Influence of childhood type II diabetes on bone formation in the growth period

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Abstract The influence of childhood type II diabetes on bone formation in the growth period was investigated using the mouse mandible, femur, and tibia. Five-week-old mice with spontaneous type II diabetes (KK-A') and C57BL/6J mice as a control group were used. Animals were randomly divided into 3 groups, and the regions were excised after 1, 5, and 13 weeks. Regarding the bone length, growth of the femur and tibia was greater in the KK-A' than in the control group, and that of the mandible varied depending on the measurement site. On P-QCT, the trabecular and cortical bone mineral densities of the femur were higher in the KK-A' than in the control group, whereas those of the head of the mandible were higher in the control. The thickness and circumferences around the exosteum and endosteum of the cortical bone were significantly different between the KK-A' and control groups, and X-SSI, Y-SSI, and Polar-SSI of the femur and head of the mandible, were significantly different. The \( \mu \)CT findings were correlated with the p-QCT findings. Bone morphometry clarified that bone formation of long bone at the early age was more active in the KK-A' than in the C57BL/6J group, and bone resorption was promoted with aging in the head of the mandible. These findings suggested that not only the bone length but also bone quality tend to increase in the growth period in childhood type II diabetes.

Introduction

The most common cause of a life style-related disease, diabetes, is obesity, and children are not exceptions. Obese children have increased as diets diversified, similarly to adults, and accompanying development of type II diabetes is considered problematic. In obesity, adipocytes excessively secrete TNF\( \alpha \), resistin, and FFA, and these induce insulin resistance, leading to diabetes, hyperlipidemia, and hypertension, the main diseases constituting metabolic syndrome and developing arteriosclerosis\(^{1,2}\). These bad adipokines inducing insulin resistance and leptin, an adipose tissue-derived factor with a food ingestion-inhibitory action similarly to insulin, are hormones closely involved in the development of obesity. Leptin strongly inhibits food ingestion and promotes energy expenditure mainly through the hypothalamic receptors\(^3\). The development of severe obesity in the presence of leptin gene or leptin receptor gene aberration has been reported\(^4-6\). It has been shown that the blood leptin level rises compared to the body fat amount in most obese persons, and it is considered that generally, obese persons are in a state with insufficient leptin activity due to leptin resistance, in addition to insulin resistance\(^7-10\). It has been clarified that the relative risk of femoral neck fracture is high despite the bone mineral density being not decreased in adults with type II diabetes, pointing out reduced bone quality\(^11-17\), but the influence of childhood type II diabetes on bone formation has not been investigated. In this study, using healthy mice (C57BL/6J)
and a mouse type II diabetes model (KK-A\textsuperscript{y}), we investigated the influence of childhood type II diabetes on bone formation in the growth period. It may be significant to raise problems concerning pediatric obesity and diabetes, which have been increasing, by investigating the influence of diabetic condition on the bone construction mechanism in children.

**Materials and Methods**

Twenty-four 5-week-old mice with spontaneous type II diabetes (KK-A\textsuperscript{y}) and C57BL/6J mice as a control group were used (48 mice in total). Animals were acclimated for 1 week and randomly divided into 3 groups comprised of 8 animals per group. All mice were given free access to CE-2 (Clea Japan) and drinking water (tap water) throughout the maintenance period. The animals were weighed every week. The 3 groups were assigned to sampling at 1, 5, and 13 weeks (1 w, 5 w, and 13 w groups, respectively). All animals were laparotomized under anesthesia induced by intraperitoneal administration of 6.4 mg/kg somnopentyl (10-fold dilution with 10% ethanol) and euthanized by exsanguination through the aorta. The head and hind limbs were excised and fixed in 70% ethanol. After fixation, the skull was laterally separated along the epicranial midline suture from the parietal bone over the mandible, and the mandible was excised. From the hind limbs, the femoris and tibiae were excised. Attached soft tissues, such as muscle, were carefully removed.

**1. Blood chemistry**

Serum was separated from exsanguinated blood and subjected to blood chemistry. The following 3 items were tested: glucose, insulin, leptin.

**2. Standardized X-ray radiographic bone length measurement of the mandible, femur, and tibia**

The femur was fixed so as to be in contact with the medial side to an X-ray film, and soft X-ray radiography was performed using an imaging system, CSM (ESM-2, Softex, Tokyo, Japan), under the following conditions: tube voltage, 28 kVp; tube current, 6 mA; acquisition time, 60 seconds; and focus-subject distance, 60 cm. Similarly, the mandible was fixed so as to be in contact with the lingual side to an X-ray film and imaged under the same conditions. The acquired film images were scanned and input into a personal computer, and the lengths of the mandible, femur, and tibia were measured using ImageJ (Image Processing and Analysis in Java). The baseline points and measurement items of the mandible and measurement points of femoral and tibial lengths were referred to the report from Fujita and Maki, et al. (Fig. 1)\textsuperscript{18,19}.

