ORIGINAL

In vitro formation of cementum in mouse molar germs cultured in newly-developed organ culture system

Naoki Fujiwara

Department of Oral Anatomy, Iwate Medical

University School of Dentistry

(Chief : Prof. Tokio Nawa)

1-3-27, Chuo-Dori, Morioka, Iwate 020, Japan

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Abstract : A new organ culture system for *in vitro* study of cementogenesis in mouse molar teeth was developed. Mandibular first molar germs at postnatal day 14 were exposed by removing off one side of the mandibular bone and were then placed cut surface down onto filter papers. The filter papers had a hole in their center, and were maintained above the bottom of the culture chambers. A serum-free, chemically-defined medium was then added, with fresh supplements at every change of the medium. This culture method maintained the three-dimensional architecture of the tooth germ as well as that of the peripheral tissues during the whole culture period. By the 43rd culture day, acellular cementum-like layer, clearly different from the periodontal ligament proper, was formed on the acellular cementum. The mineralization of the newly formed cementum-like matrix was confirmed by electron microscopy and X-ray microanalysis.

抄録：マウス臼歯のセメント質形成について *in vitro* で研究するために、新しい器官培養系を開発した。材料には生後 14 日齢マウス下顎を用い、片側の歯槽突起を取り去り第一臼歯の一部を露出させた。培養器は中央に臼歯歯胚が入る程度の穴を開けたフィルターを持つ各セルと、それを入れる 24 穴マルチディッシュで構成され、歯胚の露出部が下になるようにフィルターに載せ、無血清完全合成培地で器官培養した。培地はそのつど調合し、一日おきに交換した。培養全期間を通じ、歯胚や歯周組織の立体構築は保たれていた。培養 43 日までに有細胞セメント質様の層が形成された。電子顕微鏡、X 線微小分析により、培養によって新たに形成されたセメント質様構造は石灰化していることが確認された。

Introduction

The biological mechanisms involved in cementogenesis remain controversial. Cho and Garant¹⁾ observed the ultrastructural features of the initial differentiation the cementoblasts. Gurling and Sampson²⁾ have suggested that Hertwig's epithelial root sheath may be involved in the induction of cementogenesis. Regarding the cellular

cementogenesis, Lester^{3,4)} and Liao⁵⁾ reported that the epithelial cells as well as cementoblasts were entrapped by the newly deposited cementum. However, these previous studies showed only early cementogenesis.

Recently, MacNeil *et al.*⁶⁾ suggested that bone sialoprotein and osteopontin may be involved in cementogenesis. Bronckers *et al.*⁷⁾ also suggested that the two types of cementum, i. e., acellular and cellular cementum, differ in chemical composition and that the

cementogenic cells responsible for acellular and cellular cementum express different adhesion proteins. However, the significance of these cell adhesion proteins in cementogenesis has not been satisfactorily defined to date. In addition, the triggering factors for induction of cementogenesis are not fully understood.

Organ culture is a useful method for the investigation of tooth development, as the use of serum-free, chemically-defined media in organ culture makes it possible to assess the direct effects of bioactive molecules. However, when Slavkin *et al.*⁸⁾ cultured molar tooth germs in a serum-free, chemically-defined medium, they failed to observe root formation and cementogenesis. Bosshardt and Schroeder⁹⁾ also studied the process of *in vitro* formation of cementum in human teeth, but observed only focal precipitation of cementum matrices at a restricted area near the root surface. Thus, substantial root formation or cementum formation has not been satisfactorily achieved in previous organ culture systems.

Sakakura¹⁰⁾ and Sakakura *et al.*¹¹⁾ improved of an organ culture system and succeeded in observing *in vitro* crown formation. These researchers suggested that the maintenance of the three-dimensional architecture of tooth germs is essential for normal development in organ culture. Furthermore, it has been suggested that epithelial-mesenchymal interactions are needed for the development of cementum and the periodontal ligament¹²⁻¹⁴⁾. Accordingly, it is reasonable to assume that the maintenance of the three-dimensional architecture of surrounding tissues, including bone, and tooth germs may be required for the successful development of cementum *in vitro*.

The present paper reports on the establishment of a new organ culture system that enables normal root development *in vitro*, and provides evidence of *in vitro* cementogenesis.

Materials and Methods

1. Explants

Seventy mice, 14 days of age (ddY, average weight : 8 g) were anesthetized under ether and killed by decapitation. Mandibular bones were removed and were usually stored in Hank's balanced salt solution

at 4°C less than 1h before explantation. One side of the mandibular bones was removed by making a longitudinal cut along the incisor-mandibular joint axis to expose the first molar germ (Fig. 1A). The lingual side of the distal root was thus exposed directly to the medium.

