6	1647.6	1721.6	1721.6	4	4	1	—	
7.10	A2G2	1663.6	1663.6	1737.6	1737.6	5	4	0	—	
7.51	FcA2G2	1809.6	1809.6	1883.6	1883.6	5	4	1	—	
8.32	FcA2G2F(3)1	1955.7	1955.7	2029.6	2029.7	5	4	2	—	
ND	*FcA3G2*[c]	2012.7	2012.7	2086.6	2086.6	5	5	1	—	
ND	*A3G3*[c]	2028.7	2028.7	—	2102.7	6	5	0	—	
8.63	FcA3G3	2174.7	2174.8	2248.8	2248.8	6	5	1	—	
8.76	FcA2G2L1	2174.7	2174.8	2248.8	2248.8	6	5	1	—	
ND	*FcA2G2L1F(3)1*[c]	2319.7	2320.8	—	2394.8	6	5	2	—	
9.98	FcA4G4	2539.6	2539.9	2613.9	2613.9	7	6	1	—	
10.00	FcA3G3L1	2539.6	2539.9	2613.9	2613.9	7	6	1	—	

Continued

Table I. *Continued*

GU	Structure[a]	MALDI m/z ([M + Na]+)		ESI m/z ([M + H2PO4]−)		Composition[b]				Structure
		Found	Calculated	Found	Calculated	H	N	dH	S	
10.97	FcA4G4F	2686.4	2686.0	—	2759.9	7	6	2	—	
12.02	FcA3G3L2	2904.6	2905.0	2979.0	2979.0	8	7	1	—	
ND	*FcA3G3L3*[c]	3269.8	3270.2	3344.1	3344.1	9	8	1	—	
ND	*FcA3G3L3F(3)1*[c]	3416.3	3416.2	—	3490.2	9	8	2	—	
ND	*FcA3G3L4*[c]	3635.7	3635.3	—	3709.3	10	9	0	—	
ND	*FcA3G3L4F(3)1*[c]	3779.6	3781.4	—	3855.3	10	9	2	—	

[a]For key to structure abbreviation, see the caption of Figure 4.
[b]Key to ionic compositions: H, hexose; N, HexNAc; dH, deoxyhexose (fucose); S, sialic acid.
[c]Example of possible structures.
ND, not detected by NP-HPLC, MALDI-TOF MS or ESI MS, as indicated.

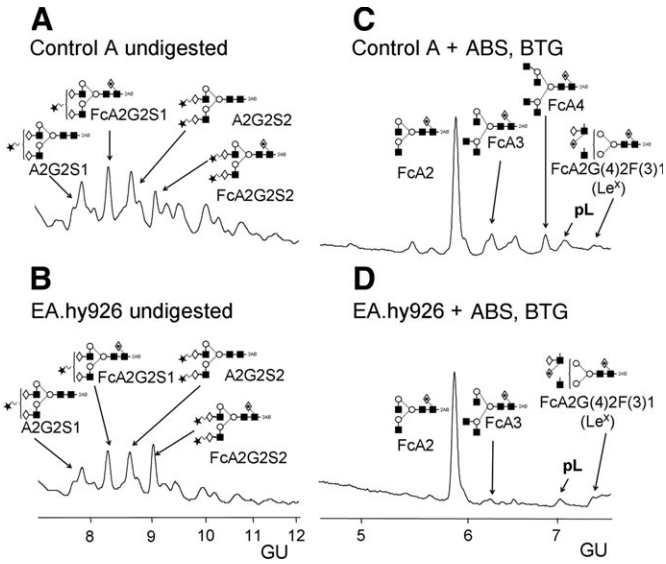


Fig. 5. NP-HPLC profiles of N-linked oligosaccharides obtained by in-gel digestion of RNase 1 from control serum A [(**A**) and (**C**)] and RNase 1 from EA.hy926 [(**B**) and (**D**)]. NP-HPLC profile of untreated oligosaccharides of control serum and EA.hy926 [(A) and (B)]. NP-HPLC profile of ABS + BTG-treated oligosaccharides of control serum and EA.hy926 [(C) and (D)]. pL indicates the peaks corresponding to partially digested poly-*N*-acetyllactosamines. Please refer to the caption of Figure 4 for key to structure abbreviations.

