Mineralization process during acellular cementogenesis in rat molars: A histochemical and immunohistochemical study using fresh-frozen sections

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Abstract

This study was designed to detect tissue non-specific alkaline phosphatase (TNSALP) by Azo-dye staining, calcium by glyoxal bis(2-hydroxyanil) (GBHA) staining, bone sialoprotein (BSP) and osteopontin (OPN) by immunoperoxidase staining in developing rat molars, and also to discuss the mineralization process during acellular cementogenesis. To restrain a reduction in histochemical and immunohistochemical reactions, fresh-frozen undemineralized sections were prepared. Where the epithelial sheath was intact, TNSALP reaction was observed in the dental follicle, but not in the epithelial sheath. With the onset of dentin mineralization, the BSP- and OPN-immunoreactive, initial cementum layer appeared. At this point cementoblasts had shown intense TNSALP reaction and GBHA reactive particles (=calcium-GBHA complex) appeared on the root surface. With further development, the reaction of TNSALP and GBHA became weak on the root surface. Previous studies have shown that the initial cementum is fibril-poor and that matrix vesicles and calciferous spherules appear on the root surface only during the initial cementogenesis. The findings mentioned above suggest that: During the initial cementogenesis cementoblasts release matrix vesicles which result in calciferous spherules, corresponding to the GBHA reactive particles. The calciferous spherules trigger the mineralization of the initial cementum. After principal fiber attachment mineralization advances along collagen fibrils without matrix vesicles.

Key words: Acellular cementum; Fresh-frozen sections; Matrix vesicles; Mineralization; Tissue non-specific alkaline phosphatase

Introduction

Cementum covers the root surface and plays important roles for the tooth-support in cooperation with the principal fibers and the alveolar bone proper. Collagen fibers are major organic cementum components and are classified into two types: extrinsic (=Sharpey's) fibers which are embedded ends of the principal fibers and intrinsic fibers of the cementum proper. Cementum is classified into several different types on the basis of the distribution of extrinsic fibers, intrinsic fibers, and cementocytes (Schroeder 1992, 1993).

Regarding noncollagenous organic matrices of the cementum, immunohistochemistry and biochemistry have identified many distinct molecules, including noncollagenous glycoproteins, proteoglycans, plasma-derived macromolecules, and several growth factors (for reviews, see Embery et al. 2000; Waddington and Embery 2001; Bosshardt 2005), and have shown that two noncollagenous glycoproteins, bone sialoprotein (BSP) and osteopontin (OPN), are major constituents of the noncollagenous matrices (McKee et al. 1996; Bosshardt et al. 1998; Nanci 1999; Bosshardt 2005). The BSP and OPN are closely associated with mineralization, matrix-matrix attachment, and cell-cell/matrix attachment in collagen-based hard tissues (for reviews, see Ganss et al. 1999; Sodek et al. 2000).

The cemento-dentinal junction, the interface between cementum and dentin, stains intensely with hematoxylin in many species. For rat molars, it is generally accepted that the cemento-dentinal junction is fibril-poor and contains more highly accumulated BSP and OPN than other parts of cementum (Paynter and Pudy 1958; Yamamoto 1986; Yamamoto and Wakita 1990; Yamamoto et al. 2000a, 2000b, 2001, 2004, 2005; Sasano et al. 2001; Tadatomo et al. 2002; Hosoya et al. 2003).

Generally, mineralized tissues including cementum have been chemically fixed and demineralized for histochemical and immunohistochemical observations. However, even though specimens are carefully fixed and demineralized, the chemicals used may cause denaturation of proteins, giving rise to a reduction in histochemical and immunohistochemical reactions. Further, to detect the precise calcium localization in mineralized tissues by light microscopy, thin (several μm thick) undemineralized sections, which are difficult to prepare, must be used.

Recently, an adhesive film method has been developed (Kawamoto and Shimizu 2000; Kawamoto 2003), in which mineralized tissues are rapidly fresh-frozen (=physically fixed), freeze-dried, and cut into thin sections. Kawamoto (2003) has confirmed that these sections can be applied to many types of light microscopic studies, such as histology, general histochemistry, enzyme histochemistry, immunohistochemistry, in situ hybridization, elemental analysis, and autoradiography for water-soluble materials.

The study here used the adhesive film method to detect tissue non-specific alkaline phosphatase (TNSALP) and calcium by histochemistry, and BSP and OPN by immunohistochemistry in developing rat molars, and to discuss the mineralization process during acellular cementogenesis. The knowledge of normal cementogenesis would be helpful in understanding other types of cementogenesis seen during clinical dental treatment.

