Intra-Articular Injection of Mitomycin C Prevents Progression of Immobilization-Induced Arthrogenic Contracture in the Remobilized Rat Knee

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Summary

This study tested whether cell cycle inhibitor mitomycin C (MMC) prevents arthrogenic contracture progression during remobilization by inhibiting fibroblast proliferation and fibrosis in the joint capsule. Rat knees were immobilized in a flexed position to generate flexion contracture. After three weeks, the fixation device was removed and rat knees were allowed to freely move for one week. Immediately after and three days after fixator removal, rats received intra-articular injections of MMC or saline. The passive extension range of motion (ROM) was measured before and after myotomy of the knee flexors to distinguish myogenic and arthrogenic contractures. In addition, both cellularity and fibrosis in the posterior joint capsule were assessed histologically. Joint immobilization significantly decreased ROMs both before and after myotomy compared with untreated controls. In saline-injected knees, remobilization increased ROM before myotomy, but further decreased that after myotomy compared with that of knees immediately after three weeks of immobilization. Histological analysis revealed that hypercellularity, mainly due to fibroblast proliferation, and fibrosis characterized by increases in collagen density and joint capsule thickness occurred after remobilization in saline-injected knees. Conversely, MMC injections were able to prevent the remobilization-enhanced reduction of ROM after myotomy by inhibiting both hypercellularity and joint capsule fibrosis. Our results suggest that joint capsule fibrosis accompanied by fibroblast proliferation is a potential cause of arthrogenic contracture progression during remobilization, and that inhibiting fibroblast proliferation may constitute an effective remedy.

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Introduction

Joint immobilization is frequently used to treat orthopedic disorders such as bone fractures and ligamentous injuries, but often causes joint contracture (Chesworth and Vandervoort 1995, Moseley *et al.* 2005, Nightingale *et al.* 2007), which is characterized by a reduced passive range of motion (ROM) of the joint (Wong *et al.* 2015). As joint contractures can lead to various types of locomotive disabilities (Bot *et al.* 2012, De Smet 2007), management of this impairment is a critical issue in the field of orthopedics.

Because joint immobilization is a major cause of joint contracture, it is clinically accepted that joint movement during remobilization is effective in improving joint contracture. Passive joint movements such as stretching are frequently applied in this context. In animal studies, however, it remains controversial whether stretching has beneficial effects on immobilization-induced joint contracture (Kondo *et al.* 2012, Okita *et al.* 2001, Usuba *et al.* 2007). In humans,

a clinical study failed to show related benefits of passive stretching (Moseley *et al.* 2005), and recent reviews supported by high-quality evidence suggest that stretching does not have a clinically important role in joint contracture treatment (Harvey *et al.* 2017a, Harvey *et al.* 2017b). More reasonable and effective treatment approaches are therefore needed.

Formation and recovery processes of immobilization-induced joint contracture have been closely examined using animal models. The responsible structures have been broadly divided into myogenic and arthrogenic factors (Nagai *et al.* 2014, Trudel and Uhthoff 2000, Trudel *et al.* 2014). Trudel *et al.* demonstrated that a myogenic factor is mainly responsible for joint contracture in the early phases of immobilization (within two weeks) and is resolved by remobilization (Trudel *et al.* 2014). In contrast, arthrogenic factors largely contribute to severe joint contracture induced by prolonged (over four weeks) immobilization, and recovery by remobilization is not expected in these cases (Trudel *et al.* 2014). Arthrogenic contracture also further progresses during remobilization following short-term (within three weeks) immobilization (Kaneguchi and Ozawa 2017, Kaneguchi *et al.* 2017, Kaneguchi *et al.* 2018a, Kaneguchi *et al.* 2018b, Kaneguchi *et al.* 2019, Trudel *et al.* 2014). To avoid irreversible joint contracture, arthrogenic contracture should therefore be targeted.

Fibrosis in the joint components is believed to be the major factor contributing to development of arthrogenic contracture in injured (Fukui *et al.* 2000, Fukui *et al.* 2001, Gao *et al.* 2017, Li *et al.* 2013a) and immobilized joints (Sasabe *et al.* 2017). For example, administration of decorin, which suppresses bioactivity of cell adhesion, as well as fibrotic regulators, such as transforming growth factor-beta, can improve restricted joint motion in the rabbit intra-articular adhesion model (Fukui *et al.* 2001). However, several studies showed development of arthrogenic contracture without fibrosis in the periarticular connective tissue in spinal cord injury and immobilization models (Hagiwara *et al.* 2010, Kaneguchi *et al.* 2017, Kaneguchi *et al.* 2018a, Kaneguchi *et al.* 2018b, Kaneguchi *et al.* 2019, Moriyama *et al.* 2007). While it therefore remains controversial whether joint fibrosis is a major factor in arthrogenic contracture, development of fibrosis is observed in the joint capsule of remobilized joints together with progression of arthrogenic contracture (Kaneguchi *et al.* 2017, Kaneguchi *et al.* 2018a,