RNase 1 purification and sequential oligosaccharide digestions of RNase 1 glycans from EA.hy926 conditioned media

EA.hy926 were cultured in DMEM 10% FBS to confluence, and RNase 1 was purified from the conditioned media after removing the FBS to avoid the presence of bovine ribonuclease. RNase 1 was concentrated and purified following the same chromatographic steps as for RNase 1 from serum samples. High-, medium-, and low-molecular mass glycoforms showed a similar electrophoretic pattern than those from serum RNase 1 (data not shown). Oligosaccharides from these fractions were released and labeled as before and pooled for glycan analysis.

The glycan profile obtained was very similar to that of the serum samples (Figure 7). The main glycan structures were also sialylated, biantennary, complex glycans, A2G2S1 and A2G2S2, with and without core fucosylation (Figure 5B). The proportion of the core fucosylated, biantennary glycans was the same as for the control serum samples (Figure 7). Digestions with ABS and BTG confirmed the presence of these structures. Again, only the lowest GU poly-*N*-acetyllactosamine structure could be detected (Figure 5D). Larger poly-*N*-acetyllactosamines were again not detectable probably due to the low amount of sample. Glycans containing the Le^x antigen and the FcA3 structure were also present. However, the FcA4 glycan detected in the serum RNase 1 was not detected in the EA.hy926 RNase 1 (Figure 5D).

The same major glycan structures were present in RNase 1 obtained from both the normal and tumor serum samples and from the conditioned medium of EA.hy926 cells, although they were detected in different proportions. Figure 7 shows that the proportion of core fucosylated forms (FcA2G2S1

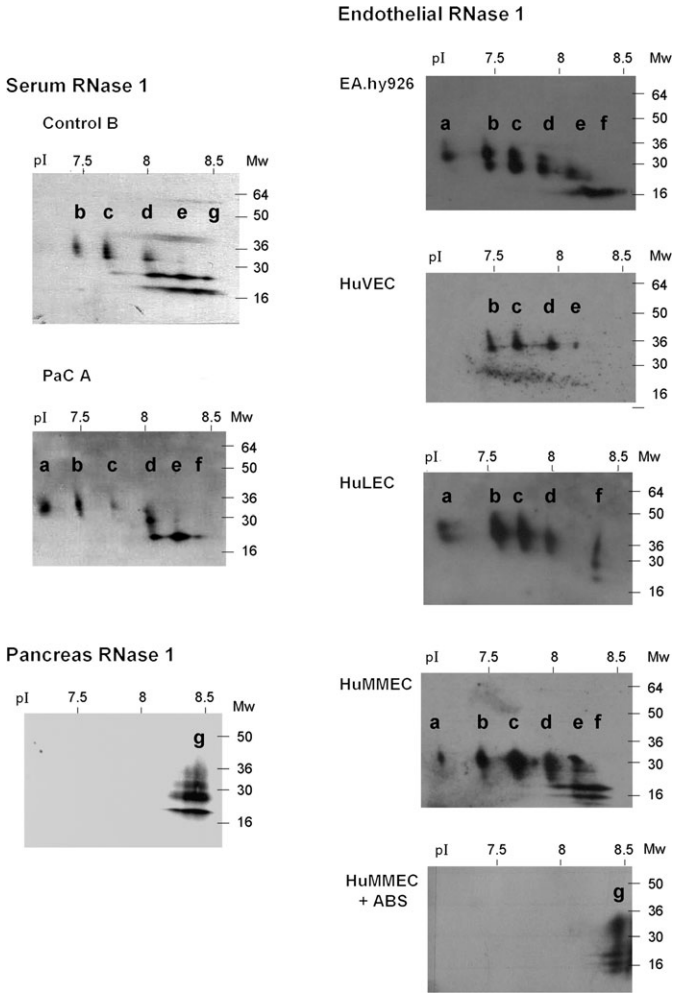


Fig. 6. 2-DE of a partially purified RNase 1 from healthy pancreas, a control serum (control B), a PaC patient serum (PaC A), and four different endothelial cell line-conditioned media. The neuraminidase digestion with ABS of one endothelial cell line-conditioned media (HuMMEC) is also shown. The 2-DE gels were transferred to PVDF membranes, and RNase 1 was detected using specific antibodies. The different RNase 1 forms detected are pooled according to their pIs (labeled a–g).

