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Hertwig's epithelial root sheath cells do not transform into cementoblasts in rat molar cementogenesis

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Summary

It is generally accepted that cementoblasts originate in the process of differentiation of mesenchymal cells of the dental follicle. Recently, a different hypothesis for the origin of cementoblasts has been proposed. Hertwig's epithelial root sheath cells undergo the epithelial-mesenchymal transformation to differentiate into cementoblasts. To elucidate whether the epithelial-mesenchymal transformation occurs in the epithelial sheath, developing rat molars were examined by keratin-vimentin and Runx2 (runt-related transcription factor 2)-keratin double immunostaining. In both acellular and cellular cementogenesis, epithelial sheath and epithelial cells derived from the epithelial sheath expressed keratin, but did not express vimentin or Runx2. Dental follicle cells and cementoblasts, however, expressed vimentin and Runx2, but did not express keratin. No cells showed coexisting keratin-vimentin or Runx2-keratin staining. These findings suggest that there is no intermediate phenotype transforming epithelial to mesenchymal cells, and that epithelial sheath cells do not generate mineralized tissue. This study concludes that the epithelial-mesenchymal transformation does not occur in Hertwig's epithelial root sheath in rat acellular or cellular cementogenesis and that the dental follicle is the origin of cementoblasts, as has been proposed in the original hypothesis.

Keywords: Cementoblasts; Hertwig's epithelial root sheath; Epithelial-mesenchymal transformation; Keratin; Runx2; Vimentin; Rat

molars

Introduction

Cementum is one of the dental mineralized tissues and plays important roles in tooth support together with the principal fibers and the alveolar bone proper. Cementum is classified into acellular and cellular cementum, and the cells in the cellular cementum are termed cementocytes. In many species, thin acellular cementum covers the cervical half and thick cellular cementum the apical half of the root.

Cementum is formed by cementoblasts, and some cementoblasts are embedded as cementocytes during cellular cementogenesis. It has been widely accepted that mesenchymal cells of the dental follicle differentiate into cementoblasts and secrete cementum matrix on the denuded root dentin after the disruption of Hertwig's epithelial root sheath. Recently, an alternative hypothesis has been proposed: Epithelial sheath cells could differentiate into cementoblasts by an epithelial-mesenchymal transformation (for reviews, see Thomas, 1995; Bosshardt, 2005; Luan et al., 2006; Zeichner-David, 2006; Foster et al., 2007).

The epithelial-mesenchymal transformation, which occurs during normal developmental processes, e.g., neural crest cell migration, sclerotome formation, cardiac cushion mesenchyme development, and palate formation, has been studied in detail (for a review, see Kang and Svoboda, 2005). For example, in palatogenesis the opposing palate shelves adhere to each other and a medial epithelial seam forms, then some seam epithelial cells disappear by the epithelial-mesenchymal transformation, and others die by apoptosis or migrate nasally or orally. This creates a confluence of connective tissue across the palate (for reviews, see Nawshad et al., 2004; Kang and Svoboda, 2005; Dudas et al., 2007).

In studies of the epithelial-mesenchymal transformation in palatogenesis, keratin has been frequently detected as a general marker of epithelial cells and vimentin as a marker of mesenchymal cells. Fitchett and Hay (1989) reported the coexpression of keratin and vimentin in the seam epithelium by immunohistochemistry, and suggested that these cells switch from epithelial to mesenchymal phenotypes. Gibbins et al. (1999) found coexpression of vimentin and keratin mRNA in the seam epithelium and supported the epithelial-mesenchymal transformation in the seam.

For cementogenesis, it is not generally agreed whether the

epithelial-mesenchymal transformation occurs in Hertwig's epithelial root sheath. We considered that if the epithelial sheath undergoes the epithelial-mesenchymal transformation, transforming epithelial cells will express both keratin and vimentin transiently before the complete transformation, and that keratin-vimentin double immunostaining is a reliable method for confirmation of the coexistence of the two proteins.