**3. Three-dimensional bone mineral density and structure analyses by pQCT (peripheral quantitative computed tomography)**

Employing pQCT (XCT Research SA+; Stratec Medizintechnik GmbH, Pforzheim, Germany), 3-
dimensional bone mineral density and structure analyses of the femoral metaphysis (1.2 mm from the distal growth plate) and the mandibular condyle (0.5 mm from the diaphysis) were performed. The positions are trabecular and cortically rich regions, respectively.

4. Bone mineral density and structure analyses by μCT
The imaged regions were the distal end of the left femur (10% from the growth plate) and left temporomandibular joint, and images were acquired using Scan Xmate-L090 (Comscantechno Co., Ltd.) under the following conditions: voltage, 75 kV; current, 100 μA; magnification power, 10.904; resolution, 9.171 μm/pixel; and slice thickness, 9.171 μm. For analytical software, 3D-BON (Ratoc System Engineering Co., Ltd.) was used.

5. Observation of bone microstructure and tissue morphometry using histological preparations
GMA resin blocks of the mouse proximal tibia and mandible were prepared. Frontal sections of the tibia and sagittal sections of the mandible with a 3-μm thickness were prepared using a microtome (LEICA RM2255) and stained with toluidine blue, and bone morphometry was performed using a morphometry system, OsteoMeasure (OsteoMetrics, Inc., USA). Regarding the position excluding the primary trabecular bone region located at 0.2–0.8 mm from the epiphyseal plate as a baseline, the measurement range of the tibia was set to the secondary trabecular bone region over a site 1.125 mm distal from the baseline. In the mandibular condyle, the measurement range was set to the region below the joint cartilage.

Statistical analysis
Values in the text and tables are represented as the mean ± standard deviation (S.D.), and those in the figures are expressed as the mean ± standard error (S.E.). Statistical significance was determined using the unpaired Student’s t-test (for equal variance) or Welch’s correction (for unequal variance). Differences at \( P < 0.05 \) were considered statistically significant.

Results
1. Blood chemistry
Significant differences were noted in GLU, insulin,
and leptin between the KK-A^Y and C57BL/6J mice at all 6, 10, and 18 weeks of age. Insulin and leptin were actively secreted in KK-A^Y, but GLU were high, and the body weight was markedly heavier than that of C57BL/6J, suggesting high insulin and leptin resistance (Fig. 2).

2. Comparison of bone length between the type II diabetes and control groups

The bone lengths of the bilateral femoris and tibiae were greater in the KK-A^Y than in C57BL/6J, showing greater growth in KK-A^Y. In the femur and tibia, significant differences were noted at all 6, 10, and 18 weeks of age (Table 1). In the mandible, growth varied among the measurement sites. The growths of Pg-Gn, Pg-Go, and Co-Pg were greater in KK-A^Y than in C57BL/6J, and the difference in Pg-Gn was significant at all ages (Table 2). In the facial profile of KK-A^Y mice imaged in mice cephalometric images, a mandibular protrusion-like profile was noted from 6 weeks of age, showing a difference in occlusion from that in the control group (Fig. 3).

3. Three-dimensional bone mineral density and structure analyses by pQCT

The femoral bone mineral density was greater in the type II diabetes than in the control group at all ages. The bone mineral density was markedly different between the groups from 1 to 5 weeks, but the trabecular bone mass and mineral density decreased with aging, and the difference between the groups was small at 13 weeks. In the femoral metaphysis, the bone mineral density decreased from 5 to 13 weeks in both groups. Similar findings were noted in the femoral cortical bone mineral density. The cortical bone thickness and exosteal circumference were greater in the KK-A^Y than in the control group.

### Table 1 Body weight and femoral and tibial length

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>6 wks</th>
<th>10 wks</th>
<th>18 wks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C57BL/6J</td>
<td>KK-A^Y</td>
<td>C57BL/6J</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>21.1 ± 0.58</td>
<td>31.7 ± 1.02**</td>
<td>25.2 ± 1.12</td>
</tr>
<tr>
<td>Femoral length (mm)</td>
<td>1.94 ± 0.04</td>
<td>2.02 ± 0.06**</td>
<td>2.11 ± 0.05</td>
</tr>
<tr>
<td>Tibial length (mm)</td>
<td>2.41 ± 0.05</td>
<td>2.35 ± 0.04*</td>
<td>2.46 ± 0.01</td>
</tr>
</tbody>
</table>

