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Expression of Transforming Growth Factor-β1 and Connective Tissue Growth Factor in the Capsule in a Rat Immobilized Knee Model

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Abstract

Background: Contracture is a very common complication of joint immobilization in daily examination, but its cause is still unknown. A fibrotic change of the capsule is suggested to be one of the main causes of the joint contracture. The goal of this study was to analyze the expression pattern of transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF), which are implicated in fibrosis in the capsule of a rat immobilized knee model.

Materials and Methods: We immobilized the unilateral knee joints of 66 rats in 150 degrees of flexion using a plastic plate and metal screws. Sham operated knee joints of 66 rats had holes drilled and screws inserted but none of them were plated. The capsule from the anterior and posterior portion of the knee joints was harvested at 3 days, 1, 2, 4, 8 and 16 weeks after immobilization and the expression patterns of TGF-β1 and CTGF were characterized using in situ hybridization and immunohistochemistry.

Results: The in situ hybridization demonstrated that the mRNAs of both TGF-β1 and CTGF increased continuously during the first 2 weeks after immobilization and then decreased. The response was relatively higher in the posterior capsule than in the anterior one. In contrast, the immunoreactivity of both TGF-β1 and CTGF increased gradually with time. The response was much stronger in the posterior capsule than in the anterior one.

Conclusions: The capsule has a potency to produce TGF-β1 and CTGF after immobilization. CTGF may play a role in causing and maintaining capsular fibrosis in collaboration with TGF-β1. The fibrotic change in the posterior capsule may have resulted in limited motion in extension in this immobilized knee model in rats. It may be possible to prevent joint contractures by somehow blocking the fibrotic process.

Introduction

A contracture is a very common complication of joint immobilization. The definition of contracture is a loss of passive and active ranges of motion of a joint [1, 2]. Though joint immobilization can be beneficial in decreasing pain and possibly joint damage in the acute phase of inflammatory arthritis [3–5], a contracture usually disturbs activities of daily living. Management of an established contracture

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includes physical therapy and surgical procedures, with equivocal functional results [6, 7]. The resulting burden of disability to the patients and the financial cost to our society are enormous [8]. Despite years of interventions from professionals involved in primary care and rehabilitation [9], the contracture remains a frequent clinical challenge.

Many experimental studies on joint immobilization have been reported [10–16]. A florid intra-articular connective tissue proliferation invading the intra-articular space has been considered a feature of contracture caused by joint immobilization [10–15]. This proliferation tissue, mainly composed of synovial lining cells, fibroblasts, fibrocytes, monocytes, adipocytes, and extracellular matrix [10–12, 16, 17], is defined as pannus [18]. Contact between the pannus and the surface of the articular cartilage has been postulated to prevent cartilage nutrition and/or mediate pathophysiological changes leading to cartilage degeneration and joint stiffness after immobilization [10, 15, 19–22]. However, there have been contradictory reports about phenomena after joint immobilization. Some reported that pannus proliferation occurred after immobilization [5, 8, 23, 24] and no contact between pannus and articular cartilage was observed [25]. Others showed that capsular stiffness, resulting in degenerative changes such as synovial atrophy, retraction, fibrosis, and adhesion, might contribute more to joint contractures than muscle function [2, 5, 10, 11, 17, 26–28].

Transforming growth factor-β (TGF-β) has been demonstrated to have multiple biological functions, especially in wound repair acceleration and experimental organ fibrosis [29]. TGF-β upregulates fibroblast proliferation and extracellular matrix synthesis and reduces matrix degradation after injury [30]. Among the three isoforms of TGF-β1, 2 and 3 in mammals, TGF-β1 is most implicated in fibrosis [31, 32]. A previous report described high expression of TGF-β and TGF-β receptors in synovial cells of human adhesive capsulitis using immunohistochemistry [33]. The concentration of TGF-β1 in synovial fluid increased after immobilization in a rabbit immobilized knee model [34]. TGF-β may be one of the factors related to joint contracture. Injection of TGF-β into the knee and ankle joints of rats induced transient synovial hyperplasia [35, 36]. Subcutaneous injection of TGF-β1 into newborn mice for 3 consecutive days caused reversible granulation tissue formation without persistent fibrosis [37].

