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Role of sialyltransferases involved in the biosynthesis of Lewis antigens in human pancreatic tumour cells

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The sialylated carbohydrate antigens, sialyl-Lewis^x and sialyl-Lewis^a, are expressed in pancreatic tumour cells and are related to their metastatic potential. While the action of the fucosyltransferases involved in the synthesis of these antigens has already been investigated, no studies have been carried out on the activity and expression of the α2,3-sialyltransferases in pancreatic tumour cells. We describe the sialyltransferase (ST) activity, mRNA expression, and analysis of the cell carbohydrate structures in four human pancreatic adenocarcinoma cell lines of a wide range of neoplastic differentiation stages and in normal human pancreatic tissues. Total ST activity measured on asialofetuin, employing a CMP fluorescent sialic acid, varied among the pancreatic cell lines and could be correlated to the expression of their cell surface antigens. However, in some of the pancreatic cell lines, no relationship could be established with their ST3Gal III and IV mRNA expression. Human pancreatic tissues also showed ST expression and activity. However, it presented a much higher expression of neutral fucosylated structures than sialylated structures. In conclusion, ST activity levels in pancreatic cells could be correlated to their expression of sialylated epitopes, which indicates their involvement in the formation of the sialyl-Lewis antigens, in addition to fucosyltransferase activities.
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Introduction

Sialyltransferases (ST) are a group of enzymes responsible for the transfer of sialic acid to the non-reducing terminal position of oligosaccharide chains of glycolipids and glycoproteins [1]. Up to 20 different human sialyltransferases have been characterized, which differ in their biochemical parameters, substrate specificities, and tissue and cell expression. They can be classified according to the specific sialic acid linkage they catalyze: α2,3-sialyltransferases ST3Gal I-VI (α2,3-bound sialic acid on β-D-galactopyranosil (Gal) residue), α2,6-sialyltransferases ST6Gal I-II (α2,6-bound sialic acid on Gal residue), α2,6-sialyltransferases ST6GalNAc I-VI (α2,6-bound sialic acid on GalNAc or GlcNAc residues) and α2,8-sialyltransferases ST8Sia I-VI (α2,8-bound sialic acid on sialic acid residues) [2].

In comparison to their normal counterparts, tumour cells often display different sialic acid content at the cell surfaces, which can be related to alterations in their pattern of sialyltransferase expression [3]. Changes in sialic acid content can also be observed in tumour-secreted glycoproteins. This is the case for α-fetoprotein [4], human pancreatic ribonuclease [5] or prostate specific antigen [6].

There are no available data regarding the activity or expression of sialyltransferases in normal and tumour pancreatic cells. On the other hand, it is already known that variations in the expression of carbohydrate antigens exist, especially Lewis antigens, as it has been determined by immunohistochemical studies.

In normal pancreas, type 1 blood group antigens, Lewis^a (Le^a), sialyl-Lewis^a (SLe^a) and Lewis^b (Le^b), are abundantly expressed and type 2 antigens are also present, like H2 and

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Lewis^y (Le^y), although the latter (Le^y) has also been detected in pancreatic cancers [7–9]. In pancreatic tumour cells, sialylated structures like SLe^a and SLe^x are predominant [8,10–13]. Le^x and related antigens (sialylated and extended chains) are frequently expressed and can be considered as cancer-associated determinants in human pancreas [14].

The presence of these tumour antigens is not restricted only to cell surface glycoproteins and glycolipids, but has also been described in secreted glycoproteins such as human pancreatic ribonuclease, which contains these sialylated Lewis antigens when secreted from metastatic tumour cells [5]. In contrast, N-glycans of RNase 1 from normal pancreatic cells presented a higher content of fucose and no sialic acid.

Some of these glycosylation changes, especially an enhanced expression of SLe^x and SLe^a in the pancreatic cell lines with metastatic potential, have been correlated to a decreased activity of α1,2-fucosyltransferases (FUT), in particular FUT1 (which transfers fucose onto the galactose of type II structures) and to an enhanced activity of the Lewis α1,3/4 fucosyltransferase, FUT3 [12,15]. As α1,2-fucosyltransferases and α2,3-sialyltransferases compete for the same substrate, the diminution of α1,2-fucosyltransferase activity in pancreatic tumour cell lines in comparison with normal tissue, could favour an enhanced activity for α2,3-sialyltransferases, giving rise to those sialylated antigens [16] .

The aim of this study is to clarify the role played by the α2,3-sialyltransferases responsible for the biosynthesis of the sialyl Lewis antigens. Therefore, an investigation was carried out regarding the expression and activity of the α2,3-sialyltransferases responsible for the biosynthesis of the sialyl Lewis antigens in four established pancreatic tumour cell lines and in human pancreatic tissues. In addition, we wanted to determine whether a correlation existed between enzyme activity, mRNA expression of those sialyltransferases and cell surface expression and immunohistochemistry of the sialylated carbohydrate antigens.