Runx2 (runt-related transcription factor2), also known as core-binding factor alpha 1(Cbfa1), was originally identified as an osteoblast-specific transcription factor essential for osteoblast differentiation (Ducy et al., 1999; Ducy, 2000). To date, Runx2 has been detected in both epithelial and mesenchymal cells in various kinds of mineralized and non-mineralized tissues (Bronckers et al., 2001). In mineralized tissues, it has been established that Runx2 regulates the differentiation of mineralization-inducing cells, viz., osteoblasts (bone), ameloblasts (enamel), odontoblasts (dentin), and cementoblasts (cementum) (D'Souza et al., 1999; Ducy et al., 1999; Ducy, 2000; Bronckers et al., 2001; Zhao et al., 2002; Camilleri and McDonald, 2006). From these, we considered that Runx2 could be used as a marker for mineralization-inducing cells in cementogenesis. If the epithelial sheath cells transform into cementoblasts, transforming epithelial cells will express both keratin and Runx2 transiently.

This study was designed on the basis of the above considerations, and to elucidate whether epithelial sheath cells transform into mesenchymal cementoblasts, developing rat molars were immunostained by keratin-vimentin and Runx2-keratin double staining and the localization of these proteins were examined in epithelial sheath cells, dental follicle cells, and cementoblasts.

Materials and methods

This study used ten 21-day-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.

Histology

After anesthesia by an intraperitoneal injection of sodium pentobarbital, the animals were perfused with 4% paraformaldehyde in 0.1M phosphate buffer (pH7.4) for 15 min. The upper jaws were dissected out, immersed in the same

fixative for 24 h, and demineralized in 5% EDTA for 4 weeks at room temperature. Then the specimens were dehydrated in a graded series of ethanols and embedded in paraffin. Next, 3- μ m-thick serial sections of the first molar were cut sagittally. Some sections were stained with hematoxylin and eosin to examine general histology of cementogenesis. Others were used for immunohistochemistry as described below.

Immunohistochemistry

Keratin-vimentin double staining

Deparaffinized sections were immersed in methanol containing 0.3 % hydrogen peroxide to inhibit endogenous peroxidase and treated with 0.5 % trypsin in 0.01M Tris-HCl buffer (pH7.6) for 25 min at 37°C. Sections were incubated with anti-keratin rabbit polyclonal antibody (pan type)(Nichirei, Tokyo, Japan) and then with Histofine Simple Stain rat MAX-PO(R)(Nichirei), and visualized by the 3, 3'-diaminobenzidine method. The immunostained sections were counter-stained with hematoxylin, mounted with glycerin, and photographed.

After removal of glycerin, sections were incubated with anti-vimentin mouse monoclonal antibody(Nichirei) and then with Histofine Simple Stain rat MAX-PO(M)(Nichirei), and visualized with a Vector® VIP substrate kit(Vector Laboratories, Burlingame, CA, U.S.A.). Following this, the sections were again mounted with glycerin and photographed.

Runx2-keratin double staining

After inhibition of endogenous peroxidase, sections were treated with trypsin for 5 min at 37°C. Then they were successively incubated with anti-Runx2 mouse monoclonal antibody (MBL, Nagoya, Japan), biotinated anti-mouse goat polyclonal antibody (DakoCytomation, Glostrup, Denmark), and streptavidin-biotin-horseradish peroxidase complex (DakoCytomation). After visualization by the 3, 3'-diaminobenzidine method, the immunostained sections were mounted with glycerin, and photographed.

After removal of glycerin, sections were treated with trypsin for 20 min at 37°C. Next, the sections were incubated with the anti-keratin antibody, and then with the Histofine Simple Stain rat MAX-PO(R), and visualized with the Vector® VIP substrate kit. Following this, the sections were counter-stained with hematoxylin, mounted with glycerin, and photographed.

