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INVESTIGATION ON THE COLLAGEN FIBER NETWORK IN HUMAN DENTAL TISSUES - TRANSMISSION AND SCANNING ELECTRON MICROSCOPY

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Abstract

The collagen fiber component of pulp, dentine and cementum, has been studied by isolating it from the inorganic component and the organic non-collagen matrix. This study was carried out on healthy molar and premolar adult teeth. Following demineralization all of the non-collagenous components of the tooth were removed by sequential treatment with H₂O₂, with different concentrations of trypsin and with EDTA. The transmission (TEM) and scanning (SEM) electron microscopy investigation confirmed that such a technique is an accurate method for isolating the collagen fibers while preserving their position and their ultrastructure in the dental tissue. The use of TEM and SEM in the observation of homogeneous samples provided further information regarding the structural characteristics of the collagen fiber network of dental tissues. Our main concern was the characterization of interodontoblastic fibers and the different distribution and arrangement of collagen fibers in different dental zones.

Key Words: Dental tissues, enzymatic digestion, removal of non-collagenous matrix, collagen fibers, transmission electron microscopy, scanning electron microscopy.

Introduction

Teeth have an anatomical structure in which tissues of different embryologic origins are tightly connected from a morphological and functional point of view. The fiber network which is present in all dental tissues of mesenchymal origin is generally recognized as having a very important role in morphogenetic processes and in determining the specific structural characteristics.

From a biochemical and immuno-histochemical point of view, research has shown that different types of collagen are present in the pulp, in the predentine and in the dentine (Magloire et al., 1982; Linde, 1985; Bronckers et al., 1989).

From a morphological point of view, special interest has been shown in investigating the fiber component present in the area between dentine and pulp. Indeed, in this zone the interconnection between the fibrillar component and the cellular odontoblastic layer gives a peculiar aspect to their structural and functional characteristics, which are as interesting as they are difficult to understand (Frank, 1966; Cahen and Frank, 1970; Ten Cate, 1978; Baume, 1980; Matthiessen et al., 1985; Thomas, 1985; Szabo et al., 1985; Sogaard-Pedersen et al., 1990; Bishop et al., 1981; Salomon et al., 1991).

With the purpose of providing new data on this topic, recent research was carried out using scanning electron microscopic (SEM) analysis of samples from the odontoblastic and predentine zones where the fibers had been isolated from the other inorganic and organic components (Sogaard-Pedersen et al., 1990). The method proposed, after demineralizing the samples, causes the glucidic component to dissociate from the collagen after treatment with H₂O₂. This treatment is followed by the removal of all non-collagenous parts by digestion with trypsin and treatment with EDTA. This method was applied for the first time in the study of cartilage collagen (Steven and Thomas, 1973). Since the above mentioned contribution only involves a rather limited area of the tooth (Sogaard-Pedersen et al., 1990) we decided to examine the efficacy of this procedure for all parts of the tooth which contain collagen. We further-
more decided to use both SEM and transmission electron microscopy (TEM) for the study of samples prepared following the same procedures, involving decalcification and enzymatic digestion, with the aim of integrating the information obtained by a morphological analysis by using convergent techniques on areas which are still problematic. For this purpose, because of the different densities and distribution of the components in the tissues to be studied, we had to find out what concentration of the enzyme after demineralization, is necessary to completely remove the organic non-collagenous part. All this without changing the layout and the structure of the collagen fibers in the different areas analyzed. The preparation of sections to be observed with TEM also required the removal of the matrix not only at the surface of the samples but also at a deeper level.

Material and Methods

Nine healthy human permanent teeth, premolars and molars which were extracted for surgical reasons from adult subjects (35-45 years old), were fixed in a 3% glutaraldehyde solution in 0.1 M sodium cacodylate buffer for 24 hours. After fixation, the teeth were demineralized with an aqueous solution of 0.5 M sodium formate and 4 M formic acid, pH 2.9 (Sogaard-Pedersen, 1990) for a period of 5-6 weeks and were then cut longitudinally. One half of each tooth was assigned to TEM while the other one was assigned to SEM observation.

After demineralization, one tooth was not subjected to the digestion procedure for the removal of the organic non-collagen matrix, but was immediately fixed and treated for TEM and SEM examination. This sample was used as a control specimen. The other demineralized teeth were digested and all organic non-collagen components were removed following the procedures formerly proposed for cartilage (Steven and Thomas, 1973) and for dentine (Sogaard-Pedersen et al., 1990) but using higher enzyme concentrations.

