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Enamel free areas in rodent molars — ultrastructure of basement membrane in rat tooth germ

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ABSTRACT **At the cusp tip of rodent molar, there is a region of dentin without an enamel cap. This region is called enamel free area (EFA). The surface collagen arrangement has been reported to differ between the EFA and the dentin covered with the enamel (DCE). To clarify the cause of this difference, we observed the ultrastructure of the basement membrane and the distal ends of the inner enamel epithelium (IEE) in rats. At 20 days prenatal, distal ends of IEE were relatively flat on both the DCE and EFA. Ultrastructurally, there was no difference between the basement membranes. At newborn, no marked changes were observed in the morphology of the distal end of IEE on the DCE or the EFA, but aperiodic microfibrils perpendicular to basal lamina were denser and longer on the DCE than the EFA. At 2 days postnatal, cytoplasmic extensions from distal end of IEE penetrated through basal lamina, and these extensions were more developed on the DCE than the EFA. On the DCE, collagen fibrils ran into and between cytoplasmic extensions and were arranged perpendicular to the surface. On the EFA, collagen fibrils ran parallel to the surface, and few collagen fibrils ran into and between cytoplasmic extensions. These findings suggested that the differences in the collagen arrangement between the EFA and DCE are associated with the developmental state of aperiodic microfibrils in the basal lamina beneath IEE and the morphology of the distal end of IEE.**

KEY WORDS: *enamel-free area, basement membrane, ultrastructure, rat molar, aperiodic microfibrils*

Introduction

At the cusp tip of the rodent molar, there is a region of dentin not covered with the enamel. Addison and Appleton (1921) named this region the enamel-free area (EFA). Though there have been only a few studies on the ultrastructure of the EFA and the dentin covered with the enamel (DCE) (Sutcliffe and Owens, 1980, 1981; Crooks *et al.*, 1983; Sakakura *et al.*, 1989; Nakamura *et al.*, 1991; Inai *et al.*, 1992; Yamamoto *et al.*, 1993), some of them have shown differences in the collagen arrangement on the surface between the EFA and DCE (Sakakura *et al.*, 1989; Nakamura *et al.*, 1991; Yamamoto *et al.*, 1993). On the DCE, collagen fibers run perpendicular to the surface. On the other hand, on the EFA, most collagen fibers complicatedly run parallel to the surface. The reason for this difference between the EFA and DCE has not been clarified. However, the following factors are considered to be associated with the collagen arrangement on the dentin surface: ultrastructure of the basement membrane (Takuma, 1967; Goto, 1974; Hurmerinta and Thesleff, 1981; Suzuki, 1985; Sawada, 1992), degree of odontoblast differentiation (Moss, 1974; Tanaka, 1987), and the direction of odontoblast process (Reith, 1968; Sisca and Provenza, 1972; Shimabara, 1986; Tanaka, 1987).

Noting the basement membrane beneath IEE observed in the early stage of tooth crown formation, we determined to clarify the relationship between the ultrastructure of the basement membrane and the collagen arrangement of the surface of the EFA and DCE.

Results

Light microscopy and transmission electron microscopy

Light microscopy at 20 days prenatal showed cuboid or short columnar IEE cells without polarization. Dental papilla cells aligned beneath IEE on the DCE, but they were scattered on the EFA (Figs. 1, 11). Transmission electron microscopy showed a nearly flat distal end of IEE on both the DCE and EFA. The basal lamina beneath IEE was clearly observed on both the DCE and EFA, but only a few aperiodic microfibrils perpendicular to the basal lamina were present. At this stage, processes of dental papilla cells were scarce near the basal lamina (Figs. 2, 3, 12, 13).

Light microscopy at newborn (22 days prenatal) showed no marked changes in IEE of the DCE and EFA or dental papilla cells compared with 20 days prenatal (Figs. 4, 14). Transmission electron microscopy revealed slight irregularity at the distal end of

Abbreviations used in this paper: DCE, dentin covered with enamel; EDTA, ethylendiaminetetraacetic acid; EFA, enamel-free area; IEE, inner enamel epithelium.

