Changes in the Subchondral Bone Affect Pain in the Natural Course of Traumatic Articular Cartilage Defects

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Abstract

Objective: Articular cartilage defect causes joint pain and finally progresses to osteoarthritis. Although the subchondral bone condition affects clinical outcomes of cartilage defects, the natural course of changes in subchondral bone and associated pain in full-thickness cartilage defects remain unknown. Therefore, we investigated the natural course of histological changes in subchondral bone and joint pain in cartilage defects using a rat model.

Design: Full-thickness cartilage defects were created at the medial femoral condyle of 10-week-old male Sprague Dawley rats. Rats were sacrificed at 3, 7, 14, 28, and 56 days postoperatively, and histological including immunohistochemistry and TRAP staining and micro-computed tomography (μCT) analyses of their knees were performed. Pain was evaluated using behavioral analysis and immunofluorescence staining of the dorsal root ganglion (DRG).

Results: The contour of the subchondral bone plate was maintained until day 3, but it was absorbed just under the cartilage defect from day 7 to 14. Starting on day 28, sclerotic changes surrounding the bone absorption area were detected. In the subchondral bone, the number of TRAP-positive cells peaked on day 14. Osteocalcin-positive cells were observed at 7 days, and their number gradually increased till day 56. Behavioral analysis showed that the total distance and the number of getting up by hind legs decreased on day 14. The number of calcitonin gene-related peptide-positive fibers in the DRG increased and was the highest on day 14.

Conclusions: The subchondral bone condition under cartilage defects dynamically changes from bone resorption to sclerosis and is related to pain level.

Key terms: articular cartilage defect; subchondral bone; pain

Introduction

Articular cartilage provides smooth joint movement and weight-bearing capabilities through its ability to absorb stress, and reduce friction, and its high resistance to wear.[1] In the clinical setting, articular cartilage injuries are often associated with joint injuries, such as ligament injury, joint dislocation, and fracture. It is difficult for articular cartilage to heal spontaneously as it is poorly vascularized and innervated, and the injury eventually progresses to osteoarthritis (OA), especially in lesions that are greater than 1.5 cm in diameter.[2] The homeostasis of articular cartilage is maintained by the subchondral bone, with interactions such as nutritional exchange and coordination of load distribution taking place.[3] Therefore, damage to the articular cartilage layer significantly affects the condition of the subchondral bone, and may introduce both sclerotic and porotic changes. Articular cartilage injury also induces joint pain, which causes functional disability of the joint and decreases daily activity.[4] Although pain originates from the synovium and subchondral bone, the experience of pain in the subchondral bone is crucial because it is directly affected by load-bearing. Thus, articular cartilage defects should be treated appropriately to improve activities of daily living by removing pain and inhibiting the progression of OA.

Appropriate treatment of articular cartilage injury is selected, according to the size of the lesion and the condition of the subchondral bone.[5] The bone marrow stimulation (BMS) technique is generally performed because of its simplicity. However, it has been reported to have poor long-term clinical outcomes due to fragile fibrocartilage tissue covering the cartilage defect.[6] Moreover, intralesional osteophytes and subchondral bone cysts have been reported as complications with this technique.[7] BMS induces endochondral ossification around the bone hole in the subchondral bone, altering bone metabolism and thus causing excessive osteogenesis and bone resorption in the subchondral bone, which affects clinical outcomes after surgery.[8] Autologous chondrocyte implantation may also be used to treat articular cartilage injury, although it was reported that the condition of the subchondral bone affects the clinical outcome.[7] Since the condition of the subchondral bone influences on the clinical outcomes, it is important to understand the changes in the subchondral bone after cartilage injury. However, the timeline of historical changes that take place in the subchondral bone after cartilage injury remains unclear. If the subchondral bone condition changes in the natural course of a cartilage injury and postoperative outcomes are affected by the condition of the subchondral bone, the timing of surgery and the selection of the surgical procedure are crucial. In addition to using imaging technology such as computed tomography (CT),

to evaluate the condition of the bone, using joint pain as an indicator of the subchondral bone condition would be a useful technique for treating articular cartilage injury. Dynamic changes in osteoclast and osteoblast differentiation occur in the subchondral bone during the pathogenesis of osteochondral lesion (OCL) and OA.[9,10] An increase in the activity of osteoclasts leads to a decrease in the threshold of the pain, and nociceptive nerve fibers, including neuropeptides, increase the subchondral bone in OA and OCL. Several studies also showed a significant relationship between joint pain and the subchondral bone condition in articular cartilage damage in the OA model. It is reported that inhibition of subchondral bone deterioration by bisphosphonate could alleviate joint pain in rat OA models.[11] Another report demonstrated that improving joint pain was reduced as the subchondral bone microarchitecture was improved by the treatment of the intermittent parathyroid hormone in the OA mice models.[12] Based on these two factors, we hypothesized that the subchondral bone condition would change dynamically after an injury to the articular cartilage and that the condition of the subchondral bone would affect pain at the joint. The purpose of this study was, therefore, to elucidate the histological changes in the subchondral bone after articular cartilage injury in the weight-bearing area and to investigate the relationship between the subchondral bone condition and pain using a rat model.