Each point represents the mean ± S.D., n = 8 of 6, 10, 18 wks of age mice. *: P<0.05, **: P<0.01

### Table 2 Cephalometry of the mandible (mm)

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>6 wks</th>
<th>10 wks</th>
<th>18 wks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C57BL/6J</td>
<td>KK-A^Y</td>
<td>C57BL/6J</td>
</tr>
<tr>
<td>Pg-Go</td>
<td>1.2 ± 0.04</td>
<td>1.33 ± 0.05**</td>
<td>1.29 ± 0.02</td>
</tr>
<tr>
<td>Pg-Gn</td>
<td>1.1 ± 0.06</td>
<td>1.12 ± 0.06*</td>
<td>1.13 ± 0.03</td>
</tr>
<tr>
<td>M1-Bl</td>
<td>0.41 ± 0.02</td>
<td>0.42 ± 0.01</td>
<td>0.45 ± 0.01</td>
</tr>
<tr>
<td>Bi-Bl</td>
<td>2.57 ± 0.07</td>
<td>2.6 ± 0.08</td>
<td>2.7 ± 0.08</td>
</tr>
<tr>
<td>Bi-Co</td>
<td>1.06 ± 0.08</td>
<td>1.1 ± 0.06**</td>
<td>1.14 ± 0.15</td>
</tr>
<tr>
<td>Bi-Id</td>
<td>2.42 ± 0.07</td>
<td>2.58 ± 0.04**</td>
<td>2.61 ± 0.09</td>
</tr>
<tr>
<td>Co-Co'</td>
<td>0.68 ± 0.02</td>
<td>0.72 ± 0.03**</td>
<td>0.69 ± 0.02</td>
</tr>
<tr>
<td>Co-Go</td>
<td>0.59 ± 0.02</td>
<td>0.61 ± 0.05</td>
<td>0.64 ± 0.02</td>
</tr>
<tr>
<td>Co-Gn</td>
<td>0.74 ± 0.02</td>
<td>0.7 ± 0.03*</td>
<td>0.73 ± 0.03</td>
</tr>
<tr>
<td>Co-Pg</td>
<td>1.44 ± 0.04</td>
<td>1.44 ± 0.07</td>
<td>1.48 ± 0.02</td>
</tr>
<tr>
<td>Il-Bl</td>
<td>0.4 ± 0.02</td>
<td>0.4 ± 0.01</td>
<td>0.49 ± 0.02</td>
</tr>
<tr>
<td>Il-Id</td>
<td>0.58 ± 0.04</td>
<td>0.61 ± 0.03</td>
<td>0.64 ± 0.02</td>
</tr>
<tr>
<td>L1-L1'</td>
<td>0.53 ± 0.02</td>
<td>0.54 ± 0.01</td>
<td>0.54 ± 0.02</td>
</tr>
<tr>
<td>L2-L2'</td>
<td>0.53 ± 0.02</td>
<td>0.48 ± 0.01**</td>
<td>0.54 ± 0.02</td>
</tr>
</tbody>
</table>

Each point represents the mean ± S.D., n = 8 of 6, 10, 18 wks of age mice. *: P<0.05, **: P<0.01
Mandibular growth was noted from 1 week (6 weeks of age) in the early stage of the disease, and occlusal changes occurred due to the rotation and elongation in the anterior lower direction of the head of the mandible in the KK-A^Y and C57BL/6J groups.