Kaneguchi *et al.* 2018b, Kaneguchi *et al.* 2019). We previously showed that anti-inflammatory treatment using the steroidal drug dexamethasone can prevent remobilization-induced arthrogenic contracture progression by suppressing joint capsule fibrosis and fibroblast proliferation (Kaneguchi *et al.* 2018b). Other studies using intra-articular adhesion models also reported that administration of anti-inflammatory agents such as celecoxib and botulinum toxin type A attenuates joint contracture and intra-articular adhesion as well as fibroblast proliferation (Baranowski *et al.* 2019, Gao *et al.* 2017, Li *et al.* 2013a). Fibroblasts produce extracellular matrix proteins such as collagens, and thus fibroblast proliferation is an important process in the development of joint fibrosis (Emami *et al.* 2012, Li *et al.* 2014). We therefore suggest that proliferation of fibroblasts triggered by inflammation plays an important role in forming joint capsule fibrosis and that inhibition of fibrosis may be essential for blocking the progression of arthrogenic contracture.

To inhibit fibroblast proliferation, we focused on the cell cycle inhibitor mitomycin C (MMC). In a clinical context, MMC is generally used as an anti-cancer drug (Kahmann *et al.* 2010). However, it also finds application pre-clinically and clinically as an anti-fibrotic drug for keloid, capsular contracture, and scarring after ocular surgery (Lane *et al.* 2003, Li *et al.* 2013b, Nava *et al.* 2017, Sidle and Kim 2011, Simman *et al.* 2003, Wang *et al.* 2012). MMC can attenuate the development of fibrosis as well as inhibit fibroblast proliferation in injury-induced intra-articular adhesion models (Kocaoglu *et al.* 2011, Li *et al.* 2013b, Wang *et al.* 2012).

Therefore, we hypothesized that remobilization-induced joint capsule fibrosis and progression of arthrogenic contracture would also be attenuated by MMC administration *via* inhibition of fibroblast proliferation. To test this hypothesis, we investigated the effects of MMC on joint ROM and histopathology in the remobilized rat knee.

Materials and Methods

Experimental animals

Twenty-five eight-week-old male Wistar rats (190-220 g; Japan SLC, Shizuoka, Japan) were used in this study. The rats were randomly divided into four groups: control (n=4), immobilization (IM: n=7), remobilization with saline injections after immobilization (RM: n=7), and remobilization with MMC injections

after immobilization (RM+M: n=7). In the control group, data from the right and left knees were treated as individual samples; therefore, we used data of eight knees from four rats as controls. Rats were housed in standard cages in a temperature-controlled room (20-25 °C) with 12 h light/dark cycles. Standard rodent chow and water were provided *ad libitum.* This experimental design was approved by the committee on animal experimentation of Hiroshima International University.

Joint immobilization and remobilization

The right knees of rats in the IM, RM, and RM+M groups were immobilized with an external fixator according to the method described in previous studies (Nagai *et al.* 2014, Ozawa *et al.* 2016). In brief, after anesthesia by intraperitoneal injection of sodium pentobarbital (32.4 mg/kg of body weight), Kirschner wires (01-132-50; MIZUHO, Tokyo, Japan) were screwed into the femur and the tibia and were fixed by wire and resin (Provinice Fast; Matsukaze, Kyoto, Japan) to immobilize knee joints at a flexion of approximately 140° (Fig. 1). During immobilization, rats could move freely using their four limbs. Knees in the control group were untreated. After three weeks, the fixation device was removed and rats in the RM and RM+M groups were allowed to recover for one week (remobilization). It is known that one week of remobilization following three weeks of immobilization induces fibrotic reactions in the joint capsule and arthrogenic contracture progression (Kaneguchi *et al.* 2017, Kaneguchi *et al.* 2018a, Kaneguchi *et al.* 2018b, Kaneguchi *et al.* 2019). Rats in the IM group were analyzed immediately after removal of the fixator and represented data from immobilization without remobilization. Therefore, data in the control and IM groups were collected at three weeks after starting the experiment, while data in the RM and RM+M groups were collected at four weeks.

MMC treatment

To inhibit remobilization-induced fibroblast proliferation, rats in the RM+M group received intra-articular injections of 0.08 mg of MMC (concentration 0.8 mg/ml). Because cell proliferation is active until three days after fixator removal (Kaneguchi *et al.* 2017), injections were given immediately after and three days after removal. The quantity of MMC administered was determined based on the literature to achieve an anti-proliferative effect (Kocaoglu *et al.* 2011). In the RM group, rats received intra-articular injections of the

same volume of saline (0.1 ml) at the same time. Thus, rats in the RM and RM+M groups received two injections.

Fig. 1. Image of joint immobilization. The right knee joint is immobilized at a flexion of approximately 140° (angle between the femur and the fibula is 40°) by an external fixator.