Materials and methods

This study used 15 3-week-old male Wistar rats weighing about 50g. The animals and tissue specimens were treated in accordance with the Guidelines of the Experimental Animal Committee, Hokkaido University Graduate School of Dental Medicine.

Preparation of fresh-frozen sections

The procedures conformed to Kawamoto and Shimizu (2000) and Kawamoto (2003). After anesthesia by intraperitoneal injection of sodium pentobarbital, the upper jaws were dissected out within one minute and rapidly frozen in cooled n-hexane(-75˚C). Then the frozen specimen was immersed in a stainless steel container filled with 5% carboxymethyl cellulose (CMC) gel (FINETEC, Japan). The container was placed in cooled n-hexane and the CMC block was completely frozen. Each frozen block was then attached to the sample stage of a Leica CM3000 Cryostat in a cryochamber at -25˚C. Next, the cutting surface of the block was covered with a polyvinylidene chloride film coated with synthetic rubber cement(Cryoglue Type I ; FINETEC), and serial sections of the first molar were cut mesio-distally with the film at a thickness of 3μm with a tungsten carbide blade (Jung TC-65; Leica, Germany). The frozen sections were then freeze-dried in a cryochamber at -25˚C for 3-5h. The dried sections were placed in a box containing silica gel to avoid condensation and removed from the cryochamber. Some sections were stained with hematoxylin and others were used for immunohistochemistry and histochemistry as described below. The stained sections were mounted with glycerin.

Immunohistochemistry

Anti-human BSP rabbit polyclonal antibody (Alexis, Switzerland) and anti-rat OPN mouse monoclonal antibody (American Research Products, USA) were used for the BSP and OPN immunodetection, respectively. We have confirmed the authenticity of the two antibodies for rats by comparison with several kinds of antibodies purchased from different companies.

Sections were immersed in methanol containing 0.3% hydrogen peroxide to inhibit endogeneous peroxidase. Then they were successively incubated with the primary antibodies, biotinated anti-rabbit swine or anti-mouse rabbit polyclonal antibody (DAKO, Japan), and streptavidin-biotin-horseradish peroxidase complex (DAKO). The sections were visualized by the 3, 3'-diaminobentizine method. Normal rabbit or mouse serum was substituted for the primary antibodies in the negative controls.

Histochemistry

TNSALP

The TNSALP　was detected by Azo-dye staining(Burstone 1962). Sections were incubated in a medium comprising 10 mg of Naphthol AS-BI phosphoric acid sodium salt(Wako, Japan) as substrate and 60 mg of Fast Red Violet LB salt(Sigma, USA) as capture agent, in 100 ml of Tris-HCl buffer(pH 8.5) for 5 min at room temperature. Control sections were incubated in the medium without substrate.

Calcium

Calcium was detected by glyoxal bis(2-hydroxyanil) (GBHA) staining that visualizes calcium

as red calcium-GBHA complex in histological sections(Kashiwa 1966; Takano et al. 1988).The procedures conformed to Takano et al. (1988). Sections were stained with 5% GBHA (Fluka, Switzerland) in 75% ethanol containing 3.4% NaOH for 5 min at room temperature. The sections were subsequently rinsed with absolute ethanol for 5 min and mounted. Prior to staining, control sections were immersed in 80% ethanol saturated with EDTA for 10 h at room temperature.

Results

The mesial root of the maxillary first molars of 3-week-old rats was examined in this study (Fig.1a, b). The mesial root displays the full range of stages of acellular cementogenesis.

General histology

At the apical end Hertwig's epithelial root sheath consisted of two or three cell layers (Fig.1c). In the cervical direction, the epithelial sheath cells became smaller and darker and the epithelial sheath disintegrated. Dental papilla cells facing the epithelial sheath differentiated into columnar odontoblasts and formed predentin (Fig.1c). Dental follicle cells approached the exposed predentin and formed dense cell population. With the onset of dentin mineralization, a hematoxylin-stained layer appeared as the initial cementum on the mineralized dentin (Fig.1d). At this point, dental follicle cells were at a short distance from the root surface, and on the basis of the location and cell shape these cells could be identified as cementoblasts.

More cervically, the cementum increased in thickness, but in the mid-root region the cemento-dentinal junction was still indiscernible (Fig.1e). Where the cementum had developed to a maximum thickness of about 3 μm in the cervical region, the cemento-dentinal junction became discernible as the most intensely hematoxylin-stained line in the cementum (Fig.1f).The periodontal ligament cells including cementoblasts became elongated and arranged in regular direction.

Immunohistochemistry

Intense immunoreactivity for BSP (Fig.2a) and OPN (Fig.2b) was detected in the acellular

cementum. Other tissues, viz., dental follicle, epithelial sheath, dentin, periodontal ligament, and dental pulp stained weakly or did not stain. The initial cementum started to form with the onset of dentin mineralization (Fig.2c), and as the cementum increased in thickness, the cemento-dentinal junction appeared as the most intensely stained line in the cementum (Fig.2d). Control sections showed no specific immunoreaction (not shown).

