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ORIGINAL ARTICLE

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Terminal end of the human odontoblast process: a study using SEM and confocal microscopy

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Abstract Towards the middle of the eighteenth century, Tomes described the presence of membranous structures of cellular origin inside the dentinal tubules. Subsequent studies have been controversial regarding the terminal end of the odontoblasts. According to Fusayama, this cellular process reaches even the dentinal-enamel junction; others, such as Brännström, believed that this cellular process is present only in the inner third of the dentin. The aim of the present study was to determine the exact area up to which the terminal ends of the odontoblasts extend. With the aid of advanced confocal laser scanning microscopy (CLSM) cylindrical structures were demonstrated inside the tubules even in the absence of odontoblasts. This would confirm that the structures previously described as cellular processes can be identified with the *lamina limitans* of the peritubular dentin. High resolution field-emission scanning electron microscopy (FE-SEM) provided further evidence that tubular structures are only seen in the inner third of the dentin, towards the pulp.

Key words Dentinal tubules · Odontoblast processes · Confocal laser scanning microscopy · Scanning electron microscopy

Introduction

Dentinal tubules house the odontoblast cytoplasmic process that is separated from the tubular wall by the so called periodontoblastic space. These cellular processes were described for the first time by Tomes in 1856 [15], who claimed that they extended up to the dentin-enamel junction (DEJ) lining the dentinal tubules throughout their length. The term "fibre of Tomes" was used as a synonym for odontoblast process until Brännström and Garberoglio showed that the odontoblast processes end always at a distance of about 0.5–0.7 mm from the pulp [2–4]. These two researchers opened up new horizons and gave new insight, albeit controversial, into the extent of the odontoblast process inside the dentin. However some authors continue to support Tomes' results that claim the odontoblast process extends along all the length of the tubule [1–3,11]. The studies favoring the latter thesis have been carried out using scanning electron microscopy (SEM) and samples in which the organic and inorganic components of the dentin were removed [3–17]. Furthermore, these same studies [3–17] reported that the results obtained by Brännström and Garberoglio [2] as well as by Thomas and Payne [12] were due to an inadequate fixation of the samples that may cause the degeneration of the most peripheral part of the cytoplasmic process [6].Conversely, those researchers who support the presence of the cellular process being limited to the inner part of the dentin [14–15] maintain that what is observed near the enamel is the *lamina limitans* [7–13]. This is an extracellular formation that covers the mineralized walls of the tubule that becomes evident after removal of the mineralized matrix of the dentin.

The aim of the present study was to observe this cellular process with the aid of SEM and advanced confocal microscopy (CLSM) to obtain a tomographic vision of the biological samples.. Deep cavities were prepared in healthy teeth in order to induce necrosis of a limited zone of odontoblasts and the subsequent production of tertiary dentin: the presence of tubular structures related to tertiary dentin is likely to imply the extracellular origin of these structures.

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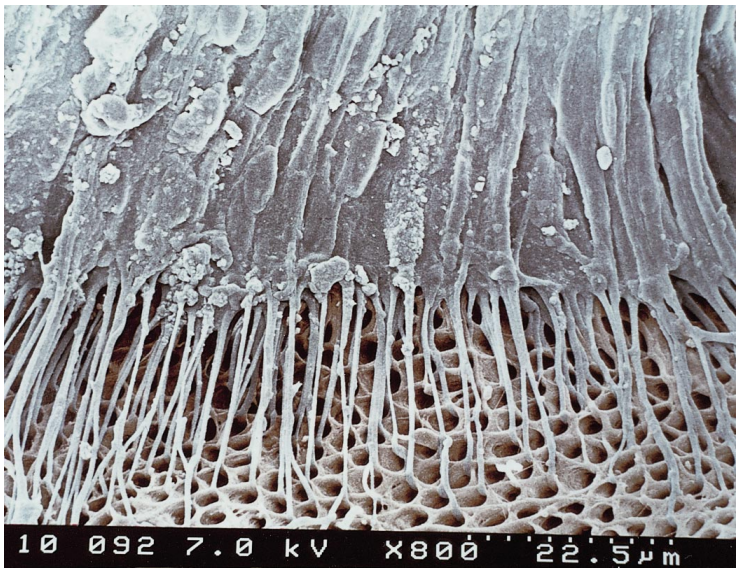