Methods

This study was performed in accordance with the Guide for Animal Experimentation and was approved by the Committee of Research Facilities for Laboratory Animal Science (A19-159).

Animals

Twenty-five male 10-week-old Sprague-Dawley rats were used in this study. The animals were housed three per cage in the animal experimentation facility under a normal 12-hour light-dark cycle with free access to food and water. Rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride (1.4 mL/kg of body weight) and xylazine (0.4 mL/kg of body weight). Then right knee joints were exposed by a parapatellar approach, and 5.0 mm × 3.0 mm of full-thickness articular cartilage defects were created in the weight-bearing area of the medial femoral condyle using a surgical knife without any damage to the subchondral bone plate according to the procedure described in a previous report.[13] The left knee joints were used without procedure as the control group. An additional five male 10-week-old rats were incised to the joint capsule using the same method and used as a sham group for behavioral analysis. After surgery,

all rats were allowed to move freely inside the cages without load restriction. One week before sacrifice, 10 μL of 2% Fluoro-gold (FG) (Fluorochrome Inc., Denver CO), a neuronal retrograde tracer, was injected into the knee joints.[14]

Histological Assessment

Five rats were sacrificed at each time point of 3, 7, 14, 28, and 56 days after surgery, and both knee joints and bilateral L4 dorsal root ganglions (DRGs) were harvested according to a procedure described previously report.[15] The knee joints were fixed in 4% paraformaldehyde phosphate-buffered saline (Wako Pure Chemical Industries Ltd.) for 24 hours at 4 ℃ and then decalcified in distilled water containing 10% ethylenediaminetetraacetic acid for 3 weeks. Each sample was embedded in paraffin and cut into 4.5-μm-thick sections along the sagittal plane, including the cartilage defect. Sections were stained using safranin-O/- fast green and hematoxylin-eosin (HE), according to standard protocols. Pathological changes in the subchondral bone below cartilage defects were graded by allocating a score ranging from 0 (best) to 3 (worst) to the subchondral bone using Aho's subchondral bone grading score. [16] A grade of 0, indicated that there was no evidence of subchondral bone sclerosis, and a grade of 3, that there was severe subchondral sclerosis and massively increased bone volume (Figure1).

Other sections were used for immunohistochemistry and tartrate-resistant acid phosphatase (TRAP) staining. The DRGs were fixed in 4% paraformaldehyde phosphate-buffered saline (Wako Pure Chemical Industries Ltd.) for 24 hours at 4 ℃ and then stored in 20% sucrose for 20 hours at 4°C. The DRGs were sliced into 8-μm sections, using a Microm HM 520 cryostat.

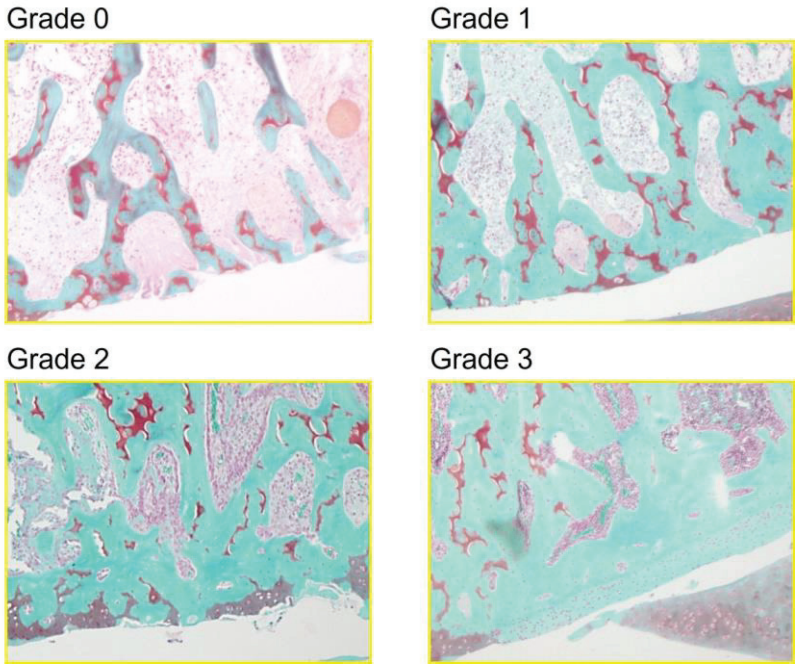


Figure 1. Aho's subchondral bone grading score. Representative images of Grades 0 – 3 in the rat model. Grade 0; No evident subchondral bone sclerosis, thin subchondral bone plate and trabecular. Grade 1; Some subchondral sclerosis and bone volume is increased. Thickened bone trabeculae can be seen. Grade 2; A distinct increase subchondral sclerosis and bone volume. Grade 3; Severe subchondral sclerosis and massively increased bone volume. Subchondral bone plate flattens.