Histochemistry

TNSALP

At the apical end, the dental follicle was TNSALP reactive and the epithelial sheath or dental papilla was not reactive (Fig.3a,b). In the cervical direction, where the initial cementogenesis started, the cementoblast population became more intensely reactive than the surrounding periodontal ligament (Fig.3b). The intense reaction appeared transiently (Fig.3c), and more cervically, the reaction on the root surface was weaker and very similar to that in the surrounding periodontal ligament (Fig.3a). Control sections showed no reaction (not shown).

Calcium

At the apical end, the dental follicle or the epithelial sheath showed no GBHA reaction (Fig.4a,b). In the cervical direction, with the onset of dentin mineralization, GBHA reactive particles about 2 μm in diameter appeared between the cementoblast population and root surface (Fig.4b). The particle appeared transiently, and more cervically, they decreased in number. In the mid-root and cervical regions no or only a few GBHA reactive particles were

observed on the root surface (Fig.4c,d). Control sections showed no GBHA reaction (not shown).

Discussion

The evaluation of fresh-frozen sections

Regarding the immunohistochemistry for BSP and OPN, the acellular cementum in fresh-frozen sections stained more intensely and specifically than in chemically fixed, EDTA-demineralized, paraffin sections (see Hosoya et al. 2003; Yamamoto et al. 2004, 2005). It is possible that some epitopes of BSP and OPN are lost and/or altered during preparation of paraffin sections, probably in the fixation and/or demineralization, and that cementum may contain more densely accumulated BSP and OPN than observed in such chemically treated sections. However, Hosoya et al. (2005) observed that EDTA-demineralized bone stained more intensely for BSP and OPN than undemineralized bone in fresh-frozen sections. At present there is no satisfactory answer to the inconsistent results in cementum and bone.

Regarding TNSALP histochemistry, mineralized tissues are usually paraformaldehyde-fixed, EDTA-demineralized, and frozen-sectioned for routine Azo-dye staining. However, because the TNSALP reaction is considerably reduced (Kawamoto and Shimizu 2000), reactivation procedures are necessary before staining (Yoshiki et al. 1972). No reactivation procedures were necessary for fresh-frozen sections prepared by the adhesive film method.

The GBHA staining method was first introduced by Kashiwa (1966). To observe reactive structures more in detail, Takano et al. (1988) applied rapid freezing, freeze substitution, and resin embedding to this original method. Although the resulting resin sections can be used for transmission electron microscopy as well as light microscopy, the preparation takes several days from freezing to sectioning.

Overall, the results here suggest that fresh-frozen sections prepared by the adhesive film method can be routinely used for histochemical and immunohistochemical studies of cementum.

The mineralization of acellular cementum

We have studied the structure and development of the acellular cementum in rat molars by light and electron microscopy (Yamamoto 1986; Yamamoto and Wakita 1990; Yamamoto et al. 2000a, 2000b, 2001, 2004, 2005), and found the following: (1) the cemento-dentinal junction is a fibril-poor layer (1-2 μm thick) and contains more densely accumulated BSP and OPN than the successively formed acellular cementum with extrinsic fibers; (2) developmentally, prior to the principal fiber attachment to the root surface, BSP and OPN are densely deposited as the initial cementum, which results in the fibril-poor cemento-dentinal junction; (3) during the initial cementogenesis, matrix vesicles and calciferous spherules appear on the root surface. After the acellular cementum is established, neither of matrix vesicles or calciferous spherules is observed and the mineralization advances along the attached principal fibers.

The findings mentioned above suggest that the mineralization process of the rat acellular cementum proceeds as follows (Yamamoto et al. 2004): The initial cementum is deficient in collagen fibrils. Under this condition, successive mineralization spreading from the mineralized dentin may not occur, and the initial cementum is mineralized independent of collagen fibrils, and calciferous spherules, which are added anew from the periodontal ligament side, induce the mineralization of the initial cementum. The initial cementum requires a dense accumulation of BSP and OPN for its mineralization, probably due to the

lack of stable mineralization media (=collagen fibrils). Once the mineralization extends to the principal fibers, the mineralization advances in the known fashion dependent on collagen fibrils (for reviews, see Limeback 1991; Linde 1995; Goldberg et al. 1995; Embery et al. 2001). Recently, Wiesmann et al. (2005) have reviewed the process of collagen mineralization more in detail in different mineralized tissues, with special emphasis on the structural relationship between developing crystallites and collagen.

