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LEPTIN EXPRESSION IN HUMAN MAMMARY EPITHELIAL CELLS AND BREAST MILK

SUSAN M. SMITH-KIRWIN[*], DARLISE M. O'CONNOR[*], JENNIFER JOHNSTON[*], ELIZABETH DE LANCEY[*], SANDRA G. HASSINK[§#], AND VICKY L. FUNANAGE[*#]

Departments of Research[*] and Pediatrics[§], Alfred I. duPont Hospital for Children, Wilmington, DE and Department of Pediatrics[#], Thomas Jefferson University, Philadelphia, PA

ABSTRACT

Leptin has recently been shown to be produced by the human placenta and potentially plays a role in fetal and neonatal growth. Many functions of the placenta are replaced by the mammary gland in terms of providing critical growth factors for the newborn. In this study, we show that leptin is produced by human mammary epithelial cells as revealed by RT/PCR analysis of total RNA from mammary gland and immunohistochemical staining of breast tissue, cultured mammary epithelial cells, and secretory epithelial cells present in human milk. We also verify that immunoreactive leptin is present in whole milk at 30- to 150-fold higher concentrations than skim milk. We propose that leptin is secreted by mammary epithelial cells in milk fat globules, which partition into the lipid portion of breast milk.

Leptin, an adipocyte-secreted protein, has been shown to be involved in regulation of energy metabolism and food intake (1). In addition, leptin has been found to be important in achieving reproductive capacity and sustaining pregnancy in the leptin-deficient mouse (2) and in enhancing normal pubertal development (3). Studies have shown that leptin concentrations rise during pregnancy and that leptin per unit fat mass is higher during pregnancy than at 3 and 6 months postpartum (4). Leptin is also produced by the human placenta (5-7) and is found in cord blood of newborns (5,8-10). Leptin concentrations in the newborn are independent of maternal levels; thus, placental leptin may serve to regulate fetal growth and development.

The function of the placenta is replaced by the mammary gland in terms of providing necessary growth factors for the newborn. Leptin has recently been demonstrated to be present in human milk and to relate to maternal adiposity (11,12). However, leptin concentrations in skim milk have been shown to be significantly lower than maternal serum leptin levels. Houseknecht et al. (11) have shown that leptin is present at a 10-fold higher concentration in whole milk as compared to skim milk. The authors speculate that leptin is derived either from the maternal circulation or produced by breast fat. However, the observation that there is no correlation between breast size and leptin concentration in milk argues against production of leptin by the adipose tissue of the breast (11). Since the mammary gland is responsible for the production of many of the nutrients, cytokines, and growth hormones present in breast milk, we hypothesized that mammary epithelial cells also synthesize and secrete leptin.

METHODS

Measurement of leptin in breast milk: All breast milk was obtained from donors who voluntarily consented to participate in the study. They were healthy women from the Research Department, between 25-35 yr of age, who planned to breast-feed and had delivered healthy full term infants. Milk was collected by either hand expression or a hand-held electric pump and stored frozen at –20 C. For leptin analysis, milk samples were thawed overnight in the refrigerator. Skim milk was prepared by centrifugation of whole milk at 13000 rpm for 10 min at 4 C to separate milk fat from the liquid phase. A spatula was used to remove the layer of fat. Milk samples were sonicated for 3-10 sec bursts with 20 sec cooling intervals using a Branson Model W180 sonicator with a stepped microtip. Radioimmunoassay (Linco Research, St. Charles, MO) for serum leptin was performed as previously described (5).

Cytospin cell preparations: Human mammary epithelial cells were prepared for cytospin from at least 20 mL breast milk. Milk was spun at 2500 rpm, and the liquid and fat layers were removed. The pelleted cells were rinsed once in HBSS and then resuspended in HBSS. The cell suspension was transferred to disposable cytofunnels with attached cytospin slides and spun at 1000 rpm for 10 min in a Cytospin 3 (Shandon Inc., Pittsburgh, PA). Slides were stored frozen at -20 C.

