Scanning Electron Microscopy of Final Enamel Formation in Rat Mandibular Incisors following Single Injections of 1-Hydroxyethylidene-1,1-Bisphosphonate

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Received September 17, 1992, and in revised form October 15, 1992

Summary. A single, high dose of 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) results in three different types of lesions along the enamel surface of the rat incisor, one of which is seen as a "bright band" crossing the final enamel surface in the scanning electron microscope (SEM). The present study presents the structural surface features of final enamel formation and its subsequent maturation in normal and HEBP-exposed rats. The position of the bright band is examined in relation to where the Tomes processes pits disappear (DTPP), where the boundary between "light" and "dark" enamel (LDB) as seen by SEM is located, and in particular, where the so-called opaque boundary (OB) is positioned. Groups of rats were given a subcutaneous dose of 0, 5, or 10 mg P/kg body wt of HEBP and killed at intervals of either 12 hours or 2 or 9 days. The mandibular incisors were processed for SEM after enzymatic digestion of enamel organ remains. Enamel surface nodules, 100-300 nm in diameter and composed of smaller units, were evident at the start of final enamel formation which was defined as the area from DTPP to LDB. With increasing maturation, the nodules merged to form a smooth surface. In HEBP-treated animals, growth and merging of these surface nodules became arrested at the time of injection resulting in an irreversible "porous" stage corresponding to that part of the surface enamel. This area—the bright band—developed incisally during the eruption of the incisor. Adjacent surface areas appeared normal, with densely packed nodules in the maturation stage. The bright band did not coincide with either LDB or OB at the time of injection as these landmarks were located about 105 and 200 μm incisally to DTPP, respectively. The width of the bright band was dose dependent (78 μm versus 105 μm at 5 and 10 mg P/kg body wt) which may indicate that HEBP carries on its effect in a few hours after administration. The findings are most likely a result of HEBP's physicochemical effect directly on crystal growth, although a cellular effect cannot be excluded.

Key words: Amelogenesis – Enamel structure – Enamel pathology – Rat incisor – Bisphosphonate – HEBP – Scanning electron microscopy.

It is well known that 1-hydroxyethylidene-1,1-bisphosphonate (HEBP) inhibits normal mineralization of skeletal and dental tissues. The mechanism of action of HEBP is assumed primarily to be of physicochemical nature, but a direct effect on various cellular processes related to matrix production in mineralizing mesenchymal tissues has also been reported (for reviews, see Fleisch [1, 2]).

A single high dose of HEBP injected subcutaneously causes a hypomineralized incremental layer in the forming enamel [3-7]. In addition to this layer we have demonstrated three different types of lesions along the enamel surface of the rat incisor [8]. One type of lesion appeared as a "bright band" across the enamel surface, as observed in the scanning electron microscope, and was suggested to be related to the stage of final enamel formation at time of injection corresponding to where the hypomineralized incremental layer within the enamel reached the outer enamel surface [8]. It was further suggested that the bright band represented an area of increased enamel porosity of the surface, indicating that final enamel formation and/or maturation might be arrested following injection of HEBP. The position of development of the bright band in relation to established landmarks on the enamel surface, such as the opaque boundary [9, 10], was not revealed. Based on a series of studies [11-13], Robinson and Kirkham [14] proposed that the opaque boundary, which separates a cracked (stage 2) and a white opaque region (stage 3) of the dried out enamel surface [9, 10], corresponds to where the ameloblasts have lost their distal processes and decreased in height.

We hypothesize that the bright band caused by HEBP exposure may reflect the position of the opaque boundary at the time of injection. To test this hypothesis, part of the present study was undertaken to define the position of the opaque boundary in relation to certain landmarks in the enamel surface, and at the same time, to describe the structural surface features of final and maturing enamel in normal and HEBP-exposed rats using scanning electron microscopy (SEM).

Materials and Methods

Forty-two male Wistar rats, weighing about 200 g, were housed in light- and temperature-controlled rooms and had free access to water and a commercial hard laboratory diet containing 25-30 ppm fluoride. The rats received a single subcutaneous injection of either 0.9% NaCl (six control animals) or HEBP in a dose of 5 or 10 mg
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Fig. 1. Schematic projection of the forming surface of rat mandibular incisor enamel at the location of final enamel formation illustrating the functional stages of amelogenesis and characteristic boundary lines at 12 hours after injecting (a) normal saline (control) or (b) HEBP (5 mg P/kg body wt). End of area of outer enamel formation (OE); area of final enamel formation (FE); start of area of enamel maturation (ME); boundary line of disappearance of Tomes’ processes pits (DTPP); boundary line between “light” and “dark” enamel (LDB); bright band (BB); incisal boundary line of bright band (BBinc). Rectangles with figure numbers indicate the approximate position of the figures in question.

P/kg body wt (18 experimental animals per group). The HEBP (disodium salt) was dissolved in 0.9% NaCl, and the injected volume of each solution was 0.05 ml/10 g body wt. Groups of six experimental and two control rats were killed in a nitrous oxide atmosphere at 12 hours, 2 days, and 9 days following injection.

Immediately following death, the mandibular incisors with adhering enamel organs were carefully dissected from the surrounding alveolar bone [15] and kept moist with 0.9% NaCl. The membranous portion of the periodontal ligaments [16] adhering to the enamel organs were pulled off with a pair of fine tweezers. For removal of enamel organ remains, the incisors were treated with 0.08% pronase (Merck, Darmstadt) in 0.9% NaCl supplemented with 0.25% CaCl₂, 2H₂O (pH 7.3) at 40°C for 30-40 minutes. After enzymatic treatment, the incisors were flushed in 0.9% NaCl and fixed overnight at room temperature in a mixed solution of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The teeth were then washed in the same buffer, dehydrated in graded ethanol, and critical-point dried in a Polaron High Pressure Bomb E3000 from carbon dioxide. The specimens were mounted on aluminum stubs using a cyan-acrylate-based glue and oxygen plasma ashed for 15 minutes in a PlasmaPrep 100 plasma chemistry unit (Nanotech Ltd., Manchester) to remove the remaining organic residues. The incisors were made conductive with silver paint and coated by evaporation with carbon on a planetary rotating stage (Edward E306A Coating System) followed by sputter coating with a 20 nm layer of gold-palladium or platinum (Polaron E5150 Film Thickness Controlled Sputter Coater). The specimens were examined with a Philips SEM 505 scanning electron microscope operated at 15 or 30 kV.

From another four rats, the mandibular incisors were removed from the jaws, treated as described by Robinson et al. [9, 10], and the position of the opaque boundary marked with a scalpel. The teeth were photographed in reflected light and finally prepared for SEM as described above.

The enamel surface of the incisor was considered fiat within apical-incisal distances of 1-2 mm. Approximate distances within this range were measured corresponding to the midsagittal plane of the tooth from surfaces oriented perpendicular to the electron beam axis at a constant working distance.

Results

Surface Features of Final Enamel in Normal Rats

At low magnification, the enamel surface of the rat incisor showed an apical “light” and an incisal “dark” part representing areas of high and low electron scattering, respectively (Fig. 2a). The boundary between the two parts (LD-boundary) was concave in incisal direction. The surface of the light part exhibited the pattern of Tomes’ processes pits characteristic of inner and outer enamel formation. Towards the LD-boundary, Tomes’ processes pits gradually obliterated and the enamel surface became rather smooth. The average distance from the location of disappearance of the pits to the LD-boundary was 105 ± 4.5 μm (n = 7) (Fig. 1a). This part of the enamel surface comprised the area of final enamel formation as defined in the SEM.