Urgent Announcement of Article Retraction

October 16, 2013

The Editorial Board of *Bioscience, Biotechnology, and Biochemistry* has recently confirmed that the review article written by Naohito AOKI, published in the *Bioscience, Biotechnology, and Biochemistry*, Vol. 70, No.9, pp2019-2027 (September 2006), is based on the papers containing a number of serious ethical problems.

Naohito AOKI

Regulation and Functional Relevance of Milk Fat Globules and Their Components in the Mammary Gland


Therefore, we have decided to retract the paper from *Bioscience, Biotechnology, and Biochemistry*.

As Editor-in-Chief, I regret the time that peer reviewers and others spent reviewing this paper. I sincerely hope and trust that there will be no repetition of this kind of problems in the future.

Kazumitsu UEDA

Editor-in-Chief,

*Bioscience, Biotechnology, and Biochemistry*
Mammary gland and epithelial cells are unique to mammals and are under the control of lactogenic hormones such as prolactin. Recent findings indicated that major components of milk fat globule membrane (MFGM) are under the control of lactogenic hormones, and that the major components butyrophilin and xanthine oxidoreductase are indispensable for milk fat secretion. Further, prolactin signaling is negatively controlled by two highly related protein tyrosine phosphatases, PTP1B and TC-PTP. Milk fat globule EGF factor 8 (MFG-E8) is one of the major components of MFGM and is upregulated during lactation. MFG-E8 is further upregulated in the involuting mammary gland. MFG-E8 on exosome-like membrane vesicles in the milk recovered from post-weaning but not lactating mammary glands exhibits higher binding activity to phosphatidylserine and apoptotic mammary epithelial cells, and serves as a link between apoptotic mammary epithelial cells and phagocytes. Recent reports using MFG-E8 deficient mice support the view that MFG-E8 is indispensable for eliminating apoptotic mammary epithelial cells during involution.

Key words: mammary gland; milk fat globule membrane; prolactin signaling; protein tyrosine phosphatase; milk fat globule EGF factor 8 (MFG-E8)

Mammary glands are unique organs developed for nursing newborn offspring. They repeat cycles of development, lactation, and involution for each pregnancy (Fig. 1). In each cycle, mammary epithelial cells (MECs) proliferate, differentiate, and die due to apoptosis.

Mammary gland development is a complex process. Normal development occurs through the influences of systemic hormonal factors such as prolactin (PRL) and locally acting growth factors that cause the mammary epithelium to proliferate and differentiate upon pregnancy. With each pregnancy, an expanded lobulo-alveolar compartment rises on the ductal compartment and differentiates to secrete milk during lactation. In the process of involution, these secretory structures undergo regression to the embryo-like state of development.

I. Mammary Epithelial Cells and Milk Fat Secretion

Milk fat, composed mainly of triglycerides, is secreted as droplets of variable sizes. In the milk of cows, 99% or more of the total lipid is found in these droplets, which are called milk fat globules (MFGs). These droplets are originally released from endoplasmic reticulum into the cytosol as microlipid droplets coated by proteins and polar lipids. Microlipid droplets can fuse with each other to form larger cytoplasmic lipid droplets. Droplets appear to be unidirectionally transported to apical cell regions by as yet unknown mechanisms that perhaps involve cytoskeletal elements. These lipid droplets appear to be secreted from the cell in which they were formed by being progressively enveloped in differentiated regions of apical plasma membrane. This process is summarized and illustrated in Fig. 2.

Abbreviations: MEC(s), mammary epithelial cell(s); PRL, prolactin; WAP, whey acidic protein; PRL-R, prolactin receptor; PTP(s), protein tyrosine phosphatase(s); MFG-E8, milk fat globule EGF factor 8; MFG(s), milk fat globule(s); MFGM, milk fat globule membrane; mAb(s), monoclonal antibody(ies); XDH, xanthine oxidoreductase; JAK, Janus kinase; STAT, signal transducers and activators of transcription; PTK(s), protein tyrosine kinase(s); TC, T-cell
Milk fat secretion is an interesting biological process essential to the maintenance of most mammalian life. Since secretion of the MFGs from mammary epithelial cells apparently takes place by a unique mechanism, this process has received attention from a number of researchers, but much is not known concerning the molecular mechanisms involved in these processes. This is due to difficulty in handling MFGM. Moreover, it is difficult to observe the fat secretion process using primary and established mammary epithelial cells in culture.

We and other groups thought that isolation and characterization of the protein components associated with MFGM might help to elucidate the milk fat secretion process. As mentioned above, it was not easy to handle MFGM and isolate its components. Hence we tried to prepare a line of monoclonal antibodies (mAbs) by immunizing a whole MFGM fraction instead of each purified component. Nine independent mAbs were successfully obtained, and using some of these mAbs, we cloned cDNAs encoding bovine MFG-E8, formerly called MGP57/53, and characterized the polysaccharides on the protein.