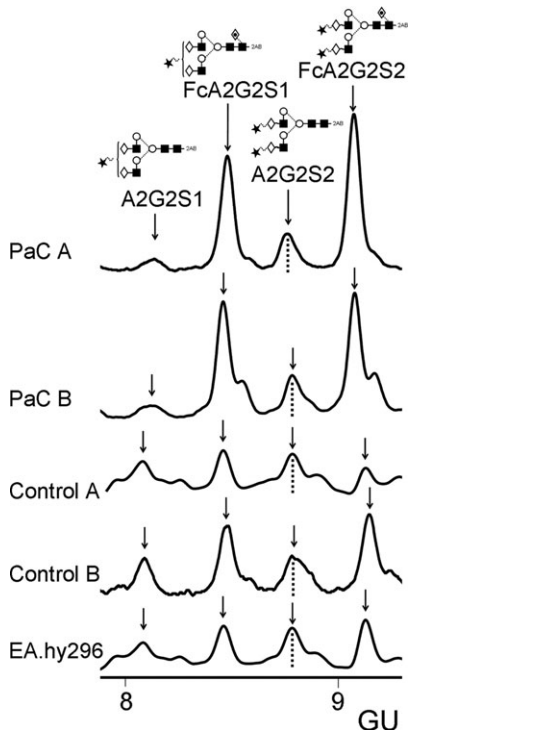


Fig. 7. NP-HPLC profiles of N-linked oligosaccharides obtained by in-gel digestion of RNase 1 from PaC serum A (high glycosylated form), PaC serum B (high glycosylated form), control serum A, control serum B, and endothelial cell line EA.hy926. The profiles are scaled against the peak marked with a dotted line. Please refer to the caption of Figure 4 for key to structure abbreviations.

and FcA2G2S2) was higher in the PaC serum samples than in control and EA.hy926 samples and that the proportions of the glycans from the control and the EA.hy936 RNase 1 were very similar.

Discussion

The study of serum RNase 1 as a possible indicator of pancreatic diseases has its basis in the fact that secreted pancreatic enzymes, such as RNase 1, can reach the blood stream through entheropancreatic recirculation (Rothman et al. 2002). As the pancreas has been described as the main organ expressing RNase 1 (Futami et al. 1997), it has been assumed that serum RNase 1 originates mainly from pancreas. Alterations in glycan processing are associated with tumor phenotype and tumor progression (Hakomori 1985). We therefore focused on the analysis of the glycosylation pattern of serum RNase 1 in normal and tumor states of the pancreas to investigate whether differences in the glycan processing of this enzyme in the tumor cells could be indicative of the disease.

In a previous work, we analyzed the glycans from RNase 1 secreted by pancreatic tumor cell lines and compared them with those of the RNase 1 from pancreas from healthy controls. Some of the glycans from the tumor cell lines contained the sialyl-Lewis^x (SLe^x) and sialyl-Lewis^a (SLe^a) antigens, which have been shown to be involved in the process of adhesion and metastasis of the tumor cells (Kannagi et al. 2004). In contrast, RNase 1 glycans from healthy pancreas were all neutral and exhibited fucosylated structures such as the Le^y antigen. The sugars mainly consisted of biantennary structures, together with some tri- and tetra-antennary ones and presented poly-*N*-acetyllactosamine extensions in the higher molecular weight RNase 1 fractions (Peracaula et al. 2003).

These significant glycosylation differences between RNase 1 from tumor cells and that from normal pancreas prompted us to compare the glycan moiety of RNase 1 obtained from the sera of PaC patients and healthy controls. Considering first the possible pancreatic origin of the serum RNase, we postulated that aberrantly glycosylated RNase 1 secreted by a pancreatic tumor should be readily detectable in the circulation. From this hypothesis, our objective was to identify a new possible marker that could be useful for the detection of PaC.

Glycan analysis of serum RNase 1

In this study, serum RNase 1 oligosaccharides from patients with pancreatic carcinoma and that from healthy controls have been compared. Although the same glycans were

detectable in healthy and tumor sera RNase 1, there was a notable increase (40%) in core fucosylation in the sialylated biantennary glycans from the PaC serum RNase 1. The proportion of noncore fucose:core fucose was 1:1.3 for control serum RNase 1 glycans, whereas it was 1:4 for PaC serum RNase 1 glycans. This increase could be explained by the tumor secreting more core fucosylated glycoforms of RNase 1, which could be detected in the serum. This difference in proportion of fucosylation could be ascribed to upregulation of the α-1,6-fucosyltransferase (FUT8) in the tumor cells (Miyoshi et al. 1999), the substrate availability (Noda et al. 2003) and/or modifications in the Golgi apparatus organization (Kellokumpu et al. 2002).

