FULL PAPER *Anatomy*

Immunohistolocalization of Carbonic Anhydrase Isozyme (CA-VI) in Bovine Mammary Glands

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ABSTRACT. The localization of bovine carbonic anhydrase isozyme VI (CA-VI) was examined immunohistochemically in bovine mammary glands during early lactation period (after 2–3 days of postpartum) and dry period (at about 2 months preparturition in adu lts), and young calves (at 30 and 150 days after birth) using specific CA-VI antiserum. The immunoreaction for anti-CA-VI antiserum was very weak in the mammary glands in young (prepubescent) calves. In dry period, CA-VI was also weakly expressed in secretory epithelial (acinar) and ductal cells. In contrast, the reaction was intense in mammary gland cells in early lactation period. Dot blotting analysis indicated that anti-CA-VI reacted positively to beastings and mature saliva, but weakly or not at all to milk during the dry per iod or calf saliva, respectively. The intense expression of CA-VI in the mammary glands in early lactation period might compensate for low levels of secretion from functionally and structurally immature salivary glands in young calves.

KEY WORDS: bovine mammary gland, CA-VI, immunohistochemical localization.

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Carbonic anhydrase VI (CA-VI) is a unique secretory isozyme that was initially discovered in the ovine parotid gland and saliva [4]. Several properties of CA-VI distinguish it from the cytoplasmic isozymes (CA-I, II, and III) [2]. CA- VI was purified from ovine [4], rat [3, 13], human [8, 12, 14, 18] and bovine saliva [1] and is thought to enhance the buffering capacity of the oral cavity [10]. Immunohistochemistry and *in situ* hybridization histochemistry have identified the acinar cells of the salivary gland as the origin of CA-VI in several species [1, 14, 15, 19]. These studies have shown that CA-VI is present in acinar cells of the mammalian parotid and submandibular glands, from where it is secreted into the saliva. Milk digestion in infant might be affected with the salivary secretion, which plays maintenance of bicarbonate levels in saliva. While, beastings might compensates for the primary immune function and the milk digestion in new born calve [25]. Beastings may keep pH levels in the infant alimentary canals of the new born calf [22]. However, CA-VI expression in the important exocrine mammary glands [9], has undergone little investigation. The present study examines the expression and distribution of CA-VI in bovine mammary glands during early lactation and during the dry period in adults and in young calves.

MATERIALS AND METHODS

CA-VI antibody: We purified CA-VI from bovine saliva as described [1]. A rabbit polyclonal antibody has been raised against purified bovine CA-VI and specific immunity to bovine CA-VI has been confirmed [1].

Tissue: We obtained biopsies from the mammary glands of 8 Holstein cows (3 in early lactation at 2–3 days postpartum, 3 in the dry period at about 2 months preparturition and 2 prepubescent calves; one 30- and one 150-day-old). The samples were immediately fixed in neutralized 10% formalin and Bouin's solution, dehydrated with a graded series of alcohols, cleared with xylene and then embedded in paraffin wax blocks that were cut into 4 μm-thick histological sections.

Immunohistochemical staining: Endogenous peroxidase activity was blocked in deparaffinized and rehydrated sections using 0.3% H_2O_2 in methanol, and immersion in normal goat serum (2% in PBS) for 20 min blocked fragment crystallizable receptors. Monospecific antisera (diluted 1:2,000) against CA-VI localized the respective isozymes in a 1 hr primary reaction. Antibody binding was visualized using the Vectastain Elite avidin-biotin-peroxidase complex kit (ABC-POD reagent kit; Vector, Burlingame, Calif., U.S.A.) and diaminobenzidine (DAB) according to the manufacturer's protocol. Mammary gland sections were stained with hematoxylin, dehydrated through a graded alcohol series, and mounted on coverslips. Samples were observed and photographed under a light microscope.

Dot blotting analysis: Beastings were collected at early lactation period: after 2–3 days of postpartum (3 animals), and whole milk during the dry period: 2 months preparturition (3 animals), and saliva from newborn calve: 2–3 days after birth (2 animals) and lactation period: after 2 months of postpartum (3 animals). All samples were stored at –20°C before dot blotting. All of these samples (40 μl) and 5%

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control Bacto skim milk (Difco Lab., Michigan, U.S.A.) were manually blotted onto Immobilon PVDC transfer membranes (MILLIPORE, Bedford. MA, U.S.A.) and blocked with 15% bovine serum albumin (Sigma–Aldrich Fine Chemicals, Missouri, U.S.A.). The membranes were incubated with anti bovine CA-VI antiserum (diluted 1:1,000) at 4°C overnight, washed with 0.1 M phosphate buffer (3 times) and first antibody reactions were detected using the Vectastain Elite avidin-biotin-peroxidase complex followed by 0.02% H_2O_2 and 0.1% diaminobenzidine (ABC-POD reagent kit; Vector, Burlingame, Calif., U.S.A.) in 0.05 M Tris-HCl (pH 7.6) for 5 min. They were colored by DAB.

