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Expression of type I collagen in the capsule of a contracture knee in a rat model

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Abstract

Contracture is a very common complication in daily examination and a fibrotic change of a capsule is suggested to be a one of the main causes. Type I collagen is a major component of a synovial capsule and also has been implicated in tissue elasticity of other organs. We immobilized the knee joints of 66 rats in 150 degrees of flexion using a plastic plate and metal screws. Sham operated knee joints had holes drilled and screws inserted but none of them were plated. The expression patterns of type I collagen were characterized using in situ hybridization and immunohistochemistry. The in situ hybridization demonstrated that the mRNA of type I collagen decreased rapidly after immobilization. However, the immunoreactivity of the capsule was not changed in the immobilized and the control groups at any time points. Other processes might be considered to evaluate the contracture capsule.

Introduction

Joint immobilization has been a frequent therapy since the time of Hippocrates [1], but it has also been one of the main causes of a joint contracture. The definition of the joint contracture is a loss of passive and active ranges of motion of a joint [2, 3]. Though the contracture usually disturbs activities of daily living in various aspects, its management is still now controversial and challenging [4, 5]. Because there is no definite therapy and prevention for the contracture, short period of immobilization is the only way to avoid joint contracture.

A large number of experimental studies on joint immobilization have been reported [6–12]. However, phenomena occurred in a joint cavity after joint immobilization have not been agreed in opinion. Some reported that connective tissue proliferation occurred after immobilization [13–16]. Others showed that capsular stiffness might contribute more to joint contractures than muscle function [6, 7, 10, 13, 17–28].

In our previous study of a rat immobilized knee model in flexion, there was slight proliferation of connective tissue in the intra-articular space. Further, elasticity of the posterior capsule detected by scanning acoustic microscopy increased after 8

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and 16 weeks of immobilization [2], which might have contributed to limitation in extension. In this respect, we hypothesized that fibrosis occurred in the posterior capsule after immobilization

Determination of structural collagens is a key to understand the elastic change of the posterior capsule. In patients with systemic scleroderma, alpha 1 (I)-collagen mRNA expression was clearly observed in their dermal fibroblasts [21]. These results should explain the elastic changes of the skin. In the synovial capsule, type I collagen is a major component and its rate is over 80 % of the collagen present [22]. We expected that the same process like systemic scleroderma might occur and that type I collagen would increase in the capsule after immobilization.

Materials and methods

Animals

The protocol for the experiments was approved by the Animal Research Committee of Tohoku University. Adult male Sprague-Dawley rats (body weight 380-400 g) were used in this study. The knee joints were immobilized at 150 degrees of flexion with an internal but extra-articular fixator for various weeks (3 days to 16 weeks) as previously described [2]. The left and right hind legs were immobilized alternately to avoid potential systematic side differences. A rigid plastic plate (POM-N, Senko Med. Co., Japan) implanted subcutaneously joined the proximal femur and the distal tibia away from the knee joint and was solidly held in place with one metal screw (Stainless Steel, Morris, J. I., Co., USA) at each end. The knee joint capsule and the joint itself were untouched. Sham operated animals had holes drilled in the femur and tibia and screws inserted but none of them were plated. The animals were allowed unlimited activity and free access to water and food. Seventy two rats (3 days, 1, 2, 4, 8, and 16 weeks, 6 rats for the immobilized group and 6 rats for the control group at each time point) were prepared for in situ hybridization and sixty rats (1, 2, 4, 8, and 16 weeks, 6 rats for each group at each time point) were prepared for immunohistochemistry. The immobilized animals and the sham operated animals made up the immobilized group and the control group, respectively.

Tissue Preparation

The rats were anesthetized and fixed with 4% paraformaldehyde with or without 0.5% glutaraldehyde in 0.1 M phosphate-buffer, pH 7.4 by perfusion through the aorta. The specimens fixed with glutaraldehyde were used for in situ hybridization. The knee joints were resected and kept in the same fixative overnight at 4°C. The fixed specimens were decalcified in 10% EDTA in 0.01 M phosphate-buffer, pH 7.4 for 2 months at 4°C. The EDTA solution was autoclaved before use. After dehydration through a graded series of ethanol solution the specimens were embedded in paraffin. The embedded tissue was cut into 5-μm sagittal sections from the

medial to the lateral side of the joint. Standardized serial sections were kept in the medial midcondylar regions of the knee. A region of analysis for in situ hybridization and immunohistochemistry was set in the anterior and posterior capsule each in each section.