Serum RNase 1 from both normal and tumor origin contains completely different glycan structures from the healthy pancreas RNase 1. Glycan structures found in serum RNase 1 are all sialylated and contain very little outer arm fucose, whereas RNase 1 from healthy pancreas contains only neutral structures and heavily fucosylated glycans with core and outer arm fucoses (Peracaula et al. 2003). 2-DE analysis of pancreas and serum RNase 1 also revealed a completely different pattern between serum and pancreas RNase 1. Several sialylated RNase 1 glycoforms were detected in serum RNase 1, whereas neutral forms were the only ones detected in healthy pancreas RNase1, which is consistent with the sequencing results.

Glycan analysis of endothelial RNase 1

Considering that the glycosylation moiety of secreted and cellular surface glycoproteins usually reflects the organ origin (Yamashita et al. 1986; Kobata 2000), the results presented here indicate that serum RNase 1, at least in the healthy state, contains very little RNase 1 from pancreatic origin. The pancreatic origin of serum RNase 1 has always been controversial, as it has been described that serum RNase 1 levels remain elevated after surgical resection of the pancreas (Peterson 1979), suggesting that RNase 1 was predominantly released in the blood stream from other organs, a hypothesis strongly supported by our present results.

With the aim of elucidating the cellular and tissue origins of serum RNase 1, we studied the glycosylation pattern of the RNase 1 produced by endothelial cells of vascular origin. RNase 1 expressed by the immortalized endothelial cell line EA.hy926, a hybrid formed by fusing HUVEC with A549/8 carcinoma cells (Edgell et al. 1983), has been purified and its carbohydrate structures analyzed by glycan sequencing. The glycan structures corresponded to those of the serum RNase 1 in proportions very similar to those in the control samples (Figure 7).

These results suggest an endothelial origin of serum RNase 1. Easy access to the blood flow of RNase 1 secreted by the endothelial cells and the important surface of the blood vessel lining (approximately 1000 m^2) in an adult human body suggest that this tissue can significantly contribute to the serum levels of RNase 1. This possibility was previously suggested by Landré et al. (2002), who described the expression by EA.hy926 cells of three different forms of RNase 1 with molecular masses 18, 26, and 28 kDa, which were due to variable site occupancy. In their study, RNase 1 expressed by EA.hy926 cells was demonstrated to come from their parental endothelial cells (HUVEC) rather than from the A549/8 carcinoma cells hybrid. Also, the molecular heterogeneity and enzymatic activity of the RNase 1 secreted by EA.hy926 was shown to be identical to the RNase 1 secreted by HUVEC and several other endothelial cell lines from different vessel and tissue origins (Landré et al. 2002). In the present study, we have also found a highly similar RNase 1 2-DE pattern among all the endothelial cell lines, suggesting that they all contain the same RNase 1 forms, which corresponded to sialylated glycoforms since they all were digested with neuraminidase to neutral RNase 1. EA.hy926 is, thus, a representative cell line from which to purify and characterize in detail the RNase 1 glycans. Therefore, the high similarity of the *N*-glycans of EA.hy926 RNase1 with those of serum RNase 1 strongly suggests that serum RNase 1 comes mainly from the endothelium.

The oligosaccharides found in this study for serum RNase 1 corresponded to some of the structures described by Hitoi et al. (1987) for urinary secretory RNase (U_L) from healthy donors. These were neutral and mono-sialylated, complex, nonbisected, mono-, bi-, tri-, and tetra-antennary glycans, with and without core fucose. Poly-*N*-acetyllactosamine structures were also observed but no outer arm fucoses or GalNAc structures (Hitoi et al. 1987). Although the amino acid sequence of U_L RNase matched with that of pancreatic RNase 1, some authors doubted the pancreatic origin of U_L RNase owing to differences in its oligosaccharide composition compared with pancreatic RNase 1 (Yamashita et al. 1986; Hitoi et al. 1987). The data presented here, which show a high similarity between serum RNase 1 and U_L RNase 1 glycan moieties, support the finding that the serum RNase 1 released to the blood stream by endothelial cells would be partially excreted through the kidney and would represent a source of urinary RNase.

