**Table 2. Numbers (%) of each osteophyte and joint space narrowing score (n = 192)***

| | Score 0 | Score 1 | Score 2 | Score 3 |
|---|---|---|---|---|
| Medial femoral osteophytes | 28 (14.6) | 42 (21.9) | 49 (25.5) | 73 (38.0) |
| Lateral femoral osteophytes | 98 (51.0) | 51 (26.6) | 32 (16.7) | 11 (5.7) |
| Medial tibial osteophytes | 19 (9.9) | 37 (19.3) | 73 (38.0) | 63 (32.8) |
| Lateral tibial osteophytes | 55 (28.6) | 74 (38.5) | 43 (22.4) | 20 (10.4) |
| Medial joint space narrowing | 23 (12.0) | 42 (21.9) | 48 (25.0) | 79 (41.1) |
| Lateral joint space narrowing | 184 (95.8) | 8 (4.2) | 0 (0) | 0 (0) |

* Osteoarthritis Research Society International atlas (4).

ratios (r = 0.844, $P < 0.001$; r = 0.797, $P < 0.001$; and r = 0.791, $P < 0.001$, respectively) (Figures 1A, B, and C).

**Association of knee condyle BMDs with age or BMI.** BMD of the medial femoral condyle increased significantly with age (r = 0.144, $P < 0.001$), whereas that of the medial tibial condyle did not (Figure 2A). Medial versus lateral condyle BMD ratios in both the femur and the tibia were positively correlated with age (r = 0.318, $P < 0.001$ and r = 0.290, $P < 0.001$, respectively) (Figure 2C), whereas BMDs of the lateral femoral and tibial condyles were negatively correlated with age (r = −0.199, $P < 0.001$ and r = −0.244, $P < 0.001$, respectively) (Figure 2B).

Medial condyle BMDs of both the femur and the tibia were positively correlated with BMI (r = 0.374, $P < 0.001$ and r = 0.475, $P < 0.001$, respectively) (Figure 2D), and similar relationships were observed between the lateral condyle BMDs of both the femur and the tibia and BMI (r = 0.260, $P < 0.001$ and r = 0.445, $P < 0.001$, respectively) (Figure 2E). The medial versus lateral condyle BMD ratio in the tibia increased significantly with BMI (r = 0.259, $P < 0.001$), whereas that in the femur did not (Figure 2F).

**Association of knee condyle BMDs with VAS pain.** Medial condyle BMDs of both the femur and the tibia increased significantly with VAS pain (r = 0.444, $P < 0.001$ and r = 0.432, $P < 0.001$, respectively) (Figure 2G), whereas lateral condyle BMDs of the femur and tibia did not (Figure 2H). Medial versus lateral condyle BMD ratios in the femur and tibia increased significantly with VAS pain (r = 0.431, $P < 0.001$ and r = 0.487, $P < 0.001$, respectively) (Figure 2I).

**Partial correlation coefficients of the condyle BMDs and condyle BMD ratios in the femur and tibia after adjusting for age and BMI.** Medial femoral and tibial condyle BMDs demonstrated a significant positive correlation with the FTA, the presence of medial and lateral osteophytes, medial JSN, lumbar spine and femoral neck BMDs, and VAS pain, and a significant negative correlation with the Knee Society knee, pain, and function scores. Lateral femoral condyle BMD demonstrated a significant negative correlation with the FTA and the presence of medial osteophytes, and a significant positive correlation with lumbar spine and femoral neck BMDs. Lateral tibial condyle BMD demonstrated a significant negative correlation with the pres-

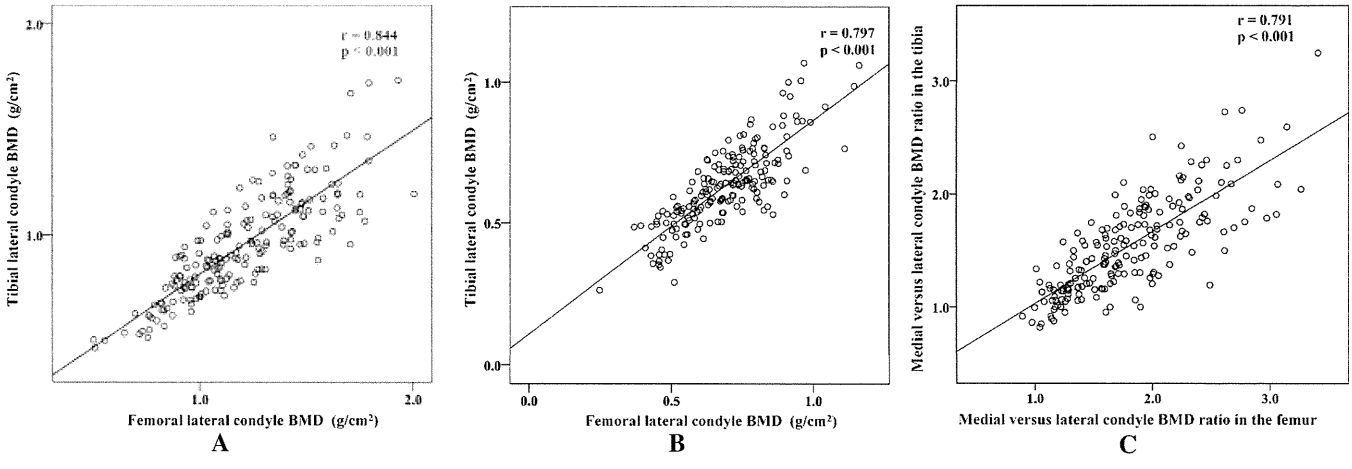


**Figure 1.** Association of **A,** medial condyle bone mineral densities (BMDs), **B,** lateral condyle BMDs, and **C,** medial versus lateral condyle BMD ratios between the femoral and tibial condyles.

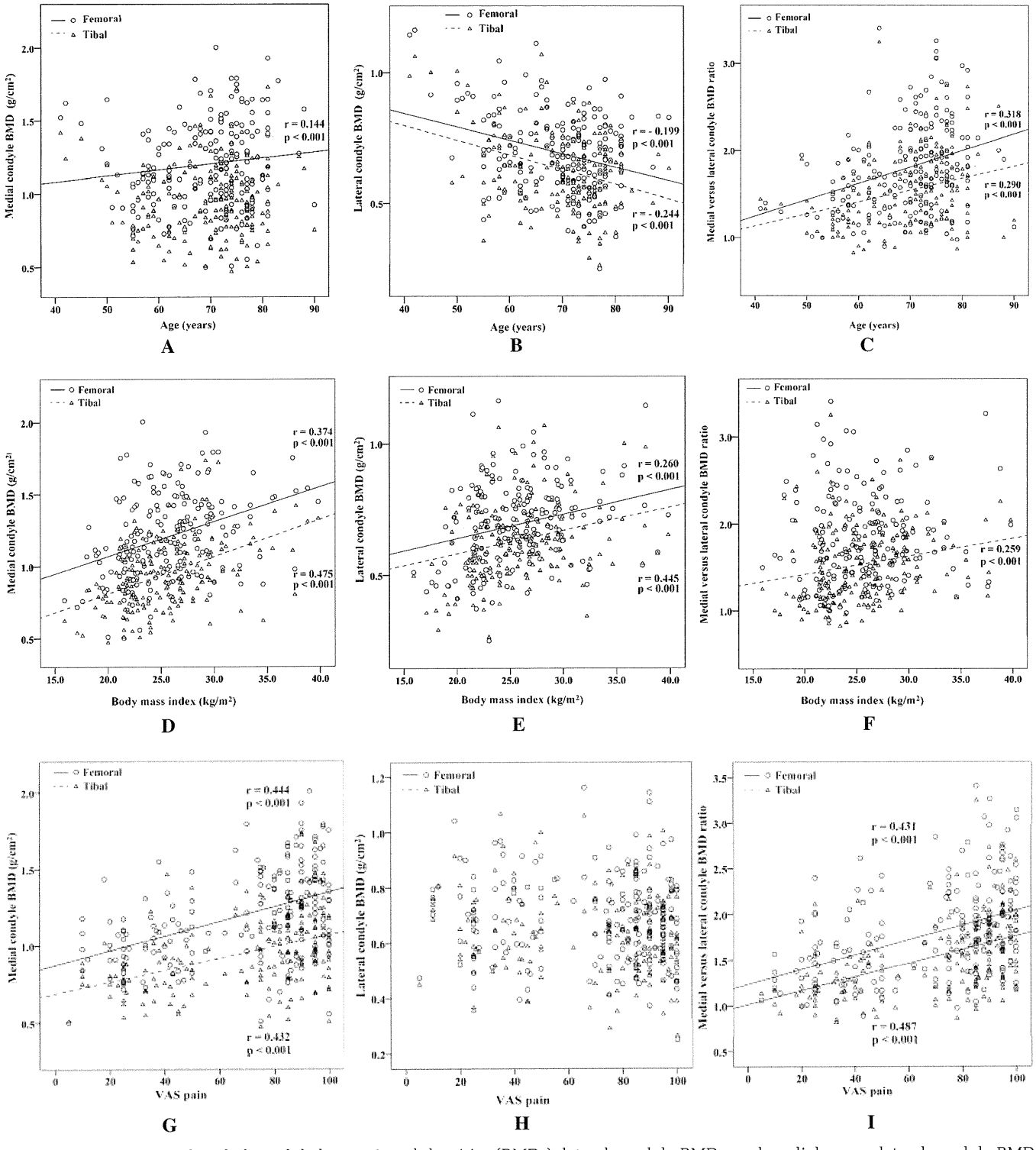


**Figure 2.** Association of medial condyle bone mineral densities (BMDs), lateral condyle BMDs, and medial versus lateral condyle BMD ratios in the femur and tibia with age (**A, B,** and **C,** respectively), body mass index (**D, E,** and **F,** respectively), or visual analog scale (VAS) pain (**G, H,** and **I,** respectively).

ence of medial osteophytes and medial JSN, and a significant positive correlation with lumbar spine and femoral neck BMDs. For the femur and tibia, the medial versus lateral condyle BMD ratio demonstrated a significant positive correlation with the FTA, the presence of medial and lateral osteophytes, medial JSN, and VAS pain, and a significant negative correlation with the Knee Society knee, pain, and function scores. Furthermore, the ratio was