Preparation of RNA probes

Digoxigenin (DIG)-labeled single strand RNA probes were prepared using the DIG RNA labeling kit (Roche, Mannheim, Germany) according to the manufacturer's instruction. A fragment encoding rat pro-alpha 1 (I)-collagen (2833-4329 bp: GenBank accession number Z78279) was obtained from total RNA of embryonic rat skin using reverse transcription followed by RT-PCR and subcloned into the pT7T3-a18 plasmid (Life Technologies; Grand Island, NY) [23]. The following oligonucleotide primers were used for the RT-PCR: upstream primer, 5′CCCAAGCTTGCGTGGT GTGGTCGGTCT3′, downstream primer, 5′GATGGAGGGAGTTTACACGA3′. The cDNA was verified by digestion with restriction enzymes and was confirmed by dideoxynucleotide sequencing. Antisense and sense riboprobes were generated by T7/HindIII and T3/EcoRI, respectively.

In Situ Hybridization

The protocol used in the present study has been reported elsewhere [24] and is only briefly described as follows. The sections were deparaffinized and washed in PBS, pH 7.4, and then immersed in 0.2 N HCl for 20 minutes. After being washed in PBS, the sections were incubated in proteinase K (20 g/ml; Roche) in PBS for 30 minutes at 37°C. The sections were then dipped in 100% ethanol and dried in air and incubated with the antisense probe or the sense control probe (400 ng/ml) in a hybridization mixture for 16 hours at 45°C. The sections were washed and treated with RNase (type 1a, 20 μg/ml; Sigma, St Louis, MO) for 30 minutes at 37°C. After washing, the hybridized probes were detected immunologically using the Nucleic Acid Detection Kit (Roche), counterstained with methyl green, and mounted with a mounting medium. Fibroblast-like cells in the capsule were counted (two to three areas per section) and positive cells were defined as same signal intensity as those of periosteum. The ratios of pro-alpha 1 (I)-collagen positive cells to the total number of cells counted (at least 100 cells) at high magnification (× 400) were calculated. The two investigators were blinded to which group of the knees were studied. The consistency of the results was verified by assessing interobserver correlations of 10 randomly chosen histological sections [13].

Immunohistochemistry

The sections were deparaffinized and washed in PBS. Endogenous immunoglobulins were blocked by incubation for 30 minutes with 10% normal goat serum (Histofine blocking reagent, Nichireibioscience, Tokyo, Japan) in PBS. The slides

were washed again in PBS and incubated with a polyclonal rabbit anti-rat type I collagen antibody (LSL LB-1102, LSL, Tokyo, dilution 1: 800) in PBS overnight at 4°C then rinsed in PBS. The slides were incubated with a goat anti-rabbit immunoglobulin antibody (1: 100, HRP conjugated, DAKO, Copenhagen, Denmark) for 30 min and then rinsed in PBS. The final detection step was carried out using 3, 3′-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich Corp.), 0.1 M imidazole, 0.03% hydrogen peroxidase as the chromogen for 10 min. Counterstaining was with methyl green for 5 min. For negative controls, normal rabbit IgG was used as a primary antibody. Cellular staining was graded as positive if specific staining was seen in slides for a given antibody and as negative if no staining or only rare cellular staining was evident [25]. Fibroblast-like cells in the capsule were counted (two to three areas per section). The ratios positive cells to the total number of cells counted (at least 100 cells) at high magnification (× 400) were calculated. The immunoreactivity of matrix staining was graded on a scale of 0-3 where: no staining was 0, weak staining 1, moderate staining 2, and strong staining 3 according to the previous report [16]. The staining intensity of endothelial cells was defined as moderate staining 2.

The two investigators were blinded to which group of the knees were studied. The consistency of the results was verified by assessing interobserver correlations of 10 randomly chosen histological sections for the ratio of positive cells [13]. All the specimens were analyzed by independent two investigators for immunoreactivity of matrix staining intensity.

