Marked Defects in the Expression and Glycosylation of $\alpha_2$-HS Glycoprotein/Fetuin-A in Plasma from Neonates with Intrauterine Growth Restriction

PROTEOMICS SCREENING AND POTENTIAL CLINICAL IMPLICATIONS

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Intrauterine growth restriction (IUGR) has been associated with increased perinatal morbidity and mortality and increased morbidity and metabolic abnormalities later in life. IUGR is characterized as the failure of a fetus to achieve his or her genetic growth potential in utero. Altered protein expression profiles associated with IUGR may be informative on the pathologic mechanisms of this condition and might reveal potential markers for postnatal complications. The aim of this study was to compare protein profiles of umbilical cord plasma from IUGR and appropriate for gestational age full-term neonates. Blood samples from doubly clamped umbilical cord at delivery from 10 IUGR and 10 appropriate for gestational age full-term neonates were analyzed by two-dimensional electrophoresis and MS. Prominent changes of the $\alpha_2$-HS glycoprotein/fetuin-A were observed in IUGR cases. Specifically we showed that these changes occur primarily at the level of post-translational modifications of the protein. Using a combination of mass spectrometry and classical biochemical assays, single and heavy chain forms of fetuin-A were found to lack the normally present O-linked sialic acids in IUGR neonates. Fetuin-A is a glycoprotein that has been associated with promotion of in vitro cell replication, fetal growth and osteogenesis, and protection from Gram-negative bacterial endotoxins. Prominent defects in glycosylation/sialylation of fetuin-A revealed by our study might be responsible for impaired function of fetuin-A, leading to deficient fetal growth, especially osteogenesis, and/or to the development of complications frequently seen later in the lives of IUGR neonates.

Received, September 4, 2007, and in revised form, November 21, 2007.

Published, MCP Papers in Press, December 7, 2007, DOI 10.1074/mcp.M700422-MCP200

Intrauterine growth restriction (IUGR) is the failure of a fetus to reach his or her genetic growth potential in utero; this situation leads to reduced fetal size and low birth weight at the time of delivery. IUGR is associated with an increased risk of perinatal morbidity and mortality (1). Furthermore individuals born with IUGR develop abnormalities characteristic of the metabolic syndrome (obesity, dyslipidemia, hypertension, impaired glucose tolerance, and type 2 diabetes mellitus) and its cardiovascular complications in later life.

Fetal growth is controlled by maternal, placental, and/or fetal factors, which consequently may also be involved in the pathogenesis of IUGR (2). Umbilical cord (UC) blood drawn from the doubly clamped umbilical cord at delivery reflects the fetal blood compartment. Along these lines, the cord blood levels of various growth factors and hormones have been related to size at birth by several groups (1, 3–5). Further investigation of differential protein expression levels in UC blood in various pathologic states will hopefully produce information on the cause and mechanisms of prenatal disorders and might reveal possible markers for postnatal complications and disease progression.

Proteomics provides information about protein expression levels, post-translational modifications, subcellular localization, and interactions (6, 7). Proteomics techniques have been applied in the investigation of the proteome of various biological systems, including human body fluids, i.e. plasma, urine, amniotic fluid, etc., in normal and diseased states (6, 8–12). Through this approach, proteins involved in cellular functions
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EXPERIMENTAL PROCEDURES

Materials—IPG strips (18 cm) and IPG buffer, pH 4–7 linear, were purchased from Amersham Biosciences. Acrylamide/piperazine solution was obtained from Biosolve (Valenswaard, The Netherlands), and the other reagents and solutions for the polyacrylamide gel preparation were supplied from Bio-Rad. Protease inhibitors mixture and proteomics grade trypsin and chymotrypsin were obtained from Roche Diagnostics. PNGase F and neuraminidase were from Sigma, and EndoH was from New England BioLabs. The colloidal Coomassie Blue staining kit was purchased from Novex (San Diego, CA). Goat polyclonal antibody against human fetuin-A and rabbit polyclonal antibody against transthyretin were from Santa Cruz Biotechnology (Santa Cruz, CA) and horseradish peroxidase-conjugated anti-goat immunoglobulins or anti-rabbit immunoglobulins were purchased from Sigma. The ECL Western blotting detection system was purchased from Pierce. All other chemicals and reagents were from Sigma.

