Distributions of Extracellular Matrix and Carbonic Anhydrase-III during Bovine Palatine Ridge Development

Hajime AMASAKI, Satoshi MATSUMOTO1, Masamine TAKANOSU, and Masayuki DAIGO

Department of Veterinary Anatomy, Nippon Veterinary and Animal Science University, 1–7–1 Kyonan-cho, Musashino 180 and
1Department of Microbiology, Yakuult Central Institute, 1796 Yaho, Kunitachi 186, Tokyo, Japan

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ABSTRACT. The present study describes histological alterations and immunohistochemical distributions of extracellular matrices (ECMs) and the carbonic anhydrase isozyme-III (CA-III) during the period of bovine palatine ridge formation. Morphogenesis of bovine palatine ridges was preceded by epidermal placodes and the mesenchymal condensation (MC). During the early stages of less than 44 cm crown rump length (CRL), fibronectin (FN) was distributed densely in the MC. Strong reactions against type I collagen (C-1) were detected outer to the FN positive site. In the stages of more than 44 cm CRL, FN and C-1 were distributed diffusely in subepithelial mesenchyme. Laminin (LN) and type IV collagen were distributed in the epithelial and endothelial basement membranes (BM) in all of the stages examined, except in the stage of 7 cm CRL, where LN was not detected only in the BM just beneath the epidermal placode. CA-III was detected in basal epithelial cells except for palatine ridge rudiments in the stages of more than 21 cm CRL. It is suggested that the expressions of LN and CA-III might play a role in the spatial determination of rudiments of bovine fetal palatine ridges.—KEY WORDS: bovine palatine ridge, CA-III, extracellular matrix.


It has been known that appendages such as feather germs [5, 10] and bovine ruminal papillae [1] are formed through molecular interactions between epithelium and mesenchyme during their development. Examples of signaling molecules include some types of collagens, fibronectin, laminin, and some types of proteoglycans. The expression and action of each extracellular matrix substance differs among organs. We have studied the specific changes in the spatial and temporal expressions of some extracellular matrices (ECMs) molecules and carbonic anhydrase isozyme-III (CA-III) in developing ruminal papillae [1].

The palatine ridges (PR) are formed from the ectoderm at an early stage of embryonic development, just after the primary and secondary fetal palates have fused. In mature animals, apical edges of PR are deflected in a caudal direction. This structure consists of two heterogeneous tissues, the epidermis and mesenchyme. Little information concerning the morphogenetic processes of the PR is available, except for that relating to the closure of the fetal palatin shelves [12].

This paper describes the temporal and spatial changes in the structural arrangements of the bovine PR and the immunohistochemical distributions of fibronectin (FN), type I collagen (C-1), type IV collagen (C-4), laminin (LN), and CA-III during the period of organogenesis.

MATERIALS AND METHODS

Fifteen bovine fetuses (Holstein) were obtained from the Omiya slaughterhouse (Saitama prefecture, Japan; 1990). Included were 15 fetuses from 7 to 60 cm in crown-rump length (CRL; Table 1). The cranial parts of the hard palate were excised and fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for two to three hrs at 4°C. All samples were rinsed in 0.01 M PBS (pH 7.2) containing a graded series of sucrose from 0 to 20%, and mounted in the tissue mount (Shiraimatu Kikai Co., Ltd., Japan).

<table>
<thead>
<tr>
<th>Sample CRL, cm</th>
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<tr>
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<tr>
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<td>5</td>
<td>16</td>
<td>10</td>
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</table>

Table 1. Bovine fetuses used in the present study.
They were frozen in dry ice-acetone, and cryosectioned at 5 μm. Each section was rinsed in 0.01 M PBS (pH 7.2) and treated in 3% BSA for 10 min at room temperature, and incubated with anti-ECMs sera or anti-equine CA-III serum for 1 hr at 37°C. The color was developed by aminoethyl carbazole (AEC) according to the ABC-methods [3].

Anti-bodies: Rabbit polyclonal sera raised against bovine FN, mouse LN, bovine C-1 and C-4, or equine CA-III were used. Their cross-reactivities against the corresponding bovine molecules have been confirmed by previous reports [1, 2].

