High-mannose-type oligosaccharides from human placental arylsulfatase A are core fucosylated as confirmed by MALDI MS

**Introduction**

Arylsulfatase A (ASA, EC 3.1.6.1) is a lysosomal glycoprotein involved in the hydrolytic degradation of various sulfatides. A deficiency of this enzyme leads to a neurological disorder—metachromatic leukodystrophy (Kolodny and Fluharty, 1995). An increase of the enzyme concentration in body fluids in lung, central nervous system, colon cancer, and malignant lymphoma patients has been reported (Laidler et al., 1991; Honke et al., 1995). Arylsulfatase A purified from various human tissues has been shown to be composed of two nonidentical subunits whose apparent molecular masses are 55–63 kDa and 49–57 kDa (Waheed et al., 1982, 1983; Bach and Neufeld, 1985). The smaller glycopeptide is a proteolytic product of the larger one. The two subunits have been suggested to be similar as shown in physicochemical and immunological experiments. Under normal conditions the bulk of ASA is retained intracellularly and transported to the lysosomes through binding to mannose 6-phosphate receptors (MPRs). The carbohydrate moiety and the phosphate groups of the intracellularly retained ASA are modified by lysosomal hydrolases. Intracellular ASA therefore does not reflect the phosphorylation state of the newly synthesized enzyme (Sommerlade et al., 1994). The carbohydrate component of ASA synthesized in tumor tissues and transformed cells has been shown to undergo increased sialylation, phosphorylation and sulfation (Nakumura et al., 1984; Waheed and Van Etten, 1985). To understand the significance of any changes in glycosylation of arylsulfatase A in cancer, it is important to know the structure of its carbohydrate component in normal tissue.

Despite numerous studies on human ASA, the structure of its glycans is not well understood. It has been shown that the concentration of arylsulfatase A increases in the body fluids of patients with some forms of cancer, and the carbohydrate component of arylsulfatase A synthesized in tumor tissues and transformed cells undergoes increased sialylation, phosphorylation and sulfation. To understand the significance of any changes in the glycosylation of arylsulfatase A in cancer, it is important to know the structure of its carbohydrate component in normal tissue. In the present study we have analyzed carbohydrate moieties of human placental arylsulfatase A using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western blotting on Immobilon P and on-blot deglycosylation using PNGase F for glycan release. Profiles of N-glycans were obtained by matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS). Oligosaccharides were sequenced using specific exoglycosidases, and digestion products were analyzed by MALDI MS and the computer matching of the resulting masses with those derived from a sequence database. Fifty micromoles (6 μg) of arylsulfatase A applied to the gel were sufficient to characterize its oligosaccharide content. The results indicated that human placental arylsulfatase A possesses only high-mannose-type oligosaccharides, of which almost half are core fucosylated. In addition, there was a minor species of high-mannose-type glycan bearing six mannose residues with a core fucose. This structure was not expected since high-mannose-type oligosaccharides basically have not been recognized as a substrate for the α1,6-fucosyltransferase.

**Key words:** arylsulfatase A/core fucosylation/high mannose type glycans/MALDI MS

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and 60.5 kDa were readily identified using molecular weight standards and Ponceau S solution staining, and the bands of interest were excised. Two small pieces of Immobilon P (sample and control) were placed into Eppendorf tubes and protein bands were destained by washing with 20 mM NaHCO₃, pH 7.0. To maintain its denatured state during deglycosylation, the sample was reduced and alkylated on the blot. Prior to deglycosylation, the piece of Immobilon P was incubated in the blocking solution to prevent nonspecific binding of PNGase F. PNGase F was used at a concentration of 60 U/ml (Tarentino et al., 1985). The removal of oligosaccharides from the sample was confirmed by the lack of reaction of deglycosylated ASA with digoxigenin-labeled GNA. Comparison of the digested protein with the control protein suggested that ASA had been completely deglycosylated. To improve spectral quality, the glycans obtained by on-blot deglycosylation were then purified with both cation and anion exchange resins prior to MALDI MS analysis. The buffer systems used for on-blot digestion and exoglycosidase sequencing were chosen to meet the criteria listed by Küster et al. (1997). Several additional precautions were taken to minimize contamination and loss of glycans during the procedure (Küster et al., 1997, 1998). Figure 2 shows MALDI MS profiles of (A) the total pool of glycans and of (B–D) the digesting products of simultaneous treatment of the sugars with mixtures of exoglycosidases. Table I shows the molecular weights measured for each glycan, the monosaccharide composition derived from this mass and carbohydrate structures deduced from these data.