Materials and methods

Cell culture

The human pancreatic adenocarcinoma cell lines used in this study were: Capan-1, Panc-1, MDAPanc-3 and MDAPanc-28. Capan-1, obtained from the American Type Culture Collection (ATCC n° HTB-79, Rockville, MD), was established from a liver metastasis of a pancreatic adenocarcinoma and was characterized histopathologically as a well-differentiated pancreatic adenocarcinoma of ductal origin [17,18]. Panc-1 derived from a mostly undifferentiated carcinoma [19] was obtained from the American Type Culture Collection (ATCC n° CRL-1469). MDAPanc-3 is a cell line established from a liver metastasis of a moderately differentiated adenocarcinoma of the head of the pancreas [20]. MDAPanc-28 was established from a poorly differentiated adenocarcinoma of the body of the pancreas presenting local invasion [21]. MDAPanc-3 and MDAPanc-28 were generous gifts from Dr. M.L. Frazier from the M. D. Anderson Cancer Center, Houston (USA).

Pancreatic adenocarcinoma cell lines were cultured to 90% confluence in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, UK) supplemented with 10% foetal bovine serum (Gibco), and 50 μg/mL Gentamicine (Gibco). The cells were kept at 37°C in a humidified atmosphere (95%) with 5%CO_2.

Tissue samples

Three normal pancreatic tissues from healthy donors (ranging from 43 to 57 years old) were provided by Dr. R. Gomis from the Hospital Clínic of Barcelona. Tissues were obtained surgically, snap-frozen in liquid nitrogen, and kept at −80°C until use. One specimen of normal pancreas used for immunohistochemistry corresponded to a woman aged 47 and was obtained from the Servei de Transplantament Hepàtic of the Hospital de la Vall d'Hebron, Barcelona. It was fixed in 10% formalin, embedded in paraffin and cut into 5 μm serial sections.

Isolation of RNA and RT-PCR for glycosyltransferases

Ninety percent confluent Capan-1, MDAPanc-3, Panc-1 and MDAPanc-28 cells growing in 75 cm^2 cell culture flasks were washed twice with PBS, dispersed with trypsin-EDTA solution (Gibco) and centrifuged at 1500 rpm for 8 min. RNA extraction was carried out from the pellets after two washes with PBS. Normal pancreatic tissue was minced for RNA extraction.

RNA purification was performed by the acid guanidium thiocyanate-phenol-chloroform method [22]. The RNA was quantified by determining absorbance at 260 nm, and its quality assessed by subjecting it to electrophoresis in citric acid urea gels. 20–25 μg of the total RNA extract was pre-treated with DNAse RNase free (DNAse RQ1) for 20 min at 37°C and then 1 μg was converted to cDNA by incubation at 37°C during 1 h 30 min, and at 70°C for 10 min, with 200 units of Superscript RNase H$^-$ Reverse Transcriptase in a 30 μL reaction volume containing 6 mM DTT, 0.4 mM dNTP and 0.5 μg OligodT. All reagents for cDNA synthesis were obtained from either Gibco or Roche (Germany).

ST and FUT expressions were examined by semiquantitative PCR using the β-actin or K-*ras* expression as internal references. PCR was performed using 1.3 μL of cDNA, 18 pmol of the forward (F) and reverse (R) primers and 18 μL of PCR SuperMix (Gibco). After a polymerase activation step (94°C for 10 min), samples were amplified for 27–40 cycles of denaturation at 94°C for 0.5–1.5 min, annealing for 0.5–1 min, and extension at 72°C for 1–1.5 min, followed by an additional extension step (72°C for 5–10 min).

The primer sequences, temperature of annealing and PCR product size were 5′-CGGATGGCTTCTGGAAATCTGT-3′(F) and 5′-TTGTGCGTCCAGGACTCTTTGA-3′ (R), 58°C, 300 bp [43] for ST3Gal III; 5′-CCCA AGAACATCCAGAGCCTCA-3′ (F) and 5′-CGTGG TGGGCTTCTGCTTAATC-3′ (R), 58°C, 458 bp [43]

for ST3Gal IV; 5′-TTGGGAGAAGGACAACCTTC-3′ (F) and 5′-CCAGGCAGCAACAGACAGTA-3′, 55°C, 647 bp [23] for ST3Gal VI; 5′-GCGCCGTGGGGACTATCTGCAGGTTA TGCC-3′(F) and 5′ CAGGCCTCTGAAGCCACGTACT-GCTGG CTC-3′ (R), 72°C, 510 bp [24] for FUT1; 5′-GAGGAATACCGCCACATCCCGGGGGAGTAC-3′ (F) and 5′-ATGGACCCCTACAAAGGTGCCCGGCCGGCT-3′, 72°C, 198 bp [25] for FUT2; and 5′-ACTGGGATATCAT-GTCCAACCCTAAGTCAC-3′ (F) and 5′-GGGCCAGGTCC-TTGGGGCTCTGGAAGTCG- 3′, 70°C, 582 bp [26] for FUT3. As a control, K-*ras* and β-actin expressions were also measured using specific primers [27,12]. The sizes of the PCR products were 265 bp for K-*ras* and 838 bp for β-actin. Amplified cDNAs were run in 2% agarose gels, stained with 0.5 μg/ml ethidium bromide, and visualized under UV light.

The intensity of the amplified cDNA bands of ST3Gal III and IV was measured using the *Quantity-One* software package (BioRad, US) and was normalized to the corresponding housekeeping gene (β-actin) for each cell line. Data were expressed as the mean $\pm$ SD of the intensity values of the PCR assays performed in triplicate.

Protein extraction

The three normal pancreatic tissues were minced with scissors, washed and homogenized with the protein extraction buffer (10 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1%Triton X-100, 10 μg/mL Leupeptin, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF)). Pancreatic tumour cells were pelleted (as described for RNA purification) and homogenized with the protein extraction buffer. Supernatants were centrifuged at 14000 *g* for 30 min at 4°C. The protein content was determined by the Bio-Rad DC-Protein Assay (Bio-Rad, USA) using bovine serum albumin (BSA) as a standard.