Each section from the knee was immunostained with an anti-osteocalcin (1:100 dilution; Santa Cruz Biotechnology, Dallas, TX), anti-substance P (1:100 dilution; Santa Cruz Biotechnology, Dallas, TX), anti-calcitonin gene-related peptide (CGRP) (1:500 dilution; Abcam, Cambridge, MA), using a 3,3'-diaminobenzidine substrate (Vector Laboratories). Osteocalcin-positive cells were evaluated at the trabecular edge, and the percentage of osteocalcin-positive cells was determined by the length of osteocalcin-positive cells divided by the length of the trabecular round, using Image J (National Institution of Health). TRAP staining was performed using a commercially available kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's protocol. TRAP-positive multinucleated cells containing more than three nuclei were identified as osteoclasts.

CGRP expression and FG were assessed using fluorescence microscopy. Sections of DRGs were incubated with rabbit anti-CGRP (1:500; Abcam, Cambridge, MA) overnight at 4 °C, and the second detection was performed with Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (1:500 dilution) for 1 hour at room temperature. CGRP-positive nerve fibers and FG-positive fibers were counted, and the ratio of the number of CGRP and FG-positive cells to the number of FG-positive cells, determined using Image

J.

Micro-Computed Tomography

Samples were analyzed using high-resolution μ-CT (SkyScan1176, Toyo Corporation, Tokyo, Japan) using the following parameters: tube voltage of 70 kV/360 μA, AI 1-mm filter, and 18-μm isotropic resolution. Images were reconstructed (NRecon, Toyo Corporation, Tokyo, Japan) for analysis using a CT analyzer (Toyo Corporation, Tokyo, Japan). The volume of interest was 5.0 mm × 3.0 mm and 1.0 mm deep in the subchondral bone lesion just below the cartilage defect, with the bone volume/tissue volume (BV/TV, %) ratio measured as previously described.[17]

Open Field Test

The open-field test was performed twice for each rat in a square arena (100 cm long, 100 cm wide, 60 cm high), based on a previous experiment by Crumeyrolle-Arias et al.;[18] just before surgery and sacrifice. Briefly, each rat was placed in one corner of an open-field arena lit in the center (500 lx) and allowed to freely explore the arena. The movements of the rats were monitored and recorded for 6 minutes. The total distance the rat traveled in the arena and the number of times of rearing, getting up by hind legs, were

calculated using specific devices (SMART, Panlab SL, Barcelona, Spain).

Statistical Analysis

All results are expressed as mean and standard deviation. The Mann-Whitney U test was used to compare the total distance travelled and number of times rearing occurred that were measured during the open field test at each time point between the cartilage defect models and sham models. Tukey Kramer's post hoc test was used to compare subchondral bone grading, TRAP-positive and CGRP-positive ratio of subchondral bone, CGRP-positive ratio of DRG, and BV/TV ratio of subchondral bone among six groups.