Table 3 Measured values of the femur and mandibular condyle on pQCT

(A) Measured values of the femur on pQCT

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6J</th>
<th>C57BL/6J</th>
<th>C57BL/6J</th>
<th>C57BL/6J</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 w (6 wks)</td>
<td>5 w (10 wks)</td>
<td>13 w (18 wks)</td>
<td>13 w (18 wks)</td>
</tr>
<tr>
<td>TRAB CNT (mg/mm)</td>
<td>0.53 ± 0.02</td>
<td>0.41 ± 0.06**</td>
<td>0.45 ± 0.05</td>
<td>0.38 ± 0.16</td>
</tr>
<tr>
<td>TRAB DEN (mg/cm³)</td>
<td>248.7 ± 14.49</td>
<td>295.32 ± 22.95*</td>
<td>301.7 ± 19.49</td>
<td>301.3 ± 42.71*</td>
</tr>
<tr>
<td>CRT CNT (mg/mm)</td>
<td>0.21 ± 0.02</td>
<td>0.71 ± 0.12**</td>
<td>0.37 ± 0.06</td>
<td>0.98 ± 0.2**</td>
</tr>
<tr>
<td>CRT DEN (mg/cm³)</td>
<td>783.36 ± 8.2</td>
<td>839.8 ± 15.6**</td>
<td>875.34 ± 4.0</td>
<td>885.36 ± 16.18**</td>
</tr>
<tr>
<td>CRT A (mm²)</td>
<td>0.27 ± 0.02</td>
<td>0.84 ± 0.14**</td>
<td>0.48 ± 0.07</td>
<td>1.11 ± 0.23**</td>
</tr>
<tr>
<td>CRT THK C (mm)</td>
<td>0.04</td>
<td>0.13 ± 0.02**</td>
<td>0.07 ± 0.01</td>
<td>0.16 ± 0.04**</td>
</tr>
<tr>
<td>PERI C (mm)</td>
<td>6.96 ± 0.10</td>
<td>7.03 ± 0.40</td>
<td>6.84 ± 0.10</td>
<td>7.28 ± 0.11**</td>
</tr>
<tr>
<td>ENDO C (mm)</td>
<td>6.71 ± 0.11</td>
<td>6.24 ± 0.43</td>
<td>6.38 ± 0.15</td>
<td>6.25 ± 0.19</td>
</tr>
<tr>
<td>x-SSI (mm³)</td>
<td>0.23 ± 0.01</td>
<td>0.42 ± 0.07**</td>
<td>0.3 ± 0.02</td>
<td>0.47 ± 0.11**</td>
</tr>
<tr>
<td>y-SSI (mm³)</td>
<td>0.26 ± 0.01</td>
<td>0.48 ± 0.06**</td>
<td>0.32 ± 0.02</td>
<td>0.62 ± 0.11**</td>
</tr>
<tr>
<td>Polar SSI (mm³)</td>
<td>0.44 ± 0.02</td>
<td>0.73 ± 0.10**</td>
<td>0.54 ± 0.03</td>
<td>0.93 ± 0.15**</td>
</tr>
</tbody>
</table>

(B) Measured values of the mandibular condyle on pQCT

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6J</th>
<th>C57BL/6J</th>
<th>C57BL/6J</th>
<th>C57BL/6J</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 w (6 wks)</td>
<td>5 w (10 wks)</td>
<td>13 w (18 wks)</td>
<td>13 w (18 wks)</td>
</tr>
<tr>
<td>TRAB CNT (mg/mm)</td>
<td>0.21 ± 0.03</td>
<td>0.23 ± 0.02</td>
<td>0.21 ± 0.01</td>
<td>0.29 ± 0.04**</td>
</tr>
<tr>
<td>TRAB DEN (mg/cm³)</td>
<td>533.88 ± 20.0</td>
<td>528.36 ± 42.7</td>
<td>595.76 ± 23.36</td>
<td>579.62 ± 31.56*</td>
</tr>
<tr>
<td>CRT CNT (mg/mm)</td>
<td>0.27 ± 0.02</td>
<td>0.22 ± 0.06</td>
<td>0.36 ± 0.03</td>
<td>0.43 ± 0.08*</td>
</tr>
<tr>
<td>CRT DEN (mg/cm³)</td>
<td>855.70 ± 12.01</td>
<td>782.54 ± 16.60*</td>
<td>937.49 ± 23.63</td>
<td>882.01 ± 16.02</td>
</tr>
<tr>
<td>CRT A (mm²)</td>
<td>0.31 ± 0.03</td>
<td>0.29 ± 0.07</td>
<td>0.38 ± 0.03</td>
<td>0.49 ± 0.08*</td>
</tr>
<tr>
<td>CRT THK C (mm)</td>
<td>0.18 ± 0.02</td>
<td>0.11 ± 0.03**</td>
<td>0.26 ± 0.04</td>
<td>0.23 ± 0.05</td>
</tr>
<tr>
<td>PERI C (mm)</td>
<td>2.32 ± 0.16</td>
<td>2.94 ± 0.25**</td>
<td>2.32 ± 0.07</td>
<td>2.88 ± 0.16**</td>
</tr>
<tr>
<td>ENDO C (mm)</td>
<td>1.18 ± 0.26</td>
<td>2.24 ± 0.30**</td>
<td>0.73 ± 0.20</td>
<td>1.46 ± 0.35**</td>
</tr>
<tr>
<td>x-SSI (mm³)</td>
<td>0.02</td>
<td>0.03 ± 0.01</td>
<td>0.02</td>
<td>0.04 ± 0.01*</td>
</tr>
<tr>
<td>y-SSI (mm³)</td>
<td>0.05 ± 0.01</td>
<td>0.10 ± 0.02</td>
<td>0.05 ± 0.01</td>
<td>0.08 ± 0.01*</td>
</tr>
<tr>
<td>Polar SSI (mm³)</td>
<td>0.06 ± 0.01</td>
<td>0.07 ± 0.02</td>
<td>0.06 ± 0.01</td>
<td>0.09 ± 0.01*</td>
</tr>
</tbody>
</table>

