Immunohistochemical and CBCT-based examination of differences between deformed and normal human condylar processes

By
Noriko KANEKO¹, Rieko ASAUMI² and Masatoshi ADACHI¹

¹Oral and Maxillofacial Surgery, The Nippon Dental University Hospital, Tokyo, Japan
²Department of Oral and Maxillofacial Radiology, School of Life Dentistry at Tokyo, The Nippon Dental University

Key Words: Immunohistochemistry, CBCT, Condylar process, VEGF, ALP

Summary: The condylar process is the most common site of mandibular fracture; however, the biomechanics of this site are not well understood. Vascular endothelial growth factor (VEGF) is a marker for vasculogenesis and the formation of bone. Alkaline phosphatase (ALP) is also a biochemical marker of bone formation. To better understand the biomechanics of the condylar process, we examined the structure of the heads and necks of human mandibles from 35 male cadavers aged 25 to 90 years (mean, 61.5 years) using cone-beam computed tomography (CBCT). In addition, we used immunohistochemistry to assess the expression of the vascular markers VEGF and CD31 as well as the bone formation marker ALP. Significant differences were observed in the thickness of the posterolateral region of the cortical bone in the head of the mandible (p < 0.05) as well as in the medial (p < 0.001) and posterior (p < 0.01) regions of the neck of the mandible. VEGF accumulated more in deformed heads than in heads with oval-shaped structures. ALP was found in numerous small cavities of cortical bone in oval-shaped heads. These differences may be related to dislocation caused by muscle tension or the occlusion of temporomandibular joint movement.

The oval-shaped region of the mandibular head is the most common site of mandible fracture and these fractures occur (with a 29.1% incidence rate) in the condyle and subcondyle regions. Mandibular fractures are most likely to occur in the neck of the mandible (16.1%) or the head of the mandible (9.1%), and these fractures are most often caused by dislocation of the head of the mandible, bilateral fracture of the condyle or molar occlusion.

The internal resistance of cortical bone with a trabecular structure is related to the cross-sectional area of the bone. The thickness of cortical bone is affected by age, tooth loss and gender. Notably, panoramic radiographs have demonstrated that bone density in the dental and molar regions of the human mandible is higher in males than in females. Therefore, the structure of cortical bone is one of the most important factors contributing to mandibular fracture. Despite these findings regarding other regions of the mandible, the thickness of the cortical bone in the head and neck of the mandible has not been determined using cone-beam computed tomography (CBCT). The detection of active osteoclastic bone resorption in cortical bone suggests the occurrence of bone matrix remodeling, which is related to bone structure. The malfunction of osteoclasts involved in bone matrix resorption has been shown to be associated with increased bone demineralization and the abnormal deposition of mineral matrices on the bone surface. These findings have been corroborated by the detection of proteins associated with revascularization and osteogenesis (e.g., VEGF and ALP) at resorption sites in bone.

Recently, VEGF was detected at the site of bone growth and was demonstrated to be an important marker of bone density maintenance. VEGF was detected in chondrocytes, and its receptor, VEGF-R, has been shown to be expressed in osteoclasts at the head of the mandible in rats. In general, angiogenesis is initiated by a remodeling of bone matrices, and the enlarged vessels in bone marrow may reflect vascular remodeling by osteoblasts. It is unknown whether vascular elements can be used as prognostic markers to predict fractures in the head and neck of the mandible. VEGF expression and the binding of VEGF to VEGF-R regulate vasculogenesis and angiogenesis. VEGF acts as a paracrine factor,
and its signals are mediated by two type III tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1), which are expressed on the surface of endothelial cells. VEGFR-2 has been shown to be the principal mediator of the mitogenic and angiogenic effects of VEGF\textsuperscript{16}. The platelet-endothelial cell adhesion molecule CD31 is also an important marker of vascular endothelial cells\textsuperscript{15}. Little is known about the structure of the head and neck of the mandible, and these regions have not been examined using CBCT images analysis or immunohistochemical analysis of vascular markers, VEGF and CD31, or bone formation markers, alkaline phosphatase (ALP).

