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

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1. Introduction

The prevalence of childhood obesity has increased worldwide over the past decade [1]. Despite evidence that human milk lowers the risk of childhood 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 adipocytes by the differentiation of adipose precursor cells; preadipocytes with increased triacylglycerol storage along with the increased adipocyte differentiation [13]. It is possible that bioactive components existing in infant remains active in their bloodstream [9–11]. Thus, it is possible to postulate that bioactive components in human milk could affect 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
sequence is the involvement of CCAAT/enhancer-binding protein (C/EBP) family. At a very early phase of adipogenesis, C/EBPα and C/EBPβ are up-regulated, which subsequently induce transcription of peroxisome proliferator-activated receptor γ (PPARγ) [15]. In addition, the down-regulation of adipocyte-specific genes, adipocyte 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 (αP2). 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-isobutyl-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-L1 adipocytes 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.
Given that whole human milk but not lipid-free milk promoted preadipocyte differentiation, we hypothesized that lipids contained in human milk were responsible for preadipocyte differentiation, as was also suggested in a previous study [13]. We then tested this hypothesis using lipid extracts from milk samples. Lipid fractions of samples with high purity and no residual peptides were obtained by liquid–liquid extraction, according to Bligh and Dyer [22]. 3T3-L1 preadipocytes treated for two days with lipid extracts from human milk accumulated more lipid droplets than cells induced by the standard induction mixture (controls) at the concentration of 1% that is equivalent to 10% whole human milk (Fig. 3A).

3.2. Human milk enhances the effects of adipogenic inducers

To determine the effect of human milk on preadipocyte differentiation during the conversion phase (transition from preadipocytes to adipocytes), 3T3-L1 preadipocyte (two days after confluence; Day 0) were treated with 10% whole human milk in the absence of the standard induction mixture of adipogenic hormones (insulin, DEX, and IBMX). Expression of differentiation markers in cells treated only with human milk was similar to control cells induced by the standard induction mixture (Fig. 2). Day 0 preadipocytes treated for three days with 10% whole milk experienced significant induction of the late adipogenesis-related genes C/EBP\(\alpha\) and adipocyte fatty acid binding protein (aP2); expression of these markers was the same level as control cells induced by the standard induction mixture (Fig. 2A–B). The appearance of lipid droplets in cells treated with human milk was more prominent than in control cells (Fig. 2C–D). Furthermore, the effect of additional treatment of standard adipogenic hormone on human milk on inducing of preadipocyte differentiation was studied. Expression of these genes was synergistically enhanced by addition of individual adipogenic reagents: insulin, DEX or IBMX (Fig. 2A–B). Besides, there was also an increase in lipid accumulation in the cells treated with the combination of human milk and each adipogenic reagent (insulin or DEX) when compared to cells treated with human milk only (Fig. 2D–F).

3.3. Adipogenic factors exist in lipid extracts from human milk

Fig. 1. Effect of human milk and commercial infant formula on confluent 3T3-L1 preadipocytes. After reaching 100% confluence (Day –2), 3T3-L1 preadipocytes were incubated with 10% whole human milk, whey or infant formula for two days. Control cells (Ctrl) were untreated. Preadipocyte specific markers (Pref1 and TIMP3) and an early adipocyte marker (C/EBP\(\beta\)) were analyzed by quantitative PCR. All data are expressed as fold increases relative to controls. Data are expressed as mean ± SD. N = 3. * indicates expression levels that differed significantly (\(p < 0.05\)) from controls. * and ** indicate values that differ significantly (\(p < 0.05\) and \(p < 0.01\), respectively) from controls; * and *** indicate values that differ significantly (\(p < 0.05\) and \(p < 0.01\), respectively) from cells treated with each adipogenic agent only. (C, D, E, F) The morphology of unstained differentiating cells was photographed at a magnification of 400 ×. The figures are representative of multiple experiments.

