

EXAMINATION OF EXTRACELLULAR MATRIX COMPONENTS
DURING HUMAN TOOTH DEVELOPMENT

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PH.D. THESIS

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INTRODUCTION

TOOTH DEVELOPMENT

Teeth develop from the oral ectoderm and neural crest derived mesenchyme under the influence of epithelial-mesenchymal interactions. The complex interactive process between epithelial and mesenchymal cells mediated by different macromolecules of the extracellular matrix (Sadler, 1985) and several diffusible signaling molecules Signaling molecules in the sonic hedgehog (Shh), fibroblast growth factor (FGF), bone morphogenetic protein (BMP), and Wnt families appear to regulate the early steps of tooth morphogenesis, and some transcription factors associated with these pathways have been shown to be necessary for tooth development (Thesleff 2001). These interactions progressively transform the tooth primordia into complex mineralized structures: the proliferation of the basal layer of oral epithelium leads to the formation of the dental lamina, which elongates into the ectomesenchyme.

The ectomesenchymel cells condense around the dental lamina and during the bud, cap and bell stages, resulting in the establishment of the characteristical form and tissues of the tooth germ. During the advanced stages of odontogenesis, mesenchymal cells differentiate into odontoblasts that synthesize the dentin matrix, whereas epithelial cells differentiate into ameloblasts that are responsible for enamel matrix formation (Tucker et al., 1999).

The extracellular space of the foetal tissues is highly hydrated, composed mainly of hyaluronan and sulphated proteoglycans, and forms a well organised network that confers special characteristics of virtually all developing organs. It has

been shown that this ECM macromolecules are not only essential for maintaining the structural integrity of developing tissues, and they also influence on several cellular processes critical to many specific developmental events such as cell proliferation, migration and differentiation (Faser et al., 1997).

Despite our increased knowledge of the regulation involved in this developmental process, the precise mechanisms of certain events, such as the 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. The enamel consists of inorganic apatite crystallites embedded in an organic matrix, and it is widely accepted that the inorganic material constitutes 96% of the weight of mature enamel. 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 (Boyde and Martin, 1982; Boyde, 1989; Daculsi and Kerebel, 1982).

Unlike the other mineralization processes of the body, the initial steps of enamel formation, i.e. crystal nucleation and growth, do not require any remodelling 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. The enamel proteins (such as amelogenins, enamelin, 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 (Robinson et al, 1998; Fincham et al., 1999). 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

HYALURONAN

STRUCTURE, SYNTHESIS AND METHABOLISM

Hyaluronan (HA), a linear unbranched acidic polysaccharide, is a ubiquitous extracellular matrix component of many soft tissues (8, 9). Despite its seemingly simple structure, with alternating N-acetyl-D-glucosamine and D-glucuronic acid residues and they are linked together through alternating beta-1,4 and beta-1,3 glycosidic bonds (Fig. 1) (Meyer et al., 1954).

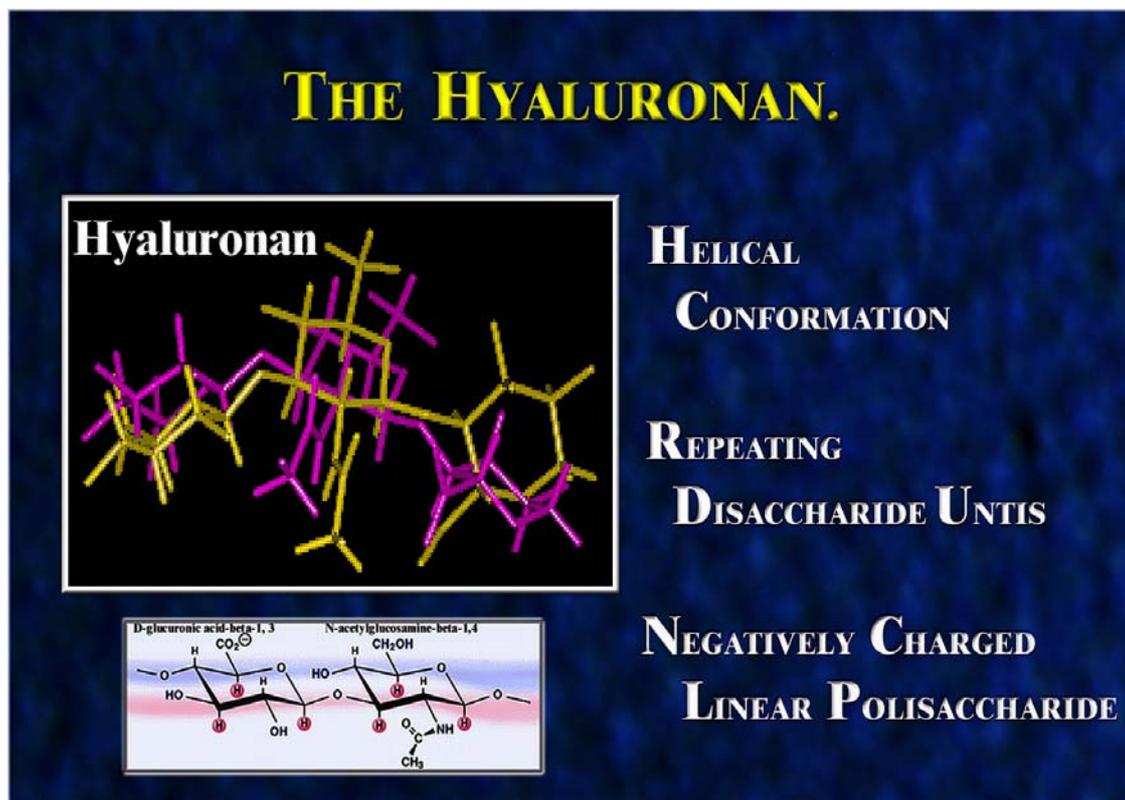


Fig. 1 The schematic drawings of Hyaluronan

The upper picture shows the three dimensional structure of HA and the lower one the monosaccharides of HA are illustrated.

It was recovered by Karl Meyer and coworkers, as a novel glycosaminoglycan (GAG) from the vitreous of eyes (Meyer and Palmer, 1934). They showed that this substance contained a uronic acid and an aminosugar, but no sulfoesters. The original name was developed from hyaloid (vitreous) + uronic acid. This marked the birth announcement for one of nature's most versatile and fascinating macromolecules. From year 70's, this macromolecule is referred to as 'Hyaluronan'.

Hyaluronan not only provides - with other extracellular matrix (ECM) constituents - the ground substance for developing tissues (10), but through specific interactions with other matrix components and cell surface receptors called hyaladherins, it is also involved in the control of cell proliferation and differentiation (Toole et al., 1984; Nehls et al. 2000; Pohl et al., 2000).

In contrast to most GAGs, which are synthesized in the Golgi network, but several studies have revealed and improved that hyaluronan is synthesized at the inner face of the cell membrane. HA is synthesized at the plasma membrane. The hyaluronan synthase has 3 isoforms. They are very compact and tightly folded proteins. All isoforms are only around 42 kDa (Weigel et al., 1997).

Hyaluronan synthase enzymes synthesize large, linear polymers of the repeating disaccharide structure of hyaluronan by alternate addition of glucuronic acid and N-acetylglucosamine to the growing chain using their activated nucleotide sugars (UDP - glucuronic acid and UDP-N-acetylglucosamine) as substrates (Weigel et al., 1997). The number of repeat disaccharides in a completed hyaluronan molecule can reach 10,000 or more, a molecular mass of ~4 million daltons. The average length of a disaccharide is ~1 nm. Thus, the length of hyaluronan is approximately equal to the diameter of a human erythrocyte.

The growing polymer is extruded through the membrane to the outside of the cell as it is being synthesized. The hyaluronan synthase must possess at least six different functional unit to produce nativ hyaluronan, as shown in Figure 3.