Other factors besides TGF-β may be involved in causing the joint contracture. Subcutaneous injection of connective tissue growth factor (CTGF) in addition to TGF-β in newborn mice produced long-term persistent fibrosis [29]. This result suggests that CTGF may play a role in causing and maintaining skin fibrosis in collaboration with TGF-β.

In our previous study, the elasticity increased in the capsule after 8 and 16 weeks of immobilization as assessed by scanning acoustic microscopy [1]. In that context, we hypothesized that fibrosis, which occurred in the capsule after immobilization, might have contributed to the limited extension observed.

In this study, we investigated expression of TGF-β1 and CTGF in the capsule during immobilization using in situ hybridization and immunohistochemistry.

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 380–400g) were used. Intra-operative and post-operative analgesia with buprenorphine (0.05 mg/kg) was injected subcutaneously. The unilateral 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 [1, 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. To keep the morphology intact, the specimens were decalcified as a whole knee joints 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 (Figure 1).

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 CTGF (876–1555 bp: GenBank accession number NM_022266) was obtained from total RNA of embryonic rat limbs using

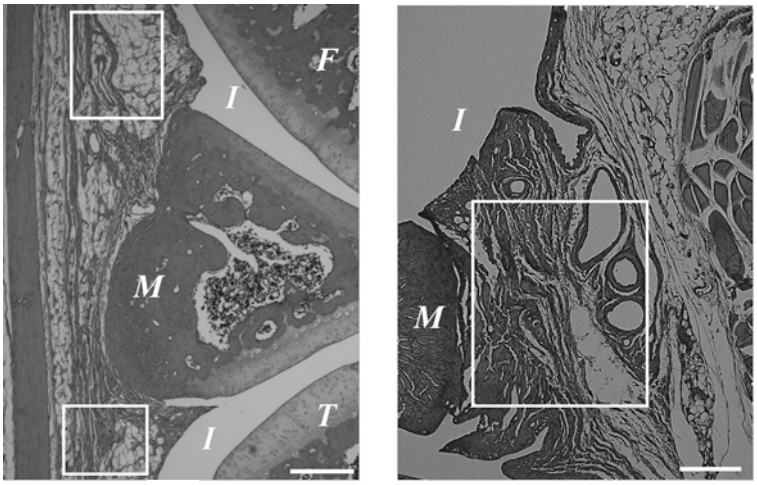

Figure 1. Microphotographs of a sagittal section of a rat knee. **A**: the anterior capsule, **B**: the posterior capsule. Squares indicate regions of analysis for in situ hybridization and immunohistochemistry. ***M***: meniscus, ***I***: intra-articular space, ***F***: Femur, ***T***: Tibia. (Scale bars = 300 μm, Original magnification × 5, hematoxylin-eosin stain).

reverse transcription followed by RT-PCR and subcloned into the pCRII TOPO (Invitrogen; Carlsbad, CA). The following oligonucleotide primers were used for the RT-PCR: upstream primer, 5′CGGGTTACCAATGACAATA3′, downstream primer, 5′GTCTTTCTCCTGGCATCTC3′. The cDNA was verified by digestion with restriction enzymes and was confirmed by dideoxynucleotide sequencing. Fragments of rat mature TGF-β1 (GenBank accession number NM_021578) were purchased from Riken Gene Bank (1230 bp, RDB1195, Saitama,P PJapan). A fragment (338–885 bp) digested with PstI was subcloned into the pBluescript II (KS+) (Stratagene, La Jolla, Calif., USA). Antisense and sense riboprobes were generated by T3/XbaI and T7/EcoRV for CTGF and T7/BamHI and Sp6/EcoRV for TGF-β1, respectively.sense and sense riboprobes were generated by T3/XbaI and T7/EcoRV for CTGF and T7/BamHI and Sp6/EcoRV for TGF-β1, respectively.

In situ hybridization

The protocol used in the present study has been reported elsewhere [38] 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 min. After being washed in PBS, the sections were incubated in proteinase K (20 μg/ml; Roche) in PBS for 30 min 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 hrs at 45°C. The sections were washed and treated with RNase (Type 1a, 20 μg/ml; Sigma, St Louis, MO) for 30 min 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 hyperchondrocyte in the growth plate. The ratios of TGF-β1 and CTGF positive cells to the total number of cells counted (at least 100 cells) at high magnification (× 400) were calculated.