**Table 3. Partial correlation coefficients in 192 women with knee osteoarthritis between the medial vs. lateral BMD ratio, condyle BMDs in the femur, and multivariable factors***

| | Partial correlation coefficients in the femoral condyles | | |
|---|---|---|---|
| | Medial condyle BMD | Lateral condyle BMD | Medial vs. lateral BMD ratio |
| Femorotibial angle, ° | 0.474† | −0.154‡ | 0.516† |
| Medial femoral osteophytes (range 0–3) | 0.442† | −0.158‡ | 0.499† |
| Lateral femoral osteophytes (range 0–3) | 0.354† | 0.001 | 0.261† |
| Medial joint space narrowing (range 0–3) | 0.507† | −0.122 | 0.504† |
| Lumbar spine BMD, gm/cm$^2$ | 0.412† | 0.491† | −0.063 |
| Femoral neck BMD, gm/cm$^2$ | 0.239† | 0.381† | −0.122 |
| Knee Society knee score (range 0–100) | −0.419† | 0.177 | −0.492† |
| Knee Society pain score (range 0–50) | −0.325† | 0.015 | −0.287† |
| Knee Society function score (range 0–100) | −0.270† | 0.094 | −0.312† |
| Visual analog scale pain (range 0–100) | 0.367† | −0.038 | 0.343† |

* Adjusted for age and body mass index. BMD = bone mineral density.
† Statistically significant at $P < 0.001$.
‡ Statistically significant at $P < 0.05$.

not correlated with lumbar spine or femoral neck BMDs (Tables 3 and 4).

## DISCUSSION

After adjusting for age and BMI, we demonstrated that in women with medial knee OA showing joint space obliteration, medial femoral and tibial condyle BMDs, medial versus lateral condyle BMD ratios, and VAS pain in both the femur and the tibia increased. In addition, after adjustment, medial versus lateral condyle BMD ratios in both the femur and the tibia demonstrated significant positive correlations with the FTA, the presence of medial and lateral osteophytes, medial JSN, and VAS pain, whereas they demonstrated significant negative correlations with the Knee Society knee, pain, and function scores. The femoral and tibial medial versus lateral condyle BMD ratios increased with more severe knee pain.

Patients with knee OA usually have increased tibial BMD (8–10). Our results demonstrated that in both the femur and the tibia of patients with joint space obliteration, medial condyle BMDs increased and lateral condyle

**Table 4. Partial correlation coefficients in 192 women with knee osteoarthritis between the medial vs. lateral BMD ratio, BMDs in the tibial condyles, and multivariable factors***

| | Partial correlation coefficients in the tibial condyle | | |
|---|---|---|---|
| | Medial condyle BMD | Lateral condyle BMD | Medial vs. lateral BMD ratio |
| Femorotibial angle, ° | 0.497† | −0.096 | 0.491† |
| Medial femoral osteophytes (range 0–3) | 0.504† | −0.172‡ | 0.600† |
| Lateral femoral osteophytes (range 0–3) | 0.361† | −0.034 | 0.334† |
| Medial joint space narrowing (range 0–3) | 0.499† | −0.193† | 0.586† |
| Lumbar spine BMD, gm/cm$^2$ | 0.450† | 0.484† | 0.008 |
| Femoral neck BMD, gm/cm$^2$ | 0.355† | 0.440† | −0.046 |
| Knee Society knee score (range 0–100) | −0.467† | 0.147 | −0.545† |
| Knee Society pain score (range 0–50) | −0.401† | −0.002 | −0.366† |
| Knee Society function score (range 0–100) | −0.278† | 0.092 | −0.352† |
| Visual analog scale pain (range 0–100) | 0.372† | −0.058 | 0.388† |

* Adjusted for age and body mass index.
† Statistically significant at $P < 0.001$.
‡ Statistically significant at $P < 0.05$.

BMDs did not change. Furthermore, the medial versus lateral condyle BMD ratios in both the femur and the tibia increased with advancing knee OA. These findings were consistent with the opinion that the medial tibial condyle BMD predicted cartilage defect increases in the medial tibial condyle (16). We adjusted our data for age and BMI on the basis of results obtained in this as well as in previous studies (15,16). After adjustment, lumbar spine BMD was significantly higher in the obliteration group compared with the narrowing group. This finding may be significant because higher lumbar spine BMD values are reportedly associated with degenerative disease (35–37).

Medial condyle BMD, lateral condyle BMD, and medial versus lateral condyle BMD ratios in both the femur and the tibia demonstrated a significant positive correlation. Previous studies have measured BMD of the tibial condyles (5,13–24). These results showed that the femoral condyles were apparently appropriate sites to measure condyle BMD, as the tibial condyles were appropriate sites.

The lateral condyle BMDs in both the femur and the tibia decreased with age, whereas the medial condyle BMD in the tibia increased with age. The decreases in lateral femoral and tibial condyle BMDs with advancing age could be attributed to general osteoporosis. We stated in a previous study that lateral tibial condyle BMD is probably a better reflection of BMD at the knee than medial tibial condyle BMD (38); furthermore, the decreased lateral condyle BMDs with age may represent a postmenopausal decrease in the general BMD. Alternatively, the finding that the medial condyle BMDs did not decrease with age might result in subchondral bone sclerosis, followed by the progression of medial knee OA with age. The increases in the medial and lateral femoral and tibial condyle BMDs with BMI were probably a reaction of the bone against weight bearing. However, the medial versus lateral condyle BMD ratio of only the tibia was correlated with BMI. Dore et al (15) found that BMD of the medial tibial condyle was negatively associated with age and positively associated with BMI. Our results demonstrated that BMD of the medial tibial condyle was not associated with age but positively associated with BMI. A possible reason for this discrepancy was that our subjects included many cases with advanced medial knee OA. In a high-resolution MRI study, it was observed that structural parameters derived from the femoral condyles could be potential markers for evaluating disease progression (12). The structure of trabecular bone in the tibia is different compared with that in the femur, and these differences in loading are caused by the convexity of the distal femur and the relative flatness of the proximal tibia (12). Although our results did not show an obvious advantage of BMD measurements in the femoral condyles, the medial versus lateral condyle BMD ratio in the femur was not affected by BMI.

After adjustment, in both the femur and the tibia, the presence of medial osteophytes was positively correlated with BMD of the medial condyle but negatively correlated with BMD of the lateral condyle. However, the presence of lateral osteophytes was positively correlated with BMD of the medial condyle but not with BMD of the lateral condyle. Dore et al (15) stated that medial tibial condyle BMD was positively correlated with the presence of medial tibial osteophytes, which was consistent with our findings. They also stated that lateral tibial condyle BMD is correlated with the presence of lateral tibial osteophytes (15); however, this result was not consistent with the results of our study, which was limited in medial knee OA.

After adjustment, subchondral BMD of the medial femoral and tibial condyles was correlated with a higher medial JSN score, indicating a relationship between changes in subchondral bone and cartilage loss (2). Moreover, higher medial condyle BMD may suggest a protective role of subchondral bone in the progression of knee OA (39). Conversely, the medial tibial BMD was higher in subjects who had cartilage defects, and it is reported that denser subchondral bone may increase the risk for developing cartilage defects (16). In addition, higher medial condyle BMD may be a good predictor of medial JSN 1 year after the onset of medial knee OA (23). Both osteophytes and JSN are correlated with higher medial tibial BMD (5), which was in agreement with our results. Our results along with previous results (18) indicate that the medial versus lateral condyle BMD ratio in the tibia was positively associated with medial JSN. Also, it was reported in previous studies that the medial versus lateral condyle BMD ratio in the tibia is negatively associated with lateral JSN (18). Our subjects were excluded if their knees had a valgus deformity, and 95.8% of knees demonstrated a score of 0 for lateral JSN. In this study, the association between the medial versus lateral condyle BMD ratio in the tibia and lateral JSN remained unclear. We agree with the opinion that among BMD values of the spine, femoral neck, and tibial condyle, tibial condyle BMD may have different determinants than those for BMD at the other sites; in addition, a higher BMD may reflect inflammatory changes in the bone, which lead to increased density (16).

After adjustment, BMDs of the medial and lateral femoral and tibial condyles correlated with those of the lumbar spine and femoral neck; this finding was consistent with that of a previous study involving the tibia (15). However, the medial versus lateral condyle BMD ratios of the femur and tibia did not correlate with BMDs of the lumbar spine and femoral neck. Regardless of BMDs at other sites, the medial versus lateral condyle BMD ratios in both the femur and the tibia may represent the severity of medial knee OA.

Lingard et al (40) reported that Knee Society pain and function scores had moderate to strong correlations with the corresponding pain and function domains of the Western Ontario and McMaster Universities Osteoarthritis Index (41) and the Medical Outcomes Study Short Form 36 (42). In this study, Knee Society pain and function scores were negatively correlated with the medial condyle BMDs and with the medial versus lateral condyle BMD ratios in the femur and tibia. VAS pain was positively correlated with the medial condyle BMDs and with the medial versus lateral condyle BMD ratios. Interestingly, the increase in

knee pain and the decrease in the function score were associated with higher BMD of the medial condyle and higher medial versus lateral condyle BMD ratios in both the femur and the tibia, but were not associated with BMD of the lateral condyle. Also, knee pain is associated with denudation of the bone (30), which was in agreement with our result that knee pain was higher in the obliteration group compared with the narrowing group, as shown in Table 1. The function score represented continuous walking distance and the ability to ascend and descend stairs. Dore et al (15) reported that BMD of the medial condyle, which indicates a response to mechanical stressors, is positively correlated with steps per day. Therefore, BMD of the medial condyle of patients with medial knee OA may be affected by the frequency or strength of weight bearing. Moreover, increased pain in patients with knee OA is associated with increased loading frequency, which is represented by steps per day (43).

