

# 周産期の牛乳腺における副甲状腺ホルモン関連蛋白質(PTHrP)mRNAの発現

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## Expression of Parathyroid Hormone-Related Protein (PTHrP) mRNA in Mammary Gland of Periparturient Cows

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**ABSTRACT.** The expression of parathyroid hormone-related protein (PTHrP) mRNA was examined in mammary gland with or without lactation, and during periparturient period in a Holstein cow and a Jersey cow. In the lactating mammary gland, PTHrP was detected in alveolar epithelial cells and the lumen by immunohistochemical analysis. The relative expression levels of PTHrP mRNA in mammary gland from lactating cows were significantly higher than those from non-lactating cows ( $P < 0.05$ ). During periparturient period, relative PTHrP mRNA level was remarkably low before the parturition in a Jersey and a Holstein cow, however, both levels were gradually increased and reached a peak level at 5–6 weeks after the parturition. In addition, the peak level in a Jersey cow was approximately 3-fold higher than that in a Holstein cow. From these results, PTHrP was synthesized and secreted in alveolar epithelial cells in mammary gland and increased subsequently with the lactation, suggesting a possible mechanism for the regulation of local calcium homeostasis.

**KEY WORDS:** bovine, lactation, mRNA, periparturient period, PTHrP.

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Parathyroid hormone-related protein (PTHrP) was initially identified as the most common cause for malignant hypercalcemia. It was also detected in various normal tissues, included mammary gland in animals. The expression of PTHrP mRNA in mammary gland increased in response to the suckling stimulus in the rat [10], showing a peak level from the day 12 to 16 of the lactation with a high milk production [15]. In cows, a large amount of PTHrP was detected in milk with an increase gradually throughout the lactation [1, 3, 5, 8, 12]. On the other hand, it has been widely accepted that PTHrP was closely related to the calcium homeostasis, especially local calcium uptake, since PTHrP bound to PTH/PTHrP receptor and showed the same biological activity of PTH [7, 9]. Therefore, many researchers considered that PTHrP in mammary gland regulated calcium uptake and resulted in the increase of calcium content in milk. In addition, Law *et al.* [5] reported that the concentration of both PTHrP and calcium in milk were higher in Jersey cows, showing a relative high occurrence of parturient hypocalcemia, than those in Holstein cows.

This study deals with the expression of PTHrP mRNA in mammary gland with or without lactation in Holstein cows and changes of its relative expression level during periparturient period in a Jersey and a Holstein cow.

### MATERIALS AND METHODS

*Lactating and non-lactating mammary glands:* Tissue samples of lactating and non-lactating mammary gland were obtained from 5 lactating Holstein cows slaughtered in a local abattoir and 5 non-lactating Holstein cows kept in

Azabu University without lactation at least for 1 year, respectively. All the cows were not pregnant and had the calving at least once. The mammary gland obtained were excised and minced under the sterile condition, and immediately frozen and stored in liquid nitrogen until use. For immunohistochemical analysis, a portion of the tissue was fixed in 10% neutral buffered formalin.

*Periparturient mammary glands:* The tissue samples of periparturient mammary gland were obtained from a Jersey cow aged 5-year-old with the calving twice and from a 3-year-old Holstein cow without the calving. The samples were collected on 2 and 3 weeks before the calving day, and on every 2 weeks at the same time (2:00 P.M.) for subsequent 8 or 9 weeks. The cows were sedated with xylazine (0.2 mg/kg B.W., i.v.) and injected procaine hydrochloride to the incision area of mammary gland as a local anesthesia. The tissue samples were stored in liquid nitrogen until use. After the sample collection, both cows were in good health and milked twice a day (8:30 A.M. and 4:30 P.M.). One Holstein cow, non-lactating and non-pregnant, was also used as a negative control for surgical invasion of mammary gland. This study was approved by the Animal Care and Use Committee of Azabu University School of Veterinary Medicine (No. 67 and No. 75).

*Immunohistochemistry:* A polyclonal anti-serum against human PTHrP [50–83] (MCR427) was obtained from immunized rabbit and purified using a MAbs Trap Kit (Amersham Bioscience, NJ, U.S.A.). Thin sections of the mammary gland tissue were deparaffinized, blocked endogenous peroxidase with 3% H<sub>2</sub>O<sub>2</sub> in methanol, and incubated with 0.1% trypsin for 30 min at 25°C. After the inhibition of nonspecific binding with 10% normal goat serum, the sections were incubated with anti-PTHrP antibody (1:800) for 16 hr at 4°C. Then, the sections were reacted with a

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Table 1. Primers for RT-PCR and competitive RT-PCR