RESULTS

Stage 1: Formation of epidermal placode (Fetuses of 7-9 cm CRL): Epidermal placodes (EP) and

Fig. 1. Histological conformation of the bovine palatine ridge during the fetal periods. H-E stain. a, b, Fetus at 7 cm CRL. a, ×650, b, ×250. Epidermal placode consists of columnar cells. The thickness of basement membrane-components of the epidermal placode site are comparable to that in the other sites. c, Fetus at 12.5 cm CRL. ×250. Epithelial cells seem stratified. The early type of PR protrudes toward the oral cavity, and slightly deflects in a caudal direction. Mesenchymal condensation is present at the apical site of the epidermal placode. d, Fetus at 44 cm CRL. ×250. The palatine ridge is well developed and the apical edge of the palatine ridge clearly deflects in a caudal direction. Mesenchymal condensation is not detected. From left to right on each figure corresponds to cranial to caudal in the specimen.

Fig. 2. Immunohistological distributions of fibronectin. a, Fetus at 7 cm CRL. ×250. Fibronectin is detected in the upper portion of the mesenchyme and the basement membranes of the palatine epithelium and the vascular endothelium. b, Fetus at 12.5 cm CRL. ×250. Fibronectin is detected mainly in the mesenchymal condensation. c, Fetus at 44 cm CRL. ×250. Fibronectin is distributed throughout the subepithelial mesenchyme. The orientation is the same as in Fig. 1.
mesenchymal condensations (MC) were detected at preforming sites of PR. The thickness of the basement membrane (BM) was comparable between the EP and others (Fig. 1-a). The epithelial layer was thin, and the epithelial cells were small in size except at the EP. At the EP sites only, there was a thick epithelial layer with cells larger in size. All epithelial cell nuclei were located at the center of cellular bodies (Figs. 1-a, 1-b). FN was detected in MC and epithelial and vascular endothelial BMs (Fig. 2-a). C-1 was distributed beneath the thin epithelial layer (Fig. 3-a). LN was detected in the BM beneath the thin epithelial layer and vascular endothelium (Fig. 4-a), but it was not detected in the BM beneath the EP. C-4 was localized throughout at the entire epithelial and vascular endothelial BMs (Fig. 5-a). CA-III was not detected anywhere (Fig. 6-a).

Stage 2: Formation of palatine ridge (PR) rudiments (Fetuses of 11–36 cm CRL): The PR rudiment had been formed. Apical parts of PR rudiments were slightly concave in a caudal direction. Mesenchymal cells gathered tightly at the apical mesenchyme of PR rudiments. The epithelial layer at the caudal slope of PR rudiments was thicker than that of the cranial one, while epithelial basal cells were

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**Fig. 3.** Immunohistological distributions of type I collagen. a; Fetus at 7 cm CRL. ×250. b; Fetus at 12.5 cm CRL. ×250. Type I collagen is distributed in the upper portion of the mesenchyme where the fibronectin is expressed. c; Fetus at 44 cm CRL. ×250. Type I collagen is detected diffusely throughout the mesenchyme. The orientation is the same as in Fig. 1.

**Fig. 4.** Immunohistological distributions of laminin. a; Fetus at 7 cm CRL. ×250. Laminin is detected in the epithelial and endothelial basement membranes except for the epidermal placode sites (arrows). b; Fetus at 12.5 cm CRL. ×250. Laminin is present in the epithelial and endothelial basement membranes. c; Fetus at 44 cm CRL. ×250. The pattern of laminin distribution is comparable to that in the earlier stage. The orientation is the same as in Fig. 1.
slightly thicker and bigger in size at the cranial slope than at the caudal one. Nuclei were located in the center of cellular bodies at the caudal slope, while they were shifted to the luminal side at the cranial slope (Fig. 1-c). FN was distributed throughout the epithelial and vascular endothelial BMs and in the subepithelial connective tissue at the MC (Fig. 2-b). C-1 was diffusely distributed throughout the subepithelial mesenchyme (Fig. 3-b). LN and C-4 were detected at the BM of the palatine epithelium and vascular endothelium (Figs. 4-b, 5-b). CA-III was not detected (Fig. 6-b), but there was a very weak reaction detected in the basal epithelial cells only in the late period of this stage (data not shown).

Stage 3: Establishment of PR (Fetuses more than 44 cm CRL): PR rudiments were arranged in a wave-shaped pattern. Apical edges of PR rudiments deflected progressively in a caudal direction. The MC was not detected. An apparent stratified squamous epithelium was seen in the cellular layer. The epithelial layer was thicker at the caudal slope than at the cranial one. The basal epithelial cell layer was thicker at the cranial slope and the caudal invaginated site than at the other caudal slope. Surface epithelial cells were cornified and some were stripped off from the apical site of PR rudiments (Fig. 1-d). FN and C-1 were diffusely distributed throughout the mesenchyme (Figs. 2-c, 3-c). The distributions of LN and C-4 were the same as seen in the earlier stages (Figs. 4-c, 5-c). CA-III was detected in palatine basal epithelial cells except in the PR rudiments (Fig. 6-c).