Each point represents the mean ± S.D., n = 8 of 6, 10, 18 wks of age mice. *, **: P < 0.05, **: P < 0.01
TRAB CNT: Trabecular bone mineral content, TRAB DEN: Trabecular bone mineral density, CRT CNT: Cortical bone mineral content, CRT DEN: Cortical bone mineral density, CRT A: Cortical bone cross-sectional area, CRT THK C: Cortical bone thickness, PERI C: Cortical bone exosteal circumference, ENDO C: Cortical bone endosteal circumference
Non-invasive bone strength indices, x-SSI, y-SSI, and Polar SSI, were also significantly different between the groups at all ages (Table 3-A). In the mandibular condyle, the cortical bone mass, cross-sectional area, and mineral density were greater in the control than in the type II diabetes group at 1 week, but the cortical bone mass and cross-sectional area in the type II diabetes group exceeded those in the control group at 5 and 13 weeks. The cortical bone mineral density was higher in the control than in the type II diabetes group at all ages, and it increased with aging in the control group, but the increase was small in the group which developed diabetes, and it rather slightly decreased from 5 to 13 weeks.

4. Bone mineral density and structure analyses by μCT

1) Bone mineral density
The relationship between the groups was similar to that observed on pQCT. In the femur, the trabecular bone mass and mineral density were greater in the type II diabetes than in the control group at all ages. In the head of the mandible, the trabecular bone mass increased with aging in both groups. The bone mineral density was greater in the control than in the type II diabetes group at all ages, and it increased with aging in the control group, but the increase was small in the group which developed diabetes, and it rather slightly decreased from 5 to 13 weeks.

2) Bone structure analysis
In the femur, the bone mineral density and trabecular thickness were significantly greater in the KK-A^Y than in the control group at all ages, and were significantly higher at 13 weeks (Table 3-B).
than in the C57BL/6J group at 1 and 5 weeks, but the differences slowly decreased from 5 to 13 weeks. Inversely, the trabecular separation was significantly larger in the C57BL/6J than in the KK-A^y group at 1 and 5 weeks, but the differences between the groups slowly decreased. In the mandibular condyle, no significant differences were noted in the bone mineral density or trabecular thickness between the 2 groups at 1 and 5 weeks, but greater in the C57BL/6J than in the KK-A^y group at 13 weeks. Inversely, the trabecular separation was significantly larger in the KK-A^y than in the C57BL/6J group at 5 and 13 weeks (Table 4).

5. Observation of bone microstructure and tissue
morphometry in histological preparation

1) Long bone (proximal tibia)
The trabecular thickness and trabecular separation increased while the trabecular number decreased with aging from week 1 to 13 in the type II diabetes group (KK-A^y). The BV/TV did not change from week 1 or 5, and slightly decreased at week 13. In the C57BL/6J control group, the BV/TV, trabecular thickness, and trabecular number slightly increased and trabecular separation slightly decreased from 1 to 5 w, and inversely, the former decreased and latter increased at 13 w. At all time-points, the BV/TV, trabecular thickness, and trabecular number were greater and trabecular separation was smaller in the KK-A^y than in the C57BL/6J control group (Fig. 5-a).

In the 1 w KK-A^y group, many resorption parameters: Oc.N/B.Pm and ES/BS, and the formation parameters: Ob.S/BS and OS/BS were observed. In the 13 w KK-A^y group, resorption parameters: Oc.N/B.Pm and ES/BS were present in a number similar to that at 5 w. As a result, in the 1 w and 13 w KK-A^y group, resorption parameters and the formation parameters were increased compared C57BL/6J mice (Fig. 6-a).

2) Mandibular condyle
In the type II diabetes group, the bone volume/total tissue volume (BV/TV) and trabecular thickness increased from 1 to 5 weeks, and then the BV/TV, trabecular thickness, and trabecular number decreased and trabecular separation increased at 13 weeks. In the control group, the BV/TV and trabecular thickness increased over the period from 1 to 13 weeks. At all time-points, the BV/TV was smaller in the KK-A^y than in the C57BL/6J control group (Fig. 5-b). In the 1 w C57BL/6J group, there were many formation parameters: Ob.S/BS and OS/BS was also active compared KK-A^y group. In the 13 w KK-A^y group, the resorption parameters: Oc.N/B.Pm and ES/BS, increased compared to those at 5 weeks. In the 13 w C57BL/6J group, bone was