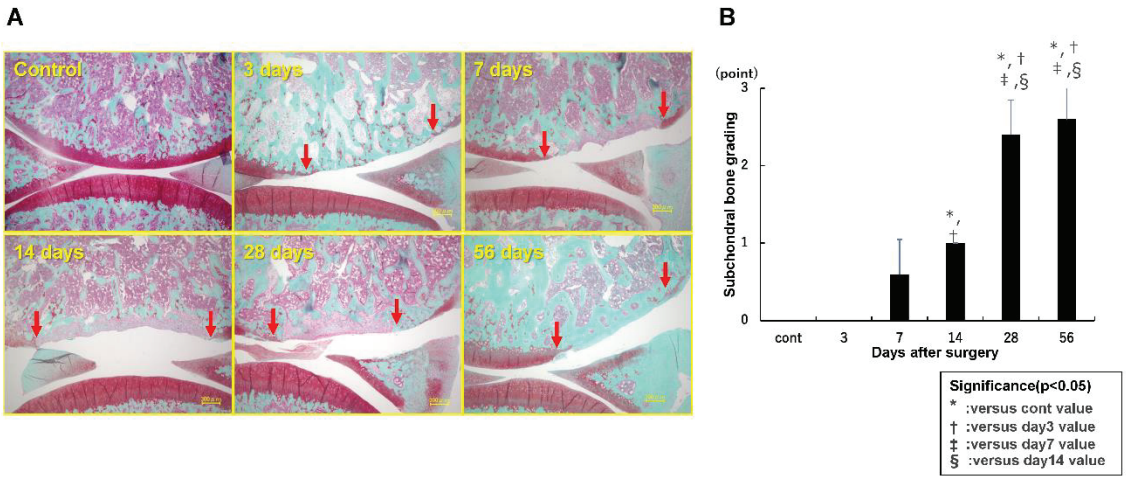
Results

Subchondral Bone Changes after Cartilage Injury

On day 3, the subchondral bone plate remained in all specimens and there was no obvious change in the subchondral bone with cartilage defect. On day 7, bone resorption was observed in the subchondral bone, which progressed until day 14. From days 28 to 56, bone sclerosis gradually progressed in the subchondral bone (Figure 2A). Aho's grading score showed no significant difference until day 7, compared with the control group (0.0 ± 0.0, 0.0 ± 0.0, and 0.6 ± 0.4 for the control group, and days 3 and 7,

193 respectively). From day 14, the score was gradually got worse (1.0 ± 0.0, 2.4 ± 0.4, and

194 2.6 ± 0.4 for days 14, 28, and 56, respectively) (Figure 2B).



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196 Figure 2. (A) histological evaluation: safranin-O staining, (B) subchondral bone grading.

197

198 The μ-CT images showed that the contour of the subchondral bone plate was

199 maintained until day 3, but it was absorbed in the area just under the cartilage defect on

200 day 7. On day 14, the depth of the area of bone absorption expanded, and the subchondral

201 bone plate disappeared, but there was no sclerotic change at the site of the cartilage defect.

202 On day 28, osteogenesis in the subchondral bone defect filled towards the articular surface,

203 with sclerotic changes surrounding the bone absorption area. On day 56, the subchondral

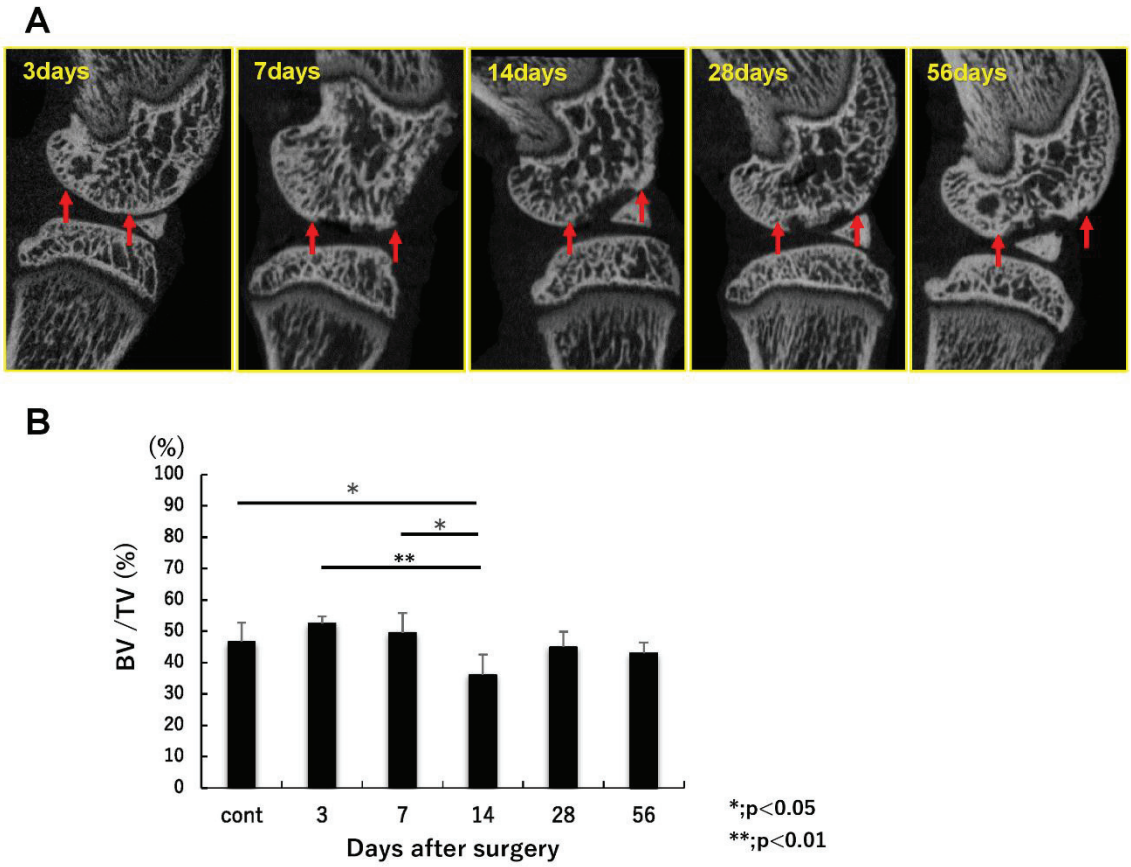
204 bone defect was filled with sclerotic bone (Figure 3A). The BV/TV decreased

205 significantly on day 14 compared with the control group on day 3 and 7 (36.1 ± 6.4% vs.

