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Histopathological changes in collagen and matrix metalloproteinase levels in articular condyle of experimental model rats with jaw deformity

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Structured Abstract

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Objective – To investigate the dynamics of the cartilage matrix in the articular condyle after removal of a side shift plate; Emergence of type I, II, and III collagen in the matrix as well as changes in levels of matrix metalloproteinase (MMP)-1, -8, and -13 that degrade collagen were studied histopathologically and immunohistochemically.

Design – Lateral displacement of the mandible was achieved by attaching a side shift plate to the anterior teeth of the maxilla in male rats at 6 weeks. The wearing period of the side shift plate was 8 weeks. Observations were made at 0, 1, 2, 4 and 8 weeks after removal.

Results – In histopathological findings, the timing of proliferation of the layer of hypertrophy varied between the bilateral sides. In immunohistochemical findings a significant decline in the expression of type II collagen in the displacement side was observed immediately after removal. Moreover, the expressions of MMPs were elevated in both sides on 0 weeks. At 1 week after removal, a significant elevated in the expression of type II collagen, MMPs was decline in both sides.

Conclusions – After removal, the levels of MMP-1, -8, and 13 were reduced and the emergence of type II collagen increased. Thus, cellular outgrowth was initiated to trigger intracartilaginous ossification to restore the cartilage matrix.

Key words: articular condyle; jaw deformity; matrix metalloproteinase 13; rat; type II collagen

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Introduction

The pathogenesis of jaw deformity has not been fully elucidated, though the causes may be multifactorial (1, 2), with malocclusion considered to be one of the contributors (3). It has been speculated that malocclusion induced by functional lateral displacement of the lower jaw during periods of growth is likely to gradually lead to skeletal displacement (4, 5). In such cases, the jaw bone can be modified to grow normally by improving the functional cause at an early stage and minimizing the deformity (6, 7).

Several studies of lateral displacement of the mandible have investigated changes of the articular condyle and mandible in experimental models, in which a side shift plate was attached to the anterior teeth to produce mandibular displacement (8, 9). However, few have reported growth changes of the jaw bone after removing the factors that cause asymmetry over an extended period. In our previous study, we investigated morphological changes of the mandible and articular condyle over a long period after inducing functional lateral displacement in Wistar male rats at 6 weeks of age and found that the mandibular length and articular condyle had a recovering tendency because of catch-up growth after plate removal (10).

Fuentes et al. (11) reported that the cartilage of the right articular condyle increased its thickness as a result of mandibular displacement to the left side in growing rats. Further, Petrovic et al. (12) found that growth suppression was manifested in the cartilage layer of the articular condyle as a result of compression stimulation to the condyle applied by use of a chin cap. On the basis of these findings, it was considered that changes in the cartilage of the articular condyle because of malocclusion might be involved in jaw deformity.

The articular condyle consists of a fibrous and cartilaginous capsule that faces the articular cavity and underlying ostein, while the cartilage of the articular condyle plays the central role in growth. The major component of that cartilage is type II collagen (13) and mechanical overload acts on the cartilage cells to produce matrix degradative enzymes. It was previously shown that various matrix metalloproteinases (MMPs) are involved in the degradation of collagen (14), while they are also considered to be involved in tissue destruction in a variety of inflammatory lesions. MMP-1 is produced by fibroblasts, synovial cells, macrophages, endothelial cells, and carcinoma cells, and degrades type I, II, III, VII, and X collagen. MMP-8 is released by neutrophils in inflammatory tissue and degrades type I, II, and III collagen. In addition, MMP-13 is observed in tumors, the cartilage of patients with osteoarthritis, the synovium of patients with rheumatism, and wounds in tissues. It degrades type I, II, III, and X collagen, and is considered to be the strongest for degrading type II collagen specifically. However, very little is known about the relationship between the recovery of the articular condyle after lateral displacement of the mandible and dynamics of the cartilage matrix of the articular condyle. Therefore, it is hypothesized that collagens and MMPs could involve with the recovery of deformed the articular condyle.

In the present study, we experimentally caused lateral displacement of the mandible by attaching a side shift plate to the anterior teeth of the maxilla in male rats at 6 weeks after birth, when endochondral ossification was active. Then, for the purpose of observing the dynamics of the cartilage matrix of the articular condyle after removing the causal factor, the emergence of type I, II, and III collagen in the matrix as well as changes in levels of MMPs that degrade collagen were studied histopathologically and immunohistochemically.

Materials and methods

Experimental animals

Forty-five male Wistar rats at 6 weeks after birth were used as experimental animals. They were randomly divided into the experimental and control groups, which each further divided into five subgroups. Each control subgroup consisted of four rats, while there were five rats in each experimental subgroup. The animals were reared in SPF clean cages at the Experimental Animal Center of Nihon University School of Dentistry at Matsudo. The softer diet (MR Powder Diet; Sankyo Labo Service Co., Tokyo, Japan), drinking water (tap water given *ad libitum*), floor mats, and cages were all sterilized before use. Both the control and experiment groups were given the softer diet. All animal experiments were performed in accordance with the Ethical Guidelines for Animal Experiments of our institution (Approval No. 05-0033).

Methods

The rats were subjected to intraperitoneal anesthesia using 0.012 ml⁄g of a mixture of Ketalar and Seractal. A side shift plate, cast using silver alloy, was attached to the upper anterior teeth for inducing mandibular displacement to the left side and then removed after 8 weeks (Fig. 1). Observation was made at 0, 1, 2, 4 and 8 weeks after removal. The left side was the displacement side and the right the non-displacement side in all of the experimental rats. The side shift plate was attached using Super Bond adhesive (San Medical Co.,

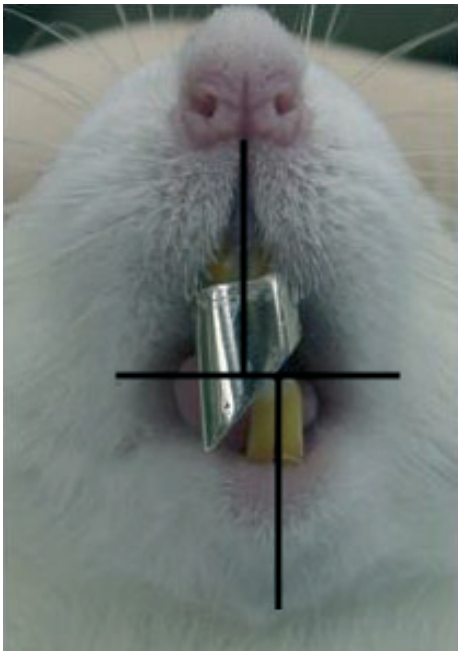

Fig. 1. Attached side shift plate in 6-week-old rat. A side shift plate, cast using silver alloy, was attached to the upper anterior teeth for inducing mandibular displacement to the left side.

Shiga, Japan) after treating all areas of the upper erupted incisor with 65% phosphate for 60 s.

