Tenascin-C Concentration in Synovial Fluid Correlates with Radiographic Progression of Knee Osteoarthritis

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ABSTRACT. ***Objective.*** Osteoarthritis (OA) is a major cause of disability and represents the most common disease in the aging population. Although the course of the disease is generally assessed using standard radiographic images, biochemical markers may be employed to detect the disease and determine the degree of severity. We developed an enzyme linked immunosorbent assay (ELISA) system using a monoclonal antibody specific for the large-splice variants of tenascin-C (TN-C) and examined whether TN-C in synovial fluid (SF) is an adequate biochemical marker of OA progression.

Methods. SF samples were obtained from knees of 74 patients with OA and 16 without OA. Based on the radiographic grading of the OA severity, the knees were divided into 3 groups: mild, moderate, and severe OA. Expression of TN-C splice variants was examined using immunoblotting. TN-C concentrations were determined by ELISA.

Results. Western blotting showed the presence of large TN-C variants in SF from severe OA. TN-C levels were 5-fold higher in OA samples compared to subjects without OA ($p < 0.0001$). TN-C levels were not different between control cases and mild OA, but increased significantly in moderate ($p = 0.0244$) and severe OA ($p < 0.0001$). After adjusting TN-C levels for age, body mass index, and sex, TN-C levels correlated with radiographic progression of knee OA ($R^2 = 0.404$, $p < 0.0001$).

Conclusion. TN-C, including the large-variant subunits, is a useful biochemical marker for OA progression in the later stages of disease. (J Rheumatol 2004;31:2021–6)

Key Indexing Terms:
OSTEOARTHRITIS TENASCIN-C SYNOVIAL FLUID ELISA

Osteoarthritis (OA) is characterized by changes in cartilage, subchondral bone, and synovium. This disease is a major cause of disability, and represents the most common disease in the aging population. The risk of disability attributable to knee OA is comparable to the risk associated with heart disease[1]. In clinical practice, the course of the disease is generally assessed using standard radiographic images of the joint, which can reveal joint space narrowing and subchondral sclerosis associated with osteophytes[2]. In addition, some biochemical markers have been examined as potential ways to detect the disease and determine the degree of severity. The candidates include proteoglycan fragments[3], cartilage oligomeric matrix protein[4], collagen II C-propeptide[5], hyaluronan[6], YKL-40[7,8], interleukin 6, tumor necrosis factor-α[9], matrix metalloproteinases (MMP), tissue inhibitor of metalloproteinases[3,10,11], pyridinoline, and deoxypyridinoline[12]. However, no marker has yet gained unrestricted acceptance in the clinical routine for the diagnosis and monitoring of OA. Only serum cartilage oligomeric matrix protein concentrations seem to parallel the radiographic progression of OA[4].

Tenascin-C (TN-C) is an extracellular matrix glycoprotein that is predominantly expressed during embryogenesis. While the expression is repressed in normal adult tissues, it reappears in association with wound healing, regeneration, or neoplastic events[13,14]. In articular cartilage, expression of TN-C is also associated with the development of, but decreases markedly during maturation of chondrocytes[15,16], and is finally almost abolished in adult cartilage[17,18]. In diseased joints including those seen in OA and rheumatoid arthritis (RA), TN-C is highly expressed in both cartilage and synovium[17-20]. Using Western blot, TN-C has also been identified in synovial fluids (SF) from patients with OA[17,21]. A recent study using an enzyme linked immunosorbent assay (ELISA) has shown that TN-C concentrations in SF correlate with the degree of cartilage degradation determined at the time of operation[22].

The amino acid sequence of TN-C subunits includes epidermal growth factor (EGF)-like repeats, fibronectin type III (FNIII)-like repeats, and a domain with a sequence similar to fibrinogen at the carboxyl terminus[23]. The sizes of the TN-C monomers vary with the alternative splicing of the FNIII repeats, which are designated A–D; the FNIII domain is inserted between the conserved repeats 5 and 6 in human TN-C[24,25]. In adults, the small TN-C variant, in which the

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alternatively spliced domains are spliced out, is barely but constitutively expressed in static tissues[25,26], while the large variants, containing the alternatively spliced FNIII domains in various combinations, are predominantly expressed in developing tissues and in pathological tissues associated with regeneration, inflammation, and tumorigenesis[13,15,25,26]. Western blot analyses of SF of joints affected by OA and RA have also shown a prominent band of the large TN-C variants[17,21]. Therefore, the large TN-C variants could be considered to be a more specific biological marker for articular damage than total TN-C.