Two investigators were blinded with regard to the group of knees that were studied. The consistency of the results was verified by assessing interobserver correlations of 10 randomly chosen histological sections according to the previous report [39].

Immunohistochemistry

The sections were deparaffinized and immersed in 3.0% hydrogen peroxide for 10 min at room temperature. After being washed in PBS, endogenous immunoglobulins were blocked by incubation for 30 min with 10% normal goat serum (Nichirei, Tokyo, Japan) in PBS. The slides were washed again in PBS for 30 min and incubated with a polyclonal rabbit anti-mouse CTGF antibody (dilution 1: 100, Abcam, Cambridge, UK) or rabbit anti-human TGF-β1 antibody (dilution 1: 400, Santa Cruz Biotechnology, Heidelberg, Germany, Cat. No. sc-146) 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 the 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 (dilution 1: 100, Santa Cruz Biotechnology, Heidelberg, Germany, Cat. No. sc-2027) 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 [33]. Fibroblast-like cells in the capsule were counted (two to three areas per section). The ratios of TGF-β1 and CTGF 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 [24]. The staining intensity of endothelial cells was defined as moderate staining 2.

Two investigators were blinded with regard to the group of knees that 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 according to the previous report [59]. All the specimens were analyzed for immunoreactivity of matrix staining intensity by two independent investigators.

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 coefficients of the total number of cells and positive cells in TGF-β1 were 0.97 and 0.99, and those in CTGF were 0.92 and 0.99, respectively. For immunohistochemistry, the interobserver correlation coefficients of the total number of cells and positive cells in TGF-β1 were 0.99 and 0.88, and those in CTGF were 0.95 and 0.96, respectively. For mean immunohistochemical scores of matrix staining intensity, the kappa coefficients were 0.88 in TGF-β1 and 0.96 in CTGF, respectively.

In situ hybridization

Strong signals were detected in both TGF-β1 and CTGF from 3 days up to 2 weeks in the immobilized group. The signals of both molecules started to decrease after 4 weeks in the immobilized group and finally reached the control level at 16 weeks. Very weak signals were observed in all the control groups (Figure 2). No hybridization signal was identified with sense probes for both molecules (data not shown). The ratio of TGF-β1 positive cells was significantly greater from 3 days to 2 weeks in the anterior capsule and from 3 days to 4 weeks in the posterior capsule of the immobilized group compared with the control group. The ratio of CTGF positive cells was significantly greater from 3 days to 2 weeks in the anterior capsule and from 3 days to 8 weeks in the posterior capsule of the immobilized group compared with the control group (Figure 3).

In the immobilized group, there were statistically significant differences with regard to TGF-β1 expression in the anterior and posterior capsules (Anterior capsule: 3 days vs. 4, 8, and 16 weeks; 1week vs. 2, 4, 8, and 16 weeks; and 2 weeks vs. 4, 8, and 16 weeks. Posterior capsule: 3 days vs. 1, 4, 8, and 16 weeks; 1 week vs. 2, 4, 8, and 16 weeks; and 2 weeks vs. 4, 8, and 16 weeks) and CTGF expression (Anterior capsule: 3 days vs. 1, 4, 8, and 16 weeks; 1 week vs. 8, and 16 week; 2 weeks vs. 4, 8, and 16 weeks; and 4 weeks vs. 8 and 16 weeks. Posterior capsule: 3 days vs. 2, 4, 8, and 16 weeks; 1week vs. 4, 8, and 16 weeks; and 2 weeks vs. 8, 16 weeks). There was no statistically significant difference between the control group in TGF-β1 and CTGF expression at any time point.