Preparation of specimens

According to the experimental schedule, the animals were placed under anesthesia, then about 250 ml of normal saline solution was injected from the left ventricle for avascularization. Perfusion fixation was performed using a 10% neutral formalin solution, and the bilateral articular condyles were extracted and refixed in a 10% neutral formalin solution at 4°C for 1 week. Following fixation, the samples were decalcified in a 10% disodium ethylenediamine tetracetic acid (EDTA, pH 7.4) at room temperature for 3 weeks. The decalcified samples were rinsed under running water and embedded in paraffin to prepare a block using a standard method. Sagittal sections of the condyle samples at 4 μm thick were sliced and stained using various techniques.

For immunohistochemical staining, Histofine Simple Stain Rat MAX-PO(R) kit (Nichirei, Tokyo, Japan) was used. For the primary antibodies, anti-rabbit type I collagen polyclonal antibody (CALBIOCHEM, dilution ratio 1:100, San Diego, CA, USA), anti-rabbit type II collagen polyclonal antibody (LSL Co., dilution ratio 1:200, Cosmo Bio, LSL, Tokyo, Japan), anti-rabbit type III collagen polyclonal antibody (LSL Co., dilution ratio 1:200), anti-rabbit MMP-1 polyclonal antibody (SANTA CRUZ, dilution ratio 1:400, Santa Cruz, CA, USA), anti-rabbit MMP-8 polyclonal antibody (SANTA CRUZ, dilution ratio 1:400), and anti-rabbit MMP-13 polyclonal antibody (SANTA CRUZ, dilution ratio 1:400) antibodies were used.

After deparaffinization and rinse with PBS, each section was left to react for 15 min in 3% H_2O_2 in methanol was added, then reactions were performed with the above-mentioned primary antibodies overnight at 4°C. After the antibody reactions, the sections were subjected to reactions with the reagent Histofine Simple Stain Rat MAX-PO(R) at room temperature for 30 min. After developing by 3,3′-diaminobenzidine tetra-hydrochloride and aminoethyl carbazole solution, they were counter-stained using Mayer's hematoxylin solution (Muto Pure Chemicals CO, Tokyo, Japan).

Histopathological observations

Tissue sections from each group were stained with hematoxylin-eosin (H-E) and for immunohistochemical staining. The sections were observed under a light microscope. Histopathological observations of the articular condyle were categorized in accordance with Kameyama's classification (15), as follows. The cartilage was classified into five layers, namely a layer of fibrous connective tissue, a cartilage layer of proliferation (layer of proliferation), a cartilage layer of differentiation (layer of differentiation), a cartilage layer of hypertrophy (layer of hypertrophy), and a layer of ossification.

Determination of areas stained for collagen and MMP

Areas of hot-spot reaction in the posterior area of the upper condyle were split into three portions at the midpoint of the horizontal line drawn from the most prominent point of the articular condyle, as reported by Sato et al., (16) and measured using Image-Pro Plus software (version 5.0: Media Cybernetics, Silver Spring, MD, USA) (Fig. 2).

Statistical methods

The values are expressed as the mean ± SD. Data were analyzed for statistical differences using the Mann-Whitney's U test (Fig. 3), and a one way analysis of

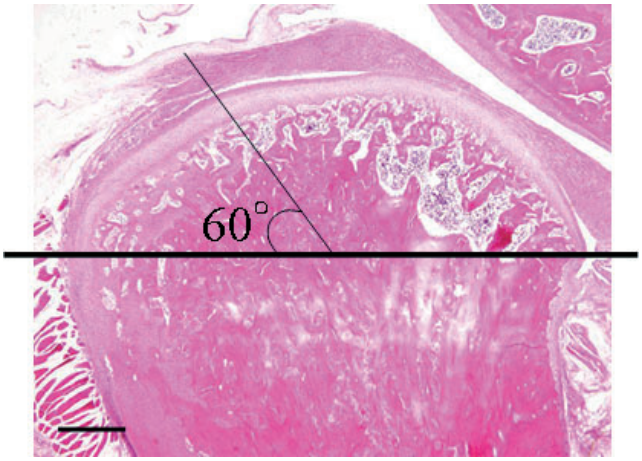

Fig. 2. Measurement area for collagen and matrix metalloproteinases. (bar: 500 μm). Deeply stained portions were measured using Image-Pro Plus software (version 5.0: Media Cybernetics) in the posterior area of the upper part of articular condyle, which was split into three portions at the midpoint of the horizontal line drawn from the most prominent part of the articular condyle.

variance (one-way ANOVA), followed by multiple comparisons with Scheffe's F test (Figs 7, 9, 11, 13, 15, and 17).

Results

Body weight

The body weight in both groups increased similarly, and significant differences were observed after attachment of the plate (the week 1–5) and removal of it (the week 9 and 10) ($p < 0.05$). However, in the end of the experiment, no significant differences were observed between the experimental and control groups (Fig. 3).

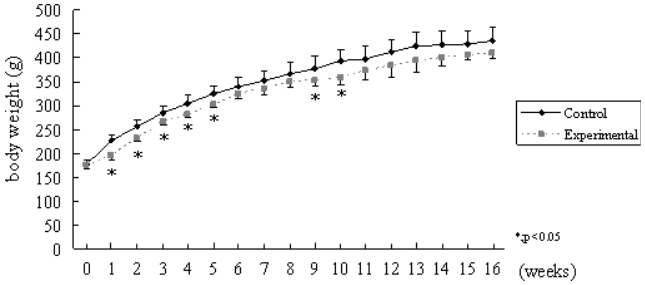

Fig. 3. Body weight. Significantly different from the corresponding control. (*$p < 0.05$, by Mann-Whitney's U test).

Morphological findings

Morphological observation of condyle

The left-ward displacement caused the resorption of the posterior region of articular condyle in displacement side (Fig. 4a, b). Then, the articular condyle has recovered at 8 weeks after removal of the plate (Fig. 4c).

Histopathological findings (H-E staining)

Control group

The superficial layer of the articular condyle was covered with thin laminated fibrous connective tissue. Beneath the fibrous connective tissue layer, a layer of proliferation, in which small oval or elliptical cellular components were densely distributed, was present. Further down, in the layer of hypertrophy, relatively large circular or elliptical cartilaginous cells were arranged in bean-like. Between the layers of proliferation and hypertrophy, the layer of differentiation was observed transitionally. Further, beneath the layer of hypertrophy, new trabecular bone arranged in a complicated mosaic structure was found (Fig. 5a–f).