ROM measurements

At the end of experimental periods, we measured ROMs according to the method of our previous study (Kaneguchi *et al.* 2015). In brief, rats were anesthetized with intraperitoneal injection of sodium pentobarbital (32.4 mg/kg of body weight). After rat hindlimbs were skinned, rats were placed in the neutral spine position and the femur was manually fixed at 90° of the hip flexion, followed by 14.6 N mm of knee extension moment. This moment stretched the knee joint close to its physiological limit (Moriyama *et al.* 2006), but did not disrupt the knee soft tissues (Trudel and Uhthoff 2000). Using a 3D motion analysis system (Kinema Tracer; Kissei Comtec, Nagano, Japan), the angle between the femur and fibula was measured as ROM before myotomy. Then, rats were sacrificed by exsanguination under sodium pentobarbital anesthesia, and knee flexor muscles were completely transected to remove the myogenic factor. Finally, ROM after myotomy was measured to evaluate arthrogenic contracture.

Histological assessment

Tissue preparation

After ROM measurements, the knee joints were harvested and immersion-fixed at flexion 90° in 0.1 M phosphate-buffered 4 % paraformaldehyde (pH 7.4) for 48 h at 4 °C. After fixation, samples were decalcified by 17.7 % ethylenediaminetetraacetic acid (pH 7.2, Osteosoft;

Merck Millipore, Darmstadt, Germany) and embedded in paraffin. Using a microtome, samples were cut into 4 μm sagittal sections at the medial mid condylar level.

Cell counting

Counting of cells was performed according to the methods described in previous studies (Kaneguchi *et al.* 2017, Kaneguchi *et al.* 2018a). In brief, sections were stained with hematoxylin and eosin. We photographed the superior, central, and inferior regions of the posterior joint capsule at 40× magnification and manually counted the number of cells. Cell numbers were then converted to cells per mm^2 of joint capsule.

Counting of fibroblasts

To visualize fibroblasts, we performed immunohistochemistry using anti-vimentin antibodies. Deparaffinized sections were treated with 1 % trypsin for 5 min at 37 °C for antigen retrieval. To quench endogenous peroxidase activity, sections were incubated with methanol containing 3 % H$_2$O$_2$ for 30 min. Nonspecific binding was blocked by 1 % normal horse serum in 0.01 M phosphate-buffered saline (PBS; pH 7.4) for 30 min. We then incubated sections with anti-vimentin antibody (1:1000 dilution; ab92547, Abcam, Cambridge, UK) for three hours at room temperature followed by rinsing with PBS. Secondary antibody (horse biotinylated anti-mouse/rabbit IgG, 1:250 dilution; BA-1400, Vector Laboratories, Burlingame, CA, USA) was added for 30 min. After rinsing with PBS, we incubated sections with a streptavidin-biotin complex (1:50 dilution; Elite ABC, Vector Laboratories) for 30 min. Finally, immunoreactivity was visualized with a Dako EnVision + kit/HRP (DAB) (Dako Japan, Tokyo, Japan), followed by counterstaining with hematoxylin. While vimentin is often used as a fibroblast marker (Abdul *et al.* 2015, Glazebrook *et al.* 2008, Krejci *et al.* 2015, Wang *et al.* 2014), it is also expressed in other cell types, including endothelial cells, macrophages, neutrophils, and lymphocytes (Evans 1998). Therefore, spindle-shaped vimentin-positive cells not detected in luminal structures were considered fibroblasts (Kaneguchi *et al.* 2018a). Counting of fibroblasts was performed in the same way as cell counting.

Calculating joint capsule collagen density

We calculated collagen density following methods described previously (Kaneguchi *et al.* 2017). In brief, sections were stained with aldehyde fuchsin-

Masson Goldner (AFMG) to identify collagen. We captured the posterior joint capsule just behind the meniscus at 20× magnification (Fig. 2A, B) and analyzed the digitized image using ImageJ software (National Institutes of Health, Bethesda, MD, USA). To isolate collagen, we extracted green color from the original color image using the Split Channels function. Using the Threshold function, an arbitrary threshold was set to determine the green stained area. Collagen density was calculated by dividing this area by the total joint capsule area.
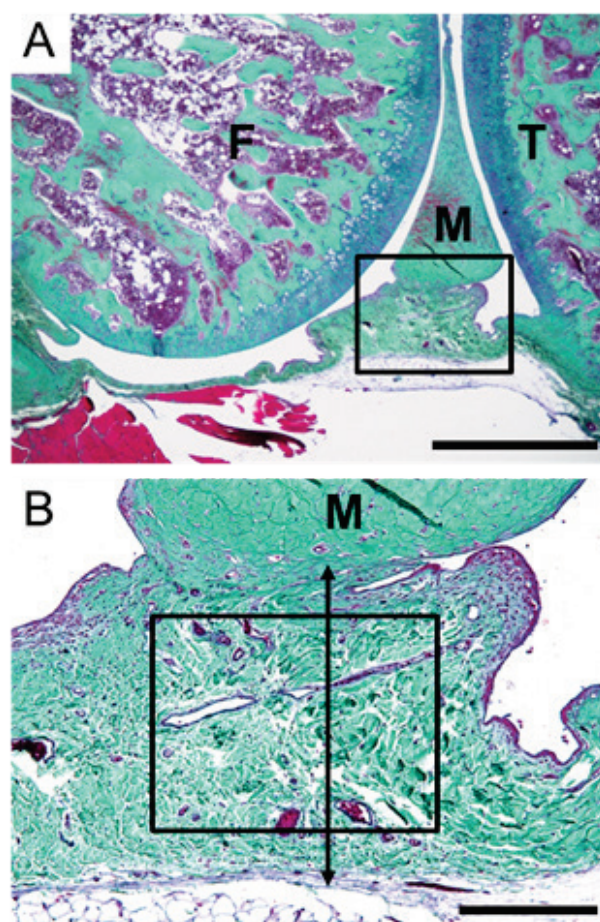

