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Facilitation of bone resorption activities in synovial lavage fluid patients with mandibular condyle fractures

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SUMMARY **The aim of this study was to investigate the bone resorption effect of the mediators delivered in joint cavity of patients with mandibular condyle fractures by detecting osteoclast markers using cellular biochemistry methods, and by analysing bone resorption activities via inducing osteoclast differentiation of the infiltrated cells from arthrocentesis. Sixteen joints in 10 patients with mandibular condyle fractures were evaluated. The control group consisted of synovial fluid (SF) samples from seven joints of four volunteers who had no clinical signs or symptoms involving the temporomandibular joint (TMJ) or disc displacement. We collected SF cells from all patients during therapeutic arthrocentesis. The infiltrating cells from TMJ SF were cultured, differentiated into tartrate-resistant acid phosphatase (TRAP)-positive osteoclast-like cells and examined bone resorption activities. We also investigated factors related to osteoclast induction of SF, using ELISA procedures. Osteoclast-like cells were induced from the SF cells obtained from all patients with condylar fractures. These multinucleated giant cells were positive for TRAP and actin, and had the ability to absorb dentin slices. The levels of macrophage colony-stimulating factor (M-CSF), prostaglandin E2 (PGE2), soluble form of receptor activator of nuclear factor kappa-B ligand (sRANKL) and osteoprotegerin (OPG), in SF samples from the patients, were significantly higher than in the controls. These findings indicate that bone resorption activities in SF from patients with mandibular condyle fractures were upregulated and may participate in the pathogenesis and wound healing.**

KEYWORDS: **mandibular condyle fractures, arthrocentesis, temporomandibular joint, synovial fluid, joint-infiltrating cells, osteoclast**

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Introduction

Condyle fractures of the mandible predispose the soft tissues of the temporomandibular joint (TMJ) to injury, while intra- and extracapsular condylar fractures are associated with haemarthrosis (1). Recent arthroscopy studies revealed that intra-articular damage generally occurs when the mandible is fractured, including haemarthrosis and shedding of the disc and joint surfaces (2, 3). These findings also suggest that haemarthrosis is the major pathology occurring in the TMJ after a condylar fracture. Furthermore, joint effusions are closely related to haemarthrosis after joint injuries, while magnetic resonance (MR) evidence of joint effusion after a condylar fracture frequently suggests haemarthrosis in that joint (4–6).

Recently, joint irrigation with saline solution (arthrocentesis) has been used therapeutically for patients with mandibular condyle fractures. Kondoh *et al.* and Nogami *et al.* found that intra-articular

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irrigation and corticosteroid injection resulted in rapid recovery of jaw function and shorter duration of distress in patients with fresh mandibular condyle fractures (7, 8). Nogami *et al.* also reported that arthrocentesis is a potent treatment modality for patients with high condyle fractures, because it decreases the interleukin (IL)-6 level, which seems to be responsible for synovitis in the TMJ after a condyle fracture (9).

Many intra-articular fracture patients eventually experience significant functional deficits, pain and stiffness from post-traumatic osteoarthritis (PTOA). PTOA occurs following a variety of joint fractures and closely related to the energy delivered to the joint at the time of injury and the resulting severity of articular destruction and surface damage (10). The improvement of the intra-articular microenvironment may be considered the most important factor leading to a good outcome.

Although pathological conditions related to a mandibular condyle fractures have been clarified, the subsequent details of wound healing, bone destruction and remodelling in the TMJ remain largely unknown. In this study, we investigate the bone resorption effect of the mediators delivered in joint cavity in patients with mandibular condyle fractures by detecting osteoclast markers using cellular biochemistry methods, and by analysing bone resorption activities via inducing osteoclast differentiation of the infiltrated cells from arthrocentesis.