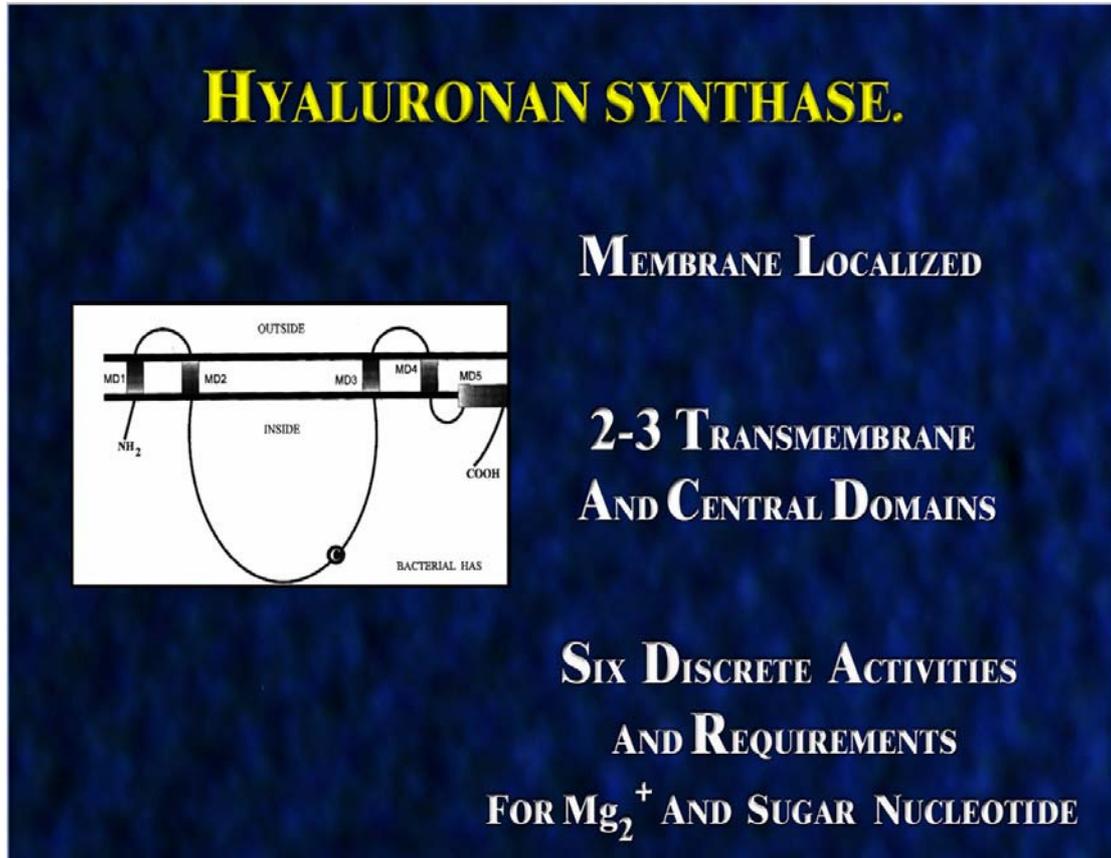


Fig. 2 The domains of HAS

The diagram shows the HAS and the it's different domains required for the enzyme to make a disaccharide unit and extend the growing hyaluronan chain.

The biggest amount of HA removed from ECM via local catabolism and/or drainage into the lymphatic system for catabolism in regional lymph nodes. The local turnover of hyaluronan must occur intracellularly within lysosomes having low pH active hyaluronidase and exoglycosydase (Culty et al., 1992; Aguiar et al., 1999; Yhou et al., 2000). In addition, numerous studies also suggest that hyaluronan internalization is mediated via different HA binfing receptors—including CD44.

CD44-MEDIATED INTERNALIZATION AND DEGRADATION OF HYALURONAN



Fig. 3 CD44-Mediated Internalization and Degradation of Hyaluronan

The part of HA bound via interaction with CD44 to the cell surface, internalized by invagination of the plasma membrane, as shown of this figure. The invaginated sample would become an endosome and subsequently fuse with a lysosome, completing the degradation of the hyaluronan into tetra and hexasaccharides by hyaluronidase and exoglycosydase (Greiling et al. 1975; Frost et al., 1996).

HYALURONAN RECEPTORS

Hyaluronan belevied to play important roles in a variety of functions within the extracellular matrix of nearly all tissues. Many of the cellular and matrix effects of HA are thought to be mediated via the interaction with different receptor proteins referred to as hyaladherins (a.k.a., “hyaluronan binding proteins”) (Aruffo et al., 1990, Hall et al., 1994, Knudson et al., 1996; Hall et al., 1994). Several have been

identified including; CD44, RHAMM, IVd4 and the Liver Endothelial Cell clearance receptor (LEC receptor) (Fig. 4).

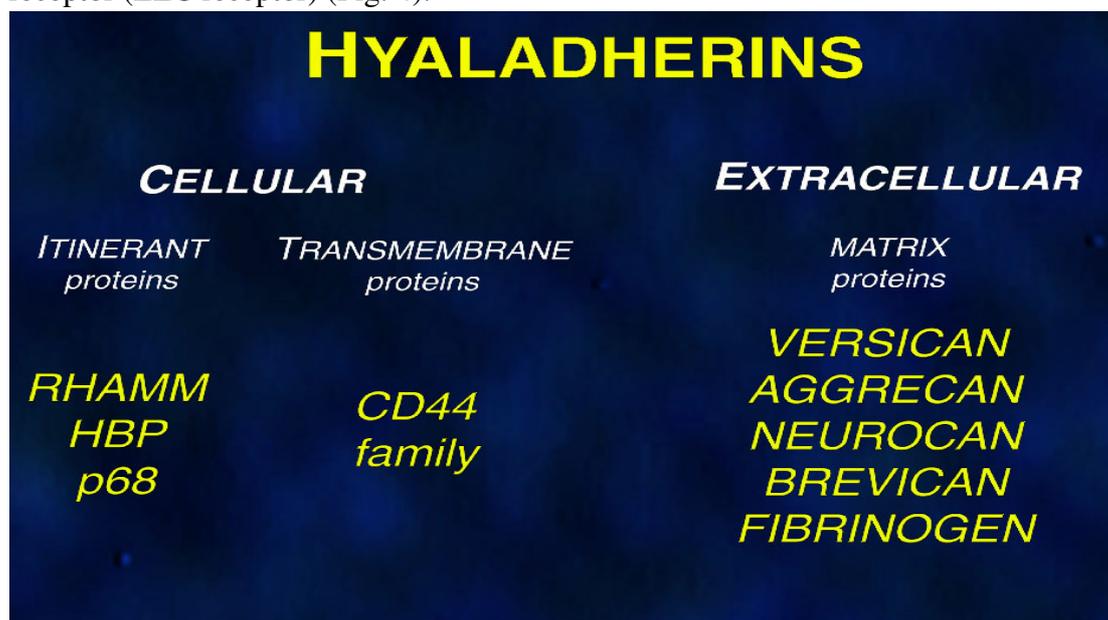


Fig. 4 The members of the hyaladherin family

One of the most well known member of this group, and the most studied to date is the cell surface receptor CD44 (also referred to as ECMRIII, H-CAM, Pgp-1) (Entwistle et al., 1996). The extracellular domain of this integral membrane protein is variably spliced, but its distal part always contains a hyaluronan binding domain. However, CD44 has several other ligands including collagens (Knutson et al., 1996), different growth factors, laminin, osteopontin, as well as serglycin. It is expressed on the surface of a variety of cell types and cell lines (Underhill, 1992, Bourguignon et al., 1999). Previous observations suggest that CD44 isoforms are involved in a wide variety of HA-mediated cellular events, such as cell migration, proliferation and differentiation which have an important role in morphogenesis (Zhou et al., 2000).

CD44 is a single-pass transmembrane glycoprotein consisting of four functional domains, (Fig). The distal extracellular domain is the region primarily responsible for the binding of hyaluronan. The membrane-proximal extracellular

domain is the primary site of alternative splicing of CD44 mRNA that produces the many isoforms of CD44. its isoforms ranging in size from 80 to 250 kDa, arise by the alternative splicing of so-called “variant” exons into the extracellular domain of this receptor. CD44 interacts with the cytoskeleton. In most isoforms of CD44 have an intracellular protein motifs that indicate a capacity for interaction with cytoskeletal proteins as well as the potential for intracellular signaling.

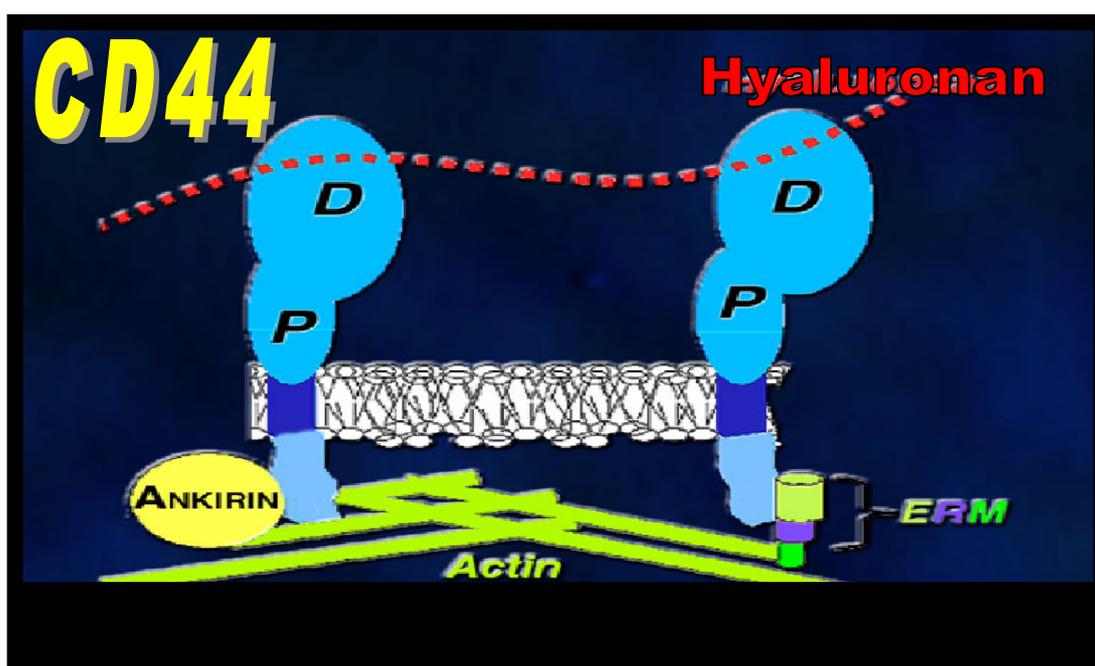


Fig. 5 Structure of CD44 and its interaction with intracellular elements.

