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In Vivo and In Vitro Expression of Connexin 43 in Human Teeth

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Gap junctions are composed of transmembrane proteins belonging to the connexin family. These proteins permit the exchange of small regulatory molecules directly between cells for the control of growth, development and differentiation. Although the presence of gap junctions in teeth has been already evidenced, the involved connexins have not yet been identified in human species. Here, we examined the distribution of connexin 43 (Cx43) in embryonic and permanent intact and carious human teeth. During tooth development, Cx43 localized both in epithelial and mesenchymal dental cells, correlated with cytodifferentiation gradients. In adult intact teeth, Cx43 was distributed in odontoblast processes. While Cx43 expression was downregulated in mature intact teeth, Cx43 appeared to be upregulated in odontoblasts facing carious lesions. In cultured pulp cells, Cx43 expression was related to the formation of mineralized nodules. These results indicate that Cx43 expression is developmentally regulated in human dental tissues, and suggest that Cx43 may participate in the processes of dentin formation and pathology.

Keywords Connexin 43, Carious, Human, Tooth, Development, Differentiation, Mineralization.

INTRODUCTION

Gap junctions are specialized regions of the cell membrane forming channels between neighboring cells. These channels permit the passage of ions and small molecules up to 1 kD between cells [1, 2] and therefore direct cell–cell communication. In excitable tissues (i.e., heart muscle), gap junctions mediate a rapid propagation of electrical signals as well as synchronization of cell activity [3]. In nonexcitable tissues, gap junctions mediate the cell to cell traffic of metabolites and may carry signaling molecules for the control of cell growth and differentiation, as well as organ formation [4–10]. Gap junctions are composed

of transmembrane proteins that belong to the connexin family. Experimental studies have shown that the inhibition of gap junction communication with antibodies to connexins results in the disruption of embryonic development [5, 11]. Furthermore, mutation of the connexin 43 was lethal due to heart formation failure [12].

Tooth development is also under the control of signaling molecules: Sequential and reciprocal interactions between the epithelium and mesenchyme lead to the differentiation of mesenchymal cells into odontoblasts and epithelial cells into ameloblasts. Odontoblasts secrete the dentin matrix while ameloblasts synthesize enamel matrix [13–15]. Mineralization takes place at the late bell stage. A differentiation gradient is observed, where undifferentiated cells are located at the cervical loop area, while differentiated cells are located in the cusp area.

Gap junctions have been reported previously to be present during odontogenesis inside the enamel organ and dental mesenchyme, respectively [16–19]. Freeze fracture and electron microscopy studies in human teeth have revealed the presence of gap junctions between odontoblasts [20, 21] and between odontoblasts and subodontoblastic cells [22].

Connexin 43 is involved in the formation of gap junctions in the developing rodent teeth [23–25]. In addition to Cx43, connexin Cx32 and connexin Cx26 are expressed in epithelial dental cells and mature odontoblasts of rats, respectively [25].

However, the specific connexins involved in the formation of gap junctions between dental cells have not been systematically explored in human species. This work was carried out to investigate the expression of connexin 43 in human teeth under normal and pathological conditions.

MATERIALS AND METHODS

Materials

Embryonic Teeth

Teeth obtained from 17- and 22-week-old fetuses were collected from 10 medical inductions from the department of

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Anatomy and Pathology (Timone Hospital, Marseilles, France). The samples were obtained in agreement with the French ethical laws and after obtaining the ethical committee agreement. The gestation age was estimated from the fetal foot length and from the last menstruation of the mother. At each age, five central incisors, five canines, and five first molars were used.

Permanent Teeth

Teeth used in this study were (a) six third molars extracted during normal orthodontic treatment of adolescents (mean age = 17 ± 1 years), and (b) six mature intact and six carious teeth (mean age = 40 ± 2 years).

Antibodies and Reagents

Anti Cx43 polyclonal antibodies were raised in rabbits against synthetic peptides and purified by affinity chromatography. These antibodies have been previously characterized [26–28] and cross-react with human tissues [26].

For the preparation of the culture media, materials were purchased from Gibco BRL (Life Technologies, Inc., Grand Island,

NY). Minimum essential medium (MEM) was supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin (Biowhittaker, Gagny, France), and 0.25 $\mu\text{g/ml}$ amphotericin B (Fungizone).