Experimental group

Immediately after removal of the side shift plate on the displacement side (0 weeks), disappearance of the layer of hypertrophy and invagination of the layer of proliferation into that area was observed. At 1 week after the removal, the cartilage layer (mainly thickening of the layers of proliferation and hypertrophy) had proliferated and new trabecular bone was observed. Eight weeks after removal, findings similar to those of the control group were observed. As for the non-displacement side, at 0 and 1 week after removal of the plate, hyalinization of the layer of hypertrophy was identified. Two weeks after removal, the cartilage layer (mainly thickening

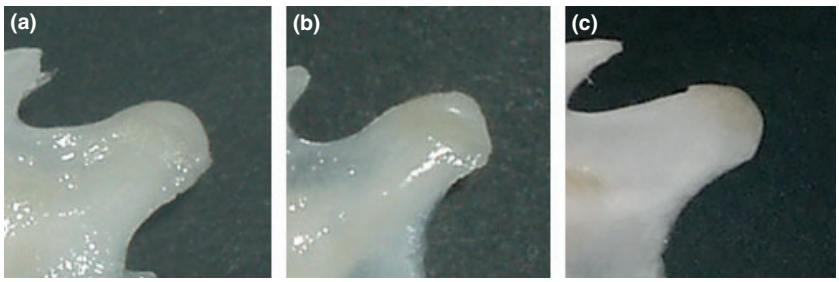

Fig. 4. Morphological observation of condyle (displacement side, left side). (a) Start. (b) 0 week after removal of the side shift plate. (c) 8 weeks after removal of the side shift plate.

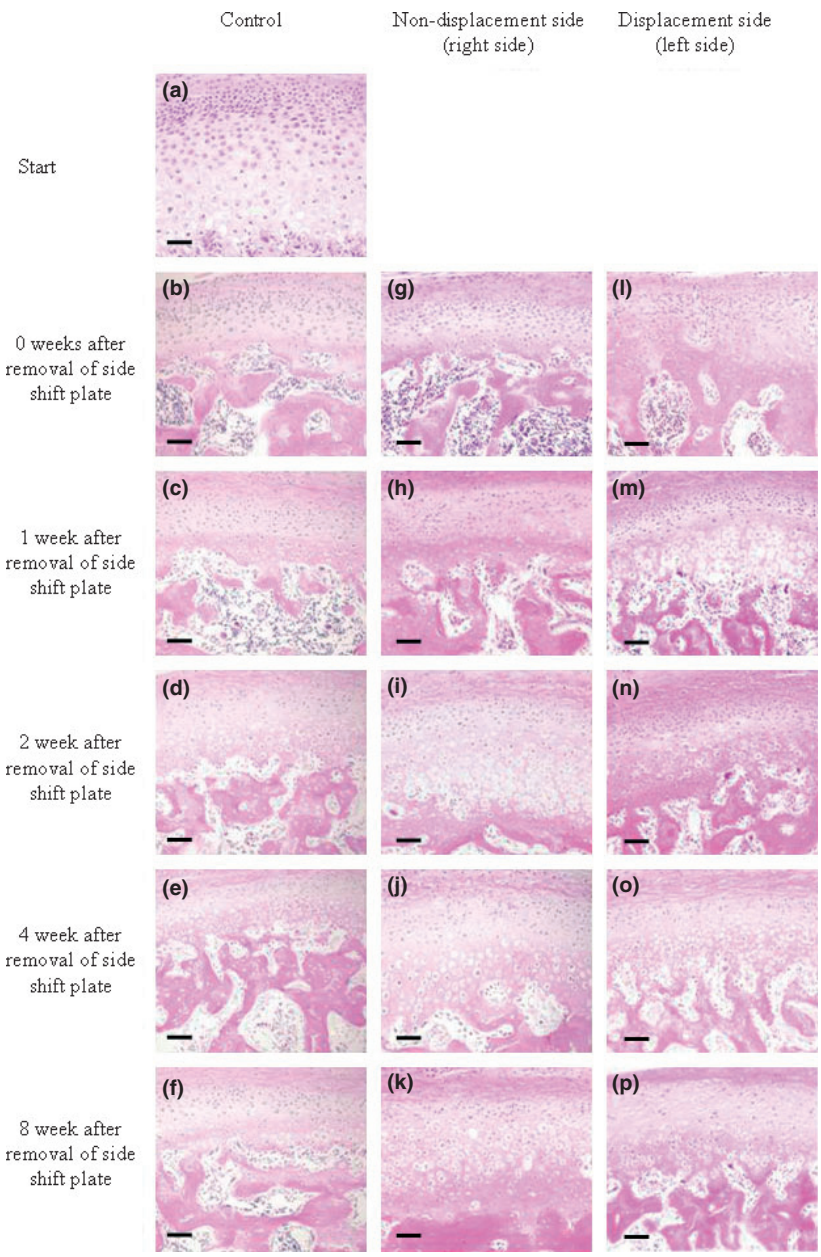

Fig. 5. Histopathological findings (hematoxylin-eosin) (bar: 50 μm). (a–f) Control. (g–k) Non-displacement side. (l–p) Displacement side.

of the layers of proliferation and hypertrophy) had proliferated. The timing of proliferation of the layer of hypertrophy varied between the bilateral sides (Fig. 5g–p).

Immunohistochemical findings

Type I collagen

Control group

The extracellular matrix of the layer of proliferation and intracellular matrix of the layer of differentiation were positively stained for type I collagen throughout the experimental period (Fig. 6a–f).

Experimental group

Immediately after removal of the side shift plate, the extracellular area matrix of the layer of proliferation and cellular area matrix of the layer of differentiation were positively stained for type I collagen in both the displacement and non-displacement sides. At 8 weeks after removal, findings similar to those of the control group were observed (Fig. 6g–p). For the areas of determination, no significant differences were noted between the experimental and control groups on 0 weeks, while the area declined thereafter. No significant differences were identified from 8 weeks in displacement side at after removal 4 weeks to the non-displacement side (Fig. 7).

