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

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**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

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**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 1, III, and V belong to the class of fibril-forming collagens. Both types 1 and 111 account for the bulk of the tissue collagen (Shuttleworth et al. 1978, 1980). Type 111 collagen is often found together with type 1. However, type III consists of fibrils of much smaller di­ameter (Lapiere et al. 1977). In human pulp, type Ill 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 1 collagen and only trace amounts of type 111 collagen (Kuo et al. 1992). During tooth development, a close relationship exists between collagen type 1 in dentin and fibronectin in immature enamel in human tooth germs. Immunohistological in­vestigations have revealed the presence of collagens type 1 and 111, 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 simili- ar 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 1 and Ill 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; Yoshibaetal. 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 his- tomorphology 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-erupt- ed, 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-Apparate- bau, 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 Picrosiri- us 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.

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**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

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*Immunohistology*

Paraffin sections of 46 teeth were examined for the immunohisto- logical 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-im- mune 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 Bio­Rad 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; Bio­Rad) 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 (Man- tel-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 1 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 Ill

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**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. xlOO

**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. xfOO

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**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. xlOO

showed a reticular network throughout the pulp. Thin fi­laments or small bundles of filaments could be observed. Blood vessels were surrounded by collagen type 111. 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 111 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.

|  |  |  |
| --- | --- | --- |
| 4-30 years | +/+ | |
| 31-50 years | +/- | +/- |
| 51-65 years | +/- | +/+ |

**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 Thick fibre bundles

Small fibre bundles

*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, 111, 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.

**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

*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.

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 11 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 11 (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 1 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 Ill is one of the main fibrillar elements present in the dental pulp matrix. Sev­eral studies have reported that types 1 and 111 collagen are present in separate fibrils. Type 1 collagen is found mainly in thick fibrils, whereas type 111 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 1 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; Tab- ata 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 (Tor- neck 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 immuno- histological 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 111. 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, 111, V, and VI are different with respect to their morphological appearance, distribution, and function throughout the pulp tissue. Therefore, the use of anti­collagen 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.

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