Changes in glycosylation in serum RNase 1

The increase in core fucosylation described for the glycans from the PaC serum RNase 1 could be due to more than one mechanism or a combination of some of them. Our results suggest that serum RNase 1 comes mainly from the endothelium. Thus, a new hypothesis should also be considered for which endothelial cells could be responsible for the alteration of the glycans of serum RNase 1 in PaC. PaC tumor cells could be producing some factors which, once released in their surroundings or the blood stream, could alter the glycosylation machinery in the endothelial cells. It has been proposed that PaC cells could induce the production of fucosylated haptoglobin from the liver in contrast to nonfucosylated haptoglobin secreted by the liver in healthy situation (Okuyama et al. 2006).

On the other hand, although RNase 1 from pancreas would probably represent only a small percentage of serum RNase 1, the release into the circulatory system of an aberrantly glycosylated RNase 1 by tumor cells during development of PaC could also be considered. Altered metabolic pathways in tumor cells could modify the glycosylation of RNase 1 before its release into the blood stream, and, although less likely, modification of the glycans after secretion in the blood plasma cannot be discounted either.

Sialylation and defucosylation of plasma glycoproteins during the acute phase response has been demonstrated in mice (Chavan et al. 2005). It is not clear how these modifications can occur, but an in situ modification of plasma

glycoproteins could explain a change in the glycans of RNase 1 from healthy pancreas, which would become less fucosylated and more sialylated. Sialyltransferases have been found in human serum in different physiological conditions (Berge et al. 1982). Sialylation of glycoproteins increases their half-lives in serum by reducing their clearance by the asialo-proteins receptor (ASGP-R) in the liver (Pricer and Ashwell 1971), an event which depends on the presence of terminal galactose residues that can bind the ASGP-R (Takamatsu et al. 2003). Glycoproteins containing α2-6 linked sialic acid are eliminated from the serum more rapidly than those glycoproteins containing sialic acid linked α2-3 (Park et al. 2005). Thus, the possibility that the pancreatic RNase 1 becomes more sialylated after its release from the pancreas cannot be excluded and could contribute to our finding that the serum RNase 1 contains sialic acid.

Conclusion

We have demonstrated that RNase 1 from EA.hy926 has a glycosylation profile very similar to that of serum RNase 1 in healthy individuals. In addition, the 2-DE analysis of RNase 1 from several endothelial cell lines and serum samples has shown a similar pattern among all of them and very different from the 2-DE pattern from healthy pancreas RNase 1. All these data strongly suggest that endothelial cells are the main source of RNase 1 in serum. We also compared the glycosylation of serum RNase 1 derived from PaC patients and healthy controls. There was a remarkable increase (40%) in core fucosylated biantennary glycans in the PaC serum RNase 1. These data suggest that there is a subset of tumor-associated glycoforms of RNase 1.

Altered core fucosylation has also been found in tumors (Ito et al. 2003) and in their secreted serum glycoproteins (Block et al. 2005), demonstrating that alterations in glycosylation pathways would be expected to affect the processing of many secreted and cell surface glycoproteins. In addition, tumors could release factors that induce changes in the glycosylation machinery of their neighboring endothelial cells, affecting also the glycan moiety of their glycoconjugates.

These glycosylation changes could be exploited to develop new markers for disease. Therefore, other methodologies are currently being developed to examine the different subforms of serum RNase 1 from more PaC patients to evaluate their usefulness as PaC markers.

Materials and methods

RNase 1 purification from serum

Sera from two PaC patients and two healthy donors were from the Hospital Universitari Dr J. Trueta (Girona, Spain), and its Ethics Committee approved their use. Patients were diagnosed for pancreatic adenocarcinoma with vascular invasion, by biopsy or image examination by the Digestive and Pathology Units. PaC patient staging was stage IVA (PaC A) and stage II–III (PaC B) according to Tumour Node Metastasis (TNM) classification. RNase 1 concentration was quantified using a sandwich enzyme-linked immunosorbent assay (ELISA). The concentrations were 2580 ng/mL for PaC A, 7370 ng/mL for PaC B, 1500 ng/mL for Control A, and 4640 ng/mL for Control B. Serum was stored at − 80 °C until use.

