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The lipid fraction of human milk initiates adipocyte differentiation in 3T3-L1 cells

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ABSTRACT

Background: The prevalence of childhood obesity has increased worldwide over the past decade. Despite evidence that human milk lowers the risk of childhood obesity, the mechanism is not fully understood.
Aims: We investigated the direct effect of human milk on differentiation of 3T3-L1 preadipocytes.
Study design and subjects: 3T3-L1 preadipocytes were treated with donated human milk only or the combination of the standard hormone mixture; insulin, dexamethasone (DEX), and 3-isobututyl-1-methylxanthine (IBMX). Furthermore, the induction of preadipocyte differentiation by extracted lipids from human milk was tested in comparison to the cells treated with lipid extracts from infant formula. Adipocyte differentiation, specific genes as well as formation of lipid droplets were examined.
Results: We clearly show that lipids present in human milk initiate 3T3-L1 preadipocyte differentiation. In contrast, this effect was not observed in response to lipids present in infant formula. The initiation of preadipocyte differentiation by human milk was enhanced by adding the adipogenic hormone, DEX or insulin. The expression of late adipocyte markers in Day 7 adipocytes that have been induced into differentiation with human milk lipid extracts was comparable to those in control cells initiated by a standard adipogenic hormone cocktail.
Conclusions: These results demonstrate that human milk contains bioactive lipids that can initiate preadipocyte differentiation in the absence of the standard adipogenic compounds *via* a unique pathway.

1. Introduction

The prevalence of childhood obesity has increased worldwide over the past decade [1]. The clinical guidelines for the prevention and treatment of pediatric obesity strongly recommend breast-feeding for at least six months based on several epidemiological studies [2]. However, despite many clinical evidences that breast-feeding is associated with lower incidence of obesity, the mechanism is not fully understood.

Adipose tissue development is the result of increased adipocyte size with increased triacylglycerol storage along with the increased adipocytes by the differentiation of adipose precursor cells; preadipocytes into mature adipocytes. Adipose stem cells get transformed to the adipose lineage during the late-fetal and post-natal period. There is also remarkable proliferation and an expansion in the number of adipocyte precursor cells through this period in response to nutritional and hormonal stimuli [3]. Furthermore, the number of adipocytes formed during this period remains throughout life [4]. Therefore, it is evident that nutritional factors during the early post-natal period can influence adipose tissue development, resulting in fat deposition at a later stage. Indeed, it has been reported that the type of early infant diet, breast-feeding or formula-feeding can cause difference in the adipose tissue development [5,6]. Hence, the programming of adipocyte precursor cells, preadipocyte by human milk is of great importance.

Human milk is unique in its nature than infant formula as it contains a variety of bioactive components such as hormones and growth factors [7] and lipids [8,9]. These components when transferred to the infant remain active in their bloodstream [9–11]. Thus, it is possible to postulate that bioactive components in human milk could affect the adipose tissue/adipocyte development among infant directly. Preadipocytes from neonatal pigs consuming maternal milk showed lower proliferation and higher differentiation of adipocytes than pigs consuming formula milk [12]. The only *in vitro* study using 3T3-L1 cells, an established cell model for the study of adipocyte physiology, showed that whole human milk can induce adipocyte differentiation [13]. It is possible that bioactive components exiting in human milk can influence the differentiation of adipocytes and infant adipose tissue; however, no identification of responsible bioactive components for adipocyte development is yet available so far.

It is essential to understand the mechanism underlying the adipocyte differentiation during the adipocyte development. The differentiation of preadipocyte into mature adipocytes requires the sequence of transcriptional factor activation [14]. The initial indication of this

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sequence is the involvement of CCAAT/enhancer-binding protein (C/EBP) family. At a very early phase of adipogenesis, C/EBPβ and C/EBPδ are up-regulated, which subsequently induce transcription of peroxisome proliferator-activated receptor γ (PPARγ) [15]. In addition, the down-regulation of preadipocyte-specific genes, preadipocyte factor-1 (Pref-1) [16] and tissue inhibitor of metalloproteinase-3 (TIMP-3) [17] is mandatory for the initiation of proper adipocyte differentiation. The coordination of these transcriptional factors leads to the final phase of adipocyte differentiation characterized by activation of adipocyte-specific genes, such as fatty-acid binding protein (aP2). It has been reported that the transcriptional sequence of adipocyte differentiation is mainly dominated by three pathways involved by insulin, glucocorticoid, and cAMP, which are commonly used as well characterized inducing hormones in *in vitro* studies [18]. Besides, it is likely that parallel pathways involving in the initiation of transcriptional network for adipocyte differentiation can be activated by other adipogenic compounds such as fatty acids and eicosanoids [19,20].

The primary objectives of this study were to investigate the effect of human milk on differentiation of 3T3-L1 preadipocytes and to identify the bioactive component(s) present in human milk responsible for modifying adipocyte physiology.

