Type X collagen in human enamel development: a possible role in mineralization

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Although type X collagen is one of the key molecules in endochondral ossification, no data are available on whether it is present in dental structures when mineralization is proceeding. We therefore monitored the appearance of type X collagen in tooth germs of human samples ranging in gestational age from 17-week-old fetuses to 9-week-old newborn. Using immunohistochemistry, ELISA techniques, and Western blotting, we show that type X collagen is present in human tooth germ during enamel maturation. Intense immunohistochemical staining for collagen type X was observed in the enamel and in the apical parts of secretory ameloblast at the bell stage when the dentine and enamel matrix were already under formation. The odontoblasts, the dentine, and the pulp were not stained. In the early (9-week) postnatal stage, the staining for collagen type X in the enamel matrix was diminished, and only a very weak signal could be detected in the secretory ameloblasts. A positive reaction for collagen type X was also observed in ELISA assay of extracts obtained from human embryonic enamel and hypertrophic cartilage samples. The Western blot analysis of the enamel demonstrated that size of the molecule detected by MoAb X53 is characteristic of the type X collagen. This correlates well with our immunohistochemical findings. Based on these data, we propose that type X collagen is one of the candidate molecules present in the enamel matrix that might be involved in mineralization of the enamel. □ *Collagen type X; human tooth development; enamel mineralization*

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Human tooth development is a highly regulated event that requires coordination of a number of interactions to progress normally (1). Despite our increased knowledge of the regulation involved in this developmental process, the precise mechanisms of certain events, such as mineralization of the tooth, are still not fully understood. The formation and mineralization of the enamel, the most highly mineralized structure in the vertebrate body, takes place during amelogenesis (2). The enamel consists of inorganic apatite crystallites embedded in an organic matrix (3), and it is widely accepted that the inorganic material constitutes 96% of the weight of mature enamel (4). A major part of the inorganic matrix is present as large, elongated hydroxyapatite crystals which are similar, but not identical, with those of other hard tissues (5).

Unlike the other mineralization processes of the body, the initial steps of enamel formation, i.e. crystal nucleation and growth, do not require any remodeling of the organic matrix. The newly secreted enamel matrix begins to mineralize immediately after elaboration, thereby forming no distinct layer of non-mineralized enamel matrix (6). The enamel proteins (such as amelogenins, enamelins, tuftelin and ameloblastin/amelin/sheathlin) are thought to be involved in many events of enamel mineralization, such as mineral ion binding and modulation of hydroxyapatite crystal growth (7–9). In various other hard tissues of the body, a number of other calcium-binding extracellular proteins and matrix vesicles have been suggested to be involved in mineralization processes (10). However, these macromolecules have not been shown in dental enamel. Type X collagen is one of those molecules (11).

Type X collagen is a product of terminally differentiated chondrocytes in regions of cartilage that undergo endochondral ossification (12–14). Its expression serves as a good marker for the hypertrophic stage of chondrocyte differentiation (15, 16). Collagen type X has been reported to be present also at bone-ligament interfaces (17), and in intervertebral endplate (18, 19). It is a non-fibrillar collagen consisting of two non-collagenous globular domains and a triple helical portion which is approximately half the length of fibril-forming collagens around 60 kDa (20). Through its carboxyl terminus, the molecule can be incorporated into supramolecular structures (21–23). The highly restricted expression of type X collagen in the extracellular matrix (ECM) of cartilage growth plate suggests that it may be involved in the process of mineralization during endochondral ossification of the epiphyses (24–26).

In mice lacking collagen type X, structural abnormalities in growth plate cartilage and trabecular bone architecture have been described with atypical distribution of matrix components within the growth plate, suggesting that collagen type X plays a role in the normal distribution of matrix vesicles and proteoglycans in ECM (27). The role

of collagen type X in mineralization was experimentally studied testing calvaria-derived osteogenic cells for their ability to mineralize eggshell membranes in vitro (28). The results of these experiments indicated that the intact collagen type X molecule does not appear to stimulate mineralization, while the removal of non-helical domains results in facilitation of cell-mediated mineralization of eggshell membranes. Our study was undertaken to investigate the expression pattern of type X collagen during human tooth development, since there are no data available on its presence or absence in dental structures during mineralization of the enamel.