Fifty picomoles (6 μg) of ASA was sufficient to identify all its glycans. Oligosaccharides were detected as [M+ Na]+ or [M + K]+ ions in the positive ion MALDI mass spectrum. The total glycan pool consisted of 9 different compositions (Figure 2A; d-Hex,HexNAc₃, m/z 1079.4; Hex,HexNAc₄, m/z 1095.4, 1111.4; d-Hex,HexNAc₄, m/z 1242.5; HexNAc, m/z 1257.5, 1273.5; d-Hex,HexNAc₅, m/z 1403.5; HexNAc, m/z 1419.5, 1435.5; d-Hex,HexNAc, m/z 1565; Hex,HexNAc, m/z 1581.5, 1598.5; Hex,HexNAc, m/z 1743.6, 1758.6). Maxima detected at m/z 1175.5, 1337.5, 1499.6, and 1661.5 contained adducts—hexose oligomers Hex₇, Hex₆, Hex₅, and Hex₄, respectively (Pitt and Gorman, 1997). Defucosylation (Figure 2B) of the sugar using bovine epididymis α-fucosidase resulted in an increase in those similar structures which were devoid of core fucose. Four glycan peaks disappeared after fucosidase treatment (m/z 1079.4, 1241.5, 1402.5, and 1564.5) and four similar structures without fucose underwent amplification (HexNAc, m/z 933.3; HexNAc, m/z 1095.3; HexNAc, m/z 1257.4 and 1273.8; HexNAc, m/z 1419.4). Simultaneous treatment of the total glycan pool with fucosidase and jack bean β-N-acetylatedaminidase (Figure 2C) resulted in the same spectrum as that received after fucosidase digestion. Treatment with the array of enzymes containing the jack bean α-mannosidase dramatically simplified the spectrum (Figure 2D). The only structure detected corresponded to HexNAc,Hex₆, m/z 609.2. These results clearly demonstrated that the total glycan pool of human placental ASA comprised only high-mannose-type oligosaccharides, of which almost half are core fucosylated.

Results

The general strategy employed in this study is outlined in Figure 1. Homogenous ASA was run on SDS–PAGE and transferred to Immobilon P sheet. SDS–PAGE enabled the concentration of the sample from dilute solutions into small areas of the gel matrix in one step and without loosing the protein of interest. Preequilibration of the slab gel permitted removal of contaminating electrophoresis buffer salts prior to electrophoretic transfer. Additionally, Western blotting allowed one to focus the samples on transfer membrane; thus immobilized proteins were more accessible to reagents. This permitted a shorter incubation and washing times for Immobilon P sheets in comparison with gel. Moreover, in the case of Immobilon P sheet, it was possible to confirm the degree of enzymatic deglycosylation by the reaction of deglycosylated ASA (sample) and nondeglycosylated ASA (control) with digoxigenin-labeled GNA. SDS was thoroughly removed during Western blotting to minimize an adverse effect on the glycosidases and to prevent subsequent interference with MALDI MS (Mock et al., 1992). The degree of electrophoretic transfer of the protein from slab gel to Immobilon P sheet was confirmed by Coomassie brilliant blue staining of the gel and this transfer was complete. The two subunits of ASA (53 kDa and 60.5 kDa) were readily identified using molecular weight standards and Ponceau S solution staining, and the bands of interest were excised. Two small pieces of Immobilon P (sample and control) were placed into Eppendorf tubes and protein bands were destained by washing with 20 mM NaHCO₃, pH 7.0. To maintain its denatured state during deglycosylation, the sample was reduced and alkylated on the blot. Prior to deglycosylation, the piece of Immobilon P was incubated in the blocking solution to prevent nonspecific binding of PNGase F. PNGase F was used at a concentration of 60 U/ml (Tarentino et al., 1985). The removal of oligosaccharides from the sample was confirmed by the lack of reaction of deglycosylated ASA with digoxigenin-labeled GNA. Comparison of the digested protein with the control protein suggested that ASA had been completely deglycosylated. To improve spectral quality, the glycans obtained by on-blot deglycosylation were then purified with both cation and anion exchange resins prior to MALDI MS analysis. The buffer systems used for on-blot digestion and exoglycosidase sequencing were chosen to meet the criteria listed by Küster et al. (1997). Several additional precautions were taken to minimize contamination and loss of glycans during the procedure (Küster et al., 1997, 1998). Figure 2 shows MALDI MS profiles of (A) the total pool of glycans and of (B–D) the digesting products of simultaneous treatment of the sugars with mixtures of exoglycosidases. Table I shows the molecular weights measured for each glycan, the monosaccharide composition derived from this mass and carbohydrate structures deduced from these data.

