Fluoride alters type I collagen expression in the early stages of odontogenesis

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Fluoride alters the expression and post-translational modifications of extracellular matrix proteins in dentin. The aim of our study was to determine the effects of fluoride on type I collagen expression during the early stages of tooth germ development in rats. Pregnant dams were divided into three groups and fed a standard diet. From the fifth day of pregnancy the three groups received tap water with, respectively, trace amounts of fluoride (C), a low fluoride concentration (F₁) or a high fluoride concentration (F₂). Changes in type I collagen expression and distribution were evaluated. The expression of type I collagen was restricted to the extracellular spaces of cells of mesenchymal origin. In the youngest animals the most intense immunoreactivity for type I collagen was detected in predentin of the F₁ group. Although the intensity of immunostaining increased in proportion to the age of the animals, the largest increase in the groups investigated was detected in the F₂ group. We concluded that a low concentration of fluoride can act as a stimulator of type I collagen deposition in the extracellular matrix of dentin, while high concentrations of fluoride have an opposite effect, acting as an inhibitor of type I collagen formation in dentin.

Key words: dentin, tooth germ development, rat

INTRODUCTION

In the early stages of tooth germ development, the formation and mineralisation of dentin is regulated by events occurring within the extracellular matrix [35]. The major structural protein of the extracellular matrix of dentin is type I collagen, making up 90% of organic matrix proteins. Its interactions with other proteins are known to play a crucial role during odontogenesis. Specifically, small amounts of type V collagen [6], another fibrillar type of collagen, interact with type I collagen to form an organic scaffold found within the dentin [32, 37]. This structure is essential to the mineralisation process that needs to occur during the development and maturation of dentin. Despite the fact that the mineralisation of dentin occurs within the type I collagen network, type I collagen fibrils alone are not able to initiate spontaneous crystal formation. The extracellular matrix of developing dentin needs first to undergo structural rearrangement to create compartments for mineral deposition [14, 29, 39]. This is achieved by non-collagenous acidic matrix proteins that account for the remaining 10% of the organic matrix proteins of dentin. They bind to the surface of type I collagen fibrils and reside in the 67 nm gap regions [13, 16]. The dentin formation and mineralisation therefore reflects the interactions between type I collagen and type V collagen, non-collagenous acidic proteins and many other specific enzymes [9, 19, 20, 33]. All of the extracellular matrix components

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of dentin are susceptible to fluoride exposure [2, 10–12, 34]. Fluoride has been recognised as an important factor that affects the mineralisation of dentin, resulting in a mixture of hypo- and hypermineralised tissue strips [38].

Controversy remains regarding fluoride’s action on dentin-producing mesenchymal cells. Veron et al. [36] reported that a 0.6 mM (25 ppm) concentration of sodium fluoride in culture medium significantly reduced the growth of dental pulp fibroblasts, while a 1.2 mM (50 ppm) concentration had a lethal effect. Others, such as Moseley et al. [26], reported that 6 mM (250 ppm) of sodium fluoride added to the organ-culture of rat dentin-pulp did not change the number of odontoblasts and pulp fibroblasts but affected protein synthesis and composition by significantly reducing the concentration of hydroxyproline incorporated into collagen fibrils [27]. This mechanism was confirmed by in vitro and in vivo studies by Helgeland [15]. Sharma [31] suggested that the decrease in collagen formation is a result of fluoride-dependent inhibition of collagen fibre maturation and an increase in collagen degradation.

It has also been reported that fluoride inhibits the expression of both α1(I) and α2(I) procollagen chains; however, the specific mechanism of fluoride action has not yet been determined [36]. Some authors have also suggested that the early phase of dentinogenesis is vulnerable to excessive amounts of fluoride [17, 23, 30]. As type I collagen is the first protein synthesised and secreted by mesenchymal lineage cells during tooth development, the aim of our study was to follow the potential changes in the expression and/or localisation of type I collagen in early tooth development. The first lower molar tooth germ of rats was used as an animal model for investigating changes in the early phase (the 1st–5th days of development) of intracellular matrix formation under various fluoride conditions.

