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Immunohistochemical studies of the periodontal membrane in primary teeth

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Abstract

Objectives. To describe the periodontal membrane of human primary teeth immunohistochemically, while focusing on the epithelial layer of Malassez, fibers, and peripheral nerves, and to compare the findings with those of a previous study of human permanent teeth. ***Material and methods.*** Nineteen human primary teeth extracted in late childhood in connection with treatment were fixed, decalcified, dehydrated, and embedded in paraffin. Paraffin sections were stained with wide spectrum screening (WSS), Vimentin, and NeuN in order to mark the epithelial layer of Malassez, fibers, and peripheral nerves. ***Results.*** For root surfaces without resorption, the epithelial rests of Malassez appeared as small scattered islands. The fibers varied from tightly packed close to the root surface to a messy and loose organization. Innervation could be seen in close proximity to the root surface. The epithelial cells of Malassez were not usually seen along root surfaces with resorption. The fibers were sparse or not present. Innervation was seen in close proximity to the root. In regions with repair of resorption lacunae, the immunohistochemical reactions for epithelial cells of Malassez, fibers, and innervation pattern could be identical to those in regions with no resorption. ***Conclusion.*** In regions without resorption, spatial organization of the periodontal membrane of primary teeth was similar to that of permanent teeth, although the number and distribution of epithelial cells and fibers differed. In regions with repair of root resorption, the epithelial cells of Malassez, fibers, and innervation appeared as root surfaces without resorption.

Key Words: *Epithelial rests of Malassez, immunohistochemistry, periodontal membrane, primary teeth, root resorption*

Introduction

In a recent study, Kjær & Nolting [1] mapped the interrelations of the epithelial layer of Malassez, fibers, and peripheral nerves in the periodontal membrane related to root surfaces of human permanent teeth. In this study, the epithelial layer of Malassez was seen as a nearly continuous layer dividing the periodontal ligament into an inner and an outer layer with completely different fiber morphology and innervation pattern. The inner layer is characterized by tightly packed fibers with no precise orientation and innervation in close proximity to the root surface, and the outer layer by fewer longitudinally organized fibers and sparse innervation. Kjær & Nolting [1] thus hypothesize that this persisting epithelial layer of Malassez is decisive in reorganization and maintenance of the periodontal membrane during eruption and tooth movement, and that it may protect the root surface from resorption.

In the periodontal ligament of human permanent teeth, it is known that the epithelial rests of Malassez are intimately related to periodontal neural endings [2]. The epithelial rests of Malassez have shown strong immunoreactivity for TrkA in rats [3]. TrkA is a high-affinity receptor for nerve growth factor (NGF) expressed in various human non-neuronal tissues. In cats, Tadokoro et al. [4] have shown that the epithelial rests of Malassez express immunoreactivity for PGP 9.5 and that nerve fibers occasionally make contact with these PGP 9.5-immunoreactive cells in the epithelial rests of Malassez. An experimental study in monkeys has indicated that this epithelium of Malassez is decisive in maintaining the thickness of the periodontal ligament [5].

In both humans and rats, it has been shown that the epithelial rests of Malassez are located adjacent to resorption areas, but never directly above an active resorption lacuna [6,7]. Persistence of an epithelial

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root sheath in the periodontal ligament of primary teeth has been described in patients with hyper-IgE syndrome [8,9]. Eruption of the permanent incisors, canines, and premolars in patients with hyper-IgE syndrome is impaired due to delayed resorption of the roots of the primary teeth. This supports the theory of a potential role of the epithelial layer of Malassez in preventing root resorption.

Few studies have focused on the connection between deviant resorption patterns in primary teeth and a tendency to resorption in permanent teeth with ectodermal morphological characteristics [10–13]. This link between resorption in both the primary and the permanent dentition supports the need for comparisons of the periodontal membrane of human primary teeth with that of human permanent teeth on a microscopic level.

The aim of the present study was to describe the epithelial layer of Malassez, fibers, and peripheral nerves in the periodontal membrane of human primary teeth and to compare the findings with those of a former, similar, study on human permanent teeth.

