Distribution of Type A and B Synoviocytes in the Adhesive and Shortened Synovial Membrane during Immobilization of the Knee Joint in Rats

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Joint immobilization is commonly used for the treatment of joint injuries and diseases, but it also causes unfavorable outcomes such as joint contracture. The purpose of this study was to examine the morphological changes of the synovial membrane that is suspected as a cause of joint contracture, and localization of type A (macrophage-like) and type B (fibroblast-like) synoviocytes in the capsule after joint immobilization. Male Sprague-Dawley rats were used in this study. Unilateral knee joints were rigidly immobilized at 150° of flexion with internal fixators for 3 days, 1, 2, 4, 8, and 16 weeks (7 rats/each immobilized group), while 42 rats were sham-operated. Sagittal sections of 5 μm were prepared from the medial midcondylar region of the knee joints and assessed with histological, histomorphometric, and immunohistochemical methods. Adhesions were observed both in the anterior and posterior synovial membranes in the immobilized group after 2 weeks. In the adhesion area, the cells were mainly composed of type A synoviocytes that were positive for CD68 and type B synoviocytes positive for prolyl 4-hydroxylase subunit beta. The length of synovial membrane in the immobilized group was significantly shorter than that in the control group after 2 and 4 weeks. After 8 weeks, the adhesion area in the immobilized group became fibrous and hypocellular. The staining intensity of hyaluronic acid-binding protein was increased after 16 weeks. Adhesion and shortening of the synovial membrane and the structural changes of the adhesion area may contribute to the development of joint contracture.

Keywords: immobilization; adhesion; synovial membrane; synoviocyte; contracture

after immobilization were characterized by adhesions and shortening of SM (Trudel et al. 1998, 2000, 2003; Moriyama et al. 2007). SM contains two different types of cells: macrophage-like cells (type A synoviocytes) and fibroblast-like cells (type B synoviocytes) (Barland et al. 1962; Graabaek 1982). Type A synoviocytes are mainly located at the superficial layer of SM and absorb cell debris and wastes in the joint cavity, whereas type B synoviocytes are mainly found at the deep layer of SM and produce hyaluronic acid and collagens (Okada et al. 1981). Though these cells do not form a true epithelium, they have protective, absorptive, and secretory functions like epithelium (Henderson and Pettipher 1985). There are no reports concerning localization of type A and B synoviocytes after joint immobilization. The purpose of this study was to elucidate the morphological changes of SM and localization of type A and B synoviocytes in immobilized rat knee joints.

Hyaluronic acid, widely distributed in the extracellular matrix of the articular cartilage and capsule, has multiple roles such as cell migration and lubrication during joint movements (Suzuki et al. 2006). We examined the distribution of hyaluronic acid-binding protein (HABP), which specifically binds hyaluronic acid, in SM after joint immobilization.

Materials and Methods

Experimental Design and Animal Procedures

The protocols for the experiments were approved by the Animal Research Committee of Tohoku University. A total of eighty-four adult male Sprague-Dawley rats aged 12 weeks old were used in this study. Anesthetized with intra-peritoneal administration of sodium pentobarbital (50 mg/kg), the unilateral knee joint was rigidly immobilized at 150° of flexion with a plastic plate and metal screws as previously described (Hagiwara et al. 2006; Ando et al. 2008). Sham-operated rats had holes drilled in the femur and tibia with metal screws, but plate was not inserted. Buprenorphine (0.05 mg/kg) was injected subcutaneously for postoperative analgesia. The immobilized rats and the sham-operated rats made up the immobilized group and the control group, respectively. Seven rats in the immobilized and the control groups each formed a one-time cohort, and 6-time cohorts (3 days, 1, 2, 4, 8, and 16 weeks) were assessed.

Tissue Preparation and Staining

At the end of the experimental period, the rats were fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS), pH 7.4 by perfusion through the ascending aorta under anesthesia. The knee joints in the control group were manually flexed at 150° during fixation (Hagiwara et al. 2006; Ando et al. 2008). Sham-operated rats had holes drilled in the femur and tibia with metal screws, but plate was not inserted. Buprenorphine (0.05 mg/kg) was injected subcutaneously for postoperative analgesia. The immobilized rats and the sham-operated rats made up the immobilized group and the control group, respectively. Seven rats in the immobilized and the control groups each formed a one-time cohort, and 6-time cohorts (3 days, 1, 2, 4, 8, and 16 weeks) were assessed.