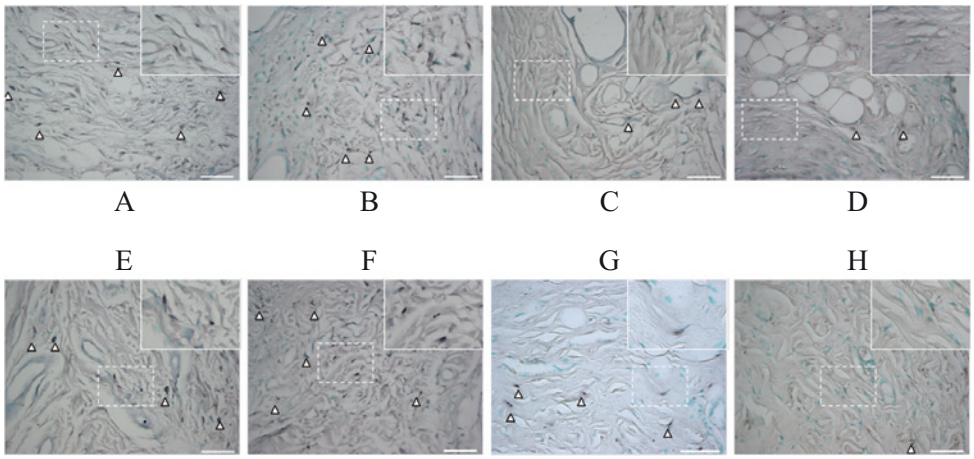

Figure 2. Expression of TGF-β1 and CTGF in the capsule (in situ hybridization). Upper row (A-D) and lower row (E-H) show TGF-β1 and CTGF expression patterns of mRNAs, respectively. A and E: the anterior capsule of the immobilized group at 1 week. B and F: the posterior capsule of the immobilized group at 1 week. C and G: the posterior capsule of the immobilized group at 8 weeks. D and H: the posterior capsule of the control group at 8 weeks. Solid line square of upper right corner in each figure is a magnification of the dotted square. Strong signals for both TGF-β1 and CTGF were observed both in the anterior and posterior capsules of the immobilized group at 1 week. The signals of both molecules were decreased in the posterior capsule of the immobilized group at 8 weeks. Weaker signals of both molecules were detected in the control group at 8 weeks. (Arrow heads indicate positive cells. Scale bars = 50 μm, original magnification × 400).

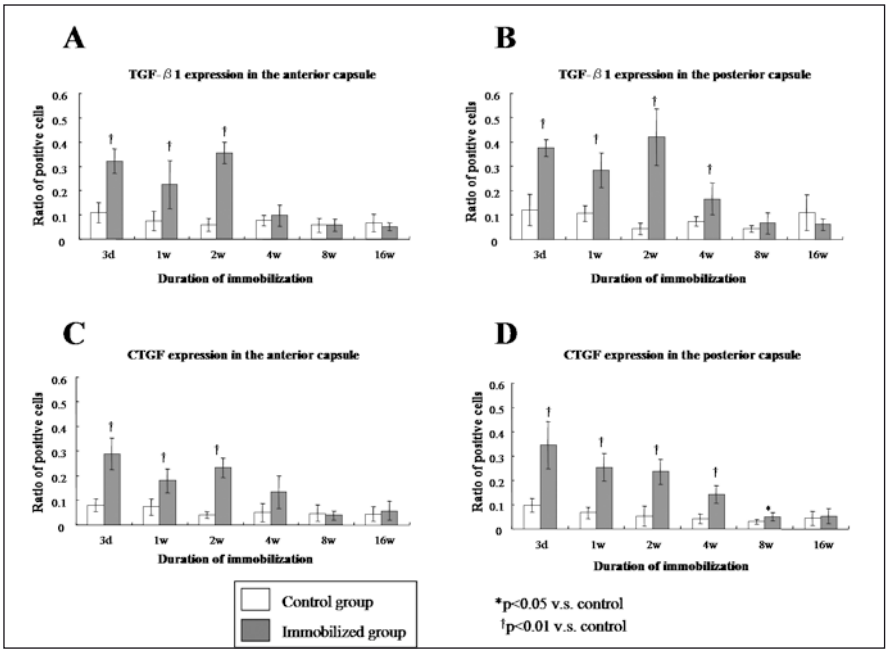

Figure 3. The ratio of TGF-β1 and CTGF positive cells in the capsule (in situ hybridization). TGF-β1 in the anterior and posterior capsules are given in **A** and **B**, and CTGF in **C** and **D**. (*p<0.05 vs. control, P†Pp<0.01 vs. control)

Immunohistochemistry

The ratio of TGF-β1 positive cells was higher at 1 week in the anterior capsule and for 2 weeks in the posterior capsule of the immobilized group when compared with the control group. The ratio of CTGF positive cells was higher from 1 to 4 weeks in the anterior capsule and from 1 to 8 weeks in the posterior capsule of the immobilized group when compared with the control group (Figure 4).