We adjusted the size of the ROIs to account for the absolute proportion of individual knees. Because men have a larger bone area and a higher BMD (20), we enrolled only women. Our ROIs have the advantage of being adjusted to account for the tibial plateau length of individual knees in the coronal plane. In addition, extension of the knee is associated with pain or swelling in many patients with rotation deformities of the tibia, which may result in the inability to achieve complete knee extension. Our semiflexed leg position allows for reproducible positioning of the tibia (20). Moreover, our femoral ROIs were not influenced by the patella.

Wada et al measured trabecular BMD beneath the subchondral plate and stated that a high BMD was attributed to a thicker trabecular bone and a microfracture in the cancellous bone (21). The ROIs of our measurements mainly detected both sclerosis of trabecular bone and width of cortical bone. Doré et al measured the area of the subchondral cortical plate and trabecular bone in the tibial condyles and found an association between subchondral BMD and medial cartilage defects in the subchondral cortical plate (16). However, Lo et al stated that DXA did not provide details on the specifics of trabecular mineralization or subchondral plate thickness (19). Wong et al found that subchondral plate thickening and subchondral collapse may be observed not only in advanced OA but also in the early stages of OA (6). Subchondral bone comprises the subchondral plate and the subarticular spongiosa. Bone proliferation results in an increase in subchondral plate thickness (3). However, the etiology of a microfracture in the subchondral bone is presently inconsistent. Microfractures occur in the calcified zone of the articular cartilage and also in the subchondral trabecular bone. At later stages, after the subchondral bone has been exposed, deformation occurs as a result of subchondral bone plate microfractures, suggesting that changes in the subchondral bone may play a larger role than that played by changes in the underlying trabeculae (3). Moreover, the association between varus inclination of the tibial plateau and low BMD suggests that microfractures may occur before the eburnation of the subchondral bone (38,44). In addition, previous experimental data have revealed that edema of the medial bone marrow, which was identified on MRI, is associated with varus deformities in patients with medial knee OA (45), but it is unknown whether edema of the bone marrow affects structural changes.

This study has a number of limitations. First, no consensus was reached on an appropriate ROI to measure BMD of the knee condyles (20). Second, we divided our patients into the JSN and obliteration groups on the basis of radiographs; however, we did not confirm the groups using MRI or arthroscopy. Third, our study, which was targeted at women with symptomatic medial knee OA, included many women with advanced knee OA; therefore, a proper investigation of patients with the early phase of knee OA would be necessary in the future. Also, men were excluded from this study, which meant that our results are only generalizable to women. Finally, this was a cross-sectional study, and future longitudinal studies are needed to confirm these results.

In conclusion, in both the femur and the tibia of women with medial knee OA, the medial condyle BMDs, medial versus lateral condyle BMD ratios, and VAS pain were significantly higher in the obliteration group compared with the narrowing group. After adjusting for age and BMI, the femoral and tibial medial versus lateral condyle BMD ratios demonstrated significant positive correlations with the FTA, the presence of medial and lateral osteophytes, medial JSN, and VAS pain, whereas it demonstrated a significant negative correlation with the Knee Society pain and function scores. Although this study was a cross-sectional study, the femoral and tibial medial versus lateral condyle BMD ratios increased with more severe knee pain and might be potential markers for monitoring the severity of medial knee OA in women.

## AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Akamatsu had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
**Study conception and design.** Akamatsu, Mitsugi, Saito.
**Acquisition of data.** Akamatsu, Taki, Kobayashi.
**Analysis and interpretation of data.** Akamatsu, Mitsugi, Saito.

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# Association between venous thromboembolism and plasma levels of both soluble fibrin and plasminogen-activator inhibitor 1 in 170 patients undergoing total hip arthroplasty

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Background and purpose **Markers of coagulation and fibrinolysis, such as soluble fibrin (SF), D-dimer, and plasminogen activator inhibitor 1 (PAI-1), have been developed in order to determine thrombotic tendency. We investigated whether these markers could be used to diagnose venous thromboembolism (VTE) in the early phase after primary total hip arthroplasty (THA).**

Methods **This prospective study involved 2 groups: an intermittent pneumatic compression (IPC) group (67 patients who underwent IPC only as prophylaxis for VTE) and a fondaparinux (FPX) group (103 patients who received IPC and FPX postoperatively). Plasma levels of SF and PAI-1 were measured on postoperative day 1. To diagnose postoperative VTE, multi-detector row computed tomography (MDCT) and duplex ultrasonography (US) were performed on postoperative day 7.**

Results **VTE was detected postoperatively in 17 cases in the IPC group (25%) and in 8 cases in the FPX group (6%). In the IPC group, plasma levels of SF and PAI-1 were higher in patients with VTE ($p < 0.01$) than in those without VTE. On the other hand, in the FPX group there were no differences in the levels of SF or PAI-1 measured before administration of FPX on postoperative day 1. The diagnostic criterion of an increase in SF or PAI-1 above the cutoff level (19.8 μg/mL and 53.5 ng/mL, respectively) provided a sensitivity of 100% and a specificity of 67% in the IPC group. In addition, when this criterion was applied to FPX patients, 7 of the 8 patients with VTE met the criterion, and there was a negative agreement rate of 48/49.**

Interpretation **Screening using the cutoff levels of SF and PAI-1 may be useful and shows high sensitivity in predicting postoperative VTE in the early phase after THA.**

Randomized clinical trials have shown that the rate of deep vein thrombosis (DVT) after total hip arthroplasty (THA) in patients who do not receive thromboprophylaxis is 42–57% (Geerts et al. 2008). The American College of Chest Physicians (ACCP) recommends routine chemoprophylaxis using anticoagulant drugs after total joint arthroplasty of the hip or knee (Geerts et al. 2008). However, chemoprophylaxis generally involves the threat of postoperative bleeding, which must be balanced against the risk of thrombotic events.

In Japan, the frequency of postoperative venous thromboembolism (VTE) after THA when no anticoagulant prophylaxis is employed is 23–42% (Fujita et al. 2000, Fuji et al. 2008). The same emphasis has been put on VTE prophylaxis as in western countries since the first Japanese guidelines for VTE prophylaxis were prepared in 2004 (The first edition guidelines for prevention of venous thromboembolism, 2004). The latest guidelines limit chemoprophylaxis to a period of 14 days after surgery, and they also suggest intermittent pneumatic compression (IPC) without any administration of anticoagulants as one choice for postoperative prophylaxis because of its preventative effects (Sugano et al. 2009, Husted et al. 2010). Thus, there is a controversy among Japanese surgeons about what kind of thromboprophylaxis should be employed after major orthopedic surgery. Also in western countries, some authors have questioned whether routine chemoprophylaxis is necessary (Callaghan et al. 2005, Dorr et al. 2007). Thus, a useful screening method to determine which patients are at high risk of postoperative VTE should be of value.

Previously, tests for global screening of the coagulation system were considered to be unhelpful in the diagnosis of thrombotic events. However, recent biochemical studies of the coagulation and fibrinolysis systems have led to the availability


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of specific and sensitive tests that can detect thrombosis. At the same time, many different markers have been found to show increased expression in clinical disorders in which there is an imbalance between coagulation and fibrinolysis. We examined acute postoperative changes in (1) soluble fibrin (SF), a complex of fibrin monomer and fibrinogen derivatives; (2) thrombin-antithrombin complex (TAT), a marker of thrombin generation; (3) D-dimer, a proteolytic fragment resulting from degradation of a fibrin clot; and (4) plasminogen-activator inhibitor 1 (PAI-1), which is the main regulator of the fibrinolysis system. In addition, we evaluated the usefulness of assaying these markers as predictors of early VTE following THA.

## Patients and methods

170 patients with a mean age of 63 (38–85) years who were scheduled for THA between 2007 and 2010 were included. Patients were excluded if they had any of the following conditions: (1) a prior THA requiring revision; (2) a previous history of VTE; (3) a pre-existing malignant tumor; or (4) renal failure (estimated glomerular filtration rate < 50 mL·min-1·1.73 m-2). Before surgery, all patients were provided with a detailed explanation of the risks and alternatives to participation in the study, and all provided written informed consent. The study was approved by the Institutional Review Board of Yokohama City University (approval no., 01-10-2007-058).

### *Prophylaxis for VTE*

There were 2 patient groups: the IPC group (67 patients who underwent primary THA between 2007 and 2008) and the fondaparinux (FPX) group (103 patients who underwent THA between 2008 and 2010). At our institution, FPX has been used routinely since September 2008. IPC was performed on all patients in both groups during surgery (under anesthesia), and the patients were given unfractionated heparin (UFH) intravenously in a single dose of 20 IU/kg of body weight (Sharrock et al. 1999). IPC was maintained postoperatively. Patients usually started walking 1–2 days after surgery. The patients in the FPX group were also given 2.5 mg of FPX subcutaneously every day for 14 days, starting on postoperative day 1.

### *Perioperative management*

All patients were operated under general anesthesia. THA using a computed tomography-based navigation system was performed through a minimally-invasive anterolateral approach with the patient in the lateral decubitus position. During surgery, all patients received up to 1,500 mL of Ringer's solution or hydroxyethyl starch. Fluid management after surgery until the next morning was routinely done with 1,000 mL of Ringer's solution and 500 mL of maintenance fluid.

Postoperative mobilization followed a protocol supervised by experienced orthopedic physiotherapists. The patients were allowed discharge on postoperative day 14, the day this mobilization protocol was completed.

### *Diagnostic methods for VTE*

Duplex ultrasonography (US) was performed on all patients 28 days before surgery in order to detect any evidence of previous DVT of the lower limb. Absent or incomplete compressibility of the vein was the diagnostic criterion. For the determination of postoperative VTE, an angiography of the pulmonary artery and deep veins on the pelvis and the lower limbs was performed on all patients on postoperative day 7, by 64-slice multidetector row computed tomography (MDCT) using a nonionic contrast agent. The diagnostic criterion for VTE was the presence of a defect of intraluminal filling because of thrombosis in the pulmonary artery or deep vein. When the presence of VTE was suspected by MDCT, a duplex US was also employed in order to confirm the presence of DVT.

Following detection of the postoperative VTE, patients with VTE were administered UFH intravenously in order to maintain the activated partial thromboplastin time at 1.5–2.5 times that of the control value. Warfarin was administered at the same time, and the international normalized ratio was maintained at 2.0–2.5.