Fig. 2. Images of morphometrical and histological analyses of the posterior joint capsule. (**A**) shows example image of the posterior knee joint stained with aldehyde fuchsin-Masson Goldner. High magnification of the box in A is shown in B. The distance between the posterior borders of the meniscus and the joint capsule (up-down arrow) was measured as posterior joint capsule thickness (**B**). In addition, collagen density (percentage of green stained area) was also measured in the posterior joint capsule (box in B). F, femur; T, tibia; M, meniscus. Scale bars=1 mm in A, 200 μm in B.

Measurement of joint capsule thickness

Using AFMG-stained sections, the thickness of the posterior joint capsule was measured according to the

methods described previously (Kaneguchi *et al.* 2017). In brief, after the posterior region of the knee joint was photographed at 2× magnification, the distance between the posterior borders of the meniscus and the joint capsule was measured as joint capsule thickness using ImageJ software (Fig. 2A, B). All analyses were performed in an unblinded manner.

Statistical analysis

We performed statistical analyses using Dr. SPSS II for Windows (SPSS Japan Inc., Tokyo, Japan) and G*Power 3.1 (University of Düsseldorf, Düsseldorf, Germany). Normality of the distribution and homoge-neity of variance were tested using the Kolmogorov-Smirnov and Levene tests, respectively. All data met normality and homoscedasticity assumptions. Therefore, one-way analysis of variance (ANOVA) and the Tukey's *post hoc* test were applied. For all tests, a P-value of<0.05 was considered statistically significant. A *post hoc* power analysis was performed using G*Power 3.1.

Results

ROM

Knee extension ROMs before myotomy were 149±4°, 91±4°, 108±4°, and 107±5° in the control, IM, RM, and RM+M groups, respectively (Fig. 3A). Compared with the control group, ROMs were significantly reduced in all joint-immobilized groups (P<0.001). Among immobilized groups, ROMs in the RM and RM+M groups were significantly larger than that in the IM group (P<0.001). Between the RM and RM+M groups, there was no difference in ROM before myotomy (P=0.952). The statistical power for ROM before myotomy was 1.00.

Knee extension ROMs after myotomy, which can indicate restriction of ROM caused by arthrogenic factor, were 161±4°, 139±8°, 128±8°, and 138±5° in the control, IM, RM, and RM+M groups, respectively (Fig. 3B). In all joint-immobilized groups, ROMs were significantly reduced compared with the control group (P<0.001). Among immobilized groups, ROM in the RM group was significantly lower than in the IM group (P=0.019). However, ROM in the RM+M group did not differ from the IM group (P=0.977), but was significantly larger than in the RM group (P=0.048). The statistical power for ROM after myotomy was 1.00.
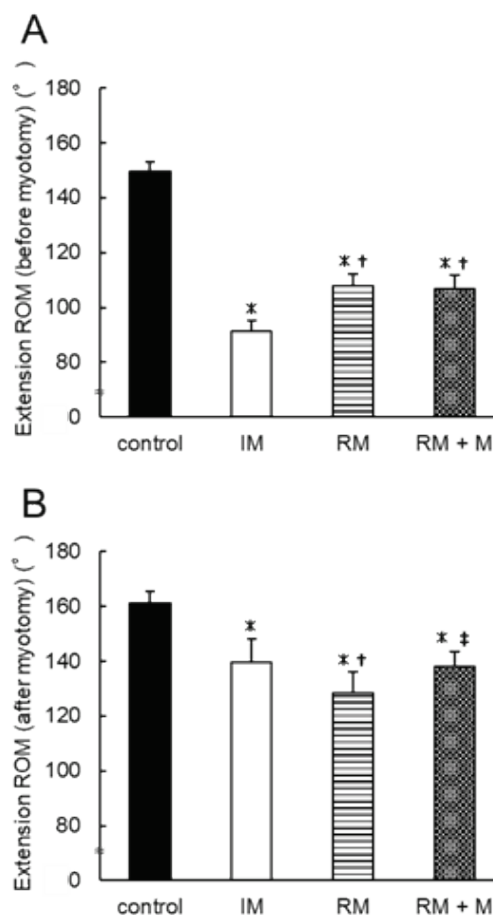