Materials and Methods

In this study, mandibles were examined from 35 human male cadavers aged 25–90 years (mean, 61.5 years), which had been donated for human dissection. For each cadaver, bilateral mandibles were studied. We selected cadavers that had more than eight occluded teeth and had no remarkable diseases such as diabetes, rheumatism or osteoporosis. We avoided specimens with post-menopausal osteoporosis by selecting only male cadavers for this study. Samples were injected with 10% formalin with return perfusion through the femoral artery. After anatomical dissection, the mandible was removed. Four samples were selected from the right and left sides of each mandible for immunohistochemical analysis.

Classification of specimens

The head of the mandible was classified based on its shape as either oval-shaped (oval-shaped head, OSH; n = 39) or deformed (deformed head, DH; n = 31). Similarly, the neck of the mandible was classified as either having a drop-like structure (DLS; n = 34) or a deformed structure (deformed neck, DN; n = 36, Fig. 1a–e). Each mandible was then classified based on the combination of its head and neck structures, thereby resulting in the following four categories: OSH-DLS (n = 18), OSH-DN (n = 16), DH-DLS (n = 14) and DH-DN (n = 22).

Measurement points

CBCT images were used to measure the thickness of cortical bone at the point with the maximal cross-sectional diameter for the following regions of the head of the mandible: the anterior surface region (ASR), the anterolateral surface region (ALSR), the lateral surface...

Fig. 1. Measurement lines and classification types for the head and neck of mandibles using CBCT images.

- a, Horizontal line across the head of the mandible at the maximal horizontal section (h) and a similar horizontal line for the neck of the mandible (n) in the CBCT image of the anterior surface of mandible head (55 years old male).
- b, Horizontal section of an oval-shaped head of a mandible (OSH, 55 years old male).
- c, Horizontal section of a drop-like structure neck of a mandible (DLS, 55 years old male).
- d, Horizontal section of a deformed head of a mandible (DH, 90 years old male).
- e, Horizontal section of a deformed neck of a mandible (DN, 90 years old male).

(Figs. 1b, c, d, e are same magnification, bar = 1 cm)
region (LSR), the anteromedial surface region (AMSR), the medial surface region (MSR), the posteromedial surface region (PMSR), the posterior surface region (PSR) and the posterolateral surface region (PLSR). Similarly, CBCT images were used to measure the thickness of cortical bone at the point with the maximal cross-sectional diameter for the following regions of the neck of the mandible: the anterior surface region (NASR), the medial surface region (NMSR), the lateral surface region (NLSR) and the posterior surface region (NPSR). For each region measured, these points were defined as the points at the center of the section of the condylar process in the head (out of 8 portions of a divided circle, each 45 degrees) and in the neck (out of 4 portions of a divided circle, each 90 degrees) (see Fig. 2a, b).

Images of the area around the temporomandibular joint (TMJ) were taken from the temporal bone of 35 human cadavers aged 25–90 years (mean, 61.5 years) in which the mandibular plane could be placed parallel to the floor using a PSR 9000N CBCT system (Asahi Roentogen Industry, Kyoto, Japan). The parameters for cone-beam scans were as follows: tube potential of 60 kV, tube current of 4 mA and imaging of cylindrical areas of $41\times 40$ mm with high resolution (voxel size of 0.1 mm). The diameter of the petrotympanic fissure at Reid’s baseline was measured using ASAHI Vision software (Asahi Roentogen Industry, Kyoto, Japan).

**Immunohistochemical staining**

The condylar process was removed and sliced into 0.1 mm sections using diamond discs. Specimens were washed with distilled water for 24 h, incubated with 3% H$_2$O$_2$ for 20 min to eliminate endogenous peroxidase activity and digested with 0.02% proteinase K (Wako, Japan) for 1 hr at 38°C. After overnight post-fixation in 4% paraformaldehyde, the samples were washed with distilled water for 50 min, and the proteinase K digestion and overnight post-fixation steps were repeated. The samples were then washed with phosphate-buffered saline (PBS) for 30 min, sequentially incubated in 2.5%, 5% and 10% sucrose in PBS and then frozen and thawed 3 times. After overnight incubation with 2% Triton X-100 in PBS at 4°C, the samples were washed 3 times with PBS for 1 hr, and the sections were incubated for 1 hr at room temperature with normal goat serum diluted 50-fold in PBS (pH 7.2) containing 0.05% Tween 20 to prevent non-specific antibody binding. After incubation with antibodies against VEGF (diluted 1:100; Lab Vision, CA, USA), alkaline phosphatase (1:200; Biogenesis, NH, USA) and CD31 (1:100; Lab Vision) or normal goat serum as a negative control, the sections were washed 3 times with PBS for 1 hr. Following the manufacturer’s protocol, sections were incubated with HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, USA). The sections were then washed 3 times with PBS for 1 hr. The staining was visualized using 0.02% H$_2$O$_2$ and

