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ORIGINAL ARTICLE

Immunolocalization of RANK and RANKL along the root surface and in the periodontal membrane of human primary and permanent teeth

MARIE-LOUISE BASTHOLM BILLE[1], BJARKE THOMSEN[1], THOMAS LEVIN ANDERSEN[2] & INGER KJÆR[1]

[1]*Department of Orthodontics, School of Dentistry, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark, and* [2]*Department of Clinical Cell Biology, University of Southern Denmark, Vejle Hospital, Vejle, Denmark*

Abstract

Objective. Root resorption, impaired tooth eruption and early tooth loss have been described in relation to diseases that involve defects in the RANK-RANKL-OPG-expression. The aim of the present immunhistochemical study was to localize and compare the reactions for RANK and membrane-bound RANKL along root surfaces and in the periodontal membrane in close proximity to the root surface of human primary and permanent teeth. ***Materials and methods.*** The material comprised extracted human teeth (11 primary teeth and six permanent teeth) from 10 different patients. Paraffin sections were prepared of each tooth and sections of each tooth were immunohistochemically stained with antibodies specific for membrane-bound RANKL and RANK. ***Results.*** The root surface and the periodontal membrane in close proximity to the root surface did not show immunoreactivity for RANKL. RANKL was only located in odontoblasts and in cells along denticles in one primary tooth. RANK was located in mononuclear cells in the pulp and in multinucleated odontoclasts along resorbed root surfaces and along resorbed dentin surfaces in the pulp in primary teeth and one permanent tooth. ***Conclusions.*** This study demonstrated RANK positivity in resorption areas in primary and permanent teeth. RANKL was positive in the pulp of one primary tooth. RANK expression in odontoclasts and RANKL expression in the pulp may indicate that RANK/RANKL play a role during resorption.

Key Words: *RANK, RANKL, root resorption, periodontal membrane*

Introduction

Root resorption is a normal physiological process in the primary dentition, whereas root resorption is a pathological process in the permanent dentition. It is unclear what initiates the resorption process in the primary dentition and what prevents the resorption process in the permanent dentition.

Immunohistochemical studies have shown that the periodontal membrane in close proximity to the root surface is similar in human primary and permanent dentitions when it comes to epithelial cells of Malassez, vimentin expression and innervation along root surfaces without resorption [1,2]. Another immunohistochemical study has shown that the expression of the extracellular matrix proteins osteopontin and bone sialoprotein in the periodontal membrane in close proximity to the root surface differs between human primary and permanent teeth [3]. Osteopontin and bone sialoprotein are seen in close proximity to the root surface in human primary teeth, but not in permanent teeth [3]. Osteoclasts and presumably also odontoclasts express membrane receptors (for example $\alpha_V\beta_3$, also called vitronectin receptor) that can bind to osteopontin and bone sialoprotein which consequently facilitate osteoclast adhesion to the bone and dentin surfaces *in vitro* [4,5]. Osteoclasts also express TRAP (tartrate-resist and acid phosphatese), which is an enzyme that presumably functions as a detachment factor. TRAP hydrolyses osteopontin and bone sialoprotein and subsequently detaches osteoclasts from the bone surface [5–7]. TRAP is also widely used as an immunohistochemical marker for osteoclasts and odontoclasts [7,8].

Most patient cases of pathological resorption in the permanent dentition cannot be explained. However, few patient cases of pathological resorption are

Correspondence: Inger Kjær, Department of Orthodontics, School of Dentistry, Faculty of Health Sciences, University of Copenhagen, 20 Nørre Alle, DK-2200 Copenhagen N, Denmark. Tel: +45 35 32 66 74. E-mail: ik@sund.ku.dk

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associated with different syndromes or diseases. One example of pathological resorption in the permanent dentition is familial expansile osteolysis, a genetic disease caused by a mutation in the gene for RANK [9,10]. RANK (receptor activator of NF-κB) is a membrane receptor in osteoclasts [11]. RANKL (receptor activator of NF-κB ligand) is a ligand, which binds to RANK and initiates osteoclast differentiation and activation [11]. RANKL exists either as a secreted ligand or as a membrane-bound ligand in osteoblasts [6]. NF-κB refers to a group of different latent regulatory proteins within the cytoplasm of most mammalian cells [12,13]. Thus, the RANK-RANKL-interaction is essential for osteoclast differentiation, activation and osteoclast survival [11]. OPG is a secreted decoy receptor for RANKL that, through binding to RANKL, inhibits osteoclasts differentiation, activation and survival [11]. Otherwise, impaired tooth eruption or early tooth loss are the main dental symptoms described in relation to diseases that involve defects in the RANK-RANKL-OPG-expression [10].