### *Blood samples*

Blood samples were obtained from peripheral veins under short fasting conditions early in the morning, on the preoperative day and on postoperative days 1, 3, 7, and 14. Plasma SF levels were measured using a latex photometric immunoassay (IATRO SF II; Mitsubishi Chemical Medience Corporation, Tokyo, Japan) with IF-43 monoclonal antibody raised against a urea-solubilized fibrin monomer. The normal upper limit for SF was < 7 μg/mL. PAI-1 was measured using a latex photometric immunoassay (LPIA-tPAI Test; Mitsubishi Chemical Medience Corporation) using a reference range of 10–50 ng/mL. Furthermore, preoperative levels of lipids such as triglycerides and total cholesterol were measured, and their association with PAI-1 was determined. Plasma D-dimer levels were also assayed with a latex photometric immunoassay (LPIA-ACE D-dimer; Mitsubishi Chemical Medience Corporation). The normal limit was < 0.7 μg/mL. TAT was measured by enzyme-linked immunosorbent assay (ELISA) with a reference range of 0.1–5.0 ng/mL (Enzygnost TATmicro; Siemens Healthcare Diagnostics Inc., Tokyo, Japan).

### *Statistics*

Statistical analyses were performed using SPSS II software. According to a Kolmogorov-Smirnov analysis, the coagulation and fibrinolysis variables showed a skewed distribution. Thus, these variables are presented as medians. The medians and interquartile ranges are plotted in the figures as a box-and-whisker plot. In the figures, the vertical bars (whiskers) represent the 5th and 95th percentiles and the horizontal bars in the boxes represent the medians. In addition, differences in

**Patient characteristics**

| Characteristics | IPC group Patients with VTE n = 17 | IPC group Patients without VTE n = 50 | FPX group Patients with VTE n = 6 | FPX group Patients without VTE n = 97 | p-value |
|---|---|---|---|---|---|
| Age, years [a] | 68 (8) | 62 (12) | 58 (8) | 61 (12) | 0.1 |
| Gender: male/female, n | 3/14 | 17/33 | 6/0 | 21/76 | 0.5 |
| Weight, kg [a] | 58 (14) | 58 (13) | 59 (14) | 58 (13) | 0.9 |
| Body mass index [a] | 24 (6) | 23 (5) | 24 (5) | 24 (5) | 0.9 |
| Primary hip disease, n | | | | | 0.8 |
| Osteoarthritis | 15 | 36 | 4 | 79 | |
| Rheumatoid arthritis | 0 | 3 | 0 | 7 | |
| ANFH | 2 | 6 | 2 | 11 | |
| PVNS | 0 | 1 | 0 | 0 | |
| Preoperative plasma levels of: | | | | | |
| Triglycerides, mg/dL [a] | 92 (35) | 108 (40) | 99 (32) | 98 (41) | 0.7 |
| Total cholesterol, mg/dL [a] | 228 (44) | 199 (31) | 202 (26) | 222 (36) | 0.7 |
| Operation length, min [a] | 182 (62) | 162 (36) | 153 (39) | 161 (37) | 0.2 |
| Blood loss, mL [a] | 557 (193) | 547 (217) | 525 (137) | 624 (265) | 0.4 |

[a] Values are mean (SD).
IPC: intermittent pneumatic compression; FPX: fondaparinux sodium; VTE: venous thromboembolism; OA: osteoarthritis; RA: rheumatoid arthritis; ANFH: avascular necrosis of femoral head; PVNS: pigmented villonodular synovitis.

the variables between patients with VTE and those without were examined statistically using Student's t-test or a two-tailed Mann-Whitney U-test. Furthermore, the chi-square test and Fisher's exact probabilities were used for the comparison between the observed and expected frequencies. The usefulness of the markers for the diagnosis of VTE was assessed by a receiver operating characteristic curve analysis. Values of $p < 0.05$ were considered statistically significant.

## Results

In the IPC group, postoperative VTE was detected in 17 of the 67 patients (25%), and the incidences of pulmonary embolism (PE) only, deep vein thrombosis (DVT) only, and both PE and DVT were 3, 12, and 2, respectively. In the FPX group, VTE was detected in 8 of the 103 patients (7%), and the incidences of PE only, DVT only, and both PE and DVT were 3, 5, and 0, respectively. The difference in the frequency of occurrence of VTE between the IPC and FPX groups was statistically significant ($p < 0.01$). All DVT occurred in the calf vein, and there were no cases of symptomatic DVT or PE. The distributions of age, sex, body mass index (BMI), duration of surgery, blood loss, and previous illness were similar in patients with and without VTE, and between the IPC and FPX groups (Table).

In the IPC group, plasma SF levels on postoperative day 1 in patients with VTE were higher than those in patients without VTE ($p < 0.01$) (Figure 1), and the median values were 38 μg/mL and 10 μg/mL, respectively. Plasma SF levels rapidly decreased to reference range by postoperative day 3. On the other hand, in the FPX group SF levels were similar in patients with and without VTE (Figure 2).

In the IPC group, plasma PAI-1 levels on postoperative day 1 in patients with VTE were higher than those in patients without VTE ($p < 0.01$) (Figure 1). The median PAI-1 values on postoperative day 1 were 93 ng/mL for patients with VTE and 40 ng/mL for patients without VTE. In addition, in both the IPC and FPX groups plasma D-dimer levels showed bimodal peaks that were evident on postoperative days 1 and 7. In the IPC group, significant differences in the plasma D-dimer levels were not seen on postoperative day 1, but they were seen on postoperative day 7 ($p < 0.01$). The median D-dimer values on day 7 were 21 μg/mL for patients with VTE and 10 μg/mL for patients without VTE (Figures 1 and 2).

IPC patients with VTE also showed higher levels of TAT on postoperative day 1 ($p < 0.05$); the median values were 24 μg/mL for patients with VTE and 14 μg/mL for patients without VTE. On the other hand, statistically significant differences in TAT levels were not found in patients of the FPX group.

To evaluate the usefulness of the markers SF, PAI-1, and TAT that were detected in the IPC group on postoperative day 1, their cutoff levels with sensitivities and specificities were determined by receiver operating characteristic (ROC) analysis (Figure 3). The cutoff level of SF was determined to be 19.8 μg/mL, with a sensitivity of 88% and a specificity of 62%. The cutoff level of PAI-1 was 53.5 ng/mL, with a sensitivity of 78% and a specificity of 72%, and that of TAT was found to be 18.1 ng/mL with a sensitivity of 85% and a specificity of 66%. Next, using a multivariate logistic regression analysis, we found that SF and PAI-1 levels on postoperative day 1 had statistically the strongest association with thrombotic tendency. Furthermore, comparison of the area under the ROC curves showed that the measurements of SF and PAI-1 on postoperative day 1 had the best performance with respect

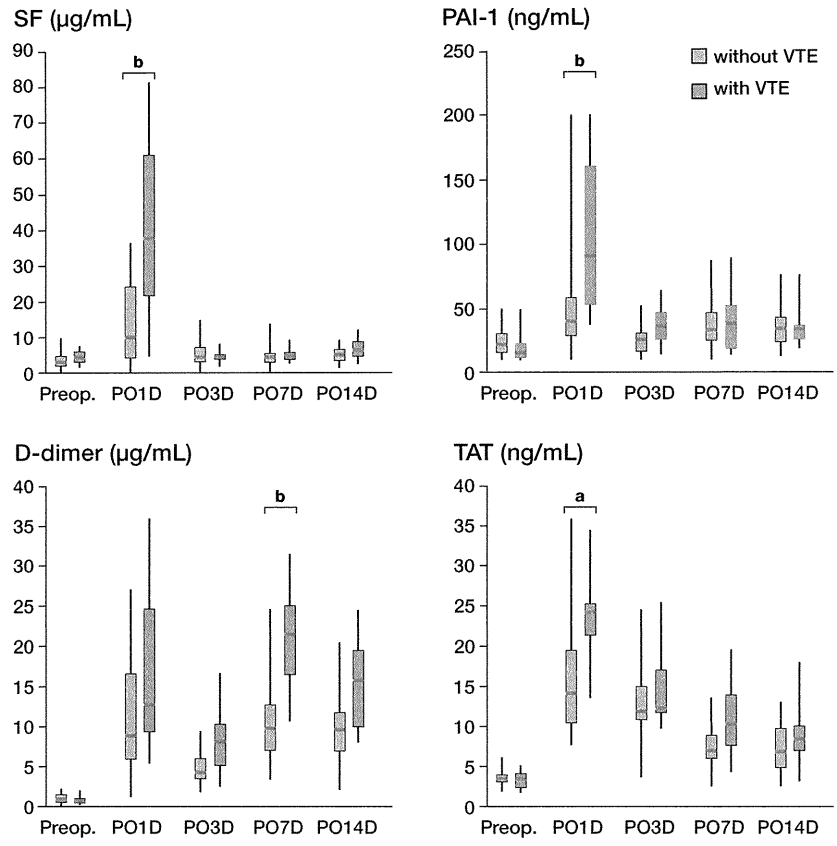


Figure 1. Changes in coagulation markers in patients who underwent intermittent pneumatic compression only. Plasma levels of soluble fibrin (SF), plasminogen activator inhibitor type 1 (PAI-1), D-dimer, and thrombin-antithrombin complex (TAT) in patients who underwent intermittent pneumatic compression alone were measured preoperatively and on postoperative days 1 (PO1D), 3 (PO3D), 7 (PO7D), and 14 (PO14D). The boxes represent the interquartile ranges. The perpendicular lines (whiskers) represent the fifth and ninety-fifth percentiles and the horizontal bars in the boxes indicate the median values. On the day after surgery, the plasma levels of SF, PAI-1, and TAT were found to be increased in the venous thromboembolism (VTE) group compared to the non-VTE group. The changes in the D-dimer levels showed bimodal peaks on postoperative days 1 and 7 in both groups. Statistically significant differences were observed in the D-dimer levels measured on postoperative day 7. [a] $p < 0.05$ and [b] $p < 0.01$.