The study here found the localization and size of GBHA reactive particles very similar to those of calciferous spherules observed in a previous study (Yamamoto 1986). This suggests that the particles correspond to the calciferous spherules. In addition, where the particles appeared, cementoblasts showed the most intense TNSALP reaction. This suggests that TNSALP is closely associated with the appearance of calciferous spherules.

The TNSALP is a multi-functional enzyme bound to the cell membrane. In mineralized tissues this enzyme has been studied in close association with biomineralization process, although its precise roles are still undetermined (for reviews, see Whyte 1994; Anderson 2003; Balcerzak et al. 2003). These reviews discuss the possible roles of TNSALP in detail, with special reference to the initial mineralization by matrix vesicles. Briefly, matrix vesicles are formed by polarized budding and pinching-off from cells (chondrocytes, osteoblasts, and odontoblasts), and are selectively located at sites of initial mineralization. The first apatitic crystals appear within matrix vesicles by the activity of phosphatases including TNSALP, all of which are concentrated in or near the matrix vesicle membrane. At this point TNSALP functions to hydrolyse pyrophosphate, an inhibitor of crystal formation. After crystals are released through the matrix vesicle membrane, they serve as nuclei (templates) for

continuous crystal formation in surrounding extracellular matrix.

Considering the above findings, it is possible to propose the mineralization process of the acellular cementum as follows: At the initial cementogenesis intense TNSALP activity appears on cementoblasts. These cells release matrix vesicles with the TNSALP activity toward the root surface. Crystal formation within matrix vesicles results in calciferous spherules, which trigger the mineralization of the initial cementum. After the principal fiber attachment, continuous crystal formation advances along the principal fibers. Although the function of TNSALP in the continuous mineralization is still unclear, this enzyme may here be less necessary than in the mineralization of the initial cementum.

Arambawatta et al. (2006) compared the structure of four different kinds of adhesive interfaces in rat alveolar bone and molars, viz., the initial attachment layer for principal fibers on developing alveolar bone, the reattachment layer for principal fibers on resorbed alveolar bone, cement lines of alveolar bone unrelated to principal fibers, and the cemento-dentinal junction, and found that all the layers are deficient in fibrils and rich in BSP and OPN. Although they have suggested that the layers undergo similar developmental process on the basis of the structural similarity, an answer to the mineralization process awaits further investigation.

The origin of the initial cementum and cementoblasts

The classical, widely accepted theory of cementogenesis explains that cementoblasts derive from mesenchymal dental follicle and generate the cementum. However, there have been different opinions: that the initial cementum is an epithelial secretory product (Paynter and

Pudy 1958; Owens 1980; Slavkin et al. 1989; Hammarstöm et al. 1996; Hammarstöm 1997); the epithelial sheath cells transform into cementoblasts to generate the initial cementum (Thomas 1995; Bosshardt and Nanci 1997; Bosshardt et al. 1998; Zeichner-David et al. 2003; Bosshardt 2005). The following points established in the study are: (1) the epithelial sheath becomes disintegrated prior to the onset of the initial cementogenesis, and where the initial cementogenesis starts, there are only a few epithelial cells on the root surface; (2) where the initial cementogenesis starts, cementoblasts have formed dense cell population; (3) dental follicle cells and cementoblasts show the TNSALP reaction consistently, but the epithelial cells show no reaction.

Related to point (1) mentioned above, Kaneko et al. (1999) have reported that the epithelial cells migrate into the periodontal ligament, or die immediately after the onset of root dentin formation in rat molars. Janones et al. (2005) immunodetected no amelogenin on the root dentin surface during root formation in rat molars. Hirata and Nakamura (2006) have reported that cytokeratin-immunoreactive epithelial cells are not immunoreactive for BSP or OPN in mouse molars. For point (2), cementoblasts have well-developed intracellular organelles, rough endoplasmic reticulum, Golgi apparatus, and secretory granules, suggesting high secretory activity (Yamamoto. 1986; Cho and Garant 1988, 1989, 2000; Diekwisch 2001). For point (3), all matrix-secreting cells show TNSALP reaction in mineralized tissues (Ten Cate 1994). Overall, it may be concluded that the classical theory can apply to the initial acellular cementogenesis in rat molars.

Adaptive, reparative, and regenerative cementogenesis is an object of interest in clinical dentistry, especially periodontics and orthodontics. However, how these events arise and

proceed is still poorly understood. This study would provide fundamental knowledge of cementum mineralization and a technical guide for future histological investigation.

