Time-Related Changes of Developing Enamel Crystals after Exposure to the Tissue Fluid in vivo: Analysis of a Subcutaneously Implanted Rat Incisor*

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Summary. To investigate the effects of tissue fluid on the growth of enamel crystals, upper and lower incisors extracted from 3-week-old Wistar rats were removed of the enamel organ, implanted subcutaneously in the dorsal portion of animals from the same litter, and harvested at 72 h or 1 week after implantation. The grafts were chemically fixed with surrounding tissues and prepared for light and electron microscopy, X-ray microanalysis, or for the immunohistochemistry of amelogenin. Mineralization of implanted enamel layers was examined by contact X-ray microangiography.

The immunoreactivities for 25 kD amelogenin in immature enamel decreased sequentially, starting from the surface to the deeper layers; by 1 week after implantation, no positive reactivities remained in the entire enamel layers at the stages of matrix formation and early maturation. In accordance with the loss of enamel proteins, immature enamel gained mineral density until it attained higher radio opacity than that of the adjacent dentin by 1 week. In contrast, the radio opacity of the full thickness of the enamel at early maturation remained low except for a superficial thin layer.

Electron microscopy revealed no sign of growth of original enamel crystals, but showed heavy precipitation of electron-dense fine granules of calcium phosphate in all layers of the secretory enamel and the superficial layer of enamel at early maturation, which showed high radio opacity. The Ca/P ratio and electron diffraction patterns of the granular materials precipitated between intrinsic enamel crystals indicated the property of hydroxy apatite or octacalcium phosphate though a characteristic ribbon-like profile of enamel crystals was lacking.

These data indicate that the enamel organ blocks exogenous mineral precipitates in growing enamel during the stage of matrix formation and plays an essential regulatory role for fine enamel crystallites to grow into large hexagonal crystals.

Enamel development is divided mainly into two stages; matrix formation and maturation. The former is characterized by the elaboration of enamel matrix proteins, and the latter by the removal of both the bulk of matrix proteins and water from, and acquisition of mineral into, the enamel for extensive crystal growth.

The mechanism whereby the mineralization of enamel matrix is maintained in a low level during the stage of matrix formation has been a matter of considerable debate. Amelogenin, which comprises a large proportion of the organic phase of immature enamel, has an inhibitory effect on the growth of enamel crystals in vitro (Doi et al., 1984). Amelogenin is thus suggested to serve to regulate the enamel crystals that keep fine ribbon-like crystals from growing into large ones, while maintaining the space for the enamel crystals to grow in later stages of maturation (Robinson et al., 1989, 1998). Bawden and Wennberg (1977) and Wennberg and Bawden (1978) examined the cellular influence on 45Ca uptake in developing enamel of rat molar tooth germs in vitro and in vivo, and concluded that the enamel organ restricts the influx of calcium ions into enamel during the stage of matrix formation. In these and other 45Ca autoradiographic studies, in which special

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care was taken in preparatory procedures to prevent any translocation of tracers, radiocalcium penetrated the thickness of the maturing enamel but was limited to the superficial layer of the enamel in the secretory stage (Engfeldt et al., 1954; Hammerström, 1967, 1971; Takano et al., 1990; Hanawa et al., 1990). Two explanations were given for the limited penetration of calcium in the superficial layer of the developing enamel: the regulated influx of calcium through the ameloblast layer being only sufficient for new crystal formation at the enamel surface (Bawden, 1989), or the presence of a diffusion barrier for calcium ions in growing enamel (Takano and Crenshaw, 1980). Takano et al. (1992) confirmed that the surface to depth diffusion rate of $^{40}$Ca in an exposed enamel dipped for a short period in $^{40}$Ca solution was same as that of the physiological diffusion rate of $^{40}$Ca in secretory stage enamel of the normal rat incisor in vivo. It is believed that the secretory ameloblast layer regulates calcium acquisition in enamel and the enamel matrix restricts calcium diffusion, thus preventing the excess mineralization of enamel during the stage of matrix formation. Even with all these data, however, the mechanism whereby the characteristic growth of enamel crystals is attained in the maturation stage remains to be explained. Whether the removal of pre-existing enamel proteins and the simultaneous influx of calcium ions fulfill the conditions that cause thin ribbon-like enamel apatite crystals to grow into large mature crystals is a question yet to be answered.

