Table 2. Femorotibial angle (FTA) before HTO, at the first evaluation, and at the latest evaluation

Classification	Number of knees	FTA (degrees)			
		Before HTO	After HTO	First evaluation[a]	Latest evaluation[b]
All Patients	48	185.4 ± 4.4	168.2 ± 2.9	169.1 ± 4.5	169.8 ± 5.2
Satisfactory group	37	185.5 ± 4.8	167.6 ± 2.8 *	168.0 ± 4.1 *	168.4 ± 4.4 **
Unsatisfactory group	11	185.3 ± 2.1	170.2 ± 2.3 *	172.7 ± 3.8 *	174.4 ± 5.2 **

Data given as mean ± standard deviation
* $P < 0.05$; ** $P < 0.01$
[a] Mean follow-up 6.5 years
[b] Mean follow-up 17.1 years

Table 3. Distribution of the radiographic osteoarthritis (OA) grade before HTO and at the latest evaluation

Classification	Number	OA grade[a]		
		Grade II	Grade III	Grade IV
Before HTO	48	8	35	5
Latest evaluation[b]	48	1	18	29**

** $P < 0.01$
[a] Radiographic OA grade according to the Kellgren-Lawrence classification
[b] Mean follow-up 17.1 years

Table 4. Distribution of the preoperative radiographic OA grade between the satisfactory group and the unsatisfactory group

Classification	Number	OA grade[a]		
		Grade II	Grade III	Grade IV
All Patients	48	8	35	5
Satisfactory group	37	8	26	3
Unsatisfactory group	11	0	9	2

[a] Radiographic OA grade according to the Kellgren-Lawrence classification

Discussion

The first purpose of this study was to evaluate our fixation methods. We used two threaded pins and figure-of-eight wire, and the basic concept of this procedure was similar to a tension band or modified tension band fixation as previously described.[17,18] Generally speaking, rigid fixation and early rehabilitation is important for good clinical outcome after HTO,[15,25] and there have been several studies concerning the primary stability of the implants for HTO.[26-28] Flamme et al.[27] tested the initial stability of the following devices: one third tubular plate with a cortical screw, blade plate with screws (Giebel's plate), bone staples, and external fixator. In their study, the highest stability was achieved by the bone staple and external fixator, while Giebel's plate and one third tubular plate were less stable. Recently, we biomechanically evaluated the initial stability of our fixation method and compared it with the bone staple, Giebel's plate, and L-buttress plate. The results of this study indicated that our method showed similar stability to Giebel's plate and the bone staple against compression and bending stress except rotational force.[29] In the present study, we additionally used cast immobilization after HTO in consideration of initial stability of our fixation method, and we clinically experienced 11 of 48 unsatisfactory cases. Furthermore, 7 of the unsatisfactory cases showed correction loss in early postoperative periods. The main reason for this early correction loss is thought to be combination of the lack of initial stability especially against rotational stress and the bone quality of the osteotomy site. Thus, we think the two threaded pins and figure-of-eight wiring fixation is an acceptable fixation procedure for HTO; however, careful attention should be paid to correction loss in the early postoperative periods.

The second purpose of the present study was to evaluate the long-term clinical results after HTO and to determine the factors related to the outcome. There are many studies about the clinical results after HTO. The

majority of authors have reported satisfactory results in the short to midterm, but these results gradually deteriorated over time, especially at more than 10 years after surgery. The reported probability of a good or excellent result after HTO was 75% to 96% after 6 years, 45% to 94% after 10 years, and 46% to 90% after more than 15 years.[3-14] In the current study, the percentage of satisfactory results (excellent or good) after HTO was 93.7% after 6 years and 77.1% after 17 years. Our results had the same tendency of deterioration over a long period as the other studies, but still maintained a favorable result up to 17 years after HTO. We think the main reason for the good clinical outcome in spite of the progression of radiographic OA is that good alignment was maintained in the majority of cases during the follow-up period and the ADL of the patients slowly deteriorated with time. Recently, Koshino et al.[14] evaluated 75 knees with a mean follow-up of 19 years and reported good or excellent results in 90% of their series. Good alignment was described as the most important factor for good long-term clinical results.[14]

There is still considerable discussion about which factors affect the long-term outcome of HTO, and the present study focused on the correction angle at the surgery and the preoperative severity of knee OA. As for the correction angle, previous studies have reported that the optimum clinical outcomes were associated with a correction of 6° to 16° valgus, and an undercorrection less than 5° was strongly related to a high failure rate.[5,8-14] In this study, the mean FTA after HTO was 167.6° in the satisfactory group and 170.2° in the unsatisfactory group. In addition, in the unsatisfactory group, progressive varus recurrence was found at the follow-up. We believe that the most important concept for HTO is to shift the loading axis from the medial compartment to the lateral compartment, and this will lead good long-term clinical outcomes in HTO. In order to achieve this safely, we recommend that we should target a valgus correction of at least 10° for medial compartment knee OA.

In western countries, the patients with advanced knee OA were primarily indicated for total knee arthroplasty. Therefore, there have been few studies that evaluate the relationship between the preoperative severity of the knee OA and the clinical result of HTO. Holden et al.[30] followed 51 knees for 10 years and found no correlation between the clinical results and the radiographic severity of the knee OA preoperatively. Rinonapoli et al.[10] evaluated 60 knees with an average follow-up of 15 years and their multivariate analysis indicated that the length of follow-up and the amount of preoperative osteoarthritis affected the clinical results. On the other hand, there have been many studies about this issue in Japan, because the preservation of range of motion is important for ADL in Japanese people. Yasuda et al.[8] found no statistical difference between the preoperative OA stage and the clinical results, but also described that no stage IV patients obtained good results. Sasazaki et al.[31] compared HTO in mild to moderate OA with advanced OA, and found no clinical difference between the two groups. They also indicated that overcorrection was effective for HTO in advanced OA cases.[31] In this study, the radiographic OA grade of the knee joint was significantly deteriorated at the mean follow-up of 17 years, but no statistical difference was observed regarding the preoperative severity of the radiographic knee OA between the satisfactory and the unsatisfactory group. Furthermore, three of five patients with preoperative Grade IV OA were included in the satisfactory group at the recent follow-up. Therefore, we agree that the mild to moderate stage is expected to have better results after HTO, but we could also expect good clinical outcomes for the advanced stage if the cartilaginous condition of the lateral compartment is acceptably preserved and the proper postoperative alignment is achieved.