Sample Preparation—The study was approved by the Ethics Committee of the Aretaieion University Hospital, Athens, Greece and was registered with the University of Athens. All parturients gave their consent to participate. Clinical profile of participating mothers and full-term IUGR and AGA infants.

|                | AGA (n = 10) | IUGR (n = 10) |
|----------------|--------------|---------------|
| Maternal age (years) | 29 ± 5       | 29 ± 3        |
| Gestational age (weeks) | 38.6 ± 1.2   | 38.5 ± 1.33   |
| Birthweight (g)       | 3276 ± 356   | 2359 ± 197    |
| Mode of delivery (n (%)) | 2 (20/8) (80) | 3 (30)/7 (70) |
| Parity (n (%))        | 3 (30)/7 (70) | 4 (40)/6 (60) |
| Gender (n (%))        | 7 (70)/3 (30) | 7 (70)/3 (30) |


a Vaginal delivery/elective cesarean section.

and pathways affected by disease (13, 14) as well as putative disease biomarkers and drug targets (10) may be identified.

In the present study, we used 2-DE followed by MS to compare UC plasma protein expression profiles from appropriate for gestational age (AGA) and IUGR full-term neonates. Multiple proteins were differentially expressed in IUGR plasma, including the human α2-HS glycoprotein/fetuin-A. We showed that in IUGR the differential expression of fetuin-A is primarily at the level of post-translational modifications of the protein. Specifically using a combination of mass spectrometry and classical biochemical assays, fetuin-A was found to be differentially glycosylated/sialylated in the plasma of IUGR compared with AGA neonates. Collectively our results establish the potential importance of fetuin-A in the pathophysiology of IUGR and generate new hypotheses regarding the role of post-translational modifications in the action of the protein and its effects during fetal and later life.

The cause of intrauterine growth restriction was identified in each one of the 10 IUGR neonates included in the study. The personal, family, and perinatal histories of each parturient; maternal ultrasound; and Doppler studies (performed every 10–15 days, starting from the 32nd gestational week) of the uterine, umbilical, and middle cerebral artery were all evaluated. In four cases, IUGR resulted from preeclampsia. In the remaining six cases, parturients suffered from pregnancy-induced hypertension or chronic diseases (anemia, hepatitis B, or thyroiditis) and had small and infarcted placentas despite exclusion of intrauterine infection. Two mothers reported smoking four to five cigarettes per day. Blood flow studies were within the normal ranges in all cases, whereas amniotic fluid volume and placental weights were reduced (the latter ranging from 255 to 400 g).

In the AGA group, mothers were healthy, one smoked up to two cigarettes per day, and another reported daily consumption of one cup of coffee. Placentas were normal in appearance and weight.

Tests for congenital infections were negative in all women of both groups, and their offspring had no symptoms of intrauterine infection or signs of genetic syndromes. One- and 5-min Apgar scores were ≥7 and ≥8 in all IUGR and AGA cases, respectively. Blood was drawn from the doubly clamped UC (mixed arteriovenous blood) at delivery, reflecting fetal state. Blood was collected in pyrogen-free tubes and was immediately centrifuged in 1000 × g for 30 min. The supernatant plasma was kept frozen at −80 °C until assay. The protein concentration in plasma samples was determined using the Bradford method with a Bio-Rad protein assay reagent kit.