Fig. 3. Changes in knee extension ROM before and after myotomy. (**A**) ROM before myotomy. In the IM group, ROM before myotomy was significantly smaller than that in the control group. In the RM and RM+M groups, ROM before myotomy partially recovered, but was still significantly smaller than that in the control group. There was no difference in ROM before myotomy between the RM and RM+M groups. (**B**) ROM after myotomy. In the IM group, ROM after myotomy was also significantly smaller than that in the control group. In the RM group, ROM after myotomy further decreased compared with the IM group. In the RM+M group, we prevented remobilization-induced progression of ROM restriction. Values are mean + standard deviation. * indicates significant difference compared with the control group (P<0.05). † indicates significant difference compared with the IM group (P<0.05). ‡ indicates significant difference compared with the RM group (P<0.05).

Cellularity

In the posterior joint capsule of the control (Fig. 4A) and IM (Fig. 4B) groups, we mainly observed spindle-shaped fibroblast-like cells and vascular endothelial cells, concluding there was no difference in cell number between these two groups (P=0.998, 3,985±867 and 4,084±569 cells/mm^2, respectively, Fig. 4E). Compared with the control and IM groups, we observed a greater cell number mainly due to proliferated spindle-shaped fibroblast-like cells in the RM group
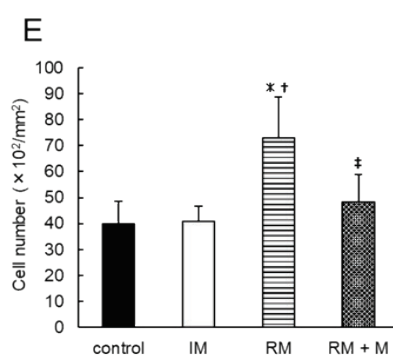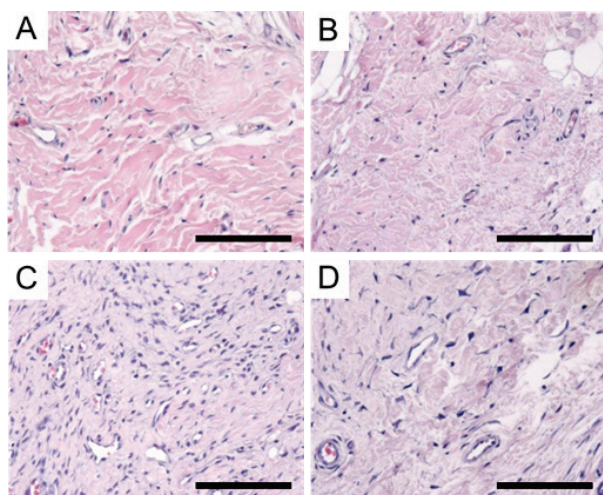
Fig. 4. Cellularity of posterior joint capsule. A-D shows the posterior knee joint capsule stained with hematoxylin and eosin. (**A**) control, (**B**) IM, (**C**) RM, and (**D**) RM+M groups. Scale bars=100 μm. E shows cell number. There was no difference in cell number between the control and IM groups. In the RM group, we observed many spindle-shaped fibroblast-like cells and, consequently, cell number was significantly higher than in the control and IM groups. Remobilization-induced hypercellularity was prevented in the RM+M group. Values are mean + standard deviation. *indicates significant difference compared with the control group (P<0.05). [†]indicates significant difference compared with the IM group (P<0.05). [‡]indicates significant difference compared with the RM group (P<0.05).
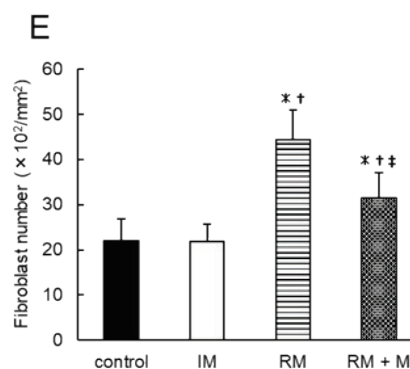
Fig. 5. Fibroblast number in the posterior joint capsule. A-D shows posterior knee joint capsule immunohistochemically stained with anti-vimentin antibody. (**A**) control, (**B**) IM, (**C**) RM, and (**D**) RM+M groups. Arrowheads indicate fibroblasts. Scale bars=50 μm. (**E**) fibroblast number. There was no difference in fibroblast number between the control and IM groups. Compared with those in the control and IM groups, the number of fibroblasts in the RM group increased. In the RM+M group, an increase in fibroblasts was partially attenuated. Values are mean + standard deviation. *indicates significant difference compared with the control group (P<0.05). [†]indicates significant difference compared with the IM group (P<0.05). [‡]indicates significant difference compared with the RM group (P<0.05).