Fig. 1 Pulp–dentinal junction – from the top: odontoblasts' cell bodies, cytoplasmic processes and predentin (scanning electron micrograph ×800)

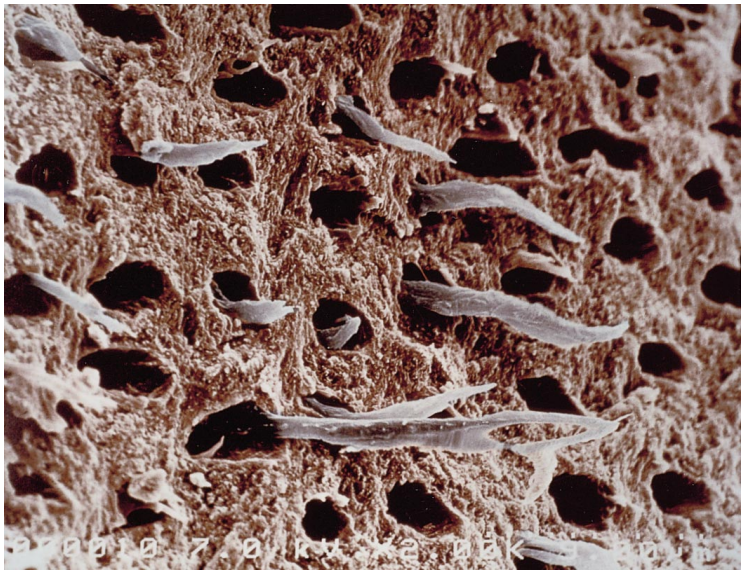

Fig. 2 Fractured dentin, 0.4 mm from the pulp. Odontoblasts' terminal ends are observed (scanning electron micrograph, ×2 000)

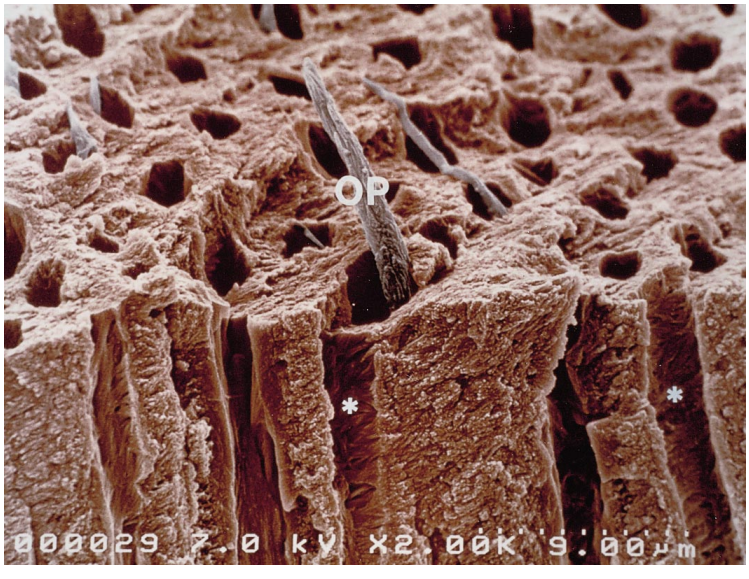

Fig. 3 Note the presence of the cytoplasmic process (*OP*) and the absence of peritubular dentin (*asterisks*) (scanning electron micrograph , ×2 000)