Few studies have focused on RANK and RANKL expression in human teeth [14–18]. To our knowledge, only one study has identified immunhistochemically the localization of RANK and RANKL in histological sections of human primary teeth [15]: RANK was seen in mono- and multinucleated odontoclasts, whereas RANKL was seen in the pulp (odontoblasts, fibroblasts) and in the periodontal membrane (fibroblasts, mono- and multinucleated cells, odontoclasts and in dentin-cementum close to resorption surfaces).

Studies have otherwise focused on isolated periodontal ligament cells in culture from human primary teeth [14], human permanent teeth [18] or both [17]; but none of these studies included histology. These studies located RANKL and RANK expression to human periodontal ligament cells (fibroblast cells and odontoclasts) [14–18]. A difference in RANKL-OPG ratio has been described between teeth with resorption compared to teeth without resorption [17]. It is not known if the immunolocalization of RANKL and RANK along root surfaces in histological sections is different in human primary teeth undergoing physiological resorption and permanent teeth without resorption.

The aim of the present immunhistochemical study was to localize and compare the reactions for membrane-bound RANKL and RANK along root surfaces and in the periodontal membrane in close proximity to the root surface of human primary and permanent teeth.

Materials and methods

Patients and histological specimens

Human teeth from 10 different patients were included in the study:

- *Primary teeth*: Eleven primary teeth from eight different children. Eight teeth from five different patients were extracted due to agenesis or ectopia of the permanent successor, and three teeth from three different patients were extracted due to secondary retention of the extracted primary tooth.
- *Permanent teeth*: Six premolars from two different adolescents were extracted in connection with and before orthodontic treatment.

All tooth material was forwarded from municipal clinics in Zealand either for diagnostic purpose or after written request. Permission was given from the biomedical research ethics committees of Copenhagen and Frederiksberg communities (KF07322471/H-KF-322471).

Fixation, decalcification and sectioning

The teeth were fixed in 4% neutral buffered formaldehyde for 2–8 days. Teeth were decalcified in either 0.5 M EDTA (ethylene diamine tetra acetic disodium salt, Tritriplex II; Merck, Darmstadt, Germany) for 6–16 weeks or in 4 M formic acid for 6–12 weeks. After decalcification, the teeth were fixed in 4% neutral buffered formaldehyde for 2 days. Teeth were dehydrated and embedded in paraffin using double staining method. Paraffin blocks with longitudinally placed teeth were serially cut in 3 μm thick sections and dried overnight at 40°C. All sections were dewaxed in xylene, inactivated using hydrogen peroxide in 99% ethanol (30% H_2O_2, 31642; Sigma-Aldrich, Broendby, Demark) and dehydrated in graded alcohols prior to immunohistochemistry.

Immunohistochemical detection of membrane-bound RANKL

Immunohistochemistry was carried out using Dako REAL EnVision ™ Detections System (K5007; Dako, Glostrup, Denmark). Sections were pre-treated in Tris-EDTA pH 9 at 60°C for 90 min and encircled with a delimiting pen (S2002; Dako). Sections were blocked by incubation in avidin and biotin blocking solution (X0590; Dako) for 2 × 10 min and in peroxidase blocking solution (S2023; Dako) for 10 min. Sections were incubated in primary antibody for 90 min using: mouse monoclonal anti-RANKL (membrane-bound RANKL) sub-type IgG_1 (ab45039; Abcam, Cambridge, UK) diluted 1:1000 in antibody diluent (S2022; Dako) and incubated in secondary antibody coupled with peroxidase (K5007; Dako). Sections were stained in Substrate Buffer/DAB+ Chromagen (K5007; Dako) for 10 min and counterstained in a staining machine (Shandon Varistain Gemini A78010402; Thermo Electron Corporation, Copenhagen, Denmark) with Carazzi's hematoxylin (Bie & Berntsen™, Herlev, Denmark). Sections were

coverslipped (Pertex 00811; Histolab, Göteborg, Sweden).