Fig. 5. Immunohistological distributions of type IV collagen. a; Fetus at 7 cm CRL. ×250. b; Fetus at 12.5 cm CRL. ×250. c; Fetus at 44 cm CRL. ×250. Type IV collagen is detected in the epithelial and endothelial basement membranes in all the gestational periods examined. The orientation is the same as in Fig. 1.

Fig. 6. Immunohistological distributions of CA-III. a; Fetus at 7 cm CRL. ×250. b; Fetus at 12.5 cm CRL. ×250. c; Fetus at 44 cm CRL. ×250. CA-III is detected in the basal palatine epithelial cells except for the sites of protrusion. The orientation is the same as in Fig. 1.
Table 2. Changes in distribution of some ECMs and CA-III and the summary of morphological descriptions during the organogenesis of the bovine fetal PR

<table>
<thead>
<tr>
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<th>stage 2 (11-36 cm CRL)</th>
<th>stage 3 (over 44 cm CRL)</th>
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<tr>
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<td>-</td>
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<tr>
<td>HE-stain*  **</td>
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<td>crsl</td>
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<td>small &amp; round</td>
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<tr>
<td>location of nucleus:</td>
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* No change in the thickness of the eosinophilic component at BM in all stages examined.
** Mesenchymal condensation and epidermal placode evident in stage 1.
*** Also thick in casl at invaginated site.

Stage 1: formation of epidermal placodes.
Stage 2: slight deflection of PR rudiments.
Stage 3: establishment of PR rudiments.
prot.: Platine ridge (PR) protrusional site.
root: Flat site except for PR protrusion.
ep: Epithelium
me: Mesenchyme
crsl: cranial slope
casl: caudal slope
+: detected
++: strongly detected
-: not detected
FN: fibronectin
C-1: type I collagen
LN: laminin
C-4: type IV collagen
CA-3: carbonic anhydrase isozyme III

Several developmental events during the bovine PR formation were summarized in Table 2.

DISCUSSION

The regularly recurrent arrangement of the superficial conformation in vivo, such as bovine ruminal papillae [1], and chick feather germs [5], probably becomes evident by one or more morphogenetic events. These events include cell proliferation and differentiation. The present study suggests that the PR was formed through sequential, but distinct morphogenetic phases: protrusion of the placode and caudal deflection of the folds. The primary fetal
bovine PR rudiment was formed as a small protrusion toward the oral cavity at early gestation as shown in Table 2. This morphogenetic event (protrusion) has been detected in chick feather germs [5] and the mouse mammary gland [4] formation. The EP and MC have been shown to determine the sites for invagination and evagination in several organs [7, 8]. The secondary caudal deflection of PR consisted of unbalanced proliferative zones. This suggests that a different polarity exists in the superficial conformation. Therefore, we recognize this morphogenetic phase as a different process from the primary protrusion. Through this secondary process caudally-directed PR would be established in mature cows.

In the EP of the primary protrusion, the epithelial cells of the cranial slope and caudal invaginating area of the secondary deflection had centrally located nuclei, which suggests that signals from the nucleus flowed both ways toward the luminal and BM sides, although actual signaling molecules could not be detected in the present study.

The FN, C-1 and C-4 did not show specific localizations in the PR forming sites. Instead, only LN showed a specific mode of distribution: they were absent from the BM just beneath the primary EP. This observation agreed with reported findings on the formation of chick feather germs [5, 10]. The absence of LN from the BM might be involved only in the primary PR protrusion, since it did not change in the secondary PR deflection. In the primary PR protrusion, the absence of LN might allow the cells to divide and migrate. Other molecules, such as proteoglycans [11], may play a role in the regulation of cellular proliferation and arrangement.

The CA-III, an isozyme of the carbonic anhydrase, the lowest enzymatic activity in the CA-isozymes, has been reported to be expressed mainly in striated muscle [2] and the bovine ruminal epithelium [6]. Amasaki et al. [1] have reported the usefulness of the CA-III as a marker for the cell function and/or differentiation in the formation of bovine ruminal papilla. In this study, CA-III was again detected in the cytoplasm of the epithelial basal cells located in all areas except for the site of protrusion in the stage when the PR was established. Acetazolamide, an inhibitor of carbonic anhydrase, induces unilateral malformations of rat limbs [9]. Therefore, the CA-III gene is actually expressed in specific places and times during PR formation, although the physiological role remains unknown.

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REFERENCES