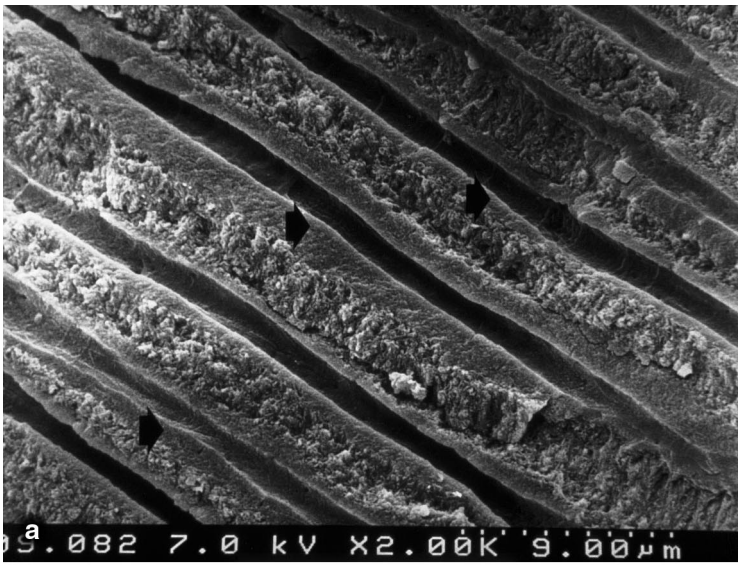

Fig. 4 a Fractured dentin, 1 mm from the pulp. Dentinal tubules are empty. Note peritubular dentin (*arrows*) (scanning electron micrograph, ×2 000).

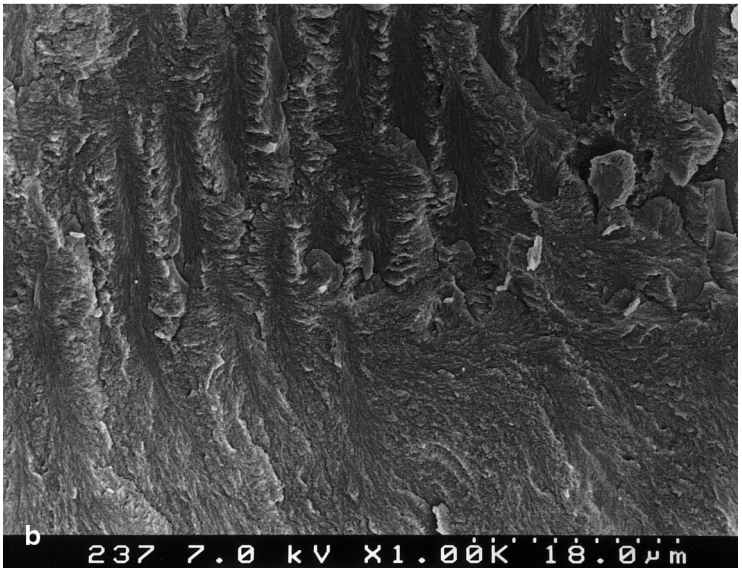

Fig. 4 b Dentin related to sectioned dentinal tubules. Note empty dentinal tubules (*top*) and atubular reparative dentin (*bottom*)(scanning electron micrograph, ×1 000)

Materials and methods

Twelve premolars removed for orthodontic reasons from five persons aged 19–22 years, were used for this study. Patients gave their informed consent to collaborate in this protocol. Before starting with the operative protocol, teeth were clinically and radiographically examined in order to exclude the presence of caries or cervical abrasions.

After isolation of each tooth with a rubber-dam, vestibular class V cavities were prepared under local anaesthesia with 2% carbocaine. The cavities measuring about 2 mm in diameter and 3 mm in depth were prepared using air/water blasts. Cavity depth was judged by either clinical observation of a thin translucent layer of the remaining dentin on the cavity floor overlying the pulp and by measuring with a periodontal probe. Cavities were subsequently restored with zinc oxide-eugenol strengthened cement maintaining the recommended powder/liquid ratio 6 to 1 by weight (IRM Caulk Dentsply; Konstanz, Germany).