Gingival epithelial cells, gingival fibroblasts, and osteoblasts on the alveolar bone were used for positive controls for the anti-keratin, anti-vimentin, and anti-Runx2 antibodies, respectively. These cells showed positive immunoreaction. Normal rabbit or mouse serum was substituted for the three primary antibodies in the negative controls. These control sections did not show any specific immunoreactivity.

Results

Division of developmental stages in cementogenesis

In the apical portion of the mesial root of the maxillary first molars, initial acellular cementogenesis had begun on the mesial side (Figure 1A, B, F) and initial cellular cementogenesis had begun on the distal side (Figure 1A, C, I). Therefore, the mesial side of the mesial root of the maxillary first molars was used for the examination of acellular cementogenesis, and the distal side was used for the examination of cellular cementogenesis. In each side the initial cementogenesis was divided into three developmental stages (Figure 1B, C): stage 1, where Hertwig's epithelial root sheath is intact; stage 2, where the epithelial sheath is disintegrating; and stage 3, where acellular or cellular cementogenesis starts.

Histology

Acellular cementogenesis

In stage 1 (Figure 1D), Hertwig's epithelial root sheath consisted of two or three cell layers. In the dental follicle, slender, undifferentiated mesenchymal cells were arranged in parallel with the epithelial sheath. In stage 2 (Figure 1E), dental papilla cells differentiated into odontoblasts and generated the initial predentin, and the epithelial sheath began to disintegrate. Dental follicle cells became large and plump and approached the exposed predentin. Dental follicle cells and epithelial cells could be roughly distinguished by differences in staining affinity and cell size and shape; however, strictly distinguishing the two kinds of cells was difficult in the hematoxylin and eosin-stained sections. In stage 3 (Figure 1F), with the onset of dentin mineralization, hematoxylin-stained, initial acellular cementum appeared on the mineralized dentin. Large cells, generally referred to as cementoblasts, were seen on the cementum.

Cellular cementogenesis

In stages 1 and 2 (Figure 1G, H), developmental aspects were similar to those of acellular cementogenesis. In stage 3 (Figure 1I), with the onset of dentin mineralization, cellular cementogenesis began. Large cementoblasts were seen on the cementum and there were cells embedded in the cementum. It was difficult to elucidate whether the embedded cells were embedded epithelial cells or embedded cementoblasts (cementocytes) in the hematoxylin and eosin-stained sections.

Immunohistochemistry

Keratin-vimentin double staining

In stage 1 of acellular cementogenesis (Figure 2A, B), the anti-keratin antibody stained the epithelial sheath, but did not stain dental follicle cells. The anti-vimentin antibody stained dental follicle cells, but did not stain the epithelial sheath. In stage 2 (Figure 2C, D), keratin-immunoreactive epithelial cells and vimentin-immunoreactive dental follicles cells mingled on the predentin. These two figures (Figure 2C, D) demonstrated that not a single cell was double-stained for keratin and vimentin. In stage 3 (Figure 2E, F), some keratin-immunoreactive epithelial cells still remained on the root. Cementoblasts was identified as vimentin-immunoreactive cells. None of the cells were double-stained for keratin and vimentin.

In stages 1 and 2 of cellular cementogenesis (not shown), the staining pattern was similar to that of the acellular cementogenesis. In stage 3 (Figure 2G, H), many vimentin-immunoreactive cementoblasts and a few keratin-immunoreactive epithelial cells were seen on the cementum. There were vimentin-immunoreactive cells (cementocytes) and keratin-immunoreactive epithelial cells in the cementum. None of the cells on or in the cementum were double-stained for keratin and vimentin.

Runx2-keratin double staining

In stage 1 of acellular cementogenesis (Figure 3A, B), the anti-Runx2 antibody stained dental follicle cells, but did not stain the epithelial sheath. In stage 2 (Figure 3C, D), Runx2-immunoreactive dental follicles cells and keratin-immunoreactive epithelial cells mingled on the predentin. These two figures (Figure 3C, D) demonstrated that not a single cell was double-stained for Runx2 and keratin. In stage 3 (Figure 3E, F), epithelial cells remaining on the root stained for only keratin and cementoblasts stained for only Runx2. None of the cells were double-stained for Runx2 and keratin.