Statistics

Statistical analysis among groups was performed using the Kruskal-Wallis test, with Bonferroni/Dunn post-hoc multiple comparisons. Differences between the experimental and control groups were compared at each time point by Mann-Whitney's U test. The interobserver coefficients were calculated with SPSS 15.0J (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SD. A value of $P < 0.05$ was accepted as statistically significant.

Results

Interobserver reliability

For in situ hybridization, the interobserver correlation coefficient of the total number of cells and positive cells was 0.98 and 0.96, respectively. For immunohistochemistry, the interobserver correlation coefficient of the total number of cells and positive cells was 0.98 and 0.97, respectively. For mean immunohistochemical scores of matrix staining intensity, the kappa coefficient was 0.89.

In Situ Hybridization

Weak signals of type I collagen was detected after 3 days both in the anterior and the posterior capsule of the immobilized group compared with the control group. The signal intensity gradually decreased over time in the control group (Figure 1). The ratio of type I collagen positive cells significantly decreased from 3 days in both the anterior and posterior capsule of the immobilized group compared with the control group. The ratio of these positive cells decreased gradually over time in the control group (Figure 2).

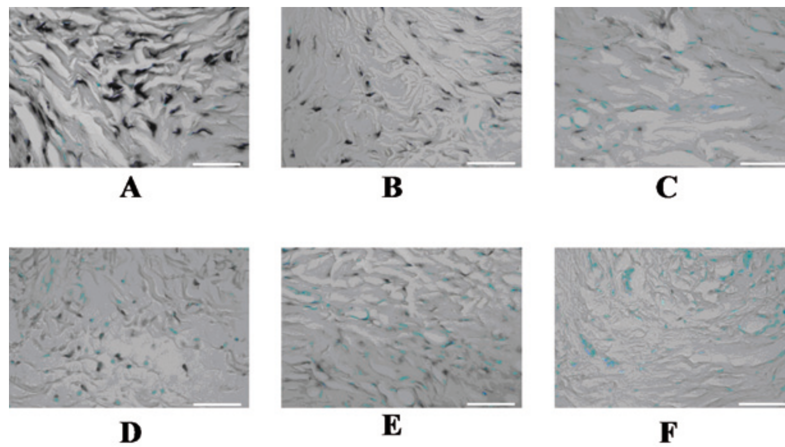

Figure 1: Expression of type I collagen in the posterior capsule (in situ hybridization). Upper row (**A-C**) and lower row (**D-F**) showed the control and the immobilized groups, respectively. **A** and **D**; 3 days, **B** and **E**; 2 weeks, **C** and **F**; 16 weeks. Weak signals of type I collagen was observed after 3 days in the posterior capsules of the immobilized group compared with the control group. The signal intensity of the molecule was gradually decreased over time in the control group. (Scale bars = 50 μm, original magnification × 400)

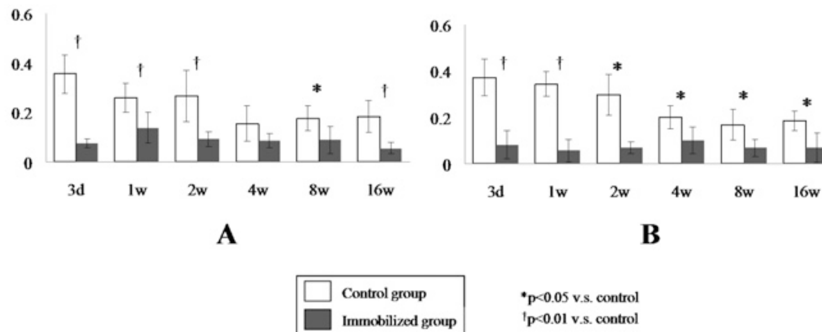

Figure 2: The ratio of type I collagen positive cells in the capsule (in situ hybridization). **A**, the anterior capsule. **B**, the posterior capsule. The ratio of type I collagen positive cells was significantly decreased from 3 days in both the anterior and posterior capsule of the immobilized group compared with the control group. The ratio of positive cells was kept at low level in the immobilized group throughout the experimental period, and it was gradually decreased over time in the control group. The ratio of positive cells was significantly lesser at 3 days, 1, 2, 8, and 16 weeks in the anterior capsule and at 3 days, 1, 2, 4, 8, and 16 weeks in the posterior capsule of the immobilized group compared with the control group.