Immunohistochemical detection of RANK

Immunohistochemical detection of RANK was carried out using DakoCytomation EnVision ™ System-HRP (K4008; Dako). Sections were pretreated in Target Retrieval Solution pH 6 (S2031; Dako) at 60°C o.n. Sections were encircled with a delimiting pen (S2002; Dako) and blocked by incubation in 0.5% w/v Casein:TBS dilution (C5890, Sigma-Aldrich) and in endogenous peroxidise (K4008; Dako) for 10 min. Sections were then incubated in primary antibody for 2 h using: rabbit polyclonal anti-RANK (sc9072; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:50 in antibody diluents (S2022; Dako) and in peroxidase-labeled polymer (K4008; Dako). Sections were stained in Substrate Cromagen AEC+ (K4008; Dako), counterstained in Carazzi's hematoxylin (Bie & Berntsen) for 30 s and cover-slipped (Kaiser's glycerol gelatine, 1.09242.0100, Bie & Berntsen (Merck).

Double-immunohistochemical detection of RANK and TRAP

Double-immunohistochemical detection of RANK and TRAP (odontoclast and osteoclast marker) was carried out on a section of a primary tooth extracted due to secondary retention. The double immunohistochemical detection was conducted as a sequential staining. Firstly, RANK was immunostained and detected as described above. Subsequently, TRAP was immunostained and detected using immungold silver enhancement, as described by Andersen et al. [19]. In short, endogenous biotin within the RANK stained sections was blocked using a biotin blocker (X0590; Dako) before the section was incubated with mouse monoclonal anti-TRAP subtype IgG_{2b} antibody (180199; Zymed/Invitrogen, Taastrup, Denmark) diluted 0.0066 mg/ml in TBS-casein for 1 h. Detection was done using biotin-conjugated goat anti-mouse IgG_{2b} (115-065-2070; Jackson ImmunoResearch, Suffolk, UK) diluted 1:250 in TBS-casein for 30 min, followed by Gold-conjugated Goat anti-biotin (Aurion™, Wargeningen, The Netherlands) diluted 1:60 in TBS-casein for 30 min. The gold particles were silver-enhanced until visible as follows: the sections were carefully washed in sterile water and then washed in 0.5% w/v hydroquinone (H9003, Sigma-Aldrich, Copenhagen, Demark) diluted in citrate buffer pH 3.8 (Sodium citrate dehydrate, 0280, J.T.Baker, AA Deventer, the Netherlands, and Citric monohydrate, 1.00244.1000, Merck). The sections were then silver-enhanced for 24 min in 0.2% w/v silver acetate (Sigma-Aldrich), 0.5% w/v hydroquinone diluted in citrate buffer pH 3.8 where the solution was changed every 8 min in order to reduce unspecific precipitation of silver grains. Finally the sections were counterstained in Carazzi's hematoxylin (Bie & Berntsen) and mounted with Kaiser's glycerol gelatine (Bie & Berntsen).

Controls

Controls for RANKL, RANK and TRAP were performed by omitting the primary antibody. Otherwise, sections were performed according to protocol.

Results

External resorption was observed in all primary teeth and one primary tooth with secondary retention showed clear signs of ankylosis. External resorption was also seen in two permanent premolars from the same patient.

Immunolocalization of membrane-bound RANKL

In general, the periodontal membrane was negative for RANKL in primary and permanent teeth (Figure 1A and B), although a weak background staining and a presumably unspecific staining was sometimes seen in the periodontal membrane in primary and permanent teeth (Figure 1B).