Material and methods

Subjects

Fracture group consisted of sixteen TMJs in 10 consecutive patients (seven males, three females; average age 31·0 (range 12–56) years) with mandibular condyle fractures were evaluated. The control group consisted of SF samples from seven TMJs of four volunteers (two males, two females; average age 26·8 (range 25–30) years) who had no clinical signs or symptoms involving the TMJ or disc displacement (Table 1). The fractures were caused by falls (60%), sports injuries (20%), assaults (10%) and traffic accidents (10%). The period between injury and first visit ranged from 0 to 6 (average 2·9) days. No patient had clinical signs or symptoms of TMJ involvement or disc displacement, and they did not exhibit habits – such as teeth grinding or clenching – before the injury.

Table 1. Characteristics of subjects

Group (No. of subjects)	No. of TMJs	Sex		Mean age (range)
		Male	Female	
Fracture (10)	16	7	3	31·0 (12–53)
Control (4)	7	2	2	26·8 (25–30)

The positions of the condylar fractures on the mandible were examined using a panoramic transcranial view and computed tomography. All positions evaluated upper neck or head condylar fracture without an associated mandibular or mandibular condyle fracture, and without dislocation. In all patients, MRI was used for diagnostic purposes before any treatment and joint effusions were evaluated on sagittal T2-weighted images.

This study was conducted in accordance with the principles of the Declaration of Helsinki and with the approval of the Ethics Committee of the Akita University School of Medicine Institutional Review Board. Informed consent for sample aspiration was obtained from each subject.

Collecting SF samples and cells

We collected TMJ synovial fluid (SF) samples from patients with mandibular condyle fractures and the control group by arthrocentesis. In all patients with high condylar fractures, arthrocentesis was performed for therapeutic purposes, the pumping procedure was performed an average of 3 days after the injury. After arthrocentesis, all patients were given NSAIDs and an antibiotic for 3 days after treatment. SF samples were collected using a pumping procedure by aspiration with a 22-gauge needle under aseptic conditions; then, the superior joint space was washed with physiological saline. The mixture of SF and saline was aspirated gently, and then re-injected a total of 10 times. Joint-infiltrating cells were harvested from the SF by centrifugation at 1900 × ***g***, and the SF supernatant was separated and stored at −80 °C until used for enzyme-linked immunosorbent assays (ELISA).

Culturing of cells from SF

Cells infiltrating the SF samples were cultured in α-minimum essential medium (α-MEM*) containing 10% heat-inactivated foetal calf serum (FCS; Gibco BRL*) and 100 U/mL penicillin–streptomycin (Gibco BRL). All cells were seeded onto eight-well chamber slides (Lab-Tek Chamber Slide)† at 1×10^6 cells/mL, and cultured in the presence of 20 ng/mL recombinant human (rh) macrophage colony-stimulating factor (M-CSF)‡ and 10^{-7} M 1,25-dihydroxy vitamin D_3 [$1,25(OH)_2D_3$]§ The cells were maintained at 37 °C in humidified air that contained 5% CO_2.

Cytochemical and immunocytochemical staining

At the end of the culture period, the cells were stained for May–Grunwald–Giemsa and tartrate-resistant acid phosphatase (TRAP) using a kit,¶ in accordance with the manufacturer's instructions. Multinucleated giant cells were fixed with cold acetone and stained immunocytochemically with rabbit polyclonal antibodies specific for actin.**

Pit formation assay

To determine the resorption activity of TRAP-positive giant cells, joint-infiltrating cells were cultured on dentin slices using the same procedure. Resorption pits were detected by staining with horseradish peroxidase-conjugated wheat germ agglutinin (WGA)-lectin (Sigma), as described (11). Briefly, the dentin slices were incubated with 50 μg/mL horseradish peroxidase-conjugated WGA-lectin in 0·1 M PBS overnight at room temperature. After three washes, the peroxidase reaction was developed for 30 min using 0·05 M Tris buffer (pH 7·6) that contained 0·02% 3,3-diaminobenzidine tetrahydrochloride (DAB)†† and 0·006% H_2O_2. The dentin slices were mounted on aluminium stubs, sputtered with gold and examined under a Hitachi S-4300 scanning electron microscope (Tokyo, Japan).