RESULTS

The reaction to anti-CA-VI antiserum was very weak in the mammary glands of young prepubescent calves. At this stage, the acinar and duct segments were histologically more immature than those at the postpartum phase (Fig. 1). Reactivity differed between early lactation period and during the dry period. In dry period, ductal and acinous epithe-

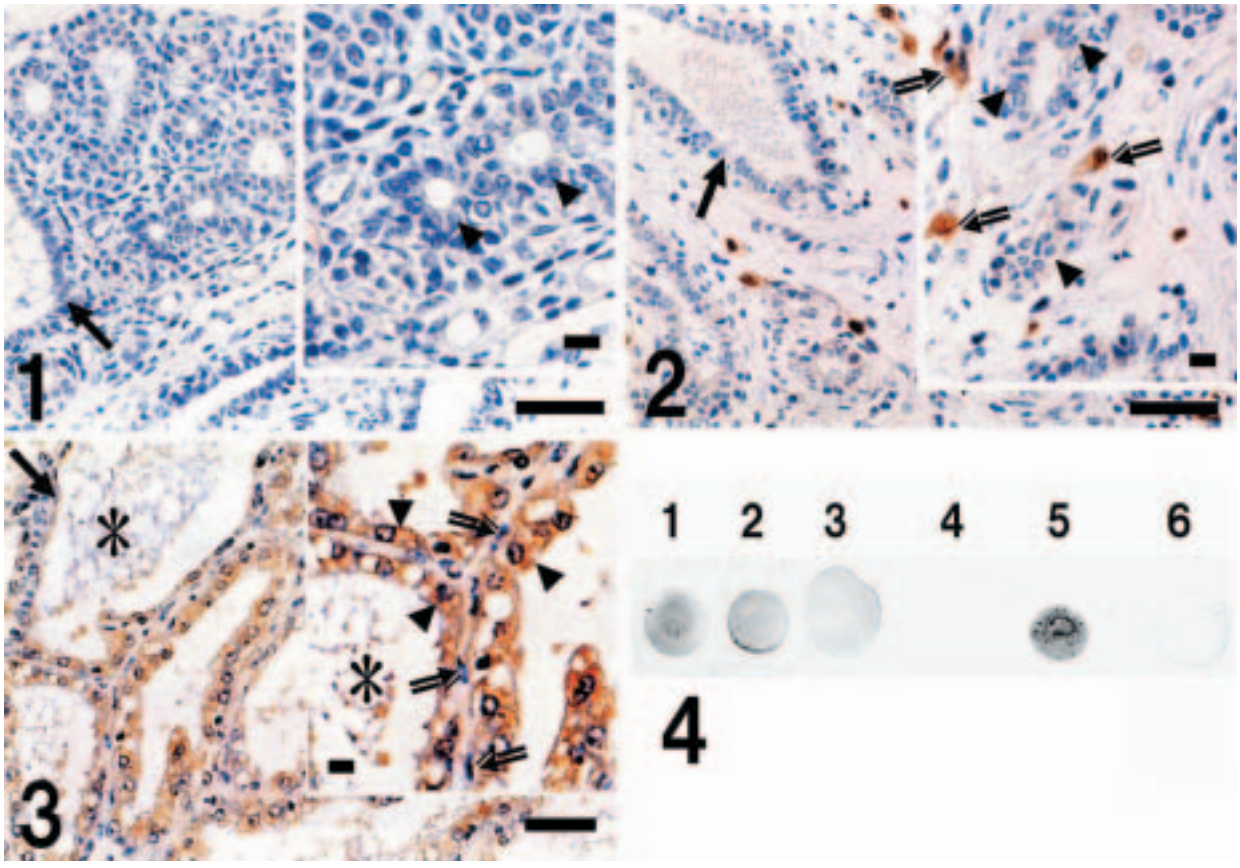

Fig. 1. Immunoreactivity to anti-CA-VI in epithelial cells of mammary glands from prepubescent 30-day-old calf is very weak. At this stage, acini and duct segments seem histologically more immature than adult structures (see also Fig. 3). Arrows show ductal epithelium. Arrowheads show immature acinar cells. Bar: 50 μm. Right square is higher magnification view. Acinar cells had a cubic immature shape without elongated cytoplasm. Bar: 10 μm.