Materials

Testicular hyaluronidase was purchased from Boehringer Mannheim (Mannheim, Germany), gelatine and guanidine HCl from Reanal (Budapest, Hungary), and Maxisorp immunoplates from Nunc Intermed Ltd. (Copenhagen, Denmark). For immunohistochemistry, Biogenex Supersensitive Detection System (San Ramon, CA, USA) was used, while horseradish peroxidase-conjugated anti-mouse secondary antibody used in ELISA assay was from DAKO A/S (Glostrup, Denmark). Polyacrylamide mini gel (BioRad, Hercules, USA), polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore, Bedford, UK), secondary biotinylated anti-mouse antibody and Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) were used for immunoblotting. All the other chemicals were of analytical grade and provided by Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany). The monoclonal antibody X53, raised against recombinant human type X collagen (29), was a kind gift from Klaus von der Mark, University of Erlangen-Nurenberg, Germany.

Sample preparation

Human tissue samples were collected according to Hungarian ethics rules at the Department of Obstetrics and Gynaecology, and the Department of Pathology, University Medical School, Debrecen, Hungary. Foetal samples were collected after legally approved artificial abortions. The age of the fetuses was calculated from anamnestic and ultrasonographic data. According to menstrual age, the gestational age of tissue material ranged from 17 weeks to 33 weeks. Postnatal samples were taken from a 9-week-old child. All samples ($n = 7$) were taken 2–6 h after death. A part of the lower jaw was removed, and the samples were cut into small blocks, each containing one tooth germ from the incisal region of the mandible. Blocks were immersed in Sainte-Marie's solution modified according to Tuckett and Morriss-Kay (99 ml 96% ethanol, 1 ml of glacial acetic acid) for 24 h at 4°C (30). After fixation, the tissue blocks were demineralized for approximately 3 weeks in 10% (w/v) EDTA solution (pH adjusted to 7.4) at 4°C, and embedded in paraffin at 54°C. A Reichert microtome equipped with a special D profile knife for hard tissues (Leica Instruments, Nussloch, Germany) was used to cut 7–8 μm thick serial sections of the mandibles parallel to the long axis of the tooth. Sections were mounted on glass slides coated with a solution of 2.5% gelatine, dewaxed in xylene, and rehydrated through descending concentrations of ethanol and distilled water.

Immunolocalization of collagen type X

Tissue sections were stained for type X collagen with the monoclonal antibody X53 that recognizes an epitope that is present in the native and pepsin-treated human collagen type X, but not in the non-collagenous NC-1 domain (29). After rehydration, the sections were washed 3 × 5 min in phosphate-buffered saline (PBS), pH 7.4, and digested with testicular hyaluronidase (1 mg/ml) in PBS for 60 min at 37°C. To prevent a possible non-specific binding of the primary antibody the sections were blocked with normal goat serum (diluted 1:20 in PBS) for 20 min at room temperature. After washings (3 × 5 min in PBS), the sections of tooth germs were incubated with the monoclonal X53 for 12 h at 4°C. After rinsings (3 × 5 min in PBS), the samples were incubated for 20 min with biotin-conjugated secondary antibody (diluted 1:100 with 1% bovine serum albumin in PBS) and with alkaline phosphatase-conjugated streptavidin (diluted 1:100 with 1% bovine serum albumin in PBS) for 20 min at room temperature. For detection, naphtol AS-MX phosphate and Fast Red TR mixture containing levamisole (1 μg/ml) were used. After staining for 30 min, the section were washed in water (3 × 5 min) and mounted in Aquamount (BDH Chemicals, Poole, UK). As a control, PBS and non-immune hybridoma cell culture medium were used instead of primary antibody.

Analysis of collagen type X with the ELISA technique

Unerupted tooth germs of the 22- and 26-week-old human fetuses were removed and dissected from the mandibular alveolar bone. Special care was taken to remove all adhering connective tissue from the sample. The enamel and dentine were separated from the other dental tissues. The separated tissues were finely ground at 4°C in Tris/Glycine buffer containing 1% SDS, and the sample was centrifuged. As a control, epiphyseal cartilage of the tibia was also removed from the same foetus. The cartilage was extracted for 48 h in 4 M guanidine HCl in the presence of protease inhibitors (10 mM EDTA, 2 mM iodoacetamide, 2 mM phenylmethylsulphonyl fluoride, 5 μg/ml soybean trypsin inhibitor and 100 mM ε-aminocaproic acid) to remove proteoglycans (31), and centrifuged.