Fig. 2. Effects of treatment with human milk and adipogenic agents on expression of aP2 and C/EBP\(\alpha\) and formation of lipids by 3T3-L1 preadipocytes. At two days post confluence, 3T3-L1 preadipocytes were treated for three days with human milk alone or in combination with IBMX, dexamethasone (DEX), or insulin (INS). Expression of aP2 (A) and C/EBP\(\alpha\) (B) were analyzed by quantitative PCR. All data are expressed as fold increases relative to cells treated with the standard induction mixture of adipogenic hormones, DEX, IBMX, and insulin (Ctrl). Data are expressed as mean ± SD. N = 3. * and ** indicate values that differ significantly (\(p < 0.05\) and \(p < 0.01\), respectively) from controls; * and *** indicate values that differ significantly (\(p < 0.05\) and \(p < 0.01\), respectively) from cells treated with each adipogenic agent only. (C, D, E, F) The morphology of unstained differentiating cells was photographed at a magnification of 400 ×. The figures are representative of multiple experiments.
preadipocyte-specific markers, Pref1 and TIMP-3, decreased to comparable levels both in cells treated with lipid extracts from human milk at the concentration of 1% in the absence of standard adipogenic hormones and in control cells induced by the standard induction mixture (Fig. 3B). Also the expression of the early adipocyte differentiation marker, C/EBPβ in cells treated with 1% human milk lipid, increased to the same level as in controls (Fig. 3C). Lipid extracts from human milk at a concentration of 0.5% and 1%, resulted in 4 and 6 fold increase in aP2 gene expression respectively, relative to control cells. In contrast, lipid extract from the commercial infant formula failed to induce significant lipid accumulation in 3T3-L1 preadipocytes (Fig. 3A), and the expression profile of adipogenic-related markers did not indicate significant adipocyte differentiation (Fig. 3B–D) Taken together, these data clearly indicate the lipids present in human milk can induce differentiation in 3T3-L1 preadipocytes.

3.4. The initiation of preadipocyte by human milk lipids is sufficient for the development into terminal differentiation.

To address whether preadipocytes treated by human milk lipids eventually develop into mature adipocytes, the expression of late adipogenic markers and the formation of lipid droplets were evaluated in Day 7 adipocytes. Postconfluent 3T3-L1 preadipocytes were treated for three days (Day 0–Day 3) with lipid extracts from

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**Fig. 3.** Effect of lipid extracts from human milk or infant formula on 3T3-L1 preadipocyte differentiation. At two days post confluence, 3T3-L1 preadipocytes were treated with human 0.5% or 1% (v/v) milk lipid or infant formula lipid for three days. (A) Intracellular lipid accumulation, visualized by oil red O staining (magnification = 400×). Expression (mRNA levels) of the preadipocyte markers, Pref1 and TIMP3 (B) and the early differentiating adipocyte marker, C/EBPβ (C) and the adipocyte specific aP2 (D). All data are expressed as fold increases relative to cells treated with the standard hormone mixture (Ctrl). Data are expressed as mean ± SD. N = 3. * and ** indicate values that differ (p < 0.05 and p < 0.01, respectively) from controls.
human milk or with the standard induction mixture (control). After that, the medium was replaced by medium containing 1.7 μM insulin and maintained for an additional four days. On Day 7, well-differentiated adipocytes were imaged by light microscopy and harvested for analysis of gene expression. Lipid droplets were formed in Day 7 adipocytes that had been treated with 0.5% (vol/vol) lipid extract from human milk, in the absence of adipogenic hormones, during preadipocyte stage (Day 0–Day 3) (Fig. 4A). The lipid droplets were slightly more abundant in cells that had been treated with the 0.5% lipid extract from human milk than in adipocytes initiated with the standard induction mixture (control). Expression of the late adipocyte markers, PPARγ and aP2, significantly increased on Day 7 in adipocytes that had been treated with 0.5% human milk lipid extracts. Expression of adipocyte marker genes in cells treated with 1% human milk lipid reached levels comparable to those observed in control cells (Fig. 4B–C). Furthermore, Day 7 adipocytes initiated by 1% human milk lipid actually had significantly higher expression of leptin mRNA than control cells (Fig. 4D). These data show that the initiation of preadipocyte differentiation by human milk lipid is sufficient for the development into mature adipocytes.

