Clinical Biochemistry 48 (2015) 167–173

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Terminal glycotope expression on milk fibronectin differs from plasma fibronectin and changes over lactation

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ARTICLE INFO

Article history:
Received 11 August 2014
Received in revised form 13 October 2014
Accepted 8 November 2014
Available online 20 November 2014

Keywords:
Fibronectin
Fucosylation
Human milk
Lactation
Lectins
O-glycans
Sialylation

ABSTRACT

Objectives: Fibronectin (FN) is a multifunctional glycoprotein appearing in various glycovariants with potential biological activities. Using lectins we analyzed the expression of terminal glycotopes on human milk fibronectin over lactation and compared it with that of the mother's plasma.

Design and methods: FN concentration and relative amounts of its fucosylated and sialylated glycovariants as well as O-glycans were analyzed in early colostrum, colostrum, transitional and mature milk samples of 132 healthy mothers by lectin-FN-ELISA using α2,3- and α2,6-sialic acid, α1,2-, α1,3-, and α1,6-fucose, and sialyl-T, asialyl-T and Tn antigen specific biotinylated *Maackia amurensis*, *Sambucus nigra*, *Ulex europaeus*, *Tetragonolobus purpureus*, *Lens culinaris*, *Artocarpus integrifolia*, *Arachis hypogaea*, and *Vicia villosa* lectins, respectively.

Results: FN concentration was almost unchanged during human milk maturation and was about 150 times lower than in plasma of lactating mothers. Milk FN elicited significantly higher expression of sialylated glycotopes including sialyl-T antigen than plasma FN, and contained fucose-linked glycans, as well as T and Tn antigens absent in plasma FN. With milk maturation the expression of α2,6-sialylated, sialyl-T, α1,6- and α1,2-fucosylated epitopes decreased in transitional milk compared with colostrum, whereas that of asialyl-T antigen increased. The expression levels of α2,3-sialyl- and α1,3-fucosyl-glycotopes and Tn antigen on FN were low and did not change over lactation.

Conclusion: The expression of terminal sugars on milk FN is different from that of plasma FN of the lactating mother and is associated with milk maturation. The analysis of degree of milk sialylation and fucosylation should be considered during control of biochemical quality of milk collected in milk banks.
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Introduction

Fibronectin (FN), a multidomain and multifunctional large glycoprotein, is a soluble component of the plasma and human milk and an insoluble component of the extracellular matrix [1–4]. Fibronectin, produced by various cells (e.g. fibroblasts, chondrocytes, lymphocytes, endothelial cells) is known to be trapped in insoluble multimeric fibrils, while that present in plasma (produced by hepatocytes) is released to the blood as a compact globular dimer [3,5]. The milk FN could originate from local synthesis by mammary epithelial cells, lymphocytes, and macrophages, and also part of it might originate from filtration of the blood [1]. FN mediates cellular interactions and plays important roles in cell adhesion, migration, proliferation, differentiation, tissue repair and extracellular matrix remodeling [5,6]. FN has a remarkably wide variety of functional activities; it binds to its biologically important natural ligands, such as collagen/gelatin, C-reactive protein, fibrin, heparin, integrins, and complement components [3,5,7]. These interactions are mediated by structural, including glycotopes, and functional domains [5,8].

Human plasma FN contains 5–9% oligosaccharides, mainly N-glycans, and to a lesser degree O-glycans [5,8]. The extent and type of FN glycosylation depend on glycoprotein origin and pathophysiological status [9,10]. The plasma and cellular FNs differ particularly in the amount of N- and O-linked glycans, number of antennae, and terminal sialic acid and fucose expression. Plasma-derived FN N-glycans can be di- and triantennary, sialylated and very slightly or nearly absent α1,6-fucosylated. In contrast, cellular FN has diantennary glycans, largely α1,6-fucosylated, but weakly sialylated [5,8,10]. The O-glycan (α2,3-sialylated Galβ1,3GalNAc-) attached to the hexapeptide VTHPGY, in the variable region of alternative-spliced FN, forms an extra domain commonly called the oncofetal IIICS epitope which is one of the effective

Abbreviations: FN, fibronectin; Jacalin, *Artocarpus integrifolia* lectin; LCA, *Lens culinaris* lectin; LTA, *Lotus tetragonolobus* lectin; MAA, *Maackia amurensis* lectin; PNA, *Arachis hypogaea* lectin; sialyl-T antigen, sialyl-Galβ1,3GalNAc; SNA, *Sambucus nigra* lectin; T antigen, Galβ1,3GalNAc; Tn antigen, GalNAc; UEA, *Ulex europaeus* lectin; VVL, *Vicia villosa* lectin.