We believe that there are two limitations in this study. The first limitation is that this is a retrospective study and the 70.6% follow-up rate is perhaps low even for the long-term periods of more than 10 years. The second limitation is that we used the JOA score for clinical evaluation. The JOA score is a good scoring system and is popular in Japan. In addition, several recent studies about HTO using this scoring system have been published in international journals.[24,32,33] However, even though the JOA score is not a worldwide universal measuring system, we believe that we can compare the result of this study with other clinical reports.

In conclusion, HTO with two threaded pins and figure-of-eight wiring fixation showed an acceptable and good clinical outcome for an average of 17 years of follow-up. The present study also suggests that the proper correction angle is necessary to achieve satisfactory long-term clinical results and HTO is considered to be indicated for the patients with a moderate to advanced stage of medial knee OA.

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References

1. Felson DT, Zhang Y. An update on the epidemiology of knee and hip osteoarthritis with a view to prevention. Arthritis Rheum 1998;41:1343–55.
2. Jackson JP, Waugh W. Tibial osteotomy for osteoarthritis of the knee. J Bone Joint Surg Br 1961;43:746–51.
3. Coventry MB. Osteotomy of the upper portion of the tibia for degenerative arthritis of the knee. A preliminary report. J Bone Joint Surg Am 1965;47:984–90.

4. Coventry MB. Upper tibial osteotomy for gonarthrosis. The evolution of the operation in the last 18 years and long-term results. Orthop Clin North Am 1979;10:191–210.
5. Rudan JF, Simurda MA. High tibial osteotomy. A prospective clinical and roentgenographic review. Clin Orthop 1990;255: 251–6.
6. Insall JN, Joseph DM, Msika C. High tibial osteotomy for varus gonarthrosis. A long-term follow-up study. J Bone Joint Surg Am 1984;66:1040–8.
7. Berman AT, Bosacco SJ, Kirshner S, Avolio A. Factors influencing long-term results in high tibial osteotomy. Clin Orthop 1991;272: 192–8.
8. Yasuda K, Majima T, Tsuchida T, Kaneda K. A ten-to 15-year follow-up observation of high tibial osteotomy in medial compartment osteoarthrosis. Clin Orthop 1992;282:186–95.
9. Coventry MB, Ilstrup DM, Wallrichs SL. Proximal tibial osteotomy. J Bone Joint Surg Am 1993;75:196–201.
10. Rinonapoli E, Mancini GB, Corvaglia A, Musiello S. Tibial osteotomy for varus gonarthrosis. Clin Orthop 1998;353:185–93.
11. Naudie D, Bourne RB, Rorabeck CH, Bourne TJ. Survivorship of the high tibial valgus osteotomy. Clin Orthop 1999;367:18–27.
12. Aglietti P, Buzzi R, Vena LM, Baldini A, Mondaini A. High tibial valgus osteotomy for medial gonarthrosis. J Knee Surg 2003;16: 21–6.
13. Sprenger TR, Doerzbacher JF. Tibial osteotomy for the treatment of varus gonarthrosis. J Bone Joint Surg Am 2003;85:469–74.
14. Koshino T, Yoshida T, Ara Y, Saito I, Saito T. Fifteen to twenty-eight years' follow-up results of high tibial valgus osteotomy for osteoarthritic knee. Knee 2004;11:439–44.
15. Koshino T, Morii T, Wada J, Saito H, Ozawa N, Noyori K. High tibial osteotomy with fixation by a blade plate for medial compartment osteoarthritis of the knee. Orthop Clin North Am 1989; 20:227–43.
16. Hofmann AA, Wyatt RW, Beck SW. High tibial osteotomy. Use of an osteotomy jig, rigid fixation, and early motion versus conventional surgical technique and cast immobilization. Clin Orthop 1991;271:212–7.
17. Weber BG, Wörsdörfer O. Zuggurtungsosteosynthese bei tibiakopfosteotomie. Z Orthop 1980;118:637–43.
18. Flamme CH, Ruhmann O, Schmolke S, Wichmann R. Long-term outcome following high tibial osteotomy with tension bend principle. Arch Orthop Trauma Surg 2003;123:12–6.
19. Koshino T, Tsuchiya K. The effect of high tibial osteotomy on osteoarthritis of the knee. Clinical and histological observations. Int Orthop 1979;3:37–45.
20. Schatzker J, Burgess RC, Glynn MK. The management of nonunions following high tibial osteotomies. Clin Orthop 1985;193: 230–3.
21. Lawrence JS, Bremner JM, Bier F. Osteo-arthrosis. Prevalence in the population and relationship between symptoms and X-ray changes. Ann Rheum Dis 1966;25:1–24.
22. Ogata K. Interlocking wedge osteotomy of the proximal tibia for gonarthrosis. Clin Orthop 1984;186:129–34.
23. Ogata K, Yoshii I, Kawamura H, Miura H, Arizono T, Sugioka Y. Standing radiographs cannot determine the correction in high tibial osteotomy. J Bone Joint Surg Br 1991;73:927–31.
24. Kanamiya T, Naito M, Hara M, Yoshimura I. The influences of biomechanical factors on cartilage regeneration after high tibial osteotomy for knees with medial compartment osteoarthritis: clinical and arthroscopic observations. Arthroscopy 2002;18: 725–9.
25. Billings A, Scott DF, Camargo MP, Hofmann AA. High tibial osteotomy with a calibrated osteotomy guide, rigid internal fixation, and early motion. J Bone Joint Surg Am 2000;82:70–9.
26. Zhang Y, Shall LM, Kiritsis PG, Wolfinbarger L, Fairclots JR. Varus tension testing of fixation devices used in proximal tibial osteotomy. Contemp Orthop 1995;30:471–6.
27. Flamme CH, Kohn D, Kirsch L, Hurschler C. Primary stability of different implants used in conjunction with high tibial osteotomy. Arch Orthop Trauma Surg 1999;119:450–5.
28. Gautier E, Thomann BW, Brantschen R, Jakob RP. Fixation of high tibial osteotomy with AO cannulated knee plate. Acta Orthop Scand 1999;70:397–9.
29. Yasumoto H, Omori G, Tanabe Y, Koga Y. Mechanical stability of different fixation procedures after high tibial osteotomy (in Japanese). J Jpn Soc Clin Biomech 2001;22:319–23.
30. Holden DL, James SL, Larson RL, Slocum DB. Proximal tibial osteotomy in patients who are fifty years old or less. J Bone Joint Surg Am 1988;70:977–82.
31. Sasazaki Y, Fujikawa K, Kobayashi T, Miwa M. Clinical results of the overcorrected high tibial osteotomy for advanced osteoarthritis of the knee (in Japanese). Bessatsu Seikeigeka 2002;42: 153–58.
32. Choi HR, Hasegawa Y, Kondo S, Shimizu T, Ida K, Iwata H. High tibial osteotomy for varus gonarthrosis; a 10- to 24-year follow-up study. J Orthop Sci 2001;6:493–7.
33. Takemae T, Omori G, Nishino K, Terajima K, Koga Y, Endo N. Three-dimensional knee motion before and after high tibial osteotomy for medial knee osteoarthritis. J Orthop Sci 2006;11: 601–6.