---

**Fig. 2. Measurement points.**

Measurement points were selected at the maximal Horizontal section for the following regions in the head (Fig. 2a) and neck (Fig. 2b) of the mandible (see Fig. 1a–e).

a, Horizontal section of an oval-shaped head of a mandible (CBCT image). ASR, anterior surface region; ALSR, anterolateral surface region; LSR, lateral surface region; AMSR, anteromedial surface region; MSR, medial surface region; PMSR, posteromedial surface region; PSR, posterior surface region and PLSR, posterolateral surface region.

b, Horizontal section of a drop-like structure neck of a mandible (CBCT image). NASR, anterior surface region; NMSR, medial surface region; NLSR, lateral surface region and NPSR, posterior surface region. For each region, measurement points were defined as the point at the center of the section of the condylar process in which the head had the greatest diameter (out of 8 portions of a divided circle, each 45 degrees) and the neck had the greatest diameter (out of four portions of a divided circle, each 90 degrees) (Figs. 2a and b are same magnification, bar = 1 cm)

---
0.1% (1 mg/ml) diaminobenzidine tetrahydrochloride in 0.1 M Tris-HCl, pH 7.2. Images were acquired using a stereomicroscope (Leica MZ 16FA; Leica Microsystems, USA) with the Leica Application Suite software (Leica Microsystems). Immunoreactivity was quantified using photo analysis computer software (Win ROOF, Mitani Co., Japan).

Statistical analysis
Data were checked for normality and equal variances. Differences in the frequency of samples among groups were analyzed using one-way analysis of variance with a post hoc Tukey’s test. A p value less than 0.05 was considered statistically significant (PASW Statistics 18, SPSS, USA).

Results
Measurement of the thickness of cortical bone via CBCT images
The thickness of the cortical bone is shown in Figure 2. Of all the regions examined, the anterolateral region was the thickest. The thickness of the anterior, lateral and anteromedial surface regions differed substantially according to their shape, and statistically significant differences in the thickness of the posterolateral region were found among the groups, with the OSH-DLS group as the control (p < 0.05). The thickness of the cortical bone was also measured in the neck of the mandible. We found a statistically significant difference in the thickness of the NMSR (p < 0.001) and the NPSR (p < 0.01) between the drop-like structure and deformed neck groups. Statistically significant differences in bone thickness between the OSH-DLS and OSH-DN groups (p < 0.05) in the NMSR, the DH-DLS and OSH-DN groups (p < 0.001) in the NMSR, the DH-DLS and OSH-DN groups (p < 0.01) in the NPSR and the DH-DLS and DH-DN groups (p < 0.01) in the NPSR were also detected (Fig. 3a, b).

Immunohistochemical analysis of ALP, VEGF and CD31
Immunohistochemical analysis showed ALP-expressing cells located in the trabecular ridge, in the marrow cavity of cortical bone and in the small cavity of the compact bone in the OSHs of the mandible, in the necks of mandibles with the DLS, in the DHs of mandibles and in the DNs of mandibles. VEGF was detected in cells around small vessels in the bone marrow cavity, in cells of the cortical bone and in the small cavity of the compact bone of the condylar process. More VEGF-positive cells were found in the DH and DN groups than in the OSH and DLS groups. CD31-positive cells were also located at the ridge of the vascular wall in the marrow cavity of all examined regions and were diffusely distributed in the bone samples from the OSH and DLS groups (Figs. 4a−h and 5a−h).