Histology and Histomorphometry

Morphological changes of SM were observed both in the anterior and posterior capsules (Fig. 1). SM is defined as a superficial cellular layer of the capsule. The length of the superior and inferior subdivisions of SM in the anterior and posterior capsule was separately measured in one section from one synoviocartilage junction to the anterior or posterior meniscus as previously reported (Fig. 1-a, b, c, d) (Trudel et al. 2000). Because adhesions between the folded SM and SM around the posterior meniscus were observed in the posterior capsule in this model, we defined the length of the postero-superior SM as the total length of the postero-superior subdivision and residual joint space (See Fig. 2C). We also measured the outside length of the posterior capsule (Fig. 1-e). Anterior and posterior capsular areas were separately measured between the SM layer and the outside capsule as previously described (Hagiwara et al. 2006; Ando et al. 2009). The sections were stained with Elastica-Masson.

![Microphotograph of a sagittal section in the medial midcondylar region of a rat knee joint. Shown is a sagittal section in the medial midcondylar region of a knee joint in the control group (control 3 days). SM length of the antero-superior (a), antero-inferior (b), postero-superior (c), postero-inferior (d), and outside of the posterior capsule (e) was separately measured. Anterior capsular area (f) and posterior capsular area (g) were also measured. Elastica-Masson staining. Scale bar = 1 mm.](image)
Synovial Adhesions after Immobilization

Immunohistochemistry of Synoviocytes and Histochemistry of HABP

To elucidate the localization of the two types of cells in SM, we used CD68 as a marker of macrophage-like type A synoviocyte (Damoiseaux et al. 1994) and prolyl 4-hydroxylase subunit beta (P4H) as a marker of fibroblast-like type B synoviocyte (Bai et al. 1986). Deparaffinized sections were predigested with 0.1% trypsin (Wako Chemicals, Tokyo, Japan) in 0.01M PBS for 30 minutes at 37°C for CD68 or heated with a microwave oven for 15 minutes at 700 W in 1,000 ml 0.01 M citrate buffer, pH 6.0 for P4H as unmasking procedures. After the inhibition of endogenous peroxidase with 1.5% H$_2$O$_2$ in distilled water for 15 minutes, nonspecific binding was eliminated with 10% normal goat serum (Nichirei, Tokyo, Japan) for 30 minutes at room temperature. The sections were then incubated with mouse anti-rat CD68 monoclonal antibody (MCA341R, Serotec, Oxford, U.K., dilution 1:100), or mouse anti-rat P4H monoclonal antibody (AF5110-1, Acris, Herford, Germany, dilution 1:20) for 24 hours at 4°C. The secondary antibody, horseradish peroxidase (HRP) conjugated goat anti-mouse immunoglobulin (IgG) (Nichirei) was applied for 1 hour at room temperature. The final detection step was carried out using 3, 3’-diaminobenzidine tetrahydrochloride (DAB) (Sigma, St. Louis, MO) in 0.1 M imidazole and 0.03% H$_2$O$_2$ as the chromogen for 10 minutes. Counterstaining was made with Carazzi’s hematoxylin. Negative control was performed using normal mouse IgG (Dako, Copenhagen, Denmark) as the primary antibody. All slides were stained in one session.

Deparaffinized sections were reacted with biotin-labeled HABP (2 µg/ml, Seikagaku, Tokyo, Japan) for 24 hours at 4°C after inhibition of endogenous peroxidase with 1.5% H$_2$O$_2$ in distilled water as mentioned above. The sections were then incubated with HRP conjugated avidin-biotin complex (ABC) (Vector, Burlingame, CA) for 90 minutes at room temperature. The slides were developed with DAB solution. Negative control was prepared by treating the sections with 2.5% bovine testicular hyaluronidase (Sigma) for 1 hour at 37°C prior to incubation with HABP.

Statistical analysis

Differences between the immobilized and control groups were compared at each time point by unpaired t-test. Data were expressed as mean ± standard deviation. A value of $P < 0.05$ was accepted as statistically significant.

Results

Histology

The histological appearance in the immobilized group was quite different from that in the control group (Fig. 2 and 3). In the postero-superior area, the number of cells in the capsule increased after 3 days and 1 week in the immobilized group compared to the control group (Fig. 2A, B and Fig. 3A, and B). Adhesions (asterisk) were observed between the postero-superior synovial fold and the SM around the posterior horn of the medial meniscus were observed after 2 weeks (Fig. 2C and 3C), and the posterior femoral articular cartilage and the meniscus were bridged by dense collagenous tissues after 4 weeks in the immobilized group (Fig. 2D). Adhesion area extended to the posterior side to diminish the residual joint space.
space after 4 weeks (Fig. 2D), and the area was replaced by fibrous and hypocellular connective tissues after 8 and 16 weeks (Fig. 2E, 2F, and 3D). The fibrous layer in the capsule also became hypocellular and SM was not distinguished after 16 weeks in the immobilized group (Fig. 2F). The anterior SM showed similar changes to the posterior SM (Fig. 3E, F). In the control group, the capsule in the antero-superior area consisted of a few cell layers of SM and fibrous layer (Fig. 3E). In the immobilized group, adhesions of SM to the articular cartilage and the meniscus were observed after 2 weeks, and the area was replaced by hypocellular loose connective tissue after 8 weeks (Fig. 3F).

