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Development of High-Sensitive ELISA Method for Detection of Adipophilin Levels in Human Colostrum and Breast Milk

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Aim: To develop and validate high-sensitive (hs) ELISA method for detection of adipophilin (adipose differentiation-related protein, ADRP) in human breast milk (BM) and to analyze adipophilin levels in BM during 12 months of lactation. *Methods:* ADRP levels were determined using hsELISA method (Biovendor-Laboratory Medicine, Inc.) in colostrum (D0) and BM of 72 mothers was collected 1, 3, 6, and 12 months following delivery (M1, 3, 6, 12). *Results:* ADRP was detectable in BM up to 12 months of lactation. Mean levels at D0 were 1.98 $\pm$ 0.12; M1, 2.83 $\pm$ 0.21; M3, 2.39 $\pm$ 0.17; M6, 2.57 $\pm$ 0.16; and at M12 3.25 $\pm$ 0.21 μg/ml. Significantly higher levels of ADRP were found in M1 and M12 when compared to D0 and in M12 when compared to M3 (overall $P = 0.0001$). No significant correlation was seen between ADRP levels in BM and adiponectin, body weight of infants, their birth length, body weight gain during the first year of life, or BMI of mothers before pregnancy. *Conclusions:* We developed and validated hsELISA for detection of ADRP in human BM. ADRP was detectable in human BM during the whole 12 months of lactation period and its levels were intraindividually well-conserved. J. Clin. Lab. Anal. 28:255–260, 2014.

Key words: adipophilin; ADRP; human breast milk; nutritional programming; ELISA

INTRODUCTION

Breast milk (BM) is a rich source of nutritional and bioactive substances and its short-term as well as long-term beneficial effects are well known. Lots of new molecules have been identified in recent years whose role is yet to be defined.

Protective role of breastfeeding from various components of metabolic syndrome is presumed, but the components of BM and mechanisms responsible for this effect were not identified so far. Possible candidate molecules include regulatory hormones of food intake and glucose/lipid metabolism, such as adiponectin, leptin, ghrelin, adipocyte fatty acid binding protein (AFABP), which were recently found in human BM (1, 2).

Adipophilin (adipose differentiation-related protein, ADRP, perilipin-2) belongs to a family of PAT proteins (perilipin, adipophilin, and TIP47) that are believed to be critical regulators of lipid accumulation in eukaryotic cells (3). It was first identified as an RNA transcript significantly induced during differentiation of cultured mouse adipogenic cell line (4, 5). The name adipophilin was

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proposed for human analog by Heid et al(6). Its molecular weight is 52 kDa and its structure similarity to perilipin led to the discovery of ADRP on the surface of lipid droplets (LDs). LDs are the major organelles for the storage of neutral lipids in cells. Basically, they are vesicules filled with triglycerides and cholesterol esters coated by a membrane, produced in endoplasmatic reticulum by a membrane envelopment process (7). PAT family proteins are involved in the coating of LDs and play a key role in regulation and secretion of cytoplasmic LDs (3).

In BM, lipids are the major energy source. Produced by epithelial (milk secreting) cells in form of LDs, they are transferred to lumen of mammary alveoli by exocytosis, during which they are wrapped in plasma membrane (called milk fat globule membrane (MFGM; (8)). ADRP is a predominant LD protein in milk. It was found in differentiating and lactating mammary gland where it was directly linked to accumulation of cytoplasmic LDs (9). Further research showed that ADRP plays a key role in sequestering triglycerides in LDs, their formation and maturation (6), and eventually their secretion in the lumen (10, 11), where it becomes part of the MFGM (12).

In previous studies, various methods of preanalytical and analytical procedures were used for detection of ADRP in BM of humans and some animal species. Usually, it was detected in defractionated MFGM after isolation of MFGs from milk cream layer using methods such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), sequence analysis, Western blotting, or mass spectrometry (13).

In this study, we established and evaluated an immunoassay for quantitative determination of ADRP in skimmed human BM. We analyzed intraindividual dynamic changes of ADRP levels in BM during 12 months of lactation and its relationship to nutritional parameters of infants and mothers as well as to other BM hormones (adiponectin, leptin, and AFABP).

