Calcification in rat developing mandibular bone demonstrated by whole mount staining, micro-computed tomography and scanning electron microscopy with energy dispersive X-ray spectroscopy

Akiko HENMI¹, Hiroshi OKATA², Yasuto MIKAMI¹, and Yasuyuki SASANO¹
¹Craniofacial Development and Regeneration, ²Periodontology and Endodontontology, Tohoku University Graduate School of Dentistry
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ABSTRACT
The study was designed to investigate calcification in developing rat mandibular bone using whole mount staining, micro-computed tomography (micro-CT) and scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX). Wistar rats at embryonic days 16, 18, and 20 and postnatal weeks 1 and 6 were used. Rats were fixed with 4% paraformaldehyde and heads were resected, frozen and sectioned for histology, then analysed with SEM-EDX. Some of the specimens were observed with micro-CT. Other rats were fixed and stained with alcian blue and alizarin red for whole mount staining. Histology and whole mount staining showed that osteoid was deposited around Meckel’s cartilage at day 15 and developed into bone at day 16. Accumulation of Ca and P was identified in the bone matrix with SEM-EDX. The area of bone expanded until week 6. The Ca/P ratio increased, whereas the C/Ca and C/P ratios decreased during development. Micro-CT demonstrated an increase in radio-opacity with bone development. The results suggest that rat mandibular bone formation is initiated around Meckel’s cartilage at day 15. Deposition and maturation of the calcium phosphate mineral increase gradually with decrease in the organic component as the rat mandible develops.

Rat mandibular bone formation begins with deposition of uncalcified bone matrix in the middle of the mandibular process, lateral to Meckel’s cartilage, at embryonic day 15 (E15). The uncalcified bone matrix calcifies at E16 (2, 3, 13, 14). Various regions of the mandibular bone differentiate and expand from the ossification site with time. The mandible has been shown to be one of the first bones to calcify in pigs (6, 10) and rabbits (4). Calcification of the bone matrix in the mandible may proceed by deposition, and then by increase and maturation of calcium phosphate minerals, as reported in calvaria (5). However, little information is available about the process of mandibular bone calcification during development.

The study was designed to investigate the process of calcification in developing rat mandibular bone. We examined the developing mandible with histology and whole mount staining to demonstrate the spatiotemporal three-dimensional architecture of developing bone. The process of calcification related to mandibular bone development is shown using micro-computed tomography (micro-CT). Analysis of the concentrations and distributions of elements that constitute the bone matrix, that is, calcium (Ca), phosphorus (P) and carbon (C) is performed by scanning electron microscope equipped with an energy dispersive X-ray spectrooscope (SEM-EDX).

MATERIALS AND METHODS

Animals. Wistar rats were used for the study. Selected developmental time points for sampling were em-
bryonic days 16 (E16), 18 (E18), and 20 (E20), and postnatal weeks 1 (W1) and 6 (W6). Rats were obtained from the SLC Corporation (Kotoh, Shizuoka, Japan). All procedures were approved by the Animal Research Committee of Tohoku University.

Tissue preparation and histology. Pregnant rats were euthanized with an overdose of isoflurane by inhalation. Embryos at E16, E18 and E20 were collected and the heads were dissected and kept in 4% paraformaldehyde in 0.01 M phosphate buffer, pH 7.4, at 4°C overnight. Postnatal W1 and W6 rats were anaesthetized with sodium pentobarbital (50 mg kg⁻¹) supplemented by isoflurane inhalation and perfused through the aorta with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Mandibles of the rats were resected and kept in the same fixative at 4°C overnight. Specimens at E16, E18, E20 and W1 were frozen with O.C.T. compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan), and W6 specimens were frozen with 4% carboxymethylcellulose sodium salt (Leica Microsystems Japan, Tokyo, Japan). Some of the specimens were examined with micro-computed tomography (micro-CT). Serial 10 μm frozen coronal sections were cut until the center of the first molar germ was exposed. The sections were stained with hematoxylin-eosin (H-E) or toluidine blue.

Micro-CT. The fixed specimens were analysed by using micro-CT (Scan Xmate-E090; Comscan, Kanagawa, Japan) under standardised conditions. The CT settings were as follows: pixel matrix, 1032 × 1032 × 1012; slice thickness, 27.7 μm; magnification, ×1.8; voltage, 80 kV; electrical current, 120 μA; electrical power, 10 W (1). The samples were reconstructed by using a software program (Tri3DBon64; Ratoc, Tokyo, Japan). The threshold for standardising micro-CT images for separating bone from soft tissue was determined, as previously reported (12).

SEM-EDX. The frozen specimen of mandible, in which the middle part of the first molar had been exposed by sectioning, was freeze-dried. The exposed region of the mandible was analysed by scanning electron microscope equipped with energy dispersive X-ray spectroscopy (SEM-EDX) (JSM-6390LA, EX-2300; JEOL, Tokyo, Japan). The low-vacuum mode was used for uncoated observation. Distributions and concentrations of Ca, P and C in the mandibular bone were examined with mapping and point analyses. Seven specimens from each time point were examined. For the point analyses, six to ten points on the bone matrix were randomly selected from each of the seven specimens, and the Ca/P, C/P and C/Ca molar ratios were calculated. The point analysis was focused on the central region of the developing bone in each specimen, where Ca and P were concentrated. The analysis was performed for each specimen under the same conditions of voltage, working distance and pressurization, that is, at 15 kV voltage, 10 mm working distance and under low pressure (30 Pa).

