Post-weaning increases in the milk-fat globule EGF-factor VIII on fat globules in mouse milk and in the uptake of the fat globules by HC11 mammary epithelial cells

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Milk fat globules (MFGs) secreted by lactating mammary gland are unique lipid surrounded by a phospholipid bi-layer. We report here post-weaning changes in MFG EGF factor VIII (MFG-E8) and annexin V-accessible phosphatidyl-L-serine on the surface of MFGs. The MFG content in milk markedly decreased to about one-half within 2 days after forced weaning, despite a slight increase in milk protein content. Immunofluorescence-staining of MFGs using anti-MFG-E8 and annexin V indicated that MFG-E8 was present on some, but not all, MFGs before weaning, whereas most of MFGs were MFG-E8-positive and annexin V-negative after weaning. Free MFG-E8 with binding activity to phosphatidyl-L-serine was present abundantly in the post-weaning milk, and indeed exhibited binding to MFGs in pre-weaning milk. MFGs were taken up by HC11 mouse mammary epithelial cells *in vitro*, and those from post-weaning milk were remarkable for such cellular uptake. Moreover, the uptake of MFGs by the cells was inhibited by an anti-MFG-E8 antibody. Taken together, these findings suggest that MFG-E8 plays a critical role in regulation of MFG dynamics after weaning or during the suckling interval through the control of MFG-epithelial cell interaction in lactating mammary glands.

Keywords: fat metabolism/involution/lactation/mammary gland/milk fat globule membrane.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; FCS, fetal calf serum; IEF, isoelectric focusing; L10, day 10 of lactation; MFG, milk fat globule; MFG-E8, milk fat

globule EGF factor VIII; MFGM, MFG membrane; PBS, phosphate-buffered saline; PC, phosphatidyl-L-choline; Ptd-Ser, phosphatidyl-L-serine; RT-PCR, reverse transcriptase-polymerase chain reaction; SDG, sucrose density gradient; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; W2, day 2 after weaning.

Milk fat globules (MFGs) are lipid droplets synthesized at the endoplasmic reticulum (ER) of mammary epithelial cells and secreted into milk by a budding-off mechanism (1). Cytosolic triglyceride droplets surrounded with outer membrane of ER move to the apical plasma membrane and are then secreted by budding of the plasma membrane, which surrounds the lipid droplet. MFGs are unique in that triglyceride droplets are surrounded with a phospholipid bi-layer of plasma membrane and differ from serum lipoproteins, which are transported from ER lumen to Golgi and secreted by exocytosis (2). Although metabolism of serum lipoproteins, including receptors on target cells, are well known, the fate of MFGs transported into the intestinal tract of neonates and remaining in mammary glands after pups' weaning are not fully understood.

Mammary involution is a biological process including morphological and functional regression of lactating mammary glands, which is induced by cessation of suckling or forced weaning. Mammary epithelial cells undergo dedifferentiation and discontinue milk synthesis and secretion at an early stage of the involution, which takes place during a few days after weaning at mid-lactation in the mouse (3). At the early stage of mouse mammary gland involution, the expression of a MFG membrane protein, MFG-E8 (MFG-EGF-Factor VIII), is up-regulated (4, 5). Moreover, MFG-E8-positive particles, presumably MFGs, are accumulated at the mammary alveolar epithelia and, at the same time, both of total and phosphatidyl-L-serine (Ptd-Ser)-reactive MFG-E8 proteins markedly increase in milk (4). Biological meanings and molecular mechanisms of these weaning-induced changes in MFG-E8 are still unknown.

In *MFG-E8*-deficient mice, lactation is normal but involution is not, *i.e.* apoptotic cells as well as presumably MFGs are not removed and the defect in scavenging of apoptotic cell debris induces

inflammatory tissue damage, resulting in functional and morphological impairment of mammary gland differentiation at the next cycle of pregnancy and gestation (6). Critical roles of MFG-E8 in clearance of apoptotic cells during mammary involution were also suggested by some other previous studies (4, 7). Apoptotic cells were engulfed in an MFG-E8-dependent manner by mammary epithelial cells as non-professional phagocytes *in vitro* (6), and the MFG-E8 from the mouse milk from involuting glands, but not lactating ones, could label apoptotic HC11 mammary epithelial cells and enhance engulfment of the labelled apoptotic cells by J774 mouse macrophages (4). However, it remains to be determined whether MFGs are engulfed similarly by mammary epithelial cells or macrophages during mammary involution.