GISA (Glycosylation ImmunoSorbent Assay)

10 ng of asialofetuin (Roche) were bound for 1 h at 37°C to 96-well polystyrene plates in coating buffer (Scil Diagnostics, Germany). After washing them twice with phosphate-buffered saline (PBS) -Tween 0.05%, plates were blocked with 1% BSA, 0.05% (v/v) Tween in PBS for 1 h at 37°C. The wells were then washed once with PBS-Tween 0.05% and were incubated for 2 h at 37°C with the different quantities of the cell protein extracts (2–20 μg) and 0.77 mg/L of CMP-5-fluoresceinyl-NeuAc (CMP-5-(N-fluoresceinylthioureido-acetyl-neuraminic acid) in 45 mM NaCl, 15 mM Tris-HCl, pH 7.2, 1% Triton X-100 and 0.1% BSA. Plates were washed four times with PBS-Tween 0.05% and an antibody against FITC (fluorescein isothiocyanate), peroxidase-conjugated 1:2000 in PBS, 0.05% Tween, 0.25% BSA was added and allowed to stand for one hour at 37°C. Plates were washed again four times with PBS-Tween 0.05% and detection was performed with 100 μl/well of 3,3′,5,5′-tetramethylbenzidine (TMB) (BM BluePOD substrate soluble, Roche). The reaction was stopped with 100 μl/well of 0.25 M H_2SO_4 and the absorption was measured at 450 nm (against a reference wavelength of 630 nm) in an automated microplate reader (BIO-TEK, USA).

Negative controls were wells without protein extract, or without the CMP-5-fluoresceinyl-NeuAc. Positive controls were wells with recombinant rat α2,6-N-Sialyltransferase (Calbiochem, Germany) instead of the cell protein extracts.

GISA (Glycosylation Immunosorbent Assay) and *in situ* glycosidase digestions

GISA was carried out as above, with an additional step: After incubation of asialofetuin with the cell protein extracts containing ST and CMP-5-fluoresceinyl-NeuAc, N-glycans were released from new sialylated asialofetuin by *in situ* digestion with 30 U/mL N-glycosidase F (PNGase F, Roche), 60 mM Tris-HCl, pH 8.5, in a volume of 100 μL for 18 h at 37°C. α2-3 linked sialic acids were digested from sialylated asialofetuin in a volume of 50 μL for 18 h at 37°C in 50 mM sodium acetate buffer pH 6.0 using 1 U/mL *Streptococcus pneumoniae* sialidase recombinant in *E. coli* (NANI) (EC 3.2.1.18) from Glyko, Inc. (USA).

Cell ELISA

Cells were seeded on a 96-well plate at a concentration of 10^4 cells/well and allowed to grow to confluence. After the cells had been washed three times with PBS (pH 7.2), they were fixed with 2% formaldehyde in PBS for 20 min at room temperature. The assay was carried out following the protocol described by Zhou *et al*. [28]. Briefly, after washing with PBS, wells were blocked with 1% BSA in PBS or polyvinyl pyrrolidone (PVP) 2% in PBS (for lectin detection). Mouse monoclonal antibodies specific against carbohydrate antigens: anti sialyl-Lewis^x, anti-Lewis^x and anti-Lewis^y (Calbiochem) at 1/50, anti sialyl-Lewis^a [29], anti-Lewis^a [30] and anti-Lewis^b [30] at 1/2, and anti-H2 [31] at 1/1000 dilutions, in 1% bovine serum albumin in PBS or the following digoxigenylated (DIG) lectins: SNA (*Sambucus nigra agglutinin*)-DIG, AAL (*Aleuria aurantia lectin*)-DIG and MAA (*Maackia amurensis agglutinin*)-DIG (Roche) 1 μg/mL in 0.1M Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, were added and incubated for 2 h at r.t. The wells were then washed with PBS and incubated with the secondary antibody: peroxidase-conjugated goat anti-(mouse Ig) serum (Jackson Immunoresearch Laboratories, Inc, USA) diluted 1/2000 in 1% BSA in PBS for 1 h at r.t., or peroxidase-conjugated anti-digoxigenin Fab fragments (Roche), 20 mU/mL in PBS for 1 h at r.t. for lectin detection. The wells were then washed with PBS and the assay was developed with 100 μl/well of 3,3′,5,5′-tetramethylbenzidine (TMB) (BM BluePOD substrate soluble, Roche). The reaction was stopped with 100 μl/well of 0.25 M H_2SO_4 and the absorption was measured at 450 nm (against a reference wavelength of 630 nm) in an automated microplate reader (BIO-TEK, USA).

Plates containing wells without primary or secondary antibodies were used as negative controls.

ELISA on cell extracts

The same pancreatic adenocarcinoma cell lines were grown until confluence in 100 mm dishes with Dulbecco's Modified Eagle Medium supplemented with 10% foetal bovine serum. Cells were washed twice with cold PBS, harvested in 0.5 mL of lysis buffer (20 mM sodium phosphate pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 5 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin), and lysed using a glass homogeniser. After centrifugation at 10000 rpm for 10 min, the protein content was determined by the Bio-Rad DC-Protein Assay using bovine serum albumin (BSA) as a standard.