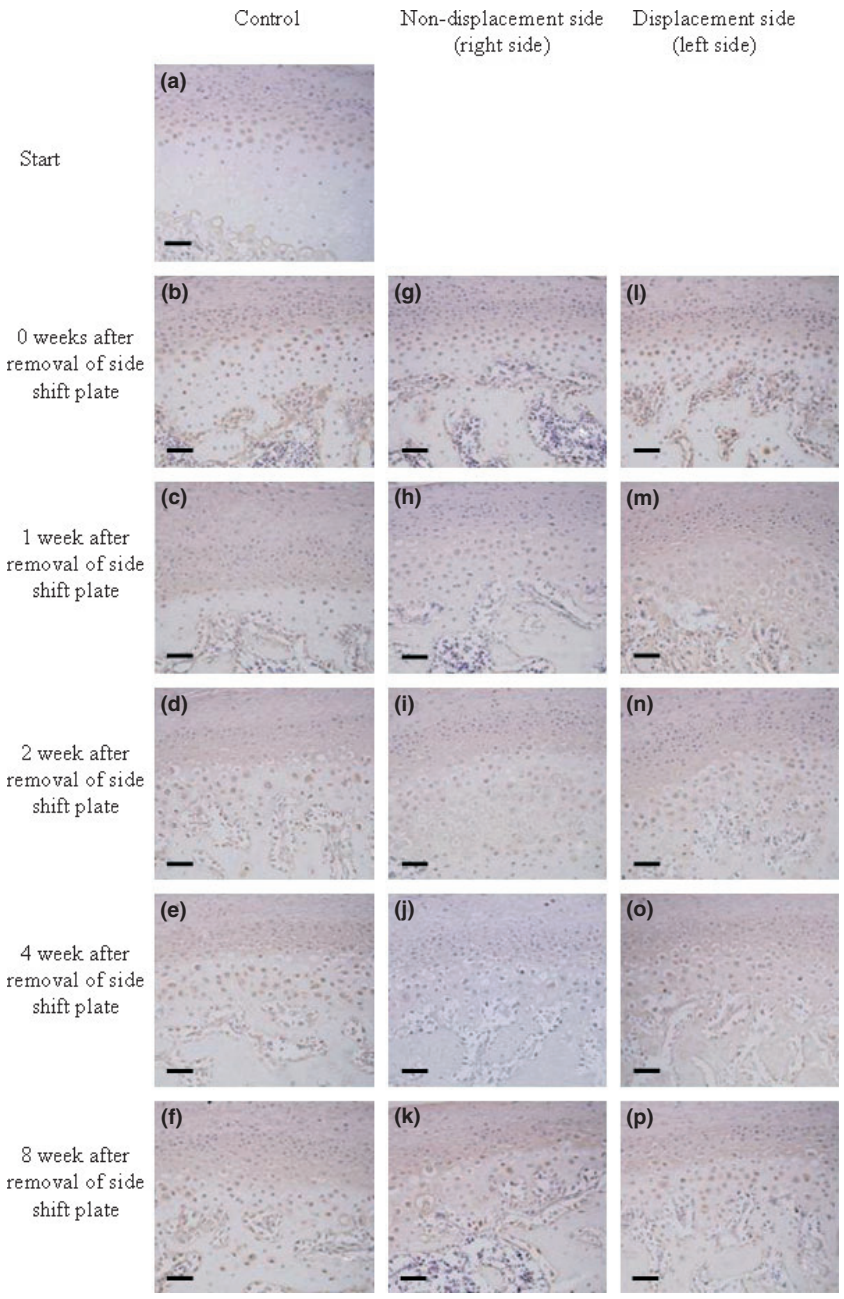

Fig. 6. Immunohistochemical findings of Type I collagen (bar: 50 μm). (a–f) Control. (g–k) Non-displacement side. (l–p) Displacement side.

Type II collagen

Control group

At the start of the experiment, positive findings for type II collagen were observed in the extracellular matrix of the layer of proliferation and cellular area matrix of the layer of differentiation. Subsequently, positive findings were identified in the extracellular matrix of the layer of proliferation and cellular area matrix of the layer of differentiation and hypertrophy throughout the experimental period (Fig. 8a–f).

Experimental group

In both the displacement and non-displacement sides, positive findings for type II collagen were identified in the extracellular matrix of the layer of proliferation and cellular area matrix of the layer of differentiation at 0 weeks after removal of the plate. At 1, 2, 4, and 8 weeks after removal, positive findings were observed in the extracellular matrix of the layer of proliferation, and cellular area matrix of the layers of differentiation and hypertrophy (Fig. 8g–p). The observed area on the displacement side was significantly smaller in

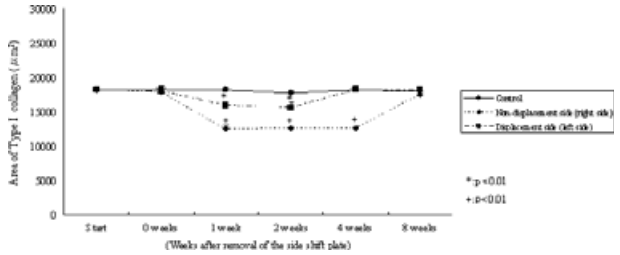

Fig. 7. Area of Type I collagen. The asterisk (+) showed significantly difference between non-displacement side and control. The asterisk (*) showed significantly difference between displacement side and control. (*$p < 0.01$, +$p < 0.01$, by one-way ANOVA).

comparison to that of the control group on 0 weeks. In the displacement sides at 1 week after removal and non-displacement sides at 2 weeks after removal, a significant increase was observed, while the area declined thereafter (Fig. 9).

Type III collagen

Control group

Positive findings were observed for type III collagen in the cellular area matrix of the layers of proliferation and differentiation throughout the experimental period (Fig. 10a–f).

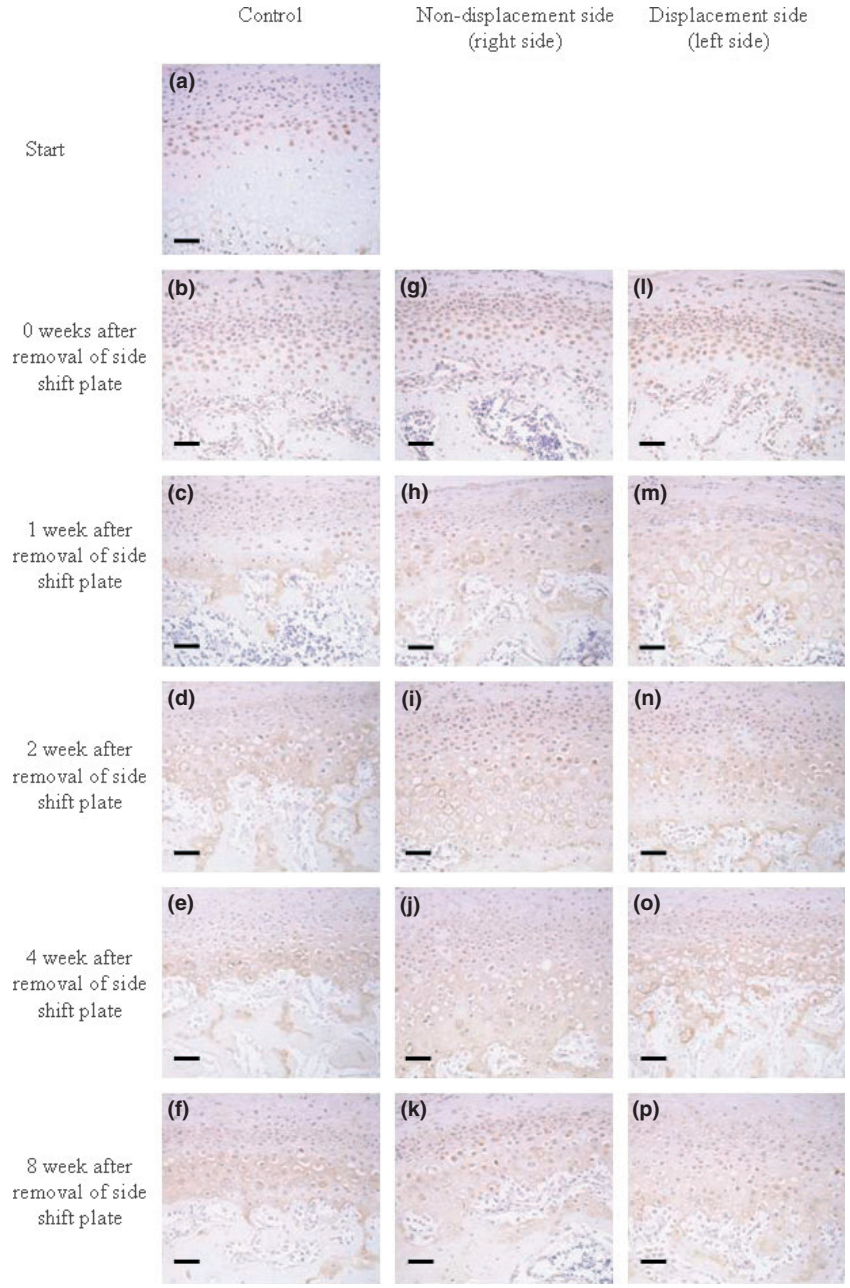

Fig. 8. Immunohistochemical findings of Type II collagen (bar: 50 μm). (a–f) Control. (g–k) Non-displacement side. (l–p) Displacement side.

