Observations on Early Development of the Murine Fetal Oral Vestibule

By

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Summary: Morphological and immuno/histochemical studies were performed on the vestibular lamina (VL) of gestational day 13 murine fetuses, using light microscopy (LM), confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM), in an effort to elucidate the early development of the oral vestibule. Histochemistry employing LM demonstrated some PAS-positive glycogen particles in embryonic cells of the VL, dental lamina (DL), the primary epithelial band connecting the VL and DL, and the related stomodeal simple epithelium. On the other hand, Gomori’s aldehyde fuchsine method stained certain cystine-containing intracellular granules and intercellular amorphous substances, particularly in the central VL. Intense immunoreactivity for CK-10 intermediate-sized filament proteins was demonstrated in suprabasal and superficial cells of the VL stratified keratinized epithelium. Conversely, reactions for CK-19 filaments were found diffusely in both VL and DL cells retaining the cytokeratin characteristic of the simple epithelium. TEM of the VL revealed an increment in keratinosomes, tonofilaments and desmosomes in the suprabasal layers shifting toward superficial flat parakeratinized cells. The TUNEL method using CLSM detected programmed cell death in the VL, while TEM provided no morphological evidence of necrosis or typical apoptotic features during VL development. The present results indicate that physiological (naturally occurring) cell death and exfoliation of the oral-gingival type multilayered keratinizing epithelium are essential for degeneration and separation of the VL, ultimately leading to formation of the oral vestibule.

Some previous studies have asserted that the dental lamina (DL) proliferates from the vestibular lamina (lip or cheek furrow band, vestibular band; VL), whereas most have found that the DL and VL are two independent epithelial diverticula which actually originate from a common primitive dental lamina – the primary epithelial band –, and that the DL develops at the lingual side of the VL (Coslet & Cohen, 1969; Kitamura, 1989). In addition, many light microscopic (LM) studies have demonstrated that the VL develops shortly after the initial appearance of the DL, and that the VL and DL originate as separate entities (Rule, 1986; Malcolm et al., 1990). The mesial diverticulum that begins at the labial/buccal side of the DL, proliferating vertically into the ectomesenchyme, is an epithelial tissue closely related to the genesis of the oral vestibule (vestibulum oris; VO). Subsequently, cells in the VL enlarge rapidly, degenerate and autolyze, resulting in an augmentative cleft between the check and the tooth-bearing area (Ten Cate, 1994).

With regards to the morphodifferentiation of the VO, it has been reported to result from different growth rates between the gum ridge, developing alveolar bone and lip primordium. However, VO formation is not due to cell death, since the VL separates at the sites showing poor adhesion between daughter cells derived from different basal cells of lip (lining mucosa) type and gum (masticatory mucosa) type epithelia (Coslet & Cohen, 1969; Pelissier et al., 1992). On the other hand, naturally-occurring programmed cell death has been elucidated as a phenomenon occurring widely in normal cell renewal, i.e. tissue turnover, during embryogenesis and pathological processes (Glückmann, 1965; Kerr et al., 1972; Trauth & Keesey, 1994). Furthermore, autophagocytosis, heterophagocytosis and early appearance of apoptotic bodies in scattered pyknotic cells have been recognized as typical morphological features in the process of apoptosis (Uchiyama, 1995). Yet many studies have reported that programmed cell death includes variants, differing from the classic definition (Clarke, 1990; Schwartz
The present histological study of the oral cavities of fetal mice was undertaken with the following aims: 1) to elucidate the fine structure study of the proliferating VL, 2) to demonstrate the histochemical and immunological features of VL components and 3) to discuss recent findings of VL epithelial cell degeneration in terms of possible mechanisms of VO development.

**Materials and Methods**

Ten pregnant Slc:ICR SPF mice (SLC, Tokyo, Japan) bearing gestational day 13 fetuses were used in the present study; the day the vaginal plug was observed was designated gestational day 0. The pregnant mice were maintained and killed under anaesthesia, and 80 fetuses were harvested by caesarean section under guidelines elaborated in the “Guide for the Care and Use of Laboratory Animals” established by Osaka Dental University.