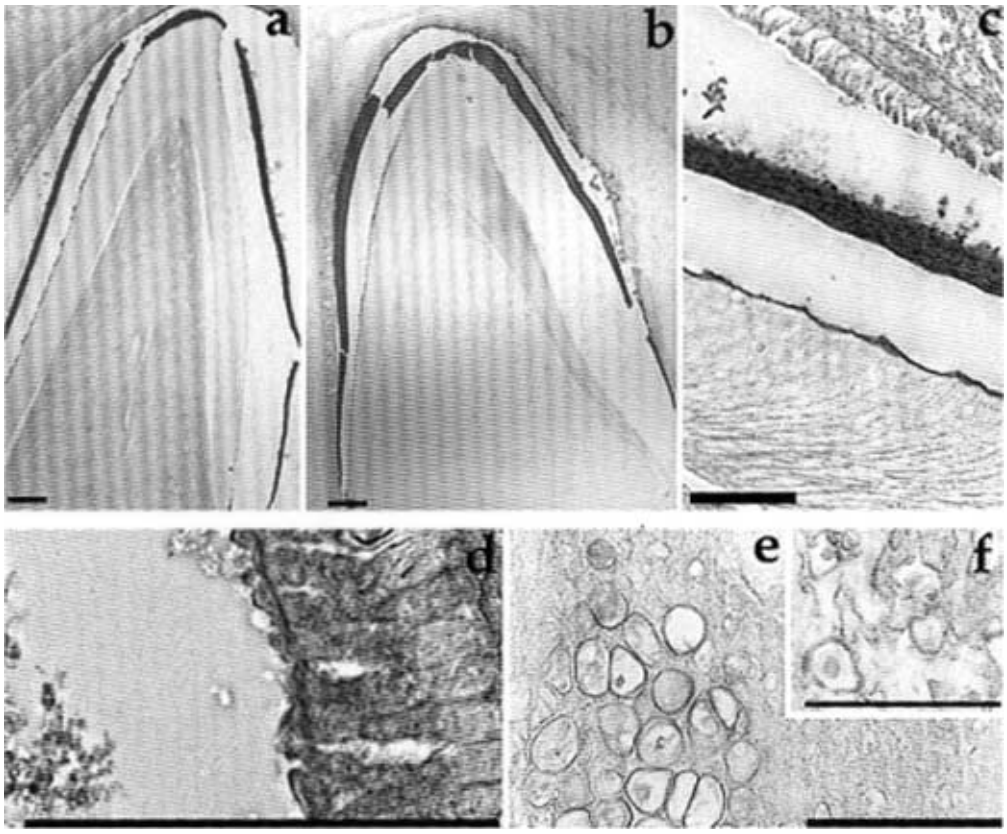

Fig. 1. Immunohistological localization of type X collagen in human tooth germ. The enamel matrix and secretory ameloblasts of the 31- and 33-week-old human fetal tooth germ were strongly stained for type X collagen (a–d). The higher magnification picture of the secretory ameloblasts shows the strong signal for type X collagen at their apical pole (d). In the Meckel's cartilage, pericellular staining of the hypertrophic chondrocytes was detected (f). In addition, a delicate extracellular staining of the hypertrophic zone of the ossifying cartilage was recorded (e). Scale bars represent 100 μm.

