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Evidence for programmed odontoblast process retraction after dentine exposure in the rat incisor

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ABSTRACT

Objective: To re-examine the morphology and potential functions of odontoblasts in intact rat incisors and after cavity preparation into dentine.
Design: Intact incisors were fixed, decalcified, snap frozen and sectioned (10 μm), before staining with rhodamine phalloidin or antibodies for cyto-skeletal proteins: vimentin and actin, ion transporter: NaK-ATPase, and dendritic cell marker: OX6. Samples with cavity were processed similarly and stained for actin and vimentin before comparing the lengths of odontoblast processes (OP) at baseline, 3 h and 24 h (n = 5 for each group).
Results: Actin was expressed through the full length of OP, while vimentin immunoreactivity was not uniform, with 4 distinct regions. OP showed morphological complexity with fine branches emanating within different regions of dentine. Novel actin-positive tree-like OP were identified within predentine which reduced in intensity and length toward the incisal portion of the tooth. Specimens with cavities showed time-dependant pulpal retraction of OP.
Conclusions: Differences in structural antibody expression suggest functional variations in OP within different regions of dentine. The role of actin positive OP in predentine is not known, but could be related to dentine deposition, cellular stability or sensing mechanisms. Cavity preparation into dentine was followed by programmed retraction of OP which could be controlled either mechanically by the spatial limitation of the OP within dentinal tubules or structurally by the presence of vimentin, in addition to actin, in the mid-dentine.

1. Introduction

Odontoblast processes (OP) are cytoplasmic extensions of the cell body, which reside within the tubular structure of dentine (Luukko, Kettunen, Fristad, & Berggreen, 2011). Ultrastructural studies report the presence of intermediate filaments and microfilaments within odontoblast cells (Od) and their processes (Nishikawa & Kitamura, 1987; Yoshiba, Yoshiba, Ejiri, Iwaku, & Ozawa, 2002). These have also been reported in immunofluorescence studies (Byers & Sugaya, 1995; Nishikawa & Sasa,1989; Sigal, Aubin, & Ten Cate, 1985) and are believed to be instrumental in structural and cytoskeletal support, cellular movement and secretion (Gunning, Ghoshdastider, Whitaker, Popp, & Robinson, 2015; Pollard & Cooper, 2009). Additionally, sodium potassium-ATPase (NaK-ATPase) has been demonstrated within the cells of the pulp, especially in subodontoblast cells (SOd), Od and OP (Alhelal, Mahdee, Eastham, Whitworth, & Gillespie, 2016; Mahdee, Alhelal, Eastham, Whitworth, & Gillespie, 2016).

The Od layer undergoes morphological, structural, and functional changes during tooth development, both in continuously growing rodent teeth (Ohshima & Yoshida, 1992) and teeth with limited growth (Couve, 1986; Yoshida & Ohshima, 1996). The degree of extension of the OP remains contentious, with a number of groups suggesting full extension to the dentino-enamel junction beneath the intact dentine surface (Grötz et al., 1998; Gunji & Kobayashi, 1983; Kagayama et al.,

Abbreviations: AP, apical papilla; BV, blood vessel; Cav, cavity; CMZ, cavity measurement zone; Cp, capillary; CPC, central pulp cells; De, dentine; DEJ, dentino-enamel junction; DL, dentinal tubule length; En, enamel; IR, immunoreactivity; L, estimated dentine thickness; Od, odontoblast cells; OEE, outer enamel epithelium; OP, odontoblast process; PAm, preameloblast; PD, predentine; POd, preodontoblast; RP, rhodamine phalloidin; SOd, subodontoblast cells; UAm, undifferentiated ameloblast; UOd, undifferentiated odontoblast; vim, vimentin

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1999; Sigal et al., 1985; Sigal, Aubin, Ten Cate, & Pitaru, 1984; Tsuchlya, Sasano, Kagayama, & Watanabe, 2002). Less information is available on the morphological complexity of OP during stages of tooth formation. Similarly, studies have shown changes in pulp innervation (Taylor, Byers, & Redd, 1988), growth factor expression (Byers, Schatteman, & Bothwell, 1990), presence of immunocompetent cells (Kawagishi, Nakakura-Ohshima, Nomura, & Ohshima, 2006), and pulp cell apoptosis (Kitamura, Kimura, Nakayama, Toyoshima, & Terashita, 2001) following dental cavity preparation, but the effects on Od and their processes are less well understood.

The specific objectives of this ex *vivo* study were to explore the development of OP and their complexities in the rat incisor, and determine their response following cavity preparation into dentine.

2. Materials and methods

All procedures on the animal used were done according to Schedule 1, UK Home Office guidelines ("Consolidated version of ASPA, 1986," 6 May 2014).

2.1. Non-cavity samples

Ten male Wistar rats, 12–13 w age (400–500 g weight) were killed in a CO_2 chamber. Half mandibles (n = 20) were dissected and sectioned to divide incisors into apical, middle and incisal pieces (Fig. 1A), with high-speed diamond burs under constant water cooling, before fixation in 4% paraformaldehyde in phosphate buffer saline for 24 h at 4 °C. Samples were demineralised and prepared in frozen blocks as described previously (Mahdee et al., 2016). Approximately 20 sagittal, 10 µm thickness sections were obtained from each block.