Two-dimensional (2-D) Gel Electrophoresis—UC plasma samples were analyzed with 2-D gel electrophoresis. In detail, 750 μg of total protein were diluted in sample buffer consisting of 50 mM Tris-HCl (pH 8.5), 7 M urea, 2 M thiourea, 2% CHAPS, 0.4% dithioerythritol (DTE), 0.2% IPG buffer (pH 4–7 linear), and 10 μl of a protease inhibitor mixture to a final volume of 250 μl. Protein samples were applied on immobilized pH 4–7 linear gradient IPG strips (18 cm) previously rehydrated for 16 h in rehydration buffer (same as sample buffer but with 8 M urea and without thiourea). Sample application was done using the cup loading method at the basic and acidic ends of the strips. Focusing was performed at 250 V for 30 min after which the voltage was gradually increased to 5000 V for 15 h and kept to 5000 V for 10 h (PROTEAN IEF Cell, Bio-Rad). After focusing, strips were equilibrated for 20 min in 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, and 0.5% DTE followed by a 20-min incubation in the same buffer containing 4% iodoacetamide instead of DTE. In the case of immunoblot analysis the second equilibration step was omitted. The second dimension was performed in 12% SDS-polyacrylamide gels (180 × 200 × 1.5 mm). The gels were run at 40 mA/ gel in an ETTAN DALT apparatus (Amersham Biosciences). In the case of immunoblot under non-reducing conditions, sample buffer without thiourea and DTE was utilized, the IPG strips were rehydrated in the absence of DTE, and prior to the second dimension, the equilibration step with DTE was omitted.

Protein Visualization and Computer Analysis—2-D gels were fixed in 50% methanol containing 5% phosphoric acid for 2 h, stained with colloidal Coomassie Blue, and scanned using the GS-800 calibrated densitometer (Bio-Rad). All gel images were analyzed using PDQuest 7.2.0 image processing software (Bio-Rad). Six 2-D gels from the IUGR and six from the AGA group were analyzed. Gel images from each group were edited, and spots were matched manually. A unique identification number was assigned to matching spots on different gels. Normalization of the spot intensities was conducted according to the total optical density in the gel (i.e., the normalized intensity was the percentage of the intensity of each spot over the sum of intensities of all detected spots in the gel). The mean value of percentage and S.E. were calculated for each spot in each group and then compared using the two-sided Student’s t test. Protein spots whose expression...
was found to be different between the two groups at the significance level of $p < 0.05$ were selected for further analysis.

Glycoprotein Detection—2-D gels were initially subjected to fluorescence staining with Pro-Q Emerald 488 glycoprotein dye (Molecular Probes) according to the manufacturer’s instructions. The gels were then stained with SYPRO Ruby fluorescence dye (Molecular Probes) for protein detection. Gel images were obtained by the use of a Typhoon 9200 laser scanner (Amersham Biosciences).

Mass Spectrometry and Protein Identification—MALDI-TOF-MS peptide analysis and protein identification were performed as described previously (12, 17). The Coomassie Blue-stained gel spots of interest were detected by the use of Melanie 4.02 software, excised from the gels with the use of Proteiner SPII (Bruker Daltonics, Bremen, Germany), and placed into 96-well microtiter plates. Protein spots were destained with 150 l of matrix solution consisting of 0.025% α-cyano-4-hydroxycinnamic acid (Sigma) and the internal standard peptides des-Arg-bradykinin (Sigma, 904.4681 Da) and adrenocorticotropic hormone fragment 18–39 (Sigma, 2465.1989 Da) in 65% ethanol, 35% acetonitrile, and 0.03% trifluoroacetic acid. Sample peptide mixtures were analyzed with a matrix-assisted laser desorption tandem time-of-flight mass spectrometer (Ultraflex II MALDI-TOF-TOF-MS, Bruker Daltonics). The peak list was created with Flexanalysis version 2.2 software (Bruker Daltonics). The signal to noise ratio was calculated by SNAP algorithm, and a threshold ratio of 2.5 was allowed. Peptide matching and protein searches were performed automatically by the use of Mascot software (Matrix Sciences Ltd., London, UK). For peptide identification monoisotopic mass accuracies were used, and a mass tolerance of 0.0025% (25 ppm) was allowed. All extraneous peaks, such as trypsin autodigests, matrix, and keratin peaks, were not considered for the protein search. Cysteine carbamidomethylation and methionine oxidation were set as fixed and variable modifications, respectively. One miscleavage was allowed. The probability score with $p < 0.05$ identified by the software was used as the criterion for the affirmative protein identification.