Serum RNase 1 was purified by three chromatographic steps. The first chromatography was a Cibacron Blue 3GA column (Sigma, St Louis, MO), which was carried out as described previously (Peracaula, Tabares, et al. 2003). Ribonuclease fractions were detected by sandwich ELISA and dialysed against the equilibration buffer of the next chromatography. HiTrapTM Heparin column (Amersham Pharmacia, Little Chalfont, UK) was performed as described previously (Peracaula et al. 2003), but changing the chromatography buffer for 25 mM Na-phosphate pH 6. Ribonuclease fractions were dialysed against milliQ water and freeze-dried. Finally, Vydac C4 (Vydac, Hesperia, CA) reversed-phase chromatography was carried out as described previously (Ribó et al. 1994).

Ribonuclease activity and sample purity were checked by zymography (Bravo et al. 1994) and by SDS–PAGE with silver staining (Blum et al. 1987), respectively.

RNase 1 quantification by sandwich ELISA

Two different polyclonal antibodies against human recombinant RNase 1 were used: polyclonal rabbit antibodies (Peracaula et al. 2000) and polyclonal chicken antibodies against human recombinant RNase 1, which were produced and purified from egg yolk (Gassmann et al. 1990). Peroxidase-conjugated donkey antichicken IgY (IgG) was from ImmunoResearch Laboratories, Inc. (West Grove, PA).

Rabbit antibodies against RNase 1 dissolved at 1.4 μg/mL in Coating Buffer (Scil Diagnostics GmbH, Viernheim, Germany) were bound for 1 h at 37 °C to 96-well polystyrene plates. After washing three times with washing buffer (0.9% NaCl solution, 0.05% Tween), plates were blocked with 1% nonfat milk, 0.05% (v/v) Tween in phosphate-buffered saline (PBS) for 1 h at 37 °C. The wells were then washed and were incubated for 1 h at 37 °C with samples containing RNase 1, diluted in PBS, 0.05% Tween. Plates were washed and incubated with chicken polyclonal antibodies against RNase 1 diluted 1:400 in PBS, 0.05% Tween for 1 h at 37 °C. Secondary antibody, peroxidase-conjugated donkey antichicken 1:4000 in PBS, 0.05% Tween, was added and allowed to stand for 1 h at 37 °C. Plates were washed and colorimetric detection was carried out using 100 μL/well of BluePeroxidase substrate soluble (Roche, Basel, Switzerland). Absorbance was read at 450 nm with a reference of 620 nm in an automated microplate reader (BIO-TEK, Winooski, VT) after stopping the reaction with 100 μL/well of 0.25 M H_2SO_4. Negative controls were wells without antigen, without the first antibody, with preimmunized chicken antibodies, and wells with lysozyme as antigen. To quantify the RNase 1 amounts, a recombinant RNase 1 (kindly donated by Dr Seno) was used to perform a curve between 0.1 and 10 ng/mL.

RNase 1 purification from conditioned cell media

EA.hy926 cells (Edgell et al. 1983) were grown in 150 cm^2 plates (Nunc, Roskilde, Denmark) to 90–95% confluence in DMEM (Gibco, Paisley, Scotland, UK) supplemented with 10% FBS (Gibco) and antibiotic antimycotic solution (Gibco) in a humidifier incubator (Heraeus, Hanau, Germany) with 5% CO_2/95% atmospheric air at 37 °C. Cells were rinsed three times in PBS and were grown 48–72 h in DMEM without FBS. More than 2 L of conditioned media

(without FBS) were collected and stored at −20 °C. RNase 1 was purified from the conditioned media as described previously for serum RNase 1 after concentrating the media using a tangential filtration membrane of 5 kDa cut-off (Millipore, Bedford, MA).

RNase 1 extraction from healthy pancreas

Human pancreas from a healthy donor was obtained from autopsy through the Hospital Josep Trueta, Girona, and its Ethics Committee approved its use. It was immediately frozen and stored at −80 °C. Ribonuclease was extracted using the method of Weickmann et al. (1981) with some modifications (Peracaula et al. 2003).

Release and purification of N-linked oligosaccharides

N-glycans were released from purified RNase 1 fractions by in situ digestion of the protein in SDS–PAGE gel bands with *N*-glycosidase F (PNGase F, Roche), as described earlier (Küster et al. 1997; Radcliffe et al. 2002). Briefly, pure ribonuclease was reduced and alkylated and run on SDS–PAGE. Protein was stained by Coomassie Blue, and bands containing the glycoprotein were excised from the gel and treated with PNGase F to release the N-linked glycans. Wide-range molecular markers were from Sigma.