Transcript	Primer	Location	Sequence (5' to 3')	Product	Accession
PTHrP	5'	74–95 nt	GTGGTGTGGAAAAACACGGTT	483 bp	AB097837
	3'	554–534 nt	TCTTTTCCTGCTCCTTGCGTT		
$\beta$ -casein	5'	130–151 nt	CCTGGTGAGATTGTGGAAAAGCC	480 bp	X06359
	3'	607–588 nt	ACTGAGAAAGGGACAGCACGG		
GAPDH	5'	186–209 nt	CACCATCTCCAGGAGCGAGATCC	578 bp	U85042
	3'	761–738 nt	GACGCCTGCTTCACCACCTTCTTG		

Table 2. Competitor prepared for competitive RT-PCR

Target (location and product)	Competitor	
	cDNA segment deleted	Product
PTHrP (74–554 nt, 481 bp)	96–285 nt (190 bp)	291 bp
$\beta$ -casein (130–607 nt, 478 bp)	152–371 nt (220 bp)	258 bp
GAPDH (186–671 nt, 576 bp)	210–391 nt (182 bp)	394 bp

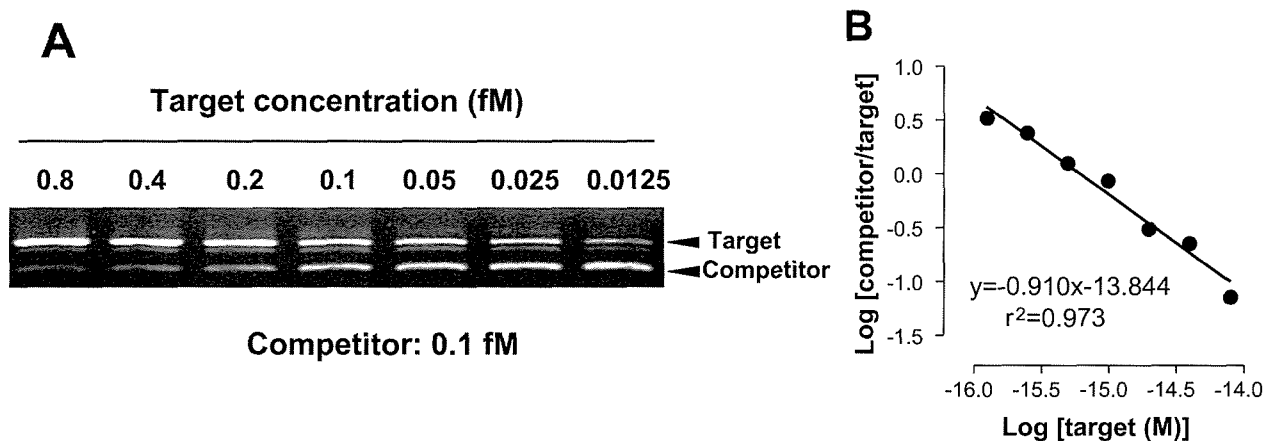


Fig. 1. Competitive RT-PCR for PTHrP using competitor template. A: Constant concentration (0.1 fM) of competitor was coamplified with various concentrations of target cDNA. The intensity of the competitor band decreased with an increase of target PTHrP cDNA band. B: Log ratios of amplified competitor and target cDNA showed linear regression curve against target cDNA. The amount of cDNA was corrected according to the amount of GAPDH cDNA.

Histfine Simple Stain Rat MAX PO MULTI (Nichirei, Tokyo, Japan), consisted of peroxidase labeled amino acid polymers, goat anti-mouse Ig, and goat anti-rabbit Ig, for 30 min at 25°C. The sections were stained with 0.02% diaminobenzidine for 5 min and counterstained with hematoxylin. Negative controls for specificity was carried out to preincubate with maltose binding protein (MBP) linked bovine PTHrP [1–141].

**RNA isolation and cDNA synthesis:** Total RNA from frozen mammary tissues was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany). One microgram of the recovered RNA was treated with DNase I (Invitrogen, CA, U.S.A.) to remove the residual DNA and reverse transcribed in 21  $\mu$ l of mixture reacted with oligo (dT) primer using a commercial kit (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen, CA, U.S.A.) to obtain first-strand cDNA. The cDNA obtained was stored at  $-80^{\circ}\text{C}$ .