### Table 4 Structural parameters of the femur and condyle using micro-CT

<table>
<thead>
<tr>
<th></th>
<th>6 wks</th>
<th>10 wks</th>
<th>18 wks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C57BL/6J</td>
<td>KK-A^y</td>
<td>C57BL/6J</td>
</tr>
<tr>
<td>Bone surface/bone volume (1/mm)</td>
<td>69.8 ± 2.6</td>
<td>58.1 ± 1.6**</td>
<td>76.6 ± 2.4</td>
</tr>
<tr>
<td>Bone volume/tissue volume (%)</td>
<td>13.4 ± 1.6</td>
<td>27.5 ± 2.8**</td>
<td>9.0 ± 0.5</td>
</tr>
<tr>
<td>Trabecular thickness (µm)</td>
<td>28.7 ± 1.0</td>
<td>34.5 ± 0.9**</td>
<td>26.1 ± 0.9</td>
</tr>
<tr>
<td>Trabecular number (1/mm)</td>
<td>4.7 ± 0.4</td>
<td>8.0 ± 0.6**</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>Trabecular separation (µm)</td>
<td>186.6 ± 19.6</td>
<td>91.7 ± 10.3**</td>
<td>266.5 ± 18.6</td>
</tr>
</tbody>
</table>

Each point represents the mean ± S.D., n = 8 of 6, 10, 18 wks of age mice. *: P<0.05, **: P<0.01
In the 1 w KK-Ay group, many trabeculae were present like islands, and round osteoblasts (red arrowhead) assumed to be activated and actively produce matrix and well-developed osteoid (red arrow) were observed. Osteoclasts (black arrowhead) and resorbed surfaces (black arrow) formed by their activity were also noted. In the 5 w, osteoclasts (black arrowhead) decreased, and osteoblasts (red arrowhead) were only occasionally present, although osteoid (red arrow) was noted, compared to those at week 1. In the 13 w, many thick trabeculae were observed. Osteoclasts (black arrowhead) were present in a number similar to that at 5 w. Osteoid (red arrow) and flat osteoblasts (red arrowhead) were also present.

In the 1 w control group (C57BL/6J), osteoid (red arrow) and activated osteoblasts (red arrowhead) were noted, and osteoclasts (black arrowhead) and resorbed surfaces (black arrow) were also observed. In the 5 w, osteoid (red arrow) development slightly progressed from that at 1 w, and activated osteoblasts (red arrowhead) were present. Osteoclasts (black arrowhead) decreased, and shallow resorbed surfaces (black arrow) were noted. In the 13 w, the bone mass decreased, and osteoclasts (black arrowhead) and resorbed surfaces (black arrow) increased compared to those at 5 w. Osteoid (red arrow) was thin, and many osteoblasts (red arrow) were flat.

Fig. 5-a  Histology (proximal tibia)

In the 1 w KK-Ay group, many trabeculae were present like islands, and round osteoblasts (red arrowhead) assumed to be activated and actively produce matrix and well-developed osteoid (red arrow) were observed. Osteoclasts (black arrowhead) and resorbed surfaces (black arrow) formed by their activity were also noted. In the 5 w, osteoclasts (black arrowhead) decreased, and osteoblasts (red arrowhead) were only occasionally present, although osteoid (red arrow) was noted, compared to those at week 1. In the 13 w, many thick trabeculae were observed. Osteoclasts (black arrowhead) were present in a number similar to that at 5 w. Osteoid (red arrow) and flat osteoblasts (red arrowhead) were also present.

In the 1 w control group (C57BL/6J), osteoid (red arrow) and activated osteoblasts (red arrowhead) were noted, and osteoclasts (black arrowhead) and resorbed surfaces (black arrow) were also observed. In the 5 w, osteoid (red arrow) development slightly progressed from that at 1 w, and activated osteoblasts (red arrowhead) were present. Osteoclasts (black arrowhead) decreased, and shallow resorbed surfaces (black arrow) were noted. In the 13 w, the bone mass decreased, and osteoclasts (black arrowhead) and resorbed surfaces (black arrow) increased compared to those at 5 w. Osteoid (red arrow) was thin, and many osteoblasts (red arrow) were flat.
Fig. 5-b Histology (mandibular condyle)

1 w KK-AY group, many activated round osteoblasts (red arrowhead) were present, and osteoid formation was active (red arrow). Many osteoclasts (black arrowhead) and resorbed surfaces (black arrow) were also noted, suggesting active metabolism. In the 5 w, osteoblasts (red arrowhead) were observed similarly to those at 1 week, but osteoid (red arrow) slightly decreased. Osteoclasts (black arrowhead) and resorbed surfaces (black arrow), also decreased. In the 13 w KK-AY group, osteoclasts (black arrowhead) and resorbed surfaces (black arrow), slightly increased compared to those at 5 weeks. Osteoid (red arrow) was also observed, but many flat osteoblasts (red arrowhead) were present, suggesting a low-level matrix production.