Weak (1) staining of TGF-β1 was observed in the anterior and posterior capsules of the control group at any time point. On the other hand, moderate (2) staining was detected in the anterior capsule of the immobilized group, which gradually increased but not as strong as in the posterior capsule. In the posterior capsule of the immobilized group, moderate (2) to strong (3) staining was observed at 4 to 16 weeks. The immunoreactivity was significantly greater from 1 to 16 weeks in the posterior capsule of the immobilized group compared with the control group (Figure 5, 6).

Regarding CTGF, immunoreactivity was much weaker than for TGF-β1 in the anterior and posterior capsules of the control group at all time points. Weak (1) staining was observed in the anterior and posterior capsules of the control group at any time point. In the immobilized group, weak (1) staining was detected in the anterior capsule. In the posterior capsule, moderate (2) staining was detected at 1 and 2 weeks and the immunoreactivity became gradually stronger (3) at 4 to 16 weeks. The immunoreactivity was significantly greater at 1, 4, 8 and 16 weeks in

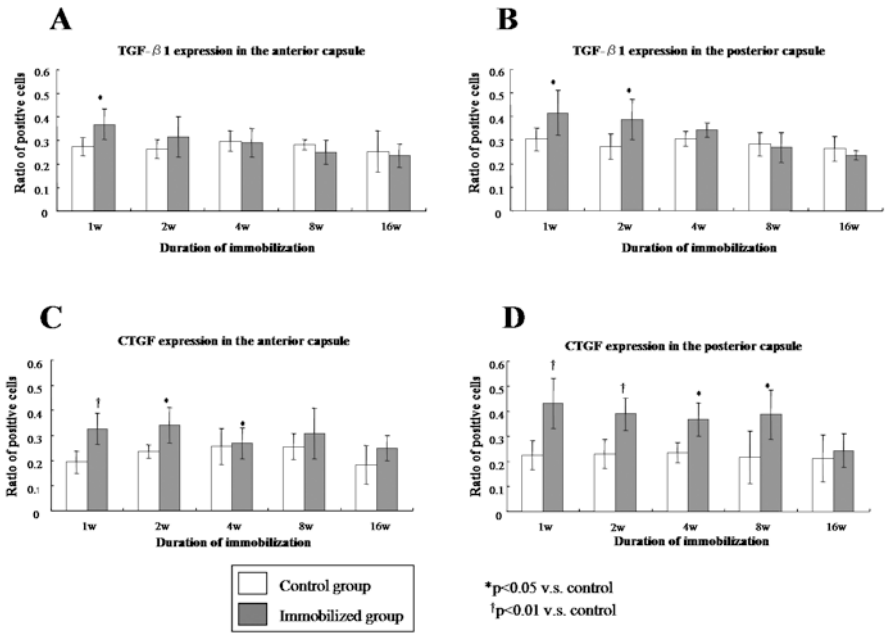

Figure 4. Ratios of TGF-β1 and CTGF positive cells in the capsule (immunohistochemistry). (*p<0.05 vs. control, †p<0.01 vs. control)