Positive reaction for RANKL was seen in only one primary tooth (Figure 2A–C). Reaction for RANKL was located in the pulp to odontoblasts (Figure 2A and B), occasionally to endothelial cells around vessels, to undefined mononucleated cells within the pulp tissue (Figure 2B) and to cells along denticles (Figure 2A and C). The RANKL positive cells around denticles were more flattened compared to the RANKL positive odontoblasts (Figure 2A–C).

Immunolocalization of RANK

The periodontal membrane along root surfaces without resorption was negative for RANK in both primary and permanent teeth (Figure 3A and B).

Weak-to-negative reaction for RANK was seen in cells resembling odontoclasts in primary teeth extracted due to ectopia or agenesis of the permanent successor (Figure 4A), whereas a much stronger reaction for RANK was seen in cells resembling odontoclasts in primary teeth extracted due to secondary retention (Figure 4B). In general, more odontoclasts/osteclasts were seen in primary teeth with secondary retention compared to primary teeth extracted in connection with ectopia or agenesis of the permanent successor. Additionally, primary teeth extracted due to secondary retention often revealed external resorption extending into the pulp. RANK

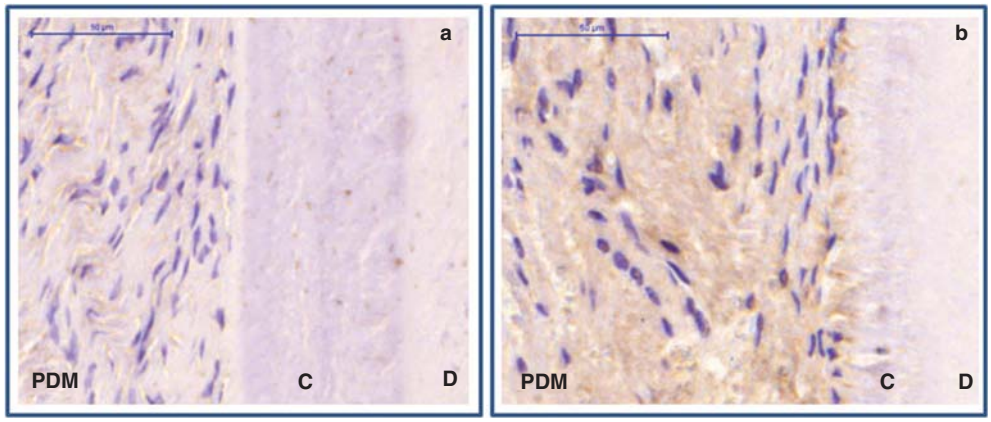

Figure 1. Photomicrographs of tooth sections from two different patients. In each photomicrograph the periodontal membrane (PDM) is seen to the left, the root cementum (C) in the middle and dentin (D) to the right. All sections have been immunohistochemically stained for membrane-bound RANKL. (a) Section of a primary molar extracted due to agenesis of the permanent successor. The periodontal membrane and the surface of the root cementum do not show brown immunoreactivity for membrane-bound RANKL. (b) Section of a premolar extracted in connection with orthodontic treatment. The periodontal membrane and the root surface do not show immunoreactivity for membrane-bound RANKL, although a slight unspecific brown background staining is seen in the periodontal membrane.

expression was seen in a resorption lacuna in one premolar. Occasionally RANK was seen in mononucleated cells in the soft tissue in the pulp or in the periodontal membrane or along root surfaces with resorption.

TRAcP/RANK

The localization of RANK in multinucleated cells was confirmed by double immunohistochemical staining together with TRAP. An extreme number