206 46.6 ± 6.1%, 52.5 ± 2.2%, and 49.5 ± 6.3% for day 14, control group, days 3 and 7,

207 respectively), and then increased to levels similar to those of the control group after day

208 28 (45.0 ± 4.9%, and 42.9 ± 3.5% for days 28 and 56, respectively) (Figure 3B).



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210 Figure 3. (A) micro-computed tomography(μCT) findings of the knee, (B)the bone

211 volume/tissue volume (BV/TV) ratio of the subchondral bone.

212

213 In the evaluation of the osteoclast activity in the subchondral bone, an increase in the

214 number of osteoclasts in the subchondral bone just under the cartilage defect was

215 observed from day 7, reaching a maximum on day 14, and then decreasing to levels

216 similar to those observed in the control group by day 56. On day 14, the number was

significantly higher than that in the control group on days 3, 7, and 56 (90.2 ± 23.9 vs. 20.4 ±5.0, 13.8±6.1, and 37.8 ± 14.8 for days 14, control group, days 3, 7 and 56, respectively) (Figure 4AB). With regard to osteogenesis, increasing numbers of osteocalcin-positive cells in the subchondral bone were observed on day 7, and the number gradually increased until day 56 (31.2% ± 3.8%, 35.6% ± 4.0%, 42.4% ± 3.4%, 47.7% ± 7.6%, 55.9% ± 7.4% for the control group, days 3, 7, 14, 28, and 56, respectively). On day 14, the osteocalcin-positive cells started accumulating, especially along the trabecular around the cystic changes. From day 7, the percentage of osteocalcin-positive cells was significantly more than that in the control group (Figure 5AB).

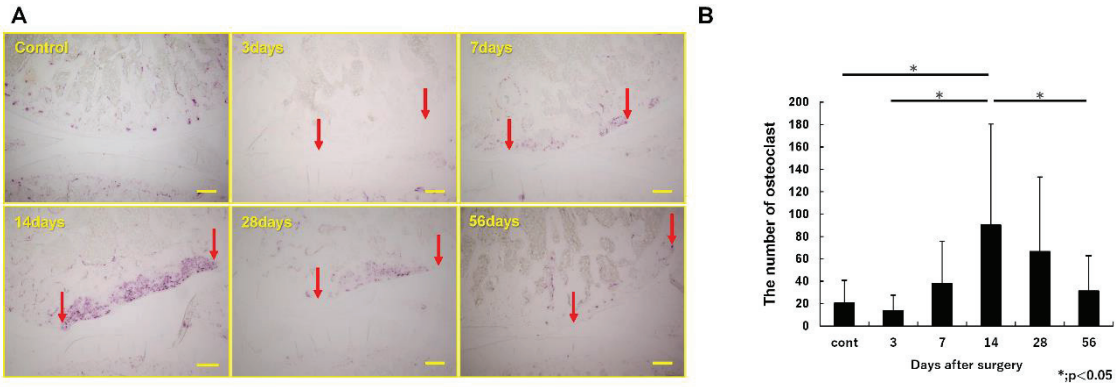
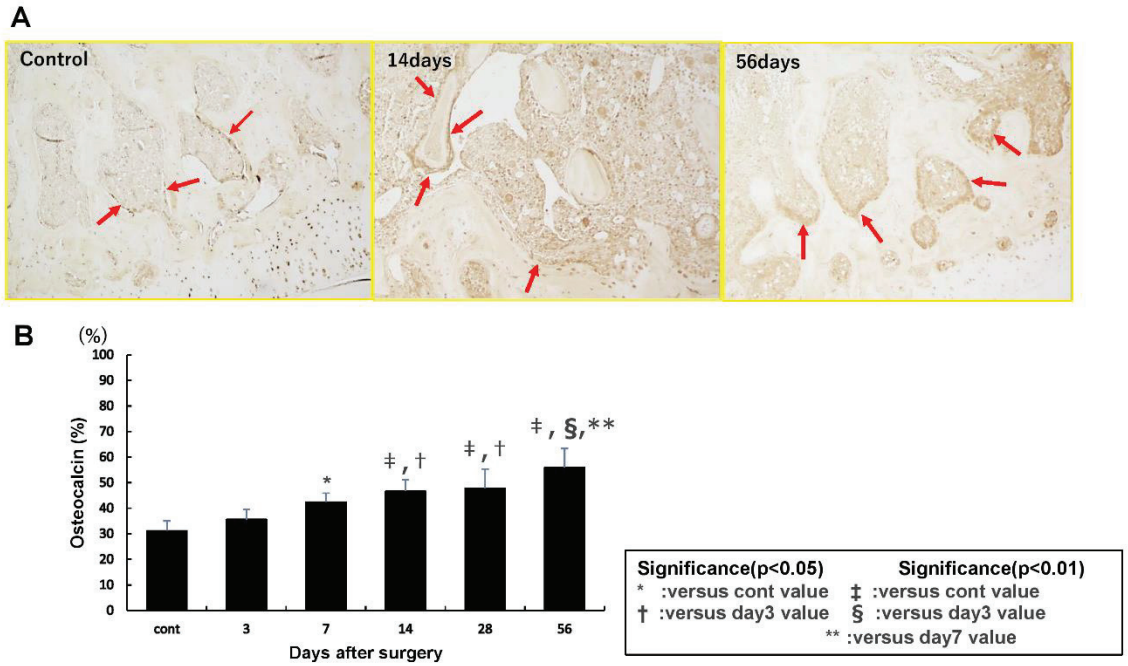