Methods

Embryonic Teeth

The teeth were immediately fixed at 4°C for 1 h in 1% paraformaldehyde prepared in phosphate-buffered saline (PBS) (Sigma Chemical Co., St. Louis, MO). They were then dipped overnight at 4°C in a 15% sucrose PBS solution. Samples were embedded in tissue-Tek O.C.T. compound (Miles, Elkhart, IN) and frozen over liquid nitrogen without prior decalcification. Serial cryostat sections (10 μm thick) were mounted on Superfrost Plus slides (CML, Nemours, France), dried at room temperature for 1 h, then processed for indirect immunohistochemistry. Mayer's hematoxylin staining was performed on some sections for histologic examination.

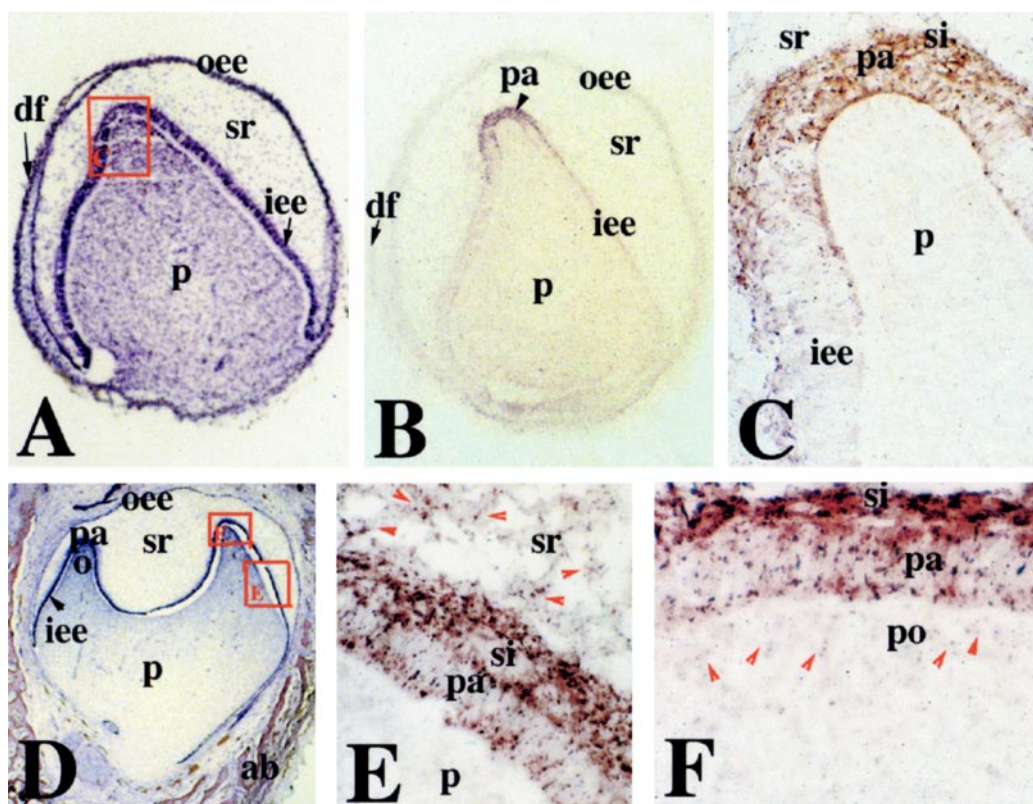


Figure 1. Immunohistochemical localization of Cx43 in embryonic human tooth germs at the bell stage of development: (A–C) Canine tooth germ of a 17-week-old embryo. (D–F) First molar tooth germ of a 22-week-old embryo. (A) Mayer's hematoxylin staining showing the different cell populations ($\times 4$). The framed area represents the portion in (C). (B) A gradient of Cx43 immunostaining: Strong labeling is evident in preameloblasts (pa) and faint immunostaining is present in inner enamel epithelium (iee) and stellate reticulum (sr) cells ($\times 4$). (C) Higher magnification of the cusp area showing the expression of Cx43 in preameloblasts and stratum intermedium (si) cells, $\times 40$. (D) Mayer's hematoxylin staining of a human first molar tooth germ at the bell stage ($\times 4$). The frames indicate the sites of panels (E) and (F). (E) Strong Cx43 immunostaining is detected in preameloblasts, stratum intermedium, and stellate reticulum (arrowheads) cells, $\times 40$. (F) In the cusp area, weak Cx43 immunostaining is also found in preodontoblasts (po; arrowheads); $\times 60$. Abbreviations: iee, inner enamel epithelium; si, stratum intermedium; sr, stellate reticulum; oee, outer enamel epithelium; df, dental follicle; p, pulp; pa, preameloblast; po, preodontoblast; ab, alveolar bone.

