Cell Tissue Res (1997) 289:145-154

Cell Tissue Research

Light-microscopical investigation of the distribution of extracellular matrix molecules and calcifications in human dental pulps of various ages

G. Hillmann, W. Geurtsen

Department of Conservative Dentistry and Periodontology, Dental School, Medical University Hannover, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany

Received: 21 August 1996 / Accepted: 13 January 1997

Abstract. The distribution of extracellular matrix molecules, especially collagen types I, III, V, and VI, in the extracellular matrix of the connective tissue of human dental pulp of various ages was studied by polarization and indirect immunofluorescence microscopy by using a conventional fluorescence microscope and a confocal laser scanning microscope. Polarization and immunofluorescence microscopy of paraffin sections showed thick fibers of collagen type I, which represented the main component of the connective tissue matrix of the dental pulp. By indirect immunofluorescence, thin fibers and small bundles of collagen type III were determined to be one of the main fibrillar elements present in the dental pulp matrix. Collagen type IV was detected by a clear intense staining of the basement membrane of blood vessels at all ages examined. Collagens type V and VI formed a dense meshwork of thin microfibrils throughout the stroma of the connective tissue of the dental pulp. These fibers were localized around blood vessels and appeared to be enriched in the subodontoblastic layer. Investigations by means of confocal laser scanning microscopy revealed fibers of collagen type VI spiralling between fully differentiated odontoblasts toward the predentin layer. With advancing age, the connective tissue matrix appeared to be condensed and aggregates of thick fiber bundles could be observed. Furthermore, the participation of various collagen types in the composition of pulp stones was shown. These calcifications and diffuse calcifications increased in frequency with advancing age in a statistically significant manner.

Key words: Teeth - Dental pulp - Immunohistology - Extracellular matrix - Collagen - Aging - Human

This study was supported by the Deutsche Gesellschaft fur Zahn-, Mund- und Kieferheilkunde

Correspondence to: G. Hillmann (Tel.: +49-511-532-4815; Fax: +49-511-532-4811)

Introduction

The connective tissue of the dental pulp is composed of several types of macromolecules and is specifically involved in the production and maintenance of dentin. In oral soft tissues, the extracellular matrix comprises four major classes of molecules, namely the collagens, proteoglycans, glycosaminoglycans, and elastin. The extracellular matrix is considered to act as an important determinator of cellular gene expression and differentiation.

Pulp fibers have been classified histologically as reticular fibers, including von Korff-fibers, collagen fibers, and elastic fibers. The classification of the pulp fibers relies on staining and morphological characteristics, but the nature of the fibrillar materials in the dental pulp remains contentious.

Of the collagens occurring in the connective tissue, types I, III, and V belong to the class of fibril-forming collagens. Both types I and III account for the bulk of the tissue collagen (Shuttleworth et al. 1978, 1980). Type III collagen is often found together with type I. However, type III consists of fibrils of much smaller diameter (Lapiere et al. 1977). In human pulp, type III collagen constitutes a large proportion (43%) of the total collagen (van Amerongen et al. 1983). On the other hand, in cell culture, human dental pulp cells produce predominantly (99%) type I collagen and only trace amounts of type III collagen (Kuo et al. 1992). During tooth development, a close relationship exists between collagen type I in dentin and fibronectin in immature enamel in human tooth germs. Immunohistological investigations have revealed the presence of collagens type I and III, tenascin, and fibronectin in the mesenchymal intercellular matrix (Garbarsch et al. 1994).

Collagen type IV is the structural backbone of all basement membranes (Yurchenko and Ruben 1987). Immunoelectron investigations have demonstrated a similiar distribution of collagen type IV and of laminin in basement membranes of blood vessels and Schwann cells in the tooth pulp of the cat. Odontoblast-associated laminin might be of significance as a guide for regener-

ating terminal pulp nerve fibers (Fried et al. 1992). Collagen type VI is a minor but ubiquitous constituent of the interstitial extracellular matrix that forms microfibrils, probably serving as a flexible anchor interconnecting collagen fibers (Bruns et al. 1986) and collagen fibers with cells (Bonaldo et al. 1990). Type III collagen, which predominates in the loose distended connective tissues, may contribute to the elasticity of the tissues (Shuttleworth et al. 1980). In contrast, type I collagen, which predominates in the dense connective tissue (Narayanan and Page 1976, 1983), is thought to stabilize tissue architecture.

Little is known about the localization and function of collagens type V and VI in human dental pulp. Type V collagen, originally isolated from fetal membranes (Burgeson et al. 1976) and later found in virtually all organs, seems to play a major role in the formation of granulation tissue (Kurita et al. 1985) and of scars in chronic inflammatory diseases (Narayanan and Engel 1983).