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Original article

Posterior condylar offset and flexion in posterior cruciate-retaining and posterior stabilized TKA

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Abstract

Background. Anterior tibial translation associated with posterior impingement has been reported to be one of the factors limiting flexion after posterior cruciate-retaining (CR) total knee arthroplasty (TKA), especially when posterior condylar offset is decreased postoperatively. On the other hand, its effect on postoperative motion in posterior-stabilized (PS) TKA remains unknown. It has been demonstrated that PS TKA exhibits a consistent posterior femoral rollback during flexion. Thus, we hypothesized that the problem of posterior impingement can be avoided by use of PS TKA. In this study, we examined the relationship between postoperative posterior condylar offset and knee flexion in CR and PS TKAs.

Methods. In this study, analysis was performed for 20 subjects who underwent bilateral TKAs (one CR and one PS TKA) as well as another group of 50 PS TKAs. All patients could be tracked for a minimum of 2 years. The range of flexion was measured before operation and at follow-up. Preoperative and postoperative posterior condylar offset was evaluated on true lateral radiographs.

Results. At the follow-up examination, the mean flexion angle was 123° in the CR knees and 131° in the PS knees with a significantly greater improvement observed for the latter group. In the roentgenographic measurement of the posterior condylar offset, no significant difference was observed between the preoperative and postoperative values both in the CR and PS knees. We divided the patients into two groups according to the change of posterior condylar offset. The first group (Group I) showed a decrease in the posterior condylar offset after surgery and the second group (Group II) showed no change or an increase. Subsequently, postoperative change in flexion was compared between Groups I and II for the CR and PS knees. A significant difference between Groups I and II was observed in the CR knees, while no difference was observed in the PS knees. The magnitude of postoperative posterior condylar offset did not correlate with an improvement in maximum flexion angle in the 50 PS knees.

Conclusions. It was shown that the magnitude of posterior condylar offset correlated with a postoperative change in flexion angle in CR knees, while no such correlation was observed in PS knees.

Introduction

Several crucial factors have been shown to influence knee flexion after total knee arthroplasty (TKA), including preoperative range of motion, surgical technique, prosthetic design, and postoperative rehabilitation.[1–9] Among those potentially influential factors, whether the posterior cruciate ligament is retained or sacrificed has been a focus of investigation, and there have been several clinical and biomechanical studies.[3–5,10–14]

We conducted a prospective comparative study of posterior cruciate-retaining (CR) and posterior stabilized (PS) TKAs in 20 patients who underwent bilateral surgeries for osteoarthritis. In this comparative study, it was shown that postoperative improvement in range of motion was significantly superior in PS TKA patients.[15]

In attempting to clarify the difference between CR and PS TKAs, flexion kinematics has been investigated through fluoroscopic analysis.[10,11,13,14,16,17] We also compared the flexion kinematics between CR and PS knees in the same group of 20 patients.[18] Our kinematic results agreed with those of the previous studies,[11,13,16,17] demonstrating anterior femoral translation in CR knees and posterior femoral rollback in PS knees during flexion.

Bellemans et al.[19] reported that the anterior tibial translation during flexion can be a factor to limit maximum flexion by posterior impingement of the tibial insert against the back of the femur. Bellemans et al.[19] defined a parameter termed "posterior condylar offset

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Brief report

Changes in biochemical markers and prediction of effectiveness of intra-articular hyaluronan in patients with knee osteoarthritis

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Summary

Objective: Intra-articular injection of hyaluronan (HA) is frequently used to treat knee osteoarthritis (OA). We studied whether HA injections induced significant changes in levels of biochemical markers in synovial fluid (SF). In addition, we investigated the possibility of predicting the effectiveness of HA based on these biochemical markers.

Methods: Twenty-eight patients with knee OA underwent five weekly intra-articular injections of HA. Knee pain was measured on visual analog scale (VAS) before and after the five injections. Levels of biochemical markers, including chondroitin 6-sulfate (C6S), chondroitin 4-sulfate (C4S), keratan sulfate (KS), and tenascin-C (TN-C), were determined before and after the five injections. Correlations between the biochemical markers before HA injection and the improvement of VAS after the five injections were evaluated.