4. Discussion

In this study, we demonstrate the effect of human milk on preadipocytes, *in vitro*, using 3T3-L1 cells. Preadipocyte differentiation response was unique to human milk and was not observed in response to commercial infant formula. Further study clearly demonstrates that the lipid component of human milk contains strong adipogenic factors, which appear to be absent in commercial infant formula. Preadipocytes incubated with human milk lipids eventually developed into mature adipocytes, as evidenced by expression of late adipocyte markers and visible adipocyte differentiation.

Human milk treatment of confluent 3T3-L1 preadipocytes reduced the expression of preadipocyte-specific markers, Pref1 and TIMP3, and initiated the expression of an early differentiating adipocyte marker; C/EBPβ. These effects were not observed in response to skimmed human milk or commercial infant formula, suggesting that preadipocyte differentiation is induced by the lipid component of human milk. In the previous study employing the same *in vitro* system using 3T3-L1 adipocytes, whole human milk also stimulated adipocyte differentiation and this effect disappeared when the lipid and cellular components were removed by centrifugation [13]. Our results are basically consistent with the findings of the previous reports.

The assumption in the previous study [13] that lipids or lipid-like components in human milk are responsible for adipocyte differentiation, is evidently confirmed by the experiments of our study. We showed that lipids extracted from human milk initiated preadipocyte conversion into mature adipocytes, evidenced by both a decrease in the preadipocyte-specific markers (Pref1 and TIMP3) and the accumulation of lipid droplets. C/EBPβ gene expression in preadipocytes treated with human milk lipids was comparable to that in cells treated with the standard adipogenic mixture, indicating that human milk lipid is sufficient for induction of preadipocyte differentiation. In contrast, lipids extracted from commercial infant formula did not induce significant preadipocyte differentiation as demonstrated by the lack of adipocyte phenotype (accumulation of lipid droplets) and the absence of an increase in the expression of adipocyte markers. These results suggest that the commercial formula lacks the bioactive lipids present in human milk. Considering that many studies have indicated significant impact of fatty acids on adipogenesis, as adipogenic hormones (n = 6 and n = 9, polyunsaturated fatty acids) [19,25,26], an independent initiator of preadipocyte differentiation (medium chain fatty acid) [27], or an inhibitor of adipocyte differentiation (n = 3 polyunsaturated fatty acid) [28], it is conjectured that fatty acids could contribute to preadipocyte differentiation. This study did not go far enough to identify bioactive lipids that are responsible for 3T3-L1 preadipocyte differentiations. Further characterization of the bioactive lipids responsible for 3T3-L1 preadipocyte differentiation is warranted and would allow the development of infant formulas that confer the same benefits as human milk and reduce the incidence of childhood obesity.

We should consider that the bioactive components present in human milk are products of enzymatic activity specific to human milk. After human milk is secreted, lipolysis, by milk lipase, starts immediately. Lipases are abundant in human milk but absent from infant formulas. Accordingly, it is possible that the bioactivity of human milk lipids may not be identified simply by comparing the
lipids present in human milk to those present in infant formulas. It is well known that handling of the human milk at higher temperature leads to formation of free fatty acid [29]. On the other hand, human milk lipases are known to be inactive below ~ 70 °C, the temperature at which our samples were stored until they were used in experiments. Therefore, it is unlikely that our human milk samples underwent additional lipolysis during storage, however, modifications by lipases present in the milk could have occurred at other stages of collection and during incubations. The process of human milk fat digestion in vivo is also worth mentioning. Lipases in human milk can cause lipolysis of milk lipids even before milk has reached the digestive phase of the intestine, resulting in significant decomposition and the production of free fatty acids [30]. In vivo the lipase in human milk aids in the digestion and absorption of human milk fat and it may have also resulted in a continual evolution of the lipid component. An investigation of changes in the lipid component and effects on adipogenesis over time would strengthen the interpretation of our results.

