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# Secretion of Parathyroid Hormone-related Protein by Bovine Mammary Cells *in vitro*

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***Abstract:***
Mammary cells were isolated from lactating cows at 1 to 6 weeks after calving and evaluated for their ability to secrete PTHrP in vitro. The tissue was enzymatically digested to release glandular acini. The digested acini were cultured on thin (1.0 mm) or thick (2.5 mm) layers of collagen. The cultures containing thick collagen were detached and allowed to contract on day 6. The culture medium consisted of M199 with prolation (8 μg/ml), insulin (5 μg/ml), cortisol (5 μg/ml), and fetal bovine serum (15%). PTHrP production was measured by N-terminal RIA and bioassay (stimulation of adenylate cyclase in the ROS 17/2.8 cell line). Medium was collected at 2-day intervals for 14 days. The cells reached confluence at 4-6 days. PTHrP production was low at day 2 (<0.5 ng/ml), but increased to peak production (2-4 ng/ml) at approximately day 6-8 of culture and remained constant until day 14. Immunoreactive and bioactive PTHrP levels in the culture medium correlated well. The cultures produced high levels of lactoferrin (500 to 3000 ng/ml) and low levels of $\alpha_{s1}$-casein (14 to 77 ng/ml).
Prolactin stimulated PTHrP production approximately 50% on days 6-14. PTHrP production was increased approximately 100% by treatment with EGF (10 ng/ml) for 2 to 4 days. Histologic evaluation of cultures on thick, contracted collagen revealed an inner layer of epithelial cells with and an outer layer of collagen containing stromal cells. These data demonstrated that mammary cells from lactating cows produced and secreted PTHrP in vitro in a regulated manner.

**Key words**: PTHrP, mammary gland, lactation

## Introduction

The lactating mammary gland produces parathyroid hormone-related protein (PTHrP) which is present in milk at concentrations 10,000-fold greater than plasma, and increases with duration of lactation [1, 4, 6, 9, 10]. The function of PTHrP in the mammary gland is uncertain, but it may facilitate transport o calcium across mammary epithelium and may be im portant in calcium homeostasis and/or intestinal cel differentiation in neonates [5, 6]. The expression o PTHrP mRNA is increased in mammary tissue b

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suckling and prolactin stimulation [12, 16] and PTHrP concentration in milk correlates with calcium content in cows [5]. PTHrP has been reported to increase blood flow to the mammary gland by vasodilatation [10].

The purpose of the present study was to investigate whether primary cultures of mammary cells from lactating cows would sustain production and secretion of PTHrP in vitro and to demonstrate the effects of serum, prolactin or epidermal growth factor, and thickness and detachment of collagen substratum on the production of PTHrP.

## Materials and Methods

### Mammary Tissue Culture

Mammary tissue was collected from three lactating Holstein dairy cows at 1 to 6 weeks after parturition. The mammary tissue was enzymatically digested to release glandular acini using collagenase, elastase, chymotrypsin, and hyaluronidase as described by Talhouk et al. [12]. Type I collagen was prepared from rat tail tendons and used to coat 24-well culture plates at either 1.0 or 2.5 mm thicknesses. Dissociated mammary acini were plated at a concentration of $5 \times 10^5$ cells/well in 1.0 ml of medium (M199 medium supplemented with 15% fetal bovine serum, insulin (5 $\mu$g/ml), hydrocortisone (5 $\mu$g/ml), bovine prolactin (8 $\mu$g/ml). The medium was collected at 2-day intervals for 14 days and stored at −20℃. On day 6, the 2.5 mm collagen gels were detached from the perimeter of the wells and allowed to contract. The 1.0 mm collagen gels were left intact throughout the experiment. The cultures on contracted 2.5 mm collagen gels were fixed in formalin, embedded in paraffin, and evaluated on hematoxylin and eosin stained sections.

### Assays for PTHrP, Lactoferrin, and $\alpha_{s1}$-casein:

PTHrP production by bovine mammary cells was measured by N-terminal radioimmunoassay [11]. PTHrP-(1-36) was radioiodinated with $^{125}$I using lodogen and separated from unlabeled peptide by reverse phase high performance liquid chromatography. Chicken anti-human PTHrP (1-36) IgG was purified from egg yolks from immunized laying hens. PTHrP bioactivity was measured by stimulation of adenylate cyclase in ROS 17/2.8 osteoblast-like cells [11]. ELISA assays for lactoferrin and $\alpha_{s1}$-casein were performed as previously described [12].