Statistical analysis. For the point analysis, seven specimens from each time point were analysed statistically with JMP Pro 10 for Windows (SAS Institute Japan Ltd., Tokyo, Japan). The data were compared by the Kruskal-Wallis test, followed by the Steel-Dwass test. The significance level was set at 0.05.

Whole mount staining. Other rats were fixed with ethanol–acetic acid, stained with alcian blue and alizarin red and treated with 0.1% potassium hydroxide to visualize the three-dimensional architecture of developing bone and cartilage in the whole mount specimen.

RESULTS

Histology showed that osteoid was deposited at E15 near Meckel’s cartilage (Fig. 1b). The osteoid was not seen in the whole mount specimen (Fig. 1a) but bone that had developed from osteoid became visible with alizarin red around Meckel’s cartilage stained with alcian blue at E16 (Fig. 1c, d). The intensity of alizarin red staining increased while the area of bone expanded with time (Fig. 1c–h). Forming bone expanded the area and organized the compact structure from E16 to W1 through intramembranous ossification (Fig. 2). Micro-CT demonstrated development of mandibular bone and increase of the bone density from E18 to W1 (Fig. 3). The mapping analysis of the elements with SEM-EDX showed that Ca and P accumulated in the bone matrix at E16, and the area expanded through to week 6, matching the region of developing bone, whereas C became localized around bone (Fig. 4). The areas with higher concentrations of Ca and P showed a lower concentration of C. Concentrations of Ca, P and C were examined with SEM-EDX point analysis. The Ca/P ratios at E18 (P = 0.02), E20 (P = 0.02) and W6 (P = 0.02) were significantly higher than at E16. In contrast, there was no significant difference between E20 and E18 (P = 0.99), W1 and E16 (P = 0.14), W1 and E18 (P = 0.38), W1 and E20 (P = 0.08), W6 and E18 (P = 0.46), W6 and E20 (P = 0.78) or W6 and...
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Fig. 1  Whole mount specimens of rat mandibles at E15 (a), E16 (c, d), E18 (e, f) and E20 (g, h) and a specimen stained with H-E of a E15 rat mandible (b). Osteoid (★) is seen around Meckel's cartilage (M) on histology at E15 (b) but not seen in the whole mount specimen (a). Mandibular bone (★), stained with alizarin red, expands around Meckel's cartilage, stained with alcian blue, during embryonic development (c–h).
Fig. 2  Histological sections stained with H-E of a rat mandible at E16 (a, b), E18 (c, d), E20 (e, f) and W1 (g, h). Figures (b), (d), (f) and (h) are at higher magnification. Mandibular bone (*) is formed around Meckel's cartilage (M) and expands during development.
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W1 (P = 0.04) and W6 (P = 0.02) were significantly lower than at E16. The ratio was also significantly lower at W6 than at E18 (P = 0.03), whereas there was no significant difference between E20 and E18 (P = 1), W1 and E18 (P = 1), W1 and E20 (P = 0.97), W6 and E20 (P = 0.11) or between W6 and W1 (P = 0.11) (Fig. 5b). The C/Ca molar ratio decreased significantly between E16 and E18 and between E18 and W6.

The C/P ratio at W6 was significantly lower than at E16 (P = 0.02). In contrast, there was no significant difference between E18 and E16 (P = 0.06), E20 and E16 (P = 0.08), E20 and E18 (P = 1), W1 and E16 (P = 0.08), W1 and E18 (P = 0.97), W1 and E20 (P = 1), W6 and E18 (P = 0.14), W6 and E20 (P = 0.14), or W6 and W1 (P = 0.38) (Fig. 5c). The C/P molar ratio at E16 significantly decreased through W6.

The medians and the interquartile ranges (IQR) for Ca/P, C/Ca and C/P for each time point are shown in Table 1.

DISCUSSION

The present histology and whole mount staining showed that osteoid is deposited at E15 and develops into bone at E16 around Meckel’s cartilage. Accumulation of Ca and P, which was identified in the bone matrix with SEM-EDX, expanded until week 6. The Ca/P ratio increased, whereas the C/Ca and C/P ratios decreased during development. Micro-CT demonstrated an increase in radio-opacity of the bone matrix in developing mandibles. The present study, using spatiotemporal three-dimensional visualization, confirmed the previous reports based on histology (2, 3, 13, 14) that rat mandibular bone formation is initiated around Meckel’s cartilage at E15, and calcification advances gradually with development of bone, increase in bone density and deposition and maturation of calcium phosphate minerals.

