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Insulin-like growth factor-I stimulates the disintegration of Hertwig's epithelial root sheath and cellular cementogenesis in mouse molars *in vitro*.

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Abstract：Previously, we have elucidated the role of insulin-like growth factor-I (IGF-I) during the early stages of tooth root formation in mandibular first molars of 5-day-old mice. We reported that IGF-I stimulates the mitotic activity in the outer enamel epithelium of the Hertwig's epithelial root sheath (HERS), resulting in the elongation of the root. In the present study, we used mandibular first molars from 20-day-old mice in order to further elucidate the role of IGF-I during the later stages of tooth root formation. The control explants showed normal development of the HERS, similar to that *in vivo*. But the explants treated with 100 ng/ml of IGF-I showed diminished cell proliferation in the inner and outer layers of the HERS. In addition, obvious disintegration of the root sheath along with the appearance of cellular cementogenesis was noted. It was considered that IGF-I signaling accelerated the changing rate, from the maintenance of the length via cell proliferation to the disintegration of the HERS layer, eventually facilitating the differentiation of cementoblasts and cellular cementogenesis. These findings indicated that IGF-I is one of the important regulatory factors throughout root formation.

Key words：cementogenesis, Hertwig's epithelial root sheath, insulin-like growth factor I (IGF-I), organ culture, tooth root formation organ culture, tooth root formation

Introduction

Root development begins after enamel and dentin are formed up to the level of the future cemento-enamel junction. The enamel organ plays an important part in root development by forming the Hertwig's epithelial root sheath (HERS), which molds the outline of the roots and induces differentiation of the odontoblasts to form the root dentin. HERS consists of an outer and an inner enamel epithelial layer without the presence of a stratum intermedium or stellate reticulum. The cells of the inner layer do not produce enamel, but they induce the

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differentiation of dental papilla cells into odontoblasts [1]. After the first layer of dentin is laid down, the HERS loses its continuity and close relationship within the surface of the tooth. The remnants of HERS persist as epithelial rests of Malassez (ERM) within the periodontal ligament (PDL) [2]. Subsequently, cementoblasts appear on the surface of the root dentin and aid in the formation of acellular and cellular cementum. In addition, the cementoblasts begin to form bundles of collagen fibers in the developing PDL, resulting in the formation of the Sharpey's fibers (the strong and thick fibers located between the cementum and the alveolar bone) [3]. Thus, the elongation and disintegration of HERS are important for root development. Previously, we had examined the mechanism of root development and the effects of growth factors on root formation using an original organ culture method that was established by our group [4-6]. By utilizing the same culture system to examine root development in molars obtained from 5-day-old mice, we found that the insulin-like growth factor-I (IGF-I) stimulated HERS elongation by up-regulating cell proliferation in the outer enamel epithelium [7]. The localization pattern of IGF receptors appeared to be similar to that of IGF-I [8], and the appearance of IGF receptors in HERS coincided with the initial stages of tooth root formation [7, 9]. Taken together, these findings suggest that IGF-I and its receptor may be involved in tooth root formation.

Cementum is the hard tissue that covers the surface of the root dentin, and maintains contact with the PDL. One of the unique characteristics of this structure includes the similarities it shares with bone; the cells and the matrix of the cementum are similar to those of bone, wherein cementoblasts and cementocytes respond to osteoblasts and osteocytes. Moreover, the cementum consists of collagen fibers, which are deposited similarly to hydroxyapatite in bone. Unlike in bone, the occurrence of remodeling in the cementum is infrequent. Furthermore, cementoblasts calcify the matrix during the initial stages of cementogenesis. It is followed by the differentiation of the cells into cementocytes, which form large areas of cementum around themselves (acellular and cellular cementum, respectively) [10, 11]. Acellular cementum is located in the cervical to the middle part of the root, while cellular cementum is mainly found in the apex of the root and in the area surrounding the root canal [12].

The cementum contains IGF-I-like molecules [11, 13]; various treatments with IGF-I, growth hormone (GH), and platelet-derived growth factors have been shown to accelerate cementogenesis and the repair of diseased periodontal ligaments *in vivo*. In addition, the characteristics of the cementum matrix and cementoblasts have been described in the literature [14-16]. The effects of GH on cellular cementum are widely known; a previous study has reported the occurrence of hypercementogenesis as a result of daily injections of human GH (hGH) in rat molars [17]. Moreover, GH has been found to induce hypercementogenesis and premature tooth eruption in a patient with pituitary gigantism [18] and to reduce cellular cementogenesis in the molars of hypophysectomized rats [19]; and the amelioration by injections of hGH to rats has been found [19, 20]. IGF-I is related to the GH, and it has been reported that a growth factor derived in the cementum matrix shows a similarity to IGF-I [13]. However, very few studies (*in vitro*) have observed the direct

influence of exogenous growth factors on cementum because it is difficult to obtain the experimental conditions *in vivo* [21]. Furthermore, the immediate effects of the exogenous factors on HERS during this stage have not been reported so far, although IGF-I and the IGF-I receptor were expressed histochemically in HERS during tooth development [7, 22].