We developed an ELISA using a monoclonal antibody mAb specific to the large-splice variants of TN-C. The purposes of this cross-sectional study were to determine the concentrations of large TN-C variants in SF from patients with OA and to evaluate a possible correlation between these concentrations and radiographic grading of OA.

MATERIALS AND METHODS

Patients and samples. SF samples (n = 74) were obtained by aspiration at the time of primary total joint arthroplasty or arthroscopic surgery on 51 women (60 knees) and 12 men (14 knees) with primary OA of the knee who fulfilled the American College of Rheumatology criteria[27]. No patient was receiving intraarticular hyaluronan or steroids. The knees were divided into 3 groups based on the radiographic grading of the OA severity described by Kellgren and Lawrence[2]: mild (grade 1 and grade 2, showing osteophytes), moderate (grade 3, showing moderate diminution of joint space), and severe (grade 4, showing a large reduction in joint space with subchondral bone sclerosis). Two independent readers blinded to the source of the data graded the knees. As a control, SF samples were also obtained from patients with knee joint pain including bruises, meniscus tears, and ligament tears during their diagnostic or therapeutic procedures (n = 16). All patients gave informed consent, and this study was approved by the local ethics committee. All patients had serum C-reactive protein concentrations within the normal range for healthy adults. The characteristics of the groups are shown in Table 1.

Monoclonal antibody specific to the large TN-C splice variants. To produce a mAb specific to the large TN-C splice variants, we first prepared a recombinant protein containing FNIII A4-D domains of human TN-C as described[26]. Human TN-C was purified from conditioned media of a glioma cell line, U251MG, as described[14]. To obtain higher efficiencies of antibody production, we immunized congenic TN-C-null mice with the recombinant protein or purified TN-C. Hybridomas were prepared by fusion of immunized mouse spleen cells with Sp2/0 myeloma cells. Positive hybridoma clones were detected by enzyme immunoassay using plates coated with native human TN-C and peroxidase-conjugated anti-mouse IgG antibodies (Biorad, Hercules, CA, USA), and cloned 3 times. The isotypes of the mAb were determined with the mouse mAb isotype kit (Amersham Lifescience, Piscataway, NJ, USA) and were IgG1κ. We used one mAb against the FNIII C domain (19C4MS) and one against the EGF-like domain (4F10TT), which has been characterized[28].

Western blot analysis. Samples of 8-fold diluted SF were resolved on sodium dodecyl sulfate-polyacrylamide gels with a 2–15% polyacrylamide gradient. The electrophoresed proteins were blotted onto Immobilon membranes (Millipore Japan, Tokyo, Japan), blocked with blocking buffer [(Tris-buffered saline, pH 7.6) and 0.5% skimmed milk] and incubated with monoclonal anti-TN-C antibody (4F10TT or 19C4MS) at 4°C overnight. The membranes were then washed in blocking buffer and treated with peroxidase-labeled goat anti-mouse IgG Fab' (1:400; MBL, Nagoya, Japan) for 1 h at room temperature. The immunoreactivity was developed in diaminobenzidine/H_2O_2 solution. The experiment was performed in duplicate.

ELISA. The SF were centrifuged at 15,000 × g for 15 min and the supernatants were stored at –80°C until analyzed. Levels of large-subunit TN-C, containing the FNIII C domain, were determined using an ELISA kit (IBL, Gunma, Japan) with 2 mAb, 4F10TT and 19C4MS. Samples were diluted 10-fold and incubated in 96-well ELISA plates coated with 19C4MS for 1 h at 37°C. After washing, horseradish peroxidase-conjugated anti-TN-C Fab' fragments (4F10TT Fab') were added. After incubation for 30 min at 4°C, the absorbance at 450 nm was determined using an ELISA plate reader. Results were calculated from the mean absorbance of duplicate wells. TN-C purified from conditioned media of human glioma cells was used to prepare a standard curve. The sensitivity of this assay is 0.38 ng/ml and the intraassay coefficient of variation ranges from 0.5% to 13.3%.

Statistical analysis. Statistical differences between the groups were evaluated by Kruskal-Wallis analysis and the Mann-Whitney U-test. Correlation was estimated by Spearman's rank correlation test and a multiple regression analysis was also performed. P values less than 0.05 were considered significant.