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Discussion

None of the studies performed so far have unequivocally defined glycan structures of arylsulfatase A. The majority of glycans were recognized as the high mannose or hybrid type with and without fucose (Fujii et al., 1992; Poretz et al., 1992; Laidler et al., 1994). Previous studies implied also the presence of complex type glycans (Gieselmann et al., 1992; Sommerlade et al., 1994). Until recently, ASA glycan structure has been characterized using indirect methods, less sensitive and less precise than those described here. High-sensitivity analysis by MALDI MS has resolved the problem of glycan type present on human placental ASA. There is no doubt that oligosaccharides of human placental ASA are only high mannose-type with and without fucose. The structural assignment has been done based on the substrate specificity of glycosidases used, including PNGase F. The product of treatment of each ASA glycan with exoglycosidase array (α-fucosidase, β-N-acetylhexosaminidase, jack bean α-mannosidase) must be assigned as GlcNAc2Man.

The previous studies indicating the presence of complex type glycans, were carried out mainly on the enzyme synthesized in transfected BHO cell with cDNA of placental ASA (Braulke et al., 1987; Gieselmann et al., 1992). However, these mutants have different glycosylation, phosphorylation, and intracellular sorting of ASA. It is noteworthy that the higher secretion of these mutants was associated with an increased processing of its oligosaccharide to an Endo H–resistant form. Gieselmann et al. (1992) assumed a priori that Endo H–resistant glycans were complex type oligosaccharides. However, it is well established that Endo H can effectively hydrolyze the chitobiose unit in mannose-containing N-linked oligosaccharides possessing at least three mannose residues, providing the α1,6-mannose arm with at least another mannose attached to it (Maley et al., 1989). This specificity of Endo H is in agreement

Fig. 1. Overview of the technique for on-blot release and analysis of glycans.

Fig. 2. Positive-ion MALDI-TOF mass spectra of oligosaccharides obtained by on-blot PNGase F digestion from 50 pmol of arylsulfatase A. All molecular weights represent monoisotopic masses of the respective [M + Na]+ or [M + K]+ ions. (A) total glycan pool, (B) after digestion with bovine epididymis α-fucosidase, (C) after digestion with α-fucosidase and jack bean β-N-acetylhexosaminidase, (D) after digestion with α-fucosidase, β-N-acetylhexosaminidase and jack bean α-mannosidase. The asterisk indicates hexose adducts. The data are analyzed in Table I.
Man$_5$GlcNAc$_2$ (1095.4, 1111.4, 1257.5, 1273.5; Figure 2A).

Moreover, the presence of fucose in the core region of glycans based on PAGE, SDS–PAGE, and Western blots did not confirm the presence of complex type glycans. Moreover, subunits of arylsulfatase A did not react with either *Datura stramonium* agglutinin (DSA) or *Ricinus communis* I agglutinin (RCA), and native placental enzyme did not bind to RCA (I)–agarose even though it contained core fucose (Laidler et al., 1994).

Another unresolved question so far has been whether the glycans present on each subunit were of the high-mannose and/or hybrid type. This uncertainty arose mainly from the fact that the enzyme showed a positive reaction with *Aleuria aurantia* lectin (AAL) and *Lens culinaris* lectin (LCA), the probes for core fucose (Poretz et al., 1992; Laidler and Lityńska, 1997). It has been established that 6-O-L fucosylation of the innermost GlcNAc is one of the later events during the oligosaccharide processing, which occurs almost exclusively in complex- and hybrid-type structures. The enzyme responsible for the addition of the core fucose (α1,6-fucosyltransferase) requires at least the presence of a β1,2GlcNAc unit on the core (α1–3Man (Schwarz and Elbein, 1985; Veynnow et al., 1991). As the core fucose was not expected, since high-mannose-type oligosaccharides have been reported not to be substrates for the α1,6-fucosyltransferase, Laidler and Lityńska (1997) did not exclude the presence of hybrid-type glycans. The early view of posttranslational glycosylation suggested that α-mannosidase I and II were found in the cis and medial Golgi, and GlcNAc-transferase I and α1,6-fucosyltransferase in the medial Golgi. Further investigation demonstrated cell-type-specific Golgi subcompartmentation and showed a different overlapping in the localization of some glycosylation enzymes (Velasco et al., 1993; Rabouille et al., 1995) and the different sequence requirements for the Golgi retention of the same enzyme in various cell types (Colley, 1997). Although no rigorous localization studies on the α1,6-fucosyltransferase have been performed, Magner et al. (1986), 1992) demonstrated that active mouse thyrotrithopeps appeared to shift the subcellular site of fucosylation partially from Golgi to RER. In addition, the investigation of the Golgi to RER recycling pathway could explain the presence of fucosyltransferase in the RER in some cell types (Doms et al., 1989; Ulmer and Palade, 1989; Lippincott-Schwartz et al., 1990). In fact, a few naturally occurring lysosomal glycoproteins (cathepsin B, cathepsin D, β-glucuronidase, and α-mannosidase) have been reported to bear core-fucosylated small oligomannose-type (Man$_9$GlcNAc$_2$ and smaller) N-glycans (Howard et al., 1982; Takahashi et al., 1983, 1984; Taniguchi et al., 1985; Kozutsumi et al., 1986; Maley et al., 1989). All of these enzymes possess a high content of oligomannose structures and bear a high percentage of large oligomannose chains (e.g., Man$_n$ GlcNAc$_m$), but only Man$_5$GlcNAc$_2$ and smaller chains were found to be fucosylated.