Assuming that IGF-I signaling is an important factor during total root development, the present study aimed to elucidate the role of this protein in cellular cementogenesis and in the cell dynamics of HERS during the late stage of root development.

Materials and methods

Animals and organ culture

The design and conditions of the animal experiments were approved by The Committee of Animal Experiments of Iwate Medical University, Morioka, Japan (#145, #30-010). The procedures for organ culture were carried out as previously described [4, 7]. Briefly, hemi-mandibles obtained from 20-day-old ddY mice (Japan SLC, Inc.; average body weight: 9.5 g) were carefully dissected in cold Hank's balanced salt solution. The oral epithelium was stripped off, and the incisors as well as the surrounding bone were dissected away. The bony piece at the bottom of the alveolar crypt was removed, and the upper half of the hemi-mandible was used as the explant. At the center of the explant, an intact first molar tooth germ with PDL (n = 5) was noted. A square-shaped cavity was made in the center of a chamber filter (Millicell-HA, Millipore Products Division, Bedford, MA, USA), which was placed upside down inside each well of a 24-well culture dish (Nunc, Roskilde, Denmark). The well was filled with BGJb medium (Fitton-Jackson modification, Sigma Chemical Co., St. Louis, USA) which was supplemented with 60 mg/l kanamycin (GibcoBRL, NY, USA), 0.1 mM L-ascorbic acid phosphate magnesium salt (Wako Pure Chemical Industries, Osaka, Japan), and 1.25 g/l bovine serum albumin (BSA, Fraction V, Sigma) up to the level of the pored filter. The explants were placed on the filter and cultured for 4, 6 and 14 days at 37℃ under a humidified atmosphere of 5% CO_2, 50% O_2, and 45% N_2. The medium was changed every other day. The medium was supplemented with recombinant human IGF-I (Chemicon International, Temecula, CA, USA) at each concentration of 10, 100, and 500 ng/ml in the experimental group, whereas no IGF-I was added to the medium in the control group (n = 5, each group). Since a concentration of 100 ng/ml IGF-I was effective for cellular cementogenesis, we performed subsequent examinations by using this concentration.

Quantitative micrograph analysis

Semi-thin sections (thickness, 2 μm) were obtained from 10 specimens in each experimental group. The sagittal sections were prepared from the medial surface of the root (medial root of mandibular first molar) to the distal surface. One section was picked up after every five sections (= 10 μm), placed on a glass slide, and stained with toluidine blue. The length and the thickness of the cellular cementum on each slide were measured in micrometers under a light microscope. We defined the range of the cellular cementum such as the maximal length in matrix embedded cementocyte-like cells, and omitted the matrix areas without any embedded cells from measurement. We calculated as the

length of the cellular cementum to the root length as a ratio.

Proliferation assay

The protocol of BrdU assay was based on a previous study [7]. Briefly, the explants (n = 5, each group) were incubated in the presence of 0.5 mg/ml BrdU for 4 h. After washing with Hank's balanced salt solution, the explants were embedded in paraffin, and serially sectioned (thickness, 5-μm). BrdU was detected using a BrdU staining kit (Calbiochem, Oncogene Research Products, Cambridge, MA, USA) according to the manufacturer's protocol, and visualized with streptavidin-peroxidase and diaminobenzidine (DAB). Specimens stained for BrdU were then counterstained weakly with hematoxylin. To compare the mitotic activities between the inner and outer layers of HERS, we counted the number of BrdU-immunopositive cells and the total number of cells in the two layers, and estimated the mitotic index.

Ultrastructural histology

The mice were fixed for 6 h at 4℃ in 2.5% glutaraldehyde that was buffered with 0.05 M sodium cacodylate (pH 7.4). The specimens (n = 5, each group) were then decalcified with 2.5% EDTA for 2 weeks at 4℃ and postfixed with 1% osmium tetroxide for 1 h at 4 ℃. Dehydration was achieved by passing the specimens through a series of graded ethanol, after which they were embedded in an Epon 812, and sectioned (thickness, 2-μm) along the bucco-lingual plane. Semi-thin sections were stained with 0.1% toluidine blue (pH 3.0) and examined via light microscopy and quantitative micrograph analysis. Ultra-thin sections were stained with 1% uranil acetate and 1% lead citrate and observed by electron microscopy.