2. Culture medium

The serum-free, chemically-defined BGJb medium (Fitton-Jackson modification, Sigma Chemical Company, St. Louis, USA), was supplemented as a final concentration with 60 mg/l kanamycin, and ITS™ Premix (Becton Dickinson Labware, Bedford, MA, USA). ITS™ Premix provides insulin and transferrin, 5 mg/ml for each, and 5 ng/ml selenious acid. A hundred mg/l ascorbic acid or 0.1 mM L-ascorbic acid phosphate magnesium salt (Wako Pure Chemical Industries, Osaka, Japan) was added to the medium prior to use. The medium was supplied with fresh supplements at every medium change. The initial pH value of the medium was adjusted to 7.4.

3. Culture procedures

The organ culture system consisted of 24-well multidishes (Nunc, Roskilde, Denmark) with culture chambers (Millicell-HA, Millipore Products Division, Bedford, MA, USA) with filters with a hole in their center (Fig. 1, B1). The hole was larger than the contour of the first molar tooth germ. The chamber was set in each well with the filter side up. Both ends of the mandibular bone of the explants were supported by the filter of the culture chamber (Fig. 1, B2), and the molar tooth germs were bathed in the culture medium through the hole in the filter. The medium described above was added to the level of the filter (Fig. 1, B3). Thus, the explants were kept at the level of the gas-medium interface. The explants were incubated for 21, 28, 35, or 43 days at 37°C in a humidified atmosphere of 5% CO₂, 50% O₂, and 45% N₂ gas mixture, with a changing of medium every other day.

4. Histological procedures

At the end of the cultivation period, some explants were immersed in 2.5% glutaraldehyde dissolved in

Fig. 1 Schematic illustration of the preparation of the explant (A), and individual steps of the culture system (B1-B3). (A) The explant is shown by the cross-hatched zone. Mandibular bone was halved along a line between the two arrows. (B1) The culture chamber is equipped with a filter paper having a hole in its center. (B2) Edges of the mandibular bone of the explant are placed on the filter paper with the cut surface down. (B3) The medium is added just to the level of the filter paper. M : medium.

0.05 M cacodylate buffer (pH 7.4) for 1.5h at 4°C. The specimens were then demineralized in 2.5% EDTA at 4°C for 4 weeks. After postfixing in 1% osmium tetroxide in the same buffer at 4°C for 1h, they were dehydrated by passage through a graded ethanol series, and embedded in Epon 812. Semi-thin and ultrathin sections of molars were cut in the bucco-lingual direction. Semi-thin sections were stained with 0.1% toluidine blue for light microscopic observation, whereas ultrathin sections were stained with uranyl

acetate and lead citrate for transmission electron microscopy.

Some explants cultivated for 43 days were immersed in periodate/lysine/paraformaldehyde fixative¹⁵⁾ at 4°C for 24h. These explants were then demineralized by soaking in Plank-Rychlo's solution at 4°C for 12-24h. After neutralization, the specimens were mounted in Tissue-Tek® O.C.T. Compound (Miles Inc. Elkhart, IN, USA), and rapidly frozen on a cryobar placed in a cryostat. Bucco-lingual cryosec-

tions 6 μm -thick were used for immunohistochemical analysis after being digested with 0.2% hyaluronidase dissolved in phosphate buffer, pH 7.4. Rabbit antisera to type-I collagen (Advance, Tokyo, Japan) and type-III collagen (LSL, Tokyo, Japan) were used as the first antisera at a dilution of 1 : 500 and 1 : 750, respectively. Normal rabbit serum (1 : 100) was used for control staining. A Vectastain *Elite* ABC kit (Vector Laboratories, Inc. Burlingame, CA, USA) was employed for the immunoreactions according to the manual provided with the kit. Reaction products were visualized by DAB reaction.

5. Electron probe X-ray microanalysis

Epoxy sections of non-demineralized organs cultured for 43 days were used for electron probe X-ray microanalysis. Two-micrometer semi-thin sections were cut and placed on a carbon specimen mount, and coated with carbon vapor. The mineral composition of the newly deposited, cementum-like matrix in the molar roots of the explants was analyzed with a JEOL JXA-8900L electron probe X-ray microanalyzer.

