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*

Panagiotis M. Karamessinis‡§, Ariadne Malamitsi-Puchner¶¶, Theodora Boutsikou¶¶, Manousos Makridakis‡, Konstantinos Vougas‡, Michael Fountoulakis¶¶, Antonia Vlahou‡, and George Chrousos‡**‡‡

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. Molecular & Cellular Proteomics 7:591–599, 2008.

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

From the ‡Division of Biotechnology and Section of Endocrinology and Metabolism, Biomedical Research Foundation, Academy of Athens, 11527 Athens, Greece, ¶¶Neonatal Division, Second Department of Obstetrics and Gynecology, University of Athens, Aretaieion Hospital, 115 28 Athens, Greece, ‡Center for Medical Genomics, F. Hoffmann-La Roche Ltd., CH-4070 Basel, Switzerland, and **First Department of Pediatrics, Athens University, Aghia Sophia Childrens Hospital, 11527 Athens, Greece

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1 The abbreviations used are: IUGR, intrauterine growth restriction; UC, umbilical cord; AGA, appropriate for gestational age; 2-D, two-dimensional; 2-DE, two-dimensional electrophoresis; DTE, dithioerythritol; A2AP, α2-antiplasmin; ALBU, serum albumin; FETUA, α2-HS glycoprotein/fetuin-A; FIBG, fibrinogen γ chain; TTHY, transthyretin; PNGase, peptide-N-glycosidase; HS, Heremans-Schmid; EndoH, endo-β-N-acetylgulcosaminidase H1.
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 αHS 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.

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 transhyretin 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 performed after obtaining written informed consent from the mothers of the fetuses. Ten asymmetric IUGR and 10 AGA full-term singleton neonates, as well as their mothers, all of Greek origin, were included in the study (Table I). Offspring of mothers with gestational pathology and with a birth weight below the 10th customized centile were characterized as IUGR. The Gestation Related Optimal Weight (15, 16) computer-generated program was used to calculate the customized centile for each pregnancy, taking into consideration significant determinants of birth weight, such as maternal height and booking weight, ethnic group, parity, gestational age, and gender.

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 uterus, 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 infected 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 x 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 μl urea, 2 μl 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 μl 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 μl 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 x 200 x 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

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**TABLE I**

Clinical profile of participating mothers and full-term IUGR and AGA infants

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

* a Vaginal delivery/ elective cesarean section.
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 Proteineer SPII (Bruker Daltonics, Bremen, Germany), and placed into 96-well microtiter plates. Protein spots were destained with 150 \( \mu l \) of 30% acetonitrile in 50 m\( M \) ammonium bicarbonate, washed with 150 \( \mu l \) of ultrapure water, and dried in a speed vacuum concentrator (MaxiDry Plus, Heto, Allerod, Denmark). Each dried gel piece was digested with 50 ng of one of the following proteases, trypsin, chymotrypsin, or Asp-N, in the appropriate enzyme buffer according to the manufacturer’s protocol. After 16 h at room temperature, peptides were extracted by adding 10 \( \mu l \) of 50% acetonitrile containing 0.3% trifluoroacetic acid to each gel piece. The peptide mixture (1.5 \( \mu l \)) was simultaneously applied on the sample target with 1 \( \mu l \) of matrix solution consisting of 0.025% \( \alpha \)-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 peptide masses 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 m\( M \) sodium bicarbonate, \( \text{pH} \) 8, containing 0.02% SDS, 10 m\( M \) 2-mercaptoethanol, and 1.5% Triton X-100. For neuraminidase digestion, the buffer used was 100 m\( M \) sodium acetate and 2 m\( M \) CaCl\(_2\), \( \text{pH} \) 5. For EndoH digestion, the buffer utilized was 50 m\( M \) sodium citrate, \( \text{pH} \) 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 same buffer without Tween 20. After washing with TBS, 0.1% Tween, membranes were incubated with horseradish peroxidase-conjugated anti-goat immunoglobulins (anti-rabbit immunoglobulins 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 \( \alpha_2 \)-HS glycoprotein/fetuin-A (FETUA), \( \alpha_2 \)-antiplasmin (A2AP), antithrombin-III, serum albumin (ALBU), transthyretin (TTHY),
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 \( \pm \) 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).