Neuraminidase, PNGase F, and EndoH Digestions—Neuraminidase, PNGase F, and EndoH digestions were performed overnight at 37 °C on UC plasma samples with the appropriate enzyme buffer. The buffer used for PNGase F digestion was 20 mM sodium bicarbonate, pH 8, containing 0.02% SDS, 10 mM 2-mercaptoethanol, and 1.5% Triton X-100. For neuraminidase digestion, the buffer used was 100 mM sodium acetate and 2 mM CaCl$_2$, pH 5. For EndoH digestion, the buffer utilized was 50 mM sodium citrate, pH 5.5, 0.05% SDS, and 0.1% 2-mercaptoethanol.

Western Blot Analysis—Equal protein amounts from UC plasma samples were separated by 10% SDS-PAGE or 2-D gel electrophoresis (as described above) under either non-reducing or reducing conditions. After electrophoresis, proteins were transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosciences) by electroblotting, and blots were blocked with 5% nonfat milk in TBS, 0.1% Tween at room temperature for 2 h. After washing, membranes were incubated overnight at 4 °C with the appropriate dilution (1:500) of polyclonal antibody against human fetuin-A or a 1:500 dilution of polyclonal antibody against transthyretin (Santa Cruz Biotechnology) in the case of transthyretin) as secondary antibodies at room temperature for 2 h. Bound antibody was detected by the ECL Western blotting detection system.

RESULTS

2-DE was used to analyze protein profiles of UC plasma from 10 AGA and 10 IUGR full-term neonates. Gels corresponding to six cases per category were analyzed by the use of image analysis software. Three hundred eighty spots were matched, and their expression levels were compared. Several differences between UC plasma from AGA and IUGR neonates were detected (Fig. 1, A and B). Specifically 20 protein spots were expressed at statistically significant different levels in the two groups (Fig. 1A); these were excised from the 2-D gels for identification by MALDI-TOF-MS peptide fingerprinting. Sixteen of these protein spots were positively identified (Table II). As shown, the protein spots corresponding to α$_2$-HS glycoprotein/fetuin-A (FETUA), α$_2$-antiplasmin (A2AP), antithrombin-III, serum albumin (ALBU), transthyretin (TTHY), and glittering.


Table II
Differentially expressed proteins in IUGR

Proteins were identified by MALDI-TOF-MS. Spot numbers are also shown in Fig. 1A. The accession numbers (from the Swiss-Prot database), the theoretical molecular mass and pI values, and the score from Mascot search, sequence coverage, and number of matching peptides are given. Scores higher than 52 indicate identity or extensive homology. The means ± S.E. of the OD of each spot expressed as the percentage of total OD in the gel (see “Experimental Procedures”) are shown. The ratio of the mean percentage of the protein spot in IUGR UC plasma (n = 6) to the mean percentage in the AGA UC plasma (n = 6) is also depicted. A ratio >1 indicates overexpression, whereas a ratio <1 indicates reduction.

Besides fetuin-A, the expression differences of TTHY were also confirmed by Western blot analysis (data not shown).