6. Normal *in vivo* development of cementum

For comparison with normal cementum development *in vivo*, 30 ddY mice of 14, 21, 23, 26, 28, and 35 days of age were anesthetized under ether and killed by decapitation. Mandibles with the first molars were removed, immersed in 2.5% glutaraldehyde dissolved in 0.05M cacodylate buffer (pH 7.4) for 1.5h at 4°C, and then demineralized in EDTA for 4-5 weeks at 4°C. After postfixing in 1% osmium tetroxide for 1h at 4°C, the explants were embedded in Epon 812. Semi-thin and ultrathin sections of the molars were cut, and examined by light and electron microscopy.

For immunohistochemical analysis, some specimens obtained for 28-day-old mice were immersed in periodate/lysine/paraformaldehyde fixative and demineralized in Plank-Rychlo's solution. After being rapidly frozen on a cryobar placed in a cryostat, cryosections of 6- μm thickness were prepared, and immunohistochemical analysis for detection of type-I and type-III collagens was performed using the same procedure as above.

Results

1. Cementogenesis *in vivo* (Control observations)

In 14-day-old mice, the crown formation of molars seemed to be almost complete. The eruption of the crown of the mandibular first molar into the oral cavity was observed at the 16th postnatal day. A thin toluidine blue-metachromatic layer, so-called initial acellular cementum, was detected on the root dentin surface in 14-day-old mice.

By day 21, a new cementum matrix that showed orthochromasia to toluidine blue was added in places to the surface of the initial acellular cementum at the apical half of the root (Fig. 2A). By day 28, the new cementum layer had thickened, especially at the apical region (Fig. 2B). It was observed that cementoblasts and principal fibers of the periodontal ligament adjacent to the acellular cementum were incorporated into the newly formed cementum matrix. However, it was difficult to judge whether epithelial cells were incorporated in the cementum matrix. The cellular cementum had further thickened by 35 days after birth (Fig. 2C). In contrast, the metachromatic acellular cementum observed on the surface of the root dentin in 14-day-old mice gradually became obscure as cementogenesis advanced, except at the cervical region.

When cellular cementum was observed by electron microscopy, cementoblasts were characterized by a polygonal shape having several cytoplasmic processes, and well-developed, rough-surfaced endoplasmic reticulum and Golgi apparatus (Fig. 3A). Cross sections of principal fiber-like thick collagen bundles were usually seen in spaces between the cytoplasmic processes of these cementoblasts (Fig. 3A). In undecalcified sections, the cementum matrix clearly showed the characteristic of mineralization. In the boundary region between the periodontal ligament and calcified cementum, there were many foci of mineral deposits (Fig. 3B).

When frozen sections of molar teeth from 28-day-old mice were immunohistochemically stained, a positive immunoreaction for type-I collagen was local-

Fig. 2 Light micrographs of roots of mouse mandibular first molars *in vivo*. (A) In 21-day-old mice, a thin layer of toluidine blue-positive matrix (arrowheads) has precipitated on the root dentin (d) showing a corrugated line against the periodontal ligament (pl). (B) The thickness of the toluidine blue-positive matrix (asterisks) has increased by 28 days. (C) Thick cellular cementum (c) can be seen at the root tip of the tooth germ at 35 days after birth. A : $\times 450$, B : $\times 420$, C : $\times 200$

ized in the cellular cementum, periodontal ligament, alveolar bone, and predentin (Fig. 7A). In contrast, the positive immunoreaction for type-III collagen was limited in the periodontal ligament (Fig. 7B).

2. Cementogenesis in organ culture

Molar teeth of the explants removed at 14 postnatal days already had roots half as long as those of adults (Fig. 4). In the precultured teeth, a very thin layer showing metachromasia to toluidine blue, i.e., an initial acellular cementum, was present on the surface of the root dentin matrix.

The explants (Fig. 5A) maintained the three-dimensional architecture of the alveolar bone, periodontal ligament, and tooth germ until the end of the cultivation period, i.e., 43 days. Hertwig's epithelial root sheath (Fig. 5B) in the apical root region and epithelial cell rests of Malassez (data not shown) in the periodontal ligament remained throughout the entire culture period. Although the dentin and predentin in the cultured tooth germs were normal in microscopic structure, it was difficult to judge whether the roots grew during the cultivation period.

On the 21st culture day, although the initial acellular cementum was observed on the root surface, there was no distinctive cellular cementum-like matrix (Fig. 5A). Between 21 and 28 days (Fig. 5B) of culture, a thin layer showing moderate orthochromasia to toluidine blue was newly formed on the surface of the acellular cementum at the root apex region. This layer had extended along the root surface by the 35th culture day (Fig. 5C). The metachromatic acellular cementum layer had gradually become obscure by this stage.