In the 1 w C57BL/6J group, there were many activated round osteoblasts (red arrowhead), and osteoid formation (red arrow) was also active. Many osteoclasts (black arrowhead) and resorbed surfaces (black arrow) were also noted, showing that the metabolic level and bone mass were similar to those in the age-matched KK-AY group. In the 5 w, osteoclasts (black arrowhead) and resorbed surfaces (black arrow), decreased compared to those at 1 week, which may have led to an increase in the bone mass. Osteoblasts (red arrowhead) and osteoid (red arrow) were also decreased compared to those at 1 week. In the 13 w, bone was markedly dense. Compared to those at 5 w, osteoclasts (black arrowhead) and resorbed surfaces (black arrow), and osteoblasts (red arrowhead) and osteoid (red arrow), were decreased.
markedly dense. Compared to those at 5 weeks, the resorption parameters: Oc.N/B.Pm and ES/BS, and the formation parameters: Ob.S/BS and OS/BS were decreased (Fig. 6-b).

**Discussion**

On blood chemistry, the blood glucose, insulin, and leptin levels were higher in the type II diabetes group at all ages. The body weight also significantly higher in the type II diabetes than in the control group at all ages, showing that the blood leptin and insulin levels were strongly correlated with the severity of obesity. The bone mineral density of the long bones increased with aging in the type II diabetes group, showing a positive correlation...
between the severity of obesity and bone mineral density. These findings suggested the presence of insulin and leptin resistance.

The tibial and femoral bone lengths became significantly different between the groups with aging. Increases in the blood insulin and leptin levels may have promoted differentiation and proliferation of osteoblasts, as described above. Particularly, leptin has been suggested to promote vascularization in enchondral ossification of long bone as its peripheral action by enhancing MMP-2 expression and activation in endothelial cells. In contrast, in the mandible, the growth amount varied among the measurement sites, suggesting elongation in the longitudinal direction due to growth of the condyle.

On p-QCT, the cortical bone mineral density and
non-invasive bone strength indices (x-SSI, y-SSI, and Polar SSI) of the femur were higher in the type II diabetes group (Table 3). In the head of the mandible, the cortical bone density was lower than that in the control group at all ages, but the non-invasive bone strength indices were higher than those in the control group. This discrepancy may have been due to the significant increases in the morphological parameters in the head of the mandible in the type II diabetes group at all ages, compared to those in the long bones. The bone mineral density was lower but the cross-sectional area and bone mass were greater in the type II diabetes than in the control group, which may have resulted in an increase in the bone strength.

On μCT, the trabecular bone mineral content and density and cross-sectional area were similar to those on p-QCT. As noted on p-QCT, the cross-sectional area increased due to local growth in the mandibular condyle in the type II diabetes group.

The histological measurement values were also correlated with the μCT and structural analysis findings. In the long bones, the bone mineral density was higher in the type II diabetes than in the control group at all ages, and one cause may have been active bone metabolism (increases in the numbers of osteoblasts, osteoid surfaces, osteoclasts, resorbed surfaces, and bone formation rate) from the early age (6 weeks of age). In contrast, bone metabolism in the mandible was similar at the early age between the type II diabetes and control groups, but it was promoted as the growth of the mandibular condyle was promoted in the type II diabetes group, resulting in the difference in the bone mineral density between the groups.

Uchikanbori et al. performed morphometry of the femur and mandible in ob/ob mice, in which leptin production is insufficient during the growth period (6–12 weeks of age) due to leptin gene mutation, and observed that the femoral length was smaller in ob/ob mice at all ages, and the anteroposterior length (longitudinal direction) of the mandible was also smaller than that in the control group from 9 to 12 weeks of age. Garris et al. measured femoral and tibial lengths at 8 and 16 weeks of age in ob/ob mice, and db/db mice with leptin receptor gene aberration, and dy/dy mice, a mouse muscular dystrophy model, and similarly observed that the lengths were smaller than those in the control group at all age.

KK-A° mice developed severe obesity and hyperglycemia on blood chemistry, compared to those in the control group, resulting in hyperinsulinemia from the early phase. Excess leptin secretion in parallel with obesity was also observed. These are characteristic symptoms of type II diabetes in children, showing that these mice are appropriate to investigate the influence of type II diabetes in the growth period.

It has recently been clarified that leptin is also secreted by chondrocytes and osteoblasts and involved in bone formation. Kume et al. clarified that leptin promotes skeletal growth in enchondral ossification by activating vascularization. Maor et al. also detected a high blood leptin level in obese immature mice with enhanced growth and showed the presence of apparent binding sites for leptin on chondrocytes in the growth center.