Distribution of ALP-, VEGF- and CD31-positive cells
ALP-positive cells were more frequent in the ALSR and AMSR and less frequent in the PMSR, PLSR and

Fig. 3. The mean thickness of the cortical bone for all measurement points for the heads and neck of mandibles (see Fig. 1a, b).

a, Data for the head of the mandible. The thickness of the PLRS was statistically significantly different among the groups (*, p < 0.05 by ANOVA), using the OSH-DLS group as a control (p < 0.05 by post hoc Tukey’s test).
b, Data for the neck of the mandible. Statistically significant differences were seen for the thickness of the NMSR (***, p < 0.001 by ANOVA) and the NPSR (**, p < 0.01 by ANOVA). Statistically significant differences in the thickness between OSH-DLS and OSH-DN groups (p < 0.05 by post hoc Tukey’s test) in the NMSR, DH-DLS and OSH-DN groups (p < 0.01 by post hoc Tukey’s test) in the NPSR, DH-DLS and OSH-DN groups (p < 0.01 by post hoc Tukey’s test) in the NPSR and DH-DLS and DH-DN groups (p < 0.01 by post hoc Tukey’s test) in the NPSR were detected.
Fig. 4. The distribution of ALP, VEGF and CD31 immunoreactivity in horizontal sections from posterior surface region of the heads of human mandibles with either an oval-shaped head (OSH) or a deformed head by immunohistochemical staining.

a, b: ALP-expression located in the trabecular ridge, in the marrow cavity of cortical bone and in the small cavity of compact bone in the OSH of the mandible (arrowheads)

c, d-1: VEGF was detected in small vessels in the bone marrow cavity, in fibroblast cells of cortical bone and in the small cavity of compact bone (arrowheads) (d-2, large magnification of d-1, bar = 0.2 mm see square)

e, f-1: CD31-positive cells were located at the ridge of the vascular wall in the marrow cavity in the anterior surface region (ASR) at the head of the mandible (arrowheads) (f-2, large magnification of f-1, bar = 0.2 mm see square)

f, h: negative controls (Figs. 4a, b, d-1, e, f-1, g, h are same magnification, bar = 1 mm)

Abbreviations; a, c, e, g, oval-shaped head of the mandible (OSH, 57 years old male); b, d-1, 2, f-1, 2, h, deformed head of the mandible (DH, 63 years old male); g, negative control for the oval-shaped head of the mandible; h, negative control for deformed head of the mandible.
Fig. 5. The distribution of ALP, VEGF and CD31 immunoreactivity in horizontal sections from the medial surface region of the necks of human mandibles with the DLS and the DN by immunohistochemical staining at the macroscopic level.

a-1, b-1: ALP-expression was detected in the trabecular ridge, in the marrow cavity of the cortical bone and in the small cavity of the compact bone in the oval-shaped head of the mandible (arrowheads) (a-2, large magnification of a-1, b-2, large magnification of b-1, bar = 0.2 mm see squares)

c, d: VEGF was also detected around small vessels in the bone marrow cavity and in the small cavity of compact bone (arrowheads)

e, f: CD31-positive cells were located at the ridge of the vascular wall in the marrow cavity in the anterior surface region of the head of the mandible (ASR) (arrowheads)

g, h: negative controls

Abbreviations; a-1, 2, c, e, g, drop-like structure neck of the mandible (DLSN, 57 years old male); b-1, 2, d, f, h, deformed neck of the mandible (DN, 63 years old male); g, negative control for a drop-like structure neck of the mandible; h, negative control for deformed neck of the mandible

(Figs. 5a-1, b-1, c, d, e, f, g, h are same magnification, bar = 1 mm)
PSR of the DH samples than in those regions of the OSH samples. In contrast, VEGF-positive cells were more frequent in the trabecular ridge and marrow cavity of cortical bone and in the small cavity of compact bone in all eight regions (ASR, LSR, MSR, PSR, ALSR, AMSR, PMSR and PLSR) of the DH group than in those of the OSH group. However, no differences were detected in the distribution of CD31-positive cells in the small cavity or marrow cavity of cortical bone.

Different distributions of these markers were found in the NASR, NMSR, NLSR and NPSR of the neck of mandibles with the DLSN and in mandibles with deformed necks. In the NLSR and NPSR, more VEGF-positive cells were located in small marrow cavities and in the large marrow cavity of compact bone in the DN compared to the DLSN. Numerous CD31-positive cells were found in the small cavity of cortical bone in all regions of a DN. ALP-positive cells were frequently detected in all regions of a DN (Table 1).

