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The effect of hypoxia on the formation of mouse incisor enamel

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ABSTRACT

Objective: The permanently growing mouse incisors exhibit all stages of tooth development along their inciso-apical axis at any time. Any disturbance or injury of the ameloblasts during enamel formation or maturation may result in permanent defects in the finished enamel since the enamel does not undergo repair or remodeling after formation. In order to increase our understanding of how hypoxia affects enamel formation, we induced severe acute hypoxia in adult mice and observed its effects on the enamel in incisors.

Design: Incisors from hypoxic mice were obtained 5 and 49 days after the hypoxic insult. Hypoxic and control incisors were dissected out and observed by scanning electron microscopy (SEM). Incisors were subsequently ground longitudinally or transversely, etched, and observed again by SEM. The nature and position of defects were considered in relation to the configuration and dynamics of the incisors.

Results: The effect of hypoxia varied considerably, among mice, among incisors, and among ameloblasts. Affected enamel showed hypoplasia with hypomineralization or hypomineralization without hypoplasia. Vascular endothelial growth factor (VEGF) showed considerably stronger labeling in hypoxic compared to control ameloblasts.

Conclusions: The present study demonstrates quantitative and qualitative defects in the enamel reflecting the vulnerability of ameloblasts toward severe acute hypoxia in mouse incisors.

1. Introduction

The etiology of developmental dental defects is multifactorial (Brook, 2009; Seow, 2014). Although amelogenesis is strictly genetically controlled, it is sensitive to environmental disturbances, resulting in different types of defects (Simmer & Hu, 2001). Any disturbance or injury of the ameloblasts during enamel formation or maturation may result in permanent defects in the finished enamel since the enamel does not undergo repair or remodeling after its formation. The type and degree of damage depend on the nature of the disturbance and on the stage of enamel development. When the ameloblasts are exposed to disturbances in the secretory stage of amelogenesis, it may cause restriction of enamel apposition and result in hypoplastic enamel (Simmer & Hu, 2001). Disturbances during the transitional and maturation stage, however, result in hypomineralized enamel of normal thickness (Simmer & Hu, 2001).

The rodent incisor undergoes attrition at its incisal end, which is compensated by continuous growth at its apical end (Harada et al., 1999; Smith & Warshawsky, 1975b). The permanently growing rodent incisor exhibits all stages of tooth development along its inciso-apical axis at any time. This characteristic makes them convenient models for the study of enamel formation, which occurs in several distinct stages along the tooth axis in incisal direction (Leblond & Warshawsky, 1979; Pindborg & Weinmann, 1959; Risnes, 1979a; Smith & Warshawsky, 1975a, 1976, 1977; Warshawsky & Smith, 1974). For this purpose, the rat incisor has for many years been extensively used, and its normal enamel structure is well established (Risnes, 1979b; Warshawsky, 1971). Fundamental similarities exist between the development of rat incisor enamel and human enamel (Warshawsky, Josephsen, Thylstrup, & Fejerskov, 1981). The distribution and structure of mouse incisor enamel resemble that of the rat (Moinichen, Lyngstadaas, & Risnes, 1996). The enamel, which covers only the labial aspect of the tooth, can be divided into four layers: a thin inner prism-free layer, inner enamel with prism decussation, i.e. transverse rows of prisms with prisms inclined medially and laterally in alternate rows, outer enamel with parallel prisms inclined incisally and a thin superficial

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prism-free layer (Moinichen et al., 1996; Warshawsky, 1971). This is also the order of formation of the layers, since the enamel formation starts at the dentin surface and proceeds in external direction.

Amelogenesis is a complex process, which appears to be sensitive to oxygen availability. Rat puppets obtained 22 days after breeding from mothers that had been exposed to a short episode of hypoxia on the 20th day of gestation, showed disturbances of secretory ameloblasts (Via, Elwood, & Bebin, 1959). Hamster molars that had been grown in vitro under low oxygen tension showed disturbances of enamel matrix production and mineralization (Bronckers, 1983). Incisors of rats that had been kept in an environment simulating an altitude of 5490 m (380 mm Hg) from an age of 7 weeks to an age of 13 weeks exhibited hypomineralized and hypoplastic incisor enamel (Angmar-Mansson & Whitford, 1990). There is also a possibility that enamel defects observed in people living at high altitudes in Thailand (Leatherwood, Burnett, Chandravejjsmarn, & Sirikaya, 1965) and Kenya (Manji, Baelum, & Fejerskov, 1986) were mistakenly classified as fluorosis (Angmar-Mansson & Whitford, 1990). Hypoxia has been suggested as a possible etiological factor, among others, for the occurrence of demarcated enamel opacities affecting one or more permanent first molars and frequently also incisors (Alaluusua, 2010; Beentjes, Weerheijm, & Groen, 2002; Jalevik, Noren, Klingberg, & Barregard, 2001; Johnsen, Krejci, Hack, & Fanaroff, 1984; van Amerongen & Kreulen, 1995), a condition also referred to as molar-incisor hypomineralization (MIH) (Weerheijm et al., 2003; Weerheijm, Jalevik, & Alaluusua, 2001).