Results: After HA injections, levels of C6S, C4S, and KS decreased significantly. Inverse correlations were observed between the levels of TN-C and C4S before HA injection and improvement of VAS after the five injections. In contrast, no significant correlation was seen between levels of C6S and KS before injections and improvement of VAS after the five injections.

Conclusion: The reduction in C6S, C4S, and KS levels after HA injections reflects that HA could help maintain normal cartilage metabolism. Our findings suggest that HA injections are effective in patients whose knees contain low levels of TN-C and C4S, reflecting an early stage of OA and limited synovitis.

Key words: Hyaluronan, Knee osteoarthritis, Biomarker, Synovial fluid.

Introduction

Osteoarthritis (OA) is one of the most prevalent diseases in the aging population. This disease is characterized by changes in cartilage, subchondral bone, and synovium. Secondary synovitis resulting from the breakdown products of cartilage and bone matrix likely modifies the symptoms of OA. Articular cartilage is composed of chondrocytes embedded in an extracellular matrix of principally type II collagen and proteoglycan aggrecan molecules[1]. The release of degraded aggrecan molecules containing glycosaminoglycans, including chondroitin sulfate (CS) and keratan sulfate (KS), from the matrix into synovial fluid (SF) can be detected and levels of these two biochemical markers are reflective of aggrecan turnover[2,3]. Adult human articular cartilage mainly contains chondroitin 6-sulfate (C6S), which is converted to chondroitin 4-sulfate (C4S) in OA cartilage[4,5]. In contrast, the synovium, meniscus, and ligaments predominantly contain the C4S isomer. KS is found almost exclusively in cartilage aggrecan[6]. Cartilage markers are divided into markers of synthesis and catabolism. Typical markers of catabolism reflect the destruction of cartilage. Catabolic markers include aggrecan molecules, CS, and KS. It is not clear whether these markers reflect degradation of mature resident proteoglycan or of newly synthesized molecules[7].

Tenascin-C (TN-C) is a hexameric glycoprotein component of the extracellular matrix. TN-C is up-regulated in many pathologic adult conditions, including tumorigenesis, regeneration, and inflammation[8–10]. We reported that TN-C levels of the SF were significantly correlated with radiographic progression of knee OA[11]. Further, we have recently shown that SF levels of TN-C were higher in patients with rheumatoid arthritis (RA) compared with patients with OA[12]. TN-C could thus be a useful biochemical marker for OA and RA.

The intra-articular injection of hyaluronan (HA) has been extensively used in the treatment of OA. In this study, we observed whether repetitive intra-articular injections of HA in patients with OA induced significant changes in SF levels of biochemical markers, including C6S, C4S, KS, and TN-C. In addition, we investigated the possibility of predicting the effectiveness of HA based on levels of these biochemical markers in the SF.

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Methods

PATIENTS AND CLINICAL ASSESSMENT

Twenty-eight patients fulfilling the American College of Rheumatology criteria of knee OA[13] underwent five weekly intra-articular injections of HA. The

cohort consisted of 19 women and nine men whose mean age and body mass index (BMI) were 76.7 years (range, 62–88 years) and 23.5 kg/m^2 (range, 17.3–31.1 kg/m^2), respectively. Most of the patients have no occupation with low activity. No concomitant steroid therapy or nonsteroidal anti-inflammatory drugs were administered. Patients were divided into four groups based on radiographic grading of the OA severity described by Kellgren and Lawrence[14]. Two independent readers blinded to the source of the data graded the knees. Two patients were grade 1, 12 were grade 2, nine were grade 3, and five were grade 4. All patients gave informed consent, and this study was approved by the local ethics committee.

Patients were treated with five weekly intra-articular injections of 1% HA (molecular weight about 900,000 Da) solution with a dosage of 2.5 mL/injection (Artz, Seikagaku Corporation, Tokyo, Japan). SF samples were collected before each HA injection. The SF was centrifuged at 15,000 × *g* for 15 min and the supernatants were stored at −80°C until analyzed.

Knee pain was measured on a 100-mm visual analog scale (VAS; 0 mm = no pain, 100 mm = worst imaginable pain) before the first injection and after five weekly injections of HA.

MEASUREMENT OF BIOCHEMICAL MARKERS

C6S and C4S in the SF were measured by high performance liquid chromatography (HPLC) as described previously[15,16]. The SF samples were diluted 10-fold and treated by a series of digestions with chondroitinase ABC and chondroitinase AC-II (Seikagaku Corporation, Tokyo, Japan). The chondroitinase digestions produce the unsaturated disaccharides, Δdi-6S and Δdi-4S, from the structure of C6S and C4S in the CS chain. After ultrafiltration of the digested solutions, the levels of Δdi-6S and Δdi-4S in the filtrates were analyzed, and the area of the peak corresponding to each unsaturated disaccharide was calculated. KS levels were determined by HPLC according to the method by Yamada *et al.*[17]. Briefly, the SF samples were diluted 10-fold and treated with keratanase II (Seikagaku Corporation) to be digested to two disaccharide isomers, namely β-galactosyl-(1-4)-6-0-sulfo-N-acetylglucosamine (L2) and β-6-0-sulfo-galactosyl-(1-4)-6-0-sulfo-N-acetylglucosamine (L4). Then, concentrations of these disaccharide isomers were determined and the sum of these levels was considered the KS level.

Levels of large-subunit TN-C containing the fibronectin type III (FNIII) C domain were determined using an enzyme-linked immunosorbent assay (ELISA) kit (IBL, Gunma, Japan) with two monoclonal antibodies, 4F10TT and 19C4MS, as described previously[11]. Samples were diluted 10-fold and incubated in 96-well ELISA plates coated with 19C4MS. After washing, horseradish peroxidase-conjugated anti-TN-C Fab' fragments (4F10TT Fab') were added. TN-C purified from conditioned media of human glioma cells was used to prepare a standard curve.