MATERIALS AND METHODS

Patients

BM samples were obtained from mothers who had delivered their children at the Department of Gynaecology and Obstetrics of the Motol University Hospital during the period of Autumn 2006 to Summer 2008. Only mothers with noncomplicated vaginal delivery of full-term infants were included and they were selected from a large cohort of mothers who collected BM samples within another prospective study concerning allergy incidence in infants. A total of 327 BM samples were collected from 72 lactating mothers. Thirty of them gave birth to a boy and 42 of them to a girl. Colostrum was collected 48 hr after the beginning of lactation (day 0, D0) and mature milk at 1 month (M1), 3 months (M3), 6 months (M6), and 12 months (M12), following delivery.

Additional data (maternal prepregnancy BMI, complications of pregnancy, infant's birth weight, birth length, sex, gestational age, and body weight at M1, M3, M6, and M12) were obtained from questionnaires and medical records. All newborns were born at term, their average birth weight was 3,472 ± 42 g, birth length was 50.2 ± 0.3 cm, and average body weight at M1, M3, M6, and M12 was 4,045 ± 53 g, 5,911 ± 86 g, 7,621 ± 110 g, and 9,590 ± 125 g, respectively (all mean ± SEM). Average BMI of mothers at the beginning of the pregnancy was 21.9 ± 0.4 kg/m^2 (mean ± SEM). All mothers declared that they did not smoke during lactation and none of the mothers was an alcohol addict. We have no detailed data on occasional alcohol intake or detailed dietary habits during pregnancy and lactation. The study was approved by The Ethical Committee of University Hospital Motol and informed consent was obtained from all participants.

Methods

BM was collected after the first morning breastfeeding (after 7:00 a.m.) into sterile tubes by either manual expression or a breast pump, divided into aliquots and frozen at −20°C for further analysis. ADRP proved to be stable at −20°C (internal unpublished data). Prior to the assay, BM samples were thawed at 4–6°C overnight and centrifuged at 2,500 × g at 4°C for 20 min for removal of the fatty layer. Commercially available ELISA kits (Biovendor-Laboratory Medicine, Inc., Brno, Czech Republic) were used for determination of adiponectin, leptin, and AFABP in skimmed BM as described previously (1, 2).

Adipophilin Assay—ELISA Test Development

We established and evaluated an immunoassay for the quantitative determination of human ADRP in human BM. The sandwich ELISA employs specific sheep polyclonal anti-human ADRP provided by Biovendor-Laboratory Medicine, Inc. and coated on microtiter wells (NUNC, Maxisorp, Thermo Fisher Scietific, Rockford, Illinois, USA): 100 μl/well, 1 μg/ml in 0.1 M carbonate buffer overnight at 4°C. The plate was washed once with Tris Buffered Saline - Tween (TBS-Tw) (0.05 M Tris-HCl; 0.15 M NaCl; pH 7.2; 0.05% (w/v) Tween 20) on the Columbus washer (Tecan Austria GmbH, Grödig, Austria). Nonspecific binding sites were blocked with 200 μl/well, 4% saccharose, 0.5% bovine serum albumin in Tris Buffered Saline (TBS) for 30 min at 25°C. After aspiration, diluted samples (500-fold with sample dilution buffer (TBS-Tw; 3% (w/v)

Triton X-100; 1% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)) or standards were pipetted in duplicates 100 μl/well. The plate was incubated for 1 hr at 25°C/room temperature. After five washes with TBS-Tw, 100 μl/well of biotin-labeled sheep polyclonal IgG (biotinylated with kit from Pierce (Thermo Fisher Scietific, Rockford, Illinois, USA)) was added and the plate was incubated for 1 hr at 25°C. Following five washes, 100 μl/well of streptavidin-horseradish peroxidase (HRP) conjugate (Amdex, GE Healthcare Bio-Sciences, Pittsburgh, Pennsylvania, USA) was added and the plate was incubated for 1 hr at 25°C. After washing, 100 μl/well of TMB substrate (KPL, Inc., Gaithersburg, Maryland, USA) was then added and the plate was incubated for another 10 min at 25°C. The reaction was stopped with 100 μl/well of sulfuric acid (0.2 M). The developed color was determined by reading the plate on the microplate reader Biotek EL808 (BioTek, Winooski, Vermont, USA) at a wavelength of 450 nm.

As the standard, we used a recombinant ADRP provided by Biovendor-Laboratory Medicine, Inc. The protein content of recombinant ADRP was determined by bicinchoninic acid (BCA) method (Sigma-Aldrich, Co., St. Louis, Missouri, USA) and its purity confirmed by SDS-PAGE (data not shown). Standards were prepared at concentrations of 30, 15, 6, 3, 1.5, 0.6, and 0.3 μg/l in sample dilution buffer and 100 μl directly pipetted into the wells.

