Production of parathyroid hormone-related protein by the rat mammary gland in pregnancy and lactation

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Rakopoulos, Maryann, Socorro J. Vargas, Matthew T. Gillespie, Patricia W. M. Ho, Hanne Diefenbach-Jäger, David D. Leaver, Vivian Grill, Jane M. Moseley, Janine A. Danks, and T. John Martin. Production of parathyroid hormone-related protein (PTHrP) by the rat mammary gland in pregnancy and lactation. Am. J. Physiol. 263 (Endocrinol. Metab. 26): E1077-E1085. 1992.—Production of parathyroid hormone-related protein (PTHrP) by the mammary gland of Sprague-Dawley rats has been examined using immunohistochemistry and in situ hybridization to detect PTHrP and PTHrP mRNA, respectively. PTHrP and PTHrP mRNA could be demonstrated in nests of epithelial cells of the developing mammary gland at day 14 of pregnancy and in the epithelial secretory cells lining the alveoli during the latter stages of pregnancy and during lactation. A specific radioimmunoassay was also used to measure the concentration of PTHrP secreted in the milk throughout lactation. The concentration of PTHrP in milk was relatively low initially but increased during the latter stages of lactation, whereas calcium concentrations remained virtually constant throughout lactation. No correlation was found between the concentrations of calcium and PTHrP in rat milk. These results show that PTHrP is present in rat milk and also in mammary tissue before parturition, and therefore it may assist in the development of the mammary gland during pregnancy.

MATERIALS AND METHODS

Experimental animals and milk sample collection. Fourteen Sprague-Dawley female rats (250–300 g, The University of Melbourne, Victoria, Australia) were pair-mated in wire cages, and the day on which a sperm plug was observed was specified as the first day of pregnancy. The day after parturition was assigned as the first day of lactation, and at that time, the number of pups in each litter was reduced to eight. Throughout pregnancy and lactation, the dams were fed standard rat feed. At each time point examined, two dams were used so that eight dams were milked and their pups weighed as follows. The dams were milked from days 1–22 postpartum, on days 1, 2, and 3 of lactation, and every third day thereafter. Litters were removed from their mothers for 4 h before milking to allow milk to accumulate in the mammary gland. Just before milking, the dams and their respective litters were weighed, and their weights were recorded as a function of milk; calcium; neonatal development

PARATHYROID HORMONE-RELATED protein (PTHrP) was identified as a result of studies in patients with malignancy-associated hypercalcemia (22). This newly characterized calciotropic factor was purified from a human lung cancer cell line, its cDNA cloned, and its gene mapped (20, 21, 27, 34). The amino-terminal sequence homology of PTHrP with PTH accounts for the ability of PTHrP to act through PTH receptors in kidney and in bone to promote bone resorption and renal conservation of calcium (13, 40, 48).

PTHrP and PTHrP mRNA have been localized in various tumors and also in a diverse range of normal tissues such as skin (7, 12), lactating mammary tissue (39), the shell gland of the chick (38), fetal sheep parathyroid glands (19, 30), areas of the nervous system (39), and the myometrium of the rat uterus (36, 37). Evidence has been presented for PTHrP production by fetal parathyroid glands acting in an endocrine fashion to promote active placental calcium transport from maternal to fetal circulation (30). However, there is no evidence to date for a physiological role of PTHrP in other tissues. In mammary and chick shell glands, substantial amounts of calcium are transported from the maternal circulation into milk or into the egg shell, suggesting a possible paracrine role for PTHrP in calcium transport. However, there are conflicting reports in different species as to whether milk PTHrP levels are correlated with calcium. In one study, a positive correlation between milk calcium and PTHrP concentrations was found in cows (17), although no correlation was observed in another study in lactating mice (23). Furthermore, a preliminary report showed that serum PTHrP was increased during lactation, coinciding with increased bone resorption in the mother (24). PTHrP produced by the mammary gland may therefore fulfill an endocrine role in the mother or, since it is secreted in the milk, in the suckling neonate.