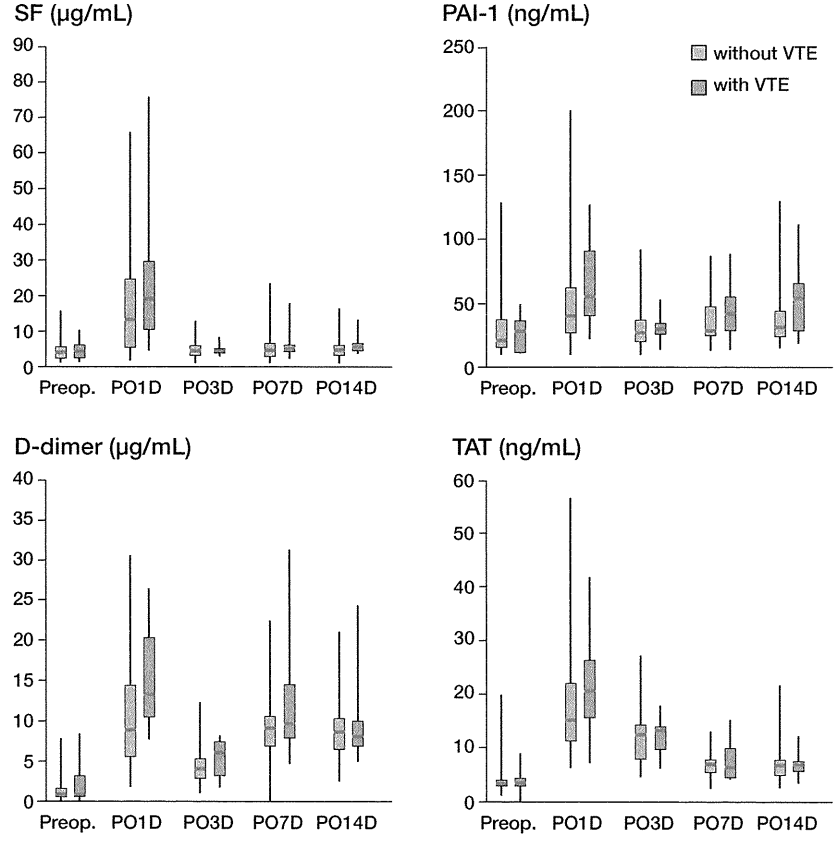


Figure 2. Changes in coagulation markers in patients who received subcutaneous injections of fondaparinux sodium. Plasma levels of soluble fibrin (SF), plasminogen activator inhibitor type 1 (PAI-1), D-dimer, and thrombin-antithrombin complex (TAT) in patients who received subcutaneous injections of fondaparinux sodium were measured preoperatively and on postoperative days 1 (PO1D), 3 (PO3D), 7 (PO7D), and 14 (PO14D). The boxes represent the interquartile ranges. The perpendicular lines represent the fifth and ninety-fifth percentiles and the horizontal bars in the boxes indicate the median values. The levels of SF, PAI-1, D-dimer, and TAT were similar in the patients in the fondaparinux group who had or did not have VTE.

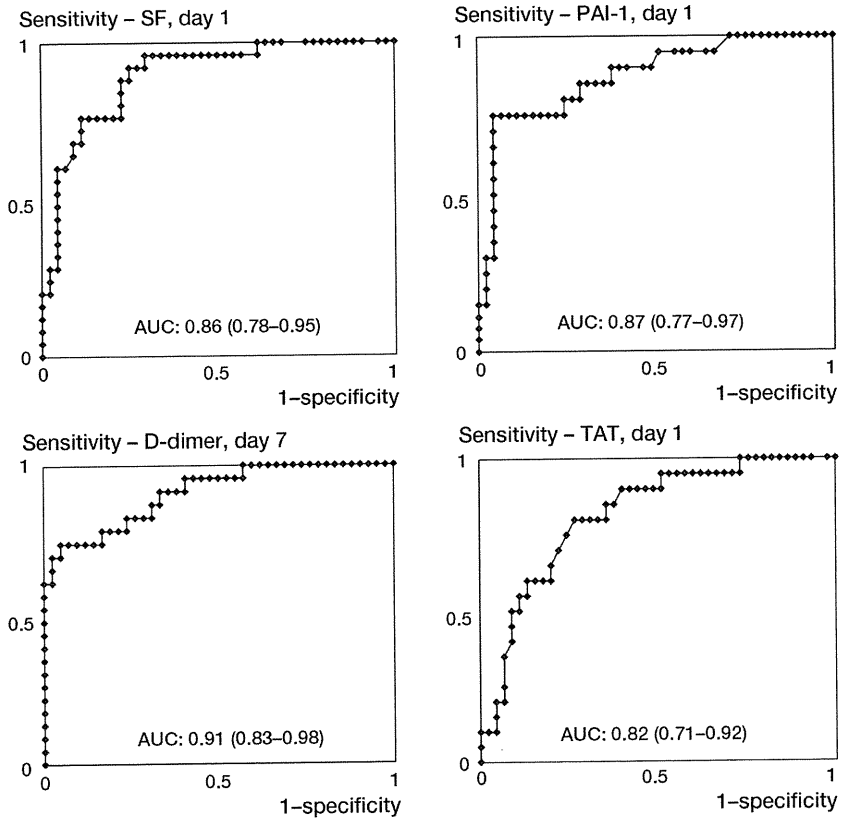


Figure 3. Receiver operating characteristic (ROC) curve analysis of the accuracy of the quantitative soluble fibrin (SF), plasminogen activator inhibitor type 1 (PAI-1), and thrombin-antithrombin complex (TAT) levels on postoperative day 1 and D-dimer levels on day 7. The area under the ROC curve (AUC) is given in each diagram with the 95% confidence interval in parentheses.

to their discrimination ability (Figure 3). Also, there was no statistically significant correlation between the levels of PAI-1 and the levels of other coagulation markers.

Figure 4 shows scatter graphs of SF and PAI-1 levels, with 2 lines at each cutoff level. These lines divide the patients into 2 groups: those with higher marker levels and those with lower levels, and these divisions provided a sensitivity of 100%, a specificity of 67%, and a positive predictive value of 50%. In addition, when this criterion was applied to patients in the FPX group, 7 of the 8 with VTE met the criterion and there was a negative agreement rate of 98.0% (48/49).

## Discussion

VTE is a common complication after total hip or knee arthroplasty, but most VTE is generally asymptomatic. Due to the lack of symptoms, the condition goes mostly unnoticed and the patient is therefore left untreated. However, approximately 10–20% of crural thrombi, most of which are asymptomatic DVT, move to the proximal veins (Labropoulos et al. 2002). Proximal propagation of a thrombus can lead to free-floating

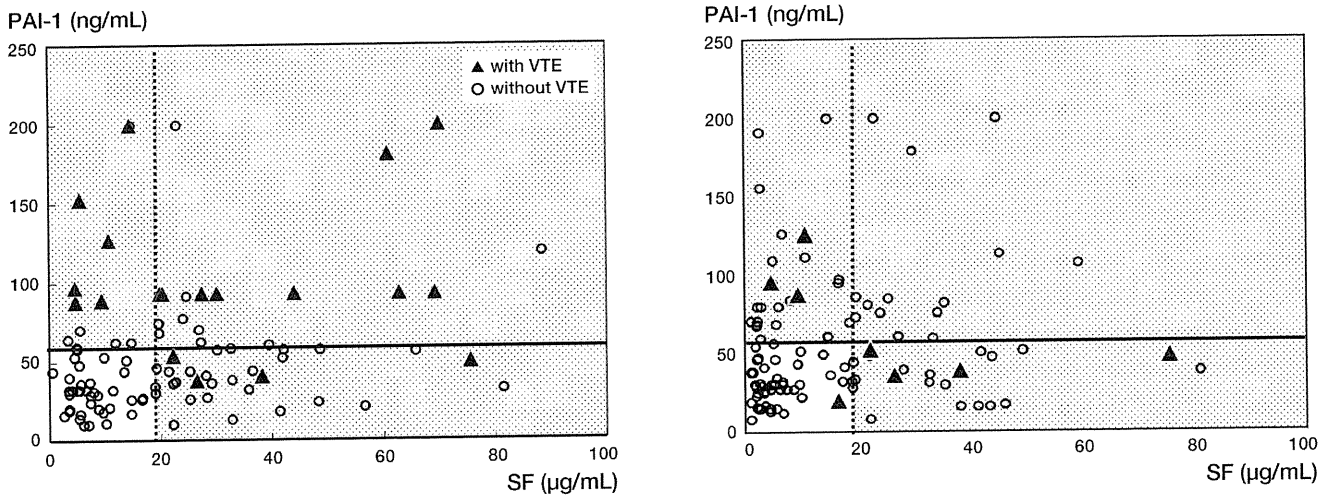


Figure 4. Discrimination of postoperative venous thromboembolism (VTE) using levels of soluble fibrin (SF) and plasminogen activator inhibitor type 1 (PAI-1). Increases in either SF or PAI-1 on postoperative day 1 above their cut-off levels provided 100% sensitivity and 67% specificity in predicting VTE when patients were not given fondaparinux sodium postoperatively (left panel). In addition, when this criterion was applied to patients who received subcutaneous injections of fondaparinux following surgery, 7 of the 8 patients with VTE met the criterion and a 98% (48/49) negative agreement rate was found (right panel).

status, resulting in critical complications after being released as a massive embolus (Baldridge et al. 1990). According to the PREVENT study (Ridker et al. 2003), the recurrence rate of VTE is approximately 7%, and long-term, low-intensity warfarin therapy reduces this risk by more than half. Thus, we suggest that all cases of VTE—including asymptomatic VTE—should be detected and treated with anticoagulants, and that an early and simple modality for screening of VTE should be established.

Highly invasive surgery has been shown to commonly result in a hypercoagulable state and increasing plasma levels of SF and TAT during the initial postoperative stage (Brueckner et al. 2003, Sudo et al. 2009). This is consistent with our present findings that plasma SF and TAT levels were elevated on postoperative day 1. Also, considering the substantial elevation of these markers in patients who developed VTE in the IPC group, we suggest that the onset of VTE is associated with a hypercoagulable state in the early phase after THA.