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Figure legends

Fig.1. General histology of the mesial root of the maxillary first molar of 3-week-old rats in hematoxylin-stained sections. (a) Overall view of the maxillary first molar (AB, alveolar bone; D, dentin; DP, dental pulp; E, enamel). Apical foramen of mesial (right) and distal (left) roots are still widely open (asterisks). Bar 1mm.(b)Overall view of the mesial root (AB, alveolar bone; D, dentin; DF, dental follicle; DP, dental pulp; E, enamel; PL, periodontal ligament). Thin acellular cementum (arrowhead) covers the root dentin. Bar 100 μm. (c) At the apical end Hertwig's epithelial root sheath (asterisk) consists of two or three cell layers. More cervically, columnar odontoblasts (OB) form predentin (PD). Where the epithelial sheath disintegrates, the epithelial cells (small arrows) become smaller and darker. Dental follicle cells (large arrows) approach the predentin surface. Bar 10 μm. (d) With the onset of dentin (D) mineralization, the hematoxylin-stained, initial cementum (arrowhead) appears on the mineralized dentin. Cementoblasts (CB) form dense cell population, keeping a short distance (asterisk) from the root surface. PD, predentin. Bar 10 μm. (e) In the mid-root region the cemento-dentinal junction is indiscernible in the cementum (arrow). D, dentin. Bar 10 μm. (f) In the most cervical region the cemento-dentinal junction (arrowhead) appears as the most intensely stained line in the cementum (between arrows). D, dentin. Bar 10 μm.

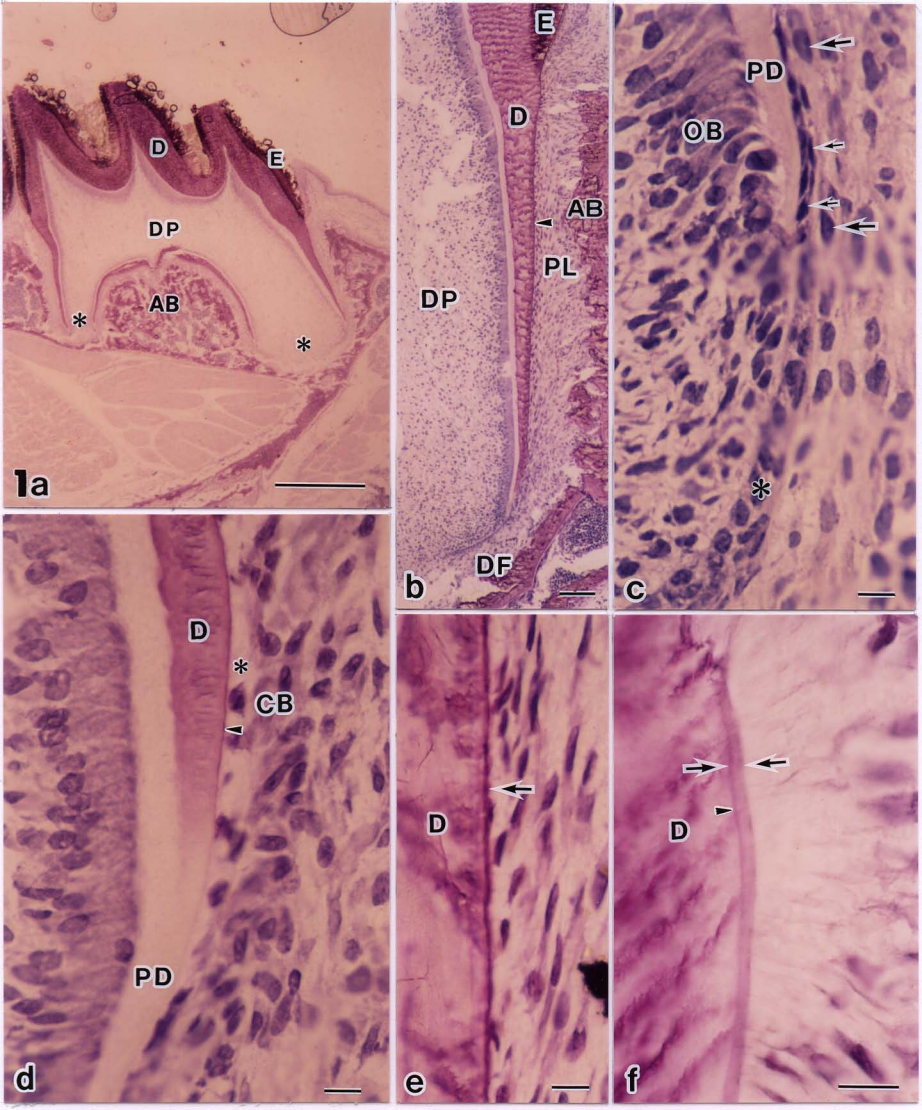
Fig.2. Sections stained with anti-BSP (a,c,d) and anti-OPN (b) antibodies. (a and b) Overall view of mesial roots (AB, alveolar bone; D, root dentin; DF, dental follicle; DP, dental pulp; E, enamel; PL, periodontal ligament). The acellular cementum (arrowheads) stains intensely for BSP (a) and OPN (b). Bars 100 μm. (c) With the onset of dentin (D) mineralization, the BSP-