(P<0.001, 7,291±1,572 cells/mm^2, 183 % of the control group, Fig. 4C). In the RM+M group (Fig. 4D), cell number was significantly smaller than in the RM group (P=0.001, 4,833±1,044 cells/mm^2, 66 % of RM group), but not significantly different from the control and IM groups (P=0.468 and 0.570, respectively, 121 % of the control group). The statistical power for cell number was 0.95.

The number of vimentin-positive fibroblasts also did not differ between the control (Fig. 5A) and IM (Fig. 5B) groups (P=1.000, 2,199±491 and 2,180±393 cells/mm^2, respectively, Fig. 5E). Vimentin-positive fibroblast number in the RM group (Fig. 5C) was significantly higher than those in the control and IM groups (P<0.001, 4,434±660 cells/mm^2, 202 % of the
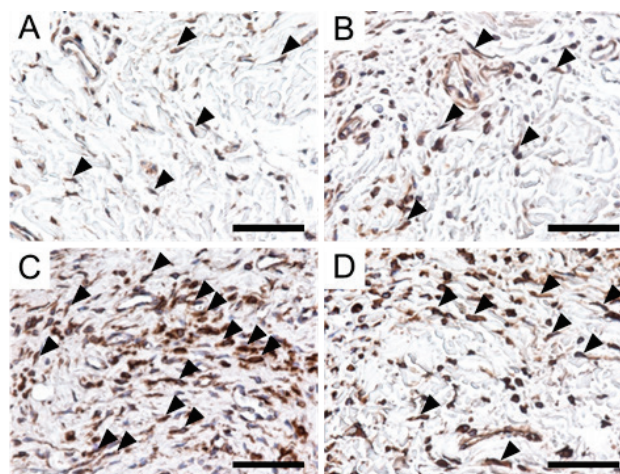
control group). Also, in the RM+M group (Fig. 5D), vimentin-positive fibroblast number was significantly higher than those in the control and IM groups (P=0.014 and 0.012, respectively, 3,149±556 cells/mm^2, 143 % of the control group). However, the number of vimentin-positive fibroblasts was significantly smaller in the RM+M group than in the RM group (P=0.001, 71 % of RM group). The statistical power for fibroblast number was 1.00.

Collagen density

In the control (Fig. 6A) and IM (Fig. 6B) groups, collagen fiber bundles in the posterior joint capsule were arranged with gaps. Collagen density was 54±4 % and 48±8 %, respectively. There was no significant difference
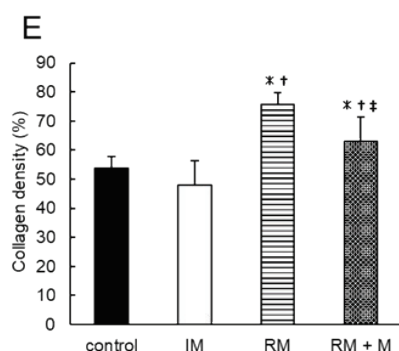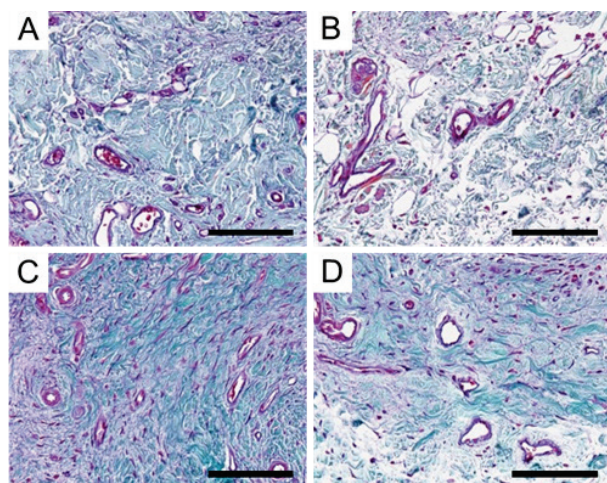
Fig. 6. Morphological changes in the posterior knee joint capsule. A–D shows posterior knee joint capsule stained with aldehyde fuchsin-Masson Goldner. (**A**) control, (**B**) IM, (**C**) RM, and (**D**) RM+M groups. Collagen is stained green. Scale bars=100 μm. (**E**) collagen density. There was no difference in collagen density between the control and IM groups. In the RM group, the gaps of collagen bundles narrowed and joint capsules became denser. Consequently, collagen density in the RM group significantly increased compared with the control and IM groups. In the RM+M group, a remobilization-induced increase in collagen density was partially attenuated. Values are mean + standard deviation. * indicates significant difference compared with the control group (P<0.05). † indicates significant difference compared with the IM group (P<0.05). ‡ indicates significant difference compared with the RM group (P<0.05).