STATISTICAL ANALYSIS

Results are presented as mean ± standard deviation. Continuous variables were compared using a paired Wilcoxon test for the differences between pre- (week 0) and post-HA injection (week 5) values. A correlation analysis was performed for VAS and SF levels of biochemical markers before injection using Spearman's rank correlation test. Correlations between levels of biochemical markers before injection and improvement of VAS were also estimated. The Kruskal–Wallis test was used to determine the difference between radiographic stages of OA and improvement of VAS. *P* values less than 0.05 were considered significant.

Results

CLINICAL RESULTS

A significant reduction in VAS was recorded after five weekly injections (27 ± 18 mm) compared with baseline (56 ± 19 mm, $P < 0.001$).

CORRELATION AMONG THE STUDY PARAMETERS BEFORE HA INJECTION

Before the first HA injection, significant correlations were found between C6S and C4S levels ($R = 0.864$, $P < 0.001$) and between C6S and KS levels ($R = 0.529$, $P = 0.006$), but not between KS and C4S levels ($R = 0.319$, $P = 0.098$). The levels of TN-C correlated with C6S ($R = 0.552$, $P = 0.004$) and C4S levels ($R = 0.713$, $P < 0.001$), but not with KS levels ($R = 0.087$, $P = 0.653$). No significant correlations between VAS and levels of biochemical markers were identified.

Table I
SF levels of C6S, C4S, KS, and TN-C before HA injection (week 0) and after five weekly injections (week 5)

	Week 0	Week 5	*P*
C6S (nmol/ml)	61.2 ± 35.8	52.8 ± 25.3	0.012
C4S (nmol/ml)	19.1 ± 6.7	17.8 ± 6.1	0.044
KS (μg/ml)	6.1 ± 3.1	5.2 ± 2.9	<0.001
TN-C (ng/ml)	37.4 ± 59.1	39.0 ± 58.1	0.411

Results are mean ± standard deviation.

CHANGES IN BIOCHEMICAL MARKERS AFTER HA INJECTIONS

The concentrations of C6S, C4S, and KS were significantly decreased after five weekly HA injections (Table I). TN-C levels showed no changes before or after HA injections (Table I).

CORRELATION BETWEEN BIOCHEMICAL MARKERS BEFORE HA INJECTION AND IMPROVEMENT OF VAS

A significant inverse correlation was observed between the TN-C levels before HA injection and improvement of VAS after five weekly HA injections (Fig. 1). The concentrations of C4S before injection also showed an inverse correlation with improvement of VAS after five weekly HA injections (Fig. 1). In contrast, no significant correlation was seen between C6S and KS levels before injection

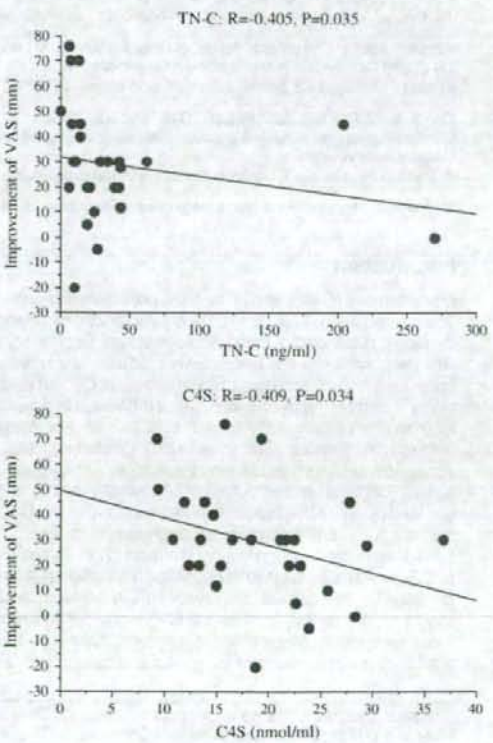

Fig. 1. Correlation between SF levels of TN-C and C4S before HA injection and improvement of VAS after five weekly injections. TN-C: $R = -0.405$, $P = 0.035$; C4S: $R = -0.409$, $P = 0.034$.

and improvement of VAS after five weekly HA injections (C6S: $P = 0.183$; KS: $P = 0.803$). Radiographic stages of OA had no significant relation with improvement of VAS ($P = 0.201$).

Discussion

Many randomized controlled studies have confirmed that intra-articular injections of HA can decrease knee pain in patients with OA[18–20]. These studies showed that the efficacy of HA treatment was superior to placebo, and the safety of HA treatment was also confirmed[18–21]. HA behaves as a viscous liquid at low shear rates and as an elastic solid at high shear rates. In addition to the rheological functions, HA has a variety of effects on cells *in vitro*. It inhibits prostaglandin E_2 synthesis induced by interleukin-1 (IL-1) and protects against proteoglycan release and cytotoxicity induced by oxygen-derived free radicals, IL-1, and mononuclear cell-conditioned medium[22]. HA induces IL-1β, tumor necrosis factor α, and insulin-like growth factor 1 messenger RNA transcript expression that are blocked by monoclonal antibodies to the HA receptor CD 44 in monocyte[23]. In cartilage, HA suppresses cartilage matrix degradation[24]. HA has been shown to increase the synthesis of proteoglycans and collagen[25]. In animal models, studies attempting to ascertain whether intra-articular injections of HA modify structural damage in the OA joint have produced conflicting results. Numerous studies have shown that HA injections prevent cartilage degradation and release of proteoglycans[26–30]. HA has a positive effect on the maintenance of cartilage matrix integrity during the development of OA as well as on the reduction of synovitis[28,31]. However, Smith *et al.*[32] showed a striking reduction in proteoglycan concentration in articular cartilage after HA injections in an OA model. This finding raises the concern that HA treatment could suppress the synthesis of proteoglycans and accelerate joint damage. There is an increasing interest in structure/disease modification as the goal in OA treatment, however, we have no universally proven structure-/disease-modifying interventions[33].