In this study, we therefore, sought to examine the effects of exposure of the growing enamel of rat incisors to the tissue fluid in vivo to gain insights into the roles of the enamel organ in enamel development, particularly for the characteristic growth of enamel apatite crystals.

**MATERIALS AND METHODS**

**Implantation of incisors**

Upper and lower incisors were extracted from 3-week-old Wistar rats (n=10) under anesthesia by an intraperitoneal injection of chloral hydrate (400 mg/kg). The enamel epithelium of the extracted incisors was carefully removed by wet aseptic gauze under a dissecting microscope to expose the whole enamel surface, including both growing and maturing enamel areas. A set of isolated incisors, two lower incisors, and two upper incisors were grafted subcutaneously into the dorsal portion of a recipient animal of the same litter under anesthesia. Sets of upper and lower incisors similarly isolated were immediately fixed by perfusion and processed for various histological examinations similarly as for experimental specimens as described in the following, and served as controls (n=3).

**Contact X-ray microradiography (CMR) of rat incisors**

The recipient animals were sacrificed at 72 h or 1 week after implantation and fixed by perfusion with a 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The grafts were removed with the adherent connective tissue and further immersed in the same fixative solution overnight at 4°C. They were then dehydrated through a graded series of ethanol, substituted with acetone, and embedded in polyester resin (Rigolac 2002) (Nissin EM Co., Tokyo) for ground sectioning. The resin blocks of rat incisors were sliced by a belt-type cutting device (EXAKT BS-300C, Norderstedt, Germany) into 400 µm thick sections along the longitudinal axis of the incisor, followed by hand grinding, and final polishing with lapping films (Maruto Instrument, Tokyo). Approximately 100 µm thick longitudinal ground sections were prepared and subjected for radiographic examination by a cabinet type X-ray apparatus (Sofron SRO-50, Sofron Ltd., Tokyo) at 15 kVp, 4 mA, 20 min.

**Light and electron microscopic observation**

Recipient animals were sacrificed by perfusion similarly as for the CMR experiment except that a mixture of 3% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) was used as the fixative. After fixation the grafts were removed with surrounding tissues, and post-fixed with 1% OsO₄ in 0.1 M phosphate buffer (pH 7.4) containing 1.5% potassium ferrocyanide. They were routinely embedded in Epon 812 resin (Taab, Aldermaston, England). Some of the specimens were decalcified with 4.13% ethylenediaminetetraacetic acid (EDTA) for 3-4 weeks at 4°C, followed by similar processing as for undecalcified specimens. Semithin and ultrathin sections parallel to the longitudinal axis of the incisor were prepared and subjected to light and electron microscopy, respectively. Ultrathin sections were stained doubly with uranyl acetate and lead citrate and examined under a H-7100 transmission electron microscope (Hitachi, Tokyo) at an acceleration voltage of 75 kV.

**X-ray microanalysis**

To detect changes in the elemental composition of the enamel of implanted rat incisors, X-ray microanalysis was carried out with an energy-dispersive spectrometer combined with an analyzer/computer system (EMAX-3770, Horiba, Kyoto), in a TEM /
STEM electron microscope (H-7100, H7110, Hitachi, Tokyo). Ultrathin sections of undecalcified specimens were carbon-coated with a Quick Carbon Coater (SC-701c) (Sanyu Denshi, Tokyo), viewed in TEM or STEM mode of microscopy for orientation, and analyzed for 100 sec (live time) with a stationary spot using a 75 kV accelerating voltage at \( \times 100,000 \) magnification. From the collected data, the Ca/P molar ratio of the mineral phase of enamel was calculated at various developmental stages and at different depths from the enamel surface. Statistical differences among the Ca/P values in different areas of analysis were made according to Mann Whitney’s U-test.

**Electron diffraction**

To characterize the mineral phase of the enamel of implanted incisors, electron micrographs of selected areas and their corresponding diffraction patterns were taken with a H-7100 transmission electron microscope (Hitachi, Tokyo) at an acceleration voltage of 75 kV and \( \times 20,000 \) magnification. In the negative diffraction messages, the diameters of bright rings or arcs (r) (if any) were measured and the values of interplanar spacing (d) calculated according to the formula of \( dr = \frac{L}{\lambda} \) (L [camera length] = 0.78 m; \( \lambda \) [wavelength of electron beam] = 0.0432 \( \text{Å} \)). The calculated values of interplanar spacing were compared with the diffraction data (1996 JCPDS-International Center for Diffraction Data) to identify the type of crystal.