Type VI collagen was originally discovered in pepsin extracts of the aorta (Chung et al. 1976). It is a glycoprotein with a short, triple helical core and large N- and C-terminal globular domains. Monomeres are assembled into tetrameres before their secretion from the cell and these are assembled end-on-end in the extracellular space into periodic micro fibrils (Engvall et al. 1986; Colombatti et al. 1987). Type VI collagen micro fibrils isolated from the dental pulp of fetal calves show long thin flexible filaments with a characteristic periodicity of about 100 nm (Shuttleworth et al. 1992). This microfibrillar collagen has a wide distribution in the connective tissue similar to that observed for type I and III collagens. Particularly strong staining has been observed in arterial vessel walls (Von der Mark et al. 1984) suggesting that this collagen forms an independent, but interconnecting, meshwork in many extracellular matrices. Collagen fibrils and fibronectin have been identified between odontoblasts and seem to be part of von Korff fibers (Higashi and Okamoto 1996; Whittaker and Adams 1972; Yoshiba et al. 1994).

Calcified structures, such as pulp stones and diffuse calcifications, are common in human dental pulp. They may occur in one or all of the teeth of one person, even in unerupted or impacted teeth. Whether they should be considered physiological or pathological remains controversial (Moss-Salentijn and Hendricks-Klyvert 1988).

In the present study, we have investigated the distributional changes of collagen types I, III, IV, V, and VI, and of calcifications in the pulp tissue of noncarious human teeth with respect to the age of the donors. The histomorphology of these structures is described by means of various light-microscopical techniques.

Materials and methods

Teeth and age relationship

This report is based on a histological survey of the evidence of calcifications in 332 human teeth. Permanent, erupted, non-erupted, single-rooted, and multirooted teeth were included. Most of the teeth were molars. They were collected over a period of 3.5 years. The teeth were either noncarious and without fillings or had only minute carious defects or minimal restorations. Informed consent was given prior to surgery.

The ages of the patients ranged from 11 to 72 years. Three age groups were constituted: group I (10-30 years), group II (31-51 years), and group III (52-72 years).

Histological examinations

Immediately after extraction, undecalcified teeth were hemisected by a sawing machine and fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.15 M cacodylate buffer at 4°C, pH 7.4. Tissues were dehydrated through graded glycol methacrylate under vacuum at 4°C and embedded in Technovit 7200 VLC (Kulzer, Wehrheim, Germany). Embedded specimens were affixed to plastic slides, cut by a sawing machine, and polished. After adhesion to a second plastic slide, thin slices of about 100 pm were obtained by using a sawing machine with a diamond saw blade (Exakt-Apparatebau, Hamburg, Germany). Thin slices of about 60 pm were produced with a grinding machine (Exakt-Apparatebau, Hamburg, Germany). Sections were stained with toluidine blue and mounted in Eukitt (Donath and Breuner 1982; Hillmann et al. 1991). Organic material, such as soft tissue of the pulp and microorganisms, were stained blue by toluidine blue. The ground sections were evaluated in a Nikon light microscope by conventional transmitted and polarized light.

For conventional paraffin histology, teeth were decalcified (10% EDTA, 14 days) after hemisectioning and fixation. Routine paraffin sections (5 pm) were prepared and stained with Picrosirius Red (Junqueira et al. 1979, 1982; Montes et al. 1980) or with hematoxylin-eosin. The Picrosirius Red staining method is easy to perform and, under polarized light, reveals restricted information about the composition of soft and hard tissues. Sections were examined in transmitted and polarized light.

---------->

Fig. 1. In the coronal pulp, thin filaments or small bundles of filaments insert into the intensely stained zone surrounding the blood vessels *(arrowheads).* The thin fibers form a reticular network and surround the blood vessels; they seem to be weakly birefringent in polarized light and are pale green. Paraffin section, Picrosirius Red staining. *Bar:* 20 pm. *375

Fig. 2. Numerous bundles of extracellular matrix molecules are strongly stained in the coronal pulp. Under polarized light, these fibre bundles appear birefringent and have a red, orange, yellow or greenish color. They are therefore probably composed of various types of collagen molecules. Paraffin section, transmitted light, Picrosirius Red staining. *Bar:* 20 pm. *375

Fig. 3. In the coronal and radicular pulp, blood vessels are surrounded by collagen type III. Fibers and bundles of fibers that are strongly stained extend between the blood vessels and the connective tissue matrix. Paraffin section, immunofluorescence microscopy. *Bar:* 20 pm. *375

Fig. 4. At sites of active secondary dentin formation, a few specimens show strong staining for collagen type III in the predentin layer. Pulp horn, paraffin section, immunofluorescence microscopy. *Bar:* 100 pm. *100

Fig. 5. The basement membranes of blood vessels are intensely stained for collagen type IV (at all ages examined). Paraffin section, immunofluorescence microscopy. *Bar:* 10 pm. ><600

Fig. 6. Fibers of type V collagen are arranged mainly in small bundles throughout the soft tissue of the pulp. Blood vessels are often surrounded by concentric layers of fibrils of this type of collagen. Paraffin section, immunofluorescence microscopy. *Bar:* 20 pm. *375