**MATERIAL AND METHODS**

### Animals

Animal care and treatment guidelines outlined by the European Community Council (1986) and protocols approved by the local ethical committee were followed.

Nine pregnant albino Wistar rats were randomly divided into three equal groups: control (C), those supplemented with a low concentration of sodium fluoride in drinking water (F), and those supplemented with a high concentration of sodium fluoride in drinking water (F). Dams were fed a standard diet containing a trace amount of fluoride. From the fifth day of pregnancy until the end of the experiment the concentration of the water drunk by the three groups was as follows: control group — 0.16 F mg/L (tap water); F, group — tap water with 11 mg/L of NaF (11 ppm); F, group — tap water with 110 mg/L of NaF (110 ppm). Sodium fluoride concentrations followed the protocol used in our previous investigations [22, 23] and that of others [24]. The high sodium fluoride concentration was calculated according to the mean body weight of the dams (22.6 mg NaF/kg b.w) and was the maximal dose at which the risk of spontaneous abortion or preterm delivery was smallest [1]. Water consumption was monitored daily. After delivery mothers and their offspring underwent the same water regimen. The experiment was performed three times.

### Tissues

During each experimental set of five consecutive days beginning from day one three pups from each group were deeply anaesthetised with Nembutal (80 mg/kg i.p. body weight) and later perfused through the ascending aorta with a physiological solution of saline (pH 7.4) containing heparin. The animals were then perfused with 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4; 4°C). They were dehydrated in 15% sucrose followed by 30% sucrose (both in 0.1 M phosphate buffer at pH 7.4 and 4°C) until equilibrium. Next all heads were frozen and sectioned in a sagittal plane using a cryostat (Young 1800; Leica, Germany). 16-µm-thick serial sections were obtained and every sixth section in each section set was routinely stained with haematoxylin and eosin (H&E), air-dried, and cover-slipped using the DPX mounting medium (Fluka, Germany).

### Immunohistochemistry

Sections were preincubated in 10% normal goat serum and 0.3% solution of Triton X-100. They were then incubated overnight at room temperature with mouse monoclonal anti-type I collagen antibodies diluted 1:200 (Sigma, USA). Subsequently, they were incubated in secondary antibodies (goat anti-mouse) conjugated with Cy-3 (Jackson, USA) at 1:600 for 1.5 h at room temperature. Sections were rinsed in PBS, mounted on slides, dried, and cover-slipped with Kaiser gelatin (Merck, USA). In the control sections the primary antibodies against type I collagen were omitted and replaced by 1% BSA buffer. In all the
groups investigated the control sections showed negative immunoreactivity to type I collagen (data not shown).

**Western blots**

Heads were cut in half in the median plane, mounted in cryostat and cut sagittally until the tooth germ of the first lower molar appeared. The whole tooth germ of the first lower molar was removed from the mandible under a microscope. One particular set was composed of both right and left first lower molar tooth germs. The tissue was manually homogenised in ice-cold buffer (20 mM Tris-HCl pH 7.5 containing 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA) containing protease inhibitors (1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin A). The protein concentration was determined using the Lowry method (Bio-Rad, USA). Equal amounts of protein extracts (40 μg) were separated on 8% polyacrylamide gel (SDS-PAGE) and transferred onto a nitrocellulose membrane (Bio-Rad, USA). After being blocked with 3% non-fat dry milk (Bio-Rad, USA) overnight, the membranes were probed with mouse monoclonal antibodies against type I collagen (Sigma, USA) for 2 hours (at a dilution of 1:1500). Rabbit anti-mouse peroxidase-conjugated IgGs (Sigma, USA) were used as a secondary antibody (2 h incubation at a dilution of 1:30,000). The membranes were then incubated with SuperSignal West Pico chemiluminescent substrate for peroxidase (Pierce, USA) and exposed to autoradiographic film. After exposure the blots were stripped with the use of “Restore” stripping buffer (Pierce, USA) and probed for β-actin, which was used as a reference protein. Mouse monoclonal antibodies anti-β-actin (Sigma, USA) were used as primary antibodies (2 h incubation at a dilution of 1:15000) and rabbit anti-mouse peroxidase-conjugated IgGs (Sigma, USA) were used as secondary antibodies (2 h incubation at a dilution of 1:50,000). Blots were again incubated with peroxidase substrate and exposed to autoradiographic film. Developed X-ray films were scanned and analysed by means of the LabWorks v.4.5 software (UVP, USA).