RESULTS

Western blot analysis. To characterize the antibodies used in this study, immunoblots of intact TN-C from human glioma cells were analyzed with the mAb 4F10TT and 19C4MS. Antibody 4F10TT labeled both TN-C variants of human glioma TN-C, while 19C4MS labeled the large variant (L) but not the small one (S; Figure 1A). Immunoblotting using recombinant fragments of cDNA encoding FNIII A4-D region of human TN-C showed that 19C4MS reacts with the proteins of FNIII A4-D and A4-C, but not A4-B and A4 (Figure 1B). Thus, the epitope recognized by 19C4MS is located on the FNIII C domain. We examined the presence of large TN-C variants, which include the FNIII C domain, in the SF of patients with OA. Antibody 4F10TT, which is specific to the EGF-like domain, reacted with all TN-C variants with molecular weights ranging from 350 to 210 kDa in

Table 1. Characteristics of the patients*.

	Control, n = 16	OA				p
		Overall, n = 74	Mild, n = 18	Moderate, n = 34	Severe, n = 22	
Age, years	26.9 (11–50)	71.5 (51–86)	66.3 (51–86)	71.4 (53–86)	75.5 (59–86)	< 0.0001
Weight, kg	63.4 (35–93)	58.8 (35–82)	59.1 (41–77)	58.4 (35–82)	58.5 (41–76)	0.5911
BMI, kg/m^2	21.8 (16.6–27.8)	25.6 (16.6–33.8)	24.7 (19.1–28.1)	25.8 (16.6–32.4)	26.2 (19.5–33.8)	0.0011
Percentage women	19	81	61	82	95	< 0.0001

* Except for the percentage of women, values are median (range).

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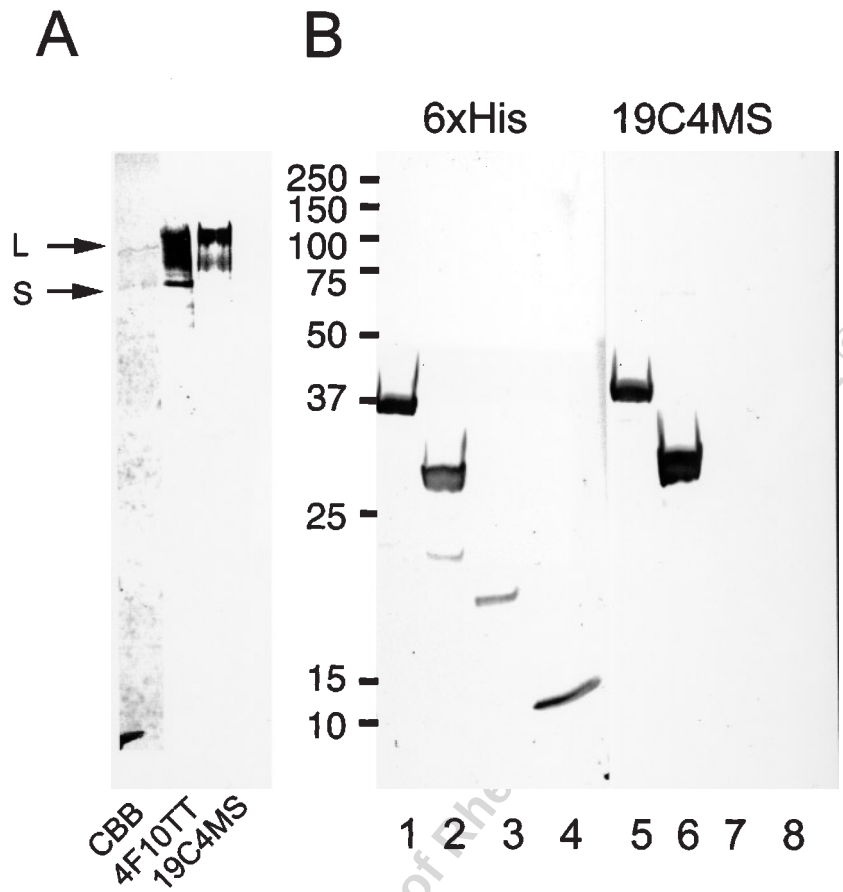

Figure 1. Characterization of mAb by immunoblotting. A. Protein staining of purified TN-C by Coomassie brilliant blue (CBB) showed 2 major bands (L and S). In immunoblotting, 4F10TT reacted with bands around 350 kDa (L) and at 210 kDa (S), while 19C4MS showed bands only around 350 kDa (L). B. Recombinant proteins FNIII A4-D (Lane 1), FNIII A4-C (Lane 2), FNIII A4-B (Lane 3), and FNIII A4 (Lane 4) with 6×His tags were visualized with an anti-6×His antibody. FNIIIA4-D (Lane 5) and FNIIIA4-C (Lane 6) were recognized by 19C4MS, but FNIII A4-B (Lane 7) and FNIII A4 (Lane 8) were not, indicating that 19C4MS can react with FNIII C repeats of human TN-C.