Measurement of M-CSF, PGE2, sRANKL and OPG levels

We also investigated factors related to osteoclast induction in SF samples from both groups. Detection of M-CSF,‡‡ prostaglandin E2 (PGE2),§§ the soluble form of receptor activator of nuclear factor kappa-B ligand (sRANKL)¶¶ and osteoprotegerin (OPG) (BioVendor Laboratory Medicine, Brno, Czech Republic) was performed using ELISA kits, according to the manufacturers' instructions. All samples and standards were assayed twice. The protein assay was performed using the bicinchoninic acid (BCA) method.*** Each detectable concentration shown was calculated per 1-mg SF total protein, as described (12). The ratio of RANKL/OPG was then calculated.

Statistical analysis

Values are presented as means ±SD. Statistical analysis was performed using the nonparametric Mann–Whitney *U*-test with SPSS ver. 16.††† *P*-values <0·05 were considered to indicate statistical significance.

Results

Quantification of joint-infiltrating cells in synovial fluid

Joint-infiltrating cells in the SF were collected by lavage and counted. There were significantly more cells in the SF from the fracture group ($2·23 \pm 1·9x10^5$ cells) compared to the control group ($0·08 \pm 0·1 \times 10^5$ cells).

Joint-infiltrating cells differentiate into multinucleated giant cells

Joint-infiltrating cells from the SF of the fracture group were cultured in the presence of 10^{-7} M 1,25 $(OH)_2D_3$ and 20 ng/mL rhM-CSF for 3 weeks, and the cell cultures had reached confluence and fibroblastic cells predominated (Fig. 1). An average of 263 ± 38·5 multinucleated giant cells/well were seen

*Gibco BRL, Gaithersburg, MD, USA
†Becton Dickinson, Franklin Lakes, NJ, USA
‡PeproTech EC, London, UK
§Calbiochem, San Diego, CA, USA.
¶Sigma Chemical, St. Louis, MO, USA
**Santa Cruz Biotechnology, Santa Cruz, CA, USA
††Dojindo Laboratories, Kumamoto, Japan
‡‡R&D Systems, Minneapolis, MN, USA
§§Neogen, Lexington, KY, USA
¶¶Biomedica, Vienna, Austria
***Pierce, Rockford, IL, USA
†††SPSS, Chicago, IL, USA

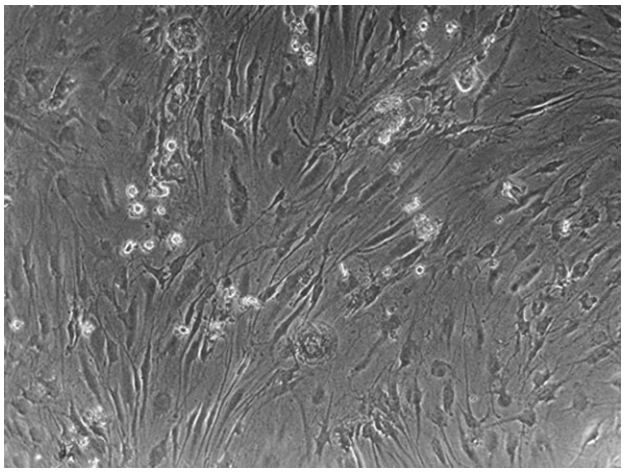

Fig. 1. Phase-contrast photomicrograph of TMJ synovial fluid cells cultured for 3 weeks in the presence of 20 ng/mL rhM-CSF and 10^{-7} M $1,25(OH)_2D_3$. Original magnification, ×100.