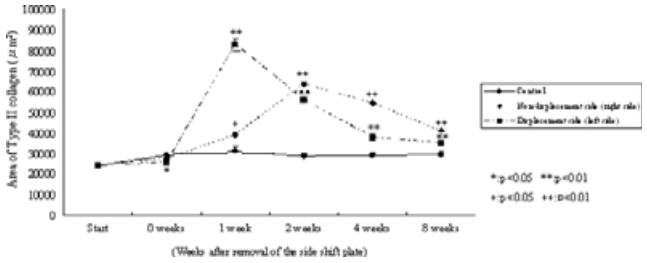

Fig. 9. Area of Type II collagen. The asterisk (+) showed significantly difference between non-displacement side and control. The asterisk (*) showed significantly difference between displacement side and control. (*$p < 0.05$, **$p < 0.01$, +$p < 0.05$, ++$p < 0.01$, by one-way ANOVA).

Experimental group

Positive findings for type III collagen were observed in the cellular area matrix of the layers of proliferation and differentiation throughout the experimental period (Fig. 10g–p). For the areas of determination, no significant differences were identified between the experimental and control groups (Fig. 11).

MMP-1

Control group

Slightly positive findings for MMP-1 were observed in the extracellular matrix of the layer of prolifer-

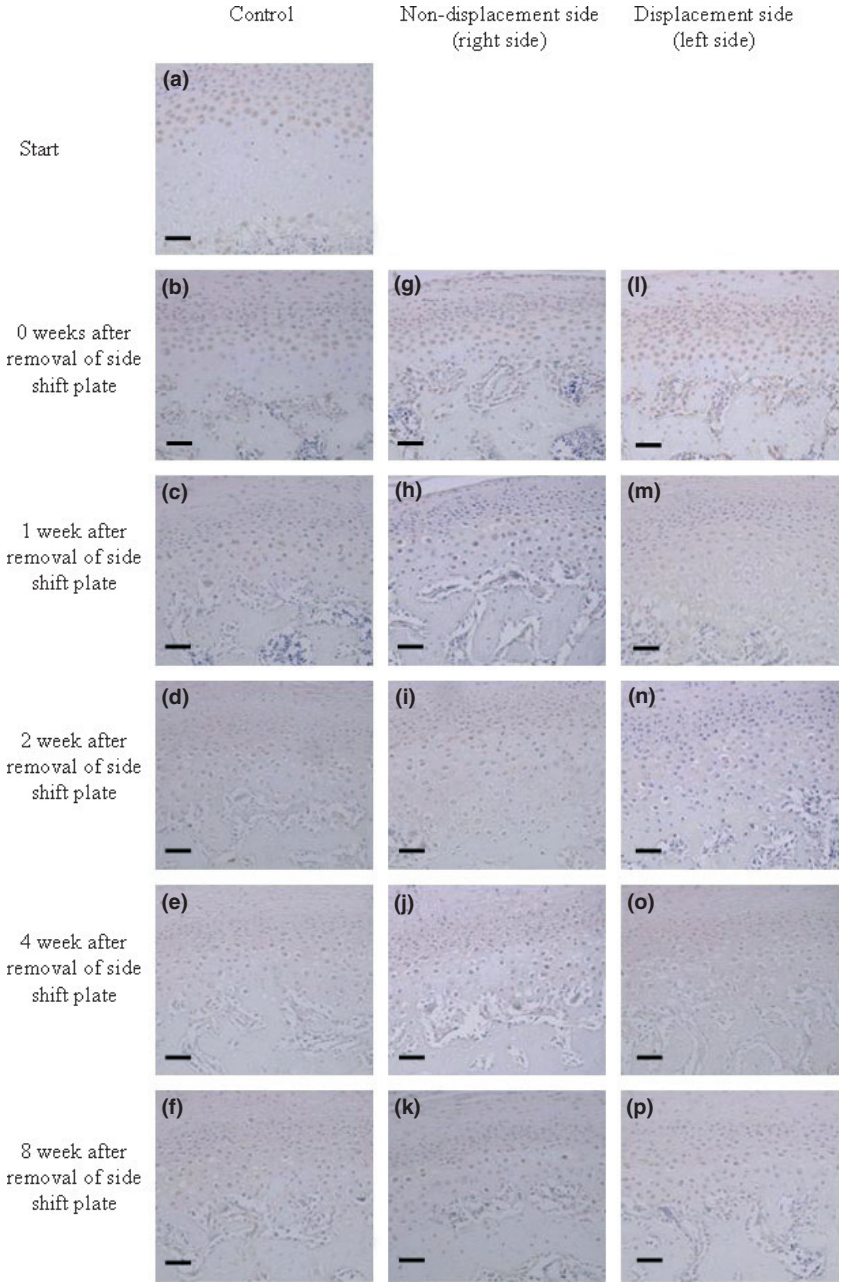

Fig. 10. Immunohistochemical findings of Type III collagen (bar: 50 μm). (a–f) Control. (g–k) Non-displacement side. (l–p) Displacement side.

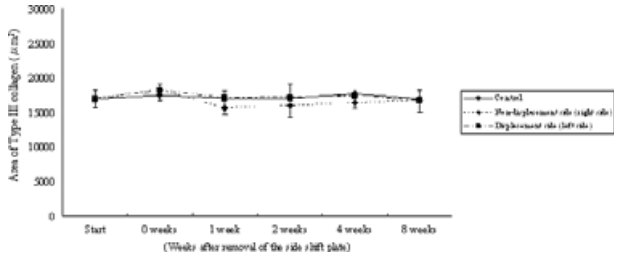

Fig. 11. Area of Type III collagen. No significant differences were observed between the experimental and control groups.

ation and cellular area matrix of the layer of differentiation throughout the experimental period (Fig. 12a–f).