### Discussion

Bone is a complex tissue with various functions and is resistant to deformation in response to both internal and external forces. Bone strength must increase to redistribute these forces or stresses by altering the bone’s microstructure through Haversian remodeling. Mandibular loading occurs during mastication. The duration and spacing of loading events are important, because bone cells become quickly saturated after approximately 30–36 loading cycles. The thickness of cortical bone is one of the most critical parameters for several loading configurations. Cortical bone is stronger during compression than during tension. The maximal compressive strength of trabecular bone is related to the square of its apparent density. Therefore, cortical bone thickness and density are key factors affecting the likelihood of bone fracture. In our study, the thickness of cortical bone in the posterolateral region differed significantly (p < 0.05) between mandibles with oval-shaped heads compared to those with deformed heads. The pterygoid fossae is the attachment point for the lateral pterygoid muscle and is opposite to the PSR, and, this site indirectly influences the PSR. Therefore, this site may be altered in order to protect against fracture due to the loss of teeth, mastication, TMJ movements or occlusion. This is in contrast to the thickness of the cortical bone in the neck of the mandible at the NMSR (p < 0.001) and NPSR (p < 0.01) sites. Lindahl (1977) reported that the neck of the mandible exhibited a higher fracture level than the head of the mandible. The dislocation of lateral override was more frequent than medial override without occlusion of tooth contact. However, this fracture site may be related to the thickness of cortical bone in the neck of the mandible with no regard to the loss of teeth. Accordingly, our results demonstrated that the medial region of the neck of the mandible is thicker than cortical bone, which may allow it to resist changing stress. Our results suggest that CBCT-based measurement of cortical bone thickness provides information that is useful in determin-
ing the risk of condylar process fracture.

VEGF is a key regulator of blood vessel growth and is expressed not only during embryonic development and differentiation but also during angiogenesis in the adult1,2,4,7,17,19. At the site of bone remodeling, VEGF expression was detected at the posterior region of the glenoid fossa (mandibular fossa) during functional appliance therapy20. In our study, VEGF was expressed in the trabecular ridge, in the marrow cavity of cortical bone and in the small cavity of compact bone at the posterolateral region in deformed heads of mandibles, thereby suggesting that bone repair or bone remodeling occurred by biological events such as mastication, occlusion or TMJ movement. The reciprocal regulation and function of endothelial cells and osteoblasts has been shown to be dependent upon the expression of VEGF during osteogenesis23. Animal studies have shown that VEGF-R is expressed in osteoblasts of the head of the mandible and contributes to the promotion of endothelial cell proliferation and osteoblast activation1). In our study, VEGF was detected at the resorption site of the trabecular ridge, in the marrow cavity of cortical bone and in the small cavity of compact bone. Resorption at these sites may occur during remodeling due to biomechanical events. Our CBCT analysis showed that the thickness of the cortical bone at the posterolateral region of the head of the mandible and in the medial and posterior regions of the neck of the mandible differed in deformed and normal mandibles. We detected strong ALP expression in the posterior regions (PSR, PLSR and PMSR) of normally shaped condylar processes, and mineral deposition may have occurred along with the ALP activity observed at these sites. In contrast, resorption and formation of bone matrix continuously occurred in deformed condylar processes. Excessive bite force may have contributed to the establishment of abnormal occlusion. In the rat TMJ, the lateral pterygoid muscle affects the differentiation of the mandibular condyle33). This site may be affected by muscle tension or the occlusion of TMJ movement in the posterior region of the head of the condylar process. VEGF expression also provides a source of materials for bone matrix formation by inducing the development of new vascular tissue in the deformed condylar processes. Thus, these changes in vascularization may also contribute to the cause of bone fractures in the condylar process.

Acknowledgements

We would like to thank Prof. Takashi Yosue (Department of Oral and Maxillofacial Radiology, School of Life Dentistry at Tokyo, The Nippon Dental University) and Prof. Iwao Sato (Department of Anatomy, School of Life Dentistry at Tokyo, The Nippon Dental University) for their unstinting kindness and leading to our research, and Assis. Prof. Yoko Miwa (Department of Anatomy, School of Life Dentistry at Tokyo, The Nippon Dental University) for kind help for immunohistochemical methods and making photographers.

References

Immunohistochemical and CBCT-based examination of differences between deformed and normal human condylar processes


34) van Hoof RF, Merkx CA and Stekelenburg EC. The different patterns of fractures of the facial skeleton in four European countries. Int J Oral Surg 1977; 6:3−11.