SF from a patient with severe OA (Figure 2, Lane 3). The main band at 350 kDa comigrated with the largest variant of human glioma TN-C (L). The smallest variant (S), which lacks the alternatively spliced FNIII repeats, was detected at 210 kDa. Faint bands at 210 kDa were detected in the sample from a patient with mild OA (Lane 2). No bands larger than 200 kDa were detected in the control sample, and degraded fragments at around 100 kDa were faintly labeled (Lane 1). Antibody 19C4MS for the large variants reacted with bands at 350 and 240 kDa, as well as with bands smaller than 200 kDa from the same severe OA sample as shown in Lane 3 (Lane 6). In the samples from both a patient with mild OA (Lane 5) and the control (Lane 4), no bands were observed. These results indicate that the level of large TN-C variants is considerably elevated in SF of joints with severe OA.

ELISA. The TN-C levels of OA samples (median 36.3 ng/ml) were roughly 5-fold higher than the control values (median 7.7 ng/ml; $p < 0.0001$). The TN-C levels were not statistically distinguishable between control and mild OA (median 14.9 ng/ml) cases, but increased significantly in moderate (median 29.9 ng/ml) and severe OA (median 136.6 ng/ml) cases, showing association with disease progression (Figure 3). In rank correlation tests, the TN-C levels also correlated with the radiographic grading levels ($R = 0.665$, $p < 0.0001$). The TN-C levels were significantly affected by age and body mass index (BMI) ($R = 0.384$, $p = 0.0003$ and $R = 0.262$, $p = 0.0134$, respectively). TN-C levels were significantly higher in women than in men (median 45.1 vs 12.9 ng/ml; $p = 0.0011$). After adjusting TN-C levels for age, BMI, and sex, the correlation with the radiographic grading levels remained statistically significant ($R^2 = 0.404$, $p < 0.0001$).

DISCUSSION

A variety of TN-C variants with masses ranging from 350 to

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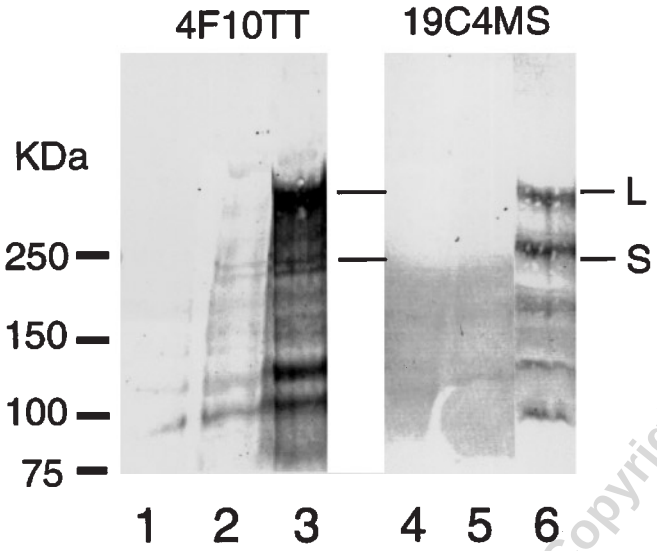

Figure 2. Representative Western blot analysis of SF using mAb 4F10TT (Lanes 1–3) and 19C4MS (Lanes 4–6) against TN-C. SF from controls (Lanes 1 and 4) and patients with mild OA (Lanes 2 and 5) and severe OA (Lanes 3 and 6) are shown. Positions of the largest (L) and the smallest (S) bands of human glioma TN-C, which comigrated on the gel, are indicated. Immunoreactive TN-C variants occur between the L and S bands, and degraded fragments are smaller than S. Joint fluid from patients with severe OA contains a considerable amount of the large variants.

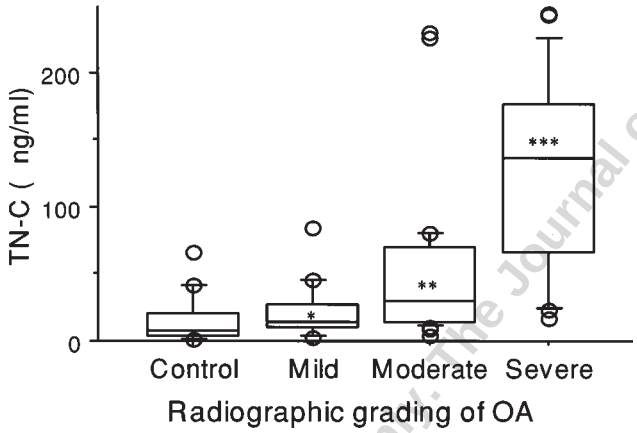

Figure 3. SF concentrations of TN-C in patients with varying severity of OA. Top, bottom, and middle lines of the box graph correspond to 75th and 25th percentile and median, respectively. Bars show the range of 10th and 90th percentiles. Each circle represents an outlier. *p = 0.0785 compared with control, **p = 0.0244 compared with mild, ***p < 0.0001 compared with moderate.