**Immunohistochemistry of amelogenin**

For immunohistochemistry, the harvested incisors were fixed with 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) followed by decalcification with 4.13% EDTA at 4°C. They were dehydrated through a graded series of dimethylformamide (DMF), and
embedded in glycer methacrylate (GMA) at 4°C under ultraviolet light.

Five hundred nm-thick GMA sections were placed on APS-coated glass slides (Matsunami, Osaka, Japan) and first blocked by 2% normal goat saline in 0.01 M phosphate buffered saline. Anti-porcine amelogenin polyclonal antibody, which is known to recognize porcine 25 kD amelogenin (kindly provided by Dr. Takashi UCHIDA of Hiroshima University), was applied at 4,000 times dilution and incubated for 60 min at room temperature. Goat-anti rabbit IgG conjugated with 5 nm colloidal gold (EY Laboratories, San Mateo, USA) was used as the secondary antibody (dilution 1:400) and incubated for 60 min at room temperature. The immunoreactions were intensified by the silver enhancement method (UCHIDA et al., 1991a, b). The sections were counter stained with methylene blue before observation.

RESULTS

Histological changes of the implanted rat incisor enamel

Contact X-ray microradiography

In the longitudinal ground section of the maxillary incisor of control animals, the radio opacity of the enamel was much lower than that of the underlying dentin throughout the stage of matrix formation and early maturation, except for the deepest thin layer of enamel along the enamel-dentin junction (Fig. 1).

By 72 h after implantation, immature enamel underwent a steep increase in radio opacity and
Fig. 3  

**a.** Microradiograph of the longitudinal ground section of the upper incisor 1 week after implantation. b, c, d, respectively correspond to the areas depicted for enlargement. T transitional stage, D dentin, E enamel, arrow incisal direction. ×23.  

**b.** Early matrix formation. Full thickness of enamel (E) shows much higher radio opacity than the adjacent dentin (D).  

**c.** Late matrix formation. The outer one-third to one-half of the enamel (double-headed arrow) displays high radio opacity comparable to the thinner enamel in b.  

**d.** Early maturation. The full thickness of enamel (E), except for the superficial layer (arrows), remains low in radio opacity. b–d: ×230

Exceeded that of the adjacent dentin near the apical end of the incisor (Fig. 2a, b). A similar increase in radio opacity also occurred in the thick enamel of the late stage of matrix formation, although the high radio opacity was limited to the surface 1/2 or 1/3 of the thickness of the enamel (Fig. 2c). The radio opacity of the enamel at the post transitional, early maturation stage remained low and was comparable to that of control specimens (Fig. 2d). At 1 week after implantation, the pattern of radio opacity of enamel remained identical to that of the 72 h group although there were significant overall increases in mineral density in enamel (Fig. 3). Even after 1 week of exposure to the tissue fluid, the radio opacity of post transitional, early maturation enamel remained low throughout the thickness except for the superficial thin layer that showed some increase in radio opacity (Fig. 3d).

**Electron microscopic observation**

In the control specimens, enamel crystallites in the surface layers of immature enamel displayed thin ribbon-like profiles approximately 4.67 ± 0.23 nm (mean ± SD) thick, being separated by an electron-lucent organic enamel matrix (Fig. 4a, c). After 72 h of implantation and exposure to the tissue fluid, the relatively wide spaces between thin ribbon-like crystallites were filled with electron-dense fine granular deposits. The density of fine granular deposits became more distinct by 1 week after implantation, and obscured the contours of pre-existing crystallites (Fig. 4a, d). In the middle and deep layers, similar fine granular, electron-dense deposits occurred but with less quantity. In the area of post transitional, early maturation, where the radio opacity of the enamel matrix remained low during the experiment periods,
granular deposits of high electron density only occurred along the superficial thin layer that showed relatively high electron density as indicated in Figure 3d.

In the control specimens, the thickness of the enamel crystals at early stages of enamel development increased toward the deeper layers as shown in Table 1. There was no significant increase in the thickness of enamel crystals in implanted specimens when compared with that of enamel crystals in the identical layer of the enamel of control specimens (Table 1). Precise measurements of the width and length of the individual crystals could not be made in this study.