**Polymerase chain reaction (PCR):** For PCR, 3 pairs of primers as shown in Table 1 were used to detect the expres-

sion of PTHrP, glyceraldehyde-3-phosphate (GAPDH), and also  $\beta$ -casein mRNA. The reaction for PCR was carried out in total volume of 20  $\mu$ l, containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 20 mM  $\text{MgCl}_2$ , 0.2 mM of each dNTP, 0.5  $\mu$ M of each primer, and 1 U of DNA polymerase mixture (Takara Ex Taq, Takara, Tokyo, Japan). After denaturation at 94°C for 1 min, the thermal cycling parameters as follows: denaturation at 94°C for 45 sec, annealing at 64°C for 45 sec, extension for 60 sec at 72°C for 35 cycles, and extension at 72°C for 5 min. The PCR products were separated on 1.5% agarose gels in 1  $\times$  TBE and visualized with ethidium bromide.

**Competitive Reverse Transcription (RT)-PCR:** The relative levels of mRNA expression were semi-quantified by the competitive RT-PCR [6]. Competitor templates were prepared for PTHrP, GAPDH, and  $\beta$ -casein gene (Table 2). Each deleted cDNA segment as the competitor was synthesized by overlap extension PCR and purified using a Suprec-02 column (Takara, Tokyo, Japan) (Fig. 1).

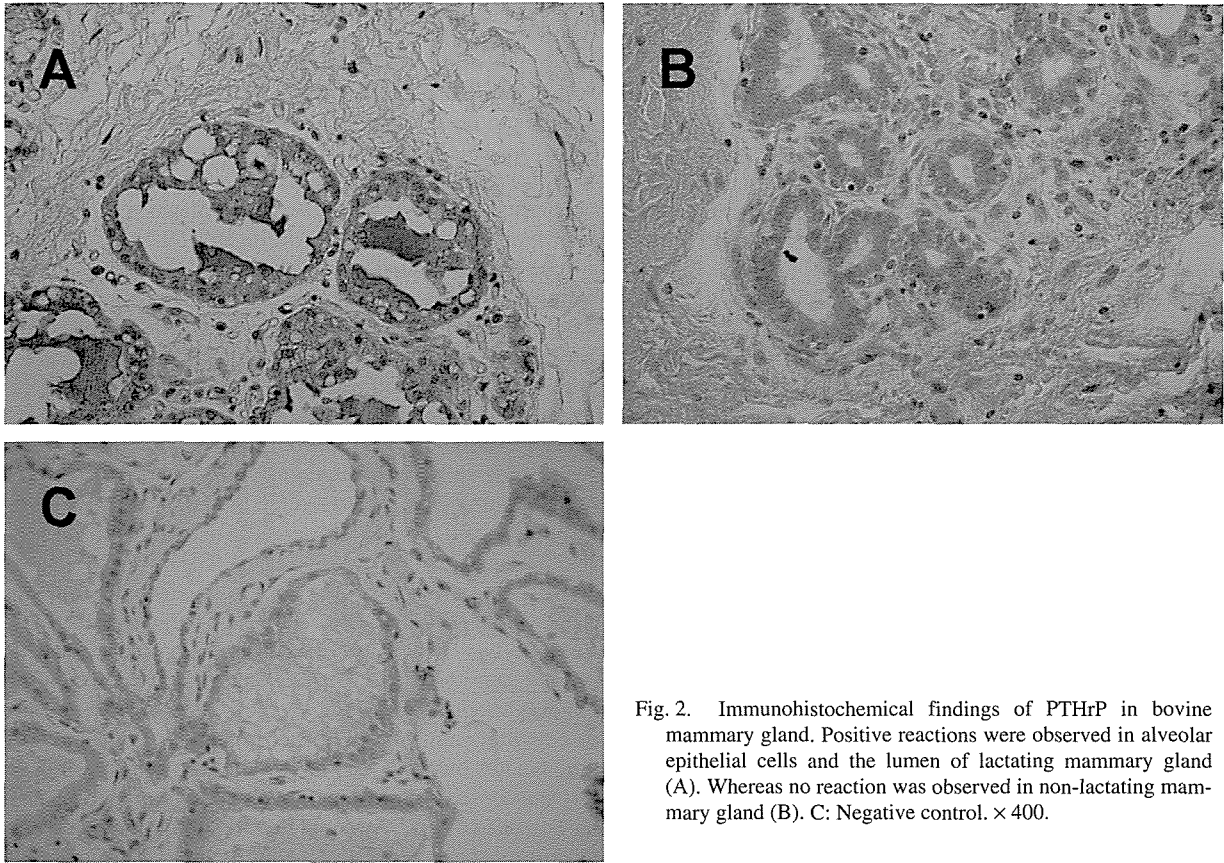


Fig. 2. Immunohistochemical findings of PTHrP in bovine mammary gland. Positive reactions were observed in alveolar epithelial cells and the lumen of lactating mammary gland (A). Whereas no reaction was observed in non-lactating mammary gland (B). C: Negative control.  $\times 400$ .