## Results

### Morphology

The enzymatically dispersed mammary acini and cells attached to the collagen gels within the first 24 hours and formed confluent sheets by days 4 to 6. After peripheral detachment of the 2.5 mm collagen gels on day 6, the cultured cells and gels contracted for 1 to 4 hours to a spherical shape and remained attached to the center of the wells. At day 14 the mammary cultures on the 2.5 mm collagen gels consisted of cuboidal to columnar mammary epithelial cells on the surface of the gels and fibroblasts and myoepithelial cells dispersed in the collagen gels. A basement membrane was observed between epithelial cells and the collagen substratum.

### PTHrP, Lactoferrin and $\alpha_{s1}$-Casein Production by Mammary Cell Cultures

The bovine mammary cells at day 2 produced low levels of PTHrP (< 0.5 ng/ml). PTHrP content in the conditioned medium increased steadily up to 2 to 4 ng/ml at day 6 to 8 and was maintained at similar concentrations until day 14. There was no significant effects of detachment and thickness of the collagen substratum on PTHrP production compared to cultures plated on thin (1.0 mm) collagen gels. Production of PTHrP was 2-fold greater in serum-supplemented medium compared to serum-free medium. The addition of 8 $\mu$g/ml of prolactin to serum-free or serum-containing medium stimulated PTHrP production approximately 50% on days 6 to 14. PTHrP bioactivity (adenylate cyclase stimulation of ROS 17/2.8 cells) correlated with PTHrP immunoreactivity in both prolactin-supplemented and prolatin-free cultures. Conditioned medium from prolactin-supplemented mammary cell cultures (day 6-14) stimulated an 8-fold increase in adenylate cyclase activity in ROS cells.

Epidermal growth factor (EGF) (10 ng/ml) was added to mammary cultures at day 8 after 2 days of exposure to serum-free medium. EGF increased PTHrP secretion 2-fold after 2 and 4 days of treatment.

The serum-supplemented mammary cell cultures (days 6 to 14) produced high levels of lactoferrin (500 to 3000 ng/ml) and low levels of $\alpha_{s1}$-casein (14 to 77 ng/ml).

## Discussion

The present study demonstrated that mammary cells from lactating cows produced PTHrP in vitro and production could be stimulated by serum, prolactin, and EGF. Thickness or detachment of the collagen substratum did not influence PTHrP production in primary cultures of the bovine mammary cells. The mammary cells grown on collagen gels produced up to 4 ng/ml PTHrP which was maintained for 14 days.

The production of PTHrP was greatest in mammary cultures after confluence. This is in contrast to normal and some malignant keratinocyte cell lines in which PTHrP production decreased after confluence [7, 15]. It has been reported that PTHrP is produced by primary cultures of mammary epithelial cells from lactating rats [2, 3]. The level of PTHrP produced by bovine mammary cells in the present study was 10 to 20-fold greater than by rat mammary cells [2].

Production of PTHrP by the lactating mammary gland is associated with mild increases in maternal plasma levels of PTHrP has been shown to increase during lactation in humans, rats and cows [5, 9, 16] and may play a role in the maintenance of the plasma concentration of ionized calcium. The expression of PTHrP mRNA by the mammary gland is dependent on prolactin in rats during lactation [13]. Prolactin treatment of bovine mammary cells resulted in stimulation of PTHrP production. The regulation of other milk-associated proteins, such as casein, are dependent on stimulation by prolactin. The results of this study demonstrated that PTHrP expression by mammary cells in vitro may be regulated similar to other milk proteins by lactogenic hormones, but also independently by growth factors, such as EGF.

Epidermal growth factor was shown to stimulate PTHrP production in this study. Epidermal growth factor has been reported to enhance differentiation of rat mammary cells and stimulate the production of PTHrP in vitro [2]. Local production of EGF by the mammary gland may directly stimulate expression of PTHrP mRNA and secretion [2].

The mammary cell cultures in this study consisted of secretory epithelium, myoepithelium and fibroblasts. The presence of multiple cell types may be important for investigating the regulation of protein synthesis and secretion by mammary epithelial cells in vitro. The function of the myoepithelial cells was apparent after detachment of the collagen gels from the perimenter of the culture wells. There was rapid contraction of the gels by myoepithelial cells. Expression of PTHrP has been reported to be stimulated by smooth muscle contraction in the uterus [14]. Parathyroid hormone-related protein may be important in expansion of mammary alveoli in the lactating mammary gland. The presence of immunoreactive PTHrP and its mRNA has been demonstrated in both mammary epithelial cells and myoepithelial cells in rats [8]. It is not known which cells in the mammary cultures of this study were responsible for the production and secretion of PTHrP. Further investigations using this model of mammary cell culture will be useful to delineate the function, regulation, and sites of production of PTHrP in mammary cells.

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