Fig. 7. Thickness in the posterior joint capsule. A–D shows posterior knee joint capsule stained with aldehyde fuchsin-Masson Goldner. (**A**) control, (**B**) IM, (**C**) RM, and (**D**) RM+M groups. Up-down arrows indicate posterior joint capsule thickness. Scale bars=200 μm. (**E**) joint capsule thickness. Compared with the control group, the posterior joint capsule in the IM group was slightly thickened, but differences were not significant (P=0.077). Joint capsule thickness in the RM group was significantly larger than those in the control and IM groups. In the RM+M group, joint capsule thickness tended to be smaller than in the RM groups (P=0.067) and was comparable to the IM group. Values are mean + standard deviation. * indicates significant difference compared with the control group (P<0.05). † indicates significant difference compared with the IM group (P<0.05).

in collagen density between the control and IM groups (P=0.324, Fig. 6E). In the RM group (Fig. 6C), the gap of collagen fiber bundles disappeared and collagen density increased to 76±4 %, significantly different to the control and IM groups (P<0.001). In the RM+M group (Fig. 6D), gaps between collagen fiber bundles were partially restored and collagen density was 63±8 %. Collagen density in the RM+M group was significantly lower than that in the RM group (P=0.006), but significantly higher than in the control and IM groups (P=0.043 and 0.001, respectively). The statistical power for collagen density was 1.00.

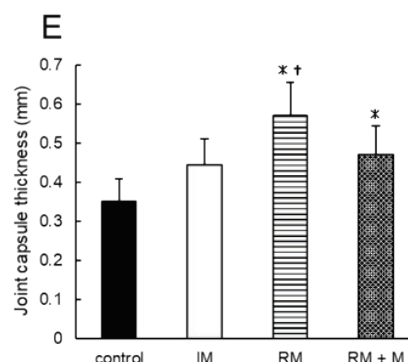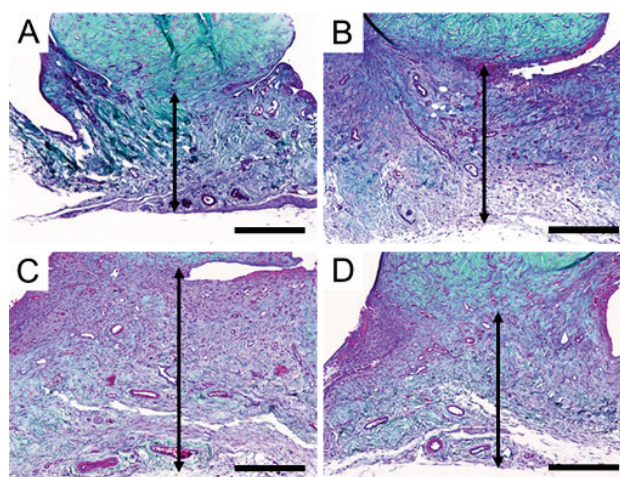*Joint capsule thickness*

In the control group (Fig. 7A), joint capsule thickness was 0.35±0.06 mm (Fig. 7E). In the IM group (Fig. 7B), the posterior joint capsule was slightly thickened (0.45±0.07 mm), but not significantly, compared with that in the control group (P=0.077). Joint capsule thickness in the RM group was 0.57±0.09 mm (Fig. 7C) and was significantly thicker than those in the control and IM groups (P<0.001 and P=0.014, respectively). In the RM+M group (Fig. 7D), joint capsule thickness tended to be lower than in the RM groups (0.47±0.07 mm, P=0.067) and was

comparable to the IM group (P=0.901). The statistical power for joint capsule thickness was 0.98.

Discussion

In this study, we tested whether MMC administration prevents the progression of remobilization-induced arthrogenic contracture by inhibiting fibroblast proliferation and fibrosis in the joint capsule. As expected, MMC injections served to attenuate progression of arthrogenic contracture by suppressing fibroblast proliferation, leading to significantly lower collagen density and a tendency to be lower joint capsule thickness. These results suggest that joint capsule fibrosis is a potential cause of arthrogenic contracture progression in remobilized joints.

Three weeks of immobilization significantly reduced ROMs both before and after myotomy. After one week of remobilization, ROM before myotomy increased but after myotomy further decreased. These results indicate that remobilization improves myogenic contracture, but aggravates arthrogenic contracture oppositely. Although immobilization-induced arthrogenic contracture is not improved by remobilization (Kaneguchi *et al.* 2017, Kaneguchi *et al.* 2018a, Kaneguchi *et al.* 2018b, Kaneguchi *et al.* 2019, Trudel *et al.* 2014), arthrogenic contracture in intra-articular adhesion model using joint immobilization with intra-articular injury partially recovers after remobilization (Baranowski *et al.* 2018). These findings suggest that arthrogenic contracture attributed to intra-articular injury may be variable in contrast with immobilization only. Intra-articular injections of MMC during remobilization could prevent further decrease in ROM after myotomy, which may be due to intra-articular (micro) injury (Kaneguchi *et al.* 2017). However, MMC injections did not improve ROM before myotomy. Our results indicate that MMC has beneficial effects on arthrogenic contracture but not on myogenic one.