Mouse MFG-E8 consists of four domains, two EGF and two C domains, the latter of which are homologous to the 'C' domain, or discoidin domain, of blood coagulation factors VIII and V (8). The first EGF domain at the N-terminus contains an integrin-binding RGD motif, while the last C domain at the C-terminus binds to Ptd-Ser (9). Human MFG-E8 (lactadherin) shows binding to membranes containing Ptd-Ser with an affinity higher than factor V or VIII and another Ptd-Ser-binding protein, annexin V, and can competitively displace the other Ptd-Ser-binding proteins for membrane binding sites (10). The C-domain of MFG-E8 secreted by activated macrophages binds to Ptd-Ser exposed on apoptotic cells, while the activated phagocytes recognize and engulf the apoptotic cells through their $\alpha v\beta 3/\beta 5$ integrin that binds to the RGD motif in the EGF domain of MFG-E8 (11). On the other hand, MFG-E8 secreted by mammary epithelial cells was identified in lipid fractions of milk from various mammalian species (8, 12–14) and characterized to be a protein associating with MFG membrane (MFGM) (15). Although MFG-E8 is presumed to bind to Ptd-Ser as a major component of MFGM, there have been almost no studies directly showing the localization of MFG-E8 on the surface of MFGs, except one in which MFGs from wild-type and *MFG-E8* gene-deficient mice were analysed by flow cytometry using anti-MFG-E8 antibody (6). Assuming that MFGs are truly labelled with MFG-E8 in milk of lactating mammary glands, a question is raised as to whether or not the MFGs labelled are engulfed by epithelial or professional phagocytic cells, not only in involuting but also lactating mammary glands.

To answer these fundamental questions, in this study, interaction of MFG-E8 with MFGs as well as triglyceride and polar lipids in the milk from pre- and post-weaning mammary glands of lactating mice were analysed by biochemical and microscopic analyses and, moreover, uptake of MFGs by HC11 mammary epithelial cells was evaluated from a viewpoint of MFG-E8 involvement. The results of these analyses suggest a post-weaning dynamic increase in labelling of MFGs with MFG-E8, a resultant decrease in exposed Ptd-Ser accessible to annexin V, and MFG-E8-dependent uptake by the mammary epithelial cells *in vitro*.

Materials and Methods

Mice care and milk sample preparation

Lactating Balb/c mice were purchased from Japan SLC (Hamamatsu, Japan). The mice were cared for according to Nagoya University guidelines for animal study. Litter size was standardized to six pups within 24 h postpartum. To induce mammary gland involution, the pups were removed from the mother at day 10 of lactation. Milk samples were collected from the mice at day 10 of lactation (L10) or 2 days after forced weaning (W2) and processed as described previously (4). For fractionation, whole milk diluted 1 : 3 in phosphate-buffered saline (PBS) was centrifuged at 15,000 *g* for 15 min at 4°C to separate fat globule fraction, milk serum (whey) and casein fractions. The fat globule fraction was diluted with the original milk-volume of PBS and used immediately as the MFG fraction. Fractionation by sucrose density gradient (SDG) ultracentrifugation was described previously (4). For filtration of milk, milk samples were first diluted in PBS, centrifuged at 15,000 *g* for 15 min at 4°C to remove casein and finally the supernatant was passed through a 0.2- μ m membrane filter.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis, 2-dimensional electrophoresis and immunoblotting

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were done as described previously (4). For 2-dimensional (2D) electrophoresis, samples were first fractionated by isoelectric focusing (IEF) using ready-to-use Immobiline Dry-Strips with linear pH gradient 3–6 (Amersham Bioscience) according to the manufacturer's instructions. After IEF, the gel strips were incubated at room temperature for 15 min in equilibration buffer, and then separated on an SDS-PAGE gel followed by CBB staining or immunoblotting using rabbit anti-MFG-E8 (16) and anti-ovalbumin (17) antibodies.

Triglyceride and protein determination

Mouse milk was diluted with PBS, and the milk triglyceride content was enzymatically measured using a Triglyceride G-test kit (Wako, Japan). Protein concentration of mouse milk samples were determined using BCA assay kit (Thermo Fisher Scientific, MA, USA).

TLC analysis for polar lipid contents in milk

Total lipids were serially extracted from 25 μ l of whole milk with 1 ml each of methanol–chloroform (1 : 2, v/v), methanol–chloroform (1 : 1, v/v), methanol–chloroform (2 : 1, v/v) and methanol–chloroform–water (2 : 1 : 0.8, v/v), according to the previously described method (18). All extracts were pooled, dried by nitrogen spray and recovered lipids were dissolved in 700 μ l of methanol. After centrifugation, an aliquot (30 μ l) of the supernatant was separated by TLC on a silica gel plate using chloroform–methanol–ammonium–water (65 : 35 : 0.8, v/v). Lipids were detected using the spray reagents of 3% CuSO₄ in 8% phosphate followed by heating at 120°C. Purified Ptd-Ser, phosphatidylcholine (PC), phosphatidylethanolamine, cholesterol, sphingomyelin and phosphatidylinositol were purchased from Sigma and used as a standard (6 μ g for each).