Fluorescent labeling of the reducing terminus of oligosaccharides and HPLC

Oligosaccharides were fluorescently labeled with 2AB (Ludger Ltd, Abingdon, UK), as described previously (Bigge et al. 1995). NP-HPLC was carried out as described (Peracaula et al. 2003). Briefly, 2AB-labeled glycans were injected in 80% acetonitrile and eluted with a linear gradient of 20–58% 50 mM ammonium formate, pH 4.4, in acetonitrile. Fluorescence was measured at 420 nm with excitation at 330 nm. 2AB-labeled glucose oligomers were run to calibrate the system (Guile et al. 1996) in order to transform retention time to GU values.

Simultaneous oligosaccharide sequencing by exoglycosidase digestions

The 2AB-labeled oligosaccharides were digested in a volume of 10 μL for 18 h at 37 °C in 50 mM sodium acetate buffer, pH 5.5, using arrays of glycosidases with different specificities: ABS (EC 3.2.1.18), 1 U/mL; NANI (EC 3.2.1.18), 1 U/mL; AMF (EC 3.2.1.111), 2 mU/mL; BKF (EC 3.2.1.51), 1 U/mL; SPG (EC 3.2.1.23), 0.1 U/mL; BTG (EC 3.2.1.23), 1 U/mL, and GuH (EC 3.2.1.30) 80 U/mL. All these enzymes were purchased from Prozyme (San Leandro, CA). XMF (EC.3.2.1.51), 1000 U/mL, from New England Biolabs (Ipswich, MA), was also used. After incubation, enzymes were removed from the samples using protein-binding filters (Amicon Micropure EZ filters, Millipore), and oligosaccharides were analyzed by NP-HPLC.

MALDI-TOF-mass spectrometry

Positive ion MALDI-time-of-flight (TOF) mass spectra were recorded with a Micromass TofSpec 2E reflectron-TOF mass spectrometer (Waters MS Technologies, Manchester, UK) fitted with delayed extraction and a nitrogen laser (337 nm). The acceleration voltage was 20 kV, the pulse voltage was 3200 V, and the delay for the delayed extraction ion source was 500 ns. Samples were prepared by adding 0.5 μL of an aqueous solution of the sample to the matrix solution (0.3 μL of a saturated solution of 2,5-dihydroxybenzoic acid in acetonitrile) on the stainless steel target plate and allowing it to dry at room temperature. The sample/matrix mixture was then recrystallized from ethanol (Harvey 1993).

Nanospray MS and MS/MS

Nanospray MS and MS/MS spectra were recorded with a Waters-Micromass hybrid quadrupole-TOF (Q-TOF) Ultima global mass spectrometer fitted with a Z-spray ESI ion source and operated in negative ion mode. Samples in water:-methanol (1:1, v:v, 5 μL) were infused with Proxeon (Proxeon Biosystems, Odense, Denmark) capillaries. Operating conditions were capillary potential, 1.2 kV; ion source temperature, 120 °C; desolvation gas, nitrogen at 50 L/h; RF-1 lens, 180 V; collision gas, argon at 0.5 bar; and a collision cell voltage appropriate to the mass of the ions being analyzed. Data acquisition and processing were conducted with MassLynx 4 software (Waters).

HPLC-ESI-mass spectrometry

Positive ion ESI LC/MS data were obtained using an LC packing Ultimate HPLC equipped with a Famos autosampler (Dionex Ltd, Leeds, UK) interfaced with the previously mentioned Q-Tof Ultima Global mass spectrometer (Waters-Micromass). Chromatographic separation was achieved using a 2 × 250 mm, microbore NP-HPLC TSK gel Amide-80 column (Hichrome, Victoria, Australia) with the same gradient and solvents as used with the standard NP-HPLC but at a lower flow rate of 40 μL/min. The mass spectrometer was operated in positive ion mode with 3 kV capillary voltage, RF-1 lens 60, source temperature 100 °C, desolvation temperature 150 °C, cone gas flow 50 L/h, and desolvation gas flow 450 L/h.