In stages 1 and 2 of cellular cementogenesis (not shown), the staining pattern was similar to that of acellular cementogenesis. In stage 3 (Figure 3G, H), cementoblasts stained for Runx2. There were Runx2-immunoreactive cells (cementocytes) and keratin-immunoreactive epithelial cells in the cementum. None of the cells on or in the cementum were double-stained for Runx2 and keratin.

Discussion

This study examined acellular and cellular cementogenesis of rat molars by keratin-vimentin and Runx2-keratin double immunostaining, and provided the following findings: (1) Hertwig's epithelial root sheath and epithelial cells derived from the sheath expressed keratin, but did not express vimentin or Runx2. (2) Dental follicle cells and cementoblasts expressed vimentin and Runx2, but did not express keratin. (3) No cells showed the coexistence of keratin-vimentin or Runx2-keratin staining. (4) The absence of Runx2 expression suggested that epithelial cells do not become mineralization-inducing cells.

Altogether, these findings suggest that the epithelial-mesenchymal transformation does not occur in the epithelial sheath and that the dental follicle is the origin of cementoblasts. This is consistent with Bronckers et al. (2001), who reported that Runx2-immunonegative epithelial sheath seemed to be disrupted by penetration of Runx2-immunopositive dental follicle cells in mouse molars, and Hosoya et al. (2006), who reported that Runx2-immunolabelling was detected in dental follicle cells, but not in the epithelial sheath in rat molars. Hirata and Nakamura (2006) also questioned the epithelial-mesenchymal transformation of the epithelial sheath in rat molars, as they found that epithelial root sheath and epithelial cells derived from the sheath were immunoreactive for keratin, but were not for bone sialoprotein or osteopontin by bone sialoprotein-keratin and osteopontin-keratin double immunostaining.

There have been several *in vivo* (Webb et al., 1996; Bosshardt et al., 1998; Bosshardt and Nanci, 1998, 2004; Lésot et al., 2000) and *in vitro* experiments (Thomas, 1995; Zeichner-David et al., 2003; Sonoyama et al., 2007) that support the epithelial-mesenchymal transformation of the epithelial sheath cells. Webb et al. (1996) found immunohistochemical coexpression of keratin and vimentin in cementoblasts on developing acellular cementum of rat molars, and suggested that these cementoblasts

are derived from the epithelial sheath. However, this opinion leaves two open questions: (1) Coexpression of the two proteins is uncertain, as the report did not use serial sectioning techniques or double staining. (2) It is uncertain that the cells reported as cementoblasts actually were cementoblasts. The cells could be epithelial cells remaining on the root.

Epithelial sheath cells were reported to surround collagen fibril bundles in cytoplasmic concave structures and to develop intracellular organelles for protein synthesis and secretion in rat molars (Bosshardt et al., 1998; Bosshardt and Nanci, 1998) and porcine teeth (Bosshardt and Nanci, 2004). From these findings, the reports suggested that the epithelial cells in question were transforming and secreting cementum matrix. However, the epithelial cells in question could change shape by thickening of fibril bundles and synthesize protein(s) other than cementum matrix proteins (discussed later).