The domains of CD44 are the following: the distal extracellular domain(D), the membrane proximal extracellular domain (P), the transmembrane domain, and the intracellular cytoplasmic domain. CD44 is known to link to the actin cytoskeleton through its interaction with particular actin-binding proteins, binding proteins associated with either the ankyrin family (Lokeshwar et al., 1994) or the ERM family (Yomenura et al, 1998).

THE TYPE X COLLAGEN

STRUCTURE AND IT'S IN MINERALIZATION

Type X collagen is a product of terminally differentiated chondrocytes in regions of cartilage that undergo endochondral ossification (Lisenmayer, 1991). Its expression serves as a good marker for the hypertrophic stage of chondrocyte differentiation (Schmid and Lisenmayer, 1985).



Fig. 6 The type X collagen expressing hypertrophic chondrocytes of the human Meckel's cartilage.

On the picture the pericellular staining of the hypertrophic chondrocytes in the Meckel's cartilage is presented. In addition to this, a delicate extracellular staining of the hypertrophic zone of the ossifying cartilage is also visible. Scale bar represents 100 μ m.

Collagen type X has been reported to be present also at bone-ligament interfaces (Niyibiyi et al., 1996; Fukuta et al., 1998), and in intervertebral endplate (Bood et al., 1997; Lammi et al., 1998). It is a non-fibrillar collagen consisting of two non-collagenous globular domains and a triple helical portion which has approximately a half of the length of fibril-forming collagens around 60 kDa (Kwan et al., 1991; Bonen and Schmid, 1991). Through its carboxyl terminus, the molecule can be incorporated into supramolecular structures (Chen et al., 1990).

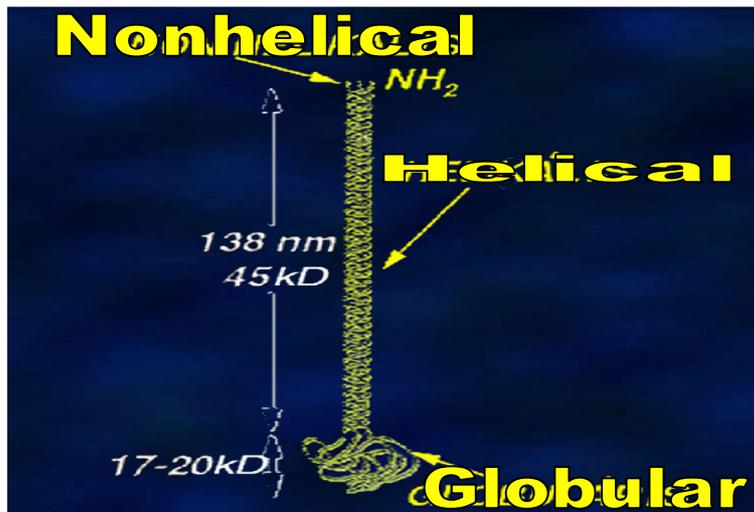


Fig. 7 The schematic drawing of type X collagen

The picture illustrates the different parts of type X collagen.

The highly restricted expression of type X collagen in the extracellular matrix of cartilage growth plate suggests that it may be involved in the process of mineralization during the endochondral ossification of the epiphyses (Gerstenfeld et al., 1991; Kirsh and von der Mark, 1991; Coe et al., 1992).

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 growth plate, suggesting that collagen type X plays a role in normal distribution of matrix vesicles and proteoglycans in ECM

(Boskey et al., 1989). 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 (Bonucci, 1989). The results of these experiments indicated that 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 the mineralization of the enamel.

THE AIM OF OUR STUDY

Although many laboratories have been attempting to identify cell membrane and ECM molecular components and their roles played during tooth development, their results mainly based on studies carried out on various vertebrate species. In only a few studies have these different factors been examined during human tooth development. Therefore our aim was to add further new information in this highly interactive and rapidly evolving field of research, using tooth germs obtained from human tissue samples. In our first study using digital image analysis we want to quantitatively describe the temporal and structural distribution pattern of extracellular matrix molecule hyaluronan /HA/ (I). In the next step of our studies, we want to analyze the expression pattern of the major hyaluronan receptor CD44 (II), finally we would wish to get evidence about the presence or absence of collagen type X in developing dental structures (III).

In our present studies we detect the expression pattern of CD44 and type X collagen in developing tooth germs obtained from human samples using anti-human antibodies raised against these different molecules and to detect hyaluronan by using a biotinylated hyaluronan binding complex. To confirm the results of the type X collagen immunohistochemical experiments, we wish to carry out Western immunoblotting and ELISA experiments of human dental structures.

MATERIAL AND METHODS

SAMPLING, FIXATION, DECALCIFICATION, EMBEDDING, CUTTING

Human samples were obtained from the Department of Obstetrics and Gynaecology, or from the Department of Pathology, University of Debrecen Medical and Health Science Centre, Debrecen, Hungary. The study protocols were reviewed and approved by the University of Debrecen Ethical Committee, and the sampling was properly carried out under the control of the University's Guidelines for Human Experimentation.

The samples were collected after legally approved artificial abortions and the authors were not involved in decisions to terminate any of these pregnancies. The age of the foetuses was calculated from anamnestic and ultrasonographic data. The distribution of different extracellular matrix molecules and CD44 was studied in foetuses with age ranging from 15 to 33 gestational weeks. The postnatal sample was taken from a 3- and 9-week-old deceased infants.

Tissues were rapidly dissected from human samples, typically within 2 hours after death. The middle part of the lower jaw was removed, containing not only a mandibular primary tooth germ; part of the bone of the mandible was also present in all samples and were immediately transferred into Sainte-Marie's fixative modified according to Tuckett and Morriss-Kay (99 ml 96% ethanol, 1 ml of glacial acetic acid) for 24 hrs at 4°C (Sainte-Marie, 1962; Truckett et al., 1988). After fixation the blocks were exposed to neutral buffered 10% (w/v) ethylenediaminetetraacetic acid (EDTA, Sigma, St. Louis, USA) solution for approximately 3 weeks at 4°C. After dehydration, tissue samples were embedded in wax at 54°C, and 2 µm thick sections were cut using a special D profile knife for hard tissue cutting (Leica Instruments,

Nussloch, Germany). Paraffin sections were placed on gelatine-coated glass slides dried overnight at 37°C.

DETECTION OF HYALURONAN

After rehydration, the sections were washed 3 times for 5 min each with phosphate-buffered saline (PBS) and treated with 1 % bovine serum albumin dissolved in PBS for 30 min at 37°C. For the specific detection of hyaluronan, we used a biotinylated hyaluronan-binding probe (bHABC) prepared from proteoglycan aggregates of bovine articular cartilage, as described in detail by TAMMI et al. (Ripellino et al., 1985; Tammi et al., 1988). The probe contains the hyaluronan-binding G1 domain of the cartilage proteoglycan, aggrecan, and link protein, which shows high affinity and specificity for hyaluronan. The bHABC probe, diluted to 2-10 µg/ml in PBS was added to the specimens and incubated for 12 h at 4°C. After washing with PBS (3x5 min), avidin-biotin peroxidase complex (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) (1:200 dilution) was added for 30 min at room temperature. The slides were washed and the bound bHABC was visualized with peroxidase and H₂O₂ using 3,3'-diaminobenzidine (DAB) for 10 min as a chromogen according to the manufacturer's instructions. The sections were dehydrated with ethanol, cleared in xylene, and mounted in DPX (Fluka, Buchs, Switzerland). The negative controls included sections incubated without bHABC or with bHABC but after treating the sections with *Streptomyces* hyaluronidase (100 IU/ml in 0.1 M sodium acetate buffer pH 5.0 at room temperature, 60 min) (ICN Pharmaceuticals, Costa Mesa, CA, USA) (Yamada et al., 1973). Further controls included bHABC with hyaluronan oligosaccharides (HA20, 3 µg/1 µg bHABC) to reveal possible non-specific binding of the bHABC (Tammi et al., 1994).