By the 35th culture day, the newly formed layer had thickened and contained sparse single cells (cementoblast-like cells) aligned parallel to the root axis (Fig. 5C). Until culture day 43, this new layer became further thickened and intensely toluidine blue positive, in the same manner as calcified cellular cementum *in vivo* (Fig. 5D). There were a number of single cells incorporated into the matrix (Fig. 5D).

Electron microscopy revealed that the cementoblast-like cells at the 35th day contained well-developed, rough-surfaced endoplasmic reticulum (Fig. 6A) and Golgi apparatus in their cytoplasm. These cells pos-

Fig. 3 Electron micrographs of a cementoblast (A) and cementum (B) in the mandibular first molar at 35th postnatal day *in vivo*. (A) Note that bundles of collagen fibrils (asterisks) are encircled by processes of the cementoblast. (B) A cellular cementum layer (c) in undecalcified section. Cementum matrix clearly shows a characteristic of mineralization. Many foci of mineral deposition are recognized at the calcification front. d : dentin. pl : periodontal ligament. A : $\times 5,250$, B : $\times 4,250$

Fig. 4 A precultured explant of a first molar with mandibular bone at 14th postnatal day as seen under the dissecting microscope. The molar tooth already possesses roots half as long as those of the adult. $\times 17$

essed a polygonal profile with numerous cytoplasmic processes in a similar manner to the cementoblasts *in vivo* (cf., Figs. 3, 6A, and 6B). In some cases these cytoplasmic processes showed slender contours with branches (Fig. 6B). While most collagen fibrils surrounding these cells exhibited a random arrangement of fibrils, those in the spaces between the cytoplasmic processes of the cementoblast-like cells showed a regular arrangement as in principal fibers (Fig. 6A and 6B). Matrix vesicles were sometimes found around the collagen fibrils. So-called afibrillar acellular cementum with granular matrix, formed by slow accretion of minerals, could not be observed in this study. In undecalcified ultrathin sections of molar teeth from the 43rd culture day, the cementum-like layer showed a very high electron density with mineral deposition (Fig. 6C). There were cementum lacuna-like spaces containing debris of presumptive cementocytes.

Fig. 5 Light micrographs of the roots of mandibular first molars *in vitro*. (A) Explant at 21st culture day. The periodontal ligament (pl) has survived as well as the alveolar bone (b) and tooth germ. No distinctive cellular cementum can be seen on the dentin (d). (B) An area of weakly positive toluidine blue matrix precipitates (arrows) is seen at the apical region of this dental root by the 28th culture day. Hertwig's epithelial root sheath (h) is maintained. (C) A layer of extracellular matrix (asterisks) is formed on the root dentin (d) by the 35th culture day. Cementoblast-like cells (arrowheads) embedded in the slightly toluidine blue-positive matrix can be seen. (D) By the 43rd culture day, the new formed layer (arrowheads) becomes thicker and intensely toluidine blue positive. A number of single cells incorporate into this matrix. p : pulp. A : $\times 110$, B : $\times 250$, C : $\times 500$, D : $\times 260$

When explants cultured for 43 days were immuno-histochemically stained with anti-type-I collagen, positive reactions were found in the newly formed cellular cementum-like matrix, periodontal ligament, alveolar bone, and predentin (Fig. 7C). In contrast, the positive reaction for type-III collagen was restricted to the periodontal ligament (Fig. 7D).

Electron probe X-ray microanalysis revealed peaks of calcium and phosphorus in the newly formed cementum-like matrix as well as in the dentin (Fig. 8A). Although the analysis was not truly quantitative, the levels of these peaks were clearly lower in the newly formed cementum-like matrix than in the dentin.

Discussion

It has been shown that when cells isolated from human or rat periodontal ligaments are cultured, mineralized nodules form in the culture dishes¹⁶⁻¹⁹. Bernstein *et al.*^{20,21}, and Beertsen and his co-workers^{22,23} reported that in the co-cultures of cells from periodontal tissue or alveolar bone with slices of dental hard tissues, collagenous or cementum-like matrices were deposited on the hard tissues²⁰⁻²³. However, these *in vitro* findings merely show restricted aspects of the phenomena involved in cementogenesis.