All teeth were extracted after 4 weeks of treatment. Immediately after extraction, they were sectioned at the center of the root in order to facilitate the penetration of the fixative. The teeth were then fixed by immersion in 2.5% glutaraldehyde in a 0.1 M phosphate buffer solution at 4°C.

SEM preparation

Half of the samples were fractured with liquid nitrogen and a razor blade after making a series of superficial notches in order to obtain sections with the longitudinal plane of fracture passing through the cavities. The samples were dehydrated using ascending concentrations of alcohol (30%, 50%, 70%, 90%, 100%) and then dried to critical point using liquid CO_2 (Balzers Union CPD 020

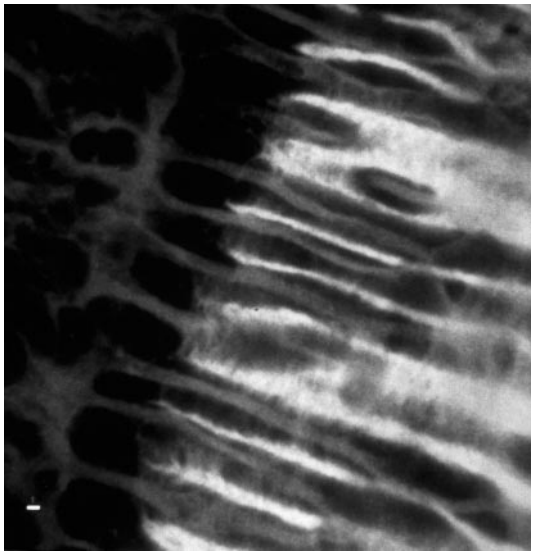

Fig. 5 Pulp–dentinal junction. Tubular structures are observed inside the dentinal tubules (confocal laser scanning micrograph, *bar*: 1 μm)

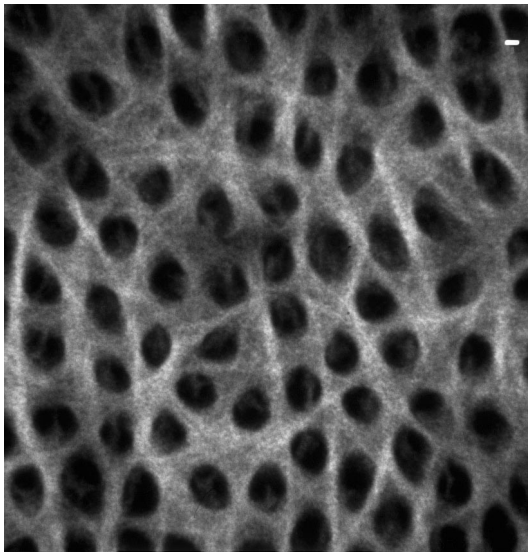

Fig. 7 Outer third of the dentin. Note the tubular structures inside the tubules (confocal laser scanning micrograph, *bar*: 1 μm)

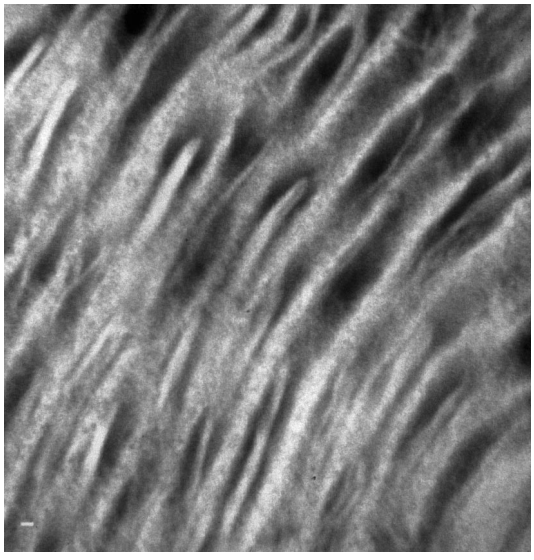

Fig. 6 Middle third of the dentin. Note the tubular structures inside the tubules (confocal laser scanning micrograph, *bar*: 1 μm)

critical point dryer; Liechtenstein), mounted on aluminum stubs with silver paste, and coated with 20 Å platinum in a sputter coater (EMITECH K550, 2 M Strumenti; Rome, Italy). Observations were made with a field-emission SEM (HITACHI S-4000; Tokyo, Japan) operated at an accelerating voltage of 7–10 kV. SEM images were taken from the outer dentinal border and progressively towards the pulp. Dentinal tubules were then photographed at different distances from the pulp, at the coronal, cervical, and radicular zones.