Counting of the number of epithelial cell rests of Malassez

For counting the number of the epithelial cell rests of Malassez (ERM), the sections of the molars after culture of 4 days and molars from 24-postnatal-day mice (n = 5, each group), were prepared in a similar manner as the study of ultrastructural histology. The ERMs around the mesial root in each group were counted in serial sections of each 10-μm after staining them toluidine blue. A ratio of the ERM number per section was calculated.

Statistical analysis

The number of BrdU-labeled cells in the HERS was evaluated in 10 sections that were picked at random. The estimated mitotic index value was indicated as the mean ± standard division (SD). The significance of the differences was evaluated by using Student's t-test. These statistical processes were performed using the Prism 5 (GraphPad) software.

Results

Effect of IGF-I during the late stage of root formation

The tooth germs of the mandibular first molars from 20-day-old mice were examined during the later stages of root formation and immediately before the onset of cellular cementogenesis. In the control group, the epithelial bilayer of the HERS and cellular cementum were observed in the apical area of the root after 14 days of culture indicating that the explant had reached the early stages of cementum deposition. Root elongation had gradually ceased by this stage.

To clear the dose dependency of the extension of the cellular cementum area, the ratio of the length of the cellular cementum to

the root length was estimated from the explants cultured in the presence of various concentrations of IGF-I (n=5 in each; Fig. 1e). Selections of IGF-I concentration referred to the previous paper [7]. The ratios were 0.19, 0.22, 0.29, and 0.20 for 0, 10, 100, and 500 ng/ml of IGF-I, respectively. This indicated that the dose dependency was oblique and that the