Recent work on characterization of proteins of the milk fat globule membrane has made more progress in revealing the nature of this coat material and has yielded information on what roles some of the constituent proteins might play in fat globule secretion. Based on studies of protein topologies and protein–protein interactions, it is likely that the protein coat on the inner face of the milk fat globule membrane comprises mainly the cytoplasmic tail of integral butyrophilin and the peripheral protein xanthine oxidoreductase (XDH). Butyrophilin is the major protein of bovine MFGM, accounting for about 20–40% of total membrane-associated protein. Butyrophilin is expressed specifically in mammary glands and is concentrated in the apical plasma membrane and the MFGM. XDH, also a major protein of MFGM, is a well-known and extensively characterized redox/purine catabolizing enzyme. In contrast to butyrophilin, XDH is a soluble cytosolic enzyme, but in milk-secreting cells, this protein is concentrated along the inner face of the apical plasma membrane and the MFGM, as revealed by immunohistochemical analysis with bovine mammary glands.

We cloned a cDNA encoding mouse butyrophilin. The intracellular part of butyrophilin was expressed as a GST-fusion protein in E. coli and purified. With this recombinant protein as bait, we demonstrated that butyrophilin specifically associates with XDH. We noticed that butyrophilin was dissociated from detergent-insoluble MFGM by treatment with a reducing reagent (Aoki et al., unpublished observation). Interestingly, it has also been reported that XDH was released from MFGM by this treatment. These data, together with the fact that disulfide isomerase is present in the MFGM fraction, suggest that disulfide bond formation...
plays a role in the interaction and envelopment of lipid droplets by the apical plasma membrane.\textsuperscript{15,16}

Further analysis using knockout mice has recently revealed that XDH and butyrophilin are indispensable for milk fat secretion. Heterologous mice with one disrupted XDH allele (XDH\textsuperscript{+/−}) displayed deficiencies in milk fat secretion with consequent accumulation of milk fat within mammary epithelial cells.\textsuperscript{17} The milk of these animals was characterized by the presence of larger lipid droplets with disrupted and incomplete MFGMs. Homozygous XDH\textsuperscript{−/−} knockout mice died within the first 6 weeks after birth.\textsuperscript{17} Milk fat secretion also was abolished in mice where butyrophilin expression was either disrupted or eliminated.\textsuperscript{18} Large amounts of triacylglycerol accumulated in the cytoplasm of secretory mammary epithelial cells, and lipid droplets escaped from the apical surface with disrupted outer membranes. Luminal spaces became engorged with unstable lipid droplets.\textsuperscript{18} These results clearly demonstrate that XDH and butyrophilin play essential roles in milk fat globule secretion, but the details of the molecular involvement of these proteins in the secretory process remain to be examined.

II. Prolactin Receptor-Mediated Signaling and Its Regulation by Protein Tyrosine Phosphatases

The polypeptide hormone prolactin is produced in the anterior pituitary.\textsuperscript{19} It regulates the activity of milk
Tyrosine phosphorylation is a fundamental mechanism for numerous important aspects of eukaryote physiology in human health and disease. Abnormalities in tyrosine phosphorylation play a role in the pathogenesis of numerous inherited and acquired human diseases, from cancer to immune deficiencies. Although it is generally accepted that tyrosine phosphorylation is regulated by the equal and balanced action of protein tyrosine kinases (PTKs) and PTPs, much more research has focused on PTKs. Recent findings have led to an emerging recognition that PTPs play specific and active, even dominant, roles in setting the levels of tyrosine phosphorylation in cells and in the regulation of many physiological processes.

Focusing on the negative regulation of PRL-mediated signaling, we first addressed the expression profiles of protein tyrosine phosphatases in mammary glands as well as mammary epithelial cells using degenerate primer sets encompassing a highly conserved PTP core sequence. With this strategy, we found that 16 different PTPs, including SHP-1 and SHP-2, were expressed in mammary glands and mammary epithelial cells and that most of them were down-regulated in lactating mammary glands, suggesting a negative role in mammary function during lactation. To extend these findings, we further investigated the involvement of each PTP in the PRL-R-mediated signaling pathway using expression constructs for PRL-R, STAT5a/b, and each PTP. We found that both of the PRL-induced tyrosine phosphorylations of STAT5a/b and promoter activation of the β-casein gene were abolished when cytosolic PTP1B was overexpressed. Nuclear translocation of STAT5 was also inhibited by PTP1B. Overexpression of PTP1B in mammary epithelial cells also resulted in dephosphorylation of PRL-activated STAT5a/b and down-regulation of β-casein gene expression upon lactogenic hormone treatment. STAT5a and STAT5b were dephosphorylated by recombinant PTP1B in vitro and were also dephosphorylated by substrate-trapping mutants of PTP1B in vivo.