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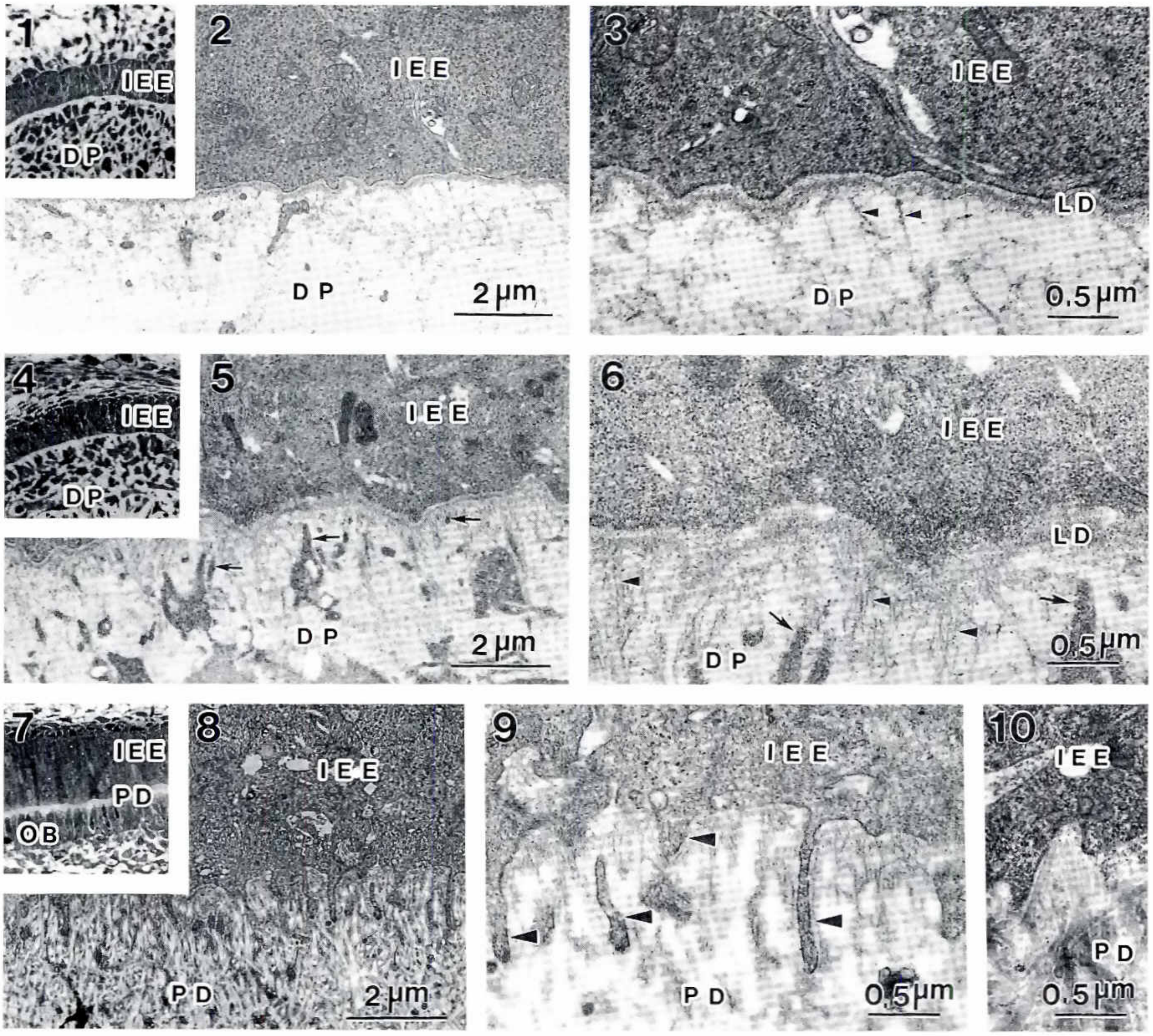
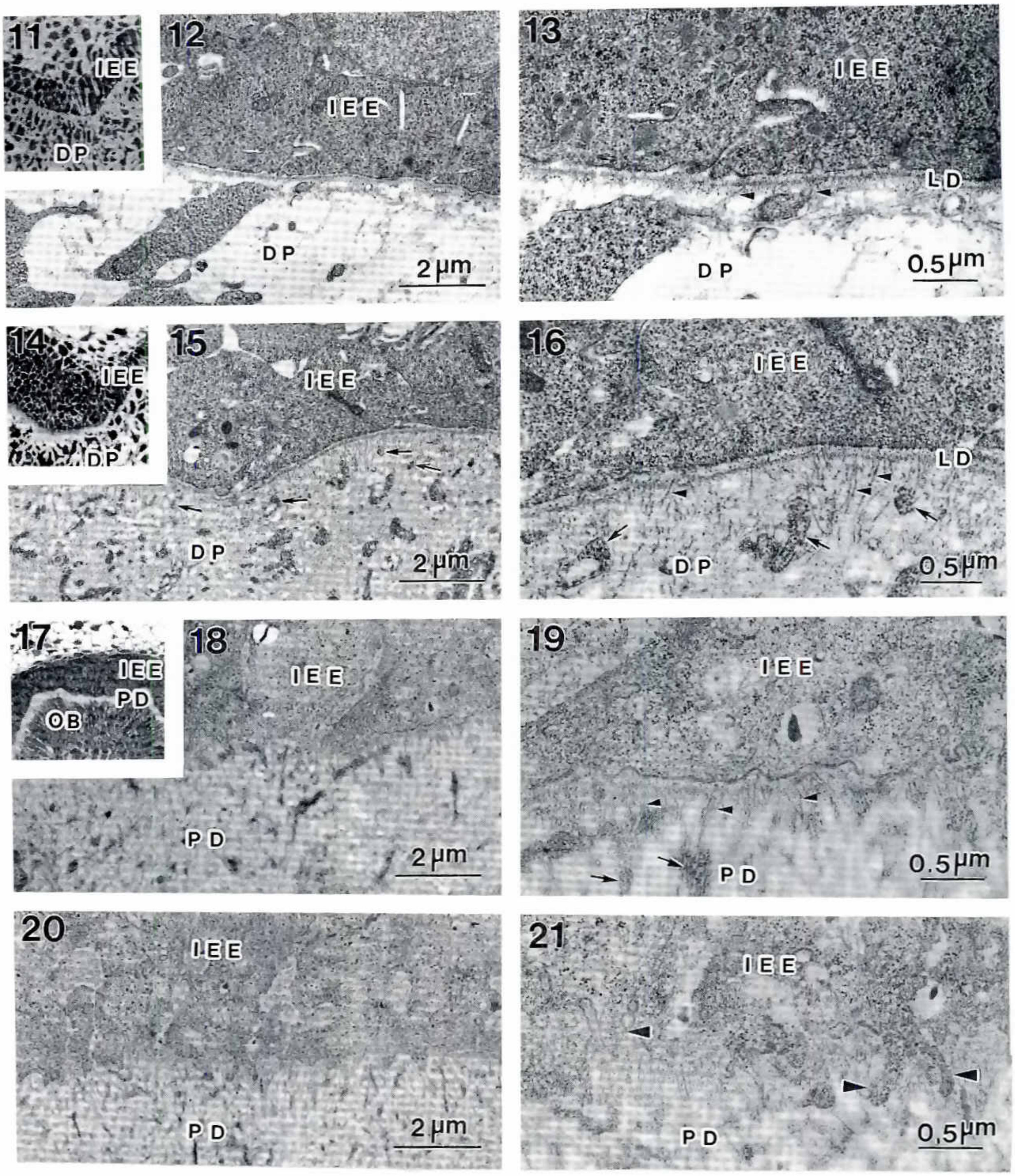