The purpose of this study was to localize PTHrP and PTHrP mRNA in both developing and lactating mammary tissue using immunohistochemistry and in situ hybridization, respectively. Secondly, in view of the conflicting evidence, a possible role for PTHrP in calcium transport from blood to milk has been investigated by examining whether milk PTHrP and calcium concentrations were correlated.

Radioimmunoassay. All milk samples were assayed at a dilution of 1:20 and 1:50 in assay buffer (0.05 M barbital buffer, pH 8.6), containing 0.5% bovine serum albumin (Commonwealth Serum Laboratories, Melbourne, Australia), 0.02% Tween 20 (Sigma Chemical, St. Louis, MO), 500 IU/ml aprotinin (Sigma), and 0.42 g/ml thimerosal [British Drug Houses (BDH) Laboratories, Poole, UK]. The radioimmunoassay was performed by using [125I]-PTHrP-(1–34) as tracer and synthetic PTHrP-(1–34) as standard. A rabbit polyclonal antibody raised against the synthetic PTHrP-(1–34) was used at a dilution of 1:10,000 (9). This antibody recognizes synthetic PTHrP-(1–34) and recombinant PTHrP-(1–84), -(1–108), and -(1–141) equally on a molar basis, but does not cross-react with PTH even at...
high concentrations (9). The sensitivity of the assay was 200 pg/ml, the intra-assay coefficient of variation was 6%, and the interassay coefficient of variation was 10.3%.

Milk calcium. Total calcium concentrations in rat milk were determined by atomic absorption spectroscopy. All samples and standards were analyzed in the presence of 1% lanthanum chloride solution (RDH Chemicals, Port Fairy, Australia), with standards being in the range of 0–15 mg/l. Milk samples were diluted 1:400 with distilled water, atomized in an air acetylene flame, and the absorbance of calcium measured at 422.7 nm.

Statistical analysis. Assay results are expressed as means ± SE unless otherwise stated, and n represents the number of rats or milk samples. The statistical significance of differences between mean values was assessed by one-way analysis of variance. When equality of variance was not assumed, the data were analyzed by nonparametric analysis of variance. The correlation between the concentrations of milk PTHrP and calcium was analyzed by linear regression, as was that between the concentration of milk PTHrP and pup weight (where r is the correlation coefficient). The level of significance was taken as P < 0.05.

Immunohistochemical localization of PTHrP. Eight of the fourteen female Sprague Dawley rats (250–300 g) were killed on days 14, 17, and 20 of pregnancy and 4 days after parturition, and their mammary glands were removed. Samples were fixed in 10% buffered Formalin overnight, processed, embedded in paraffin blocks, and sections (0.5 μm) were then cut on a Leitz 1515 rotary microtome. The peroxidase-antiperoxidase method used to stain the sections was carried out as described previously (7, 8, 19, 33). Briefly, the procedure involved preliminary blocking of endogenous peroxidase activity in the tissue with hydrogen peroxide. Sections were then incubated with non-immune rabbit serum to reduce background staining before the application of the primary antiserum. The sheep anti-human (h) PTHrP-(50–69) antiserum (19) was used at dilutions of 1:50 and 1:100. Standard rabbit anti goat immunoglobulins and goat horseradish peroxidase-antiperoxidase complex were used (Dakopatts, Copenhagen, Denmark). Peroxidase activity was demonstrated by the oxidation of 3,3'-diaminobenzidine (Sigma) to produce a brown reaction product in the cytoplasm of mammary cells.

Sections were scored as positive by qualitative comparison of the concentrations of immunoreactive PTHrP and total calcium (r = 0.286; n = 30; P > 0.05) (results not shown).

Immunohistochemistry and in situ hybridization. At day 14 of pregnancy, the mammary gland is relatively undeveloped, consisting of nests of epithelial cells separated by adipocytes and fibrous tissue. In comparison, the

alcohols, and air dried. The slides were pretreated, prehybridized, and hybridized in parallel to the frozen sections.