To validate the reliability of the assay, we tested the precision and the accuracy of the assay. To analyze the spiking recovery, human BM from two subjects with baseline ADRP levels of 0.8 and 1.2 mg/l were spiked with increasing amounts of recombinant protein (+1.0, +2.0, and +4.0 mg/l) and assayed. The mean recovery was 93%. Moreover, we tested human serum samples from another two subjects with baseline ADRP levels of 4.3 and 3.9 mg/l for dilution linearity. The mean recovery was 87%. The limit of detection of the assay was 0.05 μg/l; the intra- and interassay coefficient of variation (CV) were always less than 10%.

Statistics

Statistical analysis was performed using Prism 5.0 statistical software (Graph Pad Software, Inc. La Jolla, California, USA). Values were tested for normality of distribution. Results are reported as mean ± SEM or as median in case of non-Gaussian distribution. The differences within groups were tested using Kruskal–Wallis test with Dunn's multiple comparison test. Correlations are expressed by Spearman correlation coefficient. *P* value of less than 0.05 was considered statistically significant.

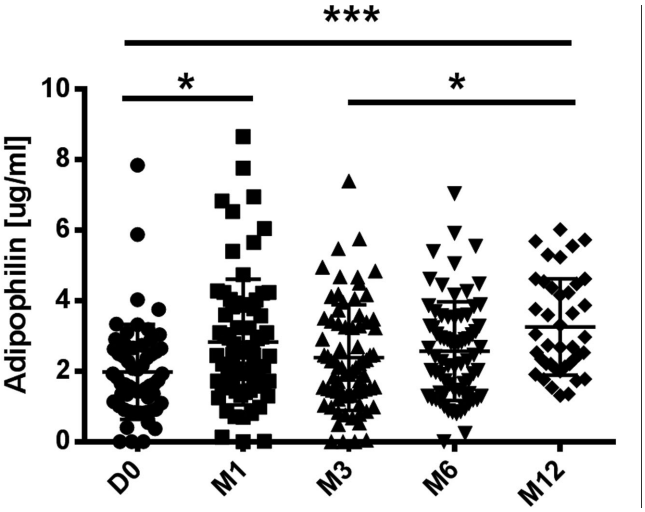

Fig. 1. Adipophilin BM levels during 12 months of lactation. D0, day 0; M1, month 1; M3, month 3; M6, month 6; M12, month 12.

RESULTS

ADRP levels were not normally distributed. ADRP levels at D0 were 1.70, M1 2.47, M3 2.12, M6 2.44, and at M12 2.73 μg/ml (median), and mean levels were at D0 1.98 ± 0.12, M1 2.83 ± 0.21, M3 2.39 ± 0.17, M6 2.57 ± 0.16, and at M12 3.25 ± 0.21 μg/ml (Fig. 1). In D0, M3, and M6 only one sample was under detection limit of the ELISA method. Significantly higher levels of ADRP were found in M1 and M12 compared to D0 and in M12 compared to M3 (overall $P = 0.0001$). Adiponectin, AFABP, and leptin BM levels and their relationship to anthropometrical parameters of infants and mothers are described in detail in a separate publication (2).

Correlations

Trend for ADRP levels was intraindividually highly conserved from M1 onwards throughout the whole lactation. Significant positive correlation was found between ADRP levels at M1 and M3 (Spearman $r = 0.3091$, $P = 0.0103$). Moreover, ADRP at M3 correlated with levels at M6 (Spearman $r = 0.2739$, $P = 0.0227$) and M12 (Spearman $r = 0.4476$, $P = 0.0043$), and levels at M6 also correlated with levels at M12 (Spearman $r = 0.3699$, $P = 0.0173$; Fig. 2). ADRP levels at M6 correlated negatively with birth weight (Spearman $r = -0.3066$, $P = 0.0083$) and birth length of infants (Spearman $r = -0.36$, $P = 0.0018$), however there was no other correlation throughout the lactation period between BM levels of ADRP and body weight of infants, their birth length or BMI of mothers at the beginning of the pregnancy. In addition, no correlation was found between body weight gain during the first year of life and BM levels of ADRP at any