**Routine light microscopy (LM) and transmission electron microscopy (TEM)**

Twenty fetuses were decapitated, the head portions were dissected, trimmed and immersed for 4 hours in chilled (0–4 °C) aldehyde fixatives (containing 2.5% glutaraldehyde and 2.0% paraformaldehyde buffered with 0.1 M sodium cacodylate: 1/2 Karnovsky’s aldehyde fixatives). After rinsing the samples by changing the buffer solution several times, they were trimmed into blocks and some were postfixed for 1 hour in chilled 1.0% osmium tetroxide (buffered with 0.1 M sodium cacodylate solution). All samples were dehydrated through a graded ethyl alcohol series, and embedded in Epon 812 following conventional methods. Semi-thin 1 μm sagittal sections were made with glass knives mounted on an LKB ultratome (LKB, Stockholm, Sweden) for the purpose of routine LM studies. The specimens were stained with triple chrome (0.5% malachite green, 80°C, 60–100 sec; 0.5% toluidine blue, 80°C, 30 sec; basic fuchsine, room temp., 10 sec), and then examined using an Olympus Vanox-S light microscope (Olympus, Tokyo, Japan). Subsequently, silver to gold color ultrathin sections containing the differentiating mandibular VL were prepared with either glass knives or a diamond knife mounted on the LKB ultratome. The ultrathin sections were picked up on parlodion-covered 150-mesh grids and stained with uranyl acetate (20 min) and lead citrate (10 min) solutions following conventional methods, and then examined and photographed under a Hitachi 7100 TEM (Hitachi, Tokyo, Japan) for ultrastructural investigation.

**LM for histochemical and immunochemical studies**

Twenty fetuses fixed in 1/2 Karnovsky’s aldehyde fixatives were embedded in paraffin employing conventional methods. Serial sagittal 1–2 μm sections of the middle portion of the head were cut using stainless steel knives mounted on an AS-500 Universal Microtome (Anglia Scientific, Cambridge, UK). Specimens containing the proliferating VL were histochemically stained with periodic acid schiff (PAS) and Gomori’s aldehyde fuchsin stains for identification of intracellular granules in the cellular components of the differentiating VL.

Another twenty samples were fixed with Carney’s solution, embedded in paraffin, sectioned and subsequently stained with monoclonal mouse anti-human cytokeratins 19 and 10 antibodies (DAKO-CK 19, RCK 108: DAKO EPOS Anti-Cytokeratin 19/HP; DAKO-CK 10, DE-K 10: DAKO EPOS Anti-Cytokeratin 10/HP; DAKO, Germany) to detect intermediate-sized filament proteins related to differentiation and maturation of epithelial cells in the VL.

**Confocal laser scanning microscopy (CLSM)**

For studying cell turnover in the differentiating VL, twenty fetuses fixed with 1/2 Karnovsky’s aldehyde fixatives were embedded in paraffin, sectioned and then studied by the TdT-mediated fluorescein-dUTP nick end-labeling (TUNEL) technique using an In situ Cell Death Detection kit, POD (Boehringer Mannheim Biochem, Germany), or an ApoTag Plus In Situ Apoptosis Detection Kit-Fluorescein (Oncor, USA). FITC (fluorescein isothiocyanate) staining for the TUNEL was co-stained with PI DNA staining (Propidium Iodine; Oncor, USA) for identification of DNA fragmentation in the cell nuclei (Gavrieli et al., 1992). The specimens were examined and the images were analysed using an Olympus LSM-GB 200 CLSM (argon ion laser, excitation wave length: 488 nm; using CH1: O515 and BP535, CH2: O575 and O590 barrier filters; Olympus, Tokyo, Japan) connected to an image analysis system (CLSM-GB 200 application ver. 2.23; Compaq Prolinea-4100 computer, USA), with the data photographed using an Avio FR-3000 film recorder (Avionics, Tokyo, Japan).

**Results**

**LM for routine examination and immuno/histochemistry**

Routine LM examination of the epon-embedded specimens stained with triple-chrome showed that
VL and DL proliferate from the thickened primary epithelial band in the anterior region of the mandibular process. Neither DL nor VL was interdigitated by the surrounding connective tissue papillae. LM showed many mitotic cells, particularly in the stratum basale and the lower spinous layer of the anterior (lip) limb and tip of the VL, as well as in the proliferating incisor DL. Some round-to-cuboidal basal cells contained certain fuchsine-stained granules. The central VL contained layers of light cells shifting towards flat superficial lining cells; the cells also contained fuchsine-reactive granules (Fig. 1). Histochemistry detected PAS-positive cells in the central portion and a superficial flat cell layer of the VL. Distinct reactions were also observed in the germinative layers of the epithelial band bridging the VL and DL and the vestibular aspect of the DL (Fig. 2a). On the other hand, weak positive Gomori's aldehyde fuchsine reactions were limited to the central VL (Fig. 2b). Monoclonal mouse anti-human cytokeratin 10 and 19 antibodies were used to study cytokeratin expression by detection of intermediate-sized filament proteins in the VL and DL. Intensely CK-10 immunoreactive cells were demonstrated in suprabasal layers and superficial flat cells of the VL, while no reactivity was detectable in the DL (Fig. 3a). In contrast, weak and diffuse reactions for CK-19 filaments were recognized in both the VL and the DL. CK-19 filament proteins were also detected in superficial VL flat cells, which shifted toward the related oral simple epithelium and the bridging oral epithelium between the VL and DL (Fig. 3b).