Experimental group

In both the displacement and non-displacement sides, positive findings for MMP-1 were observed in the cellular area matrix of the layers of proliferation and differentiation at 0 weeks after removal of the plate. Subsequently, the levels of staining declined. At 8 weeks after removal of the plate, findings similar to those of the control group were found (Fig. 12g–p). For the areas of determination in both the displacement and non-displacement sides, a significant increase was identified as compared to the area in the control group immediately after removal of the plate, while the area declined

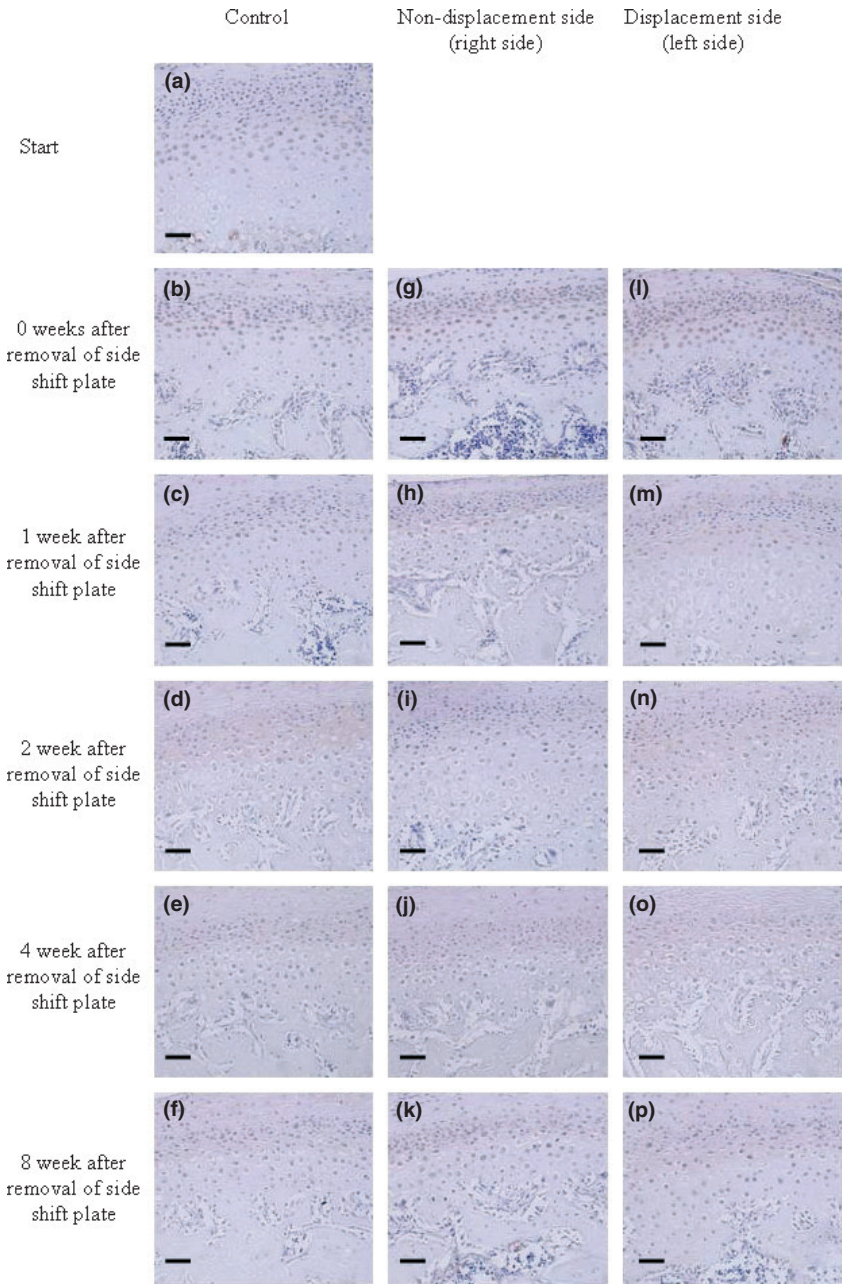

Fig. 12. Immunohistochemical findings of matrix metalloproteinase-1 (bar: 50 μm). (a–f) Control. (g–k) Non-displacement side. (l–p) Displacement side.

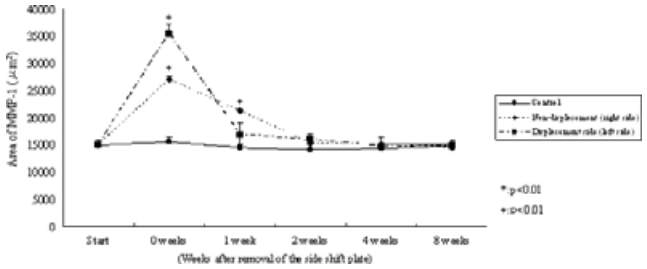

Fig. 13. Area of matrix metalloproteinase (MMP)-1. The asterisk (+) showed significantly difference between non-displacement side and control. The asterisk (*) showed significantly difference between displacement side and control. (*$p < 0.01$, +$p < 0.01$, by one-way ANOVA).

thereafter. No significant difference in the area was observed between the control and experimental groups from 2 weeks after removal in the non-displacement side and from 1 week in the displacement side (Fig. 13).

MMP-8

Control group

At the start of the experiment, positive findings for MMP-8 were observed in the extracellular matrix of the inferior region of the layer of hypertrophy. From 0 to 8 weeks after removal of the plate, slightly positive findings were found in the extracellular matrix of the layer of proliferation and cellular area matrix of the layer of differentiation (Fig. 14a–f).

Experimental group

Positive findings were observed in the extracellular matrix of the layer of proliferation and the cellular area matrix of the layer of differentiation in both the displacement and non-displacement sides immediately after removal of the plate. Subsequently, the levels of staining gradually declined. At 8 weeks after removal, findings similar to those of the control group were indicated (Fig. 14g–p). For the area of determination, a significant increase was observed in the experimental group in both the displacement and non-displacement sides as compared to the control group at 0 weeks, after which the areas declined. No significant differences were observed from 2 weeks after removal of the plate in both the displacement and non-displacement sides as compared to the control group (Fig. 15).

MMP-13

Control group

Positive findings for MMP-13 were observed in the cellular area matrix of the layer of differentiation and extracellular matrix of the layer of late hypertrophy throughout the experimental period (Fig. 16a–f).

Experimental group

Positive findings for MMP-13 were indicated in the cellular area matrix of the layer of differentiation and extracellular matrix of the layer of hypertrophy in both the displacement and non-displacement sides immediately after removal of the plate. Subsequently, positive findings were identified in the extracellular matrix of the layer of hypertrophy (Fig. 16g–p). For the area of determination, a significant increase was observed in both the displacement and non-displacement sides on 0 weeks as compared to the control group, while a decrease in the area was observed thereafter. No significant differences were observed between the groups from 2 weeks after removal of the plate in the non-displacement side and from 4 weeks in the displacement side (Fig. 17).

Discussion

The body weight in both groups increased similarly. The experimental group was decreased in the body weight compared to the control group after attachment of the plate (week 1–5) and removal of it (week 9 and 10). It was speculated that this loss of weight is based on the eating disorder by attachment of the plate and removal of it. However, in the end of the experiment, no significant differences were observed between the experimental and control groups. Therefore, we considered that the eating disorder was temporary, and the influence of the whole body on health can be disregarded (Fig. 3).