Figure 4. (A)osteoclast activity; TRAP staining (arrow: range of cartilage defect), (B)the number of osteoclast at each time.



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230 Figure 5. (A)osteoblast activity (arrow: osteocalcin-positive cells), (B)the percentage of

231 osteocalcin-positive cells.

232

233 ***Pain Assessment***

234 Compared with the sham models, cartilage defect models showed significantly

235 reduced total distance traveled in the open field test on day 14 (P = 0.008) and 56 (P =

236 0.00007) (Figure 6A), and a reduced number of times of rearing on day 14 (P = 0.01)

237 (Figure 6B). In immunofluorescence staining for CGRP of the DRG, the percentage of

238 CGRP -and FG- positive fibers increased peaked on day 14, and gradually decreased from

239 day 28. The percentage measured on day 14 (54.1% ± 4.9%) was significantly higher than

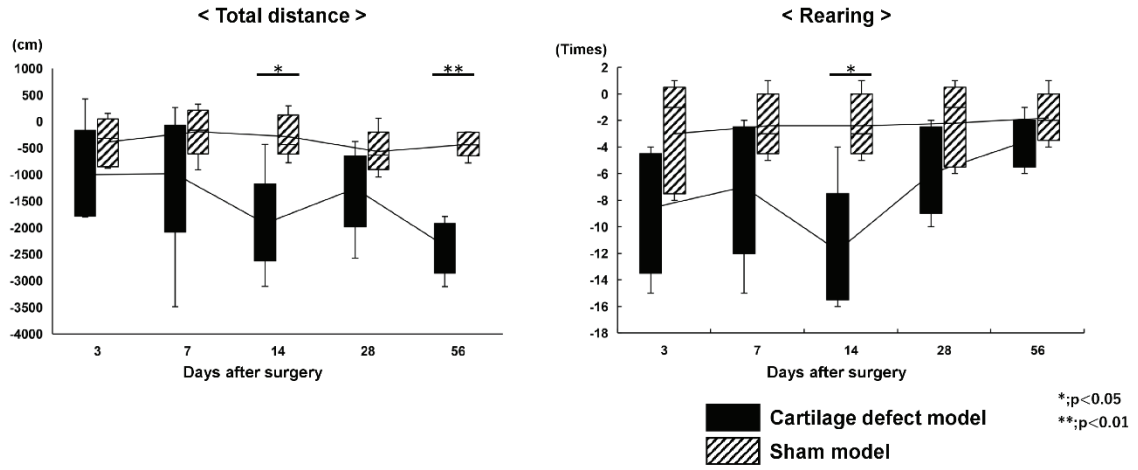
240 that of the control group, and day3 and day56 (34.5% ± 5.6%, 36.9% ± 7.6%, and 37.5%

241 ± 5.5% for the control group, and days 3 and 56, respectively) (Figure 7A, 7B).

242 Immunostaining images for CGRP and substance P of the knee are shown in Figure 7C.

243 The number of fibers stained positively for CGRP and substance P positive increased in

244 the subchondral bone under the cartilage defect on day 14.



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246 Figure 6. The results of the open field test; (A) total distance travelled, and (B) the number

247 of times rearing occurred.