The teeth were immersed in a 3% solution of H2O2 and left in the dark for 20 hours at 20°C, rinsed with water and then with a 1% solution of NaCl. Following this they were treated with trypsin (1120 BAEE units/mg) in a 1% NaHCO3 solution, pH 8.7, for 20 hours with continuous shaking at 20°C. Two different concentrations of the enzyme were used to establish the most effective one for a complete removal of the organic component without destroying the collagen fibers. Four teeth were digested in trypsin solution with a 1:50 enzyme weight to tissue weight ratio and the other four teeth with a 1:25 ratio. Thereafter, the teeth were rinsed with water and with a 1% NaCl solution and then kept in a 4% EDTA solution at pH 7.5 for 20 hours at 20°C to remove the non-collagenous proteins. Following this they were rinsed with acetic acid at 0.1 M, pH 7 and with a 1% NaCl solution to remove the residual non-collagen matrix. The whole procedure was again repeated beginning from the treatment with H2O2.

Finally the specimens were fixed in a 3% glutaraldehyde solution in sodium cacodylate buffer at 0.1 M and pH 7.4 for 12 hours, rinsed in the same buffer and post-fixed in 1% OsO4 in collidine buffer for one hour. The samples for TEM observations were dehydrated and embedded in epoxy resin. The ultrathin sections (80 nm thick) were contrasted with uranyl acetate and sodium citrate and observed with a Zeiss EM 10. The samples used in the SEM studies were dehydrated, placed in amyl acetate, critical-point dried and sputtered with gold and observed with a Philips 515.

Results

The analysis of the demineralized samples which had not undergone enzymatic digestion assured us that the inorganic component had been removed before the samples underwent the procedure used to remove the organic non-fibrillar component. In TEM, especially in the dentine, only the decalcified sample showed well preserved odontoblastic processes surrounded by the "lamina limitans" and by the periodontoblastic space containing fine matter, which was partially amorphous and partially fibrillar in nature. The dentine collagen fibers were partially hidden by the amorphous matrix in which they are contained (Fig. 1a). These observations were confirmed by SEM which showed some odontoblastic processes inside the dentinal tubules, even though they were damaged by the preparatory methods used which were not optimal for their preservation. The perfectly preserved organic intertubular matrix gives the tissue a compact look (Fig. 1b).

In the samples which had undergone treatment to remove the non-collagen organic components, the results varied with different enzyme concentrations. In the samples treated with the lower concentration of the enzyme (enzyme weight/tissue weight ratio 1:50) the removal of the non-collagenous organic components was not complete especially deep inside the samples analyzed using TEM. The pulp still contained cells or cell residues, blood vessels, myelin sheaths and odontoblasts. In the dentine, digestion seemed to have been more complete, however, odontoblastic processes sometimes remained in the dentinal tubules. Besides a thin granular layer of electron dense matter was rather commonly present with similar texture and position to the "lamina limitans" (Fig. 1c). In SEM, the fibers were still partly hidden by the amorphous matrix residues, especially in the intertubular dentine (Fig. 1d).

In the samples obtained by treatment at the higher
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**Fig. 1.** (a, b): TEM (a) and SEM (b) micrographs of dentine in demineralized samples. The organic components are still present. (c, d): TEM (c) and SEM (d) micrographs of dentine from samples treated at the lower enzyme concentration. The collagen component is still evident. In (c) an electron-dense matter is sometimes present in the dentinal tubules (arrows). The fibers are partly hidden by residual amorphous matrix. Bars = 1 µm (a); 10 µm (b); 0.5 µm (c); and 10 µm (d).

Concentration of the enzyme (enzyme weight to tissue weight ratio 1:25) only the collagenous fibers were preserved. These, when cut lengthwise, clearly showed their typical cross-sections in TEM (Fig. 2a). Although the non-fibrillar organic component had been completely removed, the architecture of the dental structures was perfectly preserved. This was not only the case for the dentine and the cementum, where the fiber network was rather dense, but also in the pulp where it was very loose. In TEM, the pulp clearly showed the entangling of collagen fibers around the removed pulpal structures: nerve fibers, cells and blood vessels. The location of blood vessels and capillaries was clearly visible due to the fiber network running longitudinally or across the luminal contour of the vessels and which was contained in the rather thick wall of the pulp vessels themselves (Fig. 2b). In samples of the pulp studied by SEM, a network of fibers woven together around the vessels could be seen clearly marking the lumen in a compact structure. This seemed denser than the samples analyzed by TEM given the different type of observation method (Fig. 2c).
Fig. 2 (above). TEM (a, b) and SEM (c) micrographs of pulp from samples treated at the higher enzyme concentration. In (a) collagen fibers with the typical cross-section. In (b), (c) collagen fibers in the wall of blood vessels. Endothelial cells and other components have been removed. In the TEM sample (b) the vascular lumen is bordered by a fibrous or finely granular matter (arrows). In the SEM figure (c) an intricate network of fibers defines the vessel lumen. Bars = 0.1 µm (a); 1 µm (b); and 10 µm (c).