RESULTS

*Immunohistochemistry:* In the lactating mammary gland, positive reactions against PTHrP were observed in alveolar epithelial cells and also in the lumen, whereas weak positive reactions were detected in some epithelial cells in the non-lactating mammary gland (Fig. 2).

*Expression of PTHrP mRNA in mammary gland:* The semi-quantified expression of PTHrP mRNA was calculated by competitive RT-PCR. The relative expression level of PTHrP mRNA in mammary gland from the lactating cows was significantly higher than that from non-lactating cows ( $P < 0.05$ ) (Fig.3).

*Changes of PTHrP mRNA expression in periparturient period:* Relative levels of PTHrP mRNA were remarkably low before the calving day in a Jersey and a Holstein cows, however, both levels were increased gradually and reached a peak at 5–6 week after the parturition (Fig. 4). The peak relative level in a Jersey cow was approximately 3-fold higher than that in a Holstein cow. Non-lactating Holstein cow showed constantly low levels during the experimental period. The relative expression level of  $\beta$ -casein mRNA was also low and increased after the parturition in both Jersey and Holstein cow (Fig. 4). The peak level, however, in a Jersey cow at 3 and 5 weeks after the parturition was extremely low compared to that in a Holstein cow.

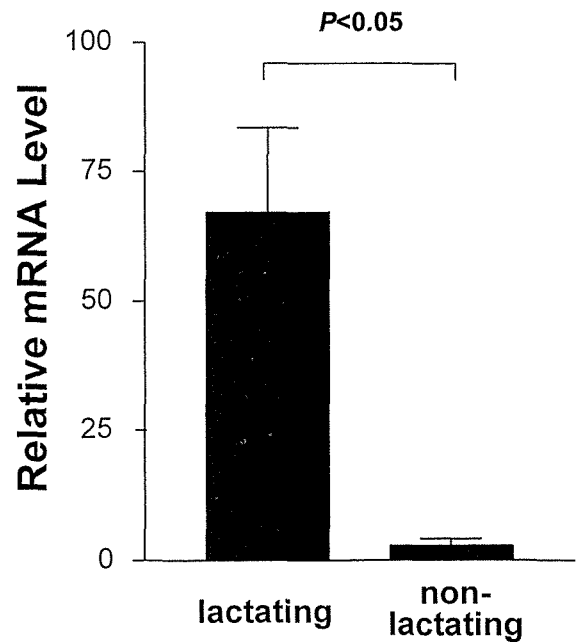


Fig. 3. Relative level of PTHrP mRNA in lactating and non-lactating mammary glands. Relative level of PTHrP mRNA was significantly higher in lactating than that in non-lactating mammary gland of Holstein cows.