and OPN (not shown)-immunoreactive layer (arrowhead) appears as the initial cementum. The epithelial sheath is demarcated between arrows. DF, dental follicle; PD, predentin. Bar 20 μm. (d) In the most cervical region the cemento-dentinal junction (arrowhead) shows the most intense immunoreactivity for BSP and OPN (not shown) in the cementum (between arrows). D, dentin. Bar 10 μm.

Fig.3. Sections showing the TNSALP reaction visualized by the Azo-dye method. (a) Overall view of the mesial root (AB, alveolar bone; D, root dentin; DF, dental follicle; DP, dental pulp; E, enamel; PL, periodontal ligament). The periodontal ligament, dental pulp, and alveolar bone surface show reaction. Bar 100 μm. (b) At the apical end the epithelial sheath (between arrows) shows no reaction and the dental follicle (DF) shows reaction. With the onset of dentin (D) mineralization, the cementoblast population shows more intense reaction (asterisk demarcated by arrowheads) than the surrounding periodontal ligament. OB, odontoblasts. PD, predentin. Bar 10 μm. (c) The intense reaction appears only in a restricted area (asterisk demarcated by arrowheads). More cervically, the reaction becomes weak. D, dentin. Bar 10 μm.

Fig.4. Sections stained with GBHA. (a) Overall view of the mesial root (AB, alveolar bone; D, root dentin; DF, dental follicle; DP, dental pulp; E, enamel; PL, periodontal ligament). All the mineralized tissues show GBHA reaction (red-stained). Bar 100 μm. (b) At the apical end the epithelial root sheath (asterisk) and dental follicle (DF) show no reaction. With the onset of dentin (D) mineralization, GBHA reactive particles (arrowheads) appear in the space between

cementoblasts and root surface (see Fig.1d). More cervically, the particles decrease in number. Bar 10 μm. (c and d) In the mid-root (c) and cervical (d) regions, no or only a few GBHA reactive particles are observed on the root surface. D, dentin. Bars 10 μm.