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http://dx.doi.org/10.1016/j.clinbiochem.2014.11.011
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markers for preterm delivery [8,10,11]. However, the role of mucin-type O-glycan has not been elucidated.

The physiological role of FN glycans is uncertain. However, they maintain the topology and domain functions of FN as well as stabilizing FN against hydrolysis [8]. Moreover, FN glycans can modulate its affinity to some substrates [5]. FN, being a glycoprotein, has a potential ability to bind some pathogens via lectin-carbohydrate or even carbohydrate-to-carbohydrate interactions. FN glycans are important for adhesion and host tissue colonization by *Borrelia burgdorferi* (which causes human Lyme disease) [12], *Campylobacter fetus* and *Campylobacter jejuni* (the most frequent bacterial causes of gastrointestinal disease and diarrhea) [13,14], and *Streptococcus pneumoniae* (the etiological agent of pneumonia) [15]. Moreover, the α1,2-fucosylated FN isoform of amniotic fluid might play a critical role in protecting the fetus and female reproductive routes against pathogenic bacteria [10], which can colonize female tissues. Soluble fucosylated FN may decoy binding sites for pathogens, thus inhibiting pathogen binding to host cell ligand [10,14,15]. However, to date there are no reports concerning the fucosylation and sialylation patterns of human milk FN over lactation and its possible roles. It can be expected that FN of human milk transferred to newborns can take part in bacteria–host interactions, bacterial colonization, and phagocytosis.

In our study, we analyzed the alternations in the expression of *Maackia amurensis* agglutinin (MAA) and *Sambucus nigra* agglutinin (SNA) reactive α2,3 and α2,6 sialyl-glycotopes and *Lens culinaris* agglutinin (LCA), *Lotus tetragonolobus* agglutinin (LTA), and *Ulex europaeus* agglutinin (UEA) reactive α1,6, α1,3, and α1,2 fucosyl-glycotopes as well as *Artocarpus integrifolia* agglutinin (Jacalin), peanut (*Arachis hypogaea*) agglutinin (PNA), and *Vicia villosa* agglutinin (VVL) reactive T and Tn antigens of human milk FN over normal lactation from the 2nd to the 50th day. The degree of α2,3 and α2,6 sialylation and α1,2, α1,3, and α1,6 fucosylation as well as T and Tn antigens patterns was determined on milk and plasma FN, extracted from samples by ELISA plates coated with polyclonal anti-FN antibodies (desialylated and defucosylated). Lectin-ELISA does not determine the structure of human FN glycans, but it allows one to analyze the expression of terminal glycotopes via interaction with lectins.

Materials and methods

Participants

Human milk samples (n = 132) were collected from healthy lactating mothers receiving regular perinatal care at the 1st and 2nd Departments of Gynecology and Obstetrics at Wroclaw Medical University, Poland. Lactating mothers were recruited for a protocol approved by the Ethics Committee at Wroclaw Medical University (KB-30/2013) (informed consent was obtained from all participants). Mothers' age ranged from 21 to 35 years. For inclusion in the study, mothers had to have a good state of health, an uncomplicated, normal pregnancy, a pre-pregnancy body mass index (in kg/m^2) between 18 and 25, and they had received regular care during pregnancy. Mothers with inflammation (e.g., mastitis or infection) as well as mothers who used tobacco products, illicit alcohol, drugs, or were pregnant with multiple fetuses were excluded.

Sample collection and preparation

Human milk samples were collected at the end of nursing (hindmilk), once per day at the same time (8.00–10.00 a.m.) by a trained nurse by complete breast emptying by manual expression. There was a significant interindividual variation in volume of hindmilk, between 5 mL for colostrum to 100 mL for mature milk. To protect the integrity of samples, immediately after collection (up to 30 min.), all milk samples were frozen in sterile plastic containers and stored at −20 °C until analysis. Before handling the milk samples were kept for 1 h at room temperature to avoid FN aggregation. Skim milk (aqueous phase) was prepared by centrifugation 3500 ×g at +4 °C for 30 min, after which the fat layer and cells were removed. All samples were assayed by the same researcher.