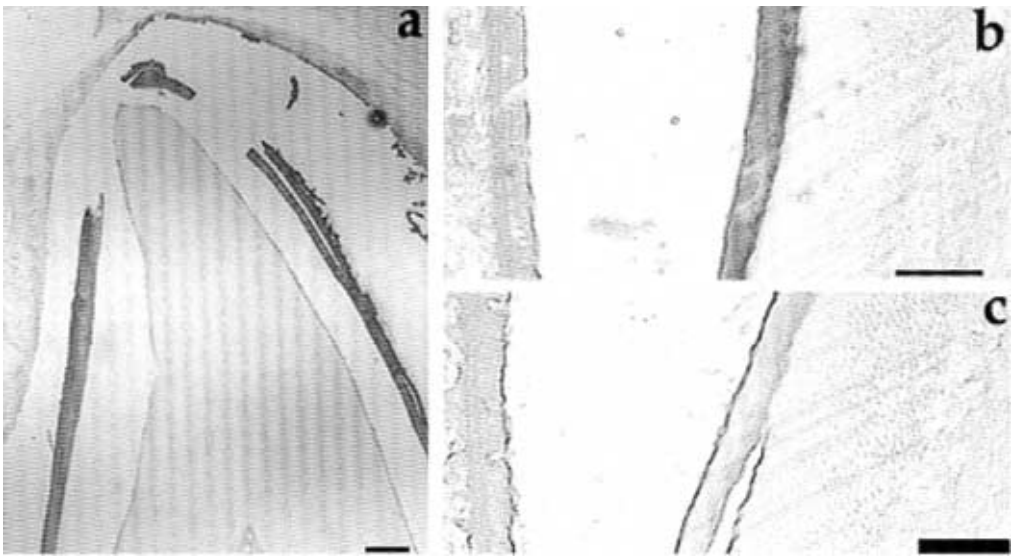

Fig. 2. Type X collagen expression in the 9-week-old newborn tooth germ. The staining intensity for type X collagen in the enamel was decreased considerably (a, b). Note the marked decrease of staining in the secretory ameloblasts, too. Similar to the earlier stages, type X collagen did not stain in the dentine, the odontoblasts, or the pulp (b). In the control sections no staining was observed in the enamel matrix when PBS or non-immune hybridoma cell culture medium were used instead of primary antibody X53 (c). Scale bars represent 100 μm.

After centrifugation, the pellets were washed and digested with pepsin (5 mg/1 g of cartilage) for 48 h at 4°C in acetic acid. The pepsin-solubilized samples were centrifuged, and the supernatants were analyzed using the ELISA technique (32). Briefly, Nunc Maxisorp immunoplates were coated with both the enamel and epiphyseal cartilage samples (10 µg protein/well). To block the non-specific binding, 1% gelatine in PBS was used. Next, the monoclonal antibody X53 was added for 2 h, and after several washes with PBS the immunoplates were incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody for 2 h at 37°C. Finally, a chromogen o-phenylene diamine was portioned, and the absorbances were measured at 492 nm with Titertek Uniscan® microplate reader (Labsystems, Helsinki, Finland). Absorbance values of 10 different wells containing the same sample were measured.

Immunoblotting

Briefly, the proteins extracted from enamel of a 22-week-old fetus were loaded on an 8% polyacrylamide mini gel. The separated proteins were electophoretically transferred onto a PVDF membrane. The free binding capacity of the membrane was blocked with 10% BSA in TBS buffer (20 mM TRIS, 500 mM NaCl, pH 7.5). After several washes in TBS 0.05% Tween 20 the primary antibody X53 was added in 1/500 dilution. Then anti-mouse Igs-biotinylated antibody was used in 1/1000 dilution followed by Vectastain ABC reaction in accordance with the manufacturer's instructions. Finally, the bands were visualized by 3,3′-diaminobenzidine.

Results

In our samples, no signal for type X collagen could be detected with the monoclonal antibody X53 in dental papilla, the stellate reticulum or the presecretory ameloblasts of the tooth germ at this developmental stage (not illustrated). At the gestational age of 31 and 33 weeks, there was an intense immunostaining for type X collagen in the enamel of tooth germs (Fig. 1a, b). The apical part of the secretory ameloblasts also stained strongly for collagen type X, whereas the basal parts of the ameloblasts were negative (Fig. 1c, d). All the other parts of the tooth germs (the dentine, the dental pulp and the odontoblasts) were negative for type X collagen (Fig. 1 a–c).

Meckel's cartilage forms mandible via endochondral ossification, which was present in the 31-week-old specimen. Extracellular matrix staining in the hypertrophic zone of the cartilage was evident in the Meckel's cartilage (Fig. 1e). Intensive pericellular staining around hypertrophic chondrocytes could also be observed with monoclonal antibody X53 (Fig. 1f). This staining pattern is typical for type X collagen in the ECM around hypertrophic chondrocytes, and confirms the specificity of the antibody.

Only a weaker positive staining for collagen type X could be observed in the enamel of the 9-week-old postnatal sample (Fig. 2a, b). The apical parts of the secretory ameloblasts showed no or very weak staining (Fig. 2b). In all negative controls no staining was noticed in the enamel matrix (Fig. 2c).