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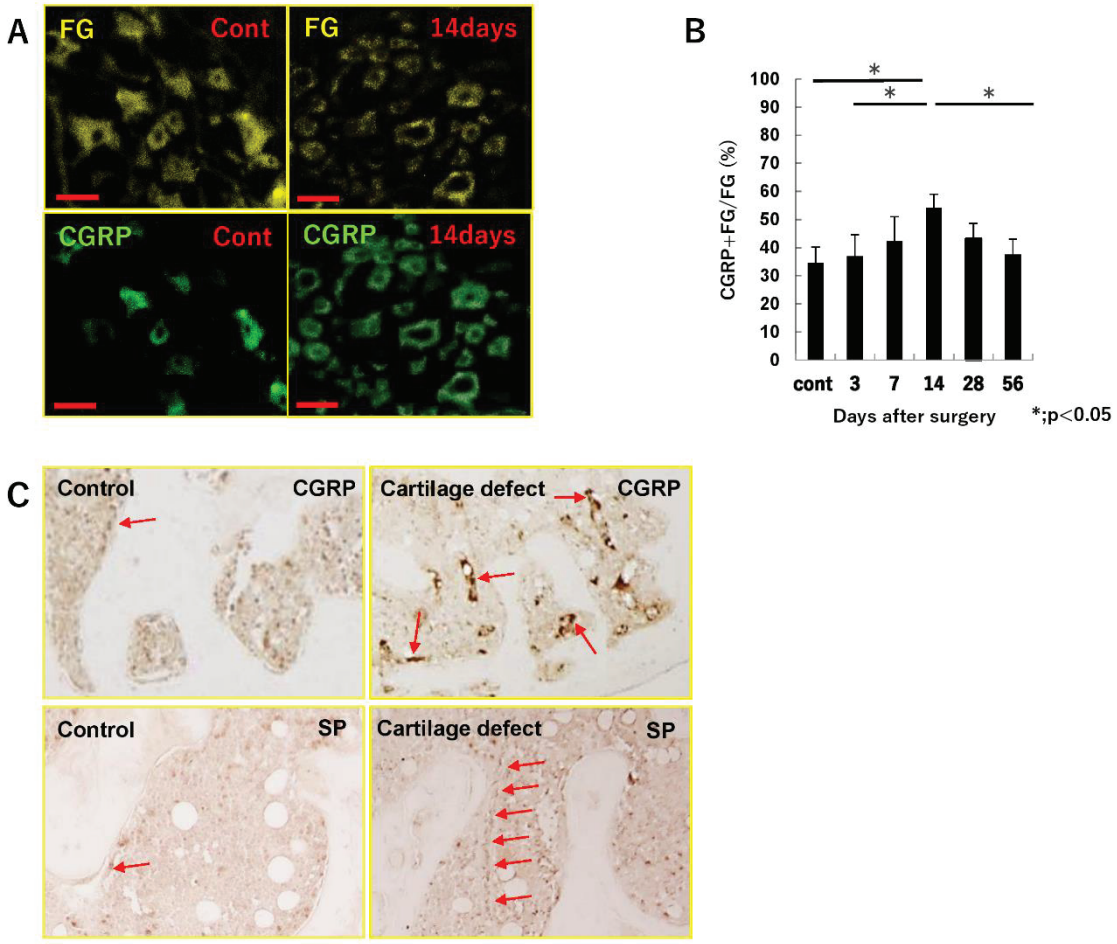

Figure 7. (A) immunofluorescence staining for Fluoro-Gold(FG) and calcitonin gene-related peptide (CGRP) of the dorsal root ganglion (DRG). The bar indicates a distance of 50 μm. (B) the percentage of CGRP-positive cells at each time. (C) immunostaining for CGRP and substance P (SP) of the knee on the control group and day 14. The bar indicates a distance of 50 μm.

Discussion

The purpose of this study was to clarify the natural changes in the subchondral bone,

and the relationship between the condition of the subchondral bone and pain in the early phase of articular cartilage defects in a rat model. Our results suggest that the activation of osteoclasts occurred from day 7, bone resorption of subchondral bone peaked on day 14, and subsequent bone sclerosis by osteoblast activation occurred from day 28. In addition, from the results of the open field test and immunofluorescence staining of the DRGs and subchondral bone, the pain level was estimated to peak at day 14, concomitant with bone absorption in the subchondral bone. Thus, the natural course of an articular cartilage defect in a rat model was revealed, and it was associated with pain levels. The findings of this study suggest that the condition of the subchondral bone can be inferred from radiographic assessment and pain level, and this will contribute to the treatment of cartilage defects in clinical practice.