DIGITAL DENSITOMETRY

Specimens were examined with a Leitz Ortholux[®] II BK microscope and a PL Fluotar 50x/0.85 N.A.-objective (Leitz, Wetzlar, Germany). The images were digitized with a 12-bit Peltier-cooled digital camera (Photometrics CH 250; Photometrics, Tucson, AZ, USA) and a KAF 1400 (Kodak, Rochester, NY, USA) charge coupled device (CCD) detector. During system calibration and measuring, each image of interest was corrected with the background using a flat fielding algorithm. Prior to the image-analysis, absorbance properties of DAB were tested between 482 and 698 nm by using a set of interference filters (Schott). The optimum wavelength for DAB was found at 543 nm. The imaging system was calibrated with neutral density filters (Schott, Wiesbaden, Germany) in the range of 0 to 3 optical density (OD) units to convert pixel gray values into standardized OD units, and all further calculations were made with OD-converted pixels (23). This conversion was done with IPLab software (v.3.5; Signal Analytics, Vienna, VA, USA). The settings of the microscope and camera were kept constant throughout the study. Spatial resolution of the system was 0.48 $\mu\text{m}/\text{pixel}$.

MEASUREMENT

The samples were placed in two groups depending on their developmental stage. Group I contained two tooth germs – with the ages of 15 and 19 wks - in an early bud stage and group II contained three tooth germs in a later developmental stage (21- and 31-wk-old fetuses and 9-wk-old newborn) when the dental hard tissue: dentine and enamel was already present.

Two sections from each individual specimen were analyzed. In group I, the extracellular matrix (ECM) of the dental papilla (DP), the dental basement membrane

(BM), the apical and the basal part of the presecretory ameloblast (ApPreA, BpPreA) was determined. In group II, the ECM of pulp (P), the odontoblast (O), the dentine (D), the apical and the basal part of the ameloblast (ApSecA, BpSecA) were measured. From each section, four randomly selected images containing the previously mentioned structures were acquired. From each picture, 15 measurements were carried out. The area of the region of interest (ROI) for all measurements was $5.64 \mu\text{m}^2$, and the area integrated mean OD values (AIOD) were calculated for each ROI, and those data were used for the presentation of the results.

STATISTICAL ANALYSIS

Within each group, the mean and SEM of the AIOD was calculated. The measuring protocol also took the possible variation in the section thickness into account.

IMMUNOHISTOCHEMICAL ANALYSIS OF CD44

The monoclonal antibody (MoAb) raised against human CD44, was a kind gift from Dr. Sirpa Jalkanen, Turku, Finland. This MoAb recognizes an epitope in the common backbone of all CD44 forms, corresponding to codons 125-235 (Goldstein et al., 1989).

To stain CD44, tissue sections were dewaxed in xylene, and rehydrated through descending concentrations of ethanol. After rehydration, the sections were washed 3 x 5 min in phosphate-buffered saline (PBS) at pH 7.4. The slides were pre-incubated in 1 % bovine serum albumin (BSA) dissolved in PBS, for 30 min at 37°C, in order to prevent a possible non-specific binding of the primary antibody. Then the

sections were incubated with the anti-CD44 MoAb diluted to 1:100 in PBS overnight at 4°C (Jalkanen et al., 1987). After rinsing in PBS (3 x 5 min), the samples were incubated for 1 h with biotinylated anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA, USA) diluted to 1:50 in 1% BSA PBS. After 3 x 5 min washing in PBS, the sections were treated with avidin-biotin peroxidase complex (Vectastain Elite ABC Kit, Vector) for 1 hour at room temperature according to the manufacture's instructions. For detection of the bound CD44 MoAb, the slides were incubated in 0.03 % H₂O₂ and 3,3'-diaminobenzidine (DAB) (0,5 mg/ml in PBS) for 5 min. The sections were washed in distilled water, dehydrated with ethanol, cleared in xylene, and mounted in DePeX (BDH Laboratory Supplies, Poole, UK). Control sections were stained otherwise in same way but the primary antibody was omitted and replaced by nonimmune IgG (IgG_{2a}, Sigma, St. Louis, USA).

IMMUNIHISTOCHEMICAL DETECTION OF TYPE X COLLEGEN

Tissue sections were stained for type X collagen with the monoclonal antibody X53 (a kind gift from K. von der Mark, Universitz of Erlangen, Nünber, Germany) that recognises an epitope that is present in the native and pepsin-treated human collagen type X, but not in non-collagenous NC-1 domain (Eerola et al., 1998). After rehydration, the sections were washed 3 x 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 x 5 min in PBS), the sections of teeth germs were incubated with the monoclonal X53 for 12 h at 4°C. After rinsings (3 x 5 min in PBS), the samples were incubated for 20 min with biotin-conjugated secondary

antibody (Biogenex Link solution, diluted 1:100 with 1% bovine serum albumin in PBS), and with alkaline phosphatase-conjugated streptavidin (Biogenex Label solution, diluted 1:100 with 1% bovine serum albumin in PBS) for 20 min at room temperature (Aigner et al., 1993). For detection, naphthol 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 x 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 ELISA TECHNIQUE

Unerupted tooth germs of the 22- and 26-wk-old human foetuses 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 was 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 aminocaproic acid) to remove proteoglycans (Linsenmayer et al., 1988), and centrifuged.

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 with ELISA technique (Wright et al., 1996). Briefly, Nunc Maxisorp immunoplates (Nunc Intermed Ltd., Copenhagen, Denmark) 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 hours, and after several washes with PBS the immunoplates were incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody for 2 hours 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.

WESTERN IMMUNOBLOT

Briefly, the proteins extracted from enamel of a 22-week-old foetus were loaded on an 8% polyacrylamide mini gel. The separated proteins were electrophoretically transferred onto a PVDF membrane. The free binding capacity of the membrane was blocked with 10% BSA in TBS buffer (20mM 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 according to manufacturer's instructions. Finally, the bands were visualized by 3,3'-diaminobenzidine (Búyás et al., 1993).

RESULTS

HYALURONAN

Group I contained two tooth germs aged 15 and 17 wks. In this stage, the developing tooth germ consisted of enamel organ and dental papilla (Figs. 8). The dental papilla showed moderate staining with bHABC (Fig. 8 a, b). The dental basement membrane, the apical part of the presecretory ameloblasts showed a strong signal, in contrast to the weakly stained basal part of these cells (Figs. 8 a, b).

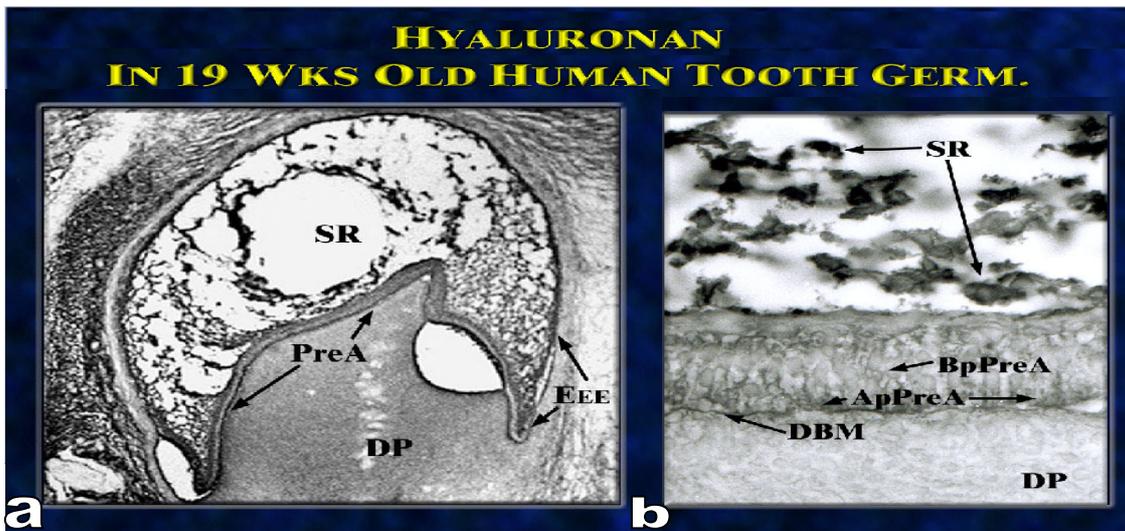


Fig. 8 Hyaluronan reaction in the cap stage human tooth germ age of 19th fetal week

(a) (b): Moderate signal is present in the dental papilla (DP), the stellate reticulum (SR), the dental basement membrane (BM). A strong reaction is seen in the apical part and weak label can be observed in the basal part of the presecretory ameloblast (ApPreA, BpPreA).

Group II contained tooth germs of human fetuses aged 21 and 31 wks, as well as a 9-wk- old postnatal sample. In the tooth germs aged 21 and 31 wks, it is the forming enamel which gave a very strong signal with bHABC (Figs. 9 a, b). This was in contrast to the enamel of the 9-wk-old newborn, where the intense staining of the

enamel matrix almost disappeared, leaving a very low signal along the borderline between the secretory ameloblasts and enamel (not illustrated). Like enamel, the apical part of the secretory ameloblasts of the 21- and 31-wk-old tooth germs showed an intense signal. The basal part of these cells displayed a definitely weaker signal (Fig. 9 b). At 9 wks, both the apical and basal parts of the secretory ameloblasts showed little or no signal at all (not illustrated). In all three specimens, the very weak signal of dentine was restricted to the dentin tubules surrounding the odontoblast processes, while no staining was observed in the rest of dentine matrix (Figs. 9 a, c). The odontoblasts showed a weak and the pulp a moderate signal, both of which gradually decreased with age (Figs. 9 a, c).