**Data analysis**

The immunohistochemically stained sections were examined with confocal laser microscopy (Radiance 2100; Bio-Rad, UK). The system was equipped with Krypton/Argon lasers mounted on an Eclipse 600 (Nikon, Japan) microscope, using the LaserSharp 2000 v.4.0 software (Bio-Rad, UK). The confocal laser scanning microscopy (CLSM) images were obtained using 20× and 60× oil immersion objective lenses of NA = 1.3 and 1.4, respectively. Statistical analysis was performed by means of the computer program Statistica v.5.0 (Statsoft, USA) and Excel 2000 (Microsoft, USA) in the following manner. All calculations were performed on spreadsheets. Data from the test fields were collected from each case and the mean value calculated. These averaged values were used as raw data for statistical analysis. For assessment of the significance of the specific a priori sequence in type I collagen immunoreactivity levels the non-parametric Jonckheere test for ordered alternatives was applied to transformed data for the C, F, and F1 groups. The significance level was set at p = 0.05.

**RESULTS**

**Immunohistochemical staining**

Positive immunostaining for type I collagen was restricted exclusively to the extracellular matrix of cells of mesenchymal origin. No staining was detected in any epithelial structures of the enamel organ; however, the F1 group exhibited slightly higher background staining.

In the early age group (newborn and one-day-old pups) (Fig. 1A–C), positive staining for type I collagen was detected in the extracellular matrix of preodontoblasts, young odontoblasts, selected cells of dental pulp and predentin. This staining was detected in all three groups. Blood vessels located in dental pulp exhibited intense immunostaining. In general, the staining for type I collagen appeared weak in the intercellular spaces between the lateral cell borders of the odontoblastic cell lineage. This staining pattern was more intense in the control group (Fig. 1A) than in the fluoride-treated (both F and F1) groups (Fig. 1B, C).

In the middle age group (of two and three days old) (Fig. 1D–F), the positive staining for type I
Figure 1. Type I collagen localisation in the youngest (A, B, C) and middle age (D, E, F) first molar tooth germ (cusp tip) from A, D — control groups; B, E — F₁ groups; C, F — F₂ groups. A, D — the control groups: Predentin (pd) stains moderately. Intercellular spaces between the lateral borders of odontoblasts (o) stain weakly. Regions accompanying blood vessels in the dental pulp (dp) stain strongly. B, E — the F₁ groups: Predentin (pd) stains strongly. Mineralising dentin accompanying dental tubules (dt) stains moderately. Spaces between the lateral borders of odontoblasts and regions of dental pulp surrounding blood vessels stain weakly. C, F — the F₂ groups: Predentin stains weakly; however, in the younger group (C) the staining is more intense than that in the older group (F). Mineralising dentin (d) is negative. Spaces surrounding the lateral borders of odontoblasts stain very weakly. Dental pulp (dp) stains moderately; a — ameloblasts; ab — alveolar bone. Scale bar 100 µm.
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collagen appeared to be stronger in all the groups examined than in the younger animals. The most intense reaction was detected in the predentin of all the groups examined; however, in the control group (Fig. 1D) the predentin layer was narrower than in the groups treated with fluoride (Fig. 1E, F). The intercellular spaces between preodontoblasts and young odontoblasts as well as the extracellular matrix surrounding the dental pulp cells stained weakly in all three groups.