Microscopic analysis of MFGs

For morphological observation of MFGs, milk samples were collected from Balb/c mice at day 10 of lactation and day 2 of involution. The samples were then immediately used for the following experiments. Milk (40 μ l) was diluted 1 : 10 with PBS and the fat globule fraction collected by centrifugation at 300 *g* for 10 min at 15°C. The fat globule fraction was washed twice in PBS and separated from PBS by centrifugation at 300 *g* for 10 min at 15°C. Finally, the fraction was suspended in 100 μ l of PBS. The fat globule fraction was then sequentially incubated with anti-MFG-E8 antibody at 4°C overnight and Alexa Fluor[®] 488 conjugated anti-rabbit IgG at room temperature for 1 h. The reactants were then centrifuged at 300 *g* for 10 min at 15°C, washed once with PBS and observed under a fluorescence microscope (IX71, OLYMPUS). For annexin V staining, MFGs were sequentially reacted with annexin V (ALEXIS Biochemical), anti-annexin V (FL-319 SantaCruz) and secondary antibody, and then observed as discussed earlier. The fluorescence intensity of MFG-E8 and annexin V signals on 40 randomly selected individual MFGs was quantified using the

Image-J software (<http://rsbweb.nih.gov/ij/>) and plotted in relation to the diameter of MFGs.

ELISA-based phospholipid-binding assay

The ELISA for MFG-E8 binding to solid-phase phospholipids was performed essentially as described previously (16). Milk samples diluted 1 : 1,000 in PBS were added to wells and bound MFG-E8 was detected with the anti-MFG-E8 antibody. For inhibition assay using liposomes, the milk serum fraction described earlier was further centrifuged at 230,000g for 30 min at 4°C twice to remove vesicular constituents. Ptd-Ser- and PC-liposomes were prepared by passing phospholipid solution through polycarbonate membrane filter (pore size of 0.2 µm) according to the previously described method (19). The liposome was added to the 1 : 1,000-diluted milk serum at the final concentration of 0.5 mM as phospholipid. Unless otherwise indicated, the ELISA values are shown after subtracting the value of control wells coated with the solvent (methanol) alone.

SDG ultracentrifugation

The mouse milk samples with and without membrane filtration were layered on a linear SDG (10–70% sucrose in PBS) prepared with Gradient Mate device (BIOCOMP, Fredericton, Canada) in a Beckman SW41 tube and was centrifuged at 200,000g for 18 h. Gradient fractions of 900 µl each were collected from the top of the tube (12 fractions in total) and subjected to trichloroacetic acid (TCA) precipitation, followed by SDS-PAGE and immunoblotting.

Gel filtration chromatography

The filtered milk serum (250 µl) was separated on a Sephacryl S-100 HR column (1.6 × 80 cm, GE healthcare) and eluted with PBS at a flow rate of 1 ml/min. Each 5 ml eluted fraction was collected and subjected to TCA precipitation followed by immunoblotting with anti-MFG-E8 antibody. The void volume and exclusion volume of several proteins (conalbumin, 75 kDa; ovalbumin, 43 kDa and ribonuclease A, 13.7 kDa) were determined using gel filtration calibration kit (GE healthcare).

Analysis of fat globule uptake by mammary HC11 cells

HC11 mammary epithelial cells were kindly given by Dr B. Groner (Georg Speyer Haus, Institute for Biomedical Research, Frankfurt am Main, Germany). The cells were cultured on cover slips placed in a 6-well plate in Dulbecco's modified Eagle's medium (DMEM, 4500 mg/l glucose, Sigma) containing 10% fetal calf serum (FCS) with 10 ng/ml of EGF (Sigma). Once confluence was reached, the cells were then incubated for 5 h at 37°C with milk samples diluted 1 : 500 with DMEM containing 10% FCS, where the cells on a cover slip were kept in contact with MFGs in the milk samples by placing the cover slip with cell monolayer upside-down. After the 5 h-incubation, the cover slip with cell layer was placed in the normal orientation in a new well and, in a few cases, incubated in a fresh DMEM/10% FCS for another 18 h, followed by fixation with 4% paraformaldehyde for 15 min at RT. The MFGs bound to HC11 cells were detected by staining with Oil red O (2.5 mg/ml in 2-butanol, diluted 6 : 4 in distilled water) or Nile Red (AdipoRed™ assay reagent, LONZA, Walkersville, MD). After staining with Nile Red, integral fluorescence intensity for each microscopic field was quantified using the Image-J software. For inhibition assay using anti-MFG-E8 antibody, milk was pre-treated with anti-MFG-E8 or control antibody (anti-V8 protease) at 4°C overnight and then added to the cell culture as above. For the RGD-peptide inhibition assay, an RGD-peptide, GRGDSP and a control peptide, GRGESF (Takara Bio, Inc., Otsu, Japan) was added to the incubation medium at a concentration of 10 µM.