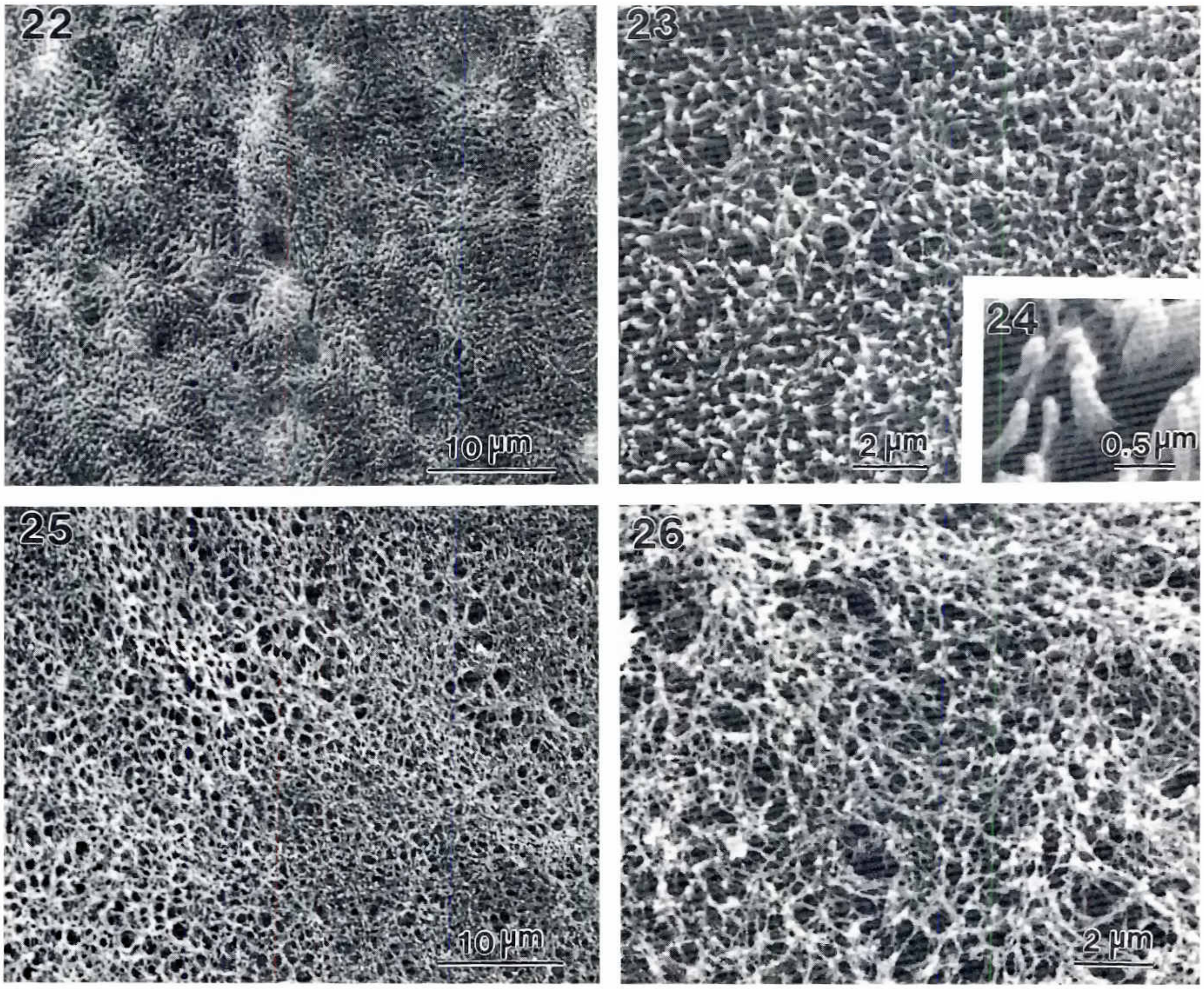
Figs. 1-10. Light micrographs and transmission electron micrographs of the DCE (1-3) at 20 days prenatal, (4-6) at newborn, (7-10) at 2 days postnatal). (1) *Light micrograph (x50, toluidine blue staining). IEE cells were cuboid or short columnar. Dental papilla cells aligned beneath IEE.* **(2)** *Transmission electron micrograph.* **(3)** *Higher magnification of Fig. 2. Distal ends of IEE were nearly flat. The lamina densa was clearly observed, but there were few aperiodic microfibrils.* **(4)** *Light micrograph (x50, toluidine blue staining). An image similar to that at 20 days prenatal was observed.* **(5)** *Transmission electron micrograph.* **(6)** *Higher magnification of Fig. 5. Distal ends of IEE were slightly irregular. Aperiodic microfibrils were increased.* **(7)** *Light micrograph (x50, toluidine blue staining). IEE cells were tall columnar and polarized. Dental papilla cells have differentiated into odontoblasts, forming the predentin.* **(8)** *Transmission electron micrograph.* **(9)** *Higher magnification of Fig. 8. Many cytoplasmic extensions from the distal end of IEE penetrated through the basal lamina.* **(10)** *Phosphotungstic acid staining. Collagen fibrils with the periodic structure ran into and between cytoplasmic extensions of IEE. Abbreviations: DP, dental papilla; IEE, inner enamel epithelium; LD, lamina densa; OB, odontoblast; PD, predentin; small arrowheads: aperiodic microfibrils; large arrowheads: cytoplasmic extensions of IEE; arrows: processes of dental papilla cells.*