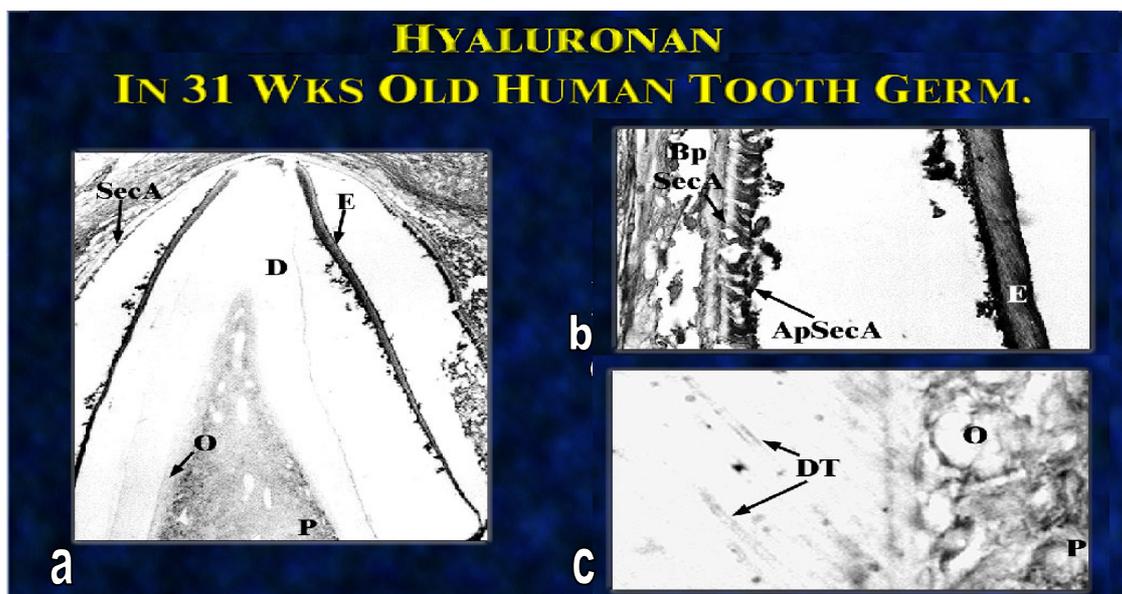


Fig. 9 Hyaluronan in the 31-wk-old human fetal tooth germ at the initiation of dentin and enamel secretion

(a): The moderate reaction of the pulp (P) is shown, (b): The very weak labelling in dentine is restricted to the dental tubuli (DT), whereas the remainder of dentine matrix (D) is not stained, (c): The apical part of the secretory ameloblast (ApSecA) and the enamel (E) are strongly labelled contrary to the weak reaction of the basal part of the secretory ameloblast (BpSecA).

The differences between the mean AIOD levels of the main structures of the developing tooth germs of group I and group II are presented in Fig. 10.

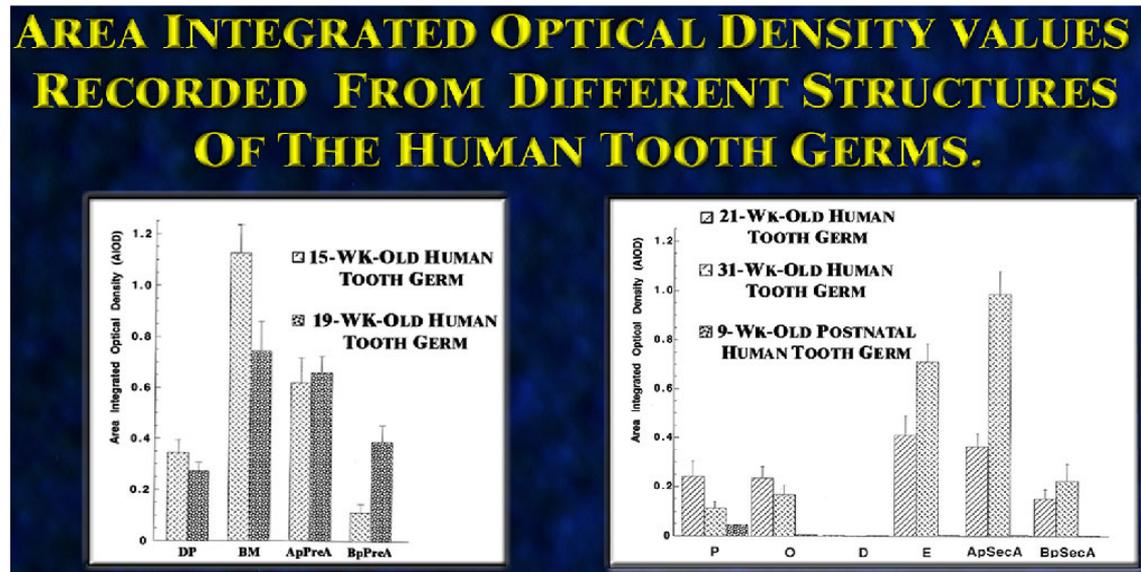


Fig. 10 Area integrated optical density (AIOD) values obtained after hyaluronan reaction in different structures of 15 and 19 week-old human fetal tooth germs and from different structures of 21-, 31- wk-old fetal, and 9-wk-old postnatal human tooth germs

Mean AIOD-s and SEM-s are shown. DP: extracellular matrix of the dental papilla; BM: the dental basement membrane; ApPreA: apical part of the presecretory ameloblasts; BpPreA: basal part of the presecretory ameloblasts. The relative hyaluronan content is significantly decreased in DP and in BM, increased in BpPreA, and remained unchanged in other structures in the function of age.

P: extracellular matrix of the pulp; O: odontoblast, D: dentin; E: enamel; ApSecA: apical part of the secretory ameloblast, BpSecA: basal part of the secretory ameloblast. Please note a gradual decrease of the relative hyaluronan content in the pulp and odontoblasts as a function of age. The relative hyaluronan content of enamel and secretory ameloblast is significantly increased between the 21-st and 31-st fetal weeks, and dramatically decreased in the early postnatal period.

No apparent signal was found either in sections pretreated with hyaluronidase (not illustrated), or in those stained with probe blocked with hyaluronan oligosaccharides. The OD values of the negative controls did not differ from the OD values of the background.

CD44

The tooth germs from early developmental stage (14-, 17- and 19-week-old) consisted of an enamel organ and dental papilla (Figs. 11). At the apical regions of these tooth germs, the presecretory ameloblasts displayed a strong reaction (Fig. 11b), but this intense signal for CD44 being seen on their plasma membrane decreased towards the root sheet region, where the presecretory ameloblast reflected to the external enamel epithelium (Fig. 11 c). Less reactivity could be detected on the cells of the stellate reticulum (Fig. 11 b). The lower layers of the oral epithelium and the proliferating cells of dental lamina showed intense reaction (Fig. 11 d, an it's inset).

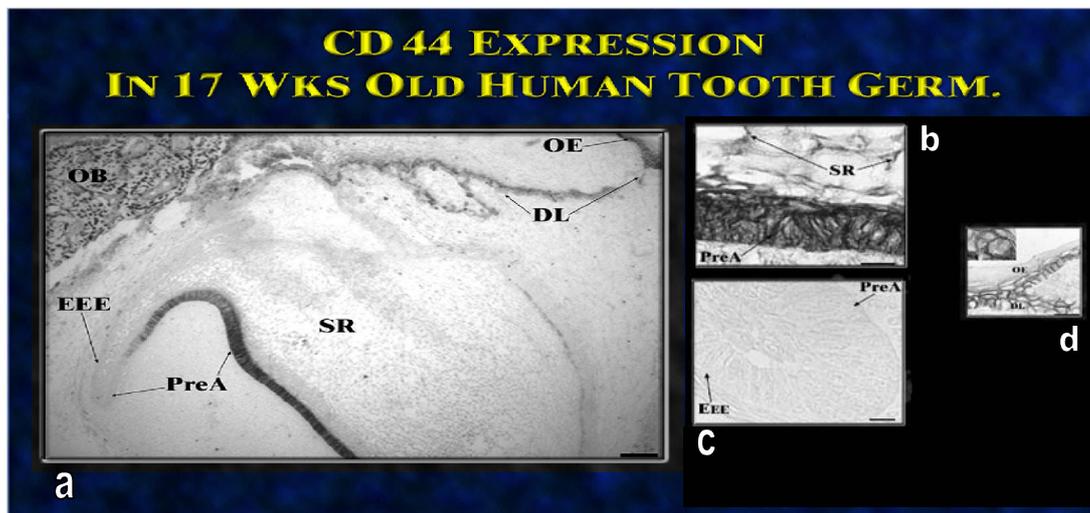


Fig. 11 CD44 expression in 17-week-old human tooth germ

(a) The forming dental lamina and the presecretory ameloblast show intense reaction. Note that the external epithelium and the presecretory ameloblasts of the

root sheet remained unstained. (b) CD44 signal in the basal and intermediate layers of the oral epithelium (OE) and in the cells of the dental lamina (DL). (c) Higher magnification micrograph of the dental lamina cells. Please note the strong CD44 staining on the plasma membrane of these proliferating cells. (d) Intense CD44 expression were detected in the presecretory ameloblasts (PreA), especially on their lateral and basal plasma membrane. The cells of the stellate reticulum (SR) show delicate immunoreactivity for CD44. Scale bars represent 100 μ m.