Immunohistology

Paraffin sections of 46 teeth were examined for the immunohistological investigation of collagens type I, III, IV, V, and VI. Specimens were prepared as described above. The age of the patients ranged from 4 to 65 years. Before immunohistological reactions were carried out, paraffin sections were dewaxed in "Roti Histol" (Roth, Karlsruhe, Germany) and, in order to enhance the antigenicity of the macromolecules, were prepared with the "Antigen Retrieval System" (Bio Genex, Calif., USA). To avoid non-specific binding of antibodies, sections were pre-incubated with non-immune mouse serum (1:20; Dakopatts, Hamburg, Germany) diluted in phosphate-buffered saline (PBS) with 1% bovine serum albumin (PBS/BSA) for 20 min at room temperature. They were then incubated with primary antibodies for indirect immunohistological characterization of the various collagen types in a moist chamber for 45 min at room temperature. Commercially available monoclonal antibodies (anti-collagen types I, III, IV, and V; Biochrom, Monosan, Berlin, Germany) and polyclonal antibodies (anti-collagen type VI; Serva, Heidelberg, Germany) were used. Their specificity was ascertained by the relevant manufacturer by means of an enzyme-linked immunosorbent assay. Secondary antibodies coupled with the fluorescent marker fluorescein isothiocyanate were used at a dilution of 1:40 in PBS/BSA. Sections were incubated for 20 min at room temperature and were mounted in Mowiol (Dartsch 1990). They were examined in a Nikon fluorescence microscope. Controls were prepared by omitting the primary antibodies to control for non-specific binding. Specimens prepared by the "Antigen Retrieval System" were carefully compared with appropriate negative controls.

Confocal microscopy

For confocal laser scanning microscopy, specimens were prepared in the same manner as for the immunohistological investigations. After mounting in Mowiol, specimens were examined with a Bio-Rad MRC 600 confocal laser scanning microscope equipped with an argon-krypton mixed gas laser and *Comos* software (version 6.0; BioRad, München, Germany). Data sets of serial optical sections were processed by *Lasersharp* software (version 1.02; Bio-Rad) and *Picture Publisher* software (version 5.0; Micrografx, München, Germany).

Qualitative and quantitative histological investigation

Two hundred teeth were examined. The location, whether coronal or apical, was recorded, and the calcifications were sorted with respect to whether they were discrete pulp stones, or whether they were free, attached to or embedded in dentin. Of the 200 teeth, 141 were used to prepare undecalcified ground sections, whereas 59 teeth were decalcified and paraffin sections were prepared. In total, 282 ground sections and 354 paraffin sections were investigated (632 histological specimens). Undecalcified ground sections were stained with toluidine blue, and paraffin sections were stained with hematoxylin-eosin. For the statistical analysis (Mantel-Haenszel-test, $P<0.05$), a tooth was rated positively for calcification if one section revealed pulp stones or diffuse calcifications. In addition, 132 teeth were used for qualitative histological investigations.

Results

Light and polarization microscopy

When stained by Picrosirius Red, matrix molecules of the dental pulp, especially the collagens, were revealed in red. These molecules appeared in the form of thick fiber bundles and thin fibers, showing a reticular network throughout the pulp. The cell-free zone (of Weil) beneath the odontoblast layer and the cell-rich zone with numerous blood vessels and fiber bundles were clearly visible. The staining reaction was strong around the blood vessels. Thin filaments or small bundles of filaments inserted into the intensely stained zone surrounding the blood vessels (Fig. 1).

Under polarized light, the thick fiber bundles appeared strongly birefringent and presented a red, orange, or yellow color. Therefore, these macromolecules seemed to be collagen type I (Fig. 2).

The thin fibers forming a reticular network and surrounding the blood vessels seemed to be weakly birefringent in polarized light and demonstrated a greenish color. Because of this appearance, these macromelcules were considered to consist mainly of collagen type III. These fine fibrils were sometimes associated with thick fibre bundles.

Immunofluorescence microscopy

Collagen type I. With the type I collagen antibodies, staining reactivity was sometimes very strong in predentin. However, the staining reactivity did not clearly exceed background staining in most of the specimens. The connective tissue of the dental pulp stained relatively weakly. Thick fiber bundles, which appeared red in polarized light, were often intensely fluorescent.

Collagen type III. The staining reactivity of this type of collagen was very strong. Thin fibers of collagen type III

---------- >

Fig. 7. Longitudinally arranged fibers of collagen type V can be seen *(large arrowheads)* adjacent to the layer of odontoblasts *(arrows).* Some thin fibers extend between the odontoblasts *(small arrowheads).* Paraffin section, immunofluorescence microscopy. *Bar:* 20 pm. x375

Fig. 8. Antibodies of type VI collagen demonstrate a microfibrillar, almost amorphous pattern. Blood vessels throughout the pulp tissue are often intensely stained for collagen type VI. Paraffin section, immunofluorescence microscopy. *Bar:* 20 pm. *375

Fig. 9. Two layers of collagen type VI can be seen adjacent to the odontoblasts. A thin line of very intense fluorecent labelling is localized directly adjacent to the odontoblast layer *(large arrowheads).* A second layer consisting of fine fibrils lies *(small arrowheads)* adjacent to this line. Pulp horn, paraffin section, immunofluorescence microscopy. *Bar:* 100 pm. xl00