A significant correlation between C6S and KS levels, but not between KS and C4S levels has been reported by others[34] and was confirmed in the present study. This correlation is interesting, as C6S and KS are most concentrated in healthy adult human articular cartilage. In contrast, in OA cartilage, there is an increased level of C4S and a decreased level of C6S and KS. The proteoglycan fragments observed in OA SF have characteristics of degradation products of proteoglycan aggrecan molecules that are released from the original resident matrix. Thus, the disease process may be characterized by a continuing attack on the original resident molecules. The reduction in glycosaminoglycan levels in severe disease is probably associated with suppression of cartilage matrix turnover and the loss of residual cartilage, as a decrease in C4S levels as well as C6S levels is also observed at this stage[5,35,36].

One of the most obvious uses of biochemical markers is their potential to shed light on the effects of the treatment on the metabolism of joint tissue. Based on changes in biochemical marker levels after HA injections, the present study showed that catabolic markers, including C6S, C4S, and KS decreased after HA injections. Several previous studies have studied SF markers in patients treated with HA injections. Creamer *et al.*[37] reported that a decrease in KS levels occurred in knees treated with HA, although their findings did not reach statistical significance. Kobayashi *et al.*[38] showed that SF levels of C4S and C6S were significantly decreased after five weekly intra-articular injections of HA. The level of intact aggrecan was stable during the series of HA injections in this study. This means that the level of degraded aggrecan alone was reduced in the SF by HA injections. The reduction in levels of CS and KS after HA injections reflects that HA could inhibit the release of proteoglycans from degenerated cartilage, down-regulate the synthesis of proteoglycans for the process of cartilage repair, and maintain normal cartilage metabolism[38,39]. Smith *et al.*[32] found favorable effects of HA treatment on suppressing proteoglycan synthesis after HA injections in an OA model.

Changes in biochemical markers during the course of HA treatment can help to determine the effectiveness of treatment. Because joint puncture is an invasive procedure, and has a risk of complications, including infection[39], determining potential nonresponders can lead to more efficient use of HA. Only one previous study approached the prediction of the effectiveness with HA injections for the treatment of knee OA, and results showed positive correlations between levels of C6S and aggrecan (molecules with HA-binding ability and a KS side chain) before HA injection and the improvement of clinical scores after injections[39]. Improvement of clinical symptoms after initiation of HA injection can be predicted by measurement of the fragments derived from aggrecan. The authors concluded that HA injections were effective for knees with high levels of aggrecan fragments, reflecting an early stage of OA in keeping with residual cartilage and chondrocyte metabolic activity. However, the markers had no significant relation with improvement of VAS. The present study is the first, to our knowledge, to show some prediction of the effectiveness of HA injections for knee pain. However, limitations of this study include a small sample size, limited follow-up period, and absence of a control group.

Low levels of TN-C and C4S at baseline were associated with decreasing pain. Previous studies revealed that TN-C levels of SF were elevated in patients with advanced OA. In addition, studies have shown that levels of TN-C and C4S were higher in patients with RA compared with OA[12,16]. It is possible that the proliferated synovium, as well as degenerated cartilage, accelerates the release of C4S into the SF. Our findings suggest that HA injections are effective for knees with low levels of TN-C and C4S, reflecting an early stage of OA and limited synovitis. Joint fluid analysis may provide useful information about the prediction of the efficacy at the time of the first injection of HA. Since blood or urine would have much wider application, further studies are needed to find blood or urine markers to predict HA effectiveness before the first injection.

Conflict of interest statement

No benefits or funds were received in support of this study.

References

1. Poole AR, Kojima T, Yasuda T, Mwale F, Kobayashi M, Laverty S. Composition and structure of articular cartilage: a template for tissue repair. Clin Orthop Relat Res 2001;391(Suppl):S26–33.
2. Poole AR, Ionescu M, Swan A, Dieppe PA. Changes in cartilage metabolism in arthritis are reflected by altered serum and synovial fluid levels of the cartilage proteoglycan aggrecan. Implications for pathogenesis. J Clin Invest 1994;94:25–33.
3. Petersson IF, Sandqvist L, Svensson B, Saxne T. Cartilage markers in synovial fluid in symptomatic knee osteoarthritis. Ann Rheum Dis 1997;56:64–7.