Our experiment has led us to somewhat confusing results, because so far nobody has detected Man$_5$GlcNAc$_2$(Fuc)GlcNAc oligosaccharide on glycoprotein. In the literature it has been debated whether a biosynthetic or degradative pathway is responsible for the formation of these...
structures (Lin et al., 1994). Taking together all of these investigations we postulate two possibilities for the processing pathway of glycan residues of human placental ASA. (1) Since trimming of Man 8GlcNAc2 to Man 5GlcNAc2 occurs in cis pathway of glycan residues of human placental ASA. (1) Since investigations we postulate two possibilities for the processing structures (Lin et al., 1994). Taking together all of these investigations we postulate two possibilities for the processing pathway of glycan residues of human placental ASA. (1) Since trimming of Man 8GlcNAc2 to Man 5GlcNAc2 occurs in cis pathway of glycan residues of human placental ASA. (1) Since investigations we postulate two possibilities for the processing

Western blotting

Electroblotting was run using a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) at 40 V constant voltage in 25 mM Tris/192 mM glycine/20% methanol, pH 8.4. Proteins (sample, 6 µg of ASA; control, 1 µg of ASA) were transferred onto an Immobilon P sheet overnight. Prior to electrophoretic transfer, the gel was pre-equilibrated in a transfer buffer for 5 min.

Protein alkylation

The procedure essentially follows the method described by Küster et al. (1998). The excised Immobilon P pieces (8 × 2 mm) were placed into Eppendorf tubes and washed twice with 20 mM NaHCO₃, pH 7.0, 15 min for each. The washes were discarded and replaced by 300 µl of fresh 20 mM NaHCO₃, pH 7.0. To this solution 20 µl of 45 mM dithiothreitol (DTT) was added and the protein reduced at 60°C for 30 min. After cooling to room temperature (RT), 20 µl of 100 mM iodoacetamide (IAA) was added and the protein alkylated for 30 min at RT in the dark. The reducing and alkylation reagents, as well as residual SDS, were then removed by incubation in 1/1 acetonitrile/fresh 20 mM NaHCO₃, pH 7.0, for 60 min. Subsequently, the membrane pieces were incubated in the blocking solution (Boehringer Mannheim Biochimica).

Materials and methods

Materials

All standard laboratory chemicals of analytical grade were purchased from Aldrich. Acetonitrile (HPLC grade), methanol (HPLC grade), and HMW Color Markers (HMW electrophoresis standard) were obtained from Sigma. Milli-Q Plus water (18.2 MΩ resistivity; Millipore) was freshly distilled prior to use. N-Glycosidase F (recombimant, cloned from Flavobacterium meningosepticum and expressed in E.coli, EC 3.2.218; 3.5.1.52), Ponceau S and digoxigenin-labeled GNA (DIG Glycan Differentiation Kit) were obtained from Roche. The column was washed with 100 µl water and dried in a SpeedVac. N-Acetyl-alpha-D-glucosaminidase (1970). Purified ASA was separated on 4.5% stacking gel and on 10% separation gel. Prior to electrophoresis, protein sample (6 µg of ASA) and control sample (1 µg of ASA) were boiled at 100°C for 10 min. The HMW protein standards (8 µl of the color markers) were boiled at 100°C for 3 min. Electrophoresis was run using a Mini-PROTEAN II cell (Bio-Rad) for ~3 h.