In tooth germs from human fetuses aged 25 and 33 weeks, as well as 3- and 9-week-old postnatal samples, with dentin and enamel already under formation, intense immunostaining for CD44 was observed in the secretory ameloblasts, odontoblasts and in the stratum intermedium (Fig. 12). The higher magnification picture of secretory ameloblasts from 3-week-old postnatal sample shows that this CD44 labelling was associated to the apical, lateral and basal plasma membrane (Fig. 12 b). Strong reactivity could be detected on the cell body of the odontoblasts. The thin Tomes processes of these cells were also positive (Fig. 12 c). In the developing pulp of the postnatal samples, close to the odontoblastic layer, a delicate staining was detected on the endothel of small blood vessels (Fig. 12 d). All the other parts of the tooth germs (the dentine, the pulp) gave no signal for CD44. Osteoblast and osteocyte, if present, showed an intensive signal on their plasma membrane and processes (not illustrated), as reported previously in bone (Pavasant *et al.* 1994). This staining pattern is typical for CD44 on these cells, and confirms the specificity of the monoclonal antibody H3.

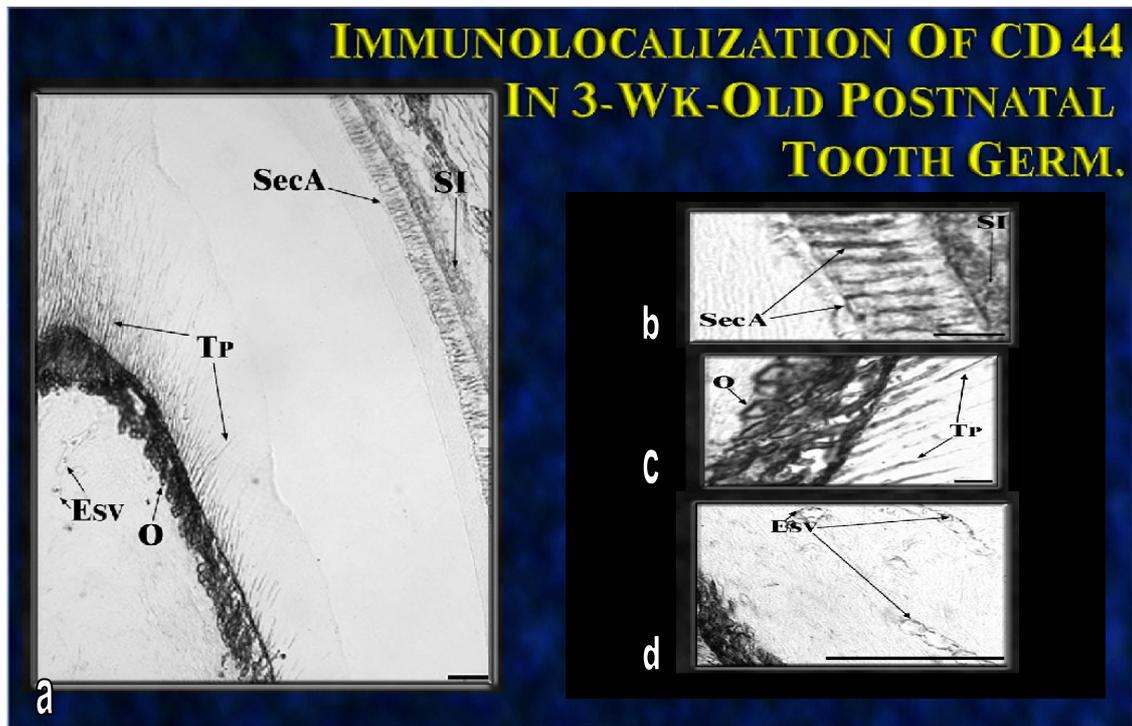


Fig. 12 Immunolocalization of CD44 in the tooth germ of a 3-week-old newborn

(a) Strong CD44 staining was detected in odontoblasts and their Tomes processes. Positive immunoreactivity was observed in the secretory ameloblasts and in the stratum intermedium. A delicate labeling for CD44 was also found within the wall of the small vessels (b) Arrowheads indicate the strong signal for CD44 at the basal, lateral and apical plasma membrane of the secretory ameloblasts (SecA). (c) Strong labeling was restricted to the odontoblasts (OdB) plasma membrane and to their Tomes processes (Tp). (d) Within the pulp, delicate immunoreactivity was detected on the endothelial (E) cells of the small vessels.

No signal was recorded from control sections incubated with nonimmune IgG instead of the primary antibody (not illustrated).

TYPE X COLLAGEN

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 wks, there was an intense immunostaining for type X collagen in the enamel of tooth germs (Fig. 13 a, 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. 13 c, d). All the other parts of the tooth germs (the dentine, the dental pulp and the odontoblasts) were negative for type X collagen (Fig. 14 a-c).

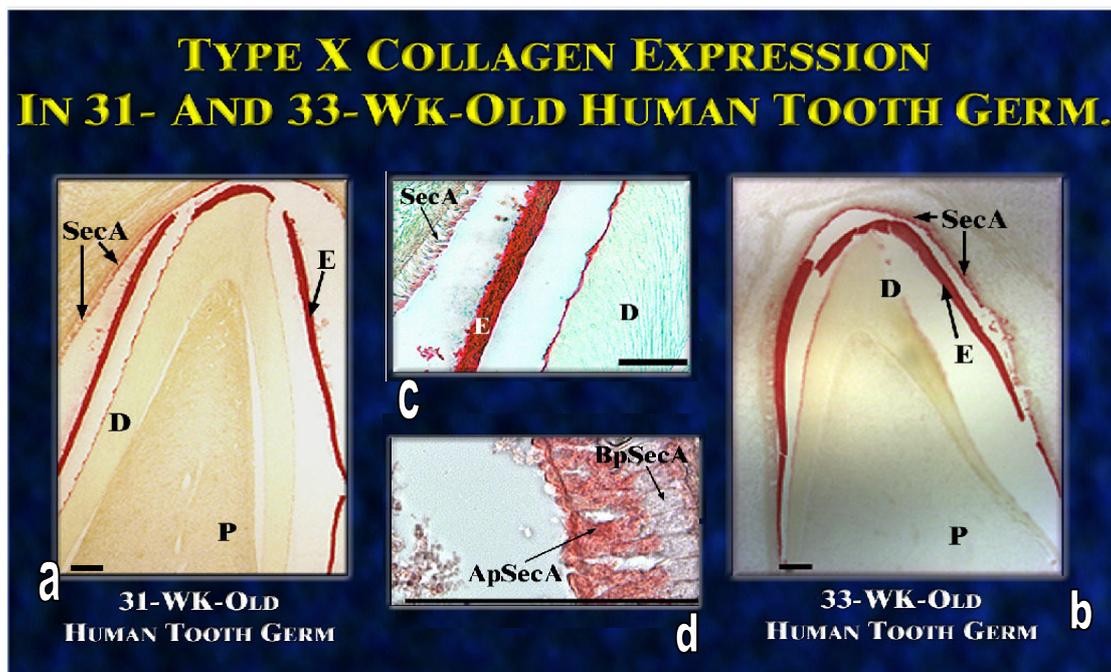


Fig. 13 Immunohistological localization of type X collagen in human tooth germ

The enamel matrix and secretory ameloblasts of the 31- and 33-wk-old human fetal tooth germ were strongly stained for type X collagen (a-d). The higher magnification pictures of the secretory ameloblasts shows the strong signal for type X collagen at their apical pole (c, d).

Meckel's cartilage forms mandible via endochondral ossification, which was present in the 31-weeks-old specimen. Extracellular matrix staining in the hypertrophic zone of the cartilage was evident in the Meckel's cartilage, see figure in the intuducton by type X collagen (Fig. 6). Intensive pericellular staining around hypertrophic chondrocytes could be also observed with monoclonal antibody X53 (Fig. 6). This staining pattern is typical for type X collagen in the extracellular matrix 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-weeks-old postnatal sample (Fig. 14 a, b). The apical parts of the secretory ameloblasts showed no or very weak staining (Fig. 14 b). In all negative controls no staining was noticed in the enamel matrix (not illustrated).