Fibrosis in joint components strongly contributes to progressing arthrogenic contracture in remobilized (Kaneguchi *et al.* 2017, Kaneguchi *et al.* 2018a, Kaneguchi *et al.* 2018b) as well as injured joints (Fukui *et al.* 2000, Fukui *et al.* 2001, Gao *et al.* 2017, Li *et al.* 2013a). Further, we observed increased joint capsule thickness and collagen density in the joint capsule following remobilization together with progressing arthrogenic contracture. Extracellular matrix proteins, such as collagen, are produced by fibroblasts. In joint

injury-induced contracture, inhibiting fibroblast proliferation by anti-proliferative agents MMC or hydroxycamptothecin can attenuate fibrosis in intra-articular adhesion sites (Li *et al.* 2013b, Liang *et al.* 2014). Therefore, it seems likely that fibroblast proliferation plays an important role in generating joint fibrosis. In our present and previous studies, we observed development of joint capsule fibrosis together with fibroblast proliferation during remobilization (Kaneguchi *et al.* 2017, Kaneguchi *et al.* 2018a, Kaneguchi *et al.* 2018b, Kaneguchi *et al.* 2019), suggesting that fibroblast proliferation contributes to fibrosis formation in remobilized joints. We previously revealed that administering the steroidal anti-inflammatory drug dexamethasone during remobilization can prevent joint capsule fibrosis by suppressing hypercellularity with increasing fibroblast numbers (Kaneguchi *et al.* 2018b). In the present study, administering MMC, which selectively prevents cell proliferation without inhibiting inflammation, was also able to attenuate joint capsule fibrosis; this prevented arthrogenic contracture progression during remobilization. We previously reported that inflammatory reactions reach their peak within one day of remobilization, but that arthrogenic contracture progression is not observed at that point (Kaneguchi *et al.* 2017). Progression of arthrogenic contracture characterized by development of joint capsule fibrosis is observed only after inflammation (Kaneguchi *et al.* 2017). These findings suggest that fibroblast proliferation (and subsequent upregulation of collagens) triggered by inflammation plays an important role in driving fibrotic processes, which induces arthrogenic contracture progression in remobilized joints.

In immobilized joints, remobilization can improve myogenic (Kaneguchi *et al.* 2017, Trudel *et al.* 2014) but not arthrogenic contracture (Kaneguchi and Ozawa 2017, Kaneguchi *et al.* 2017, Kaneguchi *et al.* 2018a, Kaneguchi *et al.* 2018b, Kaneguchi *et al.* 2019, Trudel *et al.* 2014). Therefore, effective therapeutic interventions for arthrogenic contracture are needed to avoid permanent joint contracture. Some animal and clinical studies reported the effectiveness of anti-inflammatory and anti-fibrotic agents on the attenuation of joint contracture (Baranowski *et al.* 2019, Usher *et al.* 2019). In the clinical setting, however, treatment options for joint contracture are limited to passive joint movements such as stretching, manipulation under anaesthesia (MUA), or surgical treatments (Charalambous and Morrey 2012, Wong *et al.* 2015).

Previous studies identify no positive effects of stretching on joint contracture (Harvey *et al.* 2017a, Harvey *et al.* 2017b, Moseley *et al.* 2005). MUA and surgical treatments are effective to improve joint contracture, but have the risk of complications, including nerve symptoms, heterotopic ossification, and instability (Cai *et al.* 2015, Usher *et al.* 2019). Developing new therapeutic strategies as alternatives to current treatments is therefore a critical concern (Wong *et al.* 2015). In this study, we demonstrated that intra-articular injections of the cell cycle inhibitor MMC effectively attenuates remobilization-induced joint capsule fibrosis and arthrogenic contracture progression. Inhibiting fibroblast proliferation during remobilization may become a novel therapeutic strategy in treating immobilization-induced arthrogenic contracture.

This study has some limitations. First, all analyses were performed in an unblinded manner. We thus cannot exclude the possibility of the subjective bias. Second, we immobilized knee joint using external fixator constructed by Kirschner wires, wire, and resin. Cast immobilization might be more suitable to mimic the human situation after the orthopedic disorders. Third, immunohistochemistry was performed only for the fibroblast. Both fibroblasts and myofibroblasts are major contributors to fibrotic changes (Hinz *et al.* 2012, Kendall and Feghali-Bostwick 2014). In particular, myofibroblasts expressing α-smooth muscle actin (α-SMA) have high extracellular matrix protein production capacity (Hinz *et al.* 2012, Kendall and Feghali-Bostwick 2014) and thus play a central role in joint fibrosis (Baranowski *et al.* 2019, Li *et al.* 2013b). Identification of myofibroblasts by α-SMA would give valuable information.

In conclusion, the present study demonstrated that inhibiting fibroblast proliferation by intra-articular MMC injections during remobilization can benefit the treatment of arthrogenic contracture by attenuating joint capsule fibrosis.

Conflict of Interest
There is no conflict of interest.

Acknowledgements

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