Material and methods

The material comprised 19 primary teeth (12 primary molars and 7 primary canines) from 12 children aged between 9 and 15 years. All teeth were forwarded from municipal clinics in Copenhagen and Frederiksberg communities, Denmark, after a written request. The teeth had been extracted due to agenesis of the permanent successor (4 primary molars) or to ectopia of a permanent tooth (8 primary molars and 7 primary canines). Teeth with caries, apical infections, or fillings were not included. All primary teeth included had areas with resorption. Permission was given from the biomedical research ethics committees of Copenhagen and Frederiksberg Communities (KF07322471).

Fixation, decalcification, and sectioning

The primary teeth were fixed in 4% neutral buffered formaldehyde for 2–8 days and afterwards decalcified in 0.5 M EDTA (ethylene diamine tetra acetic disodium salt, Titriplex II; Merck, Darmstadt, Germany) for 6–10 weeks.

After decalcification, the primary teeth were fixed in 4% neutral buffered formaldehyde for 2–4 days and then dehydrated and embedded in paraffin using a double embedding method: 80% ethanol – 90% ethanol – 96% ethanol – 99% ethanol – 99% ethanol/methyl salicylate – methyl salicylate – 1% celloidin in methyl salicylate – methyl salicylate/paraffin – several changes of paraffin – and finally embedded in paraffin. Paraffin blocks were serially cut into 5 μm thick sections and dried overnight at 40°C.

Every tenth section was stained with hematoxylin and eosin, washed, and dehydrated in graded alcohols using a staining machine (Shandon Varistain Gemini A78010402; Thermo Electron Corporation, Copenhagen, Denmark). Sections were then cover-slipped (Pertex 00801; Histolab, Göteborg, Sweden).

Immunohistochemistry with WSS, vimentin, and NeuN

Immunohistochemistry was carried out using the Dako REAL EnVision™ Detection System (K5007; Dako, Glostrup, Denmark). Sections were dewaxed in xylene, rehydrated in graded alcohols, and pretreated with Tris-EDTA pH 9 at 60°C for 90 min. Sections were washed in Tris-buffered saline (TBS 0.05 M Tris, 0.15 M NaCl, pH 7.6; Bie & Berntsen, Herlev, Denmark) for 5 min, encircled with a delimiting pen (S2002; Dako), and then washed twice in TBS for 5 min.

Sections were incubated for 10 min with peroxidase blocking solution (S2023; Dako) and then washed in TBS for 5 min.

Sections were incubated in primary antibodies for 60 min using: Cytokeratin wide spectrum screening (WSS Z0622; Dako) diluted 1:5000, anti-vimentin 3B4 (M7020; Dako) diluted 1:500, and anti-NeuN clone A60 (MAB377; Chemicon (Millipore), Copenhagen, Denmark) diluted 1:50. All primary antibodies were diluted in antibody diluent (S2022; Dako).

Sections were then washed twice (5 min) and incubated in secondary antibody coupled with peroxidase (K5007; Dako). Sections were washed three times (5 min) and then incubated in Substrate Buffer/DAB+ Chromagen (K5007; Dako) for 10 min and then washed in distilled water. Sections were counter stained in Carazzi's hematoxylin (Bie & Berntsen), washed, and dehydrated in graded alcohols in a staining machine (Shandon Varistain Gemini A78010402; Thermo Electron Corporation, Copenhagen, Denmark). Sections were cover-slipped (Pertex 00811; Histolab).

Control of immunohistochemistry

In the negative controls, the primary antibody was deleted, and the sections were incubated in antibody diluent (S2022; Dako) only. Sections were otherwise processed according to protocol. Positive controls of the primary antibodies used were performed on human permanent teeth, where the reactions have already been described [1].

Results

Immunohistochemistry of root surfaces without resorption

Wide spectrum screening (WSS). The epithelial layer of Malassez, seen as small scattered oval islands of cells with varied morphology, showed positive reactions for WSS. Some islands contained well-defined cells (Figure 1a), but usually they were irregular in shape with unclearly defined borders and the cells within the islands seemed dissolved. In three primary teeth, extracted owing to ectopia of the permanent successor, epithelial cells of Malassez were not visible. The epithelial layer of Malassez never appeared as a continuous or nearly continuous layer, as previously documented in permanent teeth (Figure 2a, b) [1].