Reverse transcriptase-polymerase chain reaction

Total RNAs were prepared from HC11 cells and mammary glands, and aliquots (5 µg) were reverse-transcribed with SuperScript II (Invitrogen) and random hexa-nucleotide primer in a 20 µl of total volume. After heat inactivation, aliquots of the cDNA pool were used for polymerase chain reaction (PCR) amplification. The primers used for each gene are as follows: integrin α , 5'-TAG AAT ATA GAC AGG ACC-3' and 5'-TGT ACA AGC TAG CCA CGA G-3'; integrin β 5, 5'-GCG AAA AGA TGC TCT GCA-3' and 5'-GCC GCA TGT GCA ATT GTA-3'; integrin β 3, 5'-GTA TTA CAG CTT ATT CTC CTA C-3' and 5'-TCA AAG CTG TCA

AGA TGA TG-3'; β -actin, 5'-TAA CCA ACT GGG ACG ATA TG-3' and 5'-ATA CAG GGA CAG CAC AGC CT-3'. Aliquots of the PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide.

Statistical analysis

Differences between two groups were analysed by *t*-test using Microsoft Office Excel.

Results

MFGs in milk decreased after weaning despite of MFG-E8 increase

The weaning-induced increase in Ptd-Ser-binding active MFG-E8 in milk (4) suggested some kind of changes in the structure of MFG-E8 or the interaction between MFG-E8 and MFGs after weaning. First, biochemical properties of MFG-E8 in L10 and W2 milk samples were characterized comparatively. Total proteins of L10 milk and W2 milk samples were fractionated by 2D-electrophoresis and immunoblotted with anti-MFG-E8 antibody. No significant differences in molecular masses of MFG-E8 (66- and 53-kDa bands for L and S forms, respectively) were observed between the two milk samples, but the pI ranges of MFG-E8 in the W2 milk were slightly wider than that of L10 ones (Fig. 1A), in addition to the increase in MFG-E8 spot intensity, especially of the L form. Such multiple pI isoforms and their change during the lactation period were also observed for bovine milk MFG-E8 (20). These pI variation in MFG-E8 may reflect the difference in sialylation of the glycans, as reported previously (21). Next, MFGs, as possible association counterparts of MFG-E8, were analysed in L10 and W2 milk samples. Triglyceride content, which reflects the amount of MFGs, was rapidly decreased to less than one-half 48 h after force weaning, whereas the protein content increased slightly and polar lipid composition did not change remarkably (Fig. 1B). With regard to the fat globule size, determined by diameter, a slight increase in proportion of the smaller MFGs (1–3 µm) was observed 48 h after weaning (Fig. 1C).

MFG-E8 and annexin V-reactive Ptd-Ser distributed differentially on MFGs in L10 and W2 milk

Fat globules were clearly stained with anti-MFG-E8 antibody, but at the same time, globules negative for immunostaining were also observed in L10 milk. In W2 milk, on the other hand, more MFGs were positive to the MFG-E8 immunostaining and the staining intensity appeared to be high (Fig. 2A). Ptd-Ser molecules exposed on the surface of MFGs were analysed using a Ptd-Ser-binding probe, annexin V (13), showing that surface Ptd-Ser molecules accessible by the probe were decreased 48 h after weaning (Fig. 2B). To analyse MFG-E8 on the fat globule surface more in detail, fluorescence intensity of MFG-E8 and annexin V signals for 40 randomly selected individual MFGs were quantified using the Image-J software and plotted in relation to the diameter of MFGs (Fig. 2C and D). When MFGs of a similar size were compared, the MFG-E8 signal-intensity of each MFG was markedly higher in W2 milk than in L10 milk, though there were several exceptions. In addition, the bigger the

Fig. 1 Analysis of proteins, lipids and MFGs in mouse milk collected before and after forced weaning. (A) Proteins in L10 (day 10 of lactation) and W2 (2 days after forced weaning) milk samples were analysed by IEF/SDS 2D-PAGE with CBB-staining (bottom), followed by immunoblotting with a mixture of anti-MFG-E8 and anti-ovalbumin (top). The long and short forms (horizontal bars 1 and 2, respectively) of MFG-E8 as well as an internal control, ovalbumin (Control) were identified by independent immunoblotting using each specific antibody. (B) Triglyceride (TG) and protein concentrations in L10 and W2 milk were determined, and the data were expressed as mean $\pm$ SD of independent milk samples from four mice (left). Alternatively, polar lipids in L10 and W2 milk were analysed by thin layer chromatography. CH, cholesterol; PS, phosphatidyl-L-serine (Ptd-Ser); PE, phosphatidylethanolamine; SM, sphingomyelin; PI, phosphatidylinositol; SD, standard deviation. (C) MFGs in L10 and W2 milk were observed under a phase contrast microscope and the number and size in diameter of MFGs in four independent visual fields (160 μ m $\times$ 200 μ m) were counted and expressed as mean $\pm$ SD. Scale bar: 20 μ m. Asterisks in (B) and (C) indicate a significant difference (B, TG concentration $P < 0.01$; B, Protein concentration and C, $P < 0.05$).