The pathogenesis of pain due to articular cartilage defects is not fully understood, although several factors are thought to be involved in the process. Since articular cartilage does not have a nerve fiber, surrounding tissues such as the subchondral bone and synovium may cause pain. In particular, the subchondral bone under the cartilage defect is directly affected by the loading force, which may subsequently induce changes in the bone structure.[19] Also, the mechanism of pain experienced in OA and osteochondral lesions (OCLs) is better understood than that in cartilage defects. In OCLs, pain may

develop from the pressurized fluid into the subchondral bone, which induces decreasing pH caused by osteoclasts. A low pH excites the nerve fibers present in the bone, inducing pain.[20] In OA, the subchondral bone has received much attention as a cause of pain. In previous reports, subchondral bone marrow edema-like lesions were visualized using magnetic resonance imaging, and were highly correlated with OA pain.[21,22] It has also been reported that an increase in osteoclast-mediated bone resorption induces sensory innervation in the subchondral bone and hyperexcitability of DRG neurons, which induces OA pain.[23] In clinical practice, risedronate, an osteoclast inhibitor, has been tested for use in OA and it has been shown to decrease subchondral bone marrow lesions, thus improving pain.[24,25] In this study, osteoclast activity under the cartilage defect was highest on day 14, suggesting a correlation between the course of pain and activation of osteoclasts.

Bone remodeling is continuously maintained through a tight equilibrium between osteoblast activity which is responsible for bone formation through the synthesis of bone matrix and osteoclast activity which is responsible for degrading the bone microenvironment.[26] In this study, osteoclasts situated under the cartilage defects were activated from days 7 to 14, and osteoblasts were activated from day 7, suggesting that the metabolism of the subchondral bone was enhanced after the articular cartilage defect

294 was created. It is well known that osteoclasts below the cartilage are activated due to the
295 increased load, inducing subchondral bone loss in early-stage OA, and subchondral bone
296 becomes sclerotic at a later stage of OA.[27,28] Our results suggest that subchondral bone
297 remodeling after cartilage defect is similar to that observed in the pathogenesis of OA.
298 Previous reports have shown that subchondral bone plays an important role in maintaining
299 cartilage homeostasis,[3] and damage to the subchondral bone causes the progression of
300 cartilage damage vice versa.[29] Furthermore, cartilage destruction in anterior cruciate
301 ligament transection models is secondary to subchondral bone damage. However, the
302 mechanism of the naturally-occurring OA induced by collagenase injection is suggested
303 that the absorption of subchondral bone is secondary to cartilage lesions.[30] In summary,
304 the condition of the subchondral bone is related to the condition of the cartilage, and the
305 condition of the subchondral bone affects the repair of cartilage.

306 Subchondral bone cysts are a major complication of the microfracture technique for
307 cartilage defects.[7] Our results suggest that microfracture in an osteoclast-dominated
308 situation, such as on day 14 after damage to the cartilage, may cause subchondral bone
309 cysts to develop. After an acute traumatic articular cartilage injury, the cartilage lesion
310 might best be treated within 2 weeks after injury to prevent subchondral bone
311 deterioration due to osteoclast activation by careful weight-bearing control to reduce the

influx of joint fluid. Therefore, understanding the change in the condition of the subchondral bone will result in the successful treatment of damaged cartilage defects. Imaging examinations such as CT and MRI are used to understand the condition of the subchondral bone in clinical practice. In addition, the pain level can be a monitoring tool for subchondral bone conditions. It is reported that antiresorptive drugs such as bisphosphonates are well known to be useful for OA pain and OA progression.[31,32] These findings, in addition to our results, support the hypothesis that drugs targeting osteoclasts can potentially inhibit pain and changes in the subchondral bone in the early stages after damage to the cartilage. Besides, further studies will be required to develop surgical procedures such as microfracture technique by analyses of the actual changes of the subchondral bone seen after microfracture and to investigate the feasibility of the application of biomaterials to improve the therapeutic repair outcomes including pain reduction.

This study has several limitations. First, we used rats as the animal model. Since the rate of bone metabolism differs between humans and rats, the time course of changes in the subchondral bone after cartilage injury may be different from that in humans. Moreover, many factors affect subchondral bone changes, such as age, sex, and degree or location of, or intervention for the cartilage injury. Second, all the animals could move

freely after the cartilage defect was created in their limb, which may have affected the rate of metabolism in the subchondral bone.[33] These factors may have affected the results of this study. Therefore, the bone resorption phase may start later than 2 weeks after cartilage injury. However, the process in which subchondral bone resorption occurs and then it changes toward bone formation as sclerotic change after cartilage injury remains unchanged, and cartilage injury should be appropriately treated according to the condition of the subchondral bone to achieve good clinical results.

In conclusion, the condition of the subchondral bone under an articular cartilage defect dynamically changes from bone resorption to sclerosis, and this process is related to the level of pain experienced. Appropriate intervention for the cartilage defect by taking into account the subchondral bone changes and their relationship with pain, will enable patients with articular cartilage injury to be treated successfully.

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