Vimentin. Periodontal fibers reacted positively to vimentin. In the periodontal membrane of primary teeth the number of fibers varied and had a messy organization. In a few primary teeth, the fibers were tightly packed in a zone close to the root surface (Figure 1b). This pattern is similar to the pattern of fibers previously demonstrated in permanent teeth [1]. No fibers were present in six primary teeth extracted due to ectopia of the permanent successor.

Anti-neuronal nuclei (NeuN). NeuN indicated regions with innervation, which was seen in the primary teeth located close to the root surface (Figure 1c) similar to the pattern previously seen in permanent teeth [1]. Innervation could not be detected in four primary teeth extracted because of ectopia of the permanent successor.

Immunohistochemistry of root surfaces with resorption

Wide spectrum screening (WSS). The epithelial cells of Malassez seemed to have disappeared in the apical parts of the periodontal membrane and were not usually seen along root surfaces with resorption (Figure 3a). In four primary teeth, epithelial islands of Malassez present within a resorption lacuna could be seen along root surfaces both with (Figure 4a) and without cement repair.

Vimentin. Fibers were either sparsely distributed or not observed along root surfaces with resorption (Figure 3b). In locations with repair after resorption, fibers (Figure 4b) could appear similar to fibers in locations without resorption.

Anti-neuronal nuclei (NeuN). Innervation could be seen along resorption surfaces both with (Figure 4c) and without cement repair (Figure 3c). The innervation pattern was similar to the pattern along root surfaces without resorption (Figure 1c) and it was not possible to determine any difference in innervation reaction between root surfaces with and without resorption.

Discussion

The present study indicates that the spatial interrelation between the epithelial layer of Malassez, fibers, and innervation in the periodontal

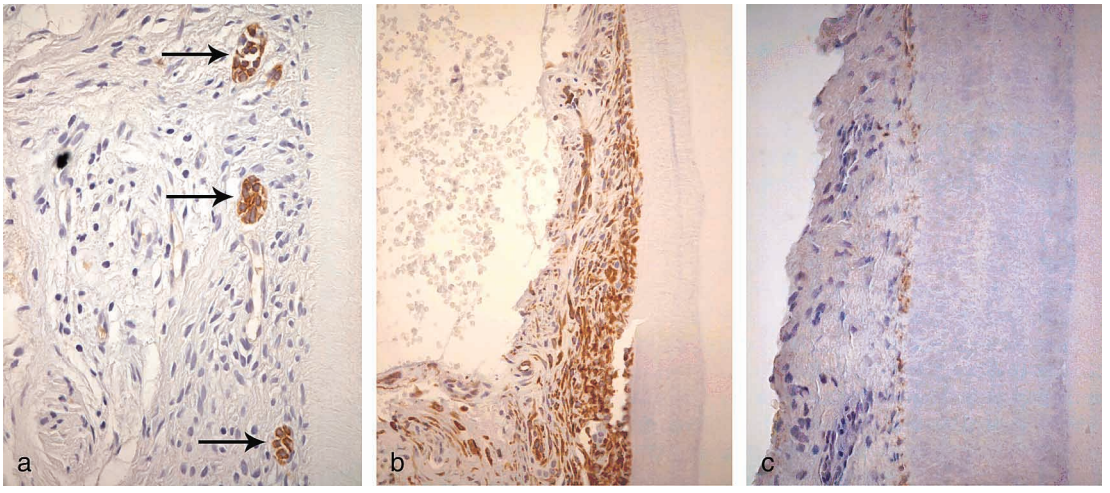

Figure 1. Sections of the periodontal membrane and root surface without resorption in human primary teeth in late childhood. In each section, the root surface is to the right and the periodontal membrane to the left. (a) Immunohistochemical staining with antibody Cytokeratin (wide spectrum screening). The epithelial cells of Malassez (*arrow*) can be seen collected in small scattered islands along the root surface. (b) Immunohistochemical staining with antibody anti-vimentin. The fibers (brown) can be seen tightly packed in a zone close to the root surface. Further distant from the root surface, the fibers are more loosely packed and have no obvious orientation. (c) Immunohistochemical staining with anti-neuronal nuclei (NeuN). Intense innervation can be seen in close proximity to the root surface. The three sections demonstrate differences in location of the epithelial cells of Malassez, fibers, and peripheral nerves.