| Spot no. | Symbol | Protein name | Accession no. | Theoretical pI/molecular mass (kDa) | MALDI-TOF-MS Score | MALDI-TOF-MS Sequence coverage | MALDI-TOF-MS Matching peptides | Unique peptides | Unmatched peptides | Expression level AGA group (mean ± S.E.) | Expression level IUGR group (mean ± S.E.) | Ratio IUGR/AGA | p |
|----------|--------|--------------|---------------|------------------------------------|-------------------|-----------------------------|-------------------------------|----------------|--------------------|-------------------------------------|-------------------------------------|---------------|----|
| 0208     | FETUA  | α2-HS glycoprotein precursor (fetuin-A) | P02765        | 5.4/39.3                           | 59                | 18                          | 5                            | 4              | 15                 | 0.85 ± 0.08                        | 0.38 ± 0.07                        | 0.45          | 0.001 |
| 1204     | FETUA  | α2-HS glycoprotein precursor (fetuin-A) | P02765        | 5.4/39.3                           | 63                | 17                          | 5                            | 4              | 3                  | 0.10 ± 0.01                        | 0.06 ± 0.02                        | 0.60          | 0.04  |
| 2303     | A2AP   | α2-Antiplasmin precursor            | P08697        | 5.9/54.5                           | 59                | 12                          | 4                            | 5              | 7                  | 0.08 ± 0.01                        | 0.04 ± 0.01                        | 0.50          | 0.015 |
| 2305     | A2AP   | α2-Antiplasmin precursor            | P08697        | 5.9/54.5                           | 71                | 26                          | 10                           | 8              | 75                 | 0.17 ± 0.01                        | 0.11 ± 0.01                        | 0.65          | 0.01  |
| 2508     | TRFE   | Serotransferrin precursor           | P02787        | 7/77                               | 202               | 40                          | 28                           | 26             | 52                 | 0.05 ± 0.02                        | 0.15 ± 0.03                        | 3.00          | 0.04  |
| 4202     | ANT3   | Antithrombin-III precursor          | P01008        | 6.3/52.6                           | 82                | 25                          | 10                           | 9              | 18                 | 0.27 ± 0.04                        | 0.12 ± 0.03                        | 0.44          | 0.015 |
| 5001     | ALBU   | Serum albumin precursor             | P02768        | 5.9/69.3                           | 150               | 36                          | 25                           | 22             | 62                 | 0.50 ± 0.02                        | 0.27 ± 0.05                        | 0.54          | 0.003 |
| 5007     | TTHY   | Transthyretin precursor             | P02766        | 5.4/15.9                           | 124               | 50                          | 7                            | 7              | 8                  | 1.00 ± 0.11                        | 0.32 ± 0.17                        | 0.32          | 0.008 |
| 5008     | APOE   | Apolipoprotein E precursor          | P02649        | 5.5/36.1                           | 124               | 45                          | 13                           | 11             | 57                 | 0.33 ± 0.08                        | 0.12 ± 0.04                        | 0.36          | 0.035 |
| 5010     | ALBU   | Serum albumin precursor             | P02768        | 5.9/69.3                           | 55                | 17                          | 12                           | 12             | 57                 | 0.18 ± 0.02                        | 0.06 ± 0.02                        | 0.33          | 0.006 |
| 5011     | ALBU   | Serum albumin precursor             | P02768        | 5.9/69.3                           | 101               | 41                          | 14                           | 13             | 57                 | 0.10 ± 0.01                        | 0.06 ± 0.01                        | 0.60          | 0.04  |
| 5102     | FIBG   | Fibrinogen γ chain precursor        | P02679        | 5.3/51.5                           | 168               | 61                          | 22                           | 19             | 54                 | 0.27 ± 0.02                        | 0.13 ± 0.04                        | 0.48          | 0.009 |
| 5104     | FIBG   | Fibrinogen γ chain precursor        | P02679        | 5.3/51.5                           | 189               | 68                          | 23                           | 21             | 63                 | 0.25 ± 0.04                        | 0.09 ± 0.03                        | 0.36          | 0.015 |
| 6101     | FIBG   | Fibrinogen γ chain precursor        | P02679        | 5.3/51.5                           | 128               | 58                          | 16                           | 16             | 70                 | 0.30 ± 0.04                        | 0.18 ± 0.06                        | 0.60          | 0.04  |
| 6902     | CFAH   | Complement factor H precursor       | P08603        | 6.3/139                            | 295               | 34                          | 38                           | 35             | 26                 | 0.13 ± 0.03                        | 0.06 ± 0.01                        | 0.46          | 0.04  |
| 7208     | ALBU   | Serum albumin precursor             | P02768        | 5.9/69.3                           | 69                | 30                          | 14                           | 14             | 70                 | 0.13 ± 0.02                        | 0.07 ± 0.01                        | 0.54          | 0.03  |
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Fig. 2. A, Western blot analysis of fetuin-A in AGA (lanes 1–5 and 10–13) and IUGR (lanes 6–9, 14, and 15) UC plasma samples. Equal protein amounts (1 μg) of UC plasma samples from AGA and IUGR neonates were separated by 10% SDS-PAGE under reducing conditions and immunoblotted with antibody against human fetuin-A. Arrows point to the IUGR-related fetuin-A isoform. Lane 7 represents one of the IUGR UC plasma samples that did not present the IUGR-specific Al/Bl row of spots in the 2-DE analysis. B–E, 2-D Western blot analysis of fetuin-A confirming the identity of group A as the heavy chain and group B as the single chain form of fetuin-A. Equal protein amounts (50 μg) of UC plasma samples from AGA (B and D) and IUGR (C and E) neonates were separated on pH 4–7 linear IPG strips. The second dimension was carried out using 10% SDS-PAGE. Plasma samples were treated under either reducing (B and C) or non-reducing conditions (D and E). After transfer, membranes were immunoblotted with antibody against human fetuin-A. Numbers on the top of the panels indicate the pl; molecular mass markers are shown on the left.