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Symbol</th>
<th>Protein name</th>
<th>Accession no.</th>
<th>Theoretical pl/molecular mass (kDa)</th>
<th>MALDI-TOF-MS</th>
<th>Expression level AGA group (mean ( \pm ) S.E.)</th>
<th>Expression level IUGR group (mean ( \pm ) S.E.)</th>
<th>Ratio = IUGR/AGA</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>0208 FETUA</td>
<td>( \alpha_2 )-HS glycoprotein precursor (fetuin-A)</td>
<td>P02765</td>
<td>5.4/39.3</td>
<td>59/18/5/4/15</td>
<td>0.85 ( \pm ) 0.08</td>
<td>0.38 ( \pm ) 0.07</td>
<td>0.45 ( \pm ) 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1204 FETUA</td>
<td>( \alpha_2 )-HS glycoprotein precursor (fetuin-A)</td>
<td>P02765</td>
<td>5.4/39.3</td>
<td>63/17/5/4/3</td>
<td>0.10 ( \pm ) 0.01</td>
<td>0.06 ( \pm ) 0.02</td>
<td>0.60 ( \pm ) 0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2303 A2AP</td>
<td>( \alpha_2 )-Antiplasmin precursor</td>
<td>P08697</td>
<td>5.9/54.5</td>
<td>59/12/4/5/7</td>
<td>0.08 ( \pm ) 0.01</td>
<td>0.04 ( \pm ) 0.01</td>
<td>0.50 ( \pm ) 0.015</td>
<td></td>
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<tr>
<td>2305 A2AP</td>
<td>( \alpha_2 )-Antiplasmin precursor</td>
<td>P08697</td>
<td>5.9/54.5</td>
<td>71/26/10/8/75</td>
<td>0.17 ( \pm ) 0.01</td>
<td>0.11 ( \pm ) 0.01</td>
<td>0.65 ( \pm ) 0.01</td>
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<tr>
<td>2508 TRFE</td>
<td>Serotransferrin precursor</td>
<td>P02787</td>
<td>7/77</td>
<td>202/40/28/26</td>
<td>0.05 ( \pm ) 0.02</td>
<td>0.15 ( \pm ) 0.03</td>
<td>3.00 ( \pm ) 0.04</td>
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<tr>
<td>4202 ANT3</td>
<td>Antithrombin-III precursor</td>
<td>P01008</td>
<td>6.3/52.6</td>
<td>82/25/10/9/18</td>
<td>0.27 ( \pm ) 0.04</td>
<td>0.12 ( \pm ) 0.03</td>
<td>0.44 ( \pm ) 0.015</td>
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<tr>
<td>5001 ALBU</td>
<td>Serum albumin precursor</td>
<td>P02768</td>
<td>5.9/69.3</td>
<td>150/36/25/22/62</td>
<td>0.50 ( \pm ) 0.02</td>
<td>0.27 ( \pm ) 0.05</td>
<td>0.54 ( \pm ) 0.003</td>
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<tr>
<td>5007 TTHY</td>
<td>Transthyretin precursor</td>
<td>P02768</td>
<td>5.4/15.9</td>
<td>124/50/7/7</td>
<td>1.00 ( \pm ) 0.11</td>
<td>0.32 ( \pm ) 0.17</td>
<td>0.32 ( \pm ) 0.008</td>
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<tr>
<td>5008 APOE</td>
<td>Apolipoprotein E precursor</td>
<td>P02649</td>
<td>5.5/36.1</td>
<td>124/45/13/11/57</td>
<td>0.33 ( \pm ) 0.08</td>
<td>0.12 ( \pm ) 0.04</td>
<td>0.36 ( \pm ) 0.035</td>
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<tr>
<td>5010 ALBU</td>
<td>Serum albumin precursor</td>
<td>P02768</td>
<td>5.9/69.3</td>
<td>55/17/12/12/57</td>
<td>0.18 ( \pm ) 0.02</td>
<td>0.06 ( \pm ) 0.02</td>
<td>0.33 ( \pm ) 0.006</td>
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</tr>
<tr>
<td>5011 ALBU</td>
<td>Serum albumin precursor</td>
<td>P02768</td>
<td>5.9/69.3</td>
<td>101/41/14/13/57</td>
<td>0.10 ( \pm ) 0.01</td>
<td>0.06 ( \pm ) 0.01</td>
<td>0.60 ( \pm ) 0.04</td>
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<tr>
<td>5102 FIBG</td>
<td>Fibrinogen ( \gamma ) chain precursor</td>
<td>P02679</td>
<td>5.3/51.5</td>
<td>168/61/22/19/54</td>
<td>0.27 ( \pm ) 0.02</td>
<td>0.13 ( \pm ) 0.04</td>
<td>0.48 ( \pm ) 0.009</td>
<td></td>
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</tr>
<tr>
<td>5104 FIBG</td>
<td>Fibrinogen ( \gamma ) chain precursor</td>
<td>P02679</td>
<td>5.3/51.5</td>
<td>189/68/23/21/63</td>
<td>0.25 ( \pm ) 0.04</td>
<td>0.09 ( \pm ) 0.03</td>
<td>0.36 ( \pm ) 0.015</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6101 FIBG</td>
<td>Fibrinogen ( \gamma ) chain precursor</td>
<td>P02679</td>
<td>5.3/51.5</td>
<td>128/58/16/16/70</td>
<td>0.30 ( \pm ) 0.04</td>
<td>0.18 ( \pm ) 0.06</td>
<td>0.60 ( \pm ) 0.04</td>
<td></td>
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<tr>
<td>6902 CFAH</td>
<td>Complement factor H precursor</td>
<td>P08603</td>
<td>6.3/139</td>
<td>295/34/38/35/26</td>
<td>0.13 ( \pm ) 0.03</td>
<td>0.06 ( \pm ) 0.01</td>
<td>0.46 ( \pm ) 0.04</td>
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<tr>
<td>7208 ALBU</td>
<td>Serum albumin precursor</td>
<td>P02768</td>
<td>5.9/69.3</td>
<td>69/30/14/14/70</td>
<td>0.13 ( \pm ) 0.02</td>
<td>0.07 ( \pm ) 0.01</td>
<td>0.54 ( \pm ) 0.03</td>
<td></td>
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</table>
apolipoprotein E, fibrinogen \( \gamma \) chain (FIBG), and complement factor H were significantly down-regulated, whereas serum transferrin was significantly up-regulated in IUGR compared with AGA UC plasma samples. Notably differences in these spots were also observed in 2-DE gels corresponding to the remaining four IUGR and 4AGA samples, which were not included in the image analyses.