4. Mourao PA. Distribution of chondroitin 4-sulfate and chondroitin 6-sulfate in human articular and growth cartilage. Arthritis Rheum 1988;31:1028–33.
5. Yamada H, Miyauchi S, Hotta H, Morita M, Yoshihara Y, Kikuchi T, *et al.* Levels of chondroitin sulfate isomers in synovial fluid of patients with hip osteoarthritis. J Orthop Sci 1999;4:250–4.
6. Thonar EJ, Lenz ME, Klintworth GK, Caterson B, Pachman LM, Glickman P, *et al.* Quantification of keratan sulfate in blood as a marker of cartilage catabolism. Arthritis Rheum 1985;28:1367–76.
7. Gamero P, Rousseau JC, Delmas PD. Molecular basis and clinical use of biochemical markers of bone, cartilage, and synovium in joint diseases. Arthritis Rheum 2000;43:953–68.
8. Chiquet-Ehrismann R, Mackie EJ, Pearson CA, Sakakura T. Tenascin: an extracellular matrix protein involved in tissue interactions during fetal development and oncogenesis. Cell 1986;47:131–9.
9. Jones PL, Jones FS. Tenascin-C in development and disease: gene regulation and cell function. Matrix Biol 2000;19:581–96.
10. Ghert MA, Qi WN, Erickson HP, Block JA, Scully SP. Tenascin-C expression and distribution in cultured human chondrocytes and chondrosarcoma cells. J Orthop Res 2002;20:834–41.
11. Hasegawa M, Hirata H, Sudo A, Kato K, Kawase D, Kinoshita N, *et al.* Tenascin-C concentration in synovial fluid correlates with radiographic progression of knee osteoarthritis. J Rheumatol 2004;31:2021–6.
12. Hasegawa M, Nakoshi Y, Muraki M, Sudo A, Kinoshita N, Yoshida T, *et al.* Expression of large tenascin-C splice variants in synovial fluid of patients with rheumatoid arthritis. J Orthop Res 2007;25:563–8.
13. Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, *et al.* Development of criteria for the classification and reporting of osteoarthritis. Classification of osteoarthritis of the knee. Arthritis Rheum 1986; 29:1039–49.
14. Kellgren JH, Lawrence JS. Radiological assessment of osteo-arthrosis. Ann Rheum Dis 1957;16:494–502.
15. Yoshida K, Miyauchi S, Kikuchi H, Tawada A, Tokuyasu K. Analysis of unsaturated disaccharides from glycosaminoglycuronan by high-performance liquid chromatography. Anal Biochem 1989;177: 327–32.
16. Shinmei M, Miyauchi S, Machida A, Miyazaki K. Quantitation of chondroitin 4-sulfate and chondroitin 6-sulfate in pathologic joint fluid. Arthritis Rheum 1992;35:1304–8.
17. Yamada H, Miyauchi S, Morita M, Yoshida Y, Yoshihara Y, Kikuchi T, *et al.* Content and sulfation pattern of keratan sulfate in hip osteoarthritis using high performance liquid chromatography. J Rheumatol 2000; 27:1721–4.
18. Wang CT, Lin J, Chang CJ, Lin YT, Hou SM. Therapeutic effects of hyaluronic acid on osteoarthritis of the knee. A meta-analysis of randomized controlled trials. J Bone Joint Surg Am 2004;86-A: 538–45.
19. Petrella RJ, Petrella M. A prospective, randomized, double-blind, placebo controlled study to evaluate the efficacy of intra-articular hyaluronic acid for osteoarthritis of the knee. J Rheumatol 2006;33: 951–6.
20. Strand V, Conaghan PG, Lohmander LS, Koutsoukos AD, Hurley FL, Bird H, *et al.* An integrated analysis of five double-blind, randomized controlled trials evaluating the safety and efficacy of a hyaluronan product for intra-articular injection in osteoarthritis of the knee. Osteoarthritis Cartilage 2006;14:859–66.
21. Stitik TP, Blacksin MF, Stiskal DM, Kim JH, Foye PM, Schoenherr L, *et al.* Efficacy and safety of hyaluronan treatment in combination therapy with home exercise for knee osteoarthritis pain. Arch Phys Med Rehabil 2007;88:135–41.
22. Brandt KD, Smith GN Jr, Simon LS. Intra-articular injection of hyaluronan as treatment for knee osteoarthritis: what is the evidence? Arthritis Rheum 2000;43:1192–203.
23. Noble PW, Lake FR, Henson PM, Riches DW. Hyaluronate activation of CD44 induces insulin-like growth factor-1 expression by a tumor necrosis factor-alpha-dependent mechanism in murine macrophages. J Clin Invest 1993;91:2368–77.
24. Homandberg GA, Hui F, Wen C, Kuettner KE, Williams JM. Hyaluronic acid suppresses fibronectin fragment mediated cartilage chondrolysis: I. *In vitro*. Osteoarthritis Cartilage 1997;5:309–19.
25. Aibe K, Ryu J, Sano S. Effects of hyaluronic acid on cartilage metabolism in free chondrocytes. J Orthop Sci 1996;1:268–76.
26. Abatangelo G, Botti P, Del Bue M, Gei G, Samson JC, Cortivo R, *et al.* Intra-articular sodium hyaluronate injections in the Pond–Nuki experimental model of osteoarthritis in dogs. I. Biochemical results. Clin Orthop Relat Res 1989;241:278–85.
27. Kikuchi T, Yamada H, Shimmei M. Effect of high molecular weight hyaluronan on cartilage degeneration in a rabbit model of osteoarthritis. Osteoarthritis Cartilage 1996;4:99–110.
28. Yoshioka M, Shimizu C, Harwood FL, Coutts RD, Amiel D. The effects of hyaluronan during the development of osteoarthritis. Osteoarthritis Cartilage 1997;5:251–60.
29. Hulmes DJ, Marsden ME, Strachan RK, Harvey RE, McInnes N, Gardner DL. Intra-articular hyaluronate in experimental rabbit osteoarthritis can prevent changes in cartilage proteoglycan content. Osteoarthritis Cartilage 2004;12:232–8.
30. Goldberg VM, Buckwalter JA. Hyaluronans in the treatment of osteoarthritis of the knee: evidence for disease-modifying activity. Osteoarthritis Cartilage 2005;13:216–24.
31. Asari A, Miyauchi S, Matsuzaka S, Ito T, Kominami E, Uchiyama Y. Molecular weight-dependent effects of hyaluronate on the arthritic synovium. Arch Histol Cytol 1998;61:125–35.
32. Smith GN Jr, Myers SL, Brandt KD, Mickler EA. Effect of intra-articular hyaluronan injection in experimental canine osteoarthritis. Arthritis Rheum 1998;41:976–85.
33. Altman RD. Structure-/disease-modifying agents for osteoarthritis. Semin Arthritis Rheum 2005;34(Suppl 2):3–5.
34. Sharif M, Osborne DJ, Meadows K, Woodhouse SM, Colvin EM, Shepstone L, *et al.* The relevance of chondroitin and keratan sulphate markers in normal and arthritic synovial fluid. Br J Rheumatol 1996;35: 951–7.
35. Campion GV, McCrae F, Schnitzer TJ, Lenz ME, Dieppe PA, Thonar EJ. Levels of keratan sulfate in the serum and synovial fluid of patients with osteoarthritis of the knee. Arthritis Rheum 1991;34:1254–9.
36. Ishiguro N, Ito T, Ito H, Iwata H, Jugessur H, Ionescu M, *et al.* Relationship of matrix metalloproteinases and their inhibitors to cartilage proteoglycan and collagen turnover: analyses of synovial fluid from patients with osteoarthritis. Arthritis Rheum 1999;42:129–36.
37. Creamer P, Sharif M, George E, Meadows K, Cushnaghan J, Shinmei M, *et al.* Intra-articular hyaluronic acid in osteoarthritis of the knee: an investigation into mechanisms of action. Osteoarthritis Cartilage 1994;2:133–40.
38. Kobayashi K, Matsuzaka S, Yoshida Y, Miyauchi S, Wada Y, Moriya H. The effects of intra-articularly injected sodium hyaluronate on levels of intact aggrecan and nitric oxide in the joint fluid of patients with knee osteoarthritis. Osteoarthritis Cartilage 2004;12:536–42.
39. Sugimoto H, Yamada H, Terada N, Kanaji A, Kato S, Date H, *et al.* Intra-articular injection of high molecular weight hyaluronan for osteoarthritis of the knee – prediction of effectiveness with biological markers. J Rheumatol 2006;33:2527–31.