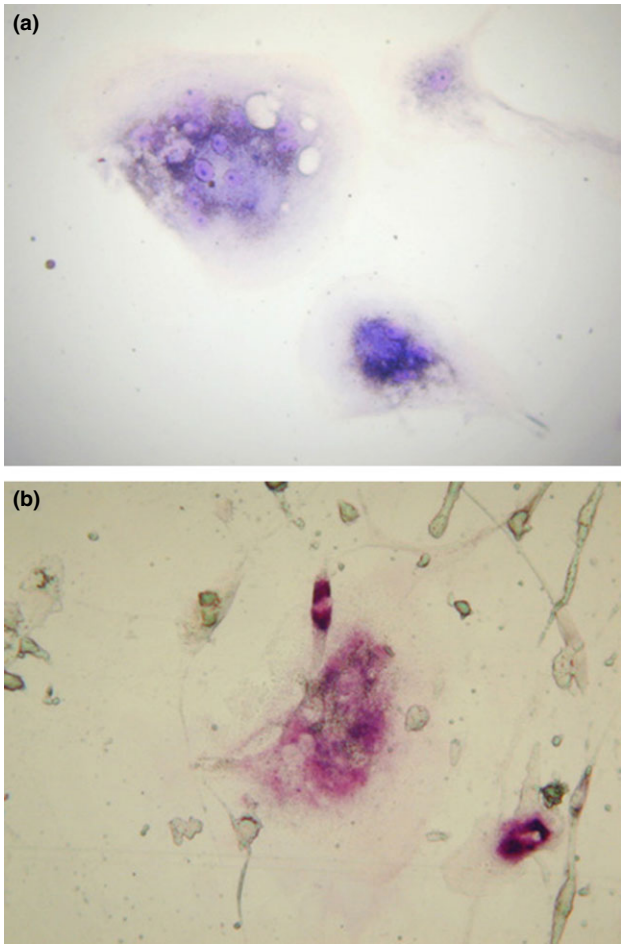

Fig. 2. May–Grunwald–Giemsa (a) and TRAP (b) staining of multinucleated giant cells. The cells induced in this culture system were multinucleated and TRAP-positive. Original magnification, ×200.

and were positive for TRAP (Fig. 2a, b). On the other hand, multinucleated cells were not inducible from cells collected from the TMJs of the control group.

Functional evidence of osteoclast differentiation

To determine the resorption activity of the TRAP-positive cells, resorption of dentin slices, a characteristic of osteoclasts, was observed after incubation for 3 weeks with cells from the fracture group SF samples. Resorption pits were clearly visible on dentin slices incubated with cells from the SF of the fracture group with lectin staining and in scanning electron micrographs (Fig. 3a, b).

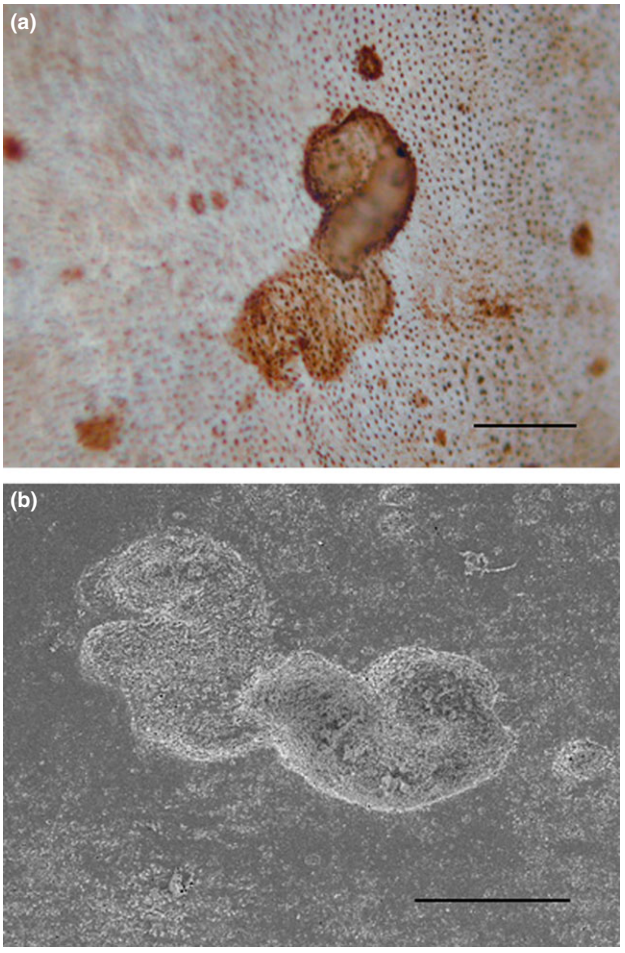

Fig. 3. Resorption pits formed by multinucleated bone-resorbing giant cells on dentin slices. Lectin staining of lacunar resorption of dentin slices on which multinucleated bone-resorbing giant cells were incubated (a). Representative scanning electron micrograph (SEM) (b). Bar, 50 μm.