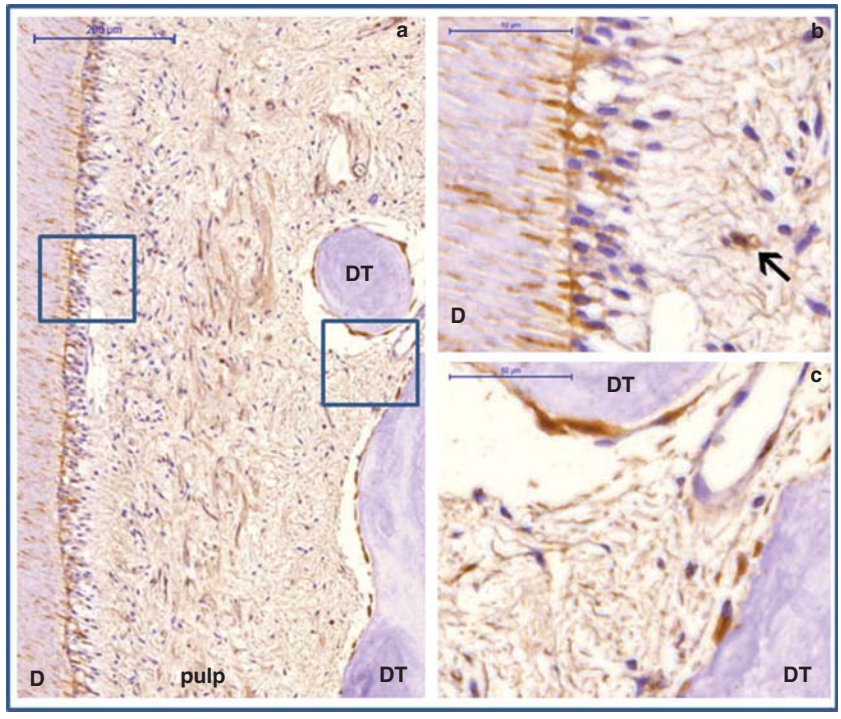

Figure 2. Photomicrographs in two magnifications of the same section from a primary molar extracted due to agenesis of the permanent successor. The section has been immunohistochemically stained for membrane-bound RANKL (brown color). (a) This photomicrograph shows a part of the pulp area (pulp) inside the crown. To the left, dentin (D) is seen with odontoblasts (stained brown) aligned along the inner dentin surface. The odontoblasts show immunoreactivity for membrane-bound RANKL (brown color). The area inside the left square has been magnified in (b). To the right, denticles (DT) are seen. Along the denticle surfaces, flattened cells (stained brown) are aligned. The area inside the right square has been magnified in (c). (b) A magnification of the area inside the left square seen in (a). Odontoblasts are seen aligned along the dentin (D) surface. The odontoblasts show immunoreactivity for RANKL (brown color). A single unspecific cell inside the pulp tissue is also immunohistochemically stained for membrane-bound RANKL (brown, indicated by a black arrow). (c) A magnification of the area inside the right square seen in (a). Flattened cells are seen aligned along the two denticle's (DT) surfaces. These flattened cells show immunoreactivity for membrane-bound RANKL (brown color).

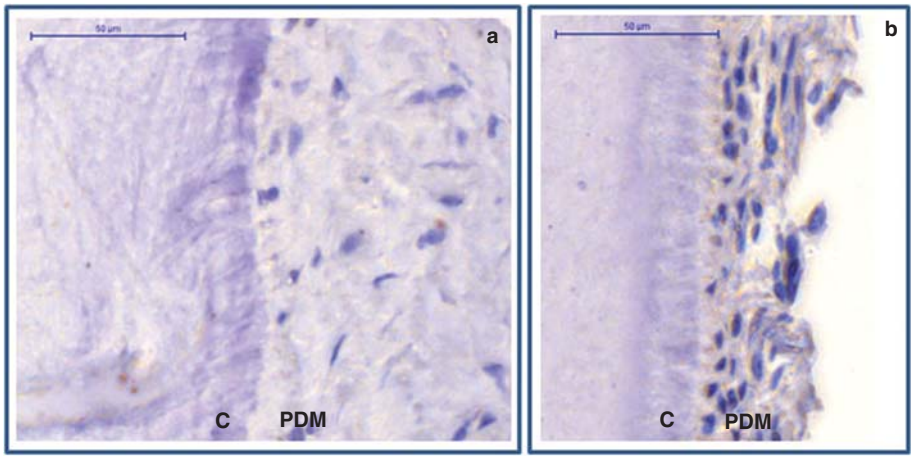

Figure 3. Photomicrographs of tooth sections from two different patients. In each photo, the root cementum (C) is in the middle and periodontal membrane (PDM) to the right. All sections have been immunohistochemically stained for RANK. (a) A section of a primary canine extracted due to ectopia of the permanent successor. The periodontal membrane and the surface of root cementum do not show red-brown immunoreactivity RANK. (b) A section of a premolar extracted in connection with orthodontic treatment. The periodontal membrane and the surface of root cementum are negative for RANK.