corroborated by Western blot of UC plasma samples using fetuin-A-specific antibody. As shown in Fig. 2A (lanes 6, 8, 9, 14, and 15), in Western blot analysis, fetuin-A was represented by two protein bands in IUGR UC plasma samples compared with only one band in the AGA samples (Fig. 2A, lanes 1–5 and 10–13). In addition, 2-D Western blot analysis confirmed the identity of all spots of groups A and B including the IUGR-related Al and Bl rows of spots as fetuin-A (Fig. 2, B and C).

The difference between the two groups (A and B) of fetuin-A protein spots was initially addressed. Fetuin-A is produced in a single chain form consisting of the A-chain, the B-chain, and a connecting peptide between them (16) (Fig. 3A, left panel). The mature protein is a two-chain form generated after cleavage between the connecting peptide and the B-chain; the two generated parts, A-chain with connecting peptide and B-chain, are held together with a disulfide bond (17–20) (Fig. 3A, right panel). The A-chain and the connecting peptide form the heavy chain, whereas the B-chain is the light chain of fetuin-A. By the use of mass spectrometry, two peptides of the B-chain of fetuin-A, with peaks at m/z 2016.0 and 2285.2, correspond-
ing to amino acids 341–361 (TVQPSVGAAAGPVVPPCPGR) and 341–363 (TVQPSVGAAAGPVVPPCPGRIR), were identified solely in the basic (B) group of spots (Fig. 3C). In contrast, peptides from the A-chain and the connecting peptide (heavy chain) were identified in both A and B groups of spots. These results suggested that the spots of group B probably are isoforms of the single chain form of the protein, whereas the spots of group A represent isoforms of the two-chain form, which lacks the B-chain, possibly due to the reducing experimental conditions. To investigate this hypothesis, 2-D Western blot analysis under non-reducing conditions was conducted; in this case, only one group of fetuin-A spots was detected in either AGA (Fig. 2D) or IUGR (Fig. 2E). The pI range of this group (4.6–5.0) is the same as that of group B (Fig. 2, B and C). This result confirmed that groups B and A correspond to fetuin-A single and heavy chain forms, respectively.

The IUGR-related fetuin-A isoforms may correspond to different proteolytic cleavage and/or post-translational modifications. To address the former, several different proteases, namely trypsin, chymotrypsin, and Asp-N, were utilized as a means to increase the peptide sequence coverage received during the MS analysis. With these enzymes, 75 and 70% sequence coverage was achieved for the upper constitutively expressed and lower IUGR-related (A and B) rows of spots, respectively. Despite the high sequence coverage, no differences were observed, justifying the molecular weight difference between the constitutively expressed and A/B isoforms of fetuin-A.

Fetuin-A is a known secreted glycoprotein with two N-linked and three O-linked carbohydrate side chains (19, 21). To test whether the IUGR-related isoforms (A/B) differed from the constitutively expressed isoforms in the glycosylation state, biochemical assays were performed. Specifically, by using appropriate fluorescent dyes that detect glycoproteins, we found that both the constitutively expressed and A/B rows of fetuin-A spots were glycosylated (data not shown). Plasma samples were then treated with various glycosidases, specifically PNGase F, neuraminidase, and EndoH, and subjected to Western blot analysis. As shown in Fig. 4A, removal of the N-linked sugars with PNGase F reduced the molecular weight of fetuin-A in both AGA (lane 5) and IUGR (lane 6) UC plasma samples but did not abolish the molecular weight shift in IUGR. In contrast, neuraminidase either alone (lane 4) or in combination with PNGase F (lane 8) led to the elimination of the molecular weight difference in IUGR samples because only one immunoreactive band was detected in both IUGR (lanes 4 and 8) and AGA UC plasma samples (lanes 3 and 7). Treatment with EndoH was used as a negative control because this enzyme is known to cleave N-glycosylated bonds that do not exist in fetuin-A (Fig. 4B, lanes 3 and 4). Collectively these data indicate that the IUGR-related fetuin-A isoforms differ in the sialic acids of the O-linked sugars. These results were confirmed by 2-D Western blot analysis of neuraminidase-treated UC plasma samples.