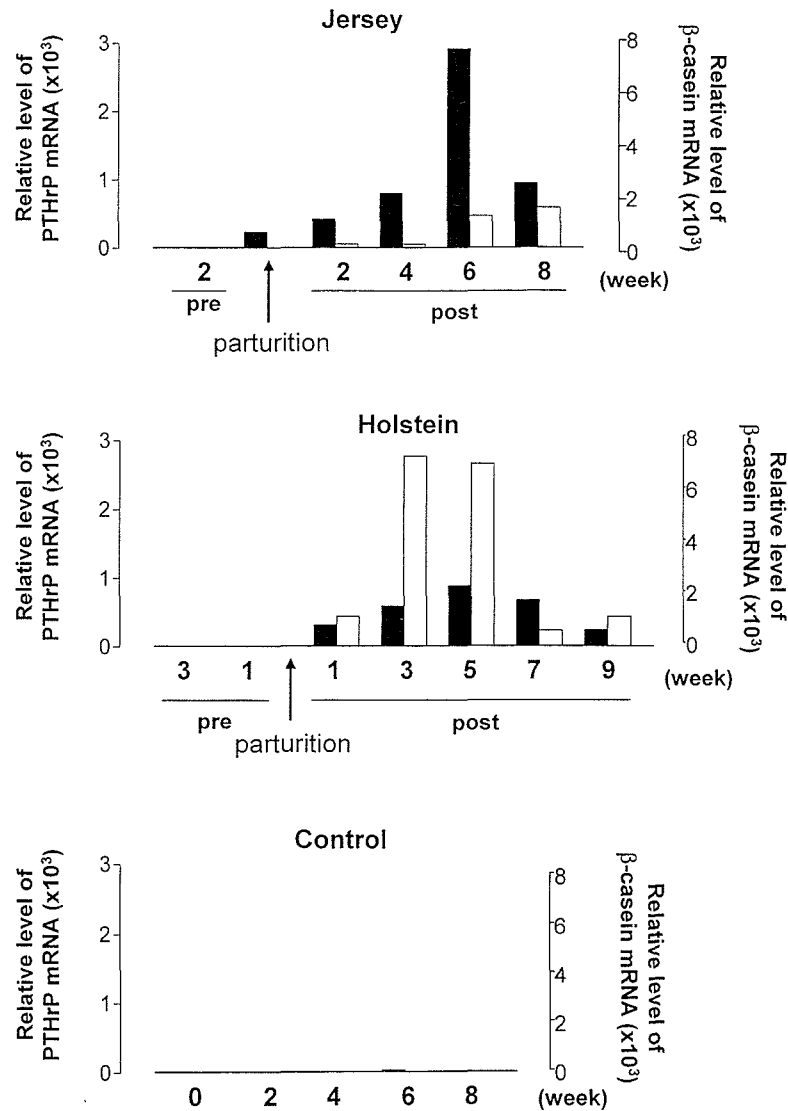


Fig. 4. Changes of relative level of PTHrP mRNA in periparturient mammary gland. Relative levels of PTHrP mRNA were remarkably low before the parturition in a Jersey and a Holstein cow, however, both levels were increased gradually and reached a peak at 5–6 weeks after the parturition. Control: non-lactating and non-pregnant Holstein cow. ■: PTHrP, □:  $\beta$ -casein.

## DISCUSSION

On the immunohistochemical findings in this study, PTHrP was detected in alveolar epithelial cells and the lumen of the lactating mammary gland. In the lactating mammary gland in cows, PTHrP was considered to be synthesized at least in alveolar epithelial cells and to be secreted into the lumen, although Kramer *et al.* [4] and Grone *et al.* [2] reported that PTHrP was detected in alveolar epithelial cells and also in duct epithelial cells and myoepithelial cells. The expression of PTHrP mRNA was reported to be detected in mammary gland [14]. In this study, the relative

level of PTHrP mRNA in the lactating mammary gland was significantly higher than that in the non-lactating cows. Thiede *et al.* [11] demonstrated that PTHrP mRNA expression in mammary gland was increased after the parturition in the rat, like as observed in a Holstein cow in this study. The increase of PTHrP mRNA in mammary gland in the rat was responded to the suckling stimulus during early stage of the lactation [10], showing a peak level from the day 12 to 16 of the lactation with a maximum milk production [15]. In contrast, PTHrP mRNA level in mammary gland was higher in a Jersey cow than that in a Holstein cow, although Jersey cows usually showed lower milk yield compared to Holstein

cows. Goff *et al.* [1] suggested that the daily PTHrP secretion from mammary epithelial cells was constant and the increase of milk PTHrP content observed in the late lactation period was resulted from the reduction of milk yield. From this hypothesis, however, PTHrP content in milk could reveal the lowest value during 3 weeks after the parturition, showing usually the highest milk yield in cows. In this study, expression of  $\beta$ -casein was also examined to compare that of PTHrP mRNA, as  $\beta$ -casein was the major secreting protein in milk. In a Jersey cow,  $\beta$ -casein mRNA level increased from 6 to 8 weeks after the parturition with a decrease of PTHrP mRNA level, whereas it reached a peak at 3 and 5 weeks after the parturition with an increase of PTHrP mRNA level in a Holstein cow. Therefore, the expression of PTHrP mRNA was considered to be independent on the milk yield of the cows. The regulation of PTHrP mRNA in mammary gland might be affected by some other factors with the lactation, but not by the milk production.

On the other hand, it has been well known that the biological activity of PTHrP was similar to PTH, regulating calcium homeostasis, especially local calcium uptake, as PTHrP also bound to PTH/PTHrP receptor [7, 9]. Law *et al.* [5] reported that the concentration of PTHrP and also calcium in milk were higher in Jersey cows than those in Holstein cows. The Jersey cows showed a tendency of the higher incidence for parturient hypocalcemia compared to the Holstein cows. Uemura *et al.* [13] reported that calcium concentration in milk was correlated with PTHrP concentration in human, although Yamamoto *et al.* [15] showed no correlation between calcium and PTHrP concentration in milk of the rat. Further studies are necessary to elucidate biological effects of PTHrP in mammary gland on the local calcium homeostasis.

From these results, PTHrP is synthesized and secreted in alveolar epithelial cells in mammary gland with a subsequent increase related to the lactation, suggesting to regulate local calcium homeostasis in mammary gland in cows.

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