Fig. 2 Immuno-fluorescence detection of the MFG-E8 and Ptd-Ser on MFGs. (A) MFG fractions prepared from each milk sample were incubated with anti-MFG-E8 antibody or a control antibody (anti-V8 protease), followed by the incubation with AlexaFluor488-conjugated secondary antibody. Typical images (fluorescence, bright and merged) were presented in each panel. (B) The MFG fractions as above were incubated with annexin V, followed by the immunostaining using anti-annexin V or the control antibody, and AlexaFluor488-conjugated secondary antibody. Typical images (fluorescence, bright and merged ones) were presented in each panel. Scale bar: 20 μm. (C, D) The fluorescence intensity of MFG-E8 and annexin V signals on randomly selected MFGs (40 MFGs) in L10 (closed circles) and W2 (open circles) milk samples was quantified and plotted for the relationship between the diameter of MFGs and the log phase of fluorescence intensity. The intensity of MFGs stained with a control antibody (anti-V8 protease) was also shown with a regression line as a baseline (dotted line).

MFG, the higher the fluorescence intensity. Such correlation of the MFG-E8 signal with the diameter of MFGs was most remarkable in MFGs from W2 milk. In contrast to the MFG-E8 signal, the annexin-V signal of MFGs in W2 milk was almost the same as that of negative control. The annexin V signal of MFGs was detected only in L10 milk.

Free MFG-E8, which is not associated with MFGs, was then analysed biochemically in the L10 and W2

milk. The size of MFGs in L10 milk was found to be mostly more than 1.0 μm (Fig. 1C). Therefore, MFG-E8 binding with MFGs was eliminated by passing milk through a membrane filter with 0.2 μm pore size. We here regard the filtrates as the filtered milk serum. Elimination of MFGs from L10 milk resulted in a remarkable decrease in MFG-E8 in the milk serum analysed by either Ptd-Ser-binding activity or immunoblotting, compared with that from W2 milk (Fig. 3A

Fig. 3 Ptd-Ser binding of MFG-free MFG-E8 in the filtered W2 milk. (A) L10 and W2 milk samples were filtered through cellulose acetate membrane (0.2 μ m pore size), and the filtrates regarded as filtered milk were subjected to immunoblotting, Ptd-Ser-binding assay for MFG-E8 and TG content analysis. The data were expressed as mean $\pm$ standard deviation (SD) of independent samples from four mice. Values with different superscript letters (a, b and c) indicate significant difference ($P < 0.01$). (B) L10 and W2 milk samples and their membrane filtrates (filtered milk) were subjected to SDG ultracentrifugation, and MFG-E8 in each density fraction was analysed by SDS-PAGE followed by immunoblotting using anti-MFG-E8 antibody. Horizontal lines in each panel indicate the fractions corresponding to two major peaks of MFG-E8 in L10 milk sample before filtration. (C) MFG-E8 in the filtered W2 milk was fractionated by gel filtration chromatography and each fraction was subjected to Ptd-Ser-binding assay (top) and immunoblotting analysis (bottom) for MFG-E8. A void volume fraction (V_0) and the fractions corresponding to the molecular-mass standard proteins are shown as arrowheads above the MFG-E8 chromatogram as determined by Ptd-Ser-binding.

and B). Under the density-gradient ultracentrifugation, MFG-E8 in the membrane filtered W2 milk distributed widely in several fractions giving densities between 0.92 and 1.09 g/ml (Fig. 3B). Size exclusion chromatography of the filtered W2 milk showed that MFG-E8 was present as both monomer and complex (or oligomer) forms in the filtered milk serum, and the complex forms of MFG-E8 tended to show specific activity on Ptd-Ser binding higher than the monomer form (Fig. 3C).

MFG-E8 in W2 milk bound to MFGs in L10 milk

Using the Ptd-Ser-binding inhibition assay, binding of free MFG-E8 in W2 milk was evaluated in L10 milk MFGs, which were anticipated to expose accessible Ptd-Ser on their surface (Fig. 2B and D). The MFG-E8 binding to Ptd-Ser adsorbed onto ELISA plates was strongly inhibited by mixing W2 milk with L10 milk, as shown by ELISA and immunoblotting (Fig. 4A). Pre-treatment of the Ptd-Ser adsorbed onto ELISA plates with L10 milk gave no effect on the subsequent binding of MFG-E8 in W2 milk (data not shown), indicating that this inhibition is not due to a masking effect of Ptd-Ser with constituents of the L10 milk.

To determine which component in L10 milk is responsible for the inhibition of Ptd-Ser-binding, L10 milk was fractionated into milk serum (whey) and MFG fractions and each fraction was incubated with W2 milk, followed by the assay for MFG-E8

Ptd-Ser-binding. Only the MFGs fraction showed strong inhibitory activity comparable with whole L10 milk (Fig. 4B). Specificity of the Ptd-Ser-binding inhibition was evaluated using liposomes instead of MFG. The Ptd-Ser-liposome, but not the PC-liposome, clearly inhibited the binding of milk MFG-E8 to the Ptd-Ser adsorbed onto ELISA plates (Fig. 4C).