210 kDa are generated by alternative splicing of FNIII A–D repeats of TN-C RNA. Expression of the small variant is low and restricted, but remains stable in quiescent tissues of adults. The larger isoforms are not expressed in normal tissues, but the expression is remarkably upregulated in pathological tissues undergoing tissue remodeling as in regeneration, inflammation, and tumorigenesis[26,29-32]. Based on immunoblotting, we found increased TN-C in synovial fluids of patients with OA and observed that the large variants are much more prevalent than the small variant. However, a previous immunoblot analysis of proteins extracted from OA cartilage of diseased joints showed the small variant is more abundant than the large variants[17]. Compared with the large variants, the small variant is known to be well incorporated into the chondroid and other matrices[32,33]. The large variants, therefore, could make up the major fraction of TN-C in SF of diseased joints. In addition, the large variants are known to be more susceptible to MMP, such as MMP-2 and MMP-7, than the small variant[34]. Our immunoblotting analysis reveals degraded TN-C fragments in the SF of OA patients, suggesting activity of MMP and other proteinases. A recent study showed that MMP-13 concentrations are elevated in the SF of OA knee joints[22]; MMP-13 can digest the large TN-C variants[35]. Our findings about the involvement of these proteinases in the progression of OA are compatible with previous studies[3,10,11].

Immunohistochemical studies have shown that TN-C is localized in articular cartilage from OA patients at the extracellular matrix underneath the surface and pericellular compartment of the chondrocytes[17,18]. Since chondrocytes produce both large and small variants during embryogenesis and in neoplasms[15,16,31], they provide a cellular source of TN-C in the SF. Immunohistochemical analysis using mAb for avian TN-C variants showed preferential expression of the large variants in articular tissues, including ligaments and the meniscus, during articular cartilage development[16]. Synovial cells in diseased joints have also been identified as a source of TN-C[19,36]. In addition, osteocytes in eroded bone surfaces of patients with severe OA may produce TN-C,

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based on *in situ* hybridization experiments during chicken bone morphogenesis and immunoblotting analyses of cultured osteoblasts and osteosarcoma that show expression of the large and small TN-C variants[37,38]. Therefore, TN-C in SF could be synthesized and secreted by a variety of cells constituting the joints.

Possible biochemical markers, each with specific advantages, for diagnosis and determination of the severity of OA have been detected in blood, urine, or SF[3-12]. Recently, TN-C levels in SF from the knees of OA patients have been shown to be one of the most reliable markers of cartilage degradation[22]. With the use of a different commercial ELISA kit to detect all TN-C variants, the levels of all TN-C variants were found to increase with advancing stages of cartilage degradation as revealed by knee surgery[22]. In this study, we also separately analyzed levels of TN-C containing the FNIII C subunits in SF from OA patients, and found that the concentration of the TN-C molecules correlates with radiographic grading of the diseased knee joints. There were differences between Schmidt-Rohlfing, *et al*[22] and our study in the method of clinical assessment of the joints (surgical findings and radiography, respectively), the stage of OA progression (earlier and later stages, respectively), and the level of TN-C specificity (all the variants or large variants, respectively). These findings, therefore, indicate a close relationship between synovial TN-C levels and OA progression. In addition, the remarkable increase of large-variant TN-C in patients with severe OA with severely damaged bone tissues suggests preferential expression of the variants by the stimulated osteocytes. Recent studies on TN-C variants have found that some large-splice variants are specific to diseased tissues and cells expressing TN-C[26]. Along this line of inquiry, we propose using immunohistochemistry and mAb against other spliced domains to explore whether TN-C variants are specific to damaged tissues during OA progression.

For this study we could not obtain SF from a control group with complete healthy knee joints. As a true control group was lacking, it seemed important to include younger patients with only minor damage to the articular cartilage not caused by OA.

Although this study is limited by the cross-sectional design of the trials, small sample size, and the differences between groups in confounding variables, we observed that tenascin-C concentrations in synovial fluid correlate with radiographic grading of knee OA, suggesting that tenascin-C is a biochemical marker of OA progression. To clarify whether tenascin-C concentrations indicate disease activity, further studies are warranted on the relationship between tenascin-C concentrations and patient prognosis and on drug efficiency.

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