Fig. 2. Acini and ductal epithelial cells of mammary glands during dry period (About 2 months preparturition) display weak immunoreactivity to anti-CA-VI. Arrows show ductal epithelium. Arrowheads show very weak reaction to CA-VI in acinar cells. Double arrows show CA-VI positive macrophage-like cells in slightly expanded interstitium. Bar: 50 μm. Right square is higher magnification view. Interstitium region is slightly expanded, but acinar cells are cubic and small with little cytoplasm. Bar: 10 μm.

Fig. 3. Acini and ductal epithelial cells of mammary glands during early lactation. Cells at about 2–3 days postpartum are immunoreactive to anti-CA-VI. Arrows show ductal epithelium. Arrowheads show active mature acinar cells in milk secretion. Immunoreaction (*) to anti-CA-VI is positive in lumen of duct and acinous cells of mammary glands. Double arrows showed myoepithelial cells that did not react to anti-CA-VI. Bar: 40 μm. Right square is higher magnification view. Acinar cells are mature and slightly varied in size with cytoplasm that is intensely positive (brownish color) to anti- CA-VI. Bar: 10 μm.

Fig. 4. Dot blotting analysis of milk and saliva by anti-bovine CA-VI antiserum. Positive reactions were detected to beastings spots at early lactation period (spots 1 and 2) and to saliva spots at lactation period: 2 months postpartum (spot 5), but weakly positive reaction was detected to milk at dry period (spot 3) and very weak to saliva at newborn calves (spot 6). Control spot: 5% skim milk was routinely negative (spot 4).

lial cells had also very weak reactivity to anti-CA-VI antiserum. Figure of acinar epithelial cells in this phase had no-elongated cytoplasm as resemble to young prepubescent period. Although, CA-VI positive some macrophage like cells were detected in the extended interstitium (Fig. 2). In contrast, the cytoplasm of the acini and ductal epithelial cells of mammary glands was intensely positive for anti-CA-VI antiserum in early lactation period. The morphology of acinar and ductal cells varied. Lumen of acinus and ducts of mammary glands was slightly expanded with some secretion products. Some lumen contents of them also had positive reaction to anti-CA-VI antiserum, but myoepithelial cells of mammary acinus were not reacted by anti-CA-VI antiserum (Fig.3).

No significant difference of immunoreactivity was found between neutralized 10% formalin and Bouin's solution.

Dot blotting analysis showed positive reactions to beasting spots during early lactation period and saliva in lactation period, but weak reactions to milk during the dry period and very weak responses to newborn saliva (Fig. 4).

DISCUSSION

Carbonic anhydrase (CA; EC 4.2.1.1) is a metalloenzyme containing zinc that catalyses the rapid hydration of bicarbonate and the dehydration of carbonic acid. It is widely distributed in mammalian tissues and participates in physiological systems such as respiration, acid-base balance, ion transport, and bone resorption, [23, 24, 26]. Several CA isozymes are expressed in a range of tissues, whereas CA-VI isozymes are expressed in a tissue- or organ-specific manner [23]. The activities of cytosolic CA-I, II, and III, mitochondrial CA-VII, secretory CA-VI, membrane-associated CA-IV, IX, XII, and XIV CA isozymes have been demonstrated [7, 23].

Carbonic anhydrase-VI might accelerate the neutralization of excess organic acids produced by microbial flora in the dental and epithelial microenvironment, and form a mutually complementary system with cytosolic CA for pH regulation on the epithelial surfaces of the human upper alimentary tract [10]. An association between CA-VI and taste and smell functions has been suggested, and CA-VI is also a trophic factor for the development of taste bud stem cells [5, 6]. The lingual von Ebner's gland also contains CA-VI [11]. CA-VI is a secretory isozyme in saliva, and its expression is specific to the parotid and submandibular glands in humans, mice, rats, sheep and cattle [1, 16, 18, 19] and to the lacrimal gland in rats and sheep [15, 17]. According to Ogawa *et al.* [15], CA-VI is synthesized in lacrimal glands, and secreted in very small amounts into lacrimal fluid. In addition, CA-VI is thought to function in maintaining the acid/base balance of the surface of the eye, akin to its role in the oral cavity.

The expression of CA-VI in human and rat mammary glands has received little focus, but Karhumaa *et al*. [9] quantified CA-VI in milk using a time resolved immunofluorometric assay and revealed an approximately eight-fold increase in the concentration in human colostrum compared with mature milk. Rat and ovine salivary glands become mature structures after weaning [16, 19–21]. Our dot blot and immunohistological findings provide evidence that CA-VI is synthesized in the bovine mammary gland and ductal cells, and that it is secreted in milk during in early lactation period. Secreted maternal CA-VI may be useful not only for the maintenance of lactate pH, but the maternal mammary gland might supply CA-VI in the lactate to supplement the immature salivary glands in newborn calves.

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