Subsequently, association of MFG-E8 from W2 milk with MFGs in L10 milk was examined by density gradient ultracentrifugation of L10 milk, W2 milk and the mixture of both. The MFG-E8 distribution profile of W2 milk was shifted to the profile of L10 milk, when W2 milk was mixed with L10 milk, *e.g.* MFG-E8 present mainly in fractions 2 and 3 of W2 milk moved to the fraction 1, which was rich in MFGs, after mixing of the two milk samples (Fig. 4D and E). Thus, MFG-E8 in W2 milk was found to possess the ability to associate with MFGs in L10 milk.

Fat globules were taken up by HC11 mammary cells in a MFG-E8 dependent manner

Interaction of MFGs with mammary epithelial cells was investigated in an *in vitro* model using HC11 mammary epithelial cells. First, L10 milk was incubated with HC11 cells and uptake of MFGs by the cells was then observed after Oil-red O staining (Fig. 5A). Oil-red O positive signals were clearly observed on the cells incubated with whole milk, but almost none were evident with membrane-filtered (MFGs depleted) milk. Such Oil-red O positive signals were decreased

Fig. 4 Binding of MFG-E8 in W2 milk to MFGs in L10 milk. (A) Ptd-Ser (PS) coated wells were incubated with W2 milk, L10 milk and the W2 milk mixed and incubated with L10 milk, respectively, and MFG-E8 bound to Ptd-Ser was measured by ELISA. Values with different superscript letters (a, b and c) indicate significant difference ($P < 0.01$). Alternatively, Ptd-Ser (PS)-, PC-coated or methanol-treated wells were incubated with L10 milk, W2 milk and the W2 milk incubated with L10 milk. After washing of the wells, SDS sample buffer was added to each well and recovered proteins were analysed by immunoblotting with anti-MFG-E8 antibody. (B) L10 milk was separated into milk serum (whey) and MFG fractions by centrifugation as described in Materials and Methods. W2 milk was mixed and incubated with the L10 milk serum, the L10 MFG fraction, the filtered L10 milk (see the legend of Fig. 3), or whole L10 milk. These milk samples were then incubated with Ptd-Ser (PS) or PC-coated wells and MFG-E8 bound to Ptd-Ser was measured by ELISA. Data were shown as mean $\pm$ standard deviation (SD) of independent L10 milk from four mice. (C) The W2-milk serum fraction prepared by ultracentrifugation was incubated with Ptd-Ser (PS)- and PC-coated wells in the presence and absence of liposome, Ptd-Ser-liposome or PC-liposome. MFG-E8 bound to the coated phospholipids was measured by ELISA. Data are shown as mean $\pm$ SD of three determinations. (D) L10 milk, W2 milk and a mixture (1 : 1 by vol.) of both milk samples were fractionated by SDG ultracentrifugation and 12 fractions (900 μ l per fraction) were collected from the top (corresponding to lanes 1–12). Distribution of MFG-E8 in these fractions was analysed by immunoblotting. (E) L10 milk was fractionated by SDG ultracentrifugation, the triglyceride concentration of each fraction was determined and expressed as mean $\pm$ standard deviation (SD) of four independent ultracentrifugation experiments. The fractions 1, 2, 6 and 7 were subjected to MFG observation under a phase contrast microscope (the right panels).

Fig. 5 MFG-E8-dependent uptake of MFGs by mammary HC11 cells and integrin expression in the cells. (A) L10 milk was incubated with HC11 cells and the bound MFGs were visualized with Oil red O staining as described in Materials and Methods. In panel d, the L10 milk was filtered before the incubation to remove MFGs. In panels b and c, the milk samples were pre-incubated with anti-MFG-E8 antibody and a control antibody (anti-V8 protease), respectively. (B) L10 and W2 milk samples were incubated with HC11 cells, in which triglyceride concentration in those samples was adjusted to equal. Bound MFGs on HC11 cells were visualized by Nile red staining, and observed under a fluorescence microscope. The number of MFGs on HC11 cells was counted using the Image-J software (the right panel). Asterisk indicates a significant difference ($P < 0.01$). (C) mRNA of several integrins in HC11 cells cultured on plate or in Engelbreth-Holm-Swarm sarcoma gel (3D) as well as mammary gland (MG) of L10 and W2 mice was analysed by RT-PCR. β -Actin mRNAs as internal control were also analysed for comparison. (D) L10-milk samples were incubated with HC11 cells in the presence of RGE-peptide (Control) and RGD-peptide. Bound MFGs on HC11 cells were visualized by Nile red staining, and observed under a fluorescence microscope. The number of MFGs on HC11 cells was counted using the Image-J software (the right panel).

remarkably when the milk was pre-incubated with anti-MFG-E8 antibody. These results imply that uptake of MFGs by HC11 cells depends on MFG-E8. Second, to examine the effect of weaning

on the uptake of the MFGs, HC11 cells were incubated with either L10 or W2 milk, appropriately diluted to equalize MFGs concentration, followed by Nile Red staining. The fluorescent signals from MFGs taken up

by HC11 cells were clearly detected for both milk samples and the fluorescent signal of W2 milk appeared to be stronger than that of L10 milk. Indeed, the integrated density of W2 milk was ~2-fold higher than those of L10 milk (Fig. 5B).