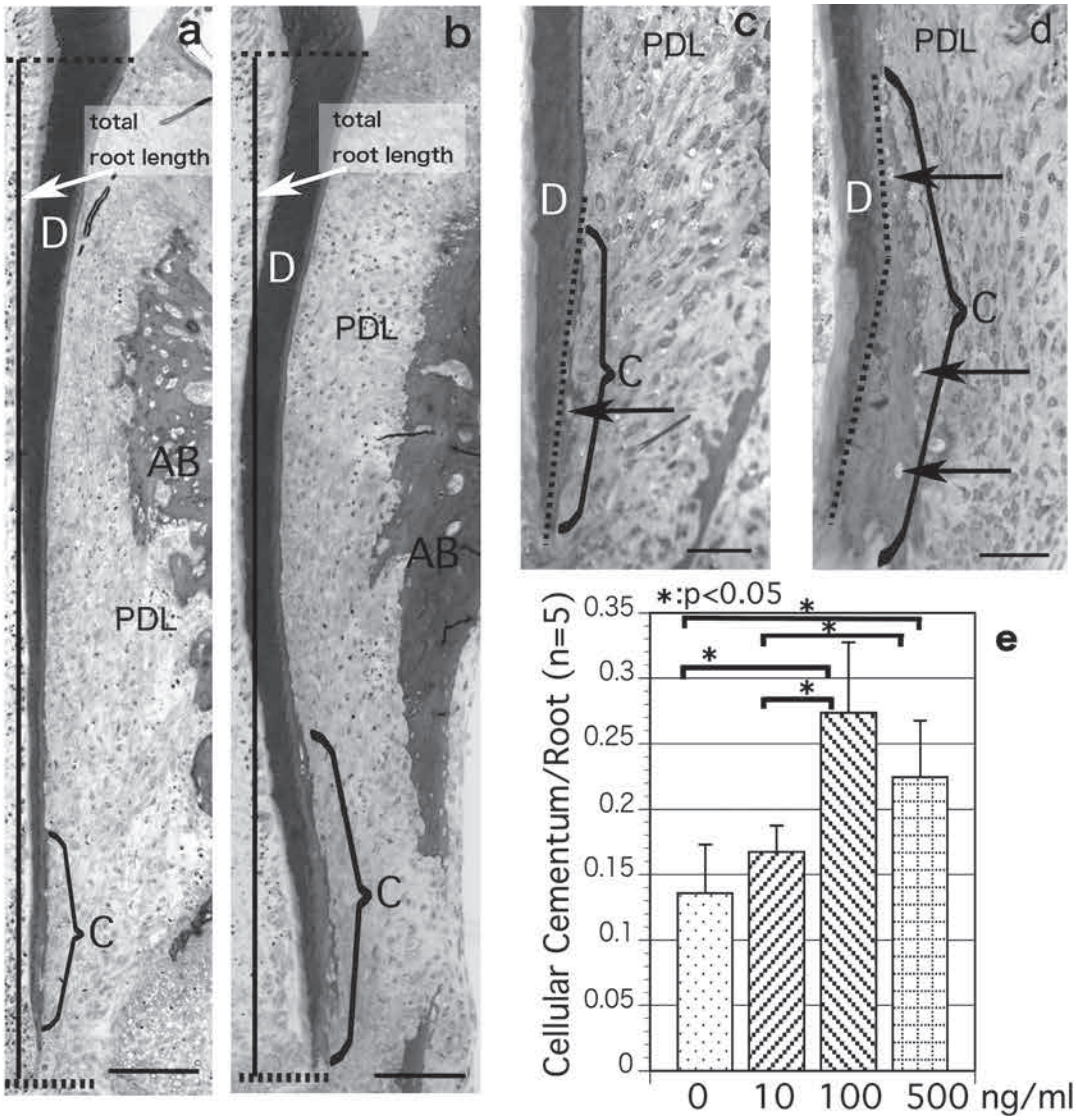

Fig. 1 : Effect of IGF-I on the development of the tooth root *in vitro*. The specimens were prepared from 20-day-old mice and cultured for 14 days with (b and d) and without (a and c) 100 ng/ml of IGF-I. One-third of the root surface was almost covered by a thick layer of cementum in the IGF-I group (b), when compared to one-fifth of the root surface in the control group (a). Histogram (e) shows the ratio of the cellular cementum length to the total length of the lingual side (cellular cementum/root) in the presence of 0 - 500 ng/ml of IGF-I. Arrows indicate cementum lacuna with cementocyte of newly developed cellular cementum, AB: alveolar bone, C: cellular cementum. D: root dentin, PDL: developing periodontal ligament. Scale bars: 100 μm (a and b), and 50 μm (c and d).

most efficient concentration was 100 ng/ml. The thickness of the cementum matrix was increased with an amount of cellular cementum formation (data not shown).

The calcification area of the cementum in the IGF-I-supplemented group (100 ng/ml IGF-I group) was wider than that in the control group. One-third of the root surface (approximately, 390 μm/1160 μm of the total length) was almost covered by cementum in the IGF-I group (Fig. 1b), compared to one-fifth of the root surface (approximately 230 μm/1150 μm total length) in the control group (Fig. 1a). The thickness of the cementum in the IGF-I group (approximately, 80 μm at the most thickness area) was thicker than that in the control group (approximately, 15 μm; Fig. 1c and d). On the other hand, no significant changes in PDL tissues were noted between the experimental and control groups. These observations indicate that IGF-I enhanced cementogenesis on the root surface during the later stages of root formation.

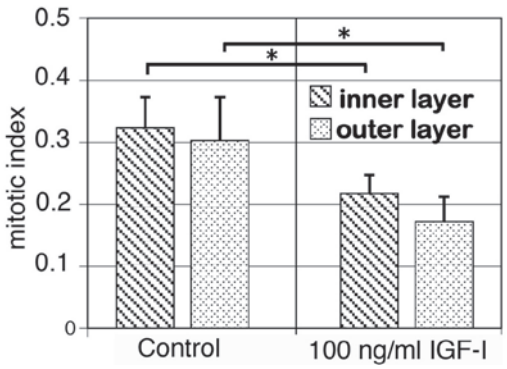

Fig.2 : Mitotic index in the total cells of the two layers of the HERS. The number of BrdU-labeled cells in the HERS (both inner and outer layers) was significantly lower in the IGF-I group when compared to those in the control group (*P<0.01).

Effect of IGF-I on cell proliferation

To examine the effect of IGF-I on cell proliferation, we conducted BrdU experiments with and without 100 ng/ml of IGF-I (n=5 in each). Both groups showed BrdU-positive cells in the HERS, dental pulp, dental follicles, and periosteum of the alveolar bone (data not shown). The numbers of BrdU-positive cells in the outer and inner layers of the HERS were counted in the sections from each group, and the mitotic indices were estimated (Fig. 2). The mitotic indices of the inner and outer layers were 3.3 and 3.0, respectively, in the control group, and 2.2 and 1.75, respectively, in the IGF-I group. These results indicated that the proliferation activity of HERS was markedly low in molars with IGF-I compared to that in the controls; this activity appeared to be suppressed by IGF-I. The mitotic index of the outer layer was slightly lower than that of the inner layer in the controls and the IGF-I group, respectively.