There was no statistical difference among the immobilized group in the anterior and posterior capsule. Among the control group, significant statistical difference was observed in the anterior and posterior capsules (Anterior capsule; 3 days vs. 4, 8, and 16 weeks. Posterior capsule; 3 days vs. 4, 8, and 16 weeks, 1 week vs. 4, 8, and 16 weeks.)

Immunohistochemistry

Weak (1) to moderate (2) immunostaining of type I collagen was detected both in the anterior and posterior capsule of the two groups. The immunoreactivity of matrix staining intensity in the anterior and posterior capsule was not changed throughout the experimental period (Figure 3, 4). No staining was identified in the negative control.

The ratio of type I collagen positive cells was significantly lesser at 2, 4, and 8 weeks in the anterior capsule and at 4, 8, and 16 weeks in the posterior capsule of the immobilized group compared with the control group (Figure 5).

For immunohistochemical scores of staining intensity, there was no significant statistical difference among the anterior and posterior capsules in the control and immobilized group. For the ratio of positive cells among the control group, significant statistical difference was observed in the anterior and posterior capsules (Anterior capsule; 2 week vs. 8 and 16 weeks, 4 weeks vs. 8 and 16 weeks. Posterior capsule; 1 week vs. 16 weeks, 2 weeks vs. 16 weeks). For the ratio of positive cells among the immobilized group, significant statistical difference was observed in the anterior and posterior capsules (Anterior capsule; 1 week vs. 8 weeks, 2 weeks vs. 8 weeks. Posterior capsule; 1 week vs. 8 and 16 weeks, 2 weeks vs. 8 and 16 weeks).

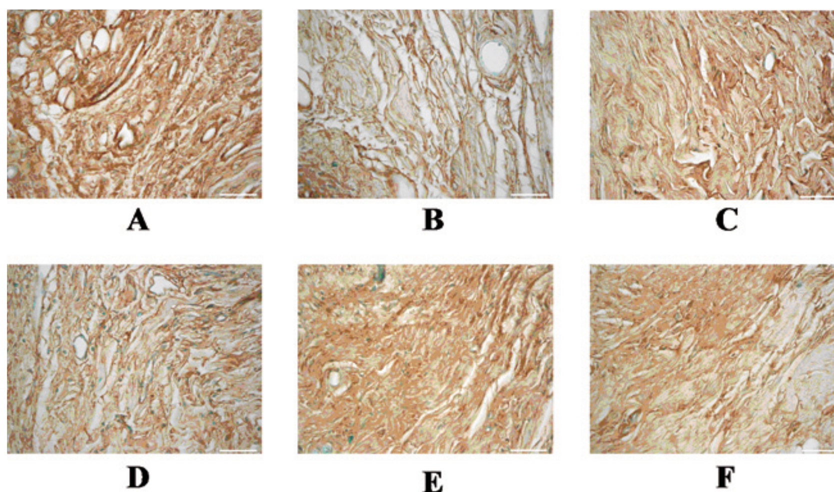

Figure 3: Immunohistostaining of the posterior capsule. Upper row (A-C) and lower row (D-G) showed the control and the immobilized groups, respectively. A and D; 1 weeks, B and E; 2 weeks, C and F; 16 weeks. Weak (1) to moderate (2) immunostaining was observed both in the posterior capsule. (Scale bars = 50 μm, original magnification × 400).

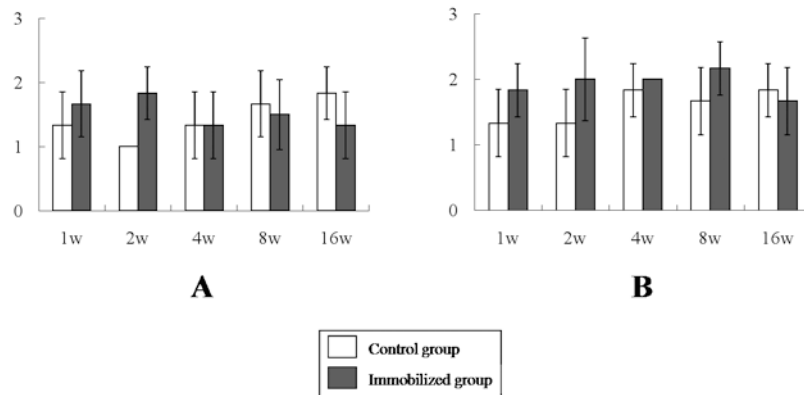

Figure 4: Immunohistochemical scores of matrix staining intensity. **A**, the anterior capsule. **B**, the posterior capsule. There was no statistical difference between the control and immobilized group both in the anterior and posterior capsule.