Cell Culture: Human Mammary Epithelial Cells (HMEC) were obtained from Clonetics (San Diego, CA) and grown in serum-free MEGM medium (Clonetics). HMEC cells were mixed 1:1 with cold undiluted Matrigel (Collaborative Research, MA) according to Gomm et al (13). Twenty-four well plates were seeded with this cellular mix and warmed to 37 C for at least 30 min before being overlaid with the growth medium. Cells were allowed to grow for up to 3 weeks in culture during which time they continued to divide and fuse to form large aggregates of cells from which ducts were commonly seen.

Immunohistochemistry: Normal frozen breast tissue was obtained from Christiana Care, Inc. (Christiana, DE), and 10 μm sections were cut and placed onto glass slides. These sections and the cytospin cell samples were fixed in Streck Tissue Fixative (Streck Laboratories Inc., Omaha, NE) for 5 min at room temperature. HMEC cells grown in Matrigel were fixed overnight at 4 C in the same fixative before the cellular gel was removed, mounted in 10% Tragacanth gum, snap-frozen in isopentane, and 10 μm sections prepared. Samples were then blocked with Peroxo-block and CAS block (Zymed, San Francisco, CA) according to the manufacturer's instructions. Superblock (Research Genetics, Huntsville, AL) was utilized to block background staining from Matrigel proteins. Polyclonal rabbit anti-leptin (RDI, Flanders, NJ) was diluted 1:100 with serum blocking buffer and incubated for 60 min at room temperature. Antibody to epithelial membrane antigen (Zymed) was used neat; milk fat globule membrane antibody (Novocastra Laboratories, Ltd., Burlingame, CA) was diluted 1:50. A kit obtained from Zymed was used for subsequent steps for the biotinylated labeled secondary antibody and colorimetric detection with either DAB or AEC chromagens.

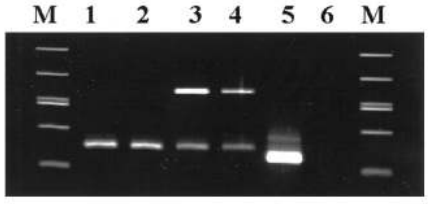

Figure 1: RT/PCR analysis of leptin mRNA in mammary gland and adipose tissue. Total RNA was reversed transcribed from either mammary gland (lanes 1,3,5) or adipose (lanes 2,4,6) tissue and amplified with PCR primers to either leptin (lanes 1-2), both leptin and β-actin (lanes 3-4) or β-casein (lanes 5-6). Markers (M) are 1000, 700, 525, 500, 400, 300 bp. The size of the leptin RT/PCR product is 348 bp; β-actin is 592 bp; β-casein is 329 bp.

RT-PCR: Human mammary gland total RNA was obtained from Clontech (Palo Alto, CA), and adipose RNA was from Invitrogen (Carlsbad, CA). One microgram of total RNA was reverse-transcribed using avian myeloblastosis virus and oligo (dT) primer (Promega, Madison, WI), according to the manufacturer's instructions. Amplification of the cDNA sequence was performed as described previously (5). For assessment of the relative levels of leptin to β-actin, a multiplex RT/PCR approach was used as initially described by Dukas et al. (14) and modified by us (5). Primer sequences for leptin and β-actin were those described previously (5). β-casein primers were: CASF1: 5'ATTCTGCCTCTTGCTCAGCCTGC3'; CASR1: 5'AGCTCTCTGAGGGTAGGGCACCAC3'. PCR products were separated on a 4% NuSieve (FMC Bioproducts, Rockland, ME) agarose gel and stained with ethidium bromide.

RESULTS

RT/PCR analysis of total RNA from mammary gland revealed the presence of leptin mRNA at a level comparable to that of adipose tissue (Fig. 1). Sequencing of the leptin cDNA product revealed complete sequence identity to that of placental and adipose leptin (GenBank accession number D9487). Figure 1 also shows expression of β-casein, a marker of mammary epithelial cell differentiation, in the mammary gland, but not the adipose tissue RNA.