Ultrastructure of HERS in the presence of IGF-I

To determine the ultrastructure of HERS in the presence of IGF-I, the explants were prepared from the mandibular first molars, and cultured for 14 days in the presence/absence of IGF-I (100 ng/ml). The explants were fixed to create ultra-thin sections, which were examined under the electron microscope. In the control group, the cells of the inner layer demonstrated a cuboidal epithelium-like morphology, while those of the outer layer showed a squamous epithelium-like morphology (Fig. 3a). The inner layer and outer layers were not separated by any structure, but the orientations, arrangements and morphologies of the two layers were markedly different from each other. Hence, it

was easy to distinguish between the cells of the inner and outer layer cells. Conversely, in the IGF-I group, cells of both layers showed disrupted cuboidal/squamous epithelium-like morphologies (Fig. 3b). Numerous spaces between the cells appeared. The basement membrane around the HERS was partially intact (Fig. 3c).

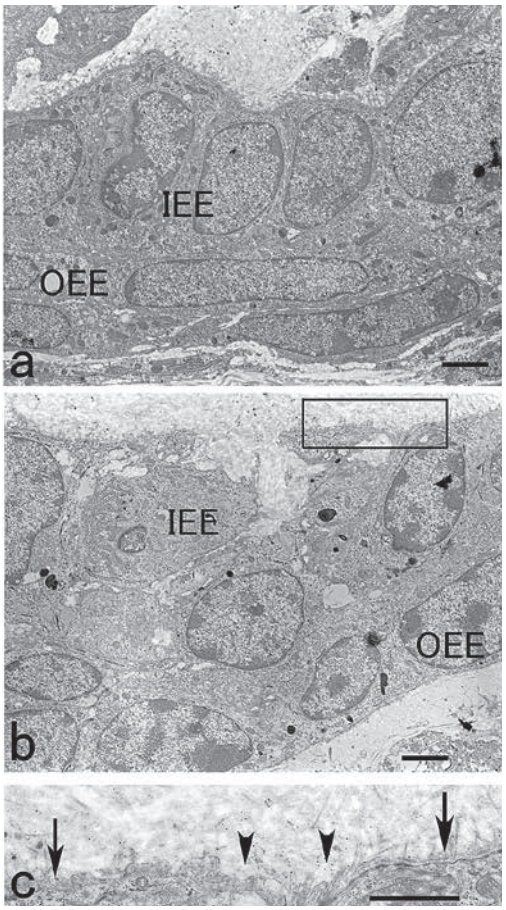

Fig.3 : Electron micrographs of the HERS layers from mandibular first molars cultured for 14 days in the control (a) and IGF-I groups (b, c). The square box in b indicates an area of higher magnification (c). IEE, inner layer of HERS; OEE, outer layer of HERS; arrowheads: areas of disintegration in the basement membrane, arrows: intact basement membrane. Scale bar: 10 μm (a, b) and 20 μm (c).

Semi-thin sections that were made from the specimens indicated that the disintegration of HERS was a frequent occurrence in the IGF-I group compared to the control group (Fig. 4a and b). In the IGF-I group, mesenchymal cell masses appeared in gaps between fragmented HERS (white arrowhead in Fig. 4b). The HERS sheets were shorter in the IGF-I group when compared to the control group (Fig.4a, b). On the other hand, the ERM-like cell masses were observed in the periodontal ligament close to the cellular cementum. Only a few ERM-like cell masses were noted in the control group (data not shown) ; nonetheless, the cell masses in the IGF-I group consisted of more than 10 cells, and were bounded by a basement membrane (Fig. 4b, c, d). Some tonofibrils were observed in the cytoplasm of the component cell (black arrowheads in Fig. 4d). The characteristics of the ERM-like cell masses were similar to those reported previously [23]. To determine the emergence ratio of ERM-like cell masses, we counted the number of ERM-like cell masses in a mesial root per section in both groups and molars obtained from 24-postnatal-day, and estimated them as an ERM ratio. The ERM ratio in the IGF-I group (1.16) showed a larger value than both that of the control group (0.34) and the molars from 24-postnatal-day (0.37) (Fig. 4e).