Expression of some species of integrins in HC11 cells as well as mouse mammary tissue was investigated by reverse transcriptase-PCR (RT-PCR) analysis (Fig. 5C). The analysis showed that $\alpha v\beta 5$ integrin was expressed by HC11 cells, regardless of the culture conditions, and also in either lactating or involuting mouse mammary glands. To evaluate the contribution of the integrin-binding RGD-motif of MFG-E8 to the uptake of MFGs by HC11 cells, MFG uptake by HC11 cells was examined in the presence of a competitive RGD-peptide and a control RGE-peptide (Fig. 5D). The RGD-peptide showed no inhibitory effect and the integrated density.

Discussion

Although knowledge on structural and functional properties of a MFG protein, MFG-E8 (also known as Guinea-pig GP-55, bovine PAS6/7, human BA46) has increased during the past 20–25 years after the first identification (14) and cDNA cloning (8), the physiological roles of MFG-E8 in breast milk, especially as a fat globule membrane component, is poorly understood. Two previous studies revealed that MFG-E8, presumably those on the surface of MFGs, accumulated at the mammary alveolar epithelium after weaning (4) and membranous materials remained in the ducts of mammary tissue during mammary gland involution of *MFG-E8* deficient mice (6), suggesting roles of MFG-E8 in the re-absorption of MFGs by epithelial cells soon after weaning, or the subsequent clearance by migrating phagocytes during latter stages of involution. In this study, we showed a weaning-associated decrease in milk triglyceride concentration, reflecting fat globule content, without quantitative and compositional changes in caseins, the major nutritional proteins in milk. This triglyceride-specific decrease suggested that a part of MFGs were selectively re-absorbed by mammary epithelial cells at some points after weaning. Slight changes in the size distribution of MFGs might result from preferential elimination of some MFG populations due to their size. A decrease in triglyceride concentration by dilution of milk with body fluids could be excluded, because protein concentration in milk was kept unchanged or rather increased slightly after weaning.

The presence of MFG-E8 on the surface of MFGs in mouse milk was shown immunocytochemically under a laser fluorescence microscope. Among MFGs in L10 milk, there were large variations in both of the MFG-E8 and annexin V signal intensities, indicating the presence of a variety of MFGs with not only MFG-E8 but also exposed and accessible Ptd-Ser on their surface. In W2 milk, on the other hand, a high correlation between the fluorescence intensity of MFG-E8 and the size of MFGs was observed, in addition to almost no annexin V signal. The virtual absence of an annexin signal on the MFGs in W2

milk could be explained by the previously reported observation (10) that human MFG-E8 bound to Ptd-Ser containing membranes with an affinity higher than some other Ptd-Ser-binding proteins including annexin V and MFG-E8 could thereby displace them for membrane binding sites. Thus, the immunocytochemical observations suggested that MFG-E8 distributed uniformly and adequately on most of MFGs in W2 milk. Such a weaning-induced transition in MFG-E8 and accessible Ptd-Ser on the MFG surface suggested that Ptd-Ser exposed on MFGs as time goes on after secretion was bound partially with MFG-E8 soon after secretion and the MFG-E8 binding to MFGs became maximal during the 2 days after weaning. However, masking of accessible Ptd-Ser by some unknown factor(s) other than MFG-E8 cannot be absolutely excluded. Cessation of suckling stimulation by weaning would stop fat biogenesis and MFG secretion by epithelial cells, and this supply disruption of fresh MFGs might result in the increase in stale and aged MFGs in breast milk. It seems likely that aged MFGs are bound with MFG-E8 through exposed Ptd-Ser in a manner similar to apoptotic bodies (22) and subsequently phagocytized by epithelial cells in involuting mammary glands. This speculation is supported by the results that the uptake of MFGs by HC11 cells was MFG-E8-dependent and dominant in W2 milk.

At the reversible stage of mouse mammary gland involution (up to 2 days after weaning) (23), mammary epithelial cells are still functionally active and can begin milk secretion again when suckling stimulation is resumed. Therefore, such phagocytized MFGs would be digested intracellularly and reused metabolically by the epithelial cells. It remains uncertain why MFGs are ahead of the other milk components, such as proteins, in the re-absorption by mammary epithelial cells. A possible interpretation is that aged MFGs are produced in breast milk during prolonged suckling intervals and need to be removed efficiently to prevent oxidative deterioration of milk fat nutrients for infants, *e.g.* docosahexaenoic acid, an important phospholipid component of the retina and brain (24). It seems likely that MFGs are exposed to some oxidative environments, because major breast-milk leukocytes are neutrophils and macrophages producing reactive oxygen species against microbial infection (25). Indeed, in one previous study using 2–3 h tissue-cultures (explants) of mammary glands from lactating mice, the level of lipid peroxidation products measured as thiobarbituric acid-reactive substances significantly increased in the mammary tissue by treatment with oxytocin (26), which is a lactation-related hormone released after suckling stimulation and which facilitates breast feeding through mammary alveolar contraction and milk ejection. Moreover, remarkable upregulation of milk ceruloplasmin within 48 h after weaning shown in our previous study (27) may indicate a rapid increase in oxidative stress in mammary glands after weaning, because serum ceruloplasmin was reported to catalytically remove hydrogen peroxide and lipid hydroperoxides in the presence of reduced glutathione (28). The MFG-E8 concentration increased in breast milk during the 48 h after weaning (Fig. 1