The immunoreactivity of tissue extracts was also tested. Collagens from the enamel, dentin, and epiphyseal cartilage from the same 22- and 26-week-old fetuses were extracted and analyzed using the ELISA method. As expected from the immunohistochemical results, only the enamel and hypertrophic cartilage extracts gave a positive reaction; the extract from dentine produced no reaction, suggesting that both the enamel and hypertrophic cartilage contained a considerable amount of collagen type X (Fig. 3a). Fig. 3b shows the Western blot analyses of the enamel obtained from the 22-week-old fetus. It demonstrates that the detected band is in the range of 60 kDa, close to the characteristic molecular weight of the type X collagen.

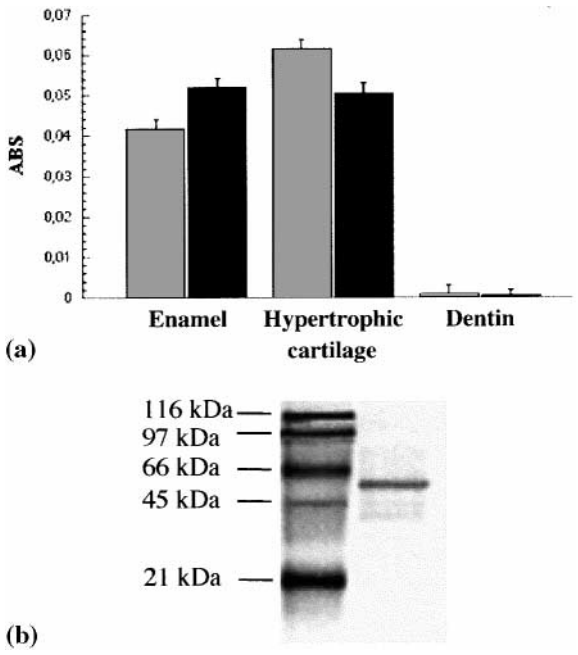

Fig. 3. Analysis of type X collagen in the enamel, dentine, and epiphyseal cartilage by ELISA assay and Western blotting. Type X collagen was extracted from the enamel, epiphyseal cartilage and dentine of 22 and 26-week-old fetuses, and analyzed using ELISA techniques. Mean absorbancies (ABS) and standard error of the mean (SEM) of parallel tests are shown after background subtraction (a). The Western blot analysis of an enamel fraction isolated from 22-week-old human developing tooth germ shows the position of the detected band, which is related to the characteristic molecular weight of the human type X collagen. Positions of molecular mass markers are indicated on the left, in kDa (b).

Discussion

In our present study, we used immunohistochemistry, ELISA techniques, and Western blot analysis to investigate the appearance of type X collagen in human tooth germs during their mineralization. We have demonstrated for the first time that type X collagen is present in the secretory ameloblasts and in the maturing enamel of the developing human tooth germ. According to our results, the presence of type X collagen is expressed temporally, so its content is highest during the fetal bell stage of tooth germ development. As shown by the intense selective immunostaining for type X collagen, the secretory ameloblasts are the cells that produce the type X collagen.

The enamel proteins—amelogenins, enamelins, tuftelin, and ameloblastin/amelin (33)—obviously participate in the mineralization process during amelogenesis. These proteins are believed to be involved in crystal nucleation and in the control of crystal growth and orientation (8, 34–37). In dentin and bone, it has been suggested that osteopontin inhibits the growth of apatite crystals, osteocalcin delays the crystal nucleation, while bone sialoprotein may play a role in the initiation of mineralization (38). These molecules have been localized in tooth cementum or dentin, and their temporal and spatial distribution correlates well with the process of mineralization (39, 40), but these molecules are not detected in the enamel.

Our results suggest that type X collagen is present in fetal enamel in considerable amounts. However, whether it interacts with the other components of the enamel and is involved in organization of the mineral deposition remains to be elucidated. And whether this interaction is directly involved at the level of mineral nuclei or some concomitant process is not clear. It is not therefore surprising to find type X collagen in the enamel matrix, since there is a proposed connection between type X collagen and mineralization during endochondral ossification. Although the function of type X collagen has not been definitively demonstrated, it is known that it can form hexagonal networks (21–23). The collagen fibrils in this network seeded with calcium ions may produce a structure which provides an optimal template for mineral deposition (41, 42).

Since type X collagen was intensively stained in the maturing enamel, we suggest that it may influence enamel crystal formation during amelogenesis. In mice lacking type X collagen, the femur bone and its trabecular structure were changed (27), but it was not specifically investigated whether the tooth development was normal in these mice. Electron microscopic investigations are under way to examine the possible function of type X collagen in enamel mineralization.

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