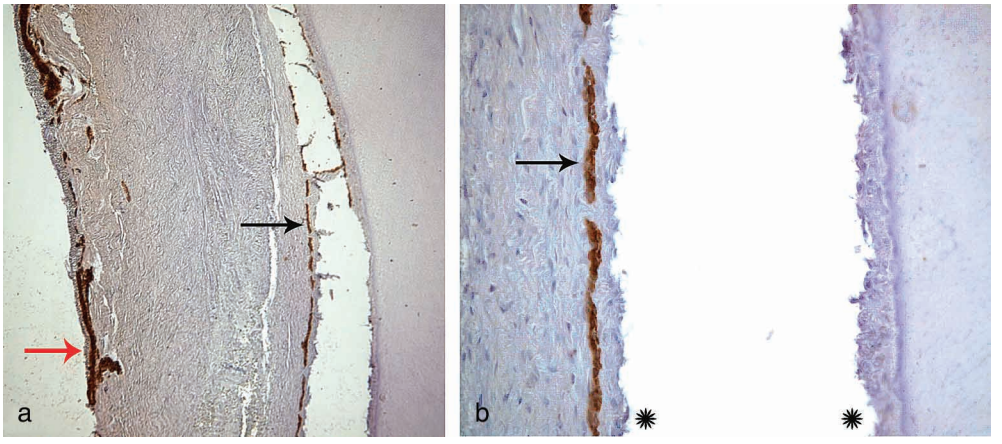

Figure 2. A section in different magnifications (a and b) of the periodontal membrane of a permanent tooth from a previous study by Kjær & Nolting [1]. The membrane is artificially torn off from the permanent tooth's root surface. The section is immunohistochemically stained with antibody Cytokeratin (wide spectrum screening). The root surface is to the right and the periodontal membrane to the left. (a) In this figure, epithelium is stained brown. This includes the epithelial layer of Malassez (*black arrow*) seen along the entire root surface. It also includes the oral epithelium (*red arrow*) seen to the left. (b) The brown color marks the epithelial layer of Malassez (*black arrow*). Distortions of the tissue may be due to extraction, sectioning, or dehydration. The two torn apart surfaces are marked with a star. Compared to the periodontal membrane of a primary tooth shown in Figure 1a the epithelial cells of Malassez are organized in a nearly continuous layer along the root surface in the periodontal membrane of this permanent tooth.

membrane of primary teeth along root surfaces without resorption is similar to that previously seen by Kjær & Nolting [1] in permanent teeth. Our study also reveals differences between the periodontal membrane of primary teeth and that of permanent teeth. The most remarkable difference is that the epithelial layer of Malassez was only seen as small scattered oval islands in primary teeth and never as a nearly continuous layer, as previously described in permanent teeth [1]. It is possible that the scattered presence of epithelial islands of Malassez in primary teeth extracted from children in late childhood can be the result of apoptosis of cells in the epithelial layer of Malassez. This can only be clarified by comparing the periodontal membrane in primary teeth extracted in late childhood versus early childhood. Extraction of human primary teeth in early childhood is seldom indicated compared to extraction of human primary teeth in late childhood. In late childhood, healthy primary teeth are extracted in connection with ectopic eruption of a permanent successor or during