In decalcified specimens of implanted incisors (1 week), transmission electron microscopy revealed crystal ghost images of intrinsic ribbon-like crystals...
in the highly mineralized surface layer of immature enamel. Relatively wide inter-crystalline spaces of surface enamel, identical to the areas of fine granular mineral deposition, were loosely filled with amorphous material (compare Fig. 4b and d).

**Ca/P molar ratio of implanted rat incisor enamel**

The Ca/P molar ratio of the mineral phase of the control enamel ranged between 0.406±0.183 (mean±SD) of the surface layer to 0.809±0.319 of the deep layer at early stages of matrix formation (Table 2, Control). During implantation, the Ca/P molar ratio in each depth of the enamel layers examined increased significantly, and the values varied from 1.046±0.687 to 1.330±0.193 (Table 2, 72h, 1w). No differences were depicted between the values of implanted, immature enamel or mature enamel that showed high radio opacity before implantation. There were significant differences in the Ca/P molar ratios between control and implanted incisors in any given depth of the enamel.

**Electron diffraction patterns of implanted rat incisor enamel**

Under the conditions used here for electron diffraction, no recognizable diffraction pattern was detected from the outermost surface layer of the control enamel at early stages of matrix formation. On the other hand, in implanted specimens, two distinct diffraction rings (d=3.50, d=2.83) could be detected from the fine granular, electron-dense deposits at comparable regions of immature enamel with those of the controls (Fig. 5). Interplanar spacing values of more distinct diffraction arcs obtained from the mature enamel of the control incisor (d=3.50, 2.83, 1.82) were practically identical to those gained from the fine granular deposits (Fig. 6).

**Immunohistochemistry of amelogenin (Fig. 7)**

In the decalcified specimens of control incisors, the remaining enamel matrix showed distinct immunoreactions for amelogenin throughout the thickness of the enamel layers until the insoluble enamel matrix finally disappeared from the mature enamel zone. The highest immunoreactivity was localized in the thin layers of enamel near the apical end of the incisor. In the thicker enamel, the surface layer showed stronger reactions than the deeper enamel.

In the implanted incisors, the immunoreactions for 25 kD amelogenin showed a gradual decrease in intensity from the surface downward and, by 72h after implantation, disappeared from the entire thickness of the thin enamel layers near the apical end as well as the enamel of early stage of maturation. Immunoreactivity remained in the thick enamel layers of the

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**Table 1.** Thickness of initial enamel crystals of rat incisors at the stage of early matrix formation.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Control (nm)</th>
<th>72h (nm)</th>
<th>1w (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface layer</td>
<td>4.67±0.23</td>
<td>5.28±0.76</td>
<td>5.01±1.12</td>
</tr>
<tr>
<td>Middle layer</td>
<td>6.83±0.74</td>
<td>6.55±0.89</td>
<td>5.64±0.69</td>
</tr>
<tr>
<td>Deep layer</td>
<td>12.19±0.986</td>
<td>13.76±2.53</td>
<td>14.76±2.79</td>
</tr>
</tbody>
</table>

**Table 2.** Average Ca/P of immature and mature enamel of rat incisor before and after implantation.

<table>
<thead>
<tr>
<th>Ca/P molar ratios of immature enamel</th>
<th>Ca/P molar ratios of mature enamel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>72h</td>
</tr>
<tr>
<td>Surface layer</td>
<td>0.406±0.183</td>
</tr>
<tr>
<td>Middle layer</td>
<td>0.529±0.235</td>
</tr>
<tr>
<td>Deep layer</td>
<td>0.809±0.319</td>
</tr>
</tbody>
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late stage of matrix formation, where a distinct decreasing gradient from the deepest layer near the enamel-dentin junction toward the enamel surface was shown. By 1 week after implantation, immunoreaction for 25 kD amelogenin was undetectable in the entire enamel.

**DISCUSSION**

It has been confirmed in the present study that subcutaneously implanted isolated rat incisors, having been removed of the enamel organ, do not show significant increases in the thickness of enamel.
crystallites after 1 week of exposure to the tissue fluid, whereas the immature enamel layers attain a drastic increase in mineral density. The high mineral density of the implanted immature enamel is attributed to the deposition of granular calcium phosphate between the intrinsic enamel crystallites.