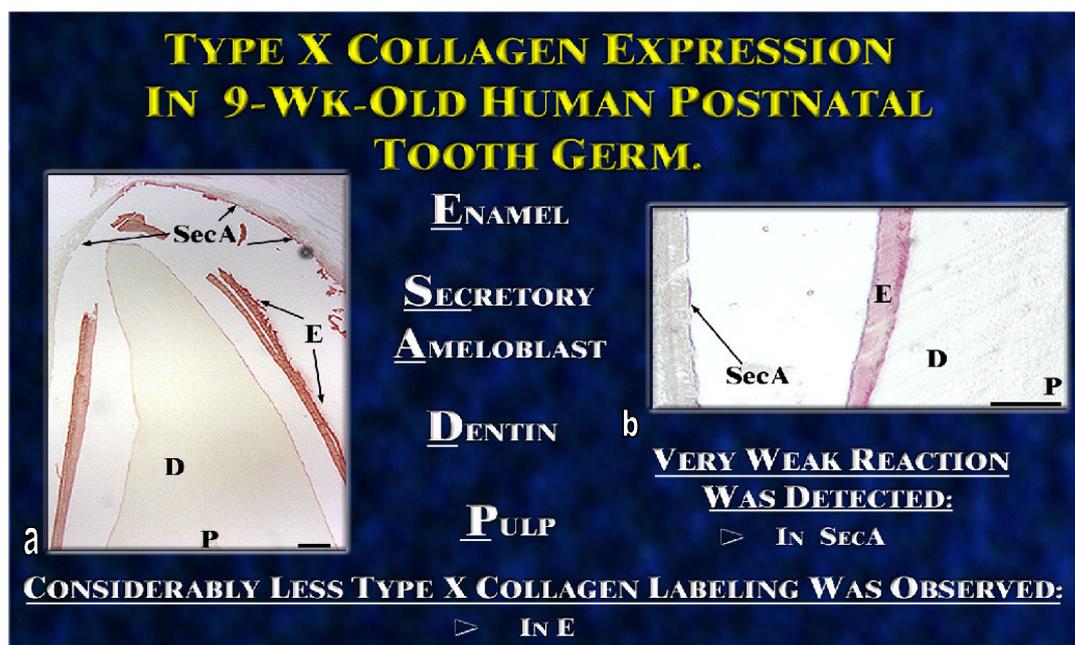


Fig. 14 Type X collagen expression in the 9-wk-old newborn tooth germ

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. Similarly to the earlier stages, type X collagen did not stain in the dentine, the odontoblasts and the pulp (b).

The immunoreactivity of tissue extracts was also tested. Collagens from the enamel, dentin and epiphyseal cartilage from the same 22- and 26-wk-old foetus were extracted and analyzed by ELISA method. As expected by immunohistochemical results, only the enamel and hypertrophic cartilage extracts gave positive reaction, whereas the extract from dentine produced no reaction, suggesting that both the enamel and hypertrophic cartilage contained a considerable amount of collagen type X (Fig. 15 a). The Fig. 15 b shows the Western blot analyses of the enamel obtained from the 22-wk-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.

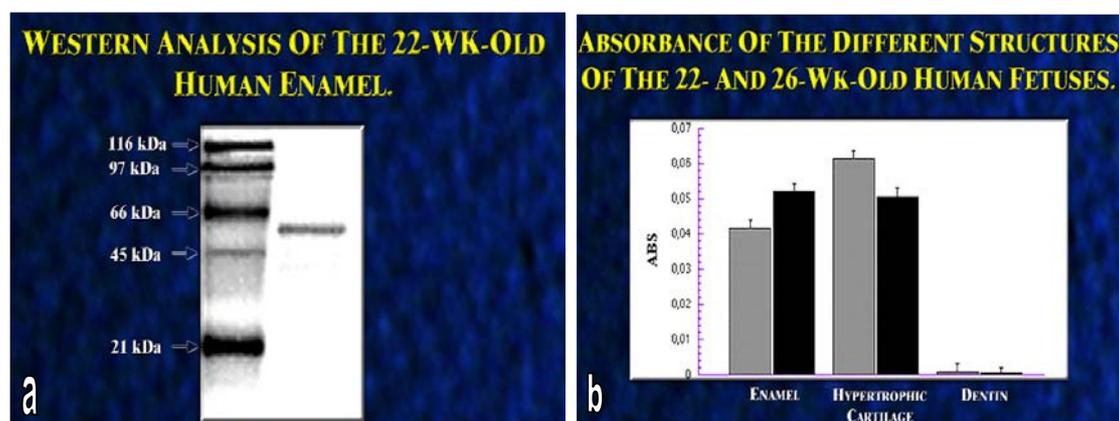


Fig. 15 Analysis of type X collagen in the enamel, dentine and epiphyseal cartilage by ELISA assay and Western blotting

Type X collagen was extracted from the enamel, epiphyseal cartilage and dentine of 22 and 26-wk-old foetuses, and analyzed by ELISA techniques. Mean absorbancies (ABS) and standard error of mean (SEM) of parallel tests are shown after background subtraction (a). The Western blot analysis of an enamel fraction isolated from 22-wk-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

ROLE OF HYALURONAN CD44 DURING TOOTH DEVELOPMENT

Using a specific histochemical reaction for HA detection, immunohistochemistry for CD 44 visualisation and digital analytical technique to describe quantitatively the HA reaction, we detected and measured the changes of HA concentrations at different developmental stages as well the spatial and temporal expression pattern of CD44 in different structures of developing human teeth.

At the early bud stage of development, the dental basement membrane contains abundant HA (Matthiessen et al., 1997). However, the present data show that the HA-positivity undergoes a substantial decrease at subsequent stages of development. It is known that the dental basement membrane at the epithelial-mesenchymal interface plays a role in transmitting signals between the inner dental epithelium and the outermost cells of the ectomesenchymal cells of dental papilla, and this signal transfer is crucial for the differentiation of odontoblasts (Kjoelby et al., 1994). Previous immunohistochemical studies revealed the presence of versican, neurocan and brevican in the dental basement membrane and also in the dental papilla (Yamauchi et al., 1997; Knudson 1993). These proteoglycan molecules belong to the aggrecan family (Knudson and Knudson, 1993). Their G1 domain specifically associates with HA, forming extracellular aggregates. HA may be an integral part of this presumably highly hydrated matrix type.

The extensive expansion of the enamel organ in the bell stage is probably connected to the high content of hyaluronan in the intercellular space of the stellate reticulum, as noted in the present and earlier studies (Matthiessen et al., 1997).

The strongest immunostaining for HA in the dental papilla found in the most active stage of development. The mesenchymal cells of dental papilla, derived from the neural crest, migrate, proliferate and differentiate into odontoblasts and pulp cells. It is likely that HA is not only a structural component of the extracellular matrix but provides instructive signal for cells in migration, proliferation, differentiation, and organogenesis (Scott, 1998). The gradual and age-dependent decrease of hyaluronan content in the dental papilla is probably associated with the stabilization of their differentiated stage and site.

The signal recorded from the dentine tubules indicates that a free extracellular space is necessary to separate the odontoblast processes from the wall of the tubule. A similar finding was observed in osteocyte canaliculi (Nakamura et al., 1996).

Our most interesting result is the strong HA reaction of ameloblasts and enamel in their early development and its nearly complete loss upon further maturation. The HA-positivity of these structures is gradually increased in the fetal period and dramatically dropped postnatally. Ameloblasts secrete and deposit hyaluronan during enamel formation, but it does not appear to be a significant component in mineralized enamel. The magnitude of the HA signal at this site and stage was not recognized in earlier studies. It was observed only along the border between ameloblasts and enamel in developing rat incisors (Chardin et al., 1990), and in close proximity to the ameloblasts in human deciduous tooth germ (Matthiessen et al., 1997). In contrast to this, our results indicate that the fetal enamel contains hyaluronan in its entire width, and the local concentration of HA increases in the fetal period of tooth development.

Amelogenins, enamelin, tuftelin and ameloblastin/amelin are the protein components of enamel. It is believed that these proteins are involved in crystal nucleation, control of growth and orientation (Robinson et al., 1998). Our results suggest that HA is also present in fetal enamel in a considerable amount. However, if it interacts with the other components and is involved in stimulation, inhibition or organization of the mineral deposition remains to be elucidated.

Due to its polyanionic nature, HA binds Ca^{2+} resulting in formation of firm HA gel under in vitro conditions (Prestwich et al., 1998) but has only moderate affinity for hydroxyapatite (Embery et al., 1979) and does not affect hydroxyapatite crystal growth (Boskey et al., 1989). In in vitro experiments, HA proved to be a potent inhibitor of aggregation of hydroxyapatite crystals (Hansen et al., 1976). We suppose that HA plays a space-filling and stabilizing role for the formation of the enamel rods, as previously demonstrated for different cells and fibers (Balázs 1998). We hope that ongoing ultrastructural investigations will contribute to a better understanding of the role of hyaluronan in enamel formation. In addition, further biochemical and morphological studies are needed to elucidate the mechanism of the disappearance of HA at the end of enamel maturation. It is possible, that in parallel with the degradation of enamel proteins by proteolytic enzymes (Moe et al., Moradian-Oldak et al., 1996), hyaluronan is depolymerized by hyaluronidase. However, we cannot exclude the possibility that some HA remains but becomes masked by organic or inorganic components in the densely packed mature enamel structure.