**Length of Synovial Membrane and Capsular Area**

No statistical difference was observed between the immobilized and control groups in the antero-superior SM length (Fig. 4A). The length of the antero-inferior SM in the immobilized group was significantly shorter than that in the control group after 4 weeks (Fig. 4B). The length of the postero-superior and postero-inferior SM in the immobilized group was significantly shorter than that in the control group after 2 weeks (Fig. 4C and 4D). The length of the postero-superior SM gradually decreased in the immobilized group (Fig. 4C). Outside length of the posterior capsule in the immobilized group was also significantly shorter than that in the control group at 8 and 16 weeks (Fig. 4E). Both in the anterior and posterior capsular areas, significant difference was not observed between the immobilized and control groups at all time points (Fig. 4F and G).

**Localization of Type A and B Synoviocytes**

In the control group, CD68 positive cells (Type A synoviocytes) were mainly located at the superficial layer of SM and at the fibrous layer (Fig. 5A, B). In the immobilized group, the superficial layer of SM was maintained at 1 week (Fig. 5C), but disappeared after 2 weeks (Fig. 5D). Both CD68 positive and negative cells were observed in the adhesion area (Fig. 5D). No apparent differences were observed in the fibrous layer of the capsule between immobilized and control groups at all time points (Fig. 5F and G).

In the control group, CD68 positive cells (Type A synoviocytes) were mainly located at the superficial layer of SM and at the fibrous layer (Fig. 5A, B). In the immobilized group, the superficial layer of SM was maintained at 1 week (Fig. 5C), but disappeared after 2 weeks (Fig. 5D). Both CD68 positive and negative cells were observed in the adhesion area (Fig. 5D). No apparent differences were observed in the fibrous layer of the capsule between immobilized and control groups at all time points (Fig. 5F and G).

P4H positive cells (Type B synoviocytes) were mainly located at the deeper layer of SM and at the fibrous layer in the control group (Fig. 5E). In the immobilized group, P4H positive cells were observed in the adhesion area (Fig. 5F).
Synovial Adhesions after Immobilization

Distribution of Hyaluronic Acid

Intense HABP reactions were observed in the matrix of the capsule (Fig. 6A). No reactivity was recognized after pretreatment with bovine testicular hyaluronidase. No apparent differences were observed in the fibrous layer of the capsule between the immobilized and control group (Fig. 6A, B). Though the staining intensity of HABP was weak at 2 weeks in the postero-superior adhesion area in the immobilized group (Fig. 6C), its reactivity increased at 16 weeks (Fig. 6D).

Discussion

We investigated the chronological changes of SM after immobilization. Adhesions and shortening of SM were observed after 2 weeks in the immobilized group. These changes were observed both in the anterior and posterior SM. After 8 and 16 weeks of immobilization, the adhesion area became more fibrous and hypocellular. These results were different from some previous reports (Sood 1971; Akeson et al. 1973; Amiel et al. 1980) but consistent with other reports (Evans et al. 1960; Thaxter et al. 1965; Finsterbush and Friedman 1973; Schollmeier et al. 1994). These controversial results can be related to the methods of immobilization. Akeson et al. (1973) could not show apparent evidence of adhesions after 9 weeks of immobilization with wires in rabbit’s knees. Our fixation method was completely rigid compared to other methods such as casting or wiring (Sood 1971; Akeson et al. 1973; Amiel et al. 1980). Even a small motion of a joint may prevent adhesions of the SM and progression of contracture. Unilateral knee joints of rats were immobilized at deeply flexed position in our immobilization model. In clinical situations, we treat a patient such as Achilles tendon rupture at deep flexed position of the ankle joint with cast, which often causes limitation in extension. Joint immobilization at extreme position is easy to evaluate the loss in ROM. The rats usually move at flexed position of the knee joint, which is one of the main reasons for choosing deep flexed position in our model.

Fig. 4. Changes in the SM length of the immobilized knee joint.