Immunohistochemical staining with a polyclonal antibody to human leptin showed leptin production by ductal epithelial cells of human breast tissue (Fig. 2A). Figure 2C shows that cultured human mammary epithelial cells maintained on a Matrigel substrate also produce leptin. Finally, leptin was shown to be present in the secretory epithelial cells of breast milk (Fig. 2D). These cells, which are the predominant cell type shed into milk after the second month of lactation (15), were identified as epithelial cells by their positive staining for epithelial membrane antigen (Fig. 2E). Other abundant cell types in human milk include neutrophils and macrophages (15); these cell types did not express leptin. Interestingly, intense immunostaining for leptin was observed in non-cellular vesicles of human milk (Fig. 2F). These vesicles also stained positively for milk fat globule protein (not shown). These results indicate that leptin is either present in, or associated with, the milk fat globules of breast milk.

In agreement with Houseknecht et al. (11), immunoreactive leptin levels were higher in whole milk as compared to skim milk (Figure 3A). However, it was critical to sonicate the milk sample to detect the high levels of leptin in whole milk. Although Houseknecht et al. (11) sonicated the whole milk samples that were used to detect both leptin and milk fat levels in study I, it was not clear whether samples were sonicated in study II, where much lower levels of leptin were reported in whole milk. In contrast, sonication of skim milk samples made no difference in immunodetection of leptin (Figure 3B). These data, together with the immunolocalization of leptin to milk fat globules described above, suggest that leptin is sequestered within these vesicles and not detectable by radioimmunoassay. Sonication disrupts the membrane vesicles and allows subsequent immunodetection of leptin.

DISCUSSION

We show in this study that human mammary epithelial cells produce and secrete leptin. Furthermore, leptin appears to be associated with milk fat globules, which partition into the lipid fraction of whole milk. These results explain why leptin is found in higher concentration in whole as compared to skim milk (11, this study). Milk fat globules are derived from the apical plasma membrane of the epithelial cell and the secretory vesicle membrane of the Golgi apparatus. The globules therefore consist of a lipid core enclosed by membrane and membrane-bound proteins that are produced in the Golgi. Leptin probably becomes part of the milk fat globule as it is processed in the Golgi apparatus. It is likely that the association of leptin with these globules confers a protective effect against degradation of leptin by the infant digestive tract. In support of this hypothesis is the study by Sato et al. (16), which showed that the intestinal absorption of a cyclosporine derivative is enhanced when administered orally in a milk fat globule membrane emulsion. Of further interest is the observation that the absorption of emulsified compounds is decreased by sonication of the milk fat globule emulsion (17).

Surprisingly, average leptin concentrations in breast milk (73.22 ± 39.03 ng/mL, mean ± SE, n=8) were higher than serum leptin levels in normal (7.5 ± 9.3 ng/mL), obese (31.3 ± 24.1 ng/mL), pregnant (29.8 ± 17.0 ng/mL), or nursing (8.93 ± 0.96 ng/mL) individuals (4,12,18). Although sonication was necessary to detect the high levels of leptin in whole breast milk, sonication of skim milk did not increase leptin levels, and values for skim milk were similar to those reported in two other studies (11,12). Thus, the mammary gland appears to produce high quantities of leptin, in agreement with our RT/PCR data (Fig. 1) showing similar levels of leptin mRNA in adipose and mammary gland tissues. Sequencing of the leptin cDNA product revealed complete sequence identity to that of placental and adipose leptin. This agrees with the study of Casabiell (12), which demonstrated that milk-borne leptin is identical to human recombinant leptin in charge, size, immunorecognition, and SDS-PAGE mobility.

The production of leptin by the mammary epithelial cell explains the lack of association between breast size (adiposity) and leptin production reported by Houseknecht et al. (11). However, leptin levels in both whole and skim milk were correlated with maternal adiposity, although the correlation with