Mandibular bone formation indicated by histology and accumulation of Ca and P begins at E16. Our SEM-EDX data demonstrated that the Ca/P molar ratio in mandibular bone increases significantly from E16 to E18 and shows no significant change from E18 through W6. The Ca/P molar ratio in bone was shown to increase with age, which leads to suggestions that bone mineral approaches the ideal hydroxyapatite stoichiometry (Ca/P = 1.67) at maturity, and the low Ca/P ratio found in immature bone is attributed to a precursor of hydroxapatite with low Ca/P (8). The composition of Ca and P may reach that of mature bone at E18 in rat mandible. Our pre-
ments showed that the distribution of Ca and P expands over the bone matrix of developing mandibular bone, whereas that of C becomes localized around bone. The point analysis of SEM-EDX showed that the C/Ca molar ratio significantly decreases between E16 and E18 and between E18 and W6, and the C/P molar ratio at E16 significantly decreases through W6 in mandibular bone development. Our previous studies using SEM-EDX showed that C/P and C/Ca

previous study reported that the Ca/P ratio in rat calvaria matures at E20, and the composition of Ca and P at E18 is still on the way to maturation (5). The chemical structure of the calcium phosphate mineral may reach maturation, or the amorphous-like structure of calcium phosphate in immature bone may transform into poorly crystalline hydroxyapatite, earlier in developing mandible than in calvaria.

The mapping analysis of SEM-EDX for the elements showed that the distribution of Ca and P expands over the bone matrix of developing mandibular bone, whereas that of C becomes localized around bone. The point analysis of SEM-EDX showed that the C/Ca molar ratio significantly decreases between E16 and E18 and between E18 and W6, and the C/P molar ratio at E16 significantly decreases through W6 in mandibular bone development. Our previous studies using SEM-EDX showed that C/P and C/Ca
Ca/P (a), C/Ca (b), C/P (c) molar ratios obtained from EDX point analysis of developing rat mandibular bone. *P < 0.05.

Table 1 Medians and interquartile ranges (IQR) of the Ca/P, C/Ca, C/P ratios of developing rat mandibular bone at E16, E18, E20, W1 and W6.

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<th>Ca/P Median</th>
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<th>C/Ca Median</th>
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<th>C/P Median</th>
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<td>6.51</td>
<td>4.70–8.45</td>
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<td>1.24–1.31</td>
<td>3.52</td>
<td>3.42–3.65</td>
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<td>1.29</td>
<td>1.26–1.31</td>
<td>3.55</td>
<td>2.54–3.77</td>
<td>4.57</td>
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<td>3.64</td>
<td>2.52–4.52</td>
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<tr>
<td>W6</td>
<td>1.35</td>
<td>1.25–1.38</td>
<td>2.31</td>
<td>1.96–2.93</td>
<td>2.82</td>
<td>2.58–4.18</td>
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ratios decrease during bone development and healing in calvaria (5, 12). Fourier transform infrared spectroscopy (FTIR) analysis indicated that the proportion of protein to mineral decreases during calvarial bone development (5). The concentration of C has been reported to decrease during developmental calcification of dentin and enamel (9). These results suggest that organic components such as proteins, represented by C, decrease, while calcium phosphate minerals, represented by Ca and P, increase as calcification proceeds in calcified tissues, bones and teeth. The present study demonstrated for the first time that the organic constituents decrease while mineral components increase in mandibular bone development. We previously reported gene expression of proteolytic enzymes, metalloproteinases (MMP) (15) and proteoglycan degrading enzymes, ADAMTS (a disintegrin and metalloprotease with thrombospondin type 1 motifs) (11) in osteoblasts and osteocytes in developing rat mandibles. Osteoblasts and osteocytes may degrade proteins and proteoglycans by using MMP and/or ADAMTS to play a role in remodeling extracellular matrices and advancing calcification during mandibular bone formation.

The putative uncalcified bone matrix, osteoid, was deposited around Meckel’s cartilage in mandible at E15, as mentioned above, whereas we did not identify osteoid in E15 calvaria (5). Osteoid may appear earlier in developing mandible than in calvaria. Meanwhile, we were unable to analyse osteoid with SEM-EDX in E15 mandibles since it was difficult to expose the localized osteoid region in the small specimen for SEM-EDX analysis. Von Kossa staining shows that osteoid is uncalcified (13, 14), while the concentration of Ca and P in osteoid is unknown. Our previous study using SEM-EDX reported that the putative uncalcified matrix of predentin contains elements of Ca and P during dentin development, while the concentration is lower than the putative calcified matrix of dentin (9). Ca and P concentrated in calcified nodules in osteoid (7) may be detected by SEM-EDX. Further investigation of the charac-
teristics of Ca and P in osteoid will be required to elucidate the mechanism of bone calcification in a future study.

In conclusion, we have analysed the atomic concentration of Ca, P and C in developing embryonic and postnatal mandibular bone using SEM-EDX for the first time. Low-vacuum SEM-EDX enabled us to analyse the concentration of C without interference from a conductive coating of carbon, gold or platinum. The study suggests that rat mandibular bone formation is initiated around Meckel’s cartilage at E15. Deposition and maturation of the calcium phosphate mineral increase gradually with decrease in the organic component as the mandible develops.

CONFLICTS OF INTEREST
All authors have no conflict of interest.

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