IEE on the DCE and a continuous basal lamina. However, aperiodic microfibrils perpendicular to the basal lamina were denser and longer than those at 20 days prenatal. In addition, many processes of dental papilla cells were present in contact with aperiodic microfibrils (Figs. 5, 6). At this stage, the distal ends of IEE on the EFA were relatively flat, and the basal lamina was continuous. Aperiodic microfibrils were denser and longer than those at 20 days prenatal but sparser and shorter than those on the DCE at the same age. As on the DCE, these aperiodic microfibrils were in contact with many processes of dental papilla cells (Figs. 15, 16).

Light microscopy at 2 days postnatal showed tall columnar IEE cells showing polarity on the DCE (Fig. 7) but short columnar IEE cells on the EFA (Fig. 17). On both the DCE and EFA, dental papilla cells had differentiated into odontoblasts, forming predentin (Figs. 7, 17). Transmission electron microscopy showed many cytoplasmic extensions from IEE on the DCE and penetration of the basal



Figs. 11-21. Light micrographs and transmission electron micrographs of the EFA (11-13) at 20 days prenatal, (14-16) at newborn, (17-21) at 2 days postnatal). (11) *Light micrograph (x50, toluidine blue staining). IEE cells were cuboid or short columnar. Dental papilla cells existed sparsely.* **(12)** *Transmission electron micrograph.* **(13)** *Higher magnification of Fig. 12. Distal ends of IEE were relatively flat. The ultrastructure of the basal lamina was similar to that in the DCE at the same stage.* **(14)** *Light micrograph (x50, toluidine blue staining). IEE cells were similar at 20 days prenatal.* **(15)** *Transmission electron micrograph.* **(16)** *Higher magnification of Fig. 15. Distal ends of IEE were relatively flat. Aperiodic microfibrils increased compared with at 20 days prenatal but less than those on the DCE.* **(17)** *Light micrograph (x50, toluidine blue staining). IEE cells were columnar. Odontoblasts that have differentiated from dental papilla cells formed predentin.* **(18)** *Transmission electron micrograph.* **(19)** *Higher magnification of Fig. 18. Distal ends of IEE were flat. The basal lamina was continuous.* **(20)** *Transmission electron micrograph.* **(21)** *Higher magnification of Fig. 20. The cytoplasmic extensions from distal end of IEE penetrated through the basal lamina. But these extensions were shorter and fewer than those on the DCE. Abbreviations: DP, dental papilla; IEE, inner enamel epithelium; LD, lamina densa; OB, odontoblasts; PD, predentin; small arrowheads: aperiodic microfibrils; large arrowheads: cytoplasmic extensions of IEE; arrows: processes of dental papilla cells.*



Figs. 22-26. The three-dimensional structure of the DCE and EFA at 20 days postnatal. (22) *Scanning electron micrograph of the DCE.* **(23)** *Higher magnification of Fig. 22.* **(24)** *Higher magnification of cone-like projection. Numerous cone-like projections consisting of fine fibrils were observed.* **(25)** *Scanning electron micrograph of the EFA.* **(26)** *Higher magnification of Fig. 25. Fine fibrils ran parallel to the surface, and formed the network structure.*