In order to increase our understanding of how hypoxia affects enamel formation, we induced hypoxia in mice and observed its effects on the enamel in incisors. Since systemic hypoxia has been found to upregulate the expression of vascular endothelial growth factor (VEGF) in cells of non-constitutive VEGF expression (Marti & Risau, 1998), we used this factor as a marker for induced hypoxia.

2. Materials and methods

2.1. Animal model

Thirty phenotypical, adult mice (CD-1 strain, 8 weeks old, 30 ±5 g body wt) were randomly selected for the study, 16 males and 14 females. Prior to experimental use, animals were given standard laboratory fodder and water ad libitum, and they were maintained on a 12 h light: dark cycle, at 21 °C with a relative humidity of 65%. The animals were kept according to the regulations of the Norwegian Gene Technology Act of 1994, and the Local Veterinary Service approved all experiments.

For hypoxia induction, animals were transferred into an airtight, transparent Plexiglas chamber of about 3000 mL volume. In a pilot study the hypoxic condition was maintained for 4 h, as suggested by Prass and colleagues (Prass et al., 2003). However, this regime appeared too severe, none of the animals survived longer than 2.5 h. Therefore, we reduced the duration of the hypoxic treatment to 2 h. A total gas flow of 400 mL/min of 92% N_2 and 8% O_2 (AGA) was established with the use of calibrated flow meter (AGA), and partial oxygen pressure was monitored using a single gas detector (O_2-meter) (BW Technologies) that was placed in the chamber during the procedure. For the control groups, room air was used at the same flow rate for 2 h. A maximum of eight mice were used per chamber procedure.

After the experimental procedure, all animals, both hypoxic and control, were transferred out of the chamber and kept under normal conditions as described above. However, immediately after the procedure one mouse from each hypoxia session was killed by cervical dislocation and their heads were processed for immunohistochemistry. After 5 days under normal conditions, the animals were sacrificed and their heads were fixed in 70% ethanol. Three hypoxic mice and three control mice were kept alive for 49 days before they were sacrificed and their heads fixed in 70% ethanol.

2.2. Immunohistochemistry

The heads of one animal from each hypoxia session were immediately immersed in 10% (v/v) buffered formalin (AppliChem, Darmstadt, Germany) and fixed for seven days. Heads were decalcified by immersion in EDTA (12.5% w/v, pH 7.4) for 5 days at room temperature. The specimens were dehydrated in a graded series of ethanol (70, 96 and 100%) and embedded in paraffin. Sections of 6 μm thickness were obtained with a motorized rotary microtome (MICROM International GmbH, Walldorf, Germany) and dried for one hour at 60 °C prior to immunohistochemical labelling.

The sections were incubated for 60 min in the presence of primary antibody to vascular endothelial growth factor (VEGF) (Abcam, Rabbit polyclonal to VEGF (ab46154)) using 1:150 dilutions. The sections used for comparison of VEGF expression in control and hypoxic mice were treated on the same slide. Control sections were incubated without primary antibody, and showed no sign of positive immunolabeling. Periodontal ligament (PDL) was used as positive control. The immunohistochemistry methods were otherwise as described previously (Khan, Sehic, Khuu, Risnes, & Osmundsen, 2013).

2.3. Scanning electron microscopy (SEM)

Maxillary and mandibular incisors were dissected out and fixed in 70% ethanol. The isolated incisors were thoroughly cleaned by dissection and by gentle brushing under running tap water. The specimens were air-dried overnight and mounted on brass cylinders with cyanoacrylate glue, sputter-coated with 30 nm gold-palladium and observed in a Philips XL30 ESEM (Philips, FEI, Netherlands) operated at 10 kV.

The incisors were later embedded in Epon. Some incisors were ground transversely, while the contralateral teeth were ground longitudinally. Grinding was performed under a stereo-microscope using grits 800 and 1200 3 M waterproof silicone carbide paper (3 M, St. Paul, MN, USA) in a specially designed apparatus (Risnes, 1985). The ground surface was then polished by grinding the specimens against the backside of the 3 M waterproof silicone carbide paper with 0.05 μm particle size alumina powder (Buehler Micropolish, Buehler, Lake Bluff, IL, USA) in water. After careful brushing under running tap water and removal of excess water, teeth were etched for 45 s in 0.1% nitric acid, air-dried overnight, sputter-coated with 30 nm gold-palladium and observed with SEM.

For the transversely ground incisors obtained after 5 days the whole procedure (grinding, polishing, etching, air-drying, sputter-coating and observing in SEM) was repeated three times, creating three transversely ground planes for observation. The first plane (T1) was about 1 mm from the incisal tip in maxillary incisors and about 2.5 mm in mandibular incisors because of the longer attrition facet in the latter. Subsequent planes (T2 and T3) were ground about 5 and 5.5 mm apical to the T1 plane in maxillary incisors and about 6 and 6.5 mm in mandibular incisors. The T2 and T3 planes were positioned in an area where disturbances in the enamel were noted when the whole teeth were observed in the SEM. In incisors obtained after 49 days a transverse plane was ground through a region of disturbed enamel in the erupted part of the incisors. Longitudinal grinding was performed to the central part of the tooth. However, achieving ideal longitudinal ground planes of mouse incisors is technically difficult due to their small size and, in mandibular incisors, due to a slight medio-lateral curvature of the teeth.