Sections were scored as positive by qualitative comparison of the number of grains overlying the cells of sections hybridized with antisense and sense probes. Sections hybridized with the sense probe were required to display only scant numbers of grains or ≤5% of those that were hybridized with the antisense probe.

RESULTS

Dam weight and pup growth. The average dam weights remained constant throughout the lactating period with only minor fluctuations (300 ± 25 g), whereas the pups gained weight at a constant rate with age (P < 0.05; n = 36–45) (results not shown).

Milk calcium and PTHrP concentrations. The concentration of PTHrP in rat milk increased over the period of lactation. The levels were relatively stable from days 1 to 6 (12 ± 3 to 16 ± 8 ng/ml) but then climbed steadily to a maximum level at day 21 of lactation (Fig. 1A).

The total calcium concentrations in milk throughout lactation are shown in Fig. 1B. Calcium concentrations increased from days 2 to 12 of lactation, the mean values ranging from 2,750 to 4,100 mg/l during this time. In contrast to PTHrP, the calcium concentrations decreased between days 18 and 21 of lactation. However, this decrease of calcium levels and increase in PTHrP levels seen nearing the end of lactation could have been due to suckling behavior, as the pups could reach solid food at this time. No significant correlation was found between the concentrations of immunoreactive PTHrP and total milk calcium (r = 0.286; n = 30; P > 0.05) (results not shown).

Fig. 1. A: concentrations of immunoreactive parathyroid hormone-related protein (PTHrP) in rat milk throughout lactation as determined by radiiodimmunoassay (means ± SE; n = 5). B: total calcium concentrations in rat milk throughout lactation as determined by atomic absorption spectroscopy (means ± SE). Calcium concentrations increased between days 2 and 12 of lactation (P < 0.01; n = 5).
17-day pregnant mammary gland has undergone significant morphological differentiation and is characterized by the proliferation of epithelial cells to form alveoli with a distinct lumen (Figs. 2, A–C). Histologically, as the mammary gland fully matures during the latter stages of pregnancy and during lactation, the mammary cells become much flatter and milk ducts increase in size and number. Specific PTHrP immunostaining, as observed with the hPTHrP-(50–69) antiserum, was seen in all mammary gland sections obtained during pregnancy and those from lactation. Specific staining of the alveola epithelial cells was readily detectable at day 17 of pregnancy.
Fig. 3. Localization of PTHrP mRNA by in situ hybridization in 14-day pregnant rat mammary gland stained with Giemsa (x500 magnification). At this stage of pregnancy mammary epithelial cells are arranged in nondifferentiated nests that will later form alveoli during course of pregnancy. A: specific hybridization to PTHrP mRNA of antisense probe showing grains in mammary epithelial cells. B: hybridization control with sense probe showing only low level background labeling.
(Fig. 2A) and of greater intensity than that observed at either day 14 or 20 of pregnancy, which were stained at the same time (results not shown). Specific staining of the alveolar epithelial cells was clearly reduced in the preabsorbed control (Fig. 2C) and absent in the nonimmune controls (Fig. 2B). In the sections in which preabsorbed antiserum was used, nonspecific staining associated with vessel walls was observed. There was minimal specific staining of the epithelial cells at day 4 of lactation, possibly due to the PTHrP being secreted into the milk (results not shown).

Specific hybridization of the antisense probe to PTHrP mRNA in light field autoradiographs is clearly shown in the mammary epithelial cells of both the pregnant (days 14 and 20, Figs. 3A and 4A, respectively) and lactating (4 days postpartum, Fig. 5A) mammary glands. The sense probe (Figs. 3B, 4B, and 5B) showed low level nonspecific hybridization. Despite the fact that the assessment of both the immunohistochemistry and in situ hybridization are qualitative, the in situ hybridization autoradiographs clearly demonstrate the presence of PTHrP mRNA in both the pregnant and lactating mammary epithelial lining cells. The level of detected hybridization to PTHrP was similar at each time point in contrast to the greater intensity of staining at day 17 by immunohistochemical localization of the antigen. The low level of hybridization in Fig. 5A may relate to the differentiation of the alveolar cells to a more flattened and extended form.