A combination of DEX, IBMX, and insulin is commonly used to initiate adipocyte differentiation in in vitro studies. The signal transduction by these chemicals has been well studied. Both glucocorticoid receptor (GR) activation by DEX [15,31] and insulin-mediated signaling via the insulin receptor [15,32] promote C/EBPβ and C/EBPα activation, resulting in the emergence of PPARγ expression; IBMX increases intracellular CAMP to enhance signal transduction. In this study, a combination treatment of whole human milk and each standard adipogenic chemical (DEX, insulin, or IBMX) showed a synergistic effect, suggesting that human milk lipids evoked an alternate differentiation pathway. One possible pathway of adipocyte differentiation is PPARγ dependent [33,34] and independent from CEBPβ/δ and/γ signals induced by the standard adipogenic cocktails. Many adipogenic components, including fatty acids and eicosanoids, are known activators of PPARγ or PPARα mediators [30,31]. Another possible pathway involved in adipocyte differentiation by human milk lipids may be a G protein–coupled receptor (GPCR) pathway. It has been reported that some orphan GPCRs are activated by free fatty acids [35–37]. Among these, GPR120 is expressed at high levels in differentiated adipocyte and adipose tissue and is activated by a long-chain fatty acid [38]. Furthermore, another GPCR, GPR 40 is expressed in preadipocytes but disappears following adipocyte differentiation [39]. Further study of the cell signaling involved in the initiation of adipocyte differentiation by human milk lipids is necessary. In order to apply our in vitro results to physiological situation, it is necessary to consider the in vivo process of lipid digestion. Lipids in human milk, primarily triacylglycerols, are digested by lipases into free fatty acids and monoglycerides. When these fatty acids enter the enterocyte, they are taken into the endoplasmic reticulum, where triglyceride is re-synthesized. Many of the candidate adipogenic compounds, including fatty acids, in human milk are not transferred directly to the circulatory system of the infant. However, there is little doubt that the circulation levels of certain fatty acids in infants depend on the concentration of them in milk [40,41]. Furthermore, many studies support that the qualitative changes (i.e. the fatty acid composition of fats) are strongly associated with pathogenic mechanisms of childhood obesity [42,43]. Thus our result that human milk lipids act as initiators of adipocyte differentiation in vitro contains important clues regarding an early determination of adiposity. The long-term consequence of 3T3-L1 adipocyte differentiation resulting from human milk-lipids also requires discussion and further investigation. It has been well known that obese children have elevated numbers of fat cells [44], and it has been suggested that the number of fat cells in a person remains constant throughout life [4]. The contribution of adipogenesis by human milk lipids in determining the adipocyte numbers one possesses later in life requires validation. Interestingly, the long chain fatty acid, palmitate (C16:0) enhances the differentiation of mouse embryonic stem cells towards the subcutaneous fat with enhanced adiponectin expression, suggesting that this fatty acid appears to play a role for programming of fat development towards a metabolically favorable profile [45]. Accordingly, it would be useful to further characterize and compare the differentiated adipocytes resulting from present in human milk or infant formula.

5. Conclusion

We showed that human milk lipids promote 3T3-L1 adipocyte differentiation. A similar differentiation response was not observed in response to infant formula. These findings demonstrate that human milk lipids contain bioactive lipids, which can influence immature adipocytes during post-neonatal development, a critical window for adipocyte programming. They also provide insight into the nature of bioactive compounds responsible for adipocyte differentiation and pathways through which they may act.

Conflict of interest statement

Authors declare no conflict of interest.

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