The normal pancreatic tissues were homogenized as described in the section on protein extraction, and the protein content was also quantified by the Bio-Rad DC-Protein Assay. 200 ng/well of each cell extract or the homogenized pancreas were bound to a 96-well plate diluted in coating buffer (Scil Diagnostics) by incubation for 1 h at 37°C. Blocking was done using 0.05% Tween, 1%BSA in PBS or PVP 2% in PBS (for lectin detection). Detection with monoclonal antibodies against carbohydrate structures or lectins was carried out following the protocols described above for the cell-ELISA, but using 0.05% Tween (v/v) in the antibodies incubation buffer. Purified proteins from Roche were used as controls: fetuin (positive control for SNA and MAA), erythropoietin (positive control for AAL) and asialofetuin (negative control for SNA and MAA) or BSA (negative control).

Immunostaining

Immunohistochemical staining was performed by the avidin-biotin-peroxidase complex technique (Vectastain Elite ABC kit; Vector Labs., USA). The working dilutions of the mouse monoclonal antibodies specific against carbohydrate antigens used were 1/50 for anti sialyl-Lewis^x, anti-Lewis^x and anti-Lewis^y (Calbiochem), 1/2 for anti sialyl-Lewis^a [29], anti-Lewis^a [30] and anti-Lewis^b [30], and 1/1000 for anti-H2 [31]. All incubations were performed at r.t. in a humid chamber.

After deparaffinization and rehydration, the sections were immersed in 3% H_2O_2 in absolute methanol for 10 min to block endogenous peroxidase activity and then washed 3 times with distilled H_2O. The slides were placed in 10 mM sodium citrate (pH 6) and microwaved on high for 10 min. Next, they were washed three times with PBS (pH 7.4), and nonserum protein block (Dako, USA) was applied for 10 min. After washing three times with PBS, normal horse serum (when staining with mouse IgG antibodies) or normal goat serum (when staining with mouse IgM antibodies) was applied for 20 min and removed by blotting. The sections were then incubated with the corresponding monoclonal antibodies against Lewis antigens diluted in normal horse/goat serum for 60 min at r.t., washed 3 times in PBS, and incubated with the biotinylated secondary antibody horse anti mouse IgG or goat anti mouse IgM for 30 min. After washing with PBS, samples were incubated for 45 min with avidin-peroxidase conjugate and then repeatedly washed with PBS. Finally, the sections were reacted with diaminobenzidine and hydrogen peroxide (stable DAB) (Research Genetics, USA) for 3 min, washed with PBS, counterstained with hematoxylin, rinsed in tap water and mounted. Preimmune serum instead of primary antibodies was used as a negative control.

Results

Sialyltransferase expression

ST3Gal III, IV and VI catalyse the formation of α2,3-linkages on type I or II chains. ST3Gal III acts preferentially on type I chains (Galβ1-3GlcNAc) and is therefore the candidate for the synthesis of sialyl-Lewis^a epitope. ST3Gal IV and VI use either glycoproteins or glycolipids containing type II chains (Galβ1-4GlcNAc) and render the precursor for sialyl-Lewis^x epitope (Figure 1).

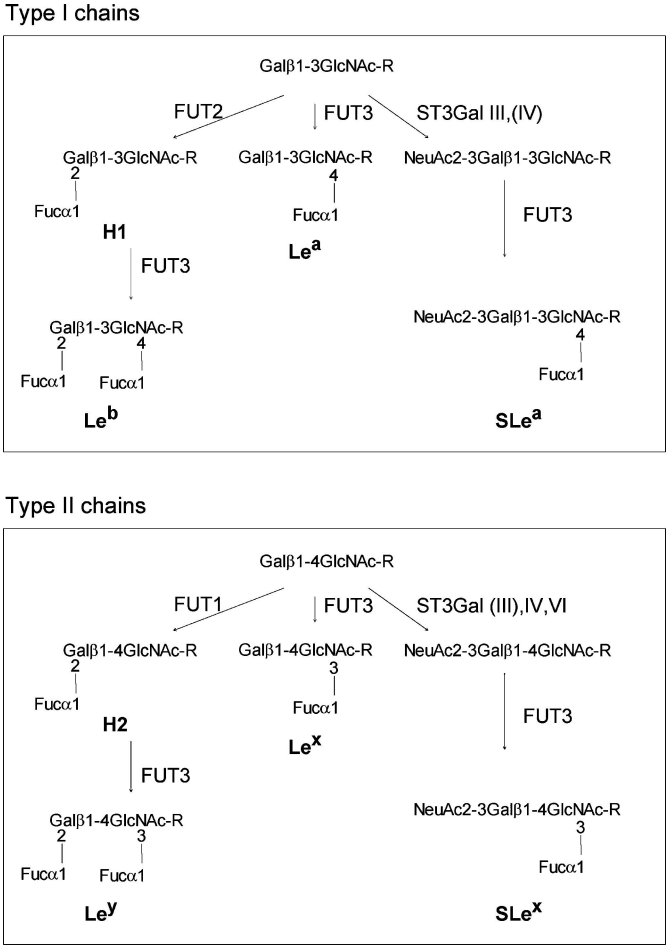

Figure 1. Structures of Lewis antigens and the glycosyltransferases involved in their synthesis. Type I and II structures refer to galactose linked β1-3 and β1-4 to GlcNAc, respectively.