In the maturation process of amelogenesis, a drastic increase in the mineral density of enamel is preceded by removal of the organic enamel matrix via the ameloblast layer, thereby leaving fluid-filled spaces for the individual crystallites to grow into large crystals (ROBINSON et al., 1998). In our subcutaneous implantation model of rat incisors, the amelogenin has been shown to disappear from both growing and premature enamel within 1 week of implantation. From the data of our experiment, it is obvious that the exposure of the enamel surface to the tissue fluid and simultaneous loss of the organic enamel matrix by passive diffusion do not promote the growth of pre-existing enamel crystallites, comparable to the events taking place in vivo controlled by the enamel organ. Instead, the exposure of the growing enamel surface to the tissue fluid in vivo allows massive precipitation of finely granular mineral deposits. It needs to be noted, however, that under the same experimental conditions, such min-
eral deposition did not occur in the post transitional, pre-maturation enamel, where active matrix formation by the ameloblasts had been arrested before implantation.

**Influence of amelogenin and serum proteins on the growth of pre-existing enamel crystals**

It has been suggested that, at the growth surface of immature enamel, nascent amelogenin and partially degraded amelogenin adsorb to the immature enamel crystals and inhibit their growth (Aoba et al., 1987; Robinson et al., 1996). In vitro data suggest that amelogenin also has facilitating effects on the growth of apatite crystals in an immobilized steady-state combined with agarose gel (Yutaka Doi, personal communication). The property of amelogenin appears comparable to that of dentin phosphophoryn, which is inhibitory on crystal formation in a soluble state but works as a crystal inducer if immobilized on agarose gel (Linde et al., 1989; Linde and Luissi, 1989; Saito et al., 1997). A more recent report has indicated the absence of such effects in recombinant human amelogenin on apatite growth by steady-state agarose gel assay (Hunter et al., 1999). Despite inconsistencies in previous data, the putative facilitating activity of immobilized amelogenin on crystal growth is intriguing and may explain the drastic mineral deposition in immature enamel of implanted incisors and lack of such precipitates in the enamel of the post transitional, early maturation stage.

Albumin has a high affinity for apatite crystals and, once adsorbed, inhibits the growth of hydroxyapatite (Garnett and Dieppe, 1990) and enamel crystals (Robinson et al., 1992, 1994, 1996). It is noteworthy that significant amounts of albumin are contained in the enamel matrix of secretory and transitional stages (Robinson et al., 1998). Under physiological conditions, however, albumin is degraded by metalloproteases or serine proteases secreted by the ameloblasts and removed from the crystal surface together with amelogenin, leading to crystal growth. The absence of growth in the immature enamel crystals in implanted incisors may be attributed to the adsorption of massive amounts of serum-derived albumin on the crystal surface due to exposure of the enamel surface to the tissue fluid. On the other hand, the amelogenin enriched in the immature enamel of implanted teeth may have been immobilized by forming a complex with various organic constituents of the tissue fluid immediately after implantation, and acquired a mineral-inducing property. This hypothesis explains the fine granular mineral deposition in immature enamel layers and lack of such mineral deposition in post transitional, pre-maturation enamel. The low mineral density of deeper enamel layers relative to the surface enamel in the late secretory stage of implanted incisors (Fig. 3c) may result from the limited diffusion of ions from surface layers where a highly mineralized layer acts as a diffusion barrier.

Amelogenin is also implicated in the growth of long and thin ribbon-like crystals in vitro (Diekwisch et al., 1993; Wen et al., 1999). The fine granular appearance of dense mineral deposits in the immature enamel of implanted teeth may be related to a lack of supply of nascent amelogenin from the secretory ameloblasts in our implant model.

**Characterization of fine granular mineral deposits**

The fine granular mineral deposits in the immature enamel of exposed and implanted incisors have been shown to be of a crystalline nature, presumably of hydroxyapatite or octacalcium phosphate, according to the diffraction patterns (Figs. 5, 6). The Ca/P ratio (1.302±0.113) of fine granular deposits is much lower than the theoretical Ca/P value of hydroxyapatite (1.67) and close to that of octacalcium phosphate (1.33). Since octacalcium phosphate is characterized by large plate-like or ribbon-like profiles (Brown et al., 1984), the mineral deposits are most likely fine crystallites of hydroxyapatite despite their low Ca/P value. Enamel apatite crystal is known for the prevalence of crystallographic defects and hence variation in Ca/P ratio.

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**REFERENCES**