A high level of SF in clinical plasma samples has been recognized to be an indicator of ongoing intravascular coagulation processes (Hamano et al. 2005). SF expresses an acute intravascular fibrin formation as well because SF is one of circulating materials growing fibrin clots. Regarding the usefulness of SF for possible diagnosis of VTE, it was previously suggested that SF levels on the day after total hip or knee arthroplasty may be valuable for prediction of postoperative VTE (Sudo et al. 2009, Niimi et al. 2010).

Similarly to SF, TAT is considered to be associated with VTE following THA (Sudo et al. 2009). Formation of TAT is, however, only an indirect measure of an activated coagulation system (Brueckner et al. 2003), and its measurement is often influenced by the peripheral blood sampling techniques used under venous occlusion. Sensitivity and specificity of TAT measurement on the day after THA have been reported to be 73% and 27%, respectively (Cofrancesco et al. 1998). Thus, TAT has been found to be inferior to other markers as a predictor of VTE (Brueckner et al. 2003, Hamano et al. 2005).

PAI-1 is the principal inhibitor and critical regulator of plasminogen activator. Several clinical studies have found that plasma PAI-1 levels may be affected by lipid levels or vascular endothelial cell injury, and these factors may therefore increase the risk of thrombosis or embolism (Hamsten et al. 1987, Juhan-Vague et al. 1987). Furthermore, PAI-1 is produced at the site of inflammation following tissue injury. It has been reported that plasma PAI-1 levels are associated with surgical invasion, and that the increased levels of the fibrinolytic inhibitor that result from this may therefore be a major contributor to fibrinolytic shutdown (Kassis et al. 1992). In the present study, plasma PAI-1 levels were substantially elevated in IPC patients with VTE, despite the absence of any significant differences in other thrombotic risk factors such as lipid levels, BMI, or pre-existing diseases. Thus, we believe that changes in the fibrinolytic system may also be associated with the development of VTE.

Plasma D-dimer levels increase after the cleavage of formed thrombi by activated plasmin, and they have been suggested to be a useful marker for the diagnosis of VTE. The sensitivity and specificity for proximal DVT were 79% and 36%, respectively. In the present study, D-dimer measurements on postoperative day 7 were different in patients with and without VTE in the IPC group. However, at this stage, VTE is already evident, and we therefore suggest that D-dimer is not useful as an early predictor of this disorder.

An imbalance between coagulation and fibrinolysis contributes to excessive fibrin deposition in the vascular wall because both systems are composed of a complex cascade of molecules and closely influence each other (Aso 2007). Our results indicate that this imbalance can be detected with a combined assay involving SF as a coagulation marker and PAI-1 as a fibrinolytic marker. As shown in Figure 4, some of the patients with VTE had high levels of SF but low PAI-1 levels, whereas some had low SF levels and high PAI-1 levels. These data suggest that the onset of VTE after THA may be due to a hypercoagulable state, a highly regulated fibrinolytic state, or both.

We think that the combined measurement of SF and PAI-1 on postoperative day 1 is a useful screening method for patients who are at high risk of developing postoperative VTE. Our findings suggest that plasma levels of SF and PAI-1 on postoperative day 1 have the potential to provide an alternative chemoprophylaxis regimen for VTE after THA. However, the high sensitivity of this screening for prediction of VTE on day 7 was observed in patients receiving perioperative heparin and short-term IPC. It is necessary to investigate this further using different thromboprophylaxis methods and various imaging modalities. In addition, investigation of late VTE, which occurs 2–3 months after surgery, should be performed to confirm whether chemoprophylaxis is necessary for low-risk patients.

The levels of SF, PAI-1, TAT, and D-dimer in patients in the FPX group were similar between patients with and without VTE. This is because the use of FPX regulated the hypercoagulable state and the formation of thrombi, even in patients whose levels of coagulation markers were high in the early phase of surgery. Also, the reason why there were no differences in the levels of D-dimer between patients with and without VTE may be that FPX functioned as a treatment agent for already-developed VTE.

In both the IPC and FPX groups, VTE may have developed in some cases before the first measurements. Thus, delayed screening for VTE—i.e. on the day after the surgery—was not helpful, and screening should ideally be performed during or immediately after the surgery. Only 1 Japanese study has examined SF levels in the perioperative period following THA, and was unable to demonstrate the usefulness of these markers during or immediately after surgery because of a variety of factors. Although our measurements were delayed, our method is one of the fastest for screening of patients who are at high risk of developing VTE.

A 64-slice MDCT was employed as the imaging modality for the detection of postoperative VTE in this study, because with this technique one can simultaneously perform both pulmonary angiography and venography of the lower extremities. In addition, it is a less invasive technique than conventional ascending venography, requires considerably less time (approximately 5–8 min), and is technically simple (Lim et al. 2004). However, MDCT has several disadvantages, such as exposure of the patient to radiation, beam-hardening artifacts around the arthroplastic joint materials, and contrast-induced nephropathy. Concerning the accuracy of DVT detection since the advent of MDCT, several studies have shown that the diagnostic ability of indirect CT venography is comparable to US (Duwe et al. 2000, Loud et al. 2001, Lim et al. 2004), and both the sensitivity and the specificity in these studies ranged from 89–100%. US has recently become a widely accepted primary modality in the diagnosis of DVT because of its advantages, including high accuracy, painlessness, noninvasiveness, and reduced cost. Furthermore, US does not require radiation or contrast materials and there are no side effects. Thus, we employed duplex US for the preoperative screening of DVT and for postoperative confirmation of DVT suspected by MDCT.

In addition to imaging modalities, other factors may have affected the incidence of postoperative VTE, such as the method of anesthesia, duration of surgery, perioperative injection of heparin, immobilization, and duration of hospital stay (Husted et al. 2010, Brueckner et al. 2003). To minimize the influence of these factors on the occurrence of VTE, mobilization and rehabilitation directed by a physiotherapist were initiated within 24 hours of surgery. All patients underwent physiotherapy, and mobilization was frequently encouraged in free time during the hospital stay. Because of the limitation that the FPX injection can only be administered by nurses or doctors during the hospital stay and cannot be self-administered by patients at home, in Japan the duration of the hospital stay is longer than in other countries. The postoperative hypercoagulation state may change if epidural anesthesia is used (Brueckner et al. 2003) or if the duration of surgery is shortened. In the present study, a computed tomography-based navigation system was used to insert the joint implant accurately in all the patients. This system generally takes time to set and register. Furthermore, MIS techniques were used for all cases. It is possible that prolonged surgery may have resulted in the hypercoagulable state. Perioperative administration of heparin may also affect the incidence of postoperative VTE or the postoperative coagulation state, although the dose of heparin was considerably low.

In conclusion, patients undergoing primary THA are at a high risk of developing VTE, which is possibly induced by a hypercoagulable or regulated fibrinolytic state during the early postoperative phase. Plasma levels of SF and PAI-1 on day 1 after THA may be of value in providing an indication of the balance between coagulation and fibrinolysis, and in predicting VTE following THA. When high levels of SF or PAI-1 are observed on the day after surgery, there is a higher risk of postoperative VTE.

YY: design of the study, data collection, literature search, and manuscript preparation. YI: design of the study, surgery, manuscript preparation, and supervision. SW and SY: collection and evaluation of data. NK: design of the study and surgery. IT, NI, HC: design of the studyand patient follow-up. TS: design of the study, manuscript preparation, and supervision.

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Inflammation Research

ORIGINAL RESEARCH PAPER

# Cyclic compression-induced p38 activation and subsequent MMP13 expression requires Rho/ROCK activity in bovine cartilage explants

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**Abstract**
*Objective* Excessive mechanical stress on the cartilage causes the degradation of the matrix, leading to the osteoarthritis (OA). Matrix metalloproteinases 13 (MMP13) is a major catalytic enzyme in OA and p38 plays an important role in its induction. However, precise pathway inducing p38 activation has not been elucidated. We hypothesized here that the small GTPase Rho and its effector ROCK might function in upper part of the mechanical stress-induced matrix degeneration pathway.
*Methods* Bovine metacarpal phalangeal articular cartilage explants were loaded with 1 MPa dynamic compression for 6 h with or without a ROCK specific inhibitor Y27632 or/and a p38 specific inhibitor SB202190. Then p38 phosphorylation and MMP13 expression were assessed by western blot or/and quantitative RT-PCR. Rho-activity was measured by pull-down assay using glutathione S-transferase fusion protein of Rho binding domain.
*Results* Cyclic compression caused Rho activation, p38 phosphorylation and MMP13 expression. Both Y27632 and SB202190 were found to block the mechanical stress-enhanced p38 phosphorylation and subsequent MMP13 expression.
*Conclusions* The present results show that p38 phosphorylation and MMP13 expression are regulated by Rho/ROCK activation, and support the potential novel pathway that Rho/ROCK is in the upper part of the mechanical stress-induced matrix degeneration cascade in cartilage comprised of p38 and MMP13.

**Keywords** Mechanical stress · Osteoarthritis · Matrix metalloproteinase · Cartilage · Rho/ROCK

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## Introduction

Alterations of the pattern of weight loading on the joint and resulting mechanical stresses to the articular cartilage tissue may be an important risk factor for initiation and progression of osteoarthritis (OA). In fact, a variety of factors such as joint instability caused by ligament injury, overuse or obesity can contribute to the alteration of the mechanical environment in the joint, and are now regarded as predisposing factors of OA. [1–6]. In healthy cartilage, chondrocytes mediate matrix remodeling through a balance between the synthesis and degradation of the extracellular matrix components. This process is regulated by cytokines, signaling molecules such as mitogen-activated protein kinases (MAPKs), transcription factors and enzymes, and these factors are influenced by the mechanical environment [2, 7, 8]. Therefore, the identification of precise cascades from mechanical stress through

degradation of the cartilage matrix is urgently needed in promoting therapeutic strategies to prevent or treat OA.