and (4)), and a considerable proportion of the MFG-E8 was free from MFGs and Ptd-Ser-binding active in W2 milk. These results suggest that secretion of MFG-E8 attains a large excess over fat globule secretion during 48 h after weaning. The Ptd-Ser-binding activity of MFG-E8 fractionated by size-exclusion chromatography and density-gradient ultracentrifugation indicated that not only the monomer form but also complex forms, possibly membrane microvesicle-associated forms, in W2 milk exhibited binding activity to the Ptd-Ser bound to ELISA plates. Moreover, MFG-E8 Ptd-Ser-binding activity in W2 milk was inhibited almost completely by adding MFGs from L10 milk and MFG-E8 in the higher density SDG fractions of W2 milk was markedly decreased by mixing with L10 milk (Fig. 4). These results suggest that both the monomer and complex forms of MFG-E8 in filtered milk serum binds to MFGs. At this moment, there is no direct evidence indicating that MFG-E8 binds to Ptd-Ser exposed on the MFG surface. However, MFG-E8 binding through Ptd-Ser is most likely, because MFGs in L10 milk-stained positive for annexin V, which binds to Ptd-Ser by competing for MFG-E8, and the number of MFG-E8 binding sites on Ptd-Ser-containing membranes was reported to depend on Ptd-Ser content of each target membrane (29). Furthermore, it seems reasonable for the long form of MFG-E8 to become dominant after weaning (Fig. 1A and (5)) for binding to Ptd-Ser on MFGs' surface, because the long form of mouse MFG-E8 was reported to bind to Ptd-Ser more strongly than the short one (11). To evaluate fat globule uptake by mammary epithelial cells, a special inverted culture was adopted. This allowed close contact of low-density, floating MFGs with HC11 cells. The inhibitory effect of an anti-MFG-E8 antibody on the MFG-uptake by HC11 cells suggested that interaction between MFGs and HC11 cells was at least MFG-E8-dependent. On the other hand, no effect of the RGD-peptide indicates that MFG uptake by HC11 cells is not simply enhanced by binding of MFG-E8-coated MFGs to RGD-containing integrins on the surface of HC11 cells. Similar results, that is, MFG-E8 dependent but RGD-peptide independent uptake, have been reported previously in a study on collagen uptake by macrophage cells in a mouse fibrosis model (30). However, MFGs resemble apoptotic bodies in their structure and components rather than collagen matrix. No inhibition by the RGD-peptide may indicate that MFG-E8 interacts with mammary epithelial cells through unknown mechanisms different from those found in the MFG-E8-binding to professional phagocytes, macrophages. It remains to be determined whether the integrins are expressed on the apical surface of polarized mammary epithelial cells *in vivo*. Nevertheless, such uptake of MFGs by mammary epithelial cells are likely to happen in the lactating mammary glands, as previously demonstrated by an earlier histological study of lactating mammary glands (31).

A previous study indicated that human MFG-E8 enhanced the uptake of apoptotic cells by macrophage cells in a dose-dependent manner but excess amounts

(above 1 µg/ml in that experimental condition) of MFG-E8 showed inhibitory effects rather than enhancing uptake (32). Free MFG-E8 in the *in vitro* MFG-uptake assay system of this study was not sufficiently excessive to inhibit MFG-HC11 cell interaction, because the milk was diluted 1 : 500. However, it is likely that large amounts of free MFG-E8 present in the W2 milk could inhibit the MFG uptake by mammary epithelial cells *in vivo*. We speculate that in the early stage of mammary gland involution MFG-E8 shows an enhancing effect on the MFG uptake by still active mammary epithelial cells until a certain time after weaning and afterward inhibits the uptake by the mammary cells, which are about to enter into apoptosis. The large amount of milk MFG-E8 at the later stage of involution may be required for the effective uptake of apoptotic mammary epithelial cells by professional phagocytes, macrophages.

In conclusion, in lactating and involuting mammary glands the supply and consumption of MFGs could be in fine balance dependent upon suckling, biogenesis, secretion and re-absorption by mammary epithelial cells. In such a delicate regulation of fat globule dynamics, MFG-E8 would play critical roles through not only the control of fat globule–cell interaction to ensure optimal clearance of apoptotic cells but also in the maintenance of the quality of minor but critical lipid components of the neonatal diet.

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Conflict of Interest

None declared.

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