of TRAP- and RANK positive cells was seen lining the dentin surface inside the pulp (Figure 5A–C). These multinucleated cells were probably odontoclasts due to the localization along the dentin surface in the pulp, but it cannot be excluded that the cells can also be osteoclasts.

Discussion

This study confirms the previously described localization of RANKL expression in the pulp in primary teeth [15,16] and RANK expression in odontoclasts [14,15,17,18]. Unlike other studies on human teeth [14,15,17,18], a clear selective RANKL expression was not seen in the periodontal membrane of human teeth in the present study. This might be explained by different choices of RANKL antibody. RANKL has been described in two forms: a soluble form and a membrane-bound form. The antibody used in the present study only localizes membrane-bound RANKL. The expression and interaction of RANKL and RANK is essential for the activation of osteoclast and presumably also odontoclasts. This study did not show any differences in expression of membrane-bound RANKL along root surfaces and in the periodontal membrane of primary and permanent teeth. In future immunohistochemical studies comparing primary and permanent teeth, it would be relevant to focus on immunolocalization of secreted RANKL and OPG along root surfaces and in the periodontal membrane in close proximity to the root surface.

Under normal circumstances with physiological external root resorption, we would not expect simultaneous internal (pulp) resorption. It was therefore surprising that membrane-bound RANKL in this study was seen in odontoblasts and in flattened cells along denticles in one primary tooth, which did not

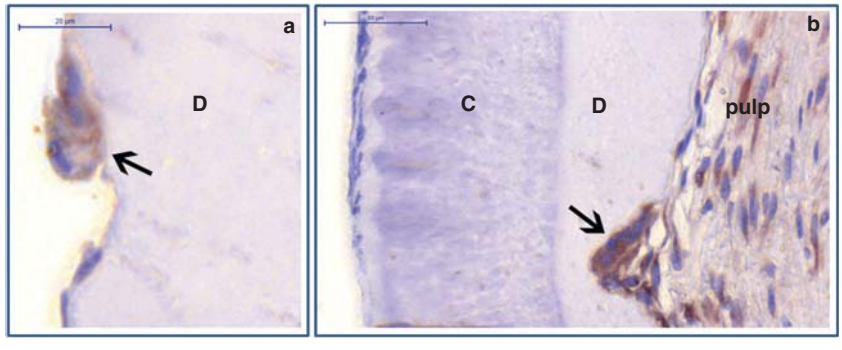

Figure 4. A photomicrograph in high magnification of tooth sections from two different patients. Both tooth sections have been immunohistochemically stained for RANK (red-brown color). (a) A section of a primary canine, which has been extracted due to ectopia of the permanent successor. The photomicrograph shows a multinucleated odontoclast (black arrow), which shows weak immunoreactivity (red coloring) for RANK. The odontoclast is seen along resorbed dentin (D) and the root surface. (b) A section of a primary molar, which has been extracted due to infraocclusion of the extracted primary molar. In this photomicrograph, the cementum (C) is seen to the left, the dentin (D) in the middle and the pulpa (pulp) to the right. The black arrow indicates a multinucleated odontoclast, which shows strong immunoreactivity for RANK (red-brown coloring). Some single nucleated cells inside the pulp also show immunoreactivity.