CLSM and light microscopy

The remaining half of the samples was demineralized in 0.3 M EDTA, washed 20 times in distilled water, treated with graded *N*-butyl alcohol, and embedded in Paraplast Plus (Sherwood Medical; London, UK).The samples were longitudinally cut in series of 10- to 20-μm-thick sections with a Leitz microtome. The sections were stained with Van Giesson's trichromic method and observed with a Zeiss Ultraphot II light microscope and with a confocal microscope (Serastro 2000 Molecular Dynamics; Birmingham, UK) using a 60× objective lens and connected to a graphic station (Silicon Graphics, Miami, Fla., USA).

Results

The odontoblast terminal ends when observed by SEM (Figs. 1,2) extended as far as 0.5–0.7 mm from the pulp-dentinal junction. Furthermore, dentinal tubules containing such cellular extents were devoid of peritubular dentin (Fig. 3) and, therefore, they appeared empty at longer distances from the pulp (Fig. 4) and showed the presence of peritubular dentin. At the same time, dentinal tubules located in zones adjacent to the prepared cavity appeared also empty. In fact, cavity preparation in these areas caused necrosis of odontoblasts and differentiation of fibroblasts with the subsequent formation of atubular tertiary dentin.

However, the samples studied by CLSM showed tubular structures, both at the pulp-dentinal junction (Fig. 5) and at the middle third of the dentin (Fig. 6) up to the DEJ (Fig. 7). These tubular structures were also observed in the cavity preparation areas, in continuity with the tertiary dentin. Figures 8 and 9 show the same field

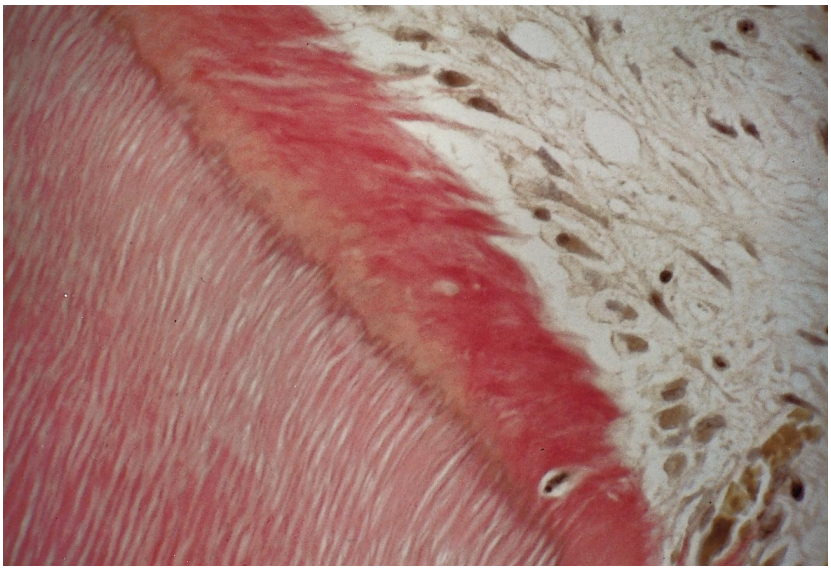

Fig. 8 Pulp–dentinal junction. Note the barrier of reparative dentin produced by differentiated cells. (light micrograph, ×250)

as seen by LM and CLSM, respectively. The tubular dentin that lies close to the reparative dentin (Figs. 10,11) shows at high magnification CLSM the same tubular structures evidenced in the healthy dentin, in zones characterized by the absence of odontoblasts.