Lésot et al. (2000) examined the expression of *Dlx-2* (*Drosophila* distal-less homeobox gene) in root formation in transgenic mice containing a *Dlx-2/LacZ* reporter construct. During the initial root formation in incisors and molars, the epithelial root sheath expressed *Dlx-2*, whereas dental follicle cells did not. During acellular cementogenesis in both teeth, the majority of cementoblasts expressed *Dlx-2*. During cellular cementogenesis in molars, the innermost cementoblasts and entrapped cementocytes expressed *Dlx-2*. These findings led Lésot et al. (2000) to suggest cementoblast subpopulations of epithelial origin. However, this opinion could be caused by a misidentification of epithelial cells remaining on the root as cementoblasts and of embedded epithelial cells as cementocytes. In mouse and rat molars, it has been established that some epithelial sheath cells remain on the root surface in acellular cementogenesis and are entrapped in the cementum in cellular cementogenesis, as shown in Fig.2G and H and in previous studies (Lester, 1969a, b; Yamamoto and Hinrichsen, 1993; Bosshardt et al., 1998; Diekwisch, 2001; Hirata and Nakamura, 2006; Luan et al., 2006).

Thomas (1995) found that cultured epithelial sheath cells of rodents were double-immunostained for keratin and vimentin under certain conditions, and suggested that the epithelial-mesenchymal transformation also occurred during *in vivo* cementogenesis. Zeichner-David et al. (2003) found coexpression of keratin and vimentin mRNA in cultured epithelial sheath cells of mouse molars. In addition, these cells appeared to have the morphological and biochemical characteristics seen in acellular

cementum-forming cells. These findings led Zeichner-David et al. (2003) to support the epithelial-mesenchymal transformation of the epithelial sheath during *in vivo* acellular cementogenesis. Sonoyama et al. (2007) found that cultured human epithelial sheath cells expressed the epithelial markers (keratin and amelogenin) and mesenchymal cell markers (vimentin, osteocalcin, and bone sialoprotein), supporting the epithelial-mesenchymal transformation of the epithelial sheath. However, it is uncertain whether the epithelial sheath cells behave similarly *in vivo* and *in vitro*, because environmental conditions are quite different.

As mentioned above, there has been no concrete evidence presented for the epithelial-mesenchymal transformation of Hertwig's epithelial root sheath, at least under *in vivo* normal cementogenesis. In the investigation here, we conclude that the epithelial sheath cells do not transform into cementoblasts in rat acellular and cellular cementogenesis, and support the original hypothesis that cementoblasts derive from the dental follicle. There have been some reports that epithelial cell rests of Malassez express mesenchymal phenotype under certain conditions *in vivo* (Hasegawa et al., 2003) and *in vitro* (Mizuno et al., 2005). To date there is no data to enable a correlation of these findings with the epithelial-mesenchymal transformation of the epithelial sheath.

In previous studies of cementum, molecules such as cementum attachment protein and cementum protein-23 had been reported as "specific cementum markers", but later they have been found in other tissues as well (for a review, see Foster et al., 2007). While these molecules may not be unique cementum markers, they may be used in addition to vimentin and Runx2 in elucidating the origin of cementoblasts.

Our conclusion does not exclude a secreting-activity of the epithelial sheath cells. Many studies have suggested that the epithelial sheath cells secrete proteins such as enamel matrix proteins, bone morphogenetic proteins, and insulin-like growth factor 1, although serious inconsistencies remain unresolved (for a review, see Foster et al., 2007). These proteins may mediate root and/or cementum formation, and do not appear to become major constituents of cementum (Luan et al., 2006). We think it likely that major interfibrillar matrices such as bone sialoprotein and osteopontin are products of dental follicle-derived cementoblasts in normal cementogenesis (Yamamoto, 1986; Cho and Garant, 1988, 1989, 2000; Diekwisch, 2001; Yamamoto and Wakita, 1990; Yamamoto et al., 2004, 2007; Hirata and

Nakamura, 2006), although there may be regions that are an exception to this, like enamel-free areas of rat molars (Bosshardt and Nanci, 1997, 1998).