Fig. 10. In the odontoblast layer of the coronal pulp, immunolocalization of collagen type VI reveals corkscrew fibers passing from the pulp between the odontoblasts *(arrowheads).* Paraffin section, immunofluorescence microscopy. *Bar:* 20 pm. x500

Fig. 11. Confocal laser scanning microscopy. Fibers of collagen type VI with a corkscrew pattern pass from the pulp into predentin parallel to the long axis of the odontoblasts *(arrowheads)* of the coronal and radicular pulp. Paraffin section. *Bar:* 20 pm. ˣ500

Fig. 12. With advancing age, thick fiber bundles of collagen can be observed throughout the pulp. Paraffin section, anti-collagen type III, immunofluorescence microscopy. *Bar:* 100 pm. xf00

Fig. 13. a Pulp stones with various arrangements of collagen fibers. Fibers are often distributed irregularly or parallel to each other. Coronal pulp, paraffin section, Picrosirius Red staining. *Bar:* 100 pm. *100. **b** Under polarized light, the colors red and green indicate the presence of collagens type I and III. Coronal pulp, paraffin section, Picrosirius Red staining. *Bar:* 100 pm. xl00

showed a reticular network throughout the pulp. Thin filaments or small bundles of filaments could be observed. Blood vessels were surrounded by collagen type III. Fibers and bundles of fibers that were strongly stained extended between the blood vessels and the connective tissue matrix (Fig. 3). At sites of active secondary dentin formation, a few specimens were strongly stained for collagen type III in the predentin layer (Fig. 4).

Collagen type IV This type of collagen was identified by intense staining of the basement membranes around numerous blood vessels at all ages investigated (Fig. 5).

Collagen type V. The reactivity of the pulpal tissue was relatively strong by indirect immunofluorescence. The fibers of collagen type V showed an intricate microfibrillar pattern of the connective tissue of the dental pulp. They were arranged mainly in small fiber bundles. Vascular membranes were negative, but blood vessels were often surrounded by concentric layers of fibrils of collagen type V. Fibers of collagen type V were arranged mainly in small fiber bundles (Fig. 6), and longitudinally arranged fibers could be observed lying adjacent to the layer of odontoblasts. Some thin fibers of collagen type V extended between the odontoblasts (Fig. 7).

Collagen type VI. A microfibrillar, almost amorphous pattern was observed in the connective tissue of the dental pulp with antibodies to type VI collagen. Intense staining of blood vessels was frequently seen (Fig. 8).

Two layers of collagen type VI below the odontoblasts could be observed. A thin line of very intense fluorescent labeling was localized directly below the odontoblast layer. A second layer consisting of fine fibrils of collagen type VI could be distinguished adjacent to this line. This layer showed a more intense fluorescent labeling than the rest of the connective tissue of the dental pulp (Fig. 9). In the odontoblast layer of the coronal region, fluorescent corkscrew fibers were found to extend from the pulp between odontoblasts. Some were also seen to penetrate the predentine. No fluorescence was detected in the calcified dentinal matrix (Fig. 10).

Confocal microscopy

As mentioned above, fibers of collagen type VI were localized in the odontoblast layer by indirect immunofluorescence. A confocal laser scanning microscope was also used to study the distribution of collagen type VI. This type of collagen was observed between the odontoblasts and appeared as corkscrew fibers passing from the pulp into the predentin parallel to the long axis of the cells (Fig. 11). A similiar distribution of collagen type VI was observed in the coronal and radicular regions.

Controls

Control sections incubated with normal serum instead of the primary antibodies showed little background staining.

Table 1. Comparison of immunostaining results with primary antibodies to various extracellular macromolecules (collagen types I, III, V, and VI) at different ages. Scoring system: +/+, intense staining; ±, moderate staining; —, weak staining

Age	Thin fibres Small fibre bundles	Thick fibre bundles
4-30 years	+/+	
31-50 years	+/-	+/-
51-65 years	+/-	+/+

Age-related changes

Extracellular matrix. The distribution of types I, III, V, and VI collagen and the age-related changes from 4 years to 65 years of age were examined. Immunofluorescence microscopy revealed that types I, III, V, and VI collagens were present in all tissues at all ages examined. The staining intensity for type I collagen was very weak. Collagens type III, V, and VI were strongly stained at all ages examined.

Most of the specimens revealed staining patterns for the various collagen types as mentioned above. However, with advancing age, thick fiber bundles of collagen type I increased in frequency and thickness. The reticular pattern of fine fibrils of collagens type III, V, and VI disappeared with advancing age and were replaced by thick fiber bundles. Thus, the connective tissue matrix appeared to be condensed and stained homogeneously at greater ages (Table 1). Aggregates of thick fiber bundles could be observed (Fig. 12). Pulp stones and diffuse calcifications increased in size with age, as did the condensation of the macromolecules of the connective tissue.