In the present experiment, we used rats as experimental animals, as they have advantages of relatively quick growth, a clearly-defined date of birth, fewer individual differences, and are readily available in large numbers and easy to rear, even though there are no similarities in the morphology of the mandible and movement of the masticatory muscle with humans.

Procedures for inducing jaw deformity associated with asymmetry of the jaw by modifying environmental factors during growth periods have been reported, such as unilateral masseter resection (17, 18), severing of the muscle-supporting nerve (19), and forcible traumatic occlusion (20). However, those procedures are largely

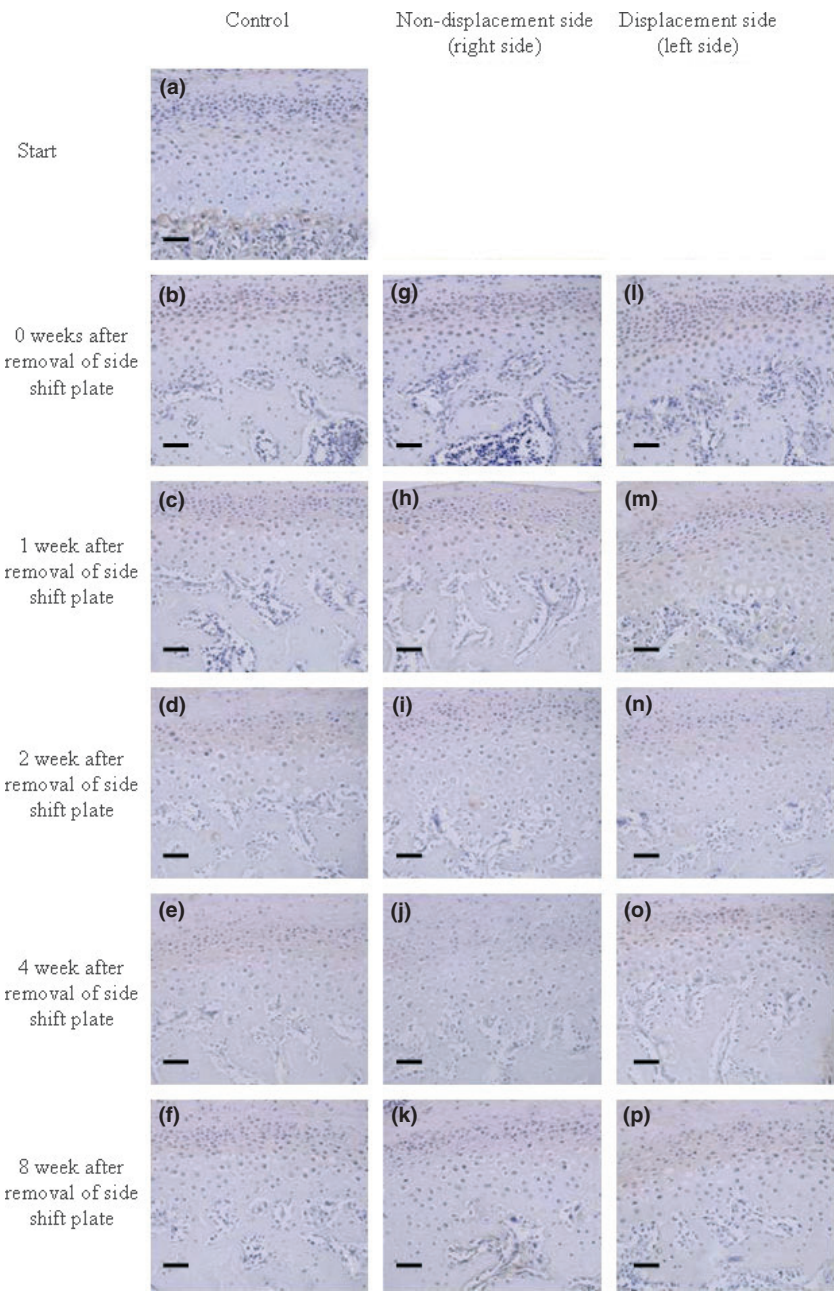

Fig. 14. Immunohistochemical findings of matrix metalloproteinase-8 (bar: 50 μm). (a–f) Control. (g–k) Non-displacement side. (l–p) Displacement side.

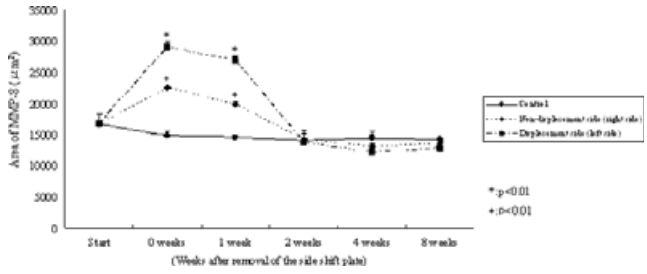

Fig. 15. Area of matrix metalloproteinase (MMP)-8. The asterisk (+) showed significantly difference between non-displacement side and control. The asterisk (*) showed significantly difference between displacement side and control. (*$p < 0.01$, +$p < 0.01$, by one-way ANOVA).

invasive and irreversible, and not suitable for investigation of morphological recovery of the jaw bone during the process of treatment for displacement. In the present study, we used a side shift plate for lateral displacement that was attached to the maxilla, as reported by Nakano (8), which is able to reproduce conditions similar to jaw displacement induced by functional factors during mandibular movement. As a result, asymmetry of the mandible and resorption of articular condyle in the present experimental group was observed 8 weeks after attaching the side shift