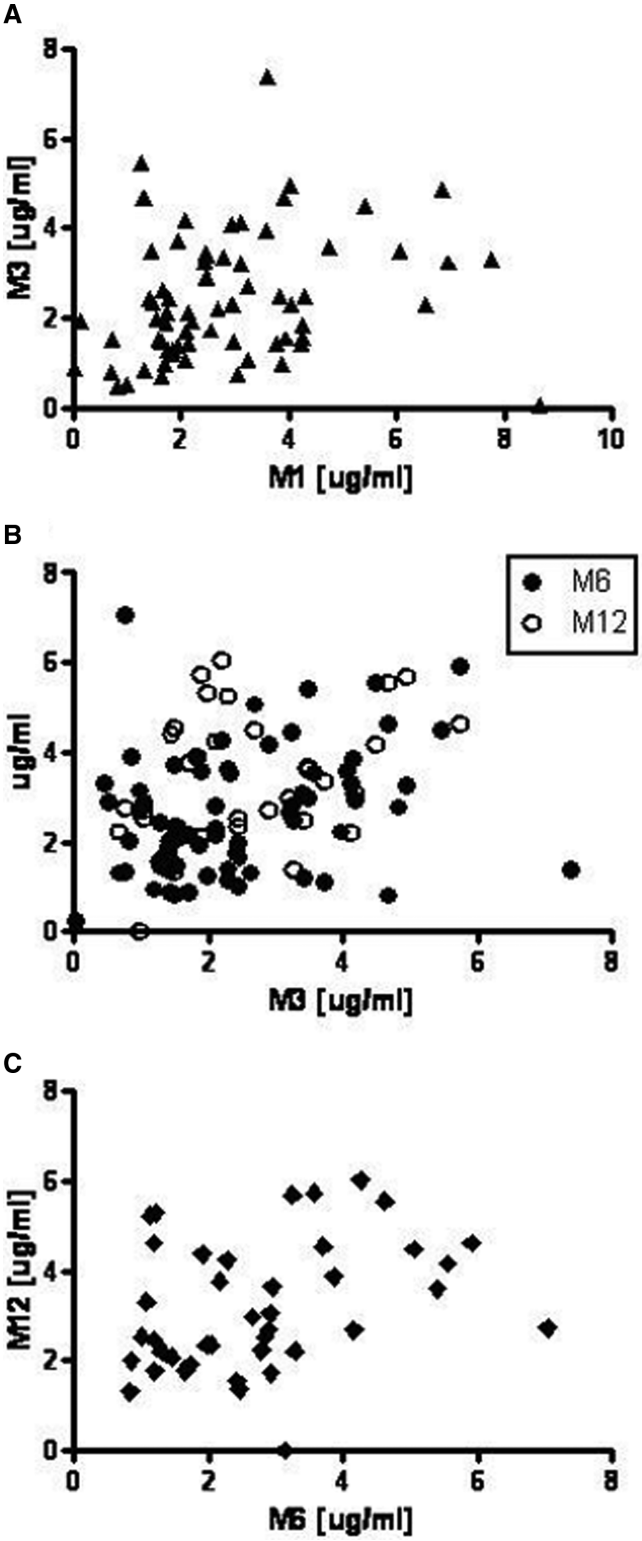

Fig. 2. Correlation between BM levels of adipophilin at M1 and M3 (A); M3 and M6, M12, respectively (B); and M6 and M12 (C). M1, month 1; M3, month 3; M6, month 6; M12, month 12.

time point up to 12 months of lactation. No significant differences were found in BM concentrations of ADRP between mothers who delivered boys versus girls at any time point up to 12 months of lactation. No correlation was found between ADRP and adiponectin, AFABP or leptin at any time point throughout the lactation period.

DISCUSSION

ADRP was previously detected in bovine (14), goat (15), rat, and human milk as a major protein constituent of the MFGM (12). Several researchers studied its potential role in BM. Animal studies revealed that ADRP is specifically localized in secretory epithelial cells of differentiating and lactating mammary gland and its increased expression is linked to accumulation of cytoplasmic LDs (9). Moreover, it is probably involved in a process of cytoplasmic LDs secretion by interacting with apical plasma membrane phospholipids (10). Scandinavian researchers recently identified exosomes in human colostrum and BM, which showed immune modulatory features. Among other molecules, ADRP was also identified by proteomic analysis of these specific vesicules (16). In most of these studies, MFGs were isolated from cream layer of BM, MFGM was then extracted and fractioned by detergent-using solutions, soluble and nonsoluble fractions were separated by centrifugation, and coat proteins were separated and detected by different procedures including SDS-PAGE, sequence analysis, Western blotting, and mass spectrometry (13).