Calcifications. The light-microscopical investigation of free pulp stones in the coronal part of the pulp of decalcified teeth indicated the presence of various collagen types that were distributed in concentric layers or in a diffuse manner. Bundles of collagen fibers were frequently arranged irregularly or parallel to each other. Pulp stones sometimes had diverse arrangements of collagen bundles. Under polarized light, the colors red and green revealed the presence of various collagen types (Fig. 13a, b). Thick fiber bundles of a range of collagen types extended from the extracellular matrix into the calcified bodies. Immunofluorescence microscopy showed a diffuse pattern of collagen type III (Fig. 14) in free pulp stones. Antibodies to collagen type VI revealed a diffuse distribution with weak staining intensities. Pulp stones localized adjacent to the orifice of the root canal demonstrated the participation of various macromolecules in the composition of the calcified structures in polarized light. This kind of calcification could cause an obstruction of the orifice of the root canal.

The investigation of undecalcified ground sections revealed free pulp stones that were characterized by various kinds of mineralization. They showed areas of concentric and lamellar calcifications.

Fig. 14. In free pulp stones, immunofluorescence microscopy reveals a diffuse pattern of collagen type III. Coronal pulp, paraffin section, immunofluorescence microscopy. *Bar:* 100 gm. *100

%

groups of ages

Fig. 15. The number of teeth with diffuse calcifications and of teeth with diffuse calcifications and pulp stones increases with advancing age. The number of teeth with pulp stones in age group II (77) and III (777) is comparable. *PS,* Pulp stones; *DC,* diffuse calcifications; 7, 11-30 years of age (94 teeth); 77, 31-51 years of age (63 teeth); 777, 52-72 years of age (43 teeth)

Diffuse calcifications could be observed in the root canal. Needle-like and cylindrically shaped calcified material occurred parallel to collagen fibers. Polarization and immunofluorescence microscopy revealed all the types of collagens examined; these were equally distributed over the calcified areas.

The quantitative evaluation of the frequency of calcifications (pulp stones), diffuse calcifications, or both in the same tooth yielded different results for the various kinds of calcifying processes. The number of teeth with diffuse calcifications and the number of teeth with pulp stones and diffuse calcifications increased with advancing age. However, the number of teeth with pulp stones in age groups II and III was comparable (Fig. 15).

A statistically significant increase ($P<0.05$) in the quantity of coronal and radicular calcifications could be determined. In group I (11-30 years), 14.9% of teeth had calcifications, in group II (31-51 years) 44.4%, and in group III (52-72 years) 65.1%.

Discussion

Considerable difficulties are encountered in characterizing fibrillar elements in connective tissues by histological staining methods. In this study, formalin-fixed and decalcified human teeth have been used to show the distribution of collagen types I, III, IV, V, and VI in the extracellular matrix of the connective tissue of the dental pulp. The "Antigen Retrieval System" has been used in order to enhance the antigenicity of the macromolecules. By employing this method, antibodies prepared for immunohistology on frozen sections give good staining results on paraffin sections of formalin-fixed tissues. Although the technical methods described in this report are relatively simple, a few precautions should be noted. Our results show that background staining may be increased by the use of the "Antigen Retrieval System", because hard tissues are prone to high backgrounds. Therefore, it is important, when evaluating this method, carefully to compare positive staining with an appropiate negative control. On the other hand, the Picrosirius Red staining method has revealed the presence of various collagen types in the soft tissue of the dental pulp and in the various kinds of calcifications. These results must be confirmed by immunohistological methods.

The immunohistological staining intensity of collagen type I is very weak in our specimens. Polarization microscopy of paraffin sections stained with Picrosirius Red (Junqueira et al. 1978) have demonstrated that collagen type I is the main component of the connective tissue matrix of dental pulp. Indirect immunofluorescence has established that collagen type III is one of the main fibrillar elements present in the dental pulp matrix. Several studies have reported that types I and III collagen are present in separate fibrils. Type I collagen is found mainly in thick fibrils, whereas type III collagen occurs in thin fibrils in the liver, skin, and tendon (Nowack et al. 1976; Fleischmajer et al. 1981) and is scattered throughout the tissue of human dental pulp but not as fibrils passing between the odontoblasts (Magloire et al 1982). However, investigations of the developing mouse molar have revealed collagen type III as a major component of interodontoblastic fibers (Shroff and Thomas 1992; Ohsaki and Nagata 1994). Our study has demonstrated the presence of collagen type III in the predentin layer but not as a component of the interodontoblastic fibers of the teeth examined. In agreement with other authors, we have shown that types I and III collagen can co-exist in the same fibrils in the pulpal tissue of human teeth (Xu et al. 1993).

Collagen type IV is intensely stained in the basement membrane of the blood vessels in all ages examined. Collagens type V and VI do not seem to be components of the basement membrane but form a dense meshwork of microfibrils throughout the stroma of the connective tissue of the dental pulp. These fibers appear to be enriched in the subodontoblastic layer; indeed, the fibers of collagen type VI may be a component of von Korff fibers. These results agree with the biochemical investigation of Shuttleworth et al. (1992).