In the case of fetuin-A, an interesting expression pattern was observed. Indeed fetuin-A was represented in all samples by multiple spots that can be distinguished, according to the pl, in two major groups, one acidic (Fig. 1, C and D, left ellipse) and one basic (Fig. 1, C and D, right ellipse) referred to as group A and B, respectively. Besides the pl differences, these two groups differed also in molecular weight with group B spots being of higher molecular weight compared with spots of group A. Interestingly in both groups, an additional row of fetuin-A protein spots with lower molecular weight was detected in eight of 10 IUGR UC plasma samples (Fig. 1D, shown with arrows, referred to as Al and Bl) but in none of the 10 AGA samples. The differential expression of fetuin-A was 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 values 2016.0 and 2285.2, correspond-
Human Fetuin-A Glycosylation in Neonates with IUGR

...iting 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 (AI and BI) rows of spots, respectively. Despite the high sequence coverage, no differences were observed, justifying the molecular weight difference between the constitutively expressed and AI/BI 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 (AI/BI) 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 AI/BI 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.

As shown in Fig. 5, two spots that correspond 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
and maintenance of protein con-
abundance spot (Fig. 5, C and D, arrows) was seen in both
samples after incubation with neuraminidase, probably repre-
senting 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 com-
plications. 2-DE-based proteomics methodologies enable us
to study global changes in expression levels and post-trans-
lational modifications of proteins in a given sample (6, 7, 11,
12). To our knowledge, the current study is the first proteom-
ics 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 pre-
viously shown to vary in the presence of abnormal fetal
growth (3–5).

Our data from 2-DE in combination with MS analysis
showed that α2-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 neo-
nates and were represented by multiple distinct spots possi-
bly 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 accumu-
lates in bone where it antagonizes the activities of transform-
ing 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 prevent-
ing 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-in-
flammatory mediator (29) and protects against lipopolysac-
charide-induced shock (30). In addition, fetuin-A has been
associated with the regulation of insulin-mediated cell signal-
ning pathways, causing insulin resistance by interacting with
the insulin receptor and inhibiting insulin-induced insulin re-
ceptor 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 sialy-
alation (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 con-
formation, protection of proteins from proteases, control of
active epitopes and antigenicity, blood clotting, embryogen-
esis, 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, desial-
ylation caused alterations in the structure and function of
glycoproteins (35, 36), affecting protein-protein interactions
(37), integrin-mediated cell adhesion (38), and immune recog-
nition (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 resi-
dues, the overall glycosylation status of fetuin-A may also
influence its cell adhesive properties in the presence of cal-
cium. IUGR has been associated with low bone mineral con-
tent and reduced bone formation (45). We may thereby hy-
pothesize 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 func-
tions regulated by fetuin-A, such as calcification and bone for-
mation (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 deficien-
cies 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 osteogen-
esis, and/or to the development of complications seen later in
the lives of IUGR neonates. These hypotheses remain to be
elucidated.
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**To whom correspondence may be addressed:** Division of Biotechnology, Biomedical Research Foundation, Academy of Athens, 4 Soronou Efeiou, 11527 Athens, Greece. Tel.: 302106597485; Fax: 302106597545; E-mail: pkaramessinis@bioacademy.gr.

†† To whom correspondence may be addressed: First Dept. of Pediatrics, University of Athens, Aghia Sophia Childrens Hospital, Thivon & Papadimantopoulou, 11527 Athens, Greece. Tel.: 302107794023; Fax: 302107759167; E-mail: chrousge@med.uoa.gr.

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