Gel electrophoresis

The gel (85 × 70 × 1 mm) was prepared in SDS conditions and using a discontinuous buffer system according to Laemmli (1970). Purified ASA was separated on 4.5% stacking gel and on 10% separation gel. Prior to electrophoresis, protein sample (6 µg of ASA) and control sample (1 µg of ASA) were boiled at 100°C for 10 min. The HMW protein standards (8 µl of the color markers) were boiled at 100°C for 3 min. Electrophoresis was run using a Mini-PROTEAN II cell (Bio-Rad) for ~3 h.
sodium acetate at pH 5.5 (Küster et al., 1997). Prior to MALDI MS, each reaction mixture was desalted using the micro-column clean-up described above.

MALDI mass spectrometry

Angiotensin II (1045.5 u), ACTH clip 18–39 (2464.2 u), bovine insulin (5733.5 u) and equine cytochrome c (12360.1 u) were used for external calibration of the mass spectrometer. The following glycans were used as oligosaccharide standards: high-mannose MAN-9; hybrid HYBR; asialo-, galactosylated tetraantennary NA4; di-sialylated, galactosylated biantennary, core substituted with fucose A2F. All calibrants (1–5 pmol/µl) were dissolved in 0.1% trifluoroacetic acid (TFA) in ultrapure Milli-Q Plus water. 2.5-dihydroxy benzoic acid (DHB) was used as MALDI matrix. The matrix was dissolved in HPLC-grade acetonitrile (1 g/l for the seed layer) (Westman et al., 1998) or in 0.1% TFA in acetonitrile/ultrapure water [1:1 v/v] (Grant K-AA/KU 12003–300), the Swedish Medical Research Council, Ricinus communis agglutinin; RCA I, Ricinus communis agglutinin I; RER, rough endoplasmic reticulum; SDS, sodium dodecyl sulfate; SLAC, serial lectin affinity chromatography; TFA, trifluoroacetic acid.

All MALDI analyses were performed with an upgraded Reflex II MALDI-TOF mass spectrometer (Bruker-Franzen Analytic Gmbh, Bremen, Germany). Samples were irradiated with a matrix seed layer. Thereafter, the 10 g/l matrix and sample solutions were mixed in a test tube 1:1, and a droplet (1 µl) of sample/matrix was deposited on the matrix seed layer. Samples were then left to dry totally in air.

All MALDI analyses were performed with an upgraded Reflex II MALDI-TOF mass spectrometer (Bruker-Franzen Analytic Gmbh, Bremen, Germany). Samples were irradiated with a 337 nm nitrogen and a laserspot ~10–20 µm in diameter. All spectra were acquired in the reflectron mode at an accelerating voltage of 20 kV. Mass spectra were analyzed using the software provided by Bruker. All spectra shown were calibrated using external calibration with a mass deviation of within 0.08%. Sample-potential/first-electrode potential ratio was optimized to achieve optimal resolution for the molecules studied. The sample probe was made of highly polished stainless steel. Since the spectrometer was equipped with a computer-controlled XY sample stage and high-definition observation optics connected to a video camera, visual inspection of the sample inside the MALDI-TOF MS was possible. This made it possible to aim the laser beam at specific sample spots and take full advantage of the small deposits on the target.

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Abbreviations

ACTH, adrenocorticotropic hormone; A2F, disialylated, galactosylated biantennary complex-type glycan with core fucose; ASA, arylsulfatase A; BHO, baby hamster ovary; DDT, diithiothreitol; DHB, dihydroxybenzoc acid; DSA, *Datura stramonium* agglutinin; Endo-H, endo-β-N-acetylgalactosaminidase H; Fuc, fucose; GC, gas-liquid chromatography; GlcNAc, N-acetylgalactosamine; GNA, *Galantus nivalis* agglutinin; ı-Hex, deoxy-hexose; Hex, hexose; HexNAc, N-acetylenohexosamine; HPAC, high pH anion-exchange chromatography; HPLC, high-performance liquid chromatography; HYBR, hybrid type glycan with bisecting GlcNAc; IAA, iodoacetamide; LCA, *Lens culinaris* agglutinin; MALDI TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Man, mannose; Man 6-P, mannose 6-phosphate; MPR, mannose 6-phosphate receptor; MAN-9, oligomannose-type glycan with nine mannose residues; NA4, asialo-, galactosylated tetraantennary complex-type glycan; NMR, nuclear magnetic resonance spectroscopy; PNGase F, peptide N-glycosidase F; RCA I, *Ricinus communis* agglutinin I; RER, rough endoplasmic reticulum; SDS, sodium dodecyl sulfate; SLAC, serial lectin affinity chromatography; TFA, trifluoroacetic acid.

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