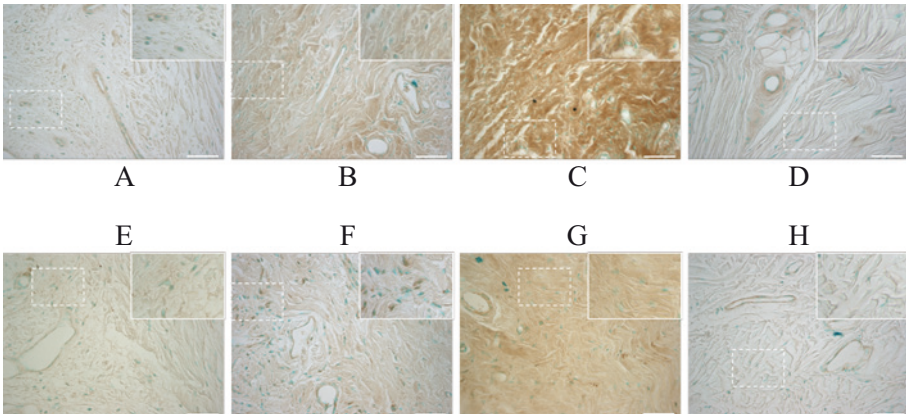

Figure 5. Immunostainings of TGF-β1 and CTGF in the capsule. Upper row (**A-D)** and lower row (**E-H)** show TGF-β1 and CTGF immunoreactivity, respectively. **A** and **E**: the anterior capsule of the immobilized group at 1week. **B** and **F**: the posterior capsule of the immobilized group at 1 week. **C** and **G**: the posterior capsule of the immobilized group at 8 weeks. **D** and **H**: the posterior capsule of the control group at 8 weeks. Solid line square of upper right corner in each figure is a magnification of the dotted square. Weak (1) immunostainings of both molecules were observed in the anterior and posterior capsules of the immobilized group at 1 week. Strong (3) immunostainings of TGF-β1 and moderate (2) to strong (3) immunostaining of CTGF were detected at 8 weeks in the immobilized group. Weak (1) immunostainings of both molecules were observed at 8 weeks in the control group. (Scale bars = 50 μm, original magnification × 400)

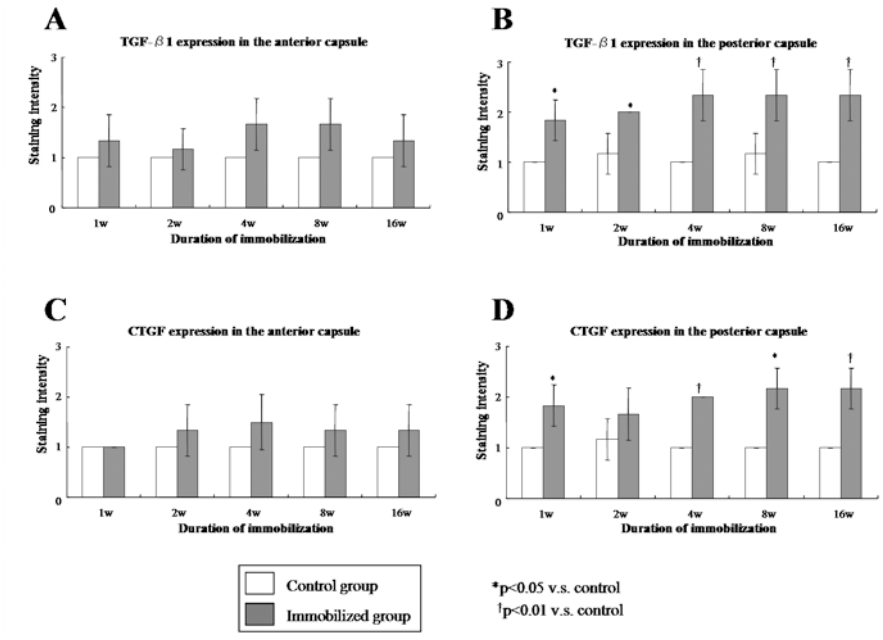

Figure 6. Immunohistochemical scores of matrix staining intensity. (*p<0.05 vs. control, †p<0.01 vs. control).

the posterior capsule of the immobilized group compared with the control group (Figure 5, 6). There was no staining for any of the molecules in the negative control (data not shown).

In the immobilized group, the ratio of TGF-β1 positive cells differed significantly with regard to the duration of the immobilization as follows: Anterior capsule; 1 week vs. 8 and 16 weeks. Posterior capsule; 1 week vs. 8 and 16 weeks, 2 weeks vs. 8 and 16 weeks. As regards ratios of CTGF positive cells: Posterior capsule; 1 and 2 weeks vs. 16 weeks. In the control group, TGF-β1 and CTGF ratios did not differ at any time point. Within the two different groups of animals, immobilized and controls, and within the two parts of the capsule, the immunohistochemical scores for staining intensity of the two molecules were similar.

Discussion

The components of joint contracture after immobilization have been classified into arthrogenic and myogenic ones, but the former has been considered as an important etiological factor after prolonged immobilization [28, 40]. The decrease in synovial intima length after immobilization in the same model as ours 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 [5]. Our previous report has shown that sound speed transmitted through the posterior capsule, which strongly correlates with the capsular elasticity, increased significantly in the immobilized group at 8 and 16 weeks compared with the control group [1]. The sound speed change indicates that the increased elasticity may be one of the causes of limited extension after prolonged immobilization in flexion. Difference in elasticity of the anterior and posterior capsules may be explained by the potential remnant mobility of the patella in the medial/lateral directions in the anterior part of the joint even after immobilization. Another possible explanation would be a difference in blood flow in the stretched side and compressed side of the joint. We actually observed a limitation in extension, which rapidly progressed for 8 weeks and advanced slowly thereafter in the immobilized group [41]. Whatever the reason, the fibrosis occurred mainly in the posterior capsule.