Shown are the SM lengths in the antero-superior (A), antero-inferior (B), postero-superior (C), postero-inferior (D) areas, outside length of the posterior capsule (E), anterior capsular area (F), and posterior capsular area (G). No statistical difference was found in the length of the antero-superior SM (A). Length of the antero-inferior SM in the immobilized group was significantly shorter after 4 weeks (B). Length of the postero-superior and postero-inferior SM in the immobilized group was significantly shorter after 2 weeks (C, D). Length of the outside length of the posterior capsule in the immobilized group was significantly shorter after 8 weeks (E). No statistical difference was observed between the two groups in the anterior capsular area (F) and the posterior capsular area (G) at all time points. Error bars = ± 1 s.d. N.S. = Not significant. **P < 0.005 and *P < 0.05 versus control.
The length of the antero-inferior, postero-superior, and postero-inferior SM in the immobilized group was significantly shorter after 2 and 4 weeks. These findings were partially consistent with the previous reports (Trudel et al. 2000; Moriyama et al. 2007). Trudel et al. (2000) showed significant shortening of SM at 4 weeks of immobilization in the anterior capsule and at 4, 8, 16, and 32 weeks of immobilization in the posterior capsule. They reported that the causes of the shortening of SM after immobilization were due to atrophy or adhesions of the synovial villi to neighboring synovial villi (Trudel et al. 2000, 2003). In addition, we clarified that the folded SM adhered not only to the articular cartilage and meniscus but also to the facing SM at deep flexed position, which induced severe shortening of SM in the posterior capsule. Outside length of the posterior capsule was also measured, and the length in the immobilized group was significantly shorter at 8 and 16 weeks. This result suggested that not only the inner layer (SM) but also the outer layer of the capsule adhered to the facing capsule at deep flexed-position. We hypothesized that proliferated adhesion area would induce an increase of the total capsular area. However, there were no statistical differences between the immobilized and control groups in the anterior and posterior capsular areas at all time points as previously reported (Trudel et al. 2000). Reduction in the concentration of glycosaminoglycans and water content after immobilization may explain the unexpected result (Akeson et al. 1973).

Type A synoviocytes absorb and degrade extracellular matrix, cell debris, and produce antigens in the synovial fluid and SM, whereas type B synoviocytes synthesize and secrete the components of the synovial fluid and extracellular matrix of the SM (Okada et al. 1981). We demonstrated that epithelium-like structures of SM disappeared after 2 weeks of immobilization and the cells in the adhesion area were mainly composed of both type A and B synoviocytes. This is the first report of localization of type A and B synoviocytes after immobilization. CD68 is a useful marker and often used for the detection of macrophage or type A synoviocytes (Damoiseaux et al. 1994; Shaw et al. 2007). However, specific markers for the detection of type B synoviocytes remain unknown. P4H is a possible marker for the detection of type B synoviocytes because type B synoviocytes were fibroblast-origin (Wilkinson et al. 1992). Wilkinson et al. (1992) concluded that P4H labeled type B synoviocytes but did not distinguish these from fibroblasts in the capsule. Both type B synoviocytes and fibroblasts in the capsule were stained in this model as previously described (Wilkinson et al. 1992). Cell numbers were increased in fibrous layer of the capsule in immobilized...
group at 3 days and 1 week. Origin of the increased cells is unknown, but to clarify the distribution of type A and B synoviocytes and other inflammatory cells in SM with some quantitative methods will give us an insight into the pathology of contracture after immobilization.

HABP specifically binds hyaluronic acid, which is the component of the synovial fluid and the extracellular matrix of the capsule (Suzuki et al. 2006). Hyaluronic acid richly existed in the matrix of the capsule both in the control and immobilized groups. Extracellular matrix in the adhesion area after 2 and 4 weeks showed weak histochemical reaction for HABP, whereas the area after 8 and 16 weeks of immobilization showed intense HABP reaction in this model. Pitsillides et al. reported that joint immobilization decreased synovial fluid hyaluronic acid and synoviocytes activity (Pitsillides et al. 1999). Decrease of hyaluronic acid secretion into the joint space, changes of the distribution of type B synoviocytes, and accumulation of hyaluronic acid into the adhesion area may explain our results. These changes may indicate conversion of the immature adhesive tissue into mature capsular tissue and affect the property of the extracellular matrix in the adhesion area and the development of the contracture formation after immobilization.

In conclusion, we showed that joint immobilization induced adhesions and shortening of SM after 2 weeks. The cells in the adhesion area were mainly composed of macrophage-like type A and fibroblast-like type B synoviocytes. After 8 and 16 weeks of immobilization, the adhesion area became fibrous and hypocellular, and the extracellular matrix of the area showed intense HABP reaction. These synovial changes may contribute to the development of contracture after joint immobilization.

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