The function of the non-fibril-forming type VI collagen is not yet known in detail (Lukinmaa and Waltimo 1992). The interodontoblastic spaces are very narrow, and the high density of intercellular junctions between odontoblasts make it difficult to identify the extracellular matrix in this layer. Scanning and transmission electron microscopical studies have revealed the presence of interodontoblastic collagen fibers, which cross the distal junctional complex of the odontoblasts and penetrate into and across the predentin (Bishop et al 1991; Salomon et al 1991; Tabata et al. 1994). By using a confocal laser scanning microscope, we have observed the three-dimensional distribution of collagen type VI in the pulp of human teeth, especially in the odontoblast layer. This type of collagen is observed between odontoblasts, appearing as a spiral fibrous structure that enters the odontoblast layer from the pulp and reaches into the predentin. These results demonstrate that collagen type VI is present in the odontoblast layer during dentinogenesis. It may be closely involved in odontoblast differentiation and dentinogenesis.

Furthermore, the present study has shown that collagens type I, III, IV, V, and VI are present in the human dental pulp throughout all ages examined. The presence of these molecules in the dental pulp suggests that these structures are involved in both development and maintenance of the pulp tissue. Our findings indicate that type V and VI collagen are important components of human dental pulp and that they might contribute to the function of cellular elements. These data suggest a significant, but as yet unknown, role of the matrix proteins, especially collagens V and VI, in the normal function and perhaps during the formation of reparative dentin (Magloire et al 1988; Higashi and Okamoto 1996) and other calcified structures.

The distinction between pulp calcifications that arise because of caries or traumatic procedures and those that are a result of the process of aging itself is problematic when pulp calcifications are investigated histologically. In order to exclude the influence of pathological processes, only caries-free teeth or teeth with small carious lesions or fillings have been investigated in our study.

The number of calcifications (pulp stones and diffuse calcifications) increases with advancing age (Sayegh and Reed 1968). The continous growth of secondary dentin with advancing age and the increase of degenerative calcifications results in a reduction of the pulp cavity (Torneck 1990).

The customary classification of calcifications includes a distinction between true and false denticles (Kronfeld 1933). In accordance with Moss-Salentijn and Hendricks-Klyvert (1988), we suggest that this classification should no longer be used, since most of the calcified structures in our study are composed of a combination of tubular dentin and atubular calcified material. Thus, the term "denticles" should only be used for calcified bodies that are formed after an inductive interaction between the pulp tissue and intrapulpal cell remnants of the Hertwig epithelial root sheath (Moss-Salentijn and Hendricks-Klyvert 1988). The term "pulp stones" should be used for polymorphous and atypical calcifications that are located within the pulpal tissue, which does not mineralize in normal conditions.

Light- and scanning electron-microscopical investigations have revealed that pulp stones are composed of concentric collagen fibers and hydroxyapatite crystals (Appleton and Williams 1973; Le May and Kaqueler 1991). Concentric and lamellar layers can be distinguished. The results presented here demonstrate the participation of various collagen types, especially collagens type I, III, and VI, in the composition of pulp stones.

The immunohistochemical identification of organic macromolecules in the calcified structures is difficult, because the antigens might be masked as a result of the high degree of mineralization. Since the number of calcifications increases with advancing age, structural changes in the organic matrix of the pulpal tissue might participate in the development of pulp stones. Calcifications may occur in a normally non-calcifying tissue, e.g., the pulp, as a result of a local breakdown of mineralization inhibitors such as proteoglycan complexes (Moss-Salentijn and Hendricks-Klyvert 1988).

Diffuse calcifications have been observed mainly in the radicular area (Bemick 1967). This type of calcification grows along organic macromolecules and in the periphery of blood vessels. Our polarization and immunohistological investigations have revealed various collagen types between the calcified structures in the root canal.

Noncarious teeth show a smaller amount of calcifications than carious teeth (Sayegh and Reed 1968). On the other hand, teeth without masticatory function (e.g., impacted molars) and teeth with a lifelong masticatory function may be distinguished (Hillmann et al. 1996). Our investigations have revealed a continuous increase in the number of teeth with diffuse calcifications and with pulp stones and diffuse calcifications. However, the number of teeth with pulp stones only is similar in age groups II and III. Thus, there is a lower number of teeth with only pulp stones at advancing age, because of the increase of diffuse calcifications in the root canal in age group II (31-51 years).

Biochemical investigations of human teeth have revealed a decrease in collagen concentration, which occurs at about the same time as eruption and root closure. However, for the rest of the life of the tooth, there is no change in collagen content (Nielsen et al. 1983). Our data suggest that all the pulp collagen, viz., types I, III, V, and VI, remains in the pulp during aging but is compressed into a smaller volume as the pulp cavity shrinks during dentinogenesis (Lechner and Kalnitsky 1981). The increasing fibril density in the pulp during dentinogenesis confirms the hypothesis of Stanley and Ranney (1962).