lamina by these extensions (Figs. 8, 9). Bundles of collagen fibril with the periodic structure ran into and between cytoplasmic extensions and were arranged perpendicular to the surface (Fig. 10). On the EFA, flat areas (Figs. 18, 19) mixed with areas with cytoplasmic extensions at the distal end of IEE. In the flat area, the basal lamina was nearly continuous, and aperiodic microfibrils were short, showing a state similar to that at newborn (Figs. 18, 19). In the area with cytoplasmic extensions, the cytoplasmic extensions penetrated through the basal lamina. However, the cytoplasmic extensions on the EFA were shorter than those on the DCE. In addition, collagen fibrils that ran into and between the cytoplasmic extensions were fewer, and cross-sectional images of collagen fibrils running parallel to the basal lamina were observed (Figs. 20, 21).

Scanning electron microscopy

On the DCE, numerous cone-like projections consisting of the fine fibrils whose apices were directed toward enamel were observed on the surface. Adjacent cone-like projections were connected by projection-forming fibrils. On the dentin surface, there were numerous dimples about 2-3 µm in diameter. Without association with the distribution of these dimples, there were small pores that seemed to be openings of dental tubules (Figs. 22, 23).

On the EFA, cone-like projections were sparse, and fibrils were complicatedly arranged parallel to the dentin surface, and formed the network structure. The surface was more porous than on the DCE (Figs. 24, 25).

Discussion

At the cusp tip of the rodent molar, there is a region of the dentin without an enamel cap. This region is generally called the enamel-free area (EFA) (Addison and Appleton, 1921). There have been a few studies on the ultrastructure of the EFA and DCE. However, the collagen arrangement on the surface has been reported to differ between the EFA and DCE (Sakakura *et al.*, 1989; Nakamura *et al.,* 1991; Yamamoto *et al.,* 1993). On the DCE, collagen fibers run perpendicular to the surface. On the EFA, most collagen fibers run parallel to the surface. This arrangement of collagen fibers on the EFA resembles that on the dentin surface of the root (Kramer, 1951; Lester, 1969; Ten Cate, 1978; Tanaka, 1987) and that on the lingual side dentin surface of rodent incisors (Kakei *et al.,* 1977; Jones and Boyde, 1984; Suzuki, 1985; Tanaka, 1987).

Takuma (1967) found that an increase of aperiodic microfibrils perpendicular to basal lamina beneath IEE synchronized with the initiation of collagen formation in the predentin, and conjectured the relationship between aperiodic microfibrils and dentin formation. Goto (1974) observed collagen formation in parallel to aperiodic microfibrils and suggested the association between these microfibrils and the direction of the collagen fiber on the dentin surface. On the other hand, Shimabara (1986) reported collagen arrangement along the long axis of odontoblast process, and suggested the direction of odontoblast processes as the major factor determining the collagen arrangement, and the direction and developmental degree of aperiodic microfibrils as an adjunctive factor. Tanaka (1987) also indicated that the differentiation degree and direction of processes at the distal end of odontoblasts determine collagen arrangement though other factors may be also involved. Our observation showed that aperiodic microfibrils perpendicular to basal lamina were denser and longer on the DCE than on the EFA. In addition, on the DCE, cytoplasmic extensions from the distal end of IEE were well developed and penetrated through the basal lamina. Collagen fibrils ran into and between these extensions and perpendicular to the surface. On the EFA, cross-sectional images of many collagen fibrils running in parallel to the EFA were observed. Poor development of aperiodic microfibrils perpendicular to basal lamina has been also reported in the root dentin and the dentin on the lingual side of rodent incisors that show collagen arrangement similar to that on the EFA (Suzuki, 1985; Tanaka, 1987). These findings suggest a close relationship between collagen arrangement on the dentin surface and the developmental state of aperiodic microfibrils perpendicular to basal lamina as well as the morphology of the distal end of IEE.

Aperiodic microfibrils have been suggested to be involved in the differentiation of the dental papilla cells to odontoblasts (Slavkin *et al.,* 1969; Thesleff, 1978; Thesleff *et al.,* 1978; Hurmerinta and Thesleff, 1981; Thesleff and Hurmerinta, 1981; Suzuki, 1985; Ruch, 1987; Tanaka, 1987; Sawada, 1992). Therefore, the degree of the development of aperiodic microfibrils may affect odontoblasts facing aperiodic microfibrils. Thus, the ultrastructural difference in the basal lamina between the DCE and EFA may cause the differences in the collagen arrangement. Moreover, it may be possible that there are ultrastructural differences between the DCE and EFA except in the collagen arrangement of the surface.