Milk samples were divided into the following groups: (1) early colostrum (days 2–3 of lactation; n = 18), (2) colostrum (days 4–7 of lactation; n = 52), (3) transitional milk (days 8–14 of lactation; n = 37), and (4) mature milk (days 15–50 of lactation; n = 25).

Additionally, plasma samples from lactating women on postpartum day 2 (n = 30) and healthy volunteers (n = 25) were included.

Determination of FN concentration

The concentration of FN was determined by ELISA, according to a slightly modified procedure described earlier [16,17] using monoclonal antibody anti-cell binding domain (CBD) of human FN (TaKaRa Shuzo Co. Ltd., Shiga, Japan). An FN preparation purchased from Sigma (St. Louis, MO, USA) was used as a standard protein.

Briefly: the monoclonal antibody directed to anti-CBD-FN (FN 30-8; M010, diluted 1:10 000) was used as a coating agent of the wells of a microtiter plate (Nalge Nunc International, Naperville, IL, USA) to bind FN from the sample. Before the analysis the aliquots of human milk and plasma samples were kept for 1 h at room temperature to avoid FN aggregation.

The milk and plasma samples need to be diluted with Tris-buffered saline containing 0.05% Tween 20 (TBS-T), 25- and 50-fold for milk and 4000- and 8000-fold for plasma samples. The samples were analyzed in two different sample dilutions, each in duplicate. The amount of FN bound by the anti-FN monoclonal antibody was quantified by rabbit anti-FN polyclonal antibodies (Sigma Chemical Co., St Louis, MO, USA, diluted 1:5 000) and by the secondary antibody peroxidase conjugated goat anti-rabbit immunoglobulins (Sigma Chemical Co., St Louis, MO, USA, diluted 1:20 000). The test was assayed by a colorimetric reaction using o-phenylenediamine dihydrochloride/H_2O_2 as the enzyme substrate and measured in a Stat Fax 2100 Microplate Reader (Awareness Technology, Inc., Palm City, FL, USA) at 492 nm with 630 nm as a reference filter.

All ELISA immunobinding and washing steps, except the coating step, were carried out in Tris-buffered saline, pH 7.3 (TBS) containing 0.1% Tween 20 (TBS-T) and 0.2% bovine albumin in TBS-T was used as a blocking agent.

The FN concentration is given in mg/L and presented as the mean ± standard deviation (SD) and median value (25th–75th percentile). The background absorbance (with buffer instead of standard or sample, but with all other reagents) ranged from 0.02 to 0.04 depending on the microtiter plate and the day of the experiment.

Lectin-based analysis of FN glycotopes

Expression of sialyl- and fucosyl-glycotopes as well as T and Tn antigens on a constant amount (50 ng) of FN was determined by lectin-FN-ELISA according to a slightly modified procedure described earlier [16, 18] using specific biotinylated lectins (Vector Laboratories, Inc., Burlingame, CA, USA) with well-described binding preferences described by Wu et al. [19]. MAA (*M. amurensis* agglutinin) and SNA (*S. nigra* agglutinin) have the abilities to bind sialic acid linked by anomeric glycosidic α2,3 and α2,6 linkages Gal/GalNAc, respectively. LCA (*L. culinaris* agglutinin), LTA (*L. tetragonolobus* agglutinin), and UEA (*U. europaeus* agglutinin) have the binding preferences to fucose linked by anomeric glycosidic α1,6, α1,3, and α1,2 linkages, respectively. PNA (*A. hypogaea* agglutinin), Jacalin (*A. integrifolia* agglutinin), and VVL (*V. villosa* agglutinin) are known to have binding preferences to asialylated T antigen (Galβ1,3GalNAc), sialylated T antigen (sialyl-Galβ1,3GalNAc), and Tn antigen (GalNAc-), respectively.

The experimental details of lectin-FN-ELISA are as follows: goat anti-human FN antibodies, oxidized with sodium periodate and desialylated

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