The expression of the α2,3-sialyltransferases, ST3Gal III, IV and VI, responsible for the biosynthesis of SLe^a and SLe^x, was studied, using semiquantitative RT-PCR, on different pancreatic human adenocarcinoma cells which represent a wide range of tumour types: well differentiated (Capan-1), moderately differentiated (MDAPanc-3), poorly differentiated (MDAPanc-28), and undifferentiated pancreatic adenocarcinoma (Panc-1). One normal pancreatic tissue was also analysed.

ST3Gal III, which acts preferentially on type I chains was expressed in all tumour cell lines and also in normal pancreas. MDAPanc-3 and normal pancreas showed the major expression levels, which were about 30–45% higher than in Panc-1 and Capan-1 cell lines, respectively. MDAPanc-28 was the cell line with the lowest expression (Figure 2).

ST3Gal IV (which acts on type II chains) was found in all the tumour cell lines, without significant differences among them, except for MDAPanc-28 where no expression was detected. Normal pancreas had around 40% less quantity of ST3Gal IV transcript compared to the positive tumour cell lines.

No expression of ST3Gal VI was observed in any of the samples (data not shown).

These results indicate that the level of the ST3Gal III and IV transcripts could not be associated with the degree of differentiation of the tumours from which these cell lines were derived.

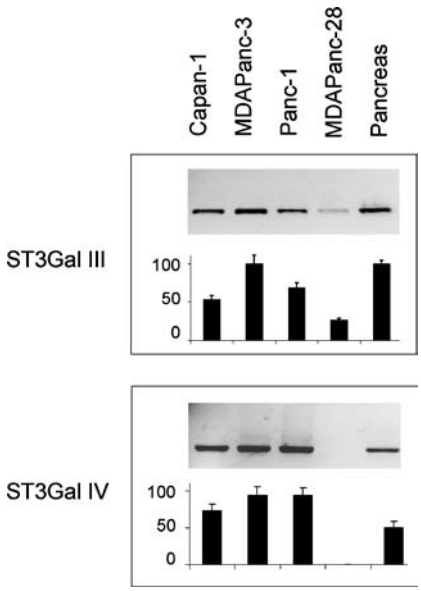

Figure 2. Expression of the human sialyltransferases ST3Gal III and IV in the indicated human pancreatic adenocarcinoma cell lines and in the normal pancreatic tissue was determined by RT-PCR. Agarose gels of the RT-PCR products and quantification of the bands of the amplified ST3Gal III and IV transcripts normalized to the housekeeping gene (β-actin). Graphs represent the mean $\pm$ SD of three experiments.

Sialyltransferase activity

The sialyltransferase activity of the protein extract from the pancreatic human adenocarcinoma cells (Capan-1, MDAPanc-3, MDAPanc-28 and Panc-1) and from pancreatic tissue was studied on asialofetuin in a GISA assay (Glycosylation ImmunoSorbent Assay). This assay consists of an ELISA, using asialofetuin as an acceptor of CMP-activated sialic acid labelled with FITC. The use of fluorescent CMP-sialic acid (CMP-5-fluoresceinyl-NeuAc) as sialyltransferase substrate allows the quantification of enzyme activity with high sensitivity [32–34]. Protein extracts containing sialyltransferases catalyse the incorporation of fluorescent sialic acid into the asialofetuin, which is detected by anti-fluorescein antibodies. Pancreatic tumour cell lines, in particular MDAPanc-3 and MDAPanc-28, presented remarkably enhanced sialyltransferase activity in comparison to other pancreatic tumour cell lines, Capan-1 and Panc-1, and normal pancreatic tissue (Figure 3).

Asialofetuin has three N-glycosylated and three O-glycosylated chains that can incorporate either α2-3 or α2-6 linked sialic acid. To distinguish between the activity of α2,3 and α2,6-sialyltransferases, sialylated asialofetuin was treated *in situ* with NANI (neuraminidase specific for digesting α2,3-bound sialic acids). The result was compared with the untreated sample after the incorporation of fluorescent-labelled sialic acid. Most of the tumour cell lines (Capan-1, Panc-1 and MDAPanc-3), except MDAPanc-28 cells, showed significant α2,3-sialyltransferase activity because the treatment with NANI reduced around 30% of the amount of sialic acid incorporated. The higher activity of α2,3-ST in these cell lines correlated to the presence of ST3Gal III and IV transcripts, which were detected in all of them. MDAPanc-28 was the cell line with minor α2,3-ST activity, which could be associated with the low or no expression of the ST3Gal III and IV transcripts, respectively. In pancreatic tissue, α2,3-ST activity was lower than in MDAPanc-3, Capan-1 or Panc-1 cell lines, and the treatment of NANI reduced the activity by only 15% (Table 1).

In order to assess whether the incorporation of sialic acid was in the N or the O-glycans, sialylated asialofetuin was treated *in situ* with PNGase (digest N-glycans) and the result was

Table 1. Results of the Glycosylation ImmunoSorbent Assay (GISA) showing percentatge of the decrease in ST activity, compared to the GISA control, after treatment with glycosidases

	% decreased after NANI treatment	*% decreased after PNGase treatment*
Capan-1	29	62
MDAPanc-3	37	20
Panc-1	21	50
MDAPanc-28	9	79.2
Pancreas	15	9

NANI (digests α2,3-bound sialic acids) and PNGase (N-glycosidase F, digests N-glycans).