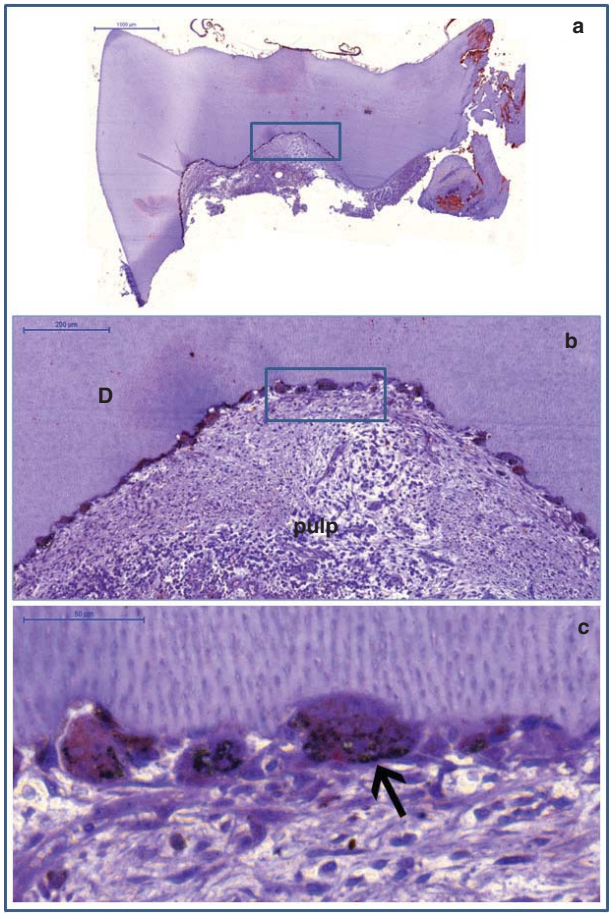

Figure 5. Photomicrographs in three magnifications of the same tooth section, which is from a primary molar extracted due to infraocclusion of the extracted primary molar. The tooth section has been immunohistochemically stained for RANK (red color) and TRAP (black color). (a) The entire primary molar in low magnification. The area inside the square has been magnified in (b). (b) A magnification of the area inside the square in (a). Odontoclasts are inside the pulp (pulp), where they are aligned along the dentin (D) surface. The odontoclasts show immunoreactivity for RANK (red color) and TRAP (black). The area inside the square has been magnified in (c). (c) This photomicrograph is a magnification of the area inside the square in (b). Multinucleated odontoclasts are seen (exemplified by a black arrow). They show immunreactivity for RANK (red color) and TRAP (black colored vesicles). This double immunoreactivity confirms that RANK immunoreactivity is seen within odontoclasts because TRAP is a widely accepted antibody used for identifying odontoclasts and osteoclasts.

show light microscopic signs of internal resorption. However, RANKL expression in the pulp has also been described previously in some human primary teeth [15,16]. A possible interaction between pulp cells and cells in the periodontal membrane during regulation of root resorption has been suggested [16]. This is an interesting aspect, which needs to be further elucidated on a larger material of primary teeth showing positive RANKL expression in the pulp.

In this study, RANK expression was seen in multinucleated cells and in mononucleated cells in primary teeth. The multinucleated cells were confirmed to be odontoclasts by double immunohistochemical detection of RANK and TRAP. RANK expression was also seen in a resorption lacuna in a permanent tooth. This might indicate similar processes during physiological resorption in primary teeth and some forms of pathological resorption in permanent teeth. A stronger RANK expression was seen in primary teeth with secondary retention compared to primary teeth extracted due to ectopia or agenesis of the permanent successor. To our knowledge this has not previously been described. The difference in RANK expression may be related to a more aggressive resorption pattern in teeth with secondary retention.

It is unclear whether odontoclasts and osteoclasts are essentially the same cell type [20]. However, studies have shown that the same cell can resorb both dentin and bone tissue *in vitro* [21]. It is known that during normal circumstances, a continuous remodeling occurs at the alveolar bone surface, whereas the root surface remains unaltered. Studies have demonstrated RANKL, presumably secreted RANKL, in the periodontal membrane [14,15,17,18], indicating a final activation of RANK-expressing odontoclasts within the periodontal membrane. In this context the differential expression of osteopontin and bone sialoprotein along root surfaces of human teeth and bone surfaces might be relevant [3]. A differential expression of extracellular matrix proteins (such as bone sialoprotein and osteopontin) along the hard tissue surfaces could explain the differential resorption pattern of hard tissue surfaces (alveolar bone and root surface) in the periodontal membrane.

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