D
E
DP
AB
1a
E
D
AB
PL
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DF
b
PD
OB
c
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D
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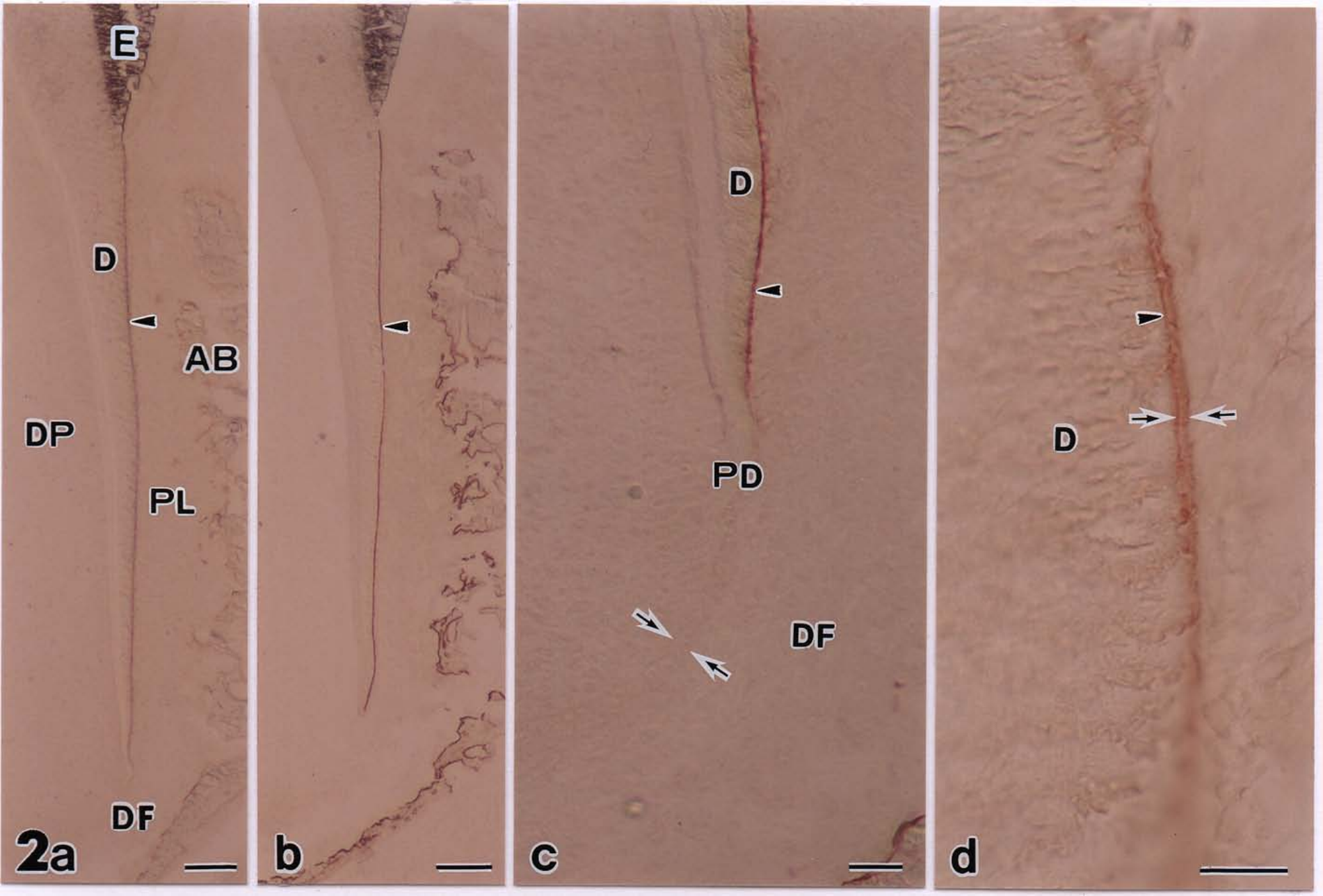
D
f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2a