It is well established that matrix metalloproteinases (MMPs) accelerate chondrocyte-mediated matrix degradation. Accumulation of MMPs in the synovial fluids due to joint injury has been considered to play an important role in the progression of OA. There are many MMPs involved in cartilage degradation. MMP13 has been considered as the major enzyme involved in OA cartilage erosion [9, 10]. Recent observations indicate that p38 is a major signaling molecule in the induction of MMP13 [11–13]. However, detailed mechanisms regulated by the p38 activity with the mechanical stresses have not been clarified. In other cell types, such as cardiomyocytes, it has been reported that small GTPase Rho could be activated by mechanical forces [14]. The Rho family of small GTP-binding proteins comprises a group of signaling molecules that are activated by a variety of biologically active substances while the GTP-bound form of Rho activates ROCK by binding to the Rho-binding domain (RBD) in ROCK. The ROCK regulates a wide range of biological processes, including reorganization of the cytoskeleton, transcriptional regulation, cell motility, mitogenesis and apoptosis [15, 16]. It has also been suggested that some major signaling molecules in cell physiology including p38 [17, 18] exists as down-stream of the Rho/ROCK signaling cascades.

Here, we focused small GTPase Rho and its effector ROCK as a primary transducer of mechanical stress. Our hypothesis is that Rho/ROCK activity is essential in the mechanical stress-induced p38 activation and MMP13 expression. To determine above hypothesis, we applied cyclic compression on bovine cartilage explants and observed Rho activity, p38 and MMP13 expressions.

## Materials and methods

### Preparation of the bovine cartilage explant and compression experiment

Full thickness explants of articular cartilage (5 mm diameter) were harvested from the condylar ridge of the metacarpophalangeal joints of freshly slaughtered calves about 10 months of age, which were donated from the local slaughterhouse. The explants were cultured in αMEM (Invitrogen, Carlsbad, CA, USA) with 10 % heat inactivated FCS, and 1 % penicillin/streptomycin (Invitrogen). All compression experiments were performed after allowing explants to equilibrate in culture for 72 h after harvest as previously reported [19]. The test and control explants were removed from adjacent sites on the joint surfaces and paired at harvest to minimize the site-dependent variations. For each experiment, the explants were placed into individual compression wells in 1 ml of culture medium. Prior to the compression treatment, the cartilage explants were precultured in serum-free culture medium composed of 0.1 %BSA (Sigma-Aldrich, St. Louis, MO, USA), DMEM/F-12, 1 % insulin-transferrin selenium (Invitrogen), 1 % sodium pyruvate (Invitrogen) and 1 % antibiotic and anti-micotic solution for 24 h to avoid unnecessary evocation of signaling molecules by FCS. We used Y27632 (Wako Pure Chemical Industries, Osaka, Japan) at 30 μM to inhibit Rho-kinase activity and SB202190 (Wako Pure Chemical Industries) at 15 μM to inhibit p38 activity. All experiments were performed at 37 °C and 5 % $CO_2$, 95 % in air. Compressive loads were applied to individual explants using the Biopress system: the Flexercell Compression Plus System, FX-4000C (Flexcell International, NC, USA). Mechanical loads were applied as a square waveform at 0.5 Hz (1 s on, 1 s off) corresponding to stress magnitudes of 1 MPa for 6 h to induce degenerating reactions as previously reported [20, 21]. All control specimens were cultured in an unloaded state.

### Fluorescent microscopic observation of the activated p38 in the compression-treated cartilage

The compression-treated cartilages were placed in Tissue-Tek cryomolds (Sakura Fine Tek, Tokyo, Japan), embedded in Tissue-Tek OCT compound and frozen on dry ice. Cryosectioning was performed on a Leica CM3050S cryomicrotome (Leica Instruments, Houston, TX, USA). Eight micrometer serial sections were adhered to Poly-prep poly-L-lysine coated slides and visualized on a microscope. Then the slides were fixed in Mildform 10 N for 1 h, washed twice with PBS and blocked by Block ace (Dainippon Pharmaceutical, Osaka, Japan) for 1 h. For immunofluorescent observation, the specimens were incubated with 1/200 diluted anti-phospho-p38 (T180/Y182) rabbit monoclonal antibody (Cell Signaling Technology, Inc., MA, USA, #4511) in 4 °C overnight. The specimens were then washed twice with PBS containing 10 % Block-ace, incubated with 1/1,000 diluted FITC-conjugated anti-rabbit IgG bovine secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After twice washing, FITC labeled specimens were stained with 1/1,000 diluted DAPI and observed using a fluorescent microscope (BZ-9000, Keyence, Osaka, Japan).

### Western blot analysis

Cartilage explants or cultured chondrocytes were collected, homogenized in SDS buffer (4 % SDS, 125 mM tris–glycine, 10 % 2-mercaptoethanol, 2 % bromophenol blue in 30 % glycerol) and subjected to polyacrylamide gel electrophoresis in the presence of SDS (SDS/PAGE) followed

by electrotransfer onto PVDF membrane (Hybond-P; Amersham Pharmacia Biotech, Buckinghamshire, UK). The blotted membranes were blocked overnight with Block ace and treated with each primary antibody overnight at 4 °C. Antibody incubations and washes were performed in 0.1 %Tween-20 in PBS throughout. Detection was realized by enhanced chemiluminescence with an ECL plus western blotting detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK) and CCD-based chemiluminescent analyzer LAS 4000. Relative expression level of Rho was quantified by normalizing western blot signals to the housekeeping protein GAPDH. Primary antibodies used in this study were as follows: anti-GAPDH mouse monoclonal antibody (Abnova, Taipei, Taiwan, 226-335); anti-phospho-p38 (T180/Y182) rabbit monoclonal antibody (Cell Signaling Technology, Inc., #4511); anti-p38 rabbit polyclonal antibody (Cell Signaling Technology, Inc., #9212); anti-MMP13 sheep polyclonal antibody (AbD Serotec, Oxford, UK, #5980-1311).

### Rho activity analysis

For Rho GTPase analysis, we performed GST pull-down of activated Rho proteins using Active Rho Pull-Down and Detection Kit (Pierce Biotechnology, IL, USA).

Briefly, explants were homogenized by handy-type homogenizer (Multipro 395; Dremel Corporation, WI, USA) in Lysis buffer. Cultured chondrocytes were washed twice with ice-cold PBS and lysed by Lysis buffer. Then the lysates were centrifuged and the supernatants were incubated with affinity gel-bound GST-Rhotekin RBD fusion protein that specifically binds GTP-Rho, and affinity purification was performed with glutathione agarose resin. Total cell lysate or pull-down material was resolved by 10 % SDS-PAGE, followed by immunoblotting with anti-Rho rabbit polyclonal antibody (Thermo Scientific Inc., MA, USA).

### Primary culture of the bovine articular chondrocyte and Rho activation by lysophosphatidic acid (LPA) treatment

Chondrocytes were isolated from the articular cartilage by enzymatic digestion with 2 mg/ml of collagenase (Wako Pure Chemical Industries, Osaka, Japan) for 12 h at 37 °C. After filtration, cells were seeded in culture plates and cultured in 10 % FCS supplemented αMEM. Cells were cultured at 37 °C and 5 % $CO_2$, 5 % $O_2$. Prior to exposure of LPA, the bovine primary chondrocytes were precultured in serum-free culture medium composed of 0.1 % BSA (Sigma-Aldrich, St. Louis, MO, USA), DMEM/F12, 1 % insulin-transferrin selenium (Invitrogen), 1 % sodium pyruvate (Invitrogen) and 1 % anti-biotic and anti-micotic solution for 24 h. To induce Rho activation, 5/10/20 μM LPA (BIOMOL International, PA, USA) was added and cultured for 6 h before analysis of the *MMP13* gene expression by quantitative RT-PCR. For inhibition experiment of p38 or ROCK signaling, SB202190 or Y27632 were added at 1/5/15 or 1/10/30 μM at the same time with LPA administration.

### RNA extraction, reverse transcription and quantitative RT-PCR (qRT-PCR) analysis

Explants or cultured chondrocytes were treated with TRIzol reagent (Invitrogen) and cDNA was prepared from total RNA using random primers under standard conditions with the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR was performed using Perfect real-time SYBR green II (Takara Bio, Inc., Shiga, Japan). PCR amplifications were performed with the Thermal Cycler Dice Real Time PCR System (Takara Bio, Inc.) at 95 °C for 10 s followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s. To quantify the relative expression of each gene, the Ct (threshold cycle) values were normalized for endogenous reference ($\Delta Ct = Ct_{MMP13} - Ct_{Gapdh\beta}$) and compared with a calibrator, using the ΔΔCt method ($\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{calibrator}$). Each primer pair used in this study were as follows:

*Gapdh* forward GTGAAGGTCGGAGTGAACG;
reverse TAAAAGCAGCCCTGGTGAC,
*MMP13* forward TCCCTTGATGCCATAACCAGTC;
reverse AACAGCTCTGCTTCAACCTGC.

### Statistical analysis of the data

Significant difference was detected by Tukey–Kramer HSD test or Student's *t* test. A *p* value of less than 0.05 was considered significant difference.