Sections from each animal were stained with rhodamine phalloidin (RP) (Molecular Probes®, Invitrogen) or with immunohistochemistry antibodies. Samples were stained with 1:500 RP in phosphate buffer saline at room temperature for 30 min in a humidified environment before washing with tris-buffered saline, tris-buffered saline-tween and tris-buffered saline for 10 min each. Vectashield hard set mounting medium with dapi (Vector Laboratories Inc, Burlingame, USA) was then applied before placing a cover slip. For immunohistochemistry, slides were stained with antibodies as previously described (Mahdee et al., 2016). This included either one or a combination of two of: mouse monoclonal anti-vimentin (vim) (1:5000, BioGenex, Launch Diagnostics cat# MU074-UC, UK), rabbit monoclonal anti-α smooth muscle actin (α-actin) (1:200, Abcam cat# ab32575, UK), rabbit monoclonal anti-NaK-ATPase enzyme (1:500, Abcam cat# Ab76020), and mouse monoclonal RT1-B antibody [OX6], a dendritic cell marker, (1:200, GeneTex, cat# GTX76190 USA).

Negative controls for immunohistochemistry staining included blocking peptides and isotype controls. Human alpha smooth muscle actin peptide (ab 211918) was specifically manufactured by Abcam as a blocking peptide for its α-actin product. The concentration of actin blocking peptide was 10:1 of the α-actin and the mixture was kept overnight with continuous agitation at 4 °C, before incubating the slides for 24 h at 4 °C. In isotype controls, both rabbit IgG monoclonal (EPR25A) isotype control (1:500 Abcam cat# ab172730) and normal mouse IgG1 (1:500 Santa Cruz Biotechnology cat# sc-3877 UK) were negative controls. Slides were incubated for 24 h before washing and staining with secondary conjugated fluorescent antibodies. Stained slides were examined as previously described (Mahdee et al., 2016). Eighty slides were examined for each stain to confirm the accuracy and consistency of the staining technique and to reveal constant staining phenomena (Gillespie, Markerink-van Ittersum, & De Vente, 2006).

2.2. Cavity samples

Teeth were harvested and sectioned as described above, with only the incisal regions included in this investigation. Groove-like cavities were prepared on labial surfaces, about 3 mm from the incisal edge, with high speed diamond burs No 009 (head diameter 0.5 mm) (Kerr Dental, Switzerland), under constant cooling from a syringe containing Dulbecco's Modified Eagle's Medium (Sigma, USA), to minimize thermal and osmotic stress. From ground section archives of rat mandibular incisors, the thickness of enamel was estimated to be 200 µm. Insertion of the bur head to half-thickness therefore resulted in a cavity of approximately 50 µm depth in dentine. Work was conducted under a Global Operating Microscope (DP Medical Systems, UK). After cavity preparation, teeth were divided into 3 groups (n = 5). Group A was immediately fixed in 4% paraformaldehyde for 24 h at 4 °C. This group also referred as '60sec' depending on the working time for cavity preparation. Other groups were incubated in a CO_2 incubator (Sanyo, Japan) at 37 °C within 5 ml Eagle's Medium, supplemented with fetal calf serum (1% Sigma) and penicillin-streptomycin (50 IU/ml- µg/ml Sigma) for 3 h (Group B), or 24 h (Group C). After incubation, samples were fixed, demineralised and sectioned as described previously. The selected cross sections (10 µm) from the centre of each cavity were stained for α-actin and vimentin as described previously, with similar negative controls. Images were captured at X100, X200, and X600

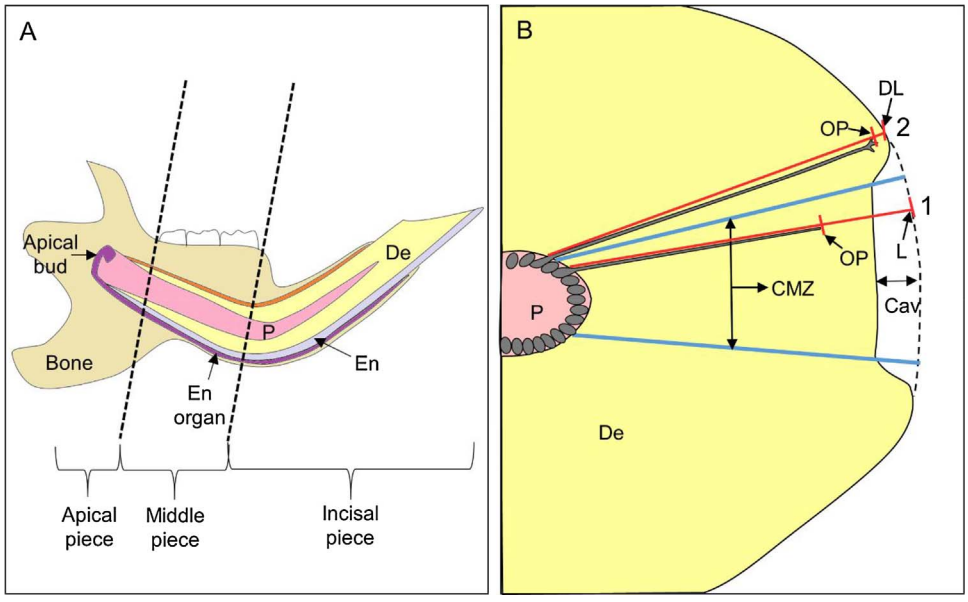

Fig. 1. Diagrams illustrate the rat mandible sectioning (A), and cavity measurement (B). P = pulp, De = dentine, and En = enamel. In A, the two dotted lines represent the sectioning positions dividing the incisor into: apical, middle and incisal pieces. B shows a tooth cross section in the cavity region, highlighting measurements within and outside the cavity measurement zone (CMZ) for post cavity-preparation (1) and normal (2) odontoblast processes (OP) respectively. The lengths of the OP were normalised either with control dentinal tubule length (DL) in intact dentine surfaces or with estimated length (L) for dentine surface before cutting.