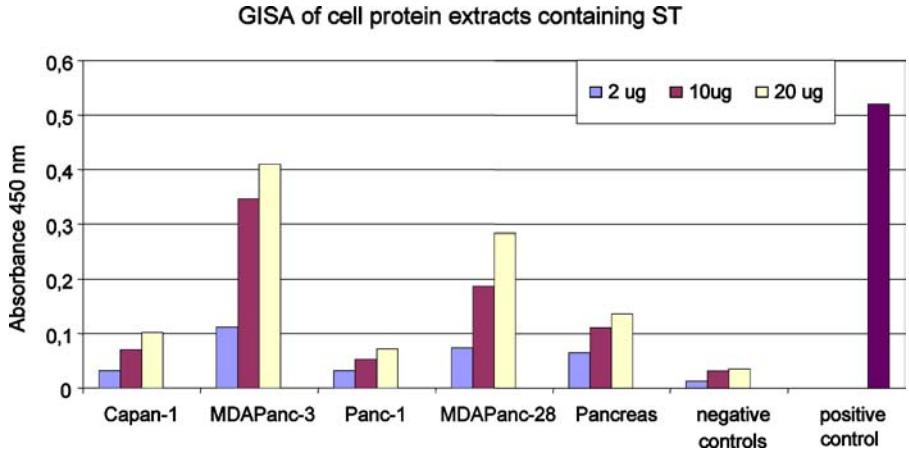

Figure 3. Sialyltransferase (ST) activity determined by GISA. ST activity of protein extracts (2, 10 and 20 μg) of Capan-1, MDAPanc-3, Panc-1, MDAPanc-28 and pancreatic tissue was measured by the level of incorporation of fluorescent sialic acid to 10 ng of asialofetuin. Positive control corresponds to 0.1 mU of recombinant rat α2,6-sialyltransferase.

compared with the untreated sample. In most of the tumour cells (Capan-1, Panc-1 and MDAPanc-28), the ST activity was reduced by more than 50% after the digestion with PNGAse, and by 80% in MDAPanc-28 (Table 1). These results indicate that these enzymes were acting predominantly on the N-glycans, of the type II chains of asialofetuin. However, in pancreatic tissue the treatment with PNGase hardly decreased the ST activity, which means that most of the sialic acid was incorporated in O-glycans.

Determination of Lewis and sialylated antigens

The diverse behaviour in sialyltransferase expression and activity present in the pancreatic tumour cell lines and in normal pancreatic tissue suggests a different expression pattern of sialylated cell surface glycoconjugates. Determination of different carbohydrate structures in these pancreatic cells was carried out by ELISA on whole cells or by ELISA on cell extracts using either antibodies against specific Lewis structures or lectins (Table 2). Immunohistochemical studies were also performed with a normal pancreas specimen.

SLe^a (α2,3-bound sialic acid on type I chains) was highly detected in MDAPanc-3 cells and in a low quantity in Capan-1 cells, whereas SLe^x (α2,3-bound sialic acid on type II chains) (see Lewis structures in Figure 1) was expressed at similar levels in both cell lines. SLe^x was weakly detected in Panc-1, and in MDAPanc-28 cells none of these structures were present. The pancreatic tumour cells containing either SLe^a or SLe^x structures, MDAPanc-3, Capan-1 and Panc-1, expressed ST3Gal III and IV and showed α2,3-ST activity at different proportions. MAA, which detects α2-3 linked sialic acid, although not in the form of SLe^a and SLe^x structures, reacted with these cells, and Capan-1 was the cell line which showed the highest response.

In MDAPanc-3 cells, the high levels of both ST3Gal III and IV correlates well to the higher α2,3-ST activity and may explain the abundance of α2,3 sialylated structures, SLe^x and SLe^a, on their cell surface. Le^a was also predominant in MDAPanc-3 cells suggesting that the Lewis αI, 3/4 fucosyltransferase activity in type I chains could also be important in these cells.

The presence of sialic acid linked α2-6 was detected by SNA lectin and reacted with all the pancreatic cells analysed, and was especially enhanced in MDAPanc-28 cells (Table 2). These cells are negative for MAA (specific for α2,3-sialic acid), SLe^x and SLe^a, which correlates well to their minor α2,3-sialyltranferase activity and low or nil expression of ST3Gal III and IV transcripts.

Table 2. Determination of Lewis and glycan structures on pancreatic cells

	Le^a	*SLe^a*	*Le^b*	*H2*	*Le^x*	*SLe^x*	*Le^y*	*SNA*	*MAA*	*AAL*
Capan-1	−	(+)	−	−	−	++	(+)	+	+	+
MDAPanc-3	++	++	−	−	−	++	+	+	(+)	+
Panc-1	−	−	−	−	−	(+)	−	+	(+)	−
MDAPanc-28	−	−	−	−	−	−	−	++	−	−
Pancreas	+	+	−	+	+	−/+	++	+	−	++

SNA (*Sambucus nigra agglutinin*); MAA (*Maackia amurensis agglutinin*); AAL (*Aleuria aurantia* lectin).
++, strongly positive; +, positive; (+), faintly positive; −, negative.