M-CSF, PGE2, sRANKL and OPG contents of SF

Using ELISA, we calculated the ratios of the optical density of SF samples from the fracture and control groups. The concentrations of M-CSF, PGE2, sRANKL and OPG were significantly higher in SF samples from the fracture group compared to the control group (Table 2). Moreover, the ratio of RANKL/OPG significantly increased in the fracture group (Fig. 4).

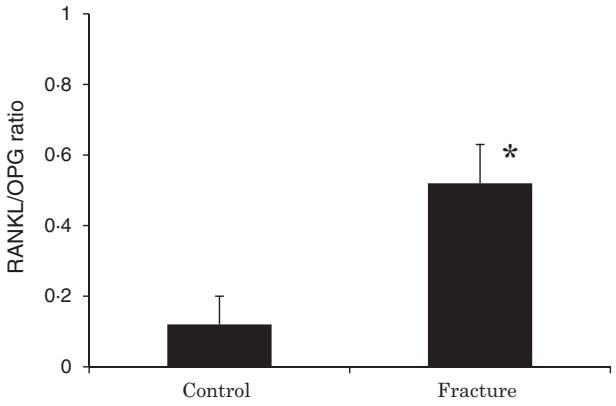

Fig. 4. Ratio of RANKL/OPG protein in SF. Each value represents mean ± SD. *$P < 0{\cdot}05$ versus control.

Discussion

Recent advances in arthroscopy and biochemical analyses of SF samples have provided new insight into the pathophysiology of temporomandibular joint disorders (TMD). Various proteinases (13, 14), neuropeptides (15) and inflammatory cytokines, including IL-1, IL-6 and tumour necrosis factor-α (TNF-α), have been detected in the SF of patients with TMD and shown to be associated with factors that influence bone destruction (16, 17). In condylar fractures, haemarthrosis and synovial hyperplasia lead to increased vascular permeability, resulting in the exudation of white blood cells, inflammatory mediators and various cytokines into the TMJ space. It is thought that these inflammatory changes recover with arthrocentesis or simple TMJ irrigation, which is defined as a lavage of the joint. Arthrocentesis is an effective treatment for TMD (18), especially in condylar fractures. Kondoh *et al.* and Nogami *et al.* showed that rapid recovery of jaw function and shorter duration of distress in patients with fresh mandibular condyle fractures by intra-articular irrigation and corticosteroid injection (7, 8), because it decreases the level of interleukin (IL)-6, which seems to be responsible for synovitis in the TMJ after a condylar fracture (9). This is likely because arthrocentesis washes out the necrotic tissue and a necrosis cells and pro-inflammatory mediators in the joint space of the early stage in the healing process by condylar fractures. Arthrocentesis excludes a floating cells in the SF, therefore, phagocytosis of necrosis cells omitted. Thereafter, haematopoietic stem cells and mesenchymal stem cells may recruit to bone fracture region. Furthermore, bone marrow stromal cells (BMSCs) are seeping in joint cavity by fracture and these cells may support differentiation to osteoclast-like cells. BMSCs has important role in modulating osteoblast and osteoclast formation (19).

Post-traumatic osteoarthritis occurs following a variety of joint injuries, it most commonly following injuries that disrupt the articular surface. The reduction of displaced articular surface fragments has been considered the most important factor leading to a good outcome (10). Usually, bone resorption activity is necessary for the healing process of the bone fracture. However, clinically, arthrocentesis may have an effect on preventing the development of PTOA by decreasing the levels of pro-inflammatory cytokines and bone resorption factors or reduction of the number of joint-infiltrating cells. Actually, patients in this study could get good healing process.