Ultrastructure of cementoblast-like cell in the presence of IGF-I

After the 14 days of culture under the IGF-I-supplemented conditions, the formation of a cementum-like matrix on the root dentin was noted, and cells were observed close to the matrix in the explants. The morphology of the cell was determined via electron microcopy (Fig. 5), and appeared to be similar to that of the cementoblast wherein several processes

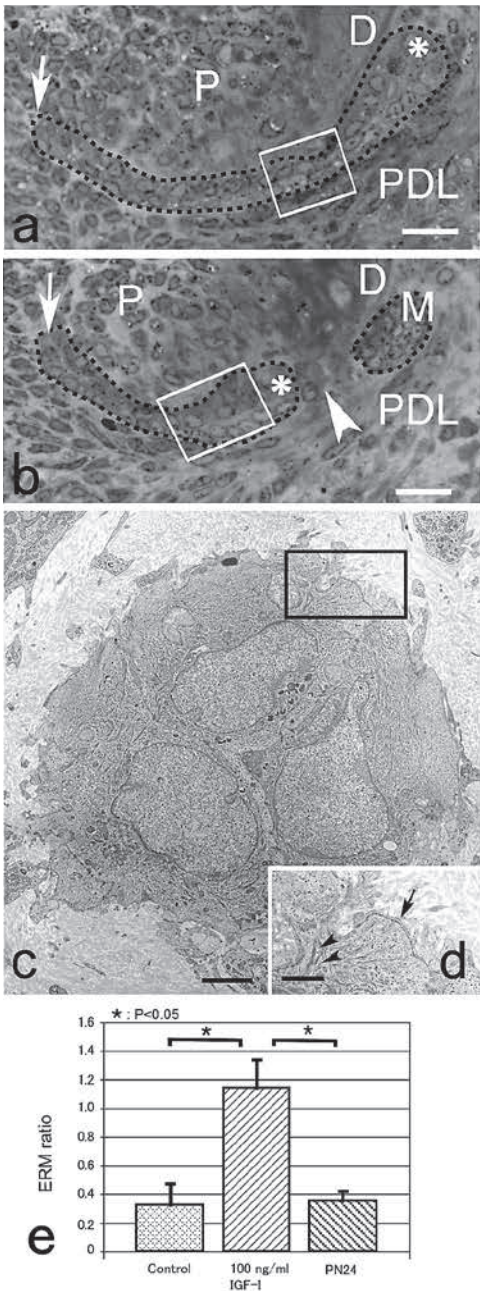

Fig.4 : Photomicrographs showing fragmented layers of the HERS in mandibular first molars in the control (a) and IGF-I groups (b-d). a and b: light microscopic observation of the root apex area. D, dentin; P, pulp; PDL, periodontal ligament; dotted line, outline of HERS; white arrow, the apex of HERS; white arrowhead, mesenchymal cells mass, which seems to enter in the space between the HERS and the ERM. Square boxes in a and b indicate the adjacent areas in Figure 4a and 4b, respectively. c and d: electron microscopic observation of ERM-like structure. d: high magnification of the area indicated in the square box in c (d). Histogram (e) shows the number of ERM-like cell masses in a mesial root per a section (ERM ratio) in both group and molars obtained from 24-postnatal-day (PN24). Black arrow, basement membrane; black arrowheads, tonofibrils. Scale bar: 10 μm (a, b), 3 μm (c), and 1.5 μm (d).

extended toward the root dentin. Well-developed organelles such as mitochondria and rough-surfaced endoplasmic reticulum were observed in the cell. Numerous collagen fibers were seen around the cells, and some of them appeared to be arranged in bundles by the processes of the cementoblast-like cells (asterisks in Fig. 5). These cells were likely to be cementoblasts, which produced the Sharpey's fibers in the periodontal ligament.

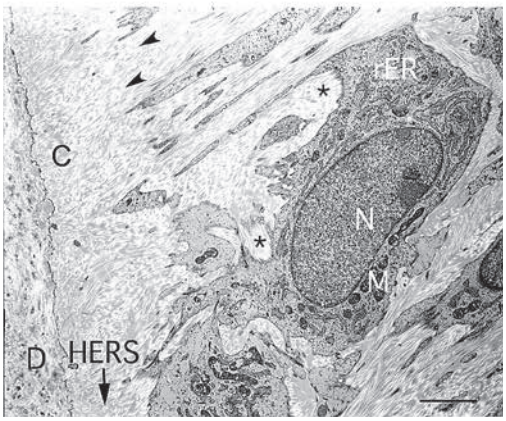

Fig.5 : Electron microscopic image of cementoblast-like cell cultured for 14 days in the IGF-I group. The cell consisted of well-developed organelles with processes extending toward the root dentin. These processes can be seen encircling bundles of collagen fibrils (asterisks). C, newly formed cementum matrix; D, dentin; M, mitochondria; N, nucleus; rER, rough endoplasmic reticulum. Scale bar: 3 μm.