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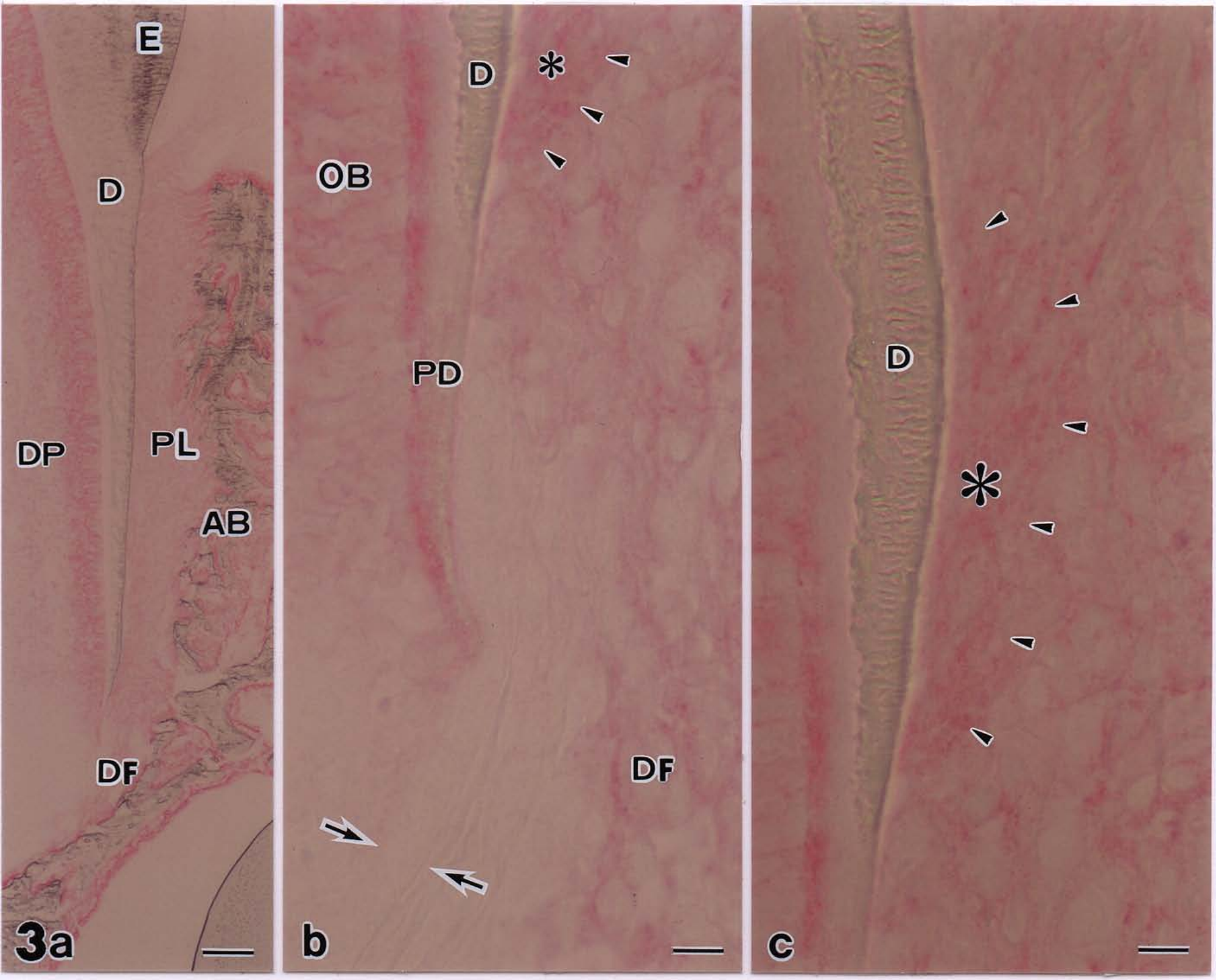
d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3a
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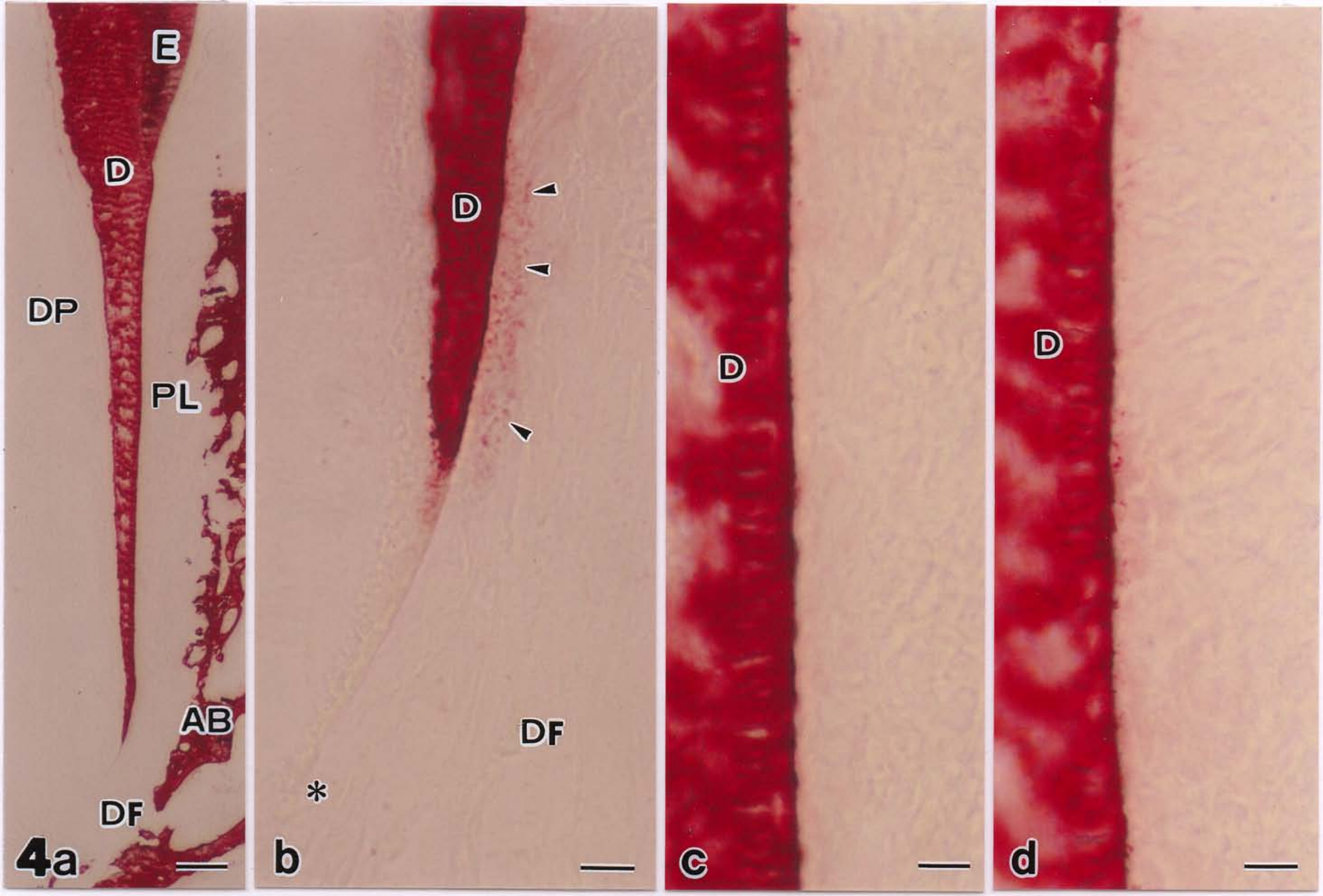
E
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