**CLSM for TUNEL techniques**

Carnoy's solution-fixed specimens were examined by the TUNEL technique. The specimens were double-stained using fluorescence *in situ* hybridization by FITC and PI costaining. CLSM analysis of TUNEL-positive nuclei indicated single cells in the early stage of DNA strand breakage and fragmentation. Many TUNEL-positive single cells were scattered throughout the VL and the surrounding mesenchyme containing embryonic cells (Fig. 4). The CLSM indicated programmed cell death in early VO development.

**TEM for morphological examination**

TEM showed the VL stratum basale to be composed of one to two layers of cuboidal cells containing large clusters of glycogen particles. Some irregular, ragged spaces giving a "moth-eaten" appearance to the cytoplasm were speculated to be sites originally occupied by glycogen particles (Fig. 5). No morphological differences between the basal cells of the lip and gum limbs of the VL were recognized. The basal cells shifted to suprabasal polygonal cells, which bound the stratum spinosum via spot desmosomes (maculae adherence). The suprabasal cells contained glycogen and small electron-dense membrane-bounded granules (keratinosomes) and tonofilaments closely associated with the desmosomes (Fig. 6). TEM indicated that the glycogen particles and keratohyaline-containing structures corresponded to the PAS-positive and Gomori's aldehyde fuchsine-reactive granules, respectively; these have been demonstrated in the germinative layers of the VL embryonic epithelium with different histochemical stains. The intracellular organelles in the cells of the germinative layers were not well developed, while many polysomes and keratinosomes were evident in the cytoplasm of prickle cells (Figs 6 & 7). TEM also revealed increments in desmosomes, tonofilaments and keratinosomes in the strata spinosum and granulosum, which shifted toward the superficial parakeratinized stratum corneum containing flat cells (Figs 6–8). No signs suggesting destruction of the intracellular membranous organelles, decrease and disappearance of filament fractions, degeneration of the cell membrane and intercellular junctional complexes or necrosis of the keratinizing epithelial cells were detected in the developing VL.

**Discussion**

The odontogeneic zone of the palatal mucosa was found to be a common morphogenetic system composed of epithelial and mesenchymal zones which are closely related to the development of the teeth, rugae palatinae and fornx vestibuli oris in a study on fetal mice. The same study spatially demonstrated that the first molar anlage was continuous with the VL and DL of the anterior diastema region, whereas the fornx vestibuli oris superior developed from the lip-furrow lined with VL at the site of fusion of the maxillary and mandibular processes (Peterková, 1985). A later study further showed that the VL developed in proximity to the maxillary incisor and mandibular molar tooth buds, though with a certain distance from the maxillary molar and mandibular incisor tooth buds (Kitamura, 1989). However, the VL and DL are usually described as two epithelial mounds generated from the common primary epithelial band (Coslet & Cohen, 1969; Malcolm et al., 1990; Ten Cate, 1994). In gestational day 13 fetuses, we focused on the positional relationships of the mandibular labial VL and incisor DL, and observed rapid proliferation of the VL and DL from the thickened primary epithelial band. LM revealed numerous mitotic epi-
thelial cells in the stratum germinativum of the VL, DL and their related oral simple epithelium.

CK-19 intermediate filament protein is reportedly a simple-epithelial keratin characteristic found in non-keratinizing stratified (multilayered) epithelium, particularly in cells that lose their epithelial polarity and thus differentiate into glandular cells (Carette et al., 1991; Ikeda & Yoshimoto, 1991a). On the other hand, CK-10 is reportedly a characteristic intermediate filament protein detectable in suprabasal layers of the keratinizing stratified epithelium but not in all simple and glandular epithelia (Van Muijen et al., 1987). In the present immunohistochemical study, we identified weak and diffuse CK-19 positive immunoreactions in the sloughing superficial layer of the VL epithelium, the epithelial band bridging the VL and DL, and also in the DL. The histology indicated that VL and DL are both epithelial diverticula retaining the cytokeratin characteristic of the stomodeal simple epithelium. Lining mucosa type cells have been demonstrated in the superficial three-quarters of the lip furrow lamina, suggesting that the boundary between the positive and negative immunoreactive cells is probably the site where the internal split of the VL takes place (Pelissier et al., 1992). However, we clearly demonstrated strong positive CK-10 immunoreactivity in the labial aspect, superficial layer and central portion of the proliferating VL, as well as some weak reactivity in the primary epithelial band. Our findings suggest an early transition of the stomodeal simple epithelium into keratinized epithelium during genesis of the VL.