2-DE for RNase 1 samples

The equipment required for 2-DE was a Protean IEF Cell from BioRad (Hercules, CA) and a Multiphor II from Amersham Pharmacia Biotech (GE Healthcare, Chalfont St Giles, UK); Immobiline DryStrips (18 cm, pH 3–10, linear), Pharmalyte (pH 3–10), ExcelGel (SDS 8–18), Buffer strips, Triton X-100, glicerol, DTT, and iodoacetamide were from GE Healthcare; urea and mineral oil were from BioRad, and SeeBlue Pre-Stained Standards were from Invitrogen (Carlsbad, CA).

First-dimension strips were reswelled overnight in rehydration buffer containing 8 M urea, 0.5% (v/v) Triton X-100, 65 mM DTT, 2% (v/v) Pharmalyte 3–10, 0.008% (w/v) Bromophenol Blue. Partially purified RNase 1 samples (between 50 and 100 ng of RNase 1, not exceeding 100 μg of total protein) were diluted in rehydration buffer to the final concentration described earlier and allowed to denature at room temperature for 1 h. The strips were electrophoresed as follows: from 0 to 300 V in 2 h, 300 V for 4 h, raised to 4000 V in 5 h, 4000 V for 3 h, raised to 8000 in 5 h, and 8000 V for 3 h until a total run of 70 kVh. The electrofocused strip was processed immediately.

Prior to the second-dimension, electrofocused strips were equilibrated with shaking for 15 min in equilibration buffer [6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 50 mM Tris/HCl pH 8.8] containing 65 mM DTT, followed by

15 min of incubation in equilibration buffer containing 25 mg/mL iodoacetamide.

The first-dimension strip gels were placed at the cathodic side of 8–18% gradient SDS–PAGE, and horizontal electrophoresis was carried out at 100 V for 45 min and 800 V for 70 min.

After electrophoresis, the separated proteins were blotted into a PVDF membrane and detected with specific polyclonal antibodies against RNase 1 as described before (Peracaula et al. 2003). The antibodies against RNase 1 (1/1000) were incubated for 1 h in blocking buffer. Secondary antibody peroxidase-conjugated goat antirabbit (Pierce, Rockford, IL) was added at 1/60 000 and incubated for 1 h. Detection was performed using the chemiluminescence kit ECL plus (GE Healthcare).

Neuraminidase and PNGase F treatments

The glycosidases used were neuraminidase from *Arthrobacter urefaciens* and recombinant *N*-glycopeptidase F (PNGase F) from *Flavobacterium meningosepticum* (Roche, Basel, Switzerland).

Between 40 and 200 ng of partially purified RNase1 was denatured at 70 °C for 10 min with 0.1% (w/v) SDS to facilitate the enzyme action. Then it was incubated for 17–18 h at 37 °C in 1% (v/v) Triton X-100, 100 mM sodium acetate, pH 5.5, containing 40 mU of neuraminidase. The reaction was stopped with the 2-DE sample buffer.

The combined treatment of both enzymes consisted in the same amount of glycoprotein denatured at 70 °C for 10 min with 0.1% (w/v) SDS and incubated for 17–18 h at 37 °C in 1% (v/v) Triton X-100, 20 mM sodium phosphate, pH 7.2, containing 40 mU of neuraminidase and 8 U of *N*-glycopeptidase F. The reaction was stopped with the 2-DE sample buffer.

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Conflict of interest statement

None declared.

Abbreviations

AMF, almond meal α-fucosidase; BKF, bovine kidney fucosidase; BTG, bovine testes β-galactosidase; DMEM, Dulbecco's Modified Eagle Medium; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray; FBS, fetal bovine serum; GU, glucose units; HPLC, high-performance liquid chromatography; HuLEC, human lung microvessel endothelial cells; HuMMEC, human mammary microvessel endothelial cells; HUVEC, human umbilical vein endothelial cells; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; NANI, *Streptococcus pneumoniae* neuraminidase; NP, normal phase; PaC, pancreatic cancer; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PNGase F, N-glycosidase F from *Flavobacterium meningosepticum*; PVDF, polyvinylidene difluoride; Q-TOF, quadrupole time-of-flight; RNase 1, Ribonuclease 1; SDS, sodium dodecyl sulfate; SPG, *Streptococcus pneumoniae* galactosidase; TNM, Tumour Node Metastasis; U_L, urinary secretory RNase; XMF, *Xanthomonas manihotis* α1–2 fucosidase; 2AB, 2-aminobenzamide; 2-DE, two-dimensional electrophoresis.

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