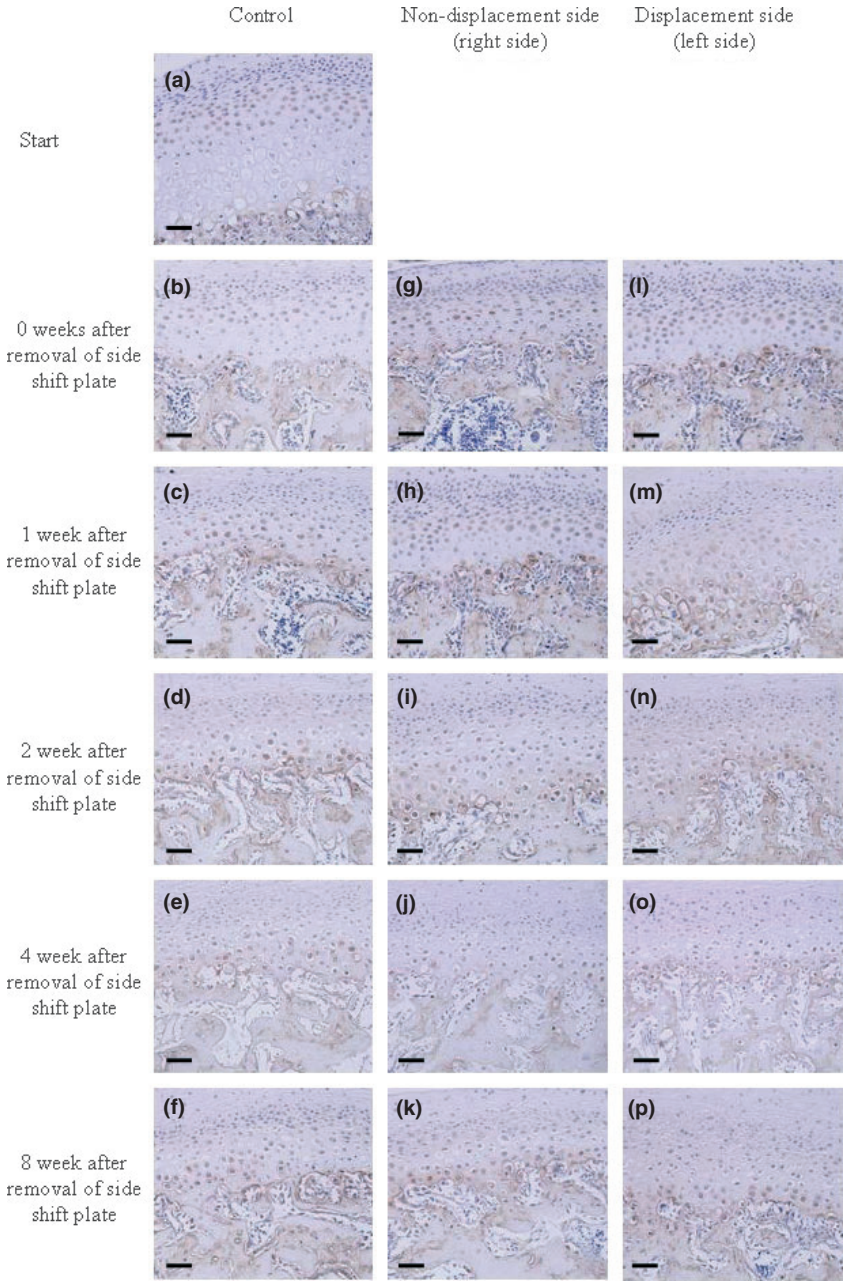

Fig. 16. Immunohistochemical findings of matrix metalloproteinase-13 (bar: 50 μm). (a–f) Control. (g–k) Non-displacement side. (l–p) Displacement side.

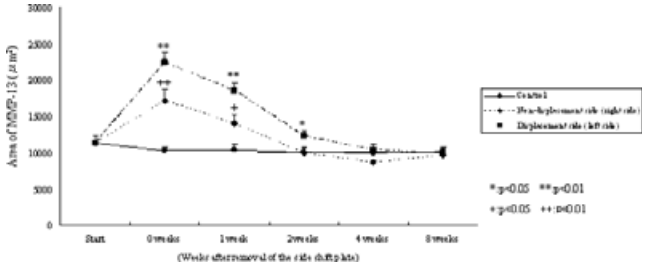

Fig. 17. Area of matrix metalloproteinase (MMP)-13. The asterisk (+) showed significantly difference between non-displacement side and control. The asterisk (*) showed significantly difference between displacement side and control. (*$p < 0.05$, **$p < 0.01$, +$p < 0.05$, ++$p < 0.01$, by one-way ANOVA).

plate. Furthermore, the resorption of posterior region of articular condyle in displacement side was admitted immediately after removal of the plate (Fig. 4). Nakano (8) also reported that the lateral displacement of the mandible caused mechanical pressure in displacement side. Accordingly, it is considered that the method is effective to experimentally induce jaw deformity associated with asymmetry.

It has been reported that type II collagen staining levels declined in the superficial layer of cartilage during in the pathological condition of osteoarthritis of the

knee joint in humans (21). Further, it has been shown that mechanical damage not only destroys the cartilage matrix directly, but also has an effect on the cellular metabolism of cartilage and further facilitates the expression of matrix degradative enzymes in the cartilage cells (22). In the present study, a significant decline in the expression of type II collagen in the displacement side was observed immediately after removal of the plate. In addition, the expressions of MMP-1, -8, and -13 were elevated in both the displacement and non-displacement on 0 weeks. Thus, we considered that overload caused by left-ward displacement of the mandible might produce MMPs and thus facilitate the degradation of type II collagen.

Furstman (23) has reported that change of mandibular joint arises on both sides when unilateral tooth extraction of the rat was performed and malocclusion arises. In the present study, changes of the joint because of malocclusion were seen bilaterally and MMP levels were elevated even in the non-displacement side. Hirota et al. (24) reported that marked intracartilaginous ossification occurred in the cartilage layer, in which intracartilaginous ossification was inhibited by use of a chin cap, after it was removed. In the present study, it was considered that intracartilaginous ossification might have been caused by cellular outgrowth associated with an increase in type II collagen and decreases in MMP-1, -8, and -13 as a result of release from the inhibition associated with left-ward displacement after removal of the side shift plate. We also speculated that so-called catch-up growth phenomenon (25) occurred. As significant differences in type II collagen levels were observed even at 8 weeks after removal of the plate, a long-term follow-up study is necessary.

As for type I collagen, no significant difference was observed immediately after removal of the plate as compared to the control group, whereas the stained area was decreased thereafter. It has been reported that type I collagen is likely involved in nucleus formation for ossification (26). Accordingly, in the present rats we considered that the decrease in type I collagen was associated with cartilage growth and contributed to ossification during the cartilage maturing process. As the levels of type III collagen did not show great changes, it was considered to not be involved in cartilage metabolism in the temporomandibular joint.

On the basis of these findings, it is considered that degradation of type II collagen was facilitated by the production of MMP-1, -8, and -13 because of overload that resulted from left-ward displacement of the mandible caused by attachment of side shift plate. After removal of the plate, the production of MMP-1, -8, and 13 was reduced and type II collagen levels increased. Thus, cellular outgrowth was initiated to trigger intracartilaginous ossification to restore the cartilage matrix.

It is also suggested that when functional lateral displacement is performed during growth periods the cartilage of the articular condyle might be restored by an increase in type II collagen, which is the result of early removal of the cause of displacement. Malocclusion, such as premature contact and cuspal interference of the teeth, is considered to be a cause of maxillofacial asymmetry. If left unchecked until the end of growth, such malocclusion will change to skeletal asymmetry (4, 5). As previously reported by Mongini et al., (6) and Vig et al., (7) removing the causes early during the growth period may be one of the effective treatments for skeletal asymmetry.

Conclusion

In the present study, histopathological changes of the articular condyle were followed for a long period after inducing functional lateral displacement by attachment of a side shift plate to the upper anterior teeth. The mandibule demonstrated left-ward displacement because of that attachment. Based on our findings, we considered that MMP-1, -8, and -13 were produced by overload and collagen degradation was facilitated. After the plate was removed, MMPs production decreased and type II collagen increased to induce cellular outgrowth, which caused intracartilaginous ossification and restored the cartilage matrix.

Therefore, during functional lateral displacement during growth, it is suggested that the cartilage of the articular condyle is restored by a decrease in MMP production and increase in type II collagen, facilitated through early removal of the causes.

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