The femoral and tibial lengths were significantly greater in the severely obese KK-A° group with a higher blood leptin level than in the control group. In the mandible, the growth in the longitudinal direction (anterior lower direction) was significantly greater. These findings indicate that, limiting to the growth period, type II diabetes-associated obesity promotes bone growth.

Leptin has recently been shown to promote growth of the early ossification center from the embryonic period. Detailed studies of the relationship between leptin and bone growth are expected.

Regarding the differences in growth between the long bones and temporomandibular joint region, differences between primary and secondary cartilages are considered. Cartilage of the mandibular condyle is embryologically classified as secondary cartilage, which develops later than primary cartilage, such as cartilage primordium of the four limbs and Meckel’s cartilage. Chondrocytes of secondary cartilage are not directly differentiated from undifferentiated mesenchymal cells, but derived from periosteum of existing bone. Growth of secondary cartilage has been shown to be strongly influenced by insulin-like growth factor (IGF-1), compared to primary cartilage. The structure and function of IGF-1 are similar to those of insulin, as the name indicates. It promotes cell proliferation through binding to IGF-1R, and exhibits blood glucose-lowering and bone growth-promoting actions in the body. The receptors are also similar with regard to the structure and properties. Ligands of these receptors combine and form heterotetramers, and stimulate local growth and metabolic regulation. It has also recently been
clarified that the blood IGF-1 level rises when insulin is deficient and blood glucose cannot be controlled, such as the condition in type I diabetes, or when hyperglycemia and body fat gain characteristic to type II diabetes occur\textsuperscript{39–42}. In mice with type II diabetes, hyperglycemia, hyperlipidemia, and insulin resistance-induced excess insulin secretion are present, for which IGF-1/insulin heterotetramers may promote growth and lower the blood glucose level.

Kojima et al.\textsuperscript{43} clarified that, in acromegaly which develops due to excess IGF-1 secretion induced by growth hormone secreted by tumors, promotion of enchondral ossification enhances bone formation and causes overgrowth of the mandible in the longitudinal direction (anteroposterior direction)\textsuperscript{44}). They raised mandibular protrusion as a characteristic of type II diabetes patients, and subsequent malocclusion is problematic. In the facial profile of KK-A\(\text{y}\) mice imaged in our study, a mandibular protrusion-like profile was noted from 6 weeks of age, showing a difference in occlusion from that in the control group. Leptin-insulin and leptin-IGF-1 feedback mechanisms have recently been suggested\textsuperscript{45–51}). The production and function of insulin and IGF-1 are promoted when they are stimulated by a high level of leptin, and, they are considered to inversely inhibit the action and level of leptin when they sense the presence of excess leptin. Considering that the elevation of the blood insulin and leptin levels and bone growth are intermittent, not persistent, these interactions may have been involved.

This study demonstrated that excess secretion of insulin and leptin apparently observed in type II diabetes during the immature period induced changes in bone growth in the growth period, which is very interesting. To elucidate the mechanisms of the relationships between leptin and IGF-1 and between leptin and diabetes, long-term observation from the onset of diabetes throughout the growth period may be necessary. The femoral trabecular and cortical bone mineral densities were higher in the type II diabetes than in the control group at all ages, as described above. On the other hand, those in the temporomandibular joint region (proximal head of the mandible) increased with aging in the control group, but decreased with aging after peaking at 10 weeks of age in the type II diabetes group. As noted in the \(\mu\)CT images, the difference from those in the long bones was an increase in the bone tissue volume (TV, mm\(^3\)) apparent from 6 weeks of age in the type II diabetes group, compared to that in the control group. The endosteal circumference of cortical bone was far greater in the type II diabetes than in the control group, as if it followed the TV.

The non-invasive indices of bone strength of the femur and mandibular condyle were significantly higher at all ages in the type II diabetes group, regardless of the bone mineral density. Reduced bone quality, represented by a high fracture rate despite the bone mineral density being higher than that in healthy subjects, is a characteristic of type II diabetes patients, and it has been a problem\textsuperscript{11–17}). It was shown that not only bone length but also bone quality increase when type II diabetes develops in the growth period in childhood, as observed in this experiment\textsuperscript{52–54}). However, this study investigated only in immature mice over the growth period. If the diabetic state continues, persistent hyperglycemia-associated glycosylation and oxidative stress may occur and reduce the quality of collagen, a bone quality factor, and low metabolic turnover and calcification may lead to vulnerable bone. However, the influence on bone metabolism after the growth period in patients with type II diabetes from childhood has not been studied, for which long-term experiments are necessary.

References