Discussion

HERS regulates the root development by promoting the differentiation of odontoblasts, and formation of cementum starts after the disintegration of HERS [5, 24]. Although there are two kinds of cementum acellular and cellular cementum, the latter appeared in the apex area of the root and the surrounding area of the root canal, and the beginning of matrix formation coincided with the ending of root elongation, as mentioned previously [3, 25]. This study explained that IGF-I signaling is an important factor during later root development, including the cellular cementogenesis and disintegration of HERS.

The existence of IGF-I in the periodontal ligament and HERS, and of IGF-I receptors in HERS, have been reported using immunohistochemistry and *in-situ* hybridization, in rats and mice [7-9, 22]. It has been reported that the expression of the IGF-I receptor was constant in HERS during tooth development [7, 9, 22], and that the neutralization antibody for IGF-I receptor down-regulated HERS elongation in early root formation stages [7]. The cementum-derived growth factor has been characterized as IGF-I-like molecules [11, 13]; moreover, the IGF in cementum has been shown to play a role in tissue homeostasis and in the attachment of the cementum to the periodontal ligaments [26]. IGF-I acts as a paracrine/autocrine factor during tooth development [9]. IGF-I signaling is considered as one of the regulating factors of root formation in molars. Root development has been reported to proceed simultaneously with that of the periodontal tissues that consisted of cementum, the periodontal ligament, and alveolar bone [25]. In the current study, we used an organ culture system that mimicked the conditions of the actual root development *in vivo*. The distinguishing effects of IGF-I were observed in cellular cementogenesis. The concentration of IGF-I in the organ culture was referred to in previous papers [7, 27]. The most effective concentration of IGF-I was 100 ng/ml, and IGF-I was found to accelerate matrix formation in the cellular cementum. The cementum matrix accumulated at the apical one-third of the root. The amount of newly formed calcified matrix on the root dentin was higher in both range and thickness in the IGF-I group compared to the controls. The morphology of the cementoblast-like cells on the newly formed cementum matrix, that was observed by electron microscopy, was similar to that observed in previous *in vivo* studies [3]. The specific markers and differentiation mechanisms of cementoblasts have not yet been uncovered [28]. We could not observe the changes in the number of cementoblasts between the control and IGF-I groups in this study.

Next, we paid attention to the changes in HERS cell growth in the IGF-I group, because we had previously reported that exogenous IGF-I accelerated the elongation of the HERS by inducting cell proliferation in the outer layer of the enamel epithelium during the early stages of root formation. At this time, the mitotic indices of the inner and outer layers were 5.09 and 5.49 in the control group [7]. However, contrary to our assumptions, the length of the HERS in the IGF-I group was lower than that in the control group (Fig. 4a, b). The mitotic index of HERS cells in the IGF-I group was also lower than that in the control group (Fig. 2). The fragmentation started at about 10-postnatal-day, then, HERS was shortened by 21-postnatal-day [29]. HERS shows several differentiated stages during root

development [30]. We considered that the effects of IGF-I were different between the early and late stages, and that IGF-I induces the decline of the HERS length by decreases of the mitosis in the later stage.

Furthermore, alterations in cell arrangement were noted in the IGF-I group. These included the partial disappearance of the basement membrane, widening of intercellular gaps, broadening of the cytoplasmic area in each cell, and was an increase in the number of organelles (Figs. 3b, c). The changes were noticeable in cells located close to the area of disintegration of the HERS (Fig. 3b and Fig. 4b). The changes of morphology indicate cell events corresponding to pre-migration [31]. It was considered that IGF-I accelerated the disintegration of the HERS before migration, and the process of a shift from root elongation to cementogenesis. It was well known that ERM was derived from disintegrated HERS, and that the mesenchymal cells that penetrated the fragmented HERS differentiate into cementoblasts [31]. In this study, ERM-like cell masses existed in both groups, but the number of masses in IGF-I group were many. Although the mechanisms of ERM formation are not really known so far, it was reported