Permanent Teeth

After extraction, intact and carious teeth were fixed in 10% neutral buffered formalin for 7 days, demineralized in sodium formiate for 21 days, and then routinely processed and embedded in paraffin wax. They were then serially sectioned (6- μ m-thick sections) and processed for immunohistochemistry.

Culture of Human Pulp Cells

Cultures were performed as previously described [29]. Briefly, after the extraction, the teeth were swabbed with 70% (v/v) alcohol and then washed with sterile PBS. They were then transferred into a laminar flow tissue culture hood in order to perform the rest of the procedures under sterile conditions. The apical part of the teeth was removed and the dental pulps were minced with scalpels and rinsed with PBS. Each dental pulp was divided into two groups: cultured without (control) or with β -glycerophosphate. In the control group, the explants were cultured in 100-mm-diameter culture dishes (Becton Dickinson Labware, Lincoln Park, NJ) in MEM medium. In the second group, the explants were cultured under the same conditions in the same medium supplemented with 2 mM β -glycerophosphate (Sigma Chemical Co., St. Louis, MO). The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂, 95% air. Confluent cultures were collected by trypsinization (0.2% trypsin and 0.02% ethylenediamine tetraacetic acid (EDTA)) and subcultured.

Immunohistochemistry on Sections and on Cell Cultures

Cultured cells were fixed with 70% ethanol for 1 h at 4°C and permeabilized for 15 min with 0.5% Triton X-100 in PBS prior to immunohistochemistry. The endogenous peroxidase activity was blocked with 3% H₂O₂ for 5 min. Immunoperoxidase staining on sections and cell cultures were done as previously described [29–30]. Paraffin sections were first deparaffinized, while cryostat sections were immediately exposed to a 0.3% solution of hydrogen peroxide in methanol and rinsed in PBS. After blocking with PBS containing 1% bovine serum albumin (BSA), the slides were rinsed in PBS and then incubated overnight at 4°C with the anti-Cx43 serum (2 μ g/ml) diluted in PBS containing 0.2% BSA. Secondary antibodies were affinity purified with goat anti-rabbit peroxidase-labeled immunoglobulins. Peroxidase was revealed by incubation with 3-amino-9-ethylcarbazole (AEC) reaction solution and then the slides were mounted with Glycergel (Dako Corporation, Carpinteria, CA).

Controls were performed by omitting the primary antibodies or the utilization of preimmune sera at the same dilution and in the same manner as the connexin 43 antibody.

RESULTS

After the extraction of the tooth germs, the histological examination showed that they were at the bell stage (Figure 1).

In the canine and central incisor tooth germs of a 17-week-old embryo, weak Cx43 immunostaining was seen in cells of the inner dental epithelium, stellate reticulum and stratum inter-