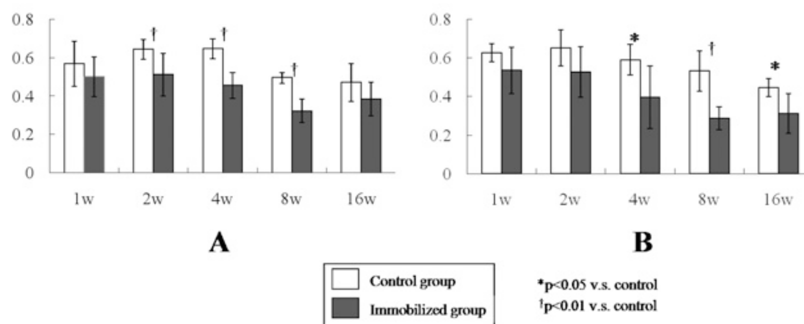

Figure 5: The number of type I collagen positive cells in the capsule (immunohistochemistry). **A**, the anterior capsule. **B**, the posterior capsule. The ratio of type I collagen positive cells of the control and immobilized group was gradually decreased over time. The ratio of positive cells was significantly lesser at 2, 4, and 8 weeks in the anterior capsule and at 4, 8, and 16 weeks in the posterior capsule of the immobilized group compared with the control group.

Discussion

The components of joint contracture after immobilization were classified into arthrogenic and myogenic ones. The arthrogenic components are lesions of bone, ligaments, capsule and synovial membrane, while the myogenic components are lesions of muscle, tendon and fascia, but the former has been considered as an important etiological factor after prolonged immobilization [20, 26]. Our previous report has shown that the sound speed, which strongly correlates with tissue elasticity, increased significantly in the posterior capsule of the immobilized group at 8 and 16 weeks compared with the control group [2]. The sound speed change indicates that the increased elasticity may be one of the causes of limited extension after prolonged immobilization in flexion.

Because collagen provides a major contribution to tissue tensile strength [27],

it is important to determine a structural change of immobilized capsule. Resulting from stress deprivation, water content (3–4%) [28], total glycosaminoglycans (20–40%) [29] and collagen mass (about 5%) [30] decreased after immobilization. The major structural collagens of the synovial capsule are type I and type III collagen and the former made up 83% of the collagen present [22]. Type I collagen has been believed to gain a tissue elasticity. In systemic scleroderma patients, alpha 1 (I)-collagen mRNA expression was clearly observed in their dermal fibroblasts [21]. Type III collagen has been localized in tissues responded to varying tensions [31]. However, we found only a few reports of immobilized joint capsule concerned with the types of structural collagen. In the canine glenohumeral joint immobilized up to 12 weeks, type III collagen increased after immobilization [32]. In the same model as ours but immobilized up to 32 weeks, type I collagen increased but type III collagen decreased using immunohistochemistry [16]. The discrepancy between the two reports regarding type III collagen may be explained that the method of immobilization was semi-rigid or rigid. The former report may have permitted joint motion and subsequently promoted repair process of the tissue. In the present study, we demonstrated a decrease of type I collagen mRNA at the early phase of immobilization and immunoreactivity was not increased after immobilization. The difference between the results of the latter report and our results may be explained that the degree of immobilization angle at surgery was greater than theirs (135 degrees v.s.150 degrees). In short, our method of immobilization might contribute to the posterior capsule in much less tension.

Our results led to the fact that fibrosis had not occurred in the capsule after immobilization. Another possibility of structural changes of the capsule after immobilization is adhesion of a synovial membrane and the capsule. In the same model as ours, a decrease in synovial intima length after immobilization suggested that adhesion of synovium villi rather than pannus proliferation was the major pathophysiological change leading to contracture, and the posterior part was more sensitive than the anterior one [13]. Decrease of water content associated with glycosaminoglycans, which retains water of extra-cellular matrix, is another possibility. Further study is needed to reveal the structural changes of the capsule after immobilization.

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