The significance of the various macromolecules in the development of pulp stones and diffuse calcifications remains to be elucidated. If techniques such as antigen retrieval can be used to visualize antigens that are otherwise undetectable, the range of useful immunohistochemical methods would be greatly expanded. Collagens type I, III, V, and VI are different with respect to their morphological appearance, distribution, and function throughout the pulp tissue. Therefore, the use of anti-collagen antibodies should provide valuable information about the function and expression of the various macromolecules during development and aging, and about pathological remodeling processes of the pulp matrix.

Acknowledgements. We thank Prof. H.-J. Kretschmann, Department of Neuroanatomy and Stomatological Anatomy, Medical University of Hannover, for kindly providing the collection of human teeth of the department, and Mrs. H. Fahlbusch and Mrs. B. Hillmann for excellent technical assistance. We also thank Dr. M. Toeroek, Department of Biometry, and Mr. W. Ko th, Department of Conservative Dentistry and Periodontology, for kindly undertaking the statistical analysis.

References

Amerongen JP van, Lemmens IG, Tonino GJM (1983) The concentration, extractability and characterization of collagen in human dental pulp. Arch Oral Biol 28:339-345

Appleton J, Williams MJR (1973) Ultrastructural observations on the calcification of human dental pulp. Cell Tissue Res 11:222-237

Bemick S (1967) Age changes in the blood supply of human teeth. J Dent Res 46:544-550

Bishop MA, Malhotra M, Yoshida S (1991) Interodontoblastic collagen (von Korff fibres) and circumpulpal dentin formation: an ultrathin serial section study in the cat. Am J Anat 191:67-73

Bonaldo P, Russo V, Bucciotto F, Doliana R, Colombatti A (1990) Structural and functional roles of the a3 chain indicate a bridging role for chicken collagen type VI in connective tissues. Biochemistry 29:1245-1254

Bruns RR, Press W, Engvall E, Timpl R, Gross J (1986) Type VI collagen in extracellular, 100 nm periodic filament and fibrils: identification by immunoelectron microscopy. J Cell Biol 103:363-404

Burgeson RE, Adli FA, Kaitila II, Hollister DW (1976) Fetal membrane collagens: identification of two new collagen alpha chains. Proc Natl Acad Sei USA 73:2579-2583

Chung E, Rhodes RK, Miller EJ (1976) Isolation of three collagenous components of probable basement membrane origin from several tissues. Biochem Biophys Res Commun 71: 1167-1174

Colombatti A, Bonaldo P, Ainger G, Bressan GM, Volpin D (1987) Biosynthesis of chick type VI collagen. I. Intracellular assembly and molecular structure. J Biol Chem 262:14454-14460

Dartsch PC (1990) Einbettungsmedien fur die Auflicht-Fluoreszenzmikroskopie. GIT Labor Mikrosk 9:450^156

Donath K, Breuner G (1982) A method for the study of undecalcified bones and teeth with attached soft tissue. J Oral Pathol 11:318-326

Engvall E, Hessle H, Klier G (1986) Molecular assembly, secretion and matrix deposition of type VI collagen. J Cell Biol 102:703-710

Fleischmajer R, Timpl R, Tuderman L, Raisher L, Wiestner M, Perlish JS, et al (1981) Ultrastructural identification of extension aminopropeptides of type I and III collagen in human skin. Proc Natl Acad Sei USA 78:7360-7364

Fried K, Risling M, Edwall L, Olgart L (1992) Immuno-electron-microscopic localization of laminin and collagen type IV in normal and denervated tooth pulp of the cat. Cell Tissue Res 270:157-164

Garbarsch C, Matthiessen ME, Olsen BE, Moe D, Kirkeby S (1994) Immunohistochemistry of the intercellular matrix components and the epithelio-mesenchymal junction of the human tooth germ. Histochem J 26:110-118

Higashi T, Okamoto H (1996) Electron microscopic study on interodontoblastic collagen fibrils in amputated canine dental pulp. J Endodontol 22:116-119

Hillmann G, Hillmann B, Donath K (1991) Enzyme, lectin and immunohistochemistry of plastic embedded undecalcified bone and other hard tissues for light microscopic investigations. Biotech Histochem 66:185-193

Hillmann G, Koth W, Geurtsen W (1996) Dentikel und diffuse Verkalkungen in unterschiedlich alten Zahnpulpen. Dtsch Zahnârztl Z 51:456—461

Junqueira LCU, Cossermelli W, Brentani R (1978) Differential staining of collagens type I, II and III by Sirius Red and polarization microscopy. Arch Histol Jpn 41:267-274

Junqueira LCU, Bignolas G, Brentani RR (1979) Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochem J 11:447—445

Junqueira LCU, Montes GS, Sanchez EM (1982) The influence of tissue section thickness on the study of collagen by the picrosirius-polarization method. Histochemistry 74:153—156

Kronfeld R (1933) Histopathology of the teeth and their surrounding structures. Lea and Febiger, Philadelphia

Kuo MYP, Lan WH, Lin SK, Tsai KS, Hahn LJ (1992) Collagen gene expression in human dental pulp cell cultures. Arch Oral Biol 37:945-952