In this study, arthrocentesis was performed for therapeutic purposes in patients with condylar fractures. Moreover, the average number of joint-infiltrating cells in the SF was significantly higher in the fracture group than the control group, indicating that inflammatory cell infiltration and vascular permeability were

Table 2. M-CSF, PGE2, sRANKL, OPG concentrations in synovial fluid

Group	M-CSF (pg/mg)	PGE2 (ng/mg)	sRANKL (pg/mg)	OPG (pg/mg)
Fracture	84·3 ± 29·3*	168 ± 91·4*	126·2 ± 32·6*	241·4 ± 41·1*
Control	27·8 ± 4·2	24 ± 26·8	12·9 ± 8·3	110·3 ± 20·2

Data are reported as means ±SD. *$P < 0{\cdot}05$ *vs.* controls.

present in the TMJs of fracture group. Therefore, the SF of fracture group may contain more haematopoietic stem cells with the capacity to differentiate into pre-osteoclasts and we consider the possibility of osteoblasts or BMSCs which seeping into joint cavity may support differentiation to osteoclasts of a haematopoietic stem cell. In the present study, we collected cells infiltrating the TMJs of fracture group and demonstrated that those cells could be induced to differentiate into TRAP-positive multinucleated bone-resorbing giant cells, which had characteristics of osteoclasts. These cells may also influence deformity of the cartilage or disc. To our knowledge, this is the first study to investigate the molecular and cellular characteristics of joint-infiltrating cells obtained from the SF of patients with mandibular condyle fractures. It is quite impossible to be accurate while collecting the synovial fluid following insertion of saline. Yet the results are important and do provide an insight towards conservative support to the healing process after condylar fractures.

Numerous studies have identified additional pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α (16, 17), which together with prostaglandins are thought to regulate osteoclastogenesis. These findings suggest that the joint microenvironment favours the differentiation of precursors into osteoclasts when the production of inflammatory cytokines is increased with a mandibular condyle fracture. More recently, the RANKL gene was cloned, and several researchers have reported that RANKL and M-CSF, which are produced by osteoblasts, bone marrow stromal cells and synovial cells, are indispensable for osteoclastogenesis (20, 21). In this study, M-CSF, PGE2 and sRANKL were detected in the SF of the fracture group. The optical density was significantly higher in the fracture group than the control group. These results suggest that these factors in the SF of condyle fracture patients activate bone resorption and osteoclastogenesis. On the other hand, M-CSF, PGE2 and sRANKL were detected very lower level in the control group. These seemed to participate in physiological bone metabolism. Furthermore, the ratio of RANKL/OPG significantly increased in the fracture group, suggesting that it has the potential to cause joint-infiltrating cells to differentiate into active osteoclasts.

Kaneyama *et al.* reported that the OPG concentrations in SF were significantly lower in patients with osteoarthritis of the TMJ, which promoted osteoclastic activity (22). By contrast, OPG protein was significantly elevated in SF samples from our fracture group, which disagrees with other recent reports. Bone fracture healing is a multistage repair process that involves complex well-orchestrated steps initiated in response to tissue injury. In the early stage, IL-6, OPG, vascular endothelial growth factor (VEGF) and bone morphogenetic proteins (BMPs) are upregulated, which indicates a central role for these factors in the initiation of cartilage and periosteal woven bone formation (23). In this study, we collected SF samples from patients with mandibular condyle fractures an average of 3 days after the injury. As the SF samples were collected in the early stage after the injuries, the higher OPG concentration is considered normal. In addition, OPG is secreted by BMSCs, osteoblast, and other cells like chondrocytes which are in the same lineage (24). In the fracture group, fracture insulted condylar surface and thus bone marrow may seeping into joint cavity which brings about osteoblasts or BMSCs into joint cavity. Therefore, the fact that OPG increased in fractured joint fluid supports this assumption.

Conclusion

The present results suggest that bone resorption activities in SF from patients with mandibular condyle fractures were upregulated and joint-infiltrating SF cells could differentiate into pre-osteoclasts. Therefore, they are thought to play important role in bone resorption and the pathogenesis of condylar fractures of the mandible. Arthrocentesis may be a potent treatment modality for patients with high condylar fractures, because it decreases the level of pro-inflammatory cytokines and bone resorption factors, which may be important and do provide an insight towards conservative support to the healing process after condylar fractures.

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Conflict of interest

The authors declare that they have no conflict of interest.

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