Normal pancreatic tissues analysed by ELISA showed slight reactivity for SNA (specific for α2-6 linked sialic acid) and also for SLe^a. SLe^x was also detected in minor quantity. The presence of the fucosylated antigens using AAL (specific for fucoses) and specific antibodies against Lewis antigens was also analysed (Table 2). Results indicated that normal pancreas was highly positive with AAL and for the difucosylated antigen Le^y. H2 epitope, precursor of Le^y, was only detected in pancreatic tissues, suggesting that α1,2-FT activity on type II chains may be important in this tissue. Other Lewis antigens, such as Le^a and Le^x, were also present, but in lower proportions. The expression of these antigens was confirmed by immunohistochemical analysis, although in the specimen analysed no detection of SLe^x was observed. The major antigens found, Le^y and H2, were present in both acinar and ductal cells (data not shown).

Lewis structures are synthesized by the combined action of fucosyltransferases and sialyltransferases. The addition of an outer α1-2 linked fucose is carried out by FUT1 that acts preferentially on type II structures, or FUT2 that acts on type I structures. In epithelial tissues the Lewis fucosyltransferase, FUT3, is involved in the synthesis of all Lewis antigens by adding α1-3/4 linked fucose on the GlcNAc of the type I and type II precursor structures.

FUT1, FUT2, and FUT3 transcripts have all been detected in normal pancreatic tissues, which accounts for the detection of most Lewis antigens in normal pancreatic cells [12]. We have studied the expression of FUT1, FUT2, and FUT3 in the tumour pancreatic cell lines (Capan-1, MDAPanc-3, and Panc-1), which display several patterns of Lewis antigen expression. FUT2 was homogeneously detected in all cell lines, while FUT1 and FUT3 were more abundantly expressed in Capan-1 and MDAPanc-3 cells (Figure 4). The low expression of FUT3 in Panc-1 cells would account for the low amount of Lewis antigens in this tumour cell line, which only express low levels of SLe^x.

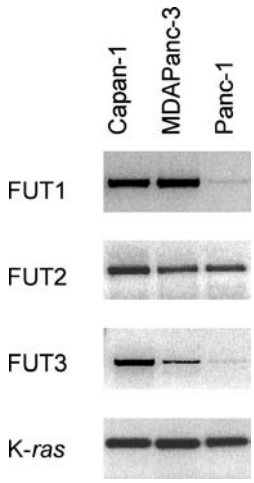

Figure 4. Expression of FUT1, FUT2 and FUT3 in the indicated human adenocarcinoma pancreatic cell lines was determined by RT-PCR. FUT1, FUT2 and FUT3 transcripts detection in Capan-1, MDAPanc-3 and Panc-1 cell lines. The K-ras expression was used as a control (bottom panel).

Discussion

Until now, the study of the expression or activity of glycosyltransferases in pancreatic tumour cells has been limited to *N*-acetylglucosaminyltransferases (GnT) and fucosyltransferases (FUT). Thus, a remarkable increase in the activity of GnT III in pancreatic adenocarcinoma tissues has been described [39]. On the other hand, it has been reported that α1,2-FUT activity is lower in metastatic pancreatic tumour cells, although no straight correlation has been observed with their FUT1 and FUT2 mRNA expression [12,16]. A decrease in α1,2-fucosyltransferase activity in metastatic pancreatic tumour cells would favour the synthesis of sialyl-$Lewis^x$ or sialyl $Lewis^a$ at the expense of difucosylated structures $Lewis^y$ or $Lewis^b$ (see Figure 1) [16]. It has also been reported that an increased expression of FUT3, responsible for the α1,3/4 fucosyltransferase activity, in the pancreatic cell line BxPC-3 would induce the synthesis of SLe^a antigen and its metastatic phenotype [15].

The synthesis of SLe^a and SLe^x requires the addition of sialic acid on the outer ends of type I and II precursor structures by α2,3-sialyltransferases (ST3Gal III, IV and VI). The occurrence of these enzymes in pancreatic cells has not yet been investigated. In order to elucidate their role in the biosynthesis of sialylated Lewis antigens in pancreatic cells, we have studied their expression, activity and the possible correlations with the cell surface glycans in several pancreatic tumour cell lines and in pancreatic tissues.

The pancreatic tumour cells investigated, which represent different stages of differentiation and metastatic potential, presented a diverse pattern of sialyltransferase expression, activity and sialylated structures. Nevertheless, the specific expression of glycoconjugates on their cell surface could be qualitatively correlated to their ST activity pattern, rather than their mRNA levels.

All pancreatic tumour cell lines displayed ST activity, although in different proportions and catalysing different sialic acid linkages. The α2-3 linked sialic acid linkage was in most of them, and α2,6 ST activity was predominant for MDAPanc-28 cells.

Capan-1 and **MDAPanc-3**, which are pancreatic tumour cell lines that were established from liver metastasis [17,20], expressed major levels of Lewis sialylated structures, showed high α2,3-ST activity and expressed ST3Gal III and IV, though in different proportions. Capan-1 expressed mostly SLe^x, while MDAPanc-3 showed high levels of both SLe^x and SLe^a.

SLe^a and SLe^x are present at high levels in metastatic cells, and they facilitate the adhesion of tumour cells to the endothelial cells of capillary vessels, initiating the process of extravasation and metastasis [35], and SLe^x expression has been reported to facilitate cancer cells to evade the immune system [36]. In addition, Capan-1 cells are capable of metastasising when

transplanted into nude mice [18,37] and express the synuclein-γ gene, involved in invasion, at similar levels as MDAPanc-3 cells [38]. MDAPanc-28 established from a carcinoma that presented local invasion also expressed the synuclein-γ gene, which is not detected in Panc-1 cells [38].