Fig. 3 (on facing page). TEM micrographs of dentine (a, b, c) and cementum (d) from samples treated with the higher concentration of trypsin. A network of collagen fibers defines the lumen of the tubules in the dentine. The fibers are more widely spaced at the amelo-dentinal junction (a) than in the circumpulpar dentine (b). In the predentine (c) the fiber diameter is smaller than in the other regions of the tissue (a, b). Fine fibrils, often with evident striated structure, lead from the collagen fibers to the lumen of the tubules (arrows). In the cementum (d) coarse bundles of the fibers intertwine. Bar = 0.5 µm.
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Fig. 4. SEM micrographs of dentine (a), interodontoblastic fibers (b, c) and cementum (d) from samples submitted to the higher enzyme concentration. In (a) it is evident the arrangement of the fiber network round the dentinal tubules. In (b) and (c) numerous fibers run parallel from the pulp (P) to the predentine (PD) crossing the odontoblastic zone (OZ). At higher magnification (c) the arrangement of the interodontoblastic collagen fibers (CF) is well resolved. In (d) the dentine (D)-cementum (C) limit with the peripheral openings of the dentinal tubules (arrows). Bars = 10 µm (a); 0.1 mm (b); 10 µm (c), and 50 µm (d).

On the basis of these considerations, we restricted our subsequent observations to the samples treated with the higher concentration of trypsin (with a enzyme weight/tissue weight ratio of 1:25).

When sectioned transversely, the tubule lumen in the dentine samples was empty with its contour clearly outlined by the collagen fibers. With TEM, it was also possible to see fine threads in the lumen of some dentinal tubules, which led from the collagen fibers to the center of the tubules themselves (Fig. 3a, b). From these images the variations in diameter and in density of the collagen fibers in the different areas of the dentine was also evident. Indeed, at the amelo-dentinal junction there was a network of more widely spaced collagen fibers which had sometimes large diameters (80-180 nm). In the circumpulpal dentine a more dense fiber network marks out the dentinal tubules. Many of these fibers (100-120 nm) were sectioned transversely and appeared...
to run parallel to the tubules. In the predentine the collagen fibers were even thinner (30-60 nm). Towards the cementum, the layout of dentine collagen fibers became more irregular. In the pre-cementum the fiber network seemed less dense than in the cementum: the fibers were not so dense and the spaces already occupied by the amorphous matrix were more ample. In the cementum, large bundles of collagen fibers were closely interwoven (Fig. 3a-d).

From SEM observations on the dentine samples it could be seen that also in this type of investigation, the complete removal of the matrix permitted a much clearer definition of the fibrous component. This appeared to be made up of fibers of which the direction was more regular, parallel or perpendicular to the tubules in the peritubular dentine and of a larger network of fibers in the intertubular dentine (Fig. 4a). Of particular interest, however, was the SEM examination undertaken in the area between the pulp and the dentine. Here, in the area occupied by the odontoblasts, which were removed by the digestion treatment, there were fibers running parallel to each other. These came from the pulp where they were connected to the sub-odontoblastic fibril network and interwoven with the predentine fibers (Fig. 4b, c).

SEM examination of the cementum samples did not offer any additional information about the fibrillar structure of this tissue compared to that obtained by conventional methods. Peripheral openings of the dentine tubules were nevertheless clearly seen at the boundary between the cementum and the dentine (Fig. 4d).

Discussion

By examining and comparing the demineralized samples with those undergoing enzyme digestion, the usefulness of this technique which has already been used to study collagen fibers in cartilage (Steven and Thomas, 1973) and for SEM study of fibril network in dentine (Sogaard-Pedersen et al., 1990) appears evident. The removal of non-collagen organic components by means of repeated treatments does not alter the structure of the examined tissues and the three-dimensional organization of the fibers remains the same. The repeated treatments with H2O2 and EDTA do not seem to be critical in this regard whereas we believe that the concentration of trypsin is a determining factor. From our observations we can conclude that a weight ratio of 1:25 between enzyme and tissue is optimal for the complete removal of the non-collagenous organic matrix, the cells and of the nerve fibers without damage to the fiber network of the different examined tissues. The fibers retain their layout and their ultrastructure, as can be seen by the clearly evident cross bandings in the collagen fibers. Our results, therefore, differ from those reported in previous studies (Sogaard-Pedersen et al., 1990) as far as regards the enzyme concentration needed to completely remove the non-collagenous organic component, but our research included other dental components with different structural characteristics. Furthermore, the investigation carried out using SEM and TEM was not only done on the surface but involved sections taken from the deeper parts of the samples. This allowed us to show that with the adopted procedure, the removal of the non-collagenous component is complete for all parts of the tooth.