Kurita K, Hashmoto Y, Takei T, Kawai T, Hayakawa T (1985) Changes in collagen types during the healing of rabbit tooth extraction wounds. J Dent Res 64:28-32

Lapiere CM, Nusgens B, Pierard GE (1977) Interaction between collagen type I and type III in conditioning bundles organization. Connect Tissue Res 5:21—29

Le May O, Kaqueler JC (1991) Scanning electron microscopic study of pulp stones in human permanent teeth. Scanning Microsc 5:257-267

Lechner JH, Kalnitsky G (1981) The presence of large amounts of type III collagen in bovine dental pulp and its significance with regard to the mechanism of dentinogenesis. Arch Oral Biol 26:265-273

Lukinmaa PL, Waltimo J (1992) Immunohistochemical localization of types I, V, and VI collagen in human permanent teeth and periodontal ligament. J Dent Res 71:391-397

Magloire H, Joffre A, Grimaud JA, Herbage D, Couble ML, Chavrier C (1982) Distribution of type III collagen in the pulp parenchyma of the human developing tooth. Histochemistry 74:319-328

Magloire H, Joffre A, Hartmann DJ (1988) Localization and synthesis of type III collagen and fibronectin in human reparative dentine. Histochemistry 88:141-149

Montes GS, Krisztan, RM, Shigihara, KM, Tokoro, R, Mourao PAS, Junqueira LCU (1980) Histochemical and morphological characterization of reticular fibers. Histochemistry 65:131-141

Moss-Salentijn L, Hendricks-Klyvert M (1988) Calcified structures in human dental pulps. J Endodontol 14:184-189

Narayanan AS, Engel LD (1983) The effect of chronic inflammation of the composition of collagen types in human connective tissue. Collagen Rel Res 3:323-334

Narayanan AS, Page RC (1976) Biochemical characterization of collagens synthesized by fibroblasts derived from normal and diseased human gingiva. J Biol Chem 251:5464-5471

Narayanan AS, Page RC (1983) Connective tissue of the periodontium: a summary of current work. Collagen Rel Res 8: 33-64

Nielsen CJ, Bentley JP, Marshall FJ (1983) Age-related changes in reducible crosslinks of human dental pulp collagen. Arch Oral Biol 28:759-764

Nowack H, Gay S, Wick G, Becker U, Timpl R (1976) Preparation and use of immunohistology of antibodies specific for type I and type III collagen and procollagen. J Immunol Methods 12:117-124

Ohsaki Y, Nagata K (1994) Type III collagen is a major component of interodontoblastic fibres of the developing mouse molar root. Anat Rec 240:308-313

Salomon JP, Septier D, Goldberg M (1991) Ultrastructure of interodontoblastic fibers in the rat molar. Arch Oral Biol 36:171-176

Sayegh FS, Reed AJ (1968) Calcifications in the dental pulp. Oral Surg Oral Med Oral Pathol 25:873-882

Shroff B, Thomas HF (1992) Investigation of the role of von Korff fibres during murine dentinogenesis. J Biol Buccale 20:139—144

Shuttleworth CA, Ward JL, Hirschmann PN (1978) The presence of type III collagen in the developing tooth. Biochem Biophys Acta 535: 348-355

Shuttleworth CA, Berry L, Wilson NHF (1980) Collagen synthesis in rabbit dental pulp fibroblast cultures. Arch Oral Biol 27: 645-650

Shuttleworth CA, Berry L, Kielty CM (1992) Microfibrillar components in dental pulp: presence of both type VI collagen- and fibrillin-containing microfibrils. Arch Oral Biol 37:1079-1084

Stanley HR, Ranney RR (1962) Age changes in the human dental pulp. I. The quantity of collagen. Oral Surg 15:1396-1404

Tabata S, Nakayama T, Funakoshi K, Yasui K, Wada K, Uemira M (1994) Collagen fibrils in the odontoblast layer of the rat incisor by scanning electron microscopy using the maceration method. Anat Rec 239:360-370

Tomeck CD (1990) The clinical significance and management of calcific pulp obliteration. Alpha-Omegan 83:50-54

Von der Mark H, Aumailley M, Wick G, Fleischmajer R, Timpl R (1984) Immunochemistry, genuine size and tissue localization of collagen VI. Eur J Biochem 142:493-502

Whittaker DK, Adams D (1972) Electron microscopic studies on von Korff fibers in the human developing tooth. Anat Rec 174:175-190

Xu LX, Ohsaki Y, Nagata K, Kurisu K (1993) Immunohistochemical studies on the distributions and age-related changes of types I and III collagen in the oral mucosa of mice. J Dent Res 72:1336-1343

Yoshiba N, Yoshiba K, Iwaku M, Nakamura H, Ozawa H (1994) A confocal laser scanning microscopic study of the immunofluorescent localization of fibronectin in the odontoblast layer of human teeth. Arch Oral Biol 39:395^100

Yurchenko PD, Ruben GC (1987) Basement membrane structure in situ: evidence for lateral association in the type VI collagen network. J Cell Biol 105:2559-2568