MDAPanc-3 also showed expression of other Lewis antigens belonging to type I structures, like Le^a, in contrast to Capan-1, which was negative for fucosylated type I structures (Le^a and Le^b), although it presented high levels of FUT3 transcript. This discrepancy could suggest low levels of the type I precursor structure (Galβ1-3GlcNAc) in Capan-1 cells. On the other hand, the major content of SLe^a in MDAPanc-3 cells with respect to Capan-1 could be explained not only by the minor level of the ST3Gal III transcript in Capan-1, but most likely by other reasons like a lower α2,3-ST activity and a probable low presence of the type I precursor structure.

Panc-1 cells expressed low amounts of SLe^x and showed no expression of other Lewis antigens, which could be related to its low ST activity and its low level of FUT3 expression. Conversely, ST3Gal III and IV transcripts were found in similar proportion to Capan-1 cells, indicating that no significant relationship could be established between the amount of ST3Gal messages and the expression of the sialylated Lewis antigens.

The different occurrence of the sialylated Lewis antigens in these pancreatic cell lines appeared to correlate to their ST activity levels rather than by their pattern of ST3Gal III and IV mRNA expression. On the other hand, differences in the activity of α1,2-fucosyltransferases, which would act as competitor enzymes of α2,3-STs for the synthesis of sialyl-Lewis antigens, could not be ruled out and could also influence in the expression of SLe^x and SLe^a, as has been described for other pancreatic cell lines [12].

MDAPanc-28, which did not express sialylated Lewis antigens but rather α2-6-bound sialic acid, detected by SNA, had increased α2,6-ST activity and low or nil levels of the ST3Gal III and IV transcripts (Figure 2).

The expression of α2-6-sialylated structures detected by SNA is controlled at multiple levels [3]. ST6Gal I is the main enzyme responsible for adding α2-6 linked sialic acid onto galactose of type II structures and it is expressed in most human tissues, including the pancreas [1,3,40]. It could compete mainly with ST3Gal IV for type II structures and its expression might influence the biosynthesis of sialyl-$Lewis^x$. On the other hand, ST6Gal I has been described as being up-regulated in human cancers, such as breast, colon, and gastric cancer [3,41–43].

The pancreatic tumour cell lines, which expressed sialyl-Lewis antigens at different levels, Capan-1, MDAPanc-3 and Panc-1, showed major α2,3-ST activity although they were also positive for SNA. However, the most important SNA staining was found in MDAPanc-28 cells, which had predominantly α2,6-ST activity, suggesting competition between α2,6-STs and α2,3-STs for similar substrates.

In contrast, **normal pancreatic tissue** expressed more fucosylated structures than pancreatic tumour cells, as determined by AAL (specific for fucoses). The most predominant Lewis antigen was the difucosylated Le^y (Table 2), which correlated to the glycans found in many secreted pancreatic glycoproteins, which are more abundant in terminal fucose structures [5,44].

Normal pancreatic tissues had also ST activity at a level comparable to the pancreatic tumour cell line Capan-1, although the ST activity was lower in transferring α2-3-bound sialic acid. There was mRNA expression of ST3Gal III and IV, but ST3Gal IV, responsible for the addition of α2-3-bound-sialic acid to type II chains to give rise to SLe^x, had a lower expression. In the normal pancreatic tissue extract, SLe^a epitope was detected and SLe^x was less expressed, whereas no detection of SLe^x was observed by immunohistochemistry. Sialylated Le^x antigens have been associated as tumour antigens of pancreatic adenocarcinoma because their expression in normal pancreatic tissue has been scarce [14]. The abundance of the precursor H2 and Le^y in normal pancreatic tissue suggests significant α1,2-fucosyltransferase activity on type II chains, which would explain the low expression of SLe^x antigen.

The increased levels of sialylated Lewis structures, implicated in cell adhesion and metastasis, and the slight presence of difucosylated Lewis structures found in Capan-1 and MDAPanc-3 cells, in relation to normal tissue, could be related to an enhanced α2,3-sialyltransferase activity in these tumour cells rather than their mRNA levels. Thus, the biosynthesis of these tumour sialylated antigens could be explained not only by variations in fucosyltransferase activities, such as a decrease in α1,2-fucosyltransferase or an increase in α1-3/4 fucosyltransferase, but also by increased activities in α2,3-sialyltransferases.

Some reports in the literature have described alterations in the glycosyltransferase expression and activity in several tumours, which presented increased sialylation in the form of SLe^x and SLe^a determinants, indicating that variations of specific glycosyltransferases may be helpful for tumour prognosis. An increased mRNA expression of several fucosyl and sialyltransferases has been reported in colorectal carcinomas [41], as well as differences in ST activity [45,46]. Gretschel *et al.* [42] described that high levels of ST3Gal III and ST6Gal I could be correlated to secondary tumour recurrence in gastric cancers. Recchi *et al.* [43] reported that high expressions of ST3Gal III and ST6Gal I could be associated with poor prognosis of human breast tumours and a prognostic value for their expression in node-negative breast cancer patients has recently been described [47]. Investigations of the expression and activity levels of α2,3-sialyltransferases in neoplastic human pancreatic tissues would be required to determine whether they could have a prognostic value for pancreatic adenocarcinoma.

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