Organ cultures of embryonic tooth germs by excel-

Fig. 6 Electron micrographs of cementoblast-like cells in an explant cultured for 35 days (A, B) and 43 days (C). Note well-developed, rough-surfaced endoplasmic reticulum (A). Although most collagen fibers surrounding the cells are oriented in random directions, those in the space between processes show a regular arrangement (A and B, asterisks). Note matrix vesicles on collagenous matrix (arrows) near the dentin (d). (B) Note slender processes of the cementoblast-like cell. (C) In undecalcified section, cementum-like layer (c) shows a very high electron density with mineral deposition on the dentin matrix (d). Cementum lacuna-like spaces (cl) exist among these matrices. pl : periodontal ligament. A : $\times 5,750$, B : $\times 8,000$, C : $\times 4,350$

Fig. 7 Immunohistochemical localization of type- I (A, C) and type-III collagens (B, D) in molar roots. (A) and (B) show sections of molars from a 28-day-old mouse, and (C) and (D), molars cultured for 43 days. Positive immunoreactions for type- I collagen are found in cementum (c), periodontal ligament (pl), bone (b), and predentin (arrowheads) in both molars *in vivo* (A) and *in vitro* (C). The immunoreactions for type-III collagen are restricted to the periodontal ligament in both molars *in vivo* (B) and *in vitro* (D). A and B : $\times 200$, C and D : $\times 500$

lent organ culture systems such as the Trowell-type²⁴⁾, flotation^{10,11)}, and their modifications^{25,26)} have been providing important information regarding the understanding of crown morphogenesis. However, for root formation including cementogenesis, no satisfactory organ culture methods have been developed to date.

In the present study, the forming of a cementum-like layer was observed on the developing root surface *in vitro* using a newly developed organ culture system. Electron microscopy indicated that the newly formed matrix contained not only proper collagen fibrils but also dense collagen bundles similar to principal fibers, in the developing periodontal membrane. In addition, there were cells incorporated into the matrix.

Cementoblast-like cells in explants showed comparable features to normal cementoblast *in vivo*²⁷⁻²⁹⁾ with well-developed cytoplasmic organelles and numerous cytoplasmic processes. Furthermore, mineralization of the matrix was confirmed by electron microscopy and electron probe X-ray microanalysis. The results of the electron microscopy also indicated that the calcification of the cellular cementum-like matrix in culture is acquired by the coalescence of mineral foci in the same manner as the cellular cementum formation *in vivo*³⁻⁵⁾, but not by the slow precipitation of granular matrix in afibrillar acellular cementum^{5,22,23)}. Also, the newly formed cementum-like matrix showed a positive immunoreaction for type- I collagen but not for type-III collagen, which is

Fig. 8 Electron probe X-ray microanalysis of the newly formed cementum and dentin *in vitro*. (A) Peaks of calcium (Ca) and phosphorus (P) are detected in both the cementum and dentin. (B) Light micrograph of a semithin section adjacent to the section used for the analysis. c : cementum, d : dentin. $\times 320$

also the case for normal cementum formed *in vivo*^{30~32}. These findings indicate that authentic cellular cementum was developed *in vitro* by our newly developed organ culture system.

The periodontal membranes *in vitro* showed affinity for anti-type-III collagen. Because type-III collagen is known to be limited to the periodontal ligament^{30~32}, it appears that the present organ culture system enabled the normal development of the periodontal ligament as well.

It is clear that the successive *in vitro* development of cementum and the periodontal ligament in the present study was accomplished by the maintenance of the special arrangement of various native cells in the dental follicle tissue of the primary explants, such

as mesenchymal cells, fibroblasts, and Hertwig's epithelial root sheath (HERS). Especially, as epithelial-mesenchymal interaction is thought to be important for the development of cementum and the periodontal ligament^{12~14}, the maintenance of normal disposition of HERS and periodontium may exert significant influence toward the normal development of these structures. Another important factor for the success in this study is that the soft tissues in the explants could be supplied with sufficient nutrients and oxygen due to the following reasons : First, explants were maintained at the level of the gas-medium interface through the hole in the filter of the culture chamber. Secondly, as one side of the mandibular bone surrounding the first molar tooth germ was removed,

the tooth germ was exposed directly to the culture medium.

In the present study, the tissues of the explants cultured for 43 days were fairly normal in their histology. This indicates that we should be able to extend further the period of organ culture to study the progression of cementogenesis. Furthermore, the combination of this newly developed culture system and media containing various bioactive factors should yield further information regarding the development of cementum and the periodontal ligament.

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