**DISCUSSION**

In the present study, PTHrP protein and PTHrP mRNA have been localized in the alveolar epithelial cells of the lactating rat mammary gland using immunohistochemistry and in situ hybridization. In previous studies in the lactating rat, Northern blot analysis was used to demonstrate that PTHrP mRNA was expressed in lactating mammary tissue, and mRNA levels were responsive to prolactin (35, 39). A novel finding in the present work is that PTHrP and PTHrP mRNA were not confined to lactating mammary tissue, but both were also detected in developing mammary tissue before the onset of lactation. Indeed, PTHrP was detectable in developing mammary tissue as early as day 14 of pregnancy, when it consisted of nests of epithelial cells separated by a fibrous stroma and primitive alveoli were just beginning to form. PTHrP is seen in a number of tissues in the developing fetus (26, 32) and is likely to have a significant role in development. During pregnancy, the mammary gland undergoes development and differentiation in a similar manner to many tissues in the fetus, and production of PTHrP during this period suggests that one possible function of PTHrP in the mammary gland is to aid in growth and/or differentiation. This hypothesis is supported by the demonstration of PTHrP production in a case of benign breast hypertrophy (15) and in a case of hypercalcemia throughout lactation (18). Furthermore, PTHrP was identified specifically in the myoepithelial cells in one of these cases (15). Our results clearly demonstrate the presence of PTHrP in the alveolar epithelium, but the resolution of the immunohistochemical staining did not allow us to conclude whether the myoepithelial cells also stain for PTHrP, possibly due to the distension of the alveoli with milk. In view of the localization of this hormone and its mRNA in smooth muscle (16, 26, 36, 38) and the relaxing effect of PTHrP on smooth muscle (25, 28, 45), it is possible that one of the functions of PTHrP in mammary tissue is to modulate the contractility of myoepithelial cells.

Milk contains large amounts of PTHrP (3, 17), suggesting that PTHrP produced by the alveolar epithelium is secreted into milk during lactation. PTHrP concentrations in milk have been found to be several orders of magnitude higher than those in the circulation of patients with humoral hypercalcemia of malignancy (3, 4, 11, 17, 24, 29, 41). In the present study, we have found PTHrP present in rat milk throughout lactation, during which time the concentrations of PTHrP increased progressively (Fig. 1A) (41, 47). The results of the localization studies together with the observation of continuous production throughout lactation suggest that PTHrP has an important physiological function in lactation. This function could be related to maintaining the secretory epithelia in the mammary gland, the transport of calcium within this gland, milk production, the contractility of the myoepithelial cells and milk expression, or in supporting the development of the neonate.

Whether PTHrP has a paracrine role in the transport of calcium into rat milk is not clear. In previous work in the cow, PTHrP and calcium concentrations in milk were significantly correlated (17), whereas no such correlation was demonstrated in rat milk in this study (r = 0.286). Calcium concentrations in milk remained virtually constant, whereas PTHrP concentrations increased progressively after the onset of lactation. It would seem logical, however, for the mammary gland to contribute to the regulation of maternal calcium metabolism so that the substantial calcium demands during lactation can be met (23). Indeed, physiological studies of calcium metabolism during lactation in the rat indicate that a factor other than 1α, 25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], and PTH is required to explain calcium turnover during lactation (2).