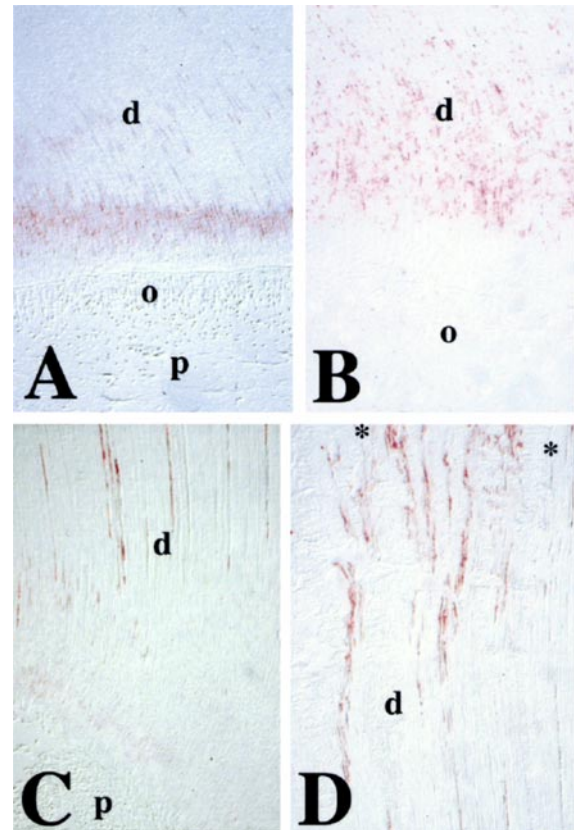


Figure 2. Immunohistochemical localization of Cx43 in intact and carious permanent human teeth. (A) In intact teeth of a 17-year-old adolescent, Cx43 immunostaining is detected in the odontoblastic processes; $\times 10$. (B) Higher magnification of (A); $\times 40$. (C) In carious teeth of 40-year-old adults, Cx43 immunoreactivity is detected in odontoblastic processes facing the carious lesion front; $\times 10$. (D) Strong Cx43 immunostaining is found in the odontoblastic processes approaching the carious front (asterisks); $\times 10$. Abbreviations: d, dentin; o, odontoblasts; p, pulp.

medium. A staining gradient was observed in the inner enamel epithelium from the cervical loop to the cusp region: the cervical loop was negative for Cx43, while the immunostaining increased toward the cusp region (Figure 1, A–C), where inner enamel epithelial cells differentiate into preameloblasts.

In the first molar of a 22-week-old embryo, Cx43 was detected in the stratum intermedium, stellate reticulum, and preameloblasts. Moreover, Cx43 staining was found in dental mesenchyme and differentiating odontoblasts (Figure 1, D–F).

In the 17-year-old developing third molars, Cx43 expression was restricted to the odontoblast processes but was not observed in the odontoblast cell bodies (Figure 2, A and B).

Connexin 43 expression in mature intact and carious teeth showed a distinctive pattern. While the labeling was completely absent in mature control samples, Cx43 immunoreactivity was observed in carious teeth as shown here (see Figure 2, C and D). The labeling was restricted to the cells located in the vicinity of carious lesions. There, Cx43 was distributed in the processes of mature odontoblasts (Figure 2, C and D).

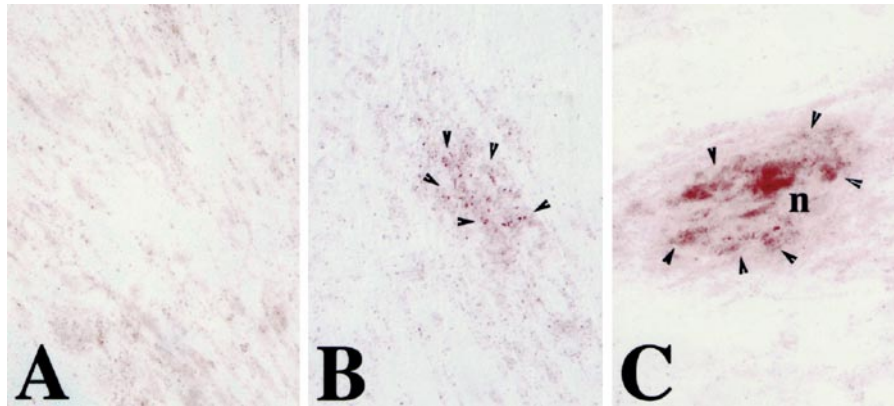


Figure 3. Cx43 immunoreactivity in cultured human dental pulp cells in vitro after β -glycerophosphate treatment. (A) Weak Cx43 immunostaining is observed in cultures of pulp cells; $\times 20$. (B) Cx 43 labeling is evident in aggregated cells involved in the formation of mineral nodules (arrowheads) $\times 20$. (C) Increased Cx43 immunostaining in cells involved in mineral matrix formation and deposition; $\times 20$. Abbreviation: n, nodule.

Connexin 43 Expression in Human Dental Pulp Cells In Vitro

This present study was based on an established primary culture of human dental pulp explants where several molecular (collagen I, osteonectin, and nestin) and mineral (Fourier transform infrared microspectroscopic analyses) forms of evidence show the formation of dentin mineralized nodules in vitro (see refs. 29 and 30). Faint Cx43 immunoreactivity was observed in monolayered pulp cells (Figure 3A), while clear immunolabelling appeared to be associated with aggregated cells during the initial stages of mineralized nodule formation (Figure 3B). Furthermore, Cx43 labeling was even more clearly evidenced and intense in well-formed mineralized nodules (Figure 3C).