In the oldest age groups (four and five days old) (Fig. 2A–F), the immunoreactivity between the groups investigated (C, F, and F) became more distinct. In general, type I collagen stained more intensely than for the younger animals. The strongest reaction for type I collagen was evident in the predentin of the F group (Fig. 2C, D). At this later age deposition of predentin was detected not only at the cusp tip region but also at the bottom of the grooves.

In routine H&E preparations the predentin of the F group was much thicker than that of the C and F groups or the F groups of younger animals. In all three groups the mineralising dentin was detected merely at the cusp tip region of the first lower molar tooth germ. Dentin showed a weak reaction for type I collagen; however, this staining was expressed more intensely in the vicinity of odontoblast processes and at the mineralisation front. The reaction for type I collagen decreased towards the dentin-enamel junction and was proportional to the progress of dentin mineralisation. This was visible in all the groups examined; however, the staining was most intense in the group treated with low fluoride concentration. The intercellular spaces between odontoblasts in the F group (Fig. 2E, F) and control (Fig. 2A, B) groups were slightly positive for type I collagen but not as evidently so as those in the F group (Fig. 2C, D). Surprisingly, a very strongly positive reaction for type I collagen was detected at the dentin-enamel junction in the F group (Fig. 2C), which appeared as a thin but extremely bright line of staining.

**DISCUSSION**

In this series of experiments we have examined changes in the deposition of type I collagen in the first lower molar tooth germ of rats supplemented with different concentrations of sodium fluoride during prenatal and postnatal life. Since fluoride has demonstrated an adverse effect on type I collagen metabolism, we focused our study on the early phases of the dentin matrix deposition. The distribution pattern and quantity of type I collagen were compared between particular groups from birth until the 5th day of age.

**The distribution of type I collagen in the first lower molar tooth germ**

Consistently with the observations of other investigators [5, 18, 21], we observed the typical distribution pattern of type I collagen in all the groups examined. Thus the signal was strongest in the predentin and dentin and considerably weaker in the intercellular spaces between preodontoblasts and odontoblasts and in the extracellular matrix surrounding dental pulp cells. Pretreatment with trypsin did not affect the intensity of the low signal from immunostaining; this may therefore be related to the masking effect of antigenic determinants [21].

**Western immunobLOTS**

Western immunobLOTS were used to assess the relative quantities of type I collagen in the first lower molar tooth germ of rats treated with various concentrations of sodium fluoride. In order to verify the differences between particular groups, we analysed all 18 samples from each age and treatment group; however, for the sake of clarity only the results from six representative groups will be discussed. These include all three samples P1C,FL,FH from the youngest (newborn) and three samples (P5C,FL,FH) from the oldest (five-day-old) animals.

Type I collagen was detected in all six samples (Fig. 3). The largest amount of type I collagen was detected in the sample of newborn rats treated with a low fluoride concentration (P1FL). The amount of type I collagen was lower in the P1C and P1FH samples respectively. In the sample of cerebral cortex used as a negative control the signal for type I collagen was almost undetectable.

In five-day-old animals the amount of type I collagen in all three samples (P5C,FL,FH) was increased in comparison with the newborn rats. The largest increase was demonstrated by the P5FL group.

The amount of type I collagen was greatest in the P5FL and P5C samples respectively. In the P5FH sample the amount of type I collagen was lower than in those from P5C and P5FL, but was still greater than the amount in the P1FH sample. The statistical data is shown in Table 1.
Figure 2. Type I collagen localisation in the oldest first molar tooth germ (cusp tip) from A, B — control group; C, D — F₁ group; E, F — F₃ group. A, B — preodontin (pd) shows strong staining. Spaces between odontoblasts (o) and dental pulp (dp) stain moderately. Mineralising dentin (d) and the dentin-enamel junction (bm) are weakly immunopositive for type I collagen (d). C, D — the F₁ groups: Preodontin (pd) is very strongly immunopositive. Mineralising dentin (d) stains moderately. Dental pulp stains weakly positive for type I collagen but more intensely than those in the control group. Dentin-enamel junction is moderately positive (bm) for type I collagen antibodies. E, F — the F₃ groups: Preodontin (pd) stains relatively weakly compared to F₁ and the control groups. Mineralising dentin (d), dental pulp (dp) and intercellular spaces between odontoblasts (o) are almost negative; a — ameloblasts; ab — alveolar bone. Scale bar 100 µm.
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The effect of fluoride on type I collagen production