At the early bud stage, while the cells of the oral epithelium expand into the underlying ectomesenchyme intense CD44 signal was expressed in the basal layer of the oral epithelium and in the proliferating cells of the growing dental lamina. The

main function of this receptor is the binding of hyaluronan to this cell layer (Miyake et al., 1990). Previous studies have reported that CD44 is expressed in multiple isoforms on a variety of cell types where it functions as a receptor for hyaluronan-mediated motility (Bourguignon et al., 1999, Yamada et al., 1999). These data suggest that CD44 may also facilitate the migration of the actively dividing cells of the dental lamina throughout the hyaluronan-rich embryonic matrix. Furthermore, the presence of CD44 in the lower layers of the oral epithelium may also be important in the maintenance of the intercellular space by anchoring HA which facilitates the transport of nutrients and metabolites between the multilayered epithelium as proposed by Tammi et al. (1998).

At the apical regions of the 14-, 17- 19-week-old tooth germs, where the presecretory ameloblasts showed a strong reaction, CD44 probably has different functions, not only to bind hyaluronan. At the epithelial-mesenchymal interface, CD44 could act as a signaling receptor, by binding to fibroblast growth factors (Kettunen et al., 1998). In turn, CD44 can mediate this signal to the cytoskeleton (Entwistle et al., 1996). It allows the hypothesis, that CD44 may trigger cytoskeletal rearrangements, which happens in presecretory ameloblast during its maturation into the secretory stage. Extracellular matrix may also modify the gene expression of the cell via the cytoskeleton (Boudreau and Bissell, 1996). It is highly probable, that like other extracellular matrix transmembrane receptors (e.g. integrins) CD44 plays a crucial role in control of cell differentiation.

One of the most interesting results of this study is that the cells of the root sheath do not express CD 44. It is well known that the cells of Hertwig's epithelial root sheath (HERS) contribute to development of the root and periodontal ligament and then disintegrate when the root is formed (Hou et al., 1999). It seems feasible,

that those presecretory ameloblasts, which participate in HERS formation, exhibit only partial characteristics of the presecretory ameloblast phenotype. Therefore it is well possible that these not fully differentiated cells do not synthesize CD44. Other possible explanation for the missing CD44 signal on the cells of the HERS that they contribute to the strong accumulation of the hyaluronan surrounding the space of the rapidly growing root sheath, as it was reported in earlier studies (Matthiessen et al., 1997, Felszeghy et al., 2000). This assumption is supported by data showing that the function of this transmembrane glycoprotein is the catabolic uptake of hyaluronan (Borland et al., 1998).

The enamel secreting ameloblasts exhibited a strong CD44 reaction, a finding in line with previous experiments (Nakamura et al., 1995, Nakamura et al., 1997, Yu et al., 1997). Here it may act as an adhesion molecule to maintain the integrity of this layer via interactions with other cell surface and basement membrane proteins such as fibronectin and laminin (Yoshida et al., 1998). The presence of CD44 on the plasma membrane of the odontoblasts, on their Tomes processes and on the apical plasma membrane of secretory ameloblast suggest that CD44 may be involved in the maintenance of the temporary HA-matrix on these cells (Nakamura et al., 1997, Felszeghy et al., 2000).

Additionally, osteoblast and osteocytes and their processes expressed abundant CD44, which is consistent with earlier studies (Nakamura et al., 1997), where CD44 was shown to play an important role in osteogenesis by forming a highly hydrated HA-matrix in the pericellular space of these cells.

In conclusion, using a monoclonal anti-human antibody Hermes 3, we have demonstrated for the first time the spatial and temporal distribution of CD44 during human tooth development and discussed its possible roles as a regulator of HA

content and metabolism in the developmental processes. Our data presented above come mainly to an understanding with earlier CD44 experiments carried out on different animal tissues. Having better information insight to the function of hyaluronan during the human tooth development we are planing ultrastructural investigations on HA and CD44 distribution in these human specimens

ROLE OF TYPE X COLLAGEN DURING ENAMEL MINERALIZATION

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.

Here we 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 temporally expressed so that its content is highest during the foetal 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, enamelines, tuftelin and ameloblastin/amelin (Robinson et al., 1988; Fincham et al., 1999) 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. 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 (Hunter et al., 1996). These molecules have been localized in tooth cementum or dentin, and their temporal and spatial

distribution correlates well with the process of mineralization (McKee et al., 1996), but these molecules are not detected in the enamel.

Our results suggest that type X collagen is also present in foetal enamel in considerable amount. However, if it interacts with the other components of the enamel and is involved in organization of the mineral deposition remains to be elucidated. Whether this interaction would be a direct involvement at the level of mineral nuclei or some concomitant process is not clear. As such, it is not so unexpected 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 (Kwan et al., 1991). The collagen fibrils in this network seeded with calcium ions may produce a structure, which provides an optimal template for mineral deposition (Apte and Olsen, 1993; Iwamoto et al, 1994).

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 (Kwan et al., 1997), 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.

SUMMARY

The cells that contribute to the enamel organ are derived from the oral epithelium whereas the mesenchymal cells of the dental papilla are derived from cranial neural crest cells. Human tooth development is a highly regulated process that requires coordination of a number of cell-cell and cell-matrix interactions.

Hyaluronan (HA) is an extracellular glycosaminoglycan found ubiquitously throughout the developing extracellular matrix. Previous observations suggest that HA is involved in a wide variety of cellular events such as cell migration, proliferation, and differentiation which have an important role in morphogenesis. Many of the cellular and matrix effects of HA are thought to be mediated via the interaction with different receptor proteins referred to as hyaladherins. One well known member of this group is the cell surface receptor CD44.

According to our current knowledge, type X collagen is a product of terminally differentiated chondrocytes in regions of cartilage that undergo endochondral ossification. 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 the endochondral ossification of the epiphyses.

Only few studies have examined the potential importance of HA and CD44 during tooth germ development. These studies were carried out mainly on different animal tissue samples or no comprehensive data were collected from different developmental stages of the human samples. The present work was undertaken to fill the remaining gaps in our knowledge about the temporal and spatial patterning of HA, CD44 during human tooth development. Since there is no data available on the

presence or absence of the type X collagen in dental structures we investigate the expression pattern of this molecule during human tooth development.

The distribution pattern of hyaluronan (HA) and its major receptor CD44 was studied by using a biotinylated HA-binding complex and anti-human CD44 MoAb. The expression level of HA was quantitated by digital image analysis. Using anti-human type X collagen MoAb immunohistochemistry, ELISA and Western immunoblot technique was carried out to investigate the expression of type X collagen during human tooth development.

Results: (1) At the cap stage, dental papilla exhibited a moderate HA-staining, while intense reaction was observed in the apical portion of presecretory ameloblasts, stellate reticulum, and in dental basement membrane. When the enamel matrix started to develop, a strong HA reaction was evident in the young enamel and the apical portion of secretory ameloblasts. No hyaluronan could be detected in the secretory ameloblasts and enamel matrix of the 9-wk-old postnatal stage. (2) The distribution of CD44 in the human tooth germs corresponds to that of hyaluronan in most locations. At the cap stage, the dental lamina displayed a strong CD44 signal. In contrast, the external enamel epithelium was negative. At the apical region of the tooth germ the presecretory ameloblasts showed an intense reaction, whereas there was no signal at the growing root sheet where these cells meet the external enamel epithelium. In the stellate reticulum, a delicate reaction was detected. The secretory ameloblasts and the stratum intermedium showed a strong cell surface CD44 signal. A strong signal was also observed on the odontoblasts and their Tomes processes. (3) Intense immunohistochemical staining for collagen type X was observed in the enamel matrix and in the apical parts of secretory ameloblast when the enamel matrix was already under formation. In the 9-week-old postnatal stage, the staining for collagen type X in

the enamel matrix was diminished, and only a weak signal could be detected in the secretory ameloblasts. 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 detected molecule is characteristic of the type X collagen.

Our results suggest different possible roles of HA played in human tooth development as follows:

(a) HA may facilitate the migration of the actively dividing cells of the dental lamina throughout the embryonic matrix.

(b) The age-dependent decrease of HA content in the dental structures is probably associated with the stabilization of their differentiated stage and site.

(c) HA may be necessary to separate the Tomes processes from the wall of the dentin tubule.

(d) In the early phase of enamel matrix development HA may play space-filling and stabilizing role for the formation of the enamel rods.

(e) Since the expression pattern of CD44 corresponds to that of hyaluronan in most locations, suggesting that this transmembrane protein play a crucial role in hyaluronan-mediated events during human tooth development.

Since type X collagen was detected in the maturing enamel, it is highly possible that it may influence enamel crystal formation during amelogenesis. Whether this is a direct involvement at the level of mineral nuclei or some concomitant process is not clear and remains to be elucidated. Since the type X collagen can form hexagonal networks, we suggest that collagen fibrils in this network seeded with calcium ions may produce a structure, which provides an optimal template for mineral deposition.

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