Additionally, the expression of cytokeratin has been found to not only correlate with germinal layer origins, but to also be related to cell functions in the development process (Viebahn et al., 1988). The pattern of cytokeratin expression by a given epithelium varies according to cell and epithelium type, developmental stage and the state of maturation (Pelissier et al., 1992). On the other hand, the mitotic rate of the gingival epithelium is reportedly one of the important factors influencing keratinization, in that where the renewal time is rapid no keratinization occurs (Cohen, 1967). In a study of the early organogenesis of the pituitary gland, it was shown that many cells immunoreactive for CK-8 and CK-19 in the presumptive anterior lobe have low proliferative activity. Conversely, delayed appearance of cytokeratins and differentiation of endocrine cells were recognized as maintaining their simple epithelial character in the presumptive intermediate lobe (Ikeda & Yoshimoto, 1991a). The heterogenous patterns of cytokeratin distribution reflect the degree of epithelial cell proliferation and differentiation; cells maintaining high proliferative activity are apt to differentiate later (Ikeda & Yoshimoto, 1991a, Ikeda & Yoshimoto, 1991b). The present immunohistochemical results suggest that the proliferating mandibular incisor enamel organ, which was diffusely stained with CK-19 antibody, will differentiate into odontogenic cells later than the VL differentiating cells showing intense reactivity to CK-10 antibody.

Histochemistry showed epithelial cells in the central part of the VL and the labial aspect of the DL to be strongly reactive to PAS staining. Gomori's aldehyde fuchsin staining also displayed a weak histochemical reaction in the central VL. Many basic fuchsin-reactive granules were also observed, particularly in the stratum basale of the fetal VL, on examination of epon-embedded specimens stained with triple-chrome. TEM evaluation suggested that the PAS- and basic fuchsin-positive granules were glycogen particles in the stratum germinativum of the VL and primary epithelial band. The small electron-dense keratinosomes related to tonofilaments and desmosomes found in the strata spinosum and granulosum were cystine-containing substances reactive to Gomori's aldehyde fuchsin staining.

Apoptosis has generally been described as one form of energy-dependent programmed cell death related to intranucleosomal DNA fragmentation (Kerr et al., 1972; Wyllie, 1987; Lo et al., 1995; Yasuda et al., 1995). Apoptotic cell death displays characteristic morphological features including individual cell shrinkage, nuclear chromatin condensation and margination as well as the appearance of membrane-bound apoptotic bodies which may be digested by autophagocytosis or heterophagocytosis, or which will degenerate in the absence of any detectable lysosomal activity (Laferla et al., 1995; Lo et al., 1995; Steller, 1995). The present fine structure study did not find histological evidence indicating plasma membrane disruption, intracellular organelle degeneration or inflammatory changes suggestive of necrosis in the keratinizing VL epithelium. Conversely, using the combination of TUNEL histochemistry and translation techniques, the CLSM demonstrated multiple areas of FITC reactivity overlapping with the PI-stained nuclei in the VL showing a keratinized stratified squamous epithelial character. The immunohistological study revealed DNA strand breaks related to programmed cell death during VL development.

On the other hand, both LM and TEM findings showed a multilayered cone-shaped distribution of the keratinizing VL epithelial cells similar to what has been described for the oral-gingival epithelium (Schroeder & Amstad-Jossi, 1979), while there were
no morphological differences between the anterior (lip) limb and posterior (gum) limb of the proliferating VL. TEM revealed many large intercellular spaces containing certain amorphous substances in the keratinizing middle and superficial layers; nevertheless there was no histological evidence indicative of poor adhesion or separation between midline cells or of VL necrosis. Our immunohistochemical results suggest that the VL is an epithelial band having a relatively long cell renewal time, which allows differentiation of keratinizing cells in the early proliferation stage, and that a form of naturally occurring cell death is involved in development of the VL. However, infiltrations of monocytes, phagocytosis and apoptotic cells in the VL were not detected by TEM.