Evaluation of the results from immunohistochemical studies from the youngest groups of animals revealed the strongest presence of type I collagen in predentin. Using Western immunoblot analysis we concluded that the highest amount of type I collagen was found in animals exposed to a low concentration of fluoride. The P1FH group had the lowest concentration of type I collagen. The older animals showed the same pattern of type I collagen concentration according to fluoride exposure. It seems possible that low concentrations of fluoride may act as a potent stimulator of type I collagen production. Similarly, others have found synthesis of dentin type I collagen to be unaffected by a fluoride concentration of up to 2–3 mM [7, 8].

In vitro observation of tissue culture supplemented with low concentration in the medium by Nakade et al. [28] confirmed that the addition of fluoride stimulated the formation of type I collagen. Veron et al. [36] showed that the addition of fluoride in concentrations up to 10 ppm altered neither the amount of type I collagen nor its gene expression. When fluoride was added in higher concentrations (25 ppm) the expression of type I collagen decreased significantly. Helgeland [15] also concluded that fluoride is responsible for a 50% decrease in the expression of type I collagen. Similarly, Araki [4] observed the formation of two types of abnormal collagen in rat incisors after a subcutaneous injection of 8.4 mg of sodium fluoride.

The effect of fluoride on predentin and dentin

In all three age groups of animals the predentin layer was thicker in the Fh groups than in the Fl and control. The increased thickness of the layer of predentin was visible in routine H&E preparation as well as in immunohistochemical studies. Since chronic exposure to fluoride demonstrated its adverse effect on calcosphere formation [3], we suspect that the increased width of predentin in the Fh groups reflects fluoride-dependent disturbances in the maturation of predentin and its rearrangement into dentin. In all the groups investigated the specific dentin-staining reaction disappeared in proportion to its mineralisation progress.

The dentin-enamel junction

In all the groups examined, especially in the Fl and controls, strong immunostaining for type I collagen was detected at the dentin-enamel junction. This may indicate that in these groups the epitopes for type I collagen remain unhidden in this area. This staining can also reflect the storage of dye, referred to as the border effect. However, a conspicuous decrease in staining in the Fh group could serve as a histological marker of impaired production of type I collagen and altered dentin mineralisation.

The role of non-collagenous proteins in type I collagen immunoreactivity

It should also be emphasised that type I collagen in dentin matrix is closely associated with non-collagenous proteins (NCPs). These proteins, synthesised by odontoblasts and released at the mineralisation front, are essential in the initiation and proper mineralisation of dentin. They bind to type I collagen fibrils and attract calcium ions simultaneously, leading to the nucleation of the mineral crystals. It is documented that fluoride exerts its adverse effect on the formation and post-translational modification of these proteins [2, 25]. It is possible that the enhancement of immunostaining for type I collagen found in animals treated with high fluoride concentration could reflect changes in NCPs, uncovering antigenic sites on collagen fibrils. Current knowledge provides diverse information about the influence that fluoride exerts on the expression of type I collagen in dentin. It is believed that in low concentrations, fluoride can act as a potent stimulator of type I collagen production. The high fluoride concentration alters the interactions between the collagenous scaffold of dentin and its non-collagenous components. Thus it is crucial that further studies are carried out to establish the doses of fluoride that are relatively safe or even beneficial for the proper development of dentin.
REFERENCES