Discussion

From the present study it is interesting to note that tubular structures inside the tubules, and previously described as odontoblast cytoplasmic extents [3–17], are observed even in the absence of odontoblasts. This fact seems to be a further demonstration that the above structures correspond to the *lamina limitans*, constituted primarily by glycosaminoglycans and, therefore, certainly of extracellular nature. Previous reports in favor of the odontoblast terminal end reaching the DEJ [1–3] were based on the demineralization and subsequent removal of the mineralized matrix of the dentin, which in our opinion, clearly shows the *lamina limitans* extending up to the enamel. Reports from recent studies [5] performed with CLSM suggest tubular structures considered as odontoblast processes up to the DEJ. These correspond to the same membranous structures observed in the present study, even in the absence of the odontoblast necrotised following cavity preparation, as is shown by the presence of tertiary dentin near the pulp-dentinal junction. In fact, Thomas and Carella [14] have reported a limiting membrane of the peritubular matrix, defined as *lamina limitans* according to the terminology used for similar structures present in the bone. This membrane contains a high content of glycosaminoglycans and glycoproteins and is, therefore, of extracellular nature as shown by recent studies carried out by transmission electron microscopy [14,16]. In the present study, SEM evidenced the presence of odontoblast processes only in the inner third of the dentin in agreement with other authors which confirms that this process extends for a distance of 0.5–0.7 mm from the pulp [2,4,14].

In addition, it is also interesting to note that the odontoblast process inside the tubule is characterized by the absence of peritubular dentin. As already mentioned, samples observed by SEM were fixed immediately after extraction and previous removal of the apexes in order to facilitate the passage of glutaraldehyde, and were subsequently fractured after immersion in liquid nitrogen. Maniatopoulus and Smith [6] disagree with previous reports based on SEM observations, that supported the presence of cellular processes limited to the inner third of the pulp [2,4,14] and ascribed these results to artifacts of cell fixation leading to the shrinkage of the most peripheral part of the cytoplasmic process. In order to ensure the fixative penetrated in the most peripheral parts of the tubule, the same authors suggested that the samples should be immersed in liquid nitrogen immediately after the extraction, thus obtaining the fracture of the sample. With this method they noted odontoblast processes reaching the dentin-enamel junction. Nevertheless, Rankin [8] has shown that immersion in liquid nitrogen (–190°C) creates an increase in intrapulpal pressure to over 1700 atm, so that all cellular material is thrust to the most peripheral parts of the tubules. Using teeth with immature apexes, and therefore, without such an increase in pressure, Rankin demonstrated that there were no cells in the most peripheral parts of the tubules [8]. Sigal et al. [9,10] demonstrated in rat and human dentins, through the use of an immunofluorescence technique, the intracellular presence of actin and tubulin which extended up to the DEJ. This supports the hypothesis that the structures actually observed with the SEM correspond to odontoblast processes that extend up to the DEJ. Nevertheless, Weber et al. [16] advised on a certain caution when using immunofluorescence staining; in fact, retraction of the cytoplasmic process may leave some cytoplasmic components attached onto the tubular walls. Besides, the

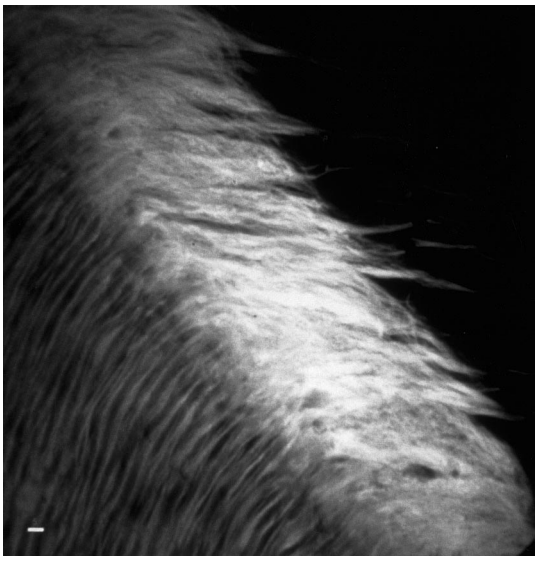

Fig. 9 Same area of Fig. 8 observed with confocal laser scanning microscopy (*bar*: 5 μm)