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Figure legends

Figure 1. Hematoxylin and eosin-stained sections. (A) Overall view of a maxillary first molar of a 21-day-old rat. Apical portions of the mesial (m) and distal (d) sides of the mesial root (M) are examined. Bar=1mm. (B and C) Magnified apical portions of the mesial (B) and distal (C) sides. Lines demarcate developmental stage1, 2, and 3 of acellular (B) and cellular (C) cementogenesis. Bar=50µm (common in B and C) (D) Stage 1 of acellular cementogenesis. Hertwig's epithelial root sheath (between arrows) separates the dental papilla (DP) and dental follicle (DF). Bar=10µm (common in D to I). (E) Stage 2 of acellular cementogenesis. Columnar odontoblasts (asterisk) generate predentin (PD). Arrowheads indicate epithelial cells and arrows indicate developing dental follicle cells. (F) Stage 3 of acellular cementogenesis. Initial acellular cementum (large arrow) appears as a thin, hematoxylin-stained layer on the dentin (D). Arrowheads indicate epithelial cells and small arrows indicate fully developed cementoblasts. PD predentin. (G) Stage 1 of cellular cementogenesis. Hertwig's epithelial root sheath (between arrows) separates the dental papilla (DP) and dental follicle (DF). (H) Stage 2 of cellular cementogenesis. Arrowheads indicate epithelial cells and arrows indicate developing dental follicle cells on the predentin (PD). (I) Stage 3 of cellular cementogenesis. Initial cellular cementum (asterisk) appears on the dentin (D). Arrows indicate fully developed cementoblasts and arrowheads indicate embedded cells. PD predentin.

Figure 2. Sections stained for keratin (A, C, E, G) and double-stained for keratin and vimentin (B, D, F, H). A and B, C and D, E and F, and G and H pairs are the same section. Keratin stains brown and vimentin purple in cytoplasm. (A and B) Stage 1 of acellular cementogenesis. Epithelial cells (arrowheads) of the epithelial sheath (between arrows) stain for only keratin, and cells (small arrows) of dental follicle (DF) stain for only vimentin. DP dental papilla. Bar=10µm (common in A to H). (C and D) Stage 2 of acellular cementogenesis. Keratin-immunoreactive epithelial cells (arrowheads) do not stain for vimentin, and vimentin-immunoreactive dental follicle cells (arrows) do not stain for keratin. PD predentin. (E and F) Stage 3 of acellular

cementogenesis. Keratin-immunoreactive epithelial cells (arrowheads) do not stain for vimentin, and vimentin-immunoreactive dental follicle cells (arrows) do not stain for keratin. D dentin. (G and H) Stage 3 of cellular cementogenesis. Large arrows indicate vimentin-immunoreactive cementoblasts. Vimentin-immunoreactive cementocytes (small arrows) and keratin-immunoreactive epithelial cells (arrowheads) are embedded in cementum (asterisk). None of the cells are double-stained. D dentin.

Figure 3. Sections stained for Runx2 (A, C, E, G) and double-stained for Runx2 and keratin (B, D, F, H). A and B, C and D, E and F, and G and H pairs are the same section. Runx2 stains brown in nuclei and keratin purple in cytoplasm. (A and B) Stage 1 of acellular cementogenesis. Epithelial cells (arrowheads) of the epithelial sheath (between arrows) stain for only keratin, and cells (small arrows) of dental follicle (DF) stain for only Runx2. DP dental papilla. Bar=10µm (common in A to H). (C and D) Stage 2 of acellular cementogenesis. Keratin-immunoreactive epithelial cells (arrowheads) do not stain for Runx2, and Runx2-immunoreactive dental follicle cells (arrows) do not stain for keratin. PD predentin. (E and F) Stage 3 of acellular cementogenesis. Keratin-immunoreactive epithelial cells (arrowheads) do not stain for Runx2, and Runx2-immunoreactive cementoblasts (arrows) do not stain for keratin. D dentin. (G and H) Stage 3 of cellular cementogenesis. Arrows indicate Runx2-immunoreactive cementoblasts. Keratin-immunoreactive epithelial cells (arrowheads) are embedded in cementum (asterisk). Runx2-immunoreactive cementocytes are not shown in these pictures. None of the cells are double-stained. D dentin.