The mammary cell has been shown to contain some of the components necessary for active calcium transport. Receptors for 1,25(OH)₂D₃ are not only present but are upregulated at the commencement of lactation (6). However, treatment with 1,25(OH)₂D₃ fails to induce a calcium-binding protein in mammary tissue (31), possibly because mammary tissue is committed to the synthesis of casein, which has substantial calcium-binding capacity. A calcium-adenosinetriphosphatase has also been demonstrated in vesicles isolated from mammary cells (44), and calcium can be shown to accumulate with casein in these vesicles (5, 6). Despite these findings, the mechanisms responsible for the control of calcium movement in the mammary cell and the contribution of PTHrP to this process remain to be elucidated.

A positive correlation between pup weight and PTHrP concentration in milk was observed in this study (r = 0.963; n = 36; P < 0.05). Even though this is likely to be a fortuitous relationship, it is possible that PTHrP may influence a specific process associated with neonatal growth. It seems unlikely that intestinal calcium transport is influenced by PTHrP in milk, as some 90% of the calcium are passively absorbed in the neonatal intestine.
Fig. 4. Localization of PTHrP mRNA by in situ hybridization in 20-day pregnant rat mammary gland stained with Giemsa (×500 magnification). At this stage in mammary gland development, secretory epithelial cells (E) are flatter in appearance and alveoli are fully functional, secreting protein-rich fluid called colostrum, accumulation of which dilates alveolar lumen (L). A: specific hybridization to PTHrP mRNA of antisense probe showing grains in mammary epithelial cells. B: hybridization control with sense probe showing only low level background labeling.
Fig. 5. Localization of PTHrP mRNA by in situ hybridization in lactating mammary tissue 4 days postpartum stained with Giemsa (×500 magnification). Lactating mammary gland is composed almost entirely of alveoli greatly distended with milk. Alveoli vary in size and consist of simple cuboidal epithelial lining (E) and myoepithelial cells, the latter of which are not able to be resolved at this magnification. A: specific hybridization to PTHrP mRNA of antisense probe showing grains in mammary epithelial cells. B: hybridization control with sense probe. Nonspecific hybridization is seen over degraded protein in lumen (L).
(1). A recent study, examining systemic effects of milk PTHrP on the neonate, found no effect of antisera directed against the amino-terminal portion of PTHrP on parameters of calcium metabolism in suckling neonatal mice (23). However, because PTHrP is expressed in many fetal tissues and is thought to be involved in cell growth and differentiation (26, 32), PTHrP ingested orally in milk could influence differentiation of the gastrointestinal tract epithelium in the suckling neonate.

PTHrP produced in lactating mammary glands may be released systematically during suckling and act in an endocrine manner on maternal target organs such as the kidney. Evidence is provided for this in a recent study by Yamamoto et al. (46), in which transient increases in the urinary excretion of phosphorus and adenosine 3′,5′-cyclic monophosphate were observed in lactating rats after suckling. These effects were not abolished by thyroparathyroidectomy and hence were not attributable to an increase in PTH secretion. It should be noted that during lactation, large amounts of maternal calcium must be available for delivery into milk, and this is associated with maternal bone loss and renal conservation of calcium (2, 14). Furthermore, the levels of PTHrP in the maternal circulation during lactation in humans are higher than in nonlactating control subjects (10). All of these observations suggest that PTHrP released by the lactating mammary gland may fulfill an endocrine function in the mother.

Using Western blot analysis, with antisera directed against the amino-terminal PTHrP sequence, we have found rat milk to contain predominantly PTHrP approximately of the same size as PTHrP-(1–108) (results not shown). This has also been shown to be the predominant size of PTHrP detected in milk of other mammalian species (17, 29). Posttranslational processing may take place in mammary epithelial cells, or cleavage of the full molecule might take place after secretion. It remains to be determined whether the remaining carboxy terminal fragment can produce any effects in the suckling neonate or on calcium metabolism in the mother, depending on whether it is secreted into the milk or into the maternal circulation. Studies on the pharmacokinetics of PTHrP during lactation should shed further light on physiological role(s) for PTHrP in maternal physiology during pregnancy and lactation, and in fetal and neonatal development. Such studies should make use of region-specific assays.

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