Separation of the VL and epithelial cells lining the nostrils has been reported to be due to poor adhesion of cells and cell death, respectively (Coslet & Cohen, 1969). Another study, however, noted apoptosis occurring in the proliferating VL epithelium (Vaahtokari et al., 1996). Some recent studies have presented histological evidence of separation and exfoliation of the reduced enamel epithelium into the oral cavity without apoptotic cell death during tooth eruption (Dan, 1997; Kaneko et al., 1997). In the present study, we showed that physiological programmed cell death in the VL is associated with an increment in epithelial debris (keratohyaline). Degeneration and disintegration, due to the ultimate breakdown of membranous structures, are essential factors in the exfoliation of VL epithelial cells. Subsequent growth of surrounding tissues and organs and contraction of the lip musculature may enhance the central split in the VL (Coslet & Cohen, 1969; Pelissier et al., 1992).

A study of the differentiation of mouse palatal epithelium demonstrated that the embryonic mesenchyme plays an important role not only in supporting differentiation, but also in actively specifying the developmental fate of its overlying epithelium (Carette et al., 1991). The epithelium plays a passive role in that it responds to the underlying mesenchyme and differentiates into phenotypes appropriate for the mesenchyme (Ferguson & Honig, 1984). On the other hand, some epithelial-mesenchymal tissue interactions have been shown to inhibit and even prevent apoptosis in dental tissues (Vaahtokari et al., 1996). The present results confirm that the VL and DL are two epithelial mounds derived from a common primary epithelial band, while they show different cytokeratin expressions and cell turnover rates. Further study is needed to elucidate factors that control the proliferation, differentiation and cell death of the VL epithelium.

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References


Abbreviations used in Figs. 1–8b

| CLSM | Confocal laser scanning microscopy |
| DL  | Dental lamina |
| K   | Keratinosome |
| SC  | Stratum corneum |
| SS  | Stratum spinosum |
| T   | Tonofilament |
| VL  | Vestibular lamina |
| D   | Desmosome |
| LM  | Light microscopy |
| SB  | Stratum basale |
| SG  | Stratum granulosum |
| St  | Stomodeaum |
| TEM | Transmission electron microscopy |

Explanation of Figures

Plate I

Fig. 1. LM of an epon-embedded specimen stained with triple-chrome. Many basic fuchsin-reactive granules (glycogen particles; arrows) are recognizable in the VL containing embryonic cells.

Fig. 2. Histochemistry of the developing VL, DL and oral simple epithelium. a. PAS-positive reactions are seen in the central portion and superficial flat cell layer of the VL, as well as in the germinative layer of the epithelial band bridging the VL and the vestibular aspect of the DL (asterisks). The basement membrane (arrowheads) of the DL and VL are also PAS reactive. Many mitotic cells (arrows) are seen, particularly in the germinative layer of the DL. b. LM of a specimen stained with Gomori’s aldehyde fuchsin, showing weak positive reactions (asterisks) only in the central portion cells with a shift toward the flat superficial cells of the VL.

Fig. 3. LM showing cytokeratin expressions in the VL. a. Intensely CK-10 immunoreactive cells (asterisks) are demonstrated in suprabasal and superficial flat cell layers of the VL; no reaction is discernible in the DL. b. Weak CK-19 filament reactions (arrows) are demonstrated in both the VL and DL. The CK-19 filament proteins are diffusely distributed in superficial flat cells of the VL, the related oral simple epithelium and the primary band bridging the VL and DL.

Fig. 4. VL examined by the TUNEL technique (stained with FITC and PI). Employing image processing, accumulation of CLSM tomograms allowed analysis of many single cells in the early stage of programmed cell death in the VL and surrounding mesenchyme.
Plate II

Fig. 5. TEM showing SB cells at the labial aspect of the VL. Basal cells contain some large clusters of glycogen particles (arrowheads) and the sites, which were originally occupied by glycogen particles (asterisks). Arrows indicate the intact basal lamina of the VL.

Fig. 6. Photo showing suprabasal polygonal cells bound to SS prickle cells via spot desmosomes (D). Accumulation of glycogen particles (asterisk) and distribution of keratohyaline-containing structures (K) are recognizable in suprabasal cells. The arrow at the upper right indicates amorphous intercellular substance in the SS. TEM suggests that the glycogen particles and K correspond to the PAS-positive and Gomori's aldehyde fuchsine-reactive granules, respectively.

Fig. 7. TEM of SG and SC, which contain flat parakeratinized cells at the superficial surfaces of the VL. Intracellular organelles are not well developed, while many polysomes and K (arrows) and T related to D are seen in the cytoplasm.

Fig. 8. Higher magnification of the SG of the VL. a. Augmentation of intercellular spaces (asterisks) is recognized in SG cells differentiating into superficial flat cells. Many D, K and T related to intercellular junctional apparatuses are demonstrated. No intercellular invasion of phagocytic cells is evident. There are no signs indicative of destruction of the cell membrane and intercellular junctions. b. Higher magnification showing D and K in the cytoplasm of granular cells.