TGF-β is known as a multi-functional regulator of cellular activity [42] and is also thought to be a potent regulator of wound healing, immune response and bone remodeling in vivo [43]. TGF-β is also a crucial regulator of ECM deposition, as it controls the expression of components of ECM, which includes type I collagen, type III collagen and fibronectin in fibroblasts [44, 45]. Though three structural isoforms of TGF-β (TGF-β1, 2, 3), encoded by three distinct genes, have been identified in mammals [46], TGF-β1 is the most implicated in fibrosis [31, 32].

A previous study showed high immunoreactivity of TGF-β in synovial cells of human adhesive capsulitis by immunohistochemistry [33]. The concentration of TGF-β1 in synovial fluid increased 7 days after immobilization in a rabbit immobilized knee model [34]. Further, intra-articular injection of TGF-β1 neutralizing

antibodies reduced adhesion in an intra-articular adhesion model in rabbits [45] and diminished fibrosis in an animal model of chronic erosive polyarthritis [47]. TGF-β1 may play an initial important role in producing a joint contracture. In our study the ratio of TGF-β1 positive cells was higher from 3 days to 2 weeks in the anterior and posterior capsules as evidenced by in situ hybridization. However, the ratio was higher in the posterior capsule of the immobilized group up to 4 weeks. In the immunohistochemical study we found that the ratio of of cells positive for TGF-β1 was higher in the posterior capsule as compared with that of the anterior capsule. Stronger immunoreactivity of TGF-β1 was detected at 4 to 16 weeks in the posterior capsule of the immobilized group compared with the anterior capsule. The difference of prolonged expression of mRNA and accumulation of TGF-β1 may be one of the causes of increased elasticity of the posterior capsule of the immobilized group detected by scanning acoustic microscopy [1]. Though stronger immunoreactivity of TGF-β1 was observed, only slight synovial hyperplasia was detected in our study.

The discrepancy in the expression pattern of in situ hybridization and immunohistochemisry can be explained as follows; in situ hybridization detects instantaneous expression of mRNA in cells and immunohistochemistry detects accumulation of proteins in cells and extra-cellular matrices. Proteins are produced by mRNA as a template and accumulated in extra-cellular matrices.

CTGF is a member of the immediate early gene family, which contains six distinct members: Cef10/Cyr61 (CCN1), CTGF/Fisp-12 (CCN2), Nov (CCN3), Elm1/WISP-1 (CCN4), Cop-1/WISP-2 (CCN5) and WISP-3 (CCN6) [48]. Because a TGF-β response element was found in the CTGF promoter [49], CTGF may be a potential downstream mediator for TGF-β signaling in fibroblasts and some of the actions of TGFβ1 in wound healing may be due to CTGF induction and action [50]. CTGF mRNA is overexpressed in a large number of fibrotic conditions, including SSc [51], keloid [52], renal fibrosi [53], inflammatory bowel disease [54], chronic pancreatitis [55], lung fibrosis [56] and liver fibrosis [57].

Subcutaneous injection of TGF-β1 into newborn mice caused a transient fibrotic response [29], which indicates that other factors besides TGF-β1 may be involved in making persistent fibrosis. Subcutaneous injection of CTGF alone had little effect, but co-injection of TGF-β1 and CTGF resulted in persistent fibrosis [29]. This result suggests that CTGF may play a role in causing and maintaining skin fibrosis in collaboration with TGF-β1, which has been named "The 2-step fibrosis hypothesis in Systemic scleroderma" [58]. Similarly, injection of TGF-β into joints of rats induced synovial hyperplasia but its reaction was reversible [35, 36]. The enhanced expression of mRNA and accumulation of proteins as TGF-β1 and CTGF in the posterior capsule of the immobilized group may support the 2-step fibrosis hypothesis.

We conclude that both TGF-β1 and CTGF are likely to play an important role in causing and maintaining a joint contracture. It may be possible to prevent joint contractures by somehow blocking the fibrotic process.

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