![Fig. 4. Western blot analysis of fetuin-A in AGA and IUGR UC plasma after digestion with glycosidases showing the elimination of the IUGR-related isoforms following treatment with neuraminidase.](image)

**Fig. 4.** Western blot analysis of fetuin-A in AGA and IUGR UC plasma after digestion with glycosidases showing the elimination of the IUGR-related isoforms following treatment with neuraminidase. A, equal protein amounts (1 μg) of UC plasma samples from AGA (lanes 1, 3, 5, and 7) and IUGR (lanes 2, 4, 6, and 8) neonates were digested with either neuraminidase (Neuase) (lanes 3 and 4), PNGase F (lanes 5 and 6), or a combination of neuraminidase and PNGase F (lanes 7 and 8). UC plasma samples from AGA and IUGR neonates in the absence of glycosidases were also loaded (lanes 1 and 2). Equal volume aliquots of the digests were separated by 10% SDS-PAGE under reducing conditions and immunoblotted with antibody against human fetuin-A. Molecular mass markers are indicated on the left. B, equal protein amounts of UC plasma samples from AGA (lanes 1 and 3) and IUGR (lanes 2 and 4) neonates were digested with EndoH, (lanes 3 and 4). AGA and IUGR UC plasma samples were also incubated in the absence of glycosidase (lanes 1 and 2). Western blot analysis was carried out as described in A.

![Fig. 5. 2-D Western blot analysis of fetuin-A in neuraminidase-treated AGA and IUGR UC plasma samples.](image)

**Fig. 5.** 2-D Western blot analysis of fetuin-A in neuraminidase-treated AGA and IUGR UC plasma samples. The elimination of the IUGR-related isoforms in both heavy and single chain forms of fetuin-A is shown. Equal protein amounts (50 μg) of UC plasma samples from AGA (C) and IUGR (D) neonates were incubated with neuraminidase. Digested proteins were separated on pH 4–7 linear IPG strips. The second dimension was carried out using 10% SDS-PAGE. Control AGA and IUGR UC plasma samples were incubated in the absence of neuraminidase (A and B). After transfer, membranes were immunoblotted with antibody against human fetuin-A. Arrows point to the desialylated single chain form of fetuin-A. Numbers on the top of the panels indicate the pI; molecular mass markers are shown on the left.

As shown in Fig. 5, two spots that corresponded to the desialylated heavy chain of fetuin-A (group A) were generated following treatment with neuraminidase in either AGA (Fig. 5C) or IUGR samples (Fig. 5D). In addition, one additional low
abundance spot (Fig. 5, C and D, arrows) was seen in both samples after incubation with neuraminidase, probably representing the desialylated single chain form (group B) of fetuin-A.

**DISCUSSION**

The comparison of protein profiles of UC plasma from AGA and IUGR full-term neonates using proteomics techniques was conducted to identify changes in protein expression that might be informative of the mechanisms underlying the IUGR state and/or serve as potential markers for its postnatal complications. 2-DE-based proteomics methodologies enable us to study global changes in expression levels and post-translational modifications of proteins in a given sample (6, 7, 11, 12). To our knowledge, the current study is the first proteomics analysis of UC plasma from AGA and IUGR full-term neonates. UC blood is a body fluid that reflects the fetal circulation compartment and hence the fetal state; expression profiles of UC blood growth factors and hormones were previously shown to vary in the presence of abnormal fetal growth (3–5).

Our data from 2-DE in combination with MS analysis showed that α1-HS glycoprotein/fetuin-A exhibits a markedly different expression pattern between AGA and IUGR UC plasma. Both the single and heavy chain forms of the protein (18) were present in the UC plasma of AGA and IUGR neonates and were represented by multiple distinct spots possibly corresponding to different post-translational modifications and/or protein polymorphisms.