DISCUSSION

This work shows the distribution of Cx43 in developing and adult human teeth. During teeth development, Cx43 is expressed in epithelial cells (i.e., preameloblasts, stratum intermedium, stellate reticulum) and differentiating odontoblasts. These results are in agreement with previous results [23, 24] in rodent dental tissues, showing that Cx43 is expressed in both epithelial and pulp cells. Cx43 expression in dental tissues is correlated to cytodifferentiation gradients. Gradients of Cx43 expression have been also reported in the developing cardiac muscle [31]. The precise role of gap junctions in such gradients remains unclear, but in the developing teeth, these gradients correspond to the differentiation status of precise dental cell populations.

The interface between the inner enamel epithelium and the dental pulp displays various changes of basement membrane components, such as type IV collagen, laminin, fibronectin, and proteoglycans, at the region of differentiating odontoblasts [32, 33]. In areas where cytodifferentiation starts, the preexisting basement membrane components may be degraded and removed and new components must be deposited. Therefore, the expression of Cx43 in both pulp and inner enamel epithelial cells and the

gradient of Cx43 toward their differentiated stages may have some role in the differentiation of these cell types.

Cx43 in Developing and Intact Adult Human Teeth

Cx43 was upregulated in pulp cells differentiating into odontoblast. Cx43 was predominantly expressed in the cuspal region of the tooth pulp. Previous studies in cat teeth showed that when the fluorescent lucifer yellow dye was introduced to odontoblasts, some subodontoblastic cells were fluorescent in addition to the odontoblastic cells, indicating that the gap junctions between the odontoblasts and the subodontoblast cells are functional [34]. Cx43 is expressed in the stratum intermedium. Few studies concerning the stratum intermedium are available, and its functional role is not known. It is believed that this layer may be essential to enamel formation, because it is absent in the part of the tooth germ that outlines the root portions of the tooth, but does not form enamel [35]. These data suggest that expression of Cx43 may play a role in both amelogenesis and dentinogenesis of human teeth.

In permanent adolescent teeth, Cx43 is detected in the odontoblast processes. Ramifications exist between the dentinal tubuli [36], permitting the contact of either odontoblast processes or nerve fibers and odontoblast processes as suggested by Holland [19]. This may indicate that connexins are involved in the direct communication between odontoblasts and/or odontoblasts and nerve fibers. In permanent adult teeth, Cx43 is downregulated. Since gap junctions are detected in adult human tissues [20, 21], this suggests that other connexins are upregulated in adult dental tissues. As a matter of fact, it has been reported that Cx32 was also expressed in epithelial cells during the late bell stage of rodent tooth development and that Cx26 was expressed in odontoblasts of the adult rat teeth [25].

Cx43 in Irritated Adult Human Teeth

Microorganisms are involved in both decalcification and proteolysis of the dentin during the process of dental caries. In

response to this irritation, the secretory activity of the odontoblasts is stimulated to elaborate reactionary dentin [37] and the tubuli become calcified. Cx43 is reexpressed in the processes of odontoblasts surrounding the carious lesion, suggesting a role for Cx43 in the hypercalcification process.

We have shown recently that dentin can be produced in vitro from cultured human pulp cells [29]. Cx43 is weakly expressed in these cells, but its expression is upregulated during mineralization processes. This is in accordance with previous findings showing increased calcium content of the extracellular matrix and enlarged mineralization nodules in osteoblastlike cells transfected with Cx43 cDNA [38]. The fact that Cx43 is important in mineralization processes has been evidenced by recent data showing that Cx43 deficiency results in delayed ossification, craniofacial abnormalities, and osteoblast dysfunctions. This is illustrated by the fact that Cx43-null mice exhibit reduced expression of specific genes and stunted mineralization in vitro, indicating that Cx43 is involved in osteogenesis and normal osteoblast functions [39]. These results suggest that Cx43 may also be involved in the secretory activity of odontoblasts in dentin production and mineralization both during tooth development and after dental injury.

In a model system where the rat teeth are forced to move (orthodontic forces), expression of Cx43 has been shown in osteoclasts and periodontal ligament cells in compression zones, and in osteoblasts and osteocytes in tension zones of the periodontal ligament. These results support the hypothesis that Cx43 may play a role in the coordination of an alveolar bone remodeling [40]. Cx43 may also play a role in the synchronization of mineral matrix deposition by odontoblasts in human teeth.

In conclusion, Cx43 expression in human dental tissues is correlated with cytodifferentiation and mineralization processes during both normal and pathologic conditions.

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