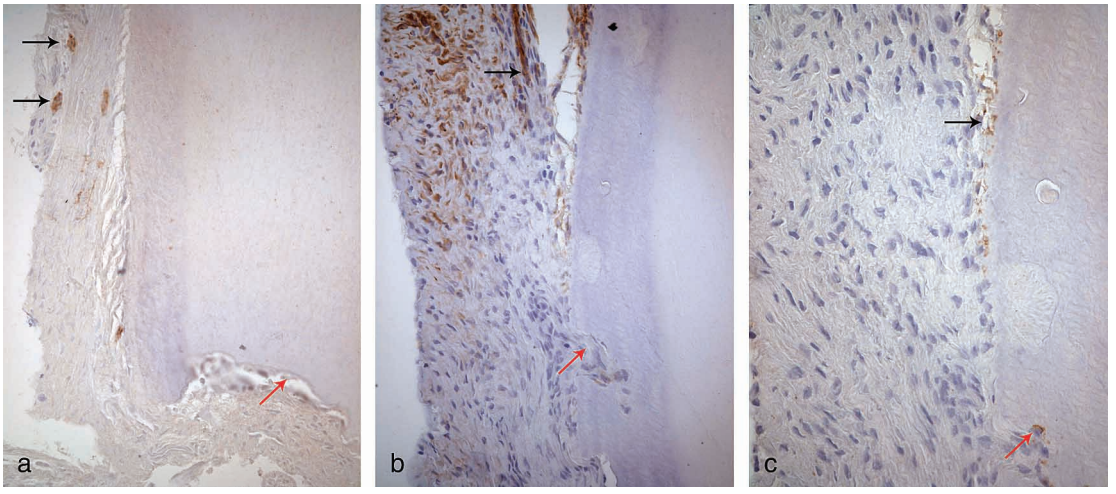

Figure 3. Sections of the periodontal membrane and root surface in human primary teeth in late childhood. Root resorption is visible in each section – the root surface to the right and the periodontal membrane to the left. (a) Immunohistochemical staining with antibody Cytokeratin (wide spectrum screening). The epithelial cells of Malassez (*black arrow*) can be seen as small scattered islands along the root surface without resorption. Compared to Figure 1a, the epithelial cells and the periodontal membrane are dissolved. Epithelial cells of Malassez are not seen apically, where root resorption has occurred (*red arrow*). (b) Immunohistochemical staining with antibody anti-vimentin. The fibers (*brown*) can be seen tightly packed in a zone close the root surface without resorption (*black arrow*). In the apical parts, where root resorption is visible, fibers have diminished (*red arrow*). (c) Immunohistochemical staining with anti-neuronal nuclei (NeuN). Intense innervation can be seen in close proximity to the root surface without resorption (*black arrow*). In the apical part, where root resorption has occurred, innervation can also be seen in close proximity to the root surface (*red arrow*).