Fetuin-A is a circulating plasma glycoprotein produced abundantly during fetal life by multiple tissues, suggesting development-associated function(s); its concentration in plasma decreases rapidly after birth, and in the adult this glycoprotein is produced mainly by the liver (22). Fetuin-A is a member of the cystatin superfamily of proteins; it accumulates in bone where it antagonizes the activities of transforming growth factor-β and the bone morphogenetic proteins (23, 24). It has a high affinity for calcium and has been implicated in bone formation and prevention of ectopic calcifications (25) by decreasing cytokine-dependent osteogenesis and preventing phosphate precipitation, respectively (23, 26). In addition, bovine fetuin has been shown to stimulate cell growth (27).

In humans, fetuin-A is a negative acute phase reactant, and its serum levels decrease significantly in response to infection and/or inflammation (28). Furthermore it acts as an anti-inflammatory mediator (29) and protects against lipopolysaccharide-induced shock (30). In addition, fetuin-A has been associated with the regulation of insulin-mediated cell signaling pathways, causing insulin resistance by interacting with the insulin receptor and inhibiting insulin-induced insulin receptor autophosphorylation and downstream signaling (31, 32). Interactions of fetuin-A with other molecules may be mediated by structural polypeptide features as well as by post-translational modifications of the protein, such as sialylation (33).

We found that in IUGR fetuin-A isoforms lacking O-linked sialic acids (21) were uniquely present. Carbohydrate residues and oligosaccharide chains play diverse and crucial roles in several biological processes, e.g., maintenance of protein conformation, protection of proteins from proteases, control of active epitopes and antigenicity, blood clotting, embryogenesis, and development (34). Sialic acids in particular are the most common terminal carbohydrate residues in the glycan moiety of glycoproteins, contributing thereby significantly to the charge and activity of proteins. Along these lines, desialylation caused alterations in the structure and function of glycoproteins (35, 36), affecting protein-protein interactions (37), integrin-mediated cell adhesion (38), and immune recognition (39). As expected, sialic acids may be involved in both physiological and pathological phenomena (40). For example, desialylation is enhanced in many clinical conditions, such as IgA nephropathy (41) and metastasis of cancer cells, possibly through alterations in cellular adhesion (42). Furthermore, in the case of circulatory proteins, loss of the sialic acid moiety may accelerate glycoprotein degradation by liver cells through binding to asialoglycoprotein receptors (43).

The reduced sialylation observed in IUGR-related fetuin-A isoforms may have a major impact on the structure and/or function of this protein. Further mechanistic studies will be necessary to address this hypothesis; nevertheless there is ample evidence pointing to this direction: in breast and lung cancer cell lines, interaction with and hence response of cells to fetuin-A require the presence of sialic acid residues on the latter as well as of Ca$$^{2+}$$ ions (33, 44) as evidenced by the lack of cell adhesion to asialofetuin. Aside from sialic acid residues, the overall glycosylation status of fetuin-A may also influence its cell adhesive properties in the presence of calcium. IUGR has been associated with low bone mineral content and reduced bone formation (45). We may thereby hypothesize that the absence of sialic acids seen in IUGR neonates may impair the interactions of fetuin-A with calcium ions and/or other proteins leading to dysregulation of functions regulated by fetuin-A, such as calcification and bone formation (23–26). Furthermore differential levels of fetuin-A have been associated with cardiovascular disease, type 2 diabetes mellitus, and metabolic syndrome (32, 46, 47), complications that have been observed in IUGR cases later in their lives (1, 2). Therefore, the IUGR-related fetuin-A isoforms might also be associated with the development of these complications.

In conclusion, we have demonstrated that marked deficiencies in glycosylation/sialylation of fetuin-A occur in UC plasma of IUGR fetuses. It is probable that in IUGR alterations in the glycosylation status may not be limited to fetuin-A but may be occurring in other proteins as well. In the case of fetuin-A, this defect may be responsible for impaired function of the protein leading to dysregulation of fetal growth, specifically osteogenesis, and/or to the development of complications seen later in the lives of IUGR neonates. These hypotheses remain to be elucidated.
**Human Fetuin-A Glycosylation in Neonates with IUGR**

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