Our comparative study on only demineralized samples and on those where the removal of the organic part was performed using different enzyme concentrations allows the following conclusions. The hypothesis regarding the non-collagenous nature of the "lamina limitans", that can be removed by using trypsin and which therefore has a high content of glycosaminoglycans appears to be confirmed (Thomas and Carella, 1984; Thomas, 1985). Furthermore, we can confirm that inside the dentinal tubules, enzymatic digestion removes first, and therefore with greater ease, the odontoblastic processes. The removal of these structures is already complete at the weakest concentration of the enzyme. The complete removal of the "lamina limitans" occurs only with the highest concentration of the enzyme, probably because of the lamina's different linkages with the collagen fibrils.

Observation by SEM of the collagen fibers which clearly lead from the pulp to the dentine allows us to confirm earlier SEM observations (Cahen and Frank, 1970; Isokawa et al., 1972; Baume, 1980; Sogaard-Pedersen et al., 1990) and more recent TEM observations (Salomon et al., 1991) on the presence of inter-odontoblastic fibers. Our method appears to be very useful in the investigation of this problem-area. Indeed, the removal of the whole non-collagenous matrix and the odontoblasts from the samples allowed us to obtain a particularly clear picture of the fiber networks in the area marking the border between pulp and dentine.

The examination by TEM of the dentine samples clearly shows the presence of collagen fibers of varying diameters and with a spatial layout that changes as it goes from the predentine to collagen fibers of varying diameters and with a spatial layout that changes as it goes from the predentine to the peripheral dentine. These observations substantiate the specific nature of the methods used to separate the collagen component from all the other dental tissue constituents as well as its usefulness for a possible comparison with histochemical and biochemical investigations to better define the origins, the nature and the morpho-functional correlations of the fibrillar structures present in the dental tissues.

According to some authors, collagen fibers are not present in the periodontoblastic spaces (Lester and Boyde, 1968) or in the peritubular dentine (Takuma and Eda, 1966). On the other hand others (Frank, 1966;
Tronstad, 1973; Thomas, 1985) support the opposite. Our observations by TEM show that fine filaments jut out from the dentinal tubule wall and lead to the lumen even after matrix digestion by the higher trypsin concentration. So we hypothesize that some material of a collagenous nature can indeed be found in the periodontoblastic space.

In our opinion an investigation integrating TEM and SEM on samples obtained using the same preparation methods is particularly useful when studying particularly complex structures such as those of the tooth.

Acknowledgements

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References


Discussion with Reviewers

S. Lindskog: The methodology is somewhat troublesome since it involves a number of chemical treatments of the specimens: fixation, decalcification, H2O2-treatment and enzyme-digestion (trypsin) without controls in every step. It is particularly surprising that the H2O2-treatment did not remove the collagenous part of the organic component of the tooth. H2O2-treatment is a commonly used technique to remove the predentine which is almost exclusively made up of collagen in order to view the mineralizing front of the dentine. Please clarify.
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Authors: We did not prepare control samples step-by-step to check the effects of chemical treatments, because the methodology is the one applied and verified by other authors cited in Materials and Methods.

We acknowledge that \( \text{H}_2\text{O}_2 \), as well as other oxidizing agents, can remove the organic components of partially calcified tissues, but it does so at concentrations and at application times much higher than those employed in our methodology. We think that the figures show with sufficient evidence the efficacy of the method in preserving the collagen structures.

S. Lindskog: The results lack an account of the effect of the chemical treatments at different distances from the tissue surfaces. This is especially interesting since the chemical treatments depend on diffusion of break-down products through the specimens.

Authors: The aim of this research was an evaluation of a methodology for a SEM/TEM morphological study of the specimens. At this stage of our work, we have not performed an evaluation of the effects of the method at some distance from the observation surfaces. Also TEM observations were made on different serial sections and we have described (see Results) the effect of the lower concentration of the enzyme deep inside the samples.

J.H.M. Wöltgens: The authors describe the application of a modified method for removal of non-collagenous components in (pre)dentine, pulp and cement by comparing TEM and SEM pictures of treated and non-treated demineralized specimens. Indeed the micrographs demonstrate mainly collagen structures; but as long as this modification is not evaluated biochemically with respect to purity and resistance of collagen, the authors have to be careful with the interpretation of their results. For example, the finding of fine filaments in the periodontal spaces does not necessary point toward the presence of collagen, but can also be the remnants of other components. Consequently the addition of a higher magnification of these filaments, showing a striated collagen structure, will increase the scientific value of this manuscript greatly.

Authors: From the morphological point of view it seemed to us that the nature, the integrity and the organization of the collagen fibers is sufficiently evident from their striated aspect showed in Fig. 2 a-d and 3.

J.H.M. Wöltgens: Did the authors also try higher enzyme concentrations?

Authors: No we did not, because we considered the proposed enzyme-tissue ratio (1:25) sufficient to completely remove the organic non-collagenous matrix.