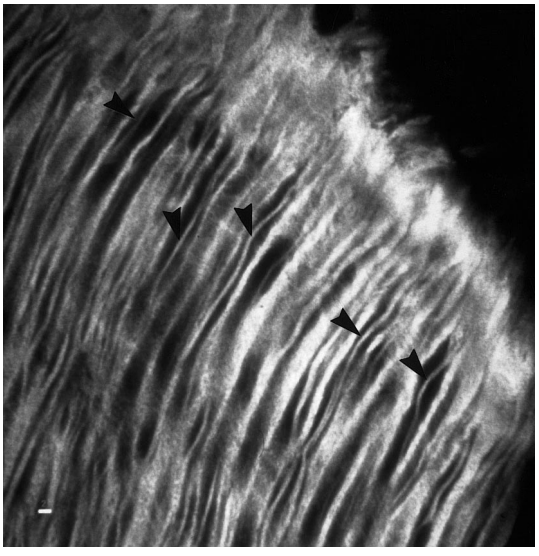

Fig. 10 Transition zone between tertiary predentin and dentin. The same tubular structures (*arrows*) described inside the tubules in the previous figures are also observed here, although in these zones there is necrosis of odontoblasts as shown by the presence of atubular tertiary dentin produced by differentiated fibroblasts (confocal laser scanning micrograph, *bar*: 2 μm)

possibility that the tubular structures observed with SEM preparations were not odontoblast processes but collagenase-resistant inner sheaths of the peritubular matrix should be considered also [16]. These structures were observed in the vicinity of tertiary dentin. However, the physiological role of the *lamina limitans* has still to be defined. In the bone, it constitutes a barrier that regulates the exchange of substances between the plasma and the mineralized matrix. Therefore, as already suggested by Thomas [14], it could have a similar function between the tubular fluid and the peritubular dentin.

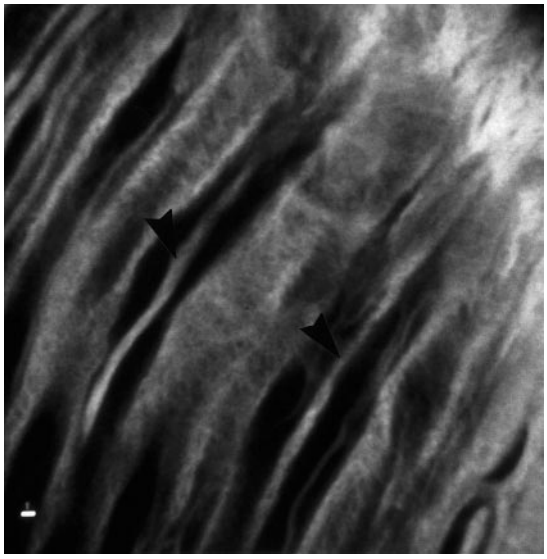

Fig. 11 Higher magnification of Fig. 10. Note the structural continuity between tertiary predentin and tubular structures (*arrows*); this fact excludes a cellular origin (confocal scanning micrograph, *bar*: 1 μm)

Conclusions

The parallel use of SEM, CLSM and light microscopy allowed the analysis of the relationship between hard dental tissues (dentin) and soft tissues (pulp). Membranous structures were observed by CLSM to extend from the pulp to the DEJ, even in the zones in which odontoblast necrosis occurred, a fact that is clearly demonstrated by the presence of the *lamina limitans* of peritubular dentin. SEM analysis confirmed once more the presence of tubular structures only in the inner third of the dentin (towards the pulp).

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