## Results

### Cyclic compression provoked small GTPase Rho activation, p38 phosphorylation and MMP13 expression

First, we determined whether MAPK p38 and its downstream molecule MMP13 could be induced by applying the cyclic compression. Phosphorylated p38 was detected at 0.5, 1, 3 and 6 h. MMP13 expressions were detected after 3 h compression (Fig. 1a). When observed by immunofluorescence with an antibody to the phosphorylated p38,

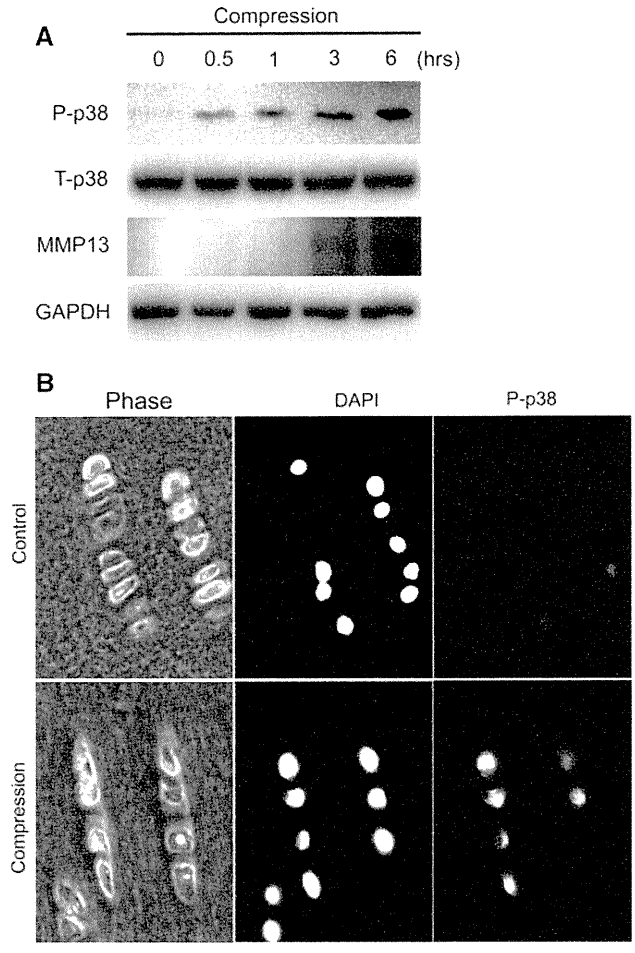


**Fig. 1** Effect of compression load to the bovine cartilage explants. **a** Western blot analysis for p38 and MMP13. Phosphorylated p38 (P-p38) was detected in all compressed samples and MMP13 expressions were detected in the samples stimulated for 3 and 6 h. T-p38 means total p38. **b** Immunofluorescent observation of the phosphorylated p38 (P-p38)

clear localization to the nuclei after 6 h continuing compression (Fig. 1b). From these results, we concluded that 6 h compression at the present condition was sufficient to initiate the mechanical stress induced matrix-degrading reaction. Then we examined Rho activity to confirm participation of Rho into the mechanical stress-induced reaction, and found that the Rho activation was initiated at 0.1 h of compression and continued to at least 6 h of compression (Fig. 2a). When the activation was quantified approximately 30 times significant increase of the activated Rho was found after 6 h of compression (Fig. 2b).

## Rho activation induced p38 phosphorylation and subsequent MMP13 expression in cultured chondrocyte

Although some major signaling molecules including p38 exist in down-stream of the Rho/ROCK signaling cascades

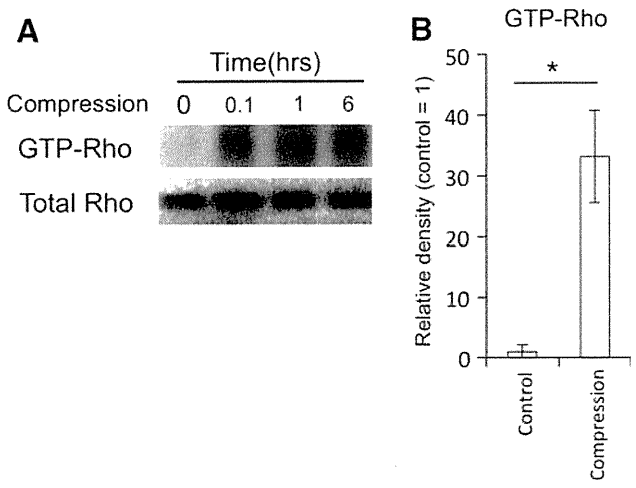


**Fig. 2** Rho activation by the cyclic compression stimulation. **a** Activated Rho was detected by GST-pull down method in compression treated samples. **b** Densitometry based quantification of GTP bounded Rho (GTP-Rho). *Asterisk* means significant differences at $p < 0.05$. *Bars* show the mean score of three independent experiments and *bars* depict SD

[17], the effect of Rho/ROCK activation on chondrocyte is still unclear. Then we examined whether Rho activation can bring p38 phosphorylation and subsequent induction of MMP13 expression in chondrocyte by stimulating the bovine primary chondrocyte with LPA, which can chemically invigorate the Rho activity. LPA dramatically activated Rho (Fig. 3a), and caused p38 phosphorylation (Fig. 3b). Furthermore, an increase of MMP13 expression in LPA treated chondrocyte was observed by western blot and qRT-PCR. Administration of SB202190, a selective inhibitor of p38 mitogen-activated protein kinase, into the LPA treated chondrocyte resulted in suppression of the MMP13 gene expression in dose dependent manner (Fig. 3c). On the other hand, adding SB202190 did not lead to diminishing of the phosphorylation status of the p38 as previously reported [22]. Rho-kinase inhibitor Y27632 administration also attenuated the enhancement of the p38 phosphorylation by the LPA treatment when observed by western blot and qRT-PCR (Fig. 3b, c). These results clearly show the possibility that the Rho activation can lead to upregulation of the p38 phosphorylation and following MMP13 expression.

## Mechanical stress induced p38 phosphorylation and MMP13 upregulation could be inhibited by administration of ROCK inhibitor

To determine whether p38 phosphorylation and MMP13 expression induced by mechanical stress could be regulated with Rho/ROCK, we applied mechanical stress on the cartilage explant in the presence of SB202190 or/and Y27632. Both SB202190 and Y27632 clearly blocked

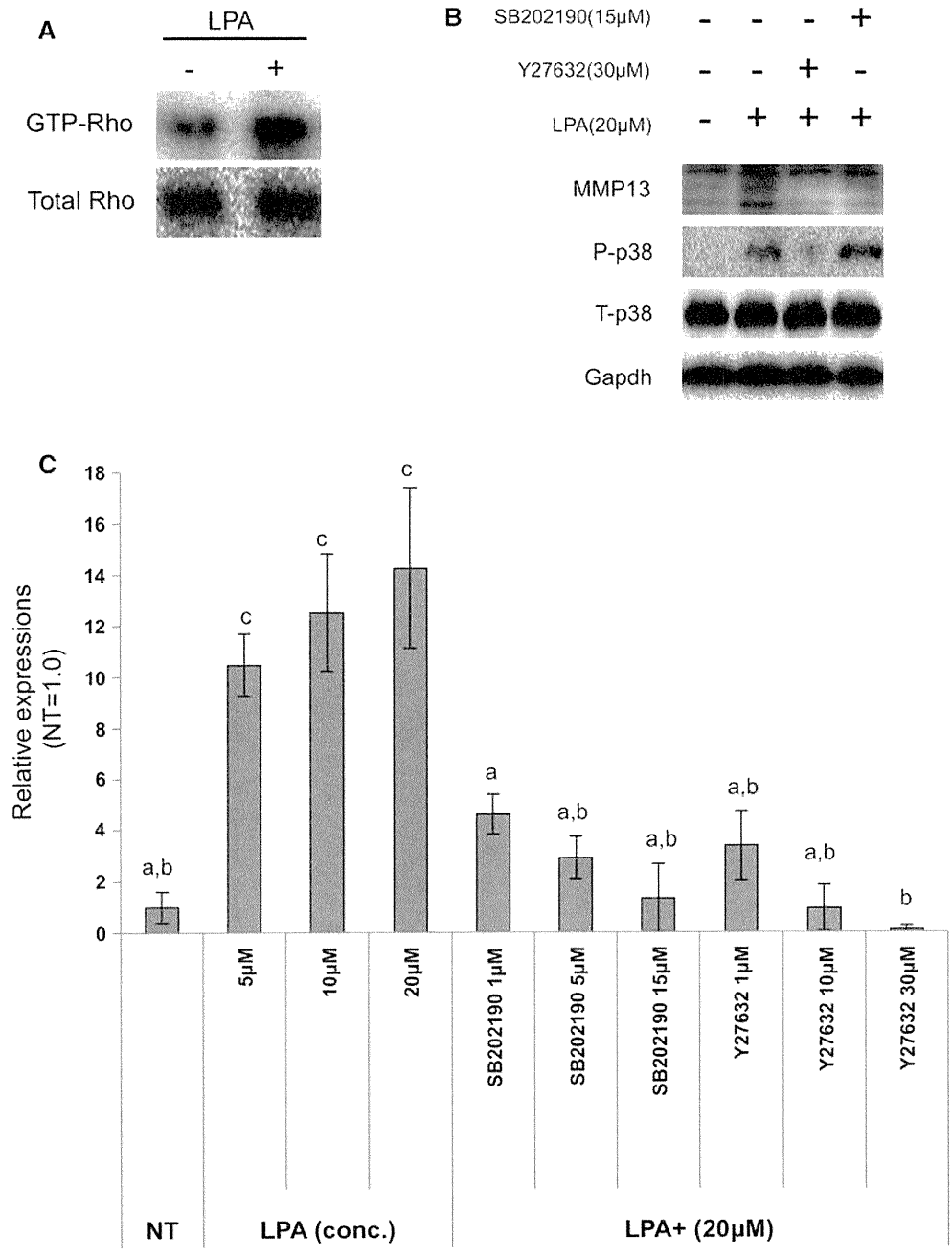


**Fig. 3** LPA administration activated Rho and p38, and induced MMP13 expression. **a** Detection of activated Rho by GST-pull down. LPA treatment induced Rho activity. **b** Western blot analysis for p38 and MMP13 of LPA treated primary chondrocyte. **c** qRT-PCR analysis for *MMP13* gene expression. *Different characters* mean significant differences between each experimental group at $p < 0.05$. NT means non-treated control. All experimental groups included the vehicle of SB202190 (DMSO) at same concentration. *Bars* show the mean score of three independent experiments and *bars* depict SD

stress-enhanced p38 phosphorylation and decreased MMP13 quantity. Interestingly, in the present western blot analysis, both activated and inactivated forms of MMP13 were decreased by addition of SB202190 or/and Y27632 (Fig. 4a). qRT-PCR analysis also demonstrated that MMP13 expressions were significantly decreased by the ROCK inhibition (Fig. 4b). At least in the present study, clear differences were observed in the strength of the inhibition effect to the MMP13 expression between the SB202190 and the Y27632. Thus it is possible to assume that the main molecular signaling of mechanical stress-induced matrix degrading reaction leads to MMP13 expression is simply through Rho/ROCK activation and p38 phosphorylation, corresponds to our hypothesis.

## Discussion

We considered here that Rho/ROCK is a critical element in the mechanotransduction pathway in the cartilage and possibly involved in the pressure overload-induced matrix degeneration reactions of the cartilage.

Recent studies have demonstrated that p38 can be an effector kinase of mechanotransduction in various types of