In this study, we used a different aproach. For quantitative detection of ADRP in BM, we estabilished and evaluated an immunoassay (hsELISA method), which is less time-consuming, commonly used and validated for detection of wide range of proteins (including cytokines, growth factors, and hormones) in BM and other biological material. As fat may interfere with ELISA method and thus practical use of full BM for this purpose is limited, we decided to measure ADRP in skimmed BM. During the sample preparation, whole milk was centrifuged, cell and cream layer was discarded and only the supernatant was used for further analysis. We are aware of the limitation of this approach with respect to the fact that ADRP is mainly present in human BM as part of lipid globule membranes. However, we were able to detect levels around 2–3 μg/ml with this method. ADRP was detectable in BM from beginning of lactation up to 12 months following delivery, with increasing trend up to first month, following the decline in third month, and another increase up to 12 months. Moreover, ADRP levels in M3 correlated with M6 and M12, which means that in some individuals the levels were generally higher at all time points

compared to other individuals and are intraindividually highly conserved.

Quantification of developmental protein changes in MFGM was performed by Reinhardt and Lippolis using shotgun proteomics (17). They found that during the transition from colostrum to mature milk, the complex of proteins associated with lipid transport, synthesis, and secretion (ADRP, butyrophilin, and xanthine dehydrogenase) were individually upregulated at day 7 in parallel compared with colostrum. These findings correspond with our results. According to them, there is an early developmental shift in milk fat transport despite higher fat content in colostrum. We speculate that increasing levels of ADRP in milk during the second half of infancy might be also related to lower frequency and longer intervals of breastfeeding and thus leading to cummulation of ADRP in BM.

Our presumption that ADRP levels in BM might be related to BMI of mothers before pregnancy was not confirmed. ADRP BM levels at M6 correlated negatively with birth weight and birth length of infants. However, this correlation seems to be of limited clinical importance, because there was no other correlation with infant's body weight or length in any time points throughout the lactation as well as their weight gain throughout the first year of life. We prospectively follow up the children to evaluate possible relationship between ADRP BM levels and anthropometrical parameters of children in the future. Interestingly, there was no correlation with regulatory hormones (adiponectin, AFABP, or leptin also detected by ELISA) at any time point throughout the lactation. Based on our data, it seems that ADRP in human BM is not related to nutritional status of infants nor mothers before pregnancy.

Data from literature show that ADRP expression and synthesis is induced by long-chain free fatty acids, especially in a condition such as fasting when lipolysis is induced and free fatty acids rise in serum (18). We do not have any available data on dietary habits of the mothers to correlate with levels of ADRP in their BM.

The role of ADRP in different organs or tissues has not been clearly estabilished so far. ADRP is a major LD protein present in all cells accumulating lipids, either normally or abnormally (19), and predominantly in mammary gland and differentiating adipose cells where it is one of the earliest markers of adipocyte differentiation (6). Although its role may not be significant in adipose tissue homeostasis, it is a major determinant of triglyceride content in the liver (20, 21). Its negative role in atherosclerosis was proposed and explained by increasing cholesterol esters stores in macrophage foams cells (22) and augmenting inflammation in macrophages by inducing expression and secretion of proinflammatory cytokines TNF-α, MCP-1, and IL-6 in these cells (23). On the other hand, ADRP is involved in maintaining insulin sensitivity in muscles, thus providing possible protective role against insulin resistance (24). It was also proposed to use ADRP as a helpful diagnostic marker for the identification of specialized differentiated cells containing LDs and for diseases associated with fat-accumulating cells, such as clear cell renal cell carcinoma (detection of ADRP in urine; (25)). It is speculated that PAT proteins might even represent therapeutic targets for intervention strategies, due to the fact that inhibition of lipogenesis exerts antineoplastic effects (26). ADRP was found in human fetal membranes (amnion epithelial, amnion fibroblasts, and chorion trophoblasts) and its presence in LDS increased with gestation and labor and was associated with enzymes involved in arachidonic acid cascade (27). This leads to the notion that LDs in fetal membranes might be foci for prostaglandin E2 production at the time of labor.

CONCLUSION

We developed a reliable and well-reproducible hsELISA method for detection of ADRP in skimmed fraction of human BM. To validate the reliability of the assay, we tested the precision and the accuracy of the assay using recombinant protein. The mean recovery, intra-, and interassay CV are very good. ADRP was detectable by ELISA method in colostrum and human BM during the whole 12 months of lactation period. Its levels show interindividual variabilities and no relation to nutritional status of the mothers or the infants. However, intraindividually, ADRP levels remain highly conserved during the whole lactation period. The role of ADRP in BM might be of little nutritional value, on the other hand, it could be involved in other processes in infant's development.

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