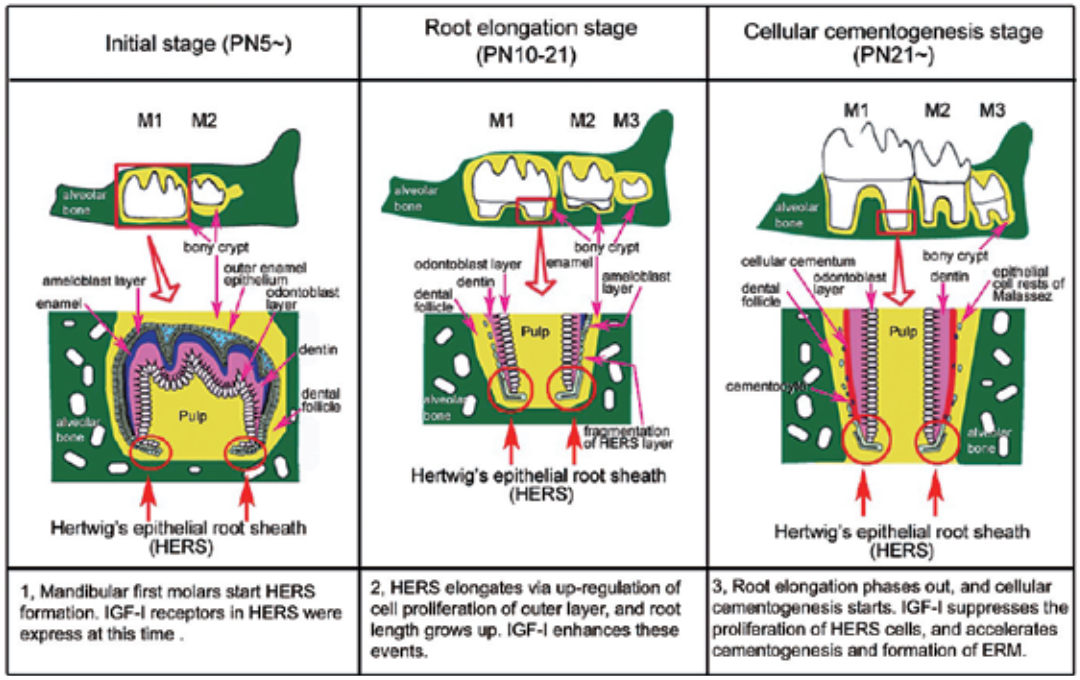

Fig.6 : Summary of root formation and the effects of IGF-I on the mandibular molars in mice. This is based on this and previous studies [7, 24, 29, 30]. The period of root formation is divided to three stages as follows; (1) initial stage, (2) root elongation stage, and (3) cellular cementogenesis stage. (1) Mandibular first molars start HERS formation at postnatal (PN) 5-day (PN5), and IGF-I receptors in HERS were expressed at that time. (2) The HERS elongates via the up-regulation of cell proliferation of the outer layer, and root length grows up. IGF-I enhances these events. A portion of HERS disintegrates at the cervical side during PN10-21. (3) The root elongation phases out until PN 21, then, the cellular cementogenesis starts at this time. IGF-I accelerates the cementogenesis. IGF-I also sup presses the proliferation of HERS cells, and stimulates the disintegration of HERS and the formation of epithelial cell rests of Malassez (ERM). M1-M3, mandibular first-third molars.

that the ERM was connected to cementoblast and showed positive reactions for a stem cell marker [23, 32]. Moreover, recent studies have advocated the new concept that HERS cells have the ability to undergo epithelial-mesenchymal transition (EMT) *in vitro* [33, 34]. The EMT cells promoted the switch in expression patterns from epithelial to mesenchymal markers, and the disappearance of the basement membrane. The EMT cells extend the cell processes into the spaces between the cells, thereafter, and move rapidly via the manner of contact inhibition of locomotion [35]. Similar phenomena in epithelial changes to mesenchymal markers were observed in the HERS cell line [34]. IGF-I has been reported as one of the factors related to EMT and regulates both cell-cell adhesion and cell migration [36, 37]. The disappearance of the basement membrane around the HERS in the IGF-I group was indicative of the early stages of EMT [38]. IGF-I might act as one of the regulators of EMT on the HERS, and be associated with ERM formation in events of the disintegration of HERS and the migration of the fragmented HERS cells into the periodontal ligament.

Taken together, it was considered that IGF-I signaling activated a transition of the developmental patterns in the HERS during the late stages of root development; that is, an accelerated changing rate from the maintenance of the length via cell proliferation to the disintegration of the HERS layer, eventually facilitating the differentiation of cementoblasts and cellular cementogenesis.

The results of the present and previous studies indicate that IGF-I is an important regulatory factor throughout the stages of root development in mouse molars [7, 9, 13], as summarized in Figure 6. Further studies exploring the gain/loss of functions *in vitro* and long-term growth under the kidney capsule *ex vivo* might aid in elucidating the mechanisms by which IGF-I regulates cementogenesis and HERS disintegration during root development.

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Conflict of interests

The authors deny any conflicts of interest related to this study.

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