To explore the possible biological functions of MFGM proteins in the mammary gland, we examined how major components, such as MFG-E8, butyrophilin, and CD36, in mammary epithelial cells were regulated during the gestation and lactation periods. With a combination of RT-PCR and Northern blot analysis, it was found that expression of these MFGM proteins was regulated during lactation, the alveolar component of the gland involutes through a process with both apoptosis and tissue remodeling, which rebuilds the gland to a virgin-like state. Under conditions of forced weaning, this process falls into two phases, an initial apoptotic phase that begins within 12 h and lasts about 72 h, and a second phase involving further apoptosis, matrix degradation, and gland remodeling. The first phase of involution (but not apoptosis) is reversible if pups are returned within the initial 2 d. During the second phase of
involution, milk accumulates locally within alveolar lumina, and the levels of systemic lactogenic hormones fall. This phase is truely apoptotic. Degradation of nuclear DNA into fragments that form ladders on agarose gels, apoptotic morphology of epithelial cells exhibiting chromatin condensation, and activation of effector caspases, all characteristic markers of apoptosis, are observed within 2 d of withdrawing pups.

Apoptotic mammary epithelial cells must be cleared immediately by phagocytes in involuting mammary glands to prevent inflammation and autoimmune response against intracellular antigens released by the dying cells. Phagocytosis of apoptotic mammary epithelial cells by both macrophages and residual living epithelial cells has been demonstrated with convincing data, and three potential fates of apoptotic mammary epithelial cells have been suggested: release into the lumen, and phagocytosis by neighboring alveolar epithelial cells or macrophages. As phagocytosis-related molecules associated with apoptotic cell uptake, various receptors and ligands such as CD14, CD36, CD68, αvβ3 integrin, ABC1 transporter, have been suggested, but the detailed mechanisms of apoptotic cell recognition by phagocytes remain to be investigated. Recently, a major MFGM component, MFG-E8, has emerged as a tethering molecule between apoptotic cells and activated macrophages.

As mentioned above, the expression of MFG-E8 in mouse mammary glands has been shown to be upregulated after parturition and to be maintained during lactation even at a later stage, day 16. Very recently, a comprehensive analysis of gene expression in mouse mammary gland involution has been conducted by the microarray technique. This indicated that MFG-E8 transcripts gradually increased to about 1.5 times in the normalized intensity within 3 d after forced weaning for 10-d lactating mice.

Our previous experimental data together with recent related ones on the structure, function, and expression of MFG-E8 suggest that MFG-E8 might also be involved in the recognition and clearance of apoptotic mammary epithelial cells during mammary involution. To this end, we first examined the expression of MFG-E8 in involuting mammary glands, where the glands undergo a substantial increase in the rate of epithelial cell apoptosis. Immunoblot and Northern blot analyses indicated that MFG-E8 at both protein and mRNA levels increased markedly in mammary glands within 3 d following either natural or forced weaning (by pup withdrawal) of lactating mice. By detailed
immunohistochemical analysis of mammary tissue cryosections, the MFG-E8 signal was detected around the epithelium of such involuting mammary glands, whereas it was almost undetectable at the early- and mid-lactation stages, though strong signals were obtained for milk fat globules stored in the alveolar lumen. Some signals double-positive to a macrophage differentiation marker, CD68, and MFG-E8 were detected in post-weaning mammary glands, though such double-positive signals were much smaller in number than the MFG-E8 single-positive ones. MFG-E8 content in milk also increased in the post-weaning mammary glands and the MFG-E8 content in a free form in post-weaning milk as measured by in vitro phosphatidylserine (PS)-binding and apoptotic HC11 cell-binding activities was much higher than that of lactation. In addition, the post-

Fig. 4. MFG-E8-Assisted Elimination of Apoptotic Mammary Epithelial Cell during Involution. During lactation, MFG-E8 is released in association with MFGM and exosome-like membrane vesicle (ELMV) (panel A). Upon weaning, MFG-E8, predominantly released from ELMVs, binds to apoptotic mammary epithelial cells (panel B) and is engulfed by macrophages and neighboring epithelial cells through the RGD-integrin bridge (panel C).
weaning milk enhanced the binding of J774 macrophages to apoptotic HC-11 cells. Sucrose density-gradient ultracentrifugation analysis revealed that such enhanced PS-binding activity of MFG-E8 was to be attributed to fractions with densities ranging from 1.05 to 1.13 g/ml, which met the criteria for exosome-like membrane vesicles, rather than MFGM fractions in the milk. Two independent groups have reported that MFG-E8 is a critical protein for mammary gland remodeling during the involution process with MFG-E8 knockout mice. A deficiency in MFG-E8 caused delayed clearance of apoptotic mammary epithelial cells as well as milk fat globules (MFGs) and impaired involution and inflammation of mammary glands. Hence weaning-induced MFG-E8 might play an important role in the recognition and engulfment of apoptotic epithelial cells by phagocytic macrophages and neighboring epithelial cells in involuting mammary glands. These processes are illustrated in Fig. 4.

IV. Concluding Remarks

The mammary gland is a dynamic and complex organ that functions in a cyclical manner during pregnancy, lactation, and involution. This review highlights the role of MFG-E8 in the remodeling of mammary glands during involution and inflammation. Weaning-induced MFG-E8 might play an important role in the recognition and engulfment of apoptotic epithelial cells by phagocytic macrophages and neighboring epithelial cells in involuting mammary glands. These processes are illustrated in Fig. 4.

References

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