2. Material and methods

2.1. Materials and reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Nacalai Tesque (Kyoto, Japan). Dexamethasone (DEX), 3-isobututyl-1-methylxanthine (IBMX), and insulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Calf serum (CS) and fetal bovine serum (FBS) were obtained from GIBCO (Grand Island, NY). Commercially prepared powdered-formulas were obtained from three different distributors (Morinaga Milk Industry Co. Ltd., MEGMILK SNOW BRAND Co. Ltd., and ICREO Co. Ltd.).

2.2. Milk collection, fractionation, and lipid extraction

Human milk samples were obtained from donors ($n = 12$): healthy, non-obese mothers of full-term, healthy infants. All mothers (gave written informed consent before participating in this study. Collection protocol was approved by the ethics committee of the Hamamatsu University School of Medicine. The range of their lactation period was 5–60 days and milk was collected by hand-expression. Individual and pooled samples were aliquot and immediately stored at −80 °C until further usage. The milk samples were used for the experiments within two months.

Milk samples were separated into lipid, whey, and casein fractions by centrifugation. To obtain each fraction, samples were first centrifuged at 4000 ×*g* for 30 min at 4 °C. The upper layer containing lipids and cells was then removed, and the aqueous layer was subjected to ultracentrifugation (150,000 ×*g*) for 1 h at 4 °C. This step resulted in a casein pellet and a supernatant portion (whey) [21].

To purify the lipid components and eliminate peptides, lipids were extracted from human milk and commercial infant formula, using a liquid–liquid extraction method described by Bligh and Dyer [22]. Briefly, 3.75 ml of a mixture of chloroform and methanol at 1.25:2.5 (vol/vol) was added to 1 ml of human milk, or infant formula adjusted from commercially prepared powdered-milk by distilled water according to the manufacturer's protocol. This was mixed with 1.25 ml chloroform, followed by the addition of 1.25 ml of water, and the samples were vortexed. Phase separation was achieved by centrifugation at 1500 ×*g* for 10 min. The lower phase, containing the total lipids, was evaporated under nitrogen and re-dissolved in 100 μl of ethanol.

2.3. Cell culture, treatment, and oil red O staining

3T3-L1 preadipocytes, obtained from the American Type Culture Collection (ATCC) (Manassas, VA), were cultured in phenol-red free DMEM with a high-glucose concentration (4.5 g/l) supplemented with 10% CS until confluent. Confluent cells were maintained in the same medium for an additional two days, after which (Day 0) cells were hormonally induced to differentiate by replacing the media with a differentiation mixture (1 μM DEX, 0.5 μM IBMX, and 1.7 μM insulin in DMEM supplemented with 10% FBS). Cells were maintained in this media for 2–3 days after which they were harvested for analysis. To assess the cells in the late stage of adipocyte differentiation, this medium was replaced after three days by medium supplemented only with 1.7 μM insulin and maintained until Day 7 before harvesting. Human milk, whey, or infant formula was added, alone or in combination with other adipogenic compound, to culture media at a final concentration of 10%. Lipid extract diluted in ethanol was added to culture media at a concentration of 0.5% or 1%. One percent lipid extract (vol/vol) was equivalent to 10% whole human milk. To assess lipid accumulation, differentiated 3T3-L1adipocytes were fixed in 10% formalin, stained with oil red O dye (Sigma-Aldrich, St. Louis, MO, USA) [23], and imaged by light microscopy.

2.4. qRT-PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Valencia, CA) and reverse transcribed into cDNA by ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). Quantitative detection of mRNA levels was performed using Applied Biosystems StepOne Plus (Foster City, CA) and THUNDERBIRD qPCR Mix (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. The primers for each gene were designed using OLIGO primer analysis software, provided by Steve Rosen and Whitehead Institute/MIT Center for Genome Research or selected from published sources [17,24]. Primer sequences are available upon request. 36B4 was used as a housekeeper transcript for 3T3-L1 cells.

2.5. Statistical analysis

The data of quantitative gene expressions were repeated thrice using the same protocol and results are expressed as mean ± standard deviation. Data were calculated using Student's t-test. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Whole breast milk induces differentiation of 3T3-L1 preadipocytes without adipogenic agents

To study the effect of breast milk on preadipocyte differentiation, confluent 3T3-L1 preadipocytes (Day −2; two days prior the induction process) were treated with whole human milk, whey of human milk, and commercial infant formula milk at a concentration of 10% (vol/vol) for two days (Fig. 1). Only treatment with whole human milk reduced expression of preadipocyte-specific markers, Pref1 and TIMP-3 significantly. This suggests that treatment with whole human milk initiates the adipocyte differentiation program. Indeed, the early adipogenic marker, CCAAT/enhancer-binding protein (C/EBP) β, increased significantly in preadipocytes treated with whole human milk for two days. This response was common across all donated milk samples, despite lactation periods ranging from several days to two months. Therefore, the human milk samples were pooled for the remaining experiments, unless otherwise noted. Similarly, all commercial infant formula milk samples from different distributors had similar findings in view of 3T3-L1 preadipocyte differentiation. Therefore, mixed infant formula milk samples were used for all the experiments.

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