It was also thought that the basal lamina concerned the differentiation of IEE cells to ameloblasts (Slavkin *et al.,* 1969; Nawa *et al.,* 1980; Hurmerinta and Thesleff, 1981; Tanaka, 1987). IEE cells on the EFA do not differentiate into typical ameloblasts as observed in the DCE. However, they secrete slight enamel-like matrix (Johannesen, 1961; Slavkin *et al.,* 1968; Sutcliffe and Owens, 1980; Diab and Zaki, 1985; Nakamura *et al.,* 1986, 1991; Sakakura *et al.,* 1989; Inai *et al.,* 1992) and further differentiate into cells that absorb this matrix (Sutcliffe and Owens, 1980, 1981; Diab and Zaki, 1985, 1991). Nawa *et al.* (1980) reported that differentiation of IEE in cultured tooth germ occurs with an increase in aperiodic microfibrils. In our study, well developed aperiodic microfibrils were also observed on the DCE. These findings suggest the involvement of aperiodic microfibrils in the differentiation of IEE.

On the DCE, many cytoplasmic extensions were observed at the distal end of IEE. And bundles of collagen fibrils with the periodic structure ran into and between these extensions. These bundles of collagen fibrils with the periodic structure corresponded to cone-like projections observed by scanning electron microscopy. Yamamoto (1992) reported microfibril cones composed of collagen fibrils existing on the dentin surface covered with the enamel of human permanent and deciduous anterior teeth and that the distribution and arrangement of microfibril cones contribute to enamel rod formation. The cone-like projections we observed on the DCE with scanning electron microscopy resembled microfibril cones. It seemed likely that the formation of cone-like projections consisting of collagen fibrils with the periodic structure relate to the enamel formation.

It is known that the components of the dental basement membrane are type IV collagen, laminin, fibronectin and heparansulphate proteoglycan (Lesot *et al.,* 1981; Thesleff *et al.,* 1981). The authors intend to clarify the localization and the function of these components of the basement membrane on the EFA in the future.

Materials and Methods

Fifty-two wistar rats from 20 days prenatal to 5 or 20 days postnatal (day of vaginal plug = day 0) were used and mandibular first molars were observed.

Embryos were removed from pregnant rats under ethanol anesthesia, and rats after birth were slaughtered by decapitation under ethanol anesthesia. Immediately, mandibulars were dissected out and fixed in 0.1 M phosphate-buffered 2.5% glutaraldehyde +2% paraformaldehyde (pH 7.2) solution at 4°C for 12 h and rinsed in 0.1 M phosphate buffer (pH 7.2) containing 7% sucrose. Some specimens were decalcified with 10% EDTA (pH 7.2) containing 7% sucrose for 2-3 weeks at 4°C. These specimens were postfixed with 1% osmium tetroxide solution for 1 h at 4°C, dehydrated in graded ethanol, and embedded in Epon 812. After trimming to observe the mandibular first molar, ultrathin sections were prepared using an LKB ultramicrotome and double stained with uranyl acetate and lead citrate (some were stained with phosphotungstic acid). The EFA and DCE were observed by means of transmission electron microscope (Hitachi H-600). According to the reports by Kato (1977) and Taniguchi (1981), semithin sections of the specimens were stained with 1% toluidine blue and observed using a light microscope, and the depressed area in the cusp tip of the tooth germ was considered to be the EFA.

In the rats 20 days after birth, unerupted first molars were dissected out of mandibulars, fixed with 0.1 M phosphate-buffered 2.5% glutaraldehyde +2% paraformaldehyde (pH 7.2), immersed in 5% hypochlorite solution to remove the soft tissue on the EFA, decalcified with 10% EDTA, and dehydrated with graded ethanol. After critical point drying and ion sputter coating with platinum, the EFA and DCE surfaces were observed from above by means of scanning electron microscope (Hitachi S-800).

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