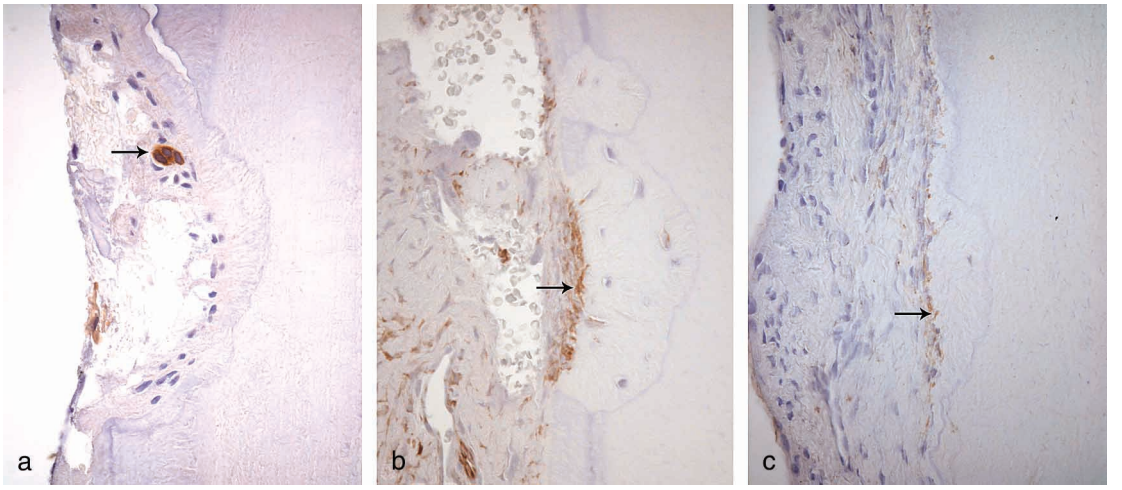

Figure 4. Sections of the periodontal membrane and root surface in human primary teeth in late childhood – the root surface to the right and the periodontal membrane to the left. Repair of former root resorption can be seen in each section. (a) Immunohistochemical staining with antibody Cytokeratin (wide spectrum screening). A small epithelial island of Malassez (*arrow*) can be seen in a former resorption lacuna, where repair is also visible. (b) Immunohistochemical staining with antibody anti-vimentin. The fibers (*arrow*) are clearly visible close to the root surface in the area of a former resorption lacuna. (c) Immunohistochemical staining with anti-neuronal nuclei (NeuN). Intense innervation can be seen in close proximity to the root surface with resorption and repair (*arrow*). The innervation pattern is similar to the innervation pattern in Figure 1c and 3c.

orthodontic treatment in cases with agenesis of the permanent successor.

Some of the primary teeth in this study were from regions with agenesis of the permanent successor. It is not known whether the presence of agenesis of the permanent successor influences the quality and quantity of the epithelial rests of Malassez in the periodontal membrane of the primary tooth. The present study does not explain differences in the periodontal membrane of primary teeth with and without permanent successor.

It is well known that root resorption is a normal physiological process in primary teeth during eruption of the permanent successor, whereas resorption in permanent teeth is a pathological process.

The sparse number of epithelial cells of Malassez might indicate reduced protection of the root in primary teeth compared to permanent teeth. It is possible that the epithelial cells of Malassez in the periodontal membrane of primary teeth are predetermined for apoptosis prior to eruption of the permanent successor, or gradually disappear during eruption of the permanent successor. The few epithelial rests of Malassez in the apical parts and along root surfaces with resorption could explain why primary teeth undergo physiological resorption. In this context, it is interesting that apoptosis of epithelial cells within the rests of Malassez has been documented in rats by TUNEL staining [14].

The persistence of an epithelial root sheath in the periodontal membrane of primary teeth not undergoing root resorption in patients with hyper-IgE syndrome [8,9] also indicates a protective role of the epithelial rests of Malassez against root resorption.

Primary molars can persist for several years in regions with agenesis of the succeeding premolar if the permanent teeth in the dentition in general do not show morphological deviations such as invaginations, taurodontia, short or slender roots [11]. Hertwig's epithelial root sheath, which develops into the epithelial layer of Malassez, determines the morphology of the root [15]. It is possible that dentitions with anatomical deviations in crown and root morphology also dispose microscopic deviations in the epithelial layer of Malassez. If so, this could explain why dentitions with different morphology are exposed differently to root resorption [10–13].

Several studies on vertebrates have indicated that the epithelial rests of Malassez maintain periodontal membrane function and regeneration [5,7,16,17]. This could explain why epithelial islands of Malassez were occasionally seen in resorption lacunae in the present study.

Our study documents that tightly packed fibers are only visible along root surfaces without resorption and surfaces repaired after resorption. Only sparsely distributed fibers were demonstrated in the periodontal membrane along root surfaces with resorption. It is hypothesized that the tightly packed fibers may protect the root surface just as periost protects the bone. Accordingly, it is assumed that these fibers have a secondary role in the resorption process.

Our study documents that innervation is present along root surfaces both with and without resorption, which might indicate that innervation regulates physiological resorption. In this context, it is interesting that an intimate relation has been demonstrated between peripheral nerves and the epithelial rests of Malassez on an electron-microscopic level [2].

The present study demonstrates differences in the periodontal membrane of human primary teeth

compared to a previous study of human permanent teeth [1], i.e. differences that should be taken into account in future studies on primary teeth, where resorption is a normal physiological process in contrast to permanent teeth, which do not resorb under normal conditions. An interesting finding is that the distribution of epithelial cells, fibers, and innervation pattern occur in regions where resorption has been repaired, indicating that re-establishment of the periodontal membrane is possible after root resorption. This perspective needs to be further elucidated. Re-establishment of the periodontal membrane is an important perspective in cases where root resorption has occurred in the permanent dentition.

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