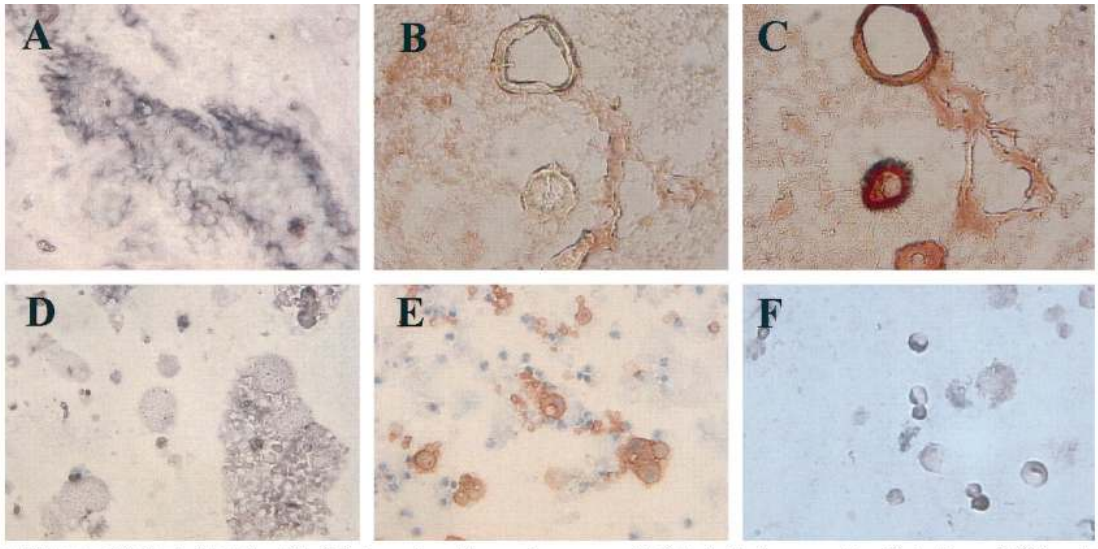

Figure 2. Immunohistochemical detection of leptin in human breast tissue and mammary epithelial cells. Sections were stained for leptin or epithelial membrane antigen as described in the Methods. Colorimetric detection was with DAB (brownish purple stain) for panels A, D, and F or AEC (red stain) for panels B, C, and E. (A) Human breast tissue stained for leptin, counterstained with methyl green; (B) Cultured HMEC on Matrigel, no primary antibody; (C) Cultured HMEC on Matrigel stained with leptin; (D, E) Epithelial cells from cytospins of 6 mo post-partum breast milk stained with either (D) leptin, counterstained with methyl green or (E) epithelial membrane antigen, counterstained with Mayer's hematoxylin; (F) milk fat globules from cream of breast milk stained with leptin. (A-E), 245X magnification; (F), 612X magnification

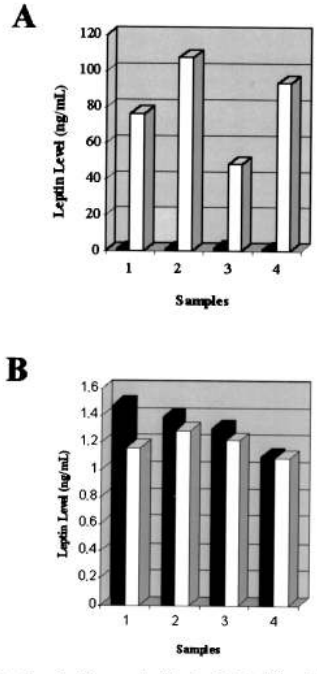

Figure 3. Effect of sonication on leptin levels in (A) whole and (B) skim milk. Milk samples were prepared and analyzed for leptin levels by radioimmunoassay as described in the Methods. (O) sonicated; (●) not sonicated.

whole milk was lower than skim milk (11). This finding may be explained by the known influence of adipose tissue on development of the mammary gland (19). Furthermore, adipocytes secrete factors known to influence mammary epithelial cell differentiation both in vivo and in vitro (20-22). Thus, women with low serum leptin levels may have less well-developed glandular structure and lower leptin levels in their milk. Additional studies are needed to prove this hypothesis

The low level of leptin that is detected in skim milk may arise from the maternal circulation and not the mammary gland. This would account for the high correlation between leptin levels in maternal serum and skim milk (11). It also would explain why sonication did not increase leptin levels in skim milk. Furthermore, Casabiell et al. (12) have shown that ^{125}I-leptin injected ip into lactating rats enters breast milk (only skim milk was analyzed) and is found in the stomach and serum of the nursing pups. However, the predominant source of leptin in breast milk appears to be the mammary gland. Putative roles of leptin in breast milk include a neonatal growth factor (5) and a regulator of neonatal food intake (12). It thus becomes critical to determine what factors regulate leptin production in the mammary gland and potentially impact on growth and development of the neonate.

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