Regulation of Tenascin-C Expression by Tumor Necrosis Factor-α in Cultured Human Osteoarthritis Chondrocytes

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ABSTRACT. ***Objective.*** Expression of tenascin-C reappears in articular cartilage of persons with osteoarthritis (OA), while it is almost abolished in normal mature cartilage. Tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, is upregulated in OA cartilage and is involved in the progression of OA, and stimulates tenascin-C expression in other types of cells. We investigated regulation of tenascin-C expression by TNF-α through nuclear factor-κB (NF-κB) in OA cartilage *in vivo* and *in vitro*.

Methods. Human articular cartilages were obtained from patients with OA and immunofluorescence examination of tenascin-C and the activated RelA subunit was performed. Cultured chondrocytes isolated from human OA cartilage were treated with TNF-α and with SN50. Activation of RelA subunit of NF-κB was examined by immunolabeling. Changes in tenascin-C protein concentrations were determined by immunofluorescence of cells after monensin treatment and Western blot analysis of the cell lysates, and mRNA levels were analyzed by quantitative real-time polymerase chain reaction.

Results. Increased intensity of tenascin-C staining was observed in the damaged cartilage compared with normal cartilage. Activated RelA staining in chondrocyte nuclei was prominent in tenascin-C-positive areas of OA cartilage. Treatment of cultured chondrocytes by TNF-α induced translocation of activated RelA to the nuclei, followed by upregulation of tenascin-C expression in both mRNA and protein. Treatment with SN50 inhibited increases of RelA and tenascin-C expression in chondrocytes.

Conclusion. TNF-α stimulated tenascin-C expression through NF-κB signaling with RelA activation in cultured OA chondrocytes, suggesting involvement of tenascin-C in OA cartilage remodeling. (First Release Dec 1 2007; J Rheumatol 2008;35:147–52)

Key Indexing Terms:
TENASCIN-C TUMOR NECROSIS FACTOR-α OSTEOARTHRITIS CHONDROCYTES

Tenascin-C, a member of the extracellular matrix glycoprotein family, consists of 6 similar subunits linked in their amino-terminal domain disulfide bonds[1]. Its expression is very restricted in normal adult tissues and reappears in association with wound healing, inflammatory processes, or neoplasia in a number of tissues[2-6]. In the lesions, tenascin-C promotes migration and proliferation of parenchymal and/or stromal cells[7-11]. In articular cartilage, tenascin-C expression is also associated with development of cartilage, but decreases markedly during the maturation of chondrocytes, and is finally almost abolished in adult articular cartilage[12-14]. In diseased joints including those with osteoarthritis (OA), tenascin-C was highly reexpressed in cartilage[15-18]. We have also demonstrated a correlation between the levels of tenascin-C in joint fluids and severity of OA apparent on radiographs[19].

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OA is characterized by degradation of cartilage[20]. It is now generally accepted that secretion of proinflammatory cytokines, including interleukin 1ß (IL-1ß) and tumor necrosis factor-α (TNF-α), by chondrocytes causes loss of cartilage matrix, resulting from the upregulation of enzymes, such as matrix metalloproteases (MMP) and aggrecanase, in chondrocytes themselves degrading the cartilage[21-26]. It is known that explants from OA cartilage are more susceptible to the effects of proinflammatory cytokines than explants from nonarthritic cartilage[27,28]. Proinflammatory cytokines are known to elicit tenascin-C expression in various cells[29-32]. Studies have also shown that IL-1ß upregulates tenascin-C expression in chondrocytes of OA cartilage[16,32]. IL-1ß and TNF-α play major roles in the inflammatory response via the activation of a variety of transcription factors such as nuclear factor-κB (NF-κB)[23,33]. NF-κB is a ubiquitous protein that specifically binds to DNA consensus sequences, activating its transcription. When the cytokine stimuli induce the phosphorylation of an inhibitory subunit and its subsequent degradation, the RelA subunit of NF-κB is activated and becomes capable of migrating to the nucleus, where it recognizes the consensus sequences in DNA. IL-1ß previously stimulated the increasing of RelA activation in human articular chondrocytes[34]. These observations led to the hypothesis that TNF-α may also induce tenascin-C production in OA chondrocytes, resulting

in tenascin-C upregulation in diseased cartilage via active RelA.

We observed the distribution of tenascin-C and activated RelA in human OA tissues using an immunofluorescence technique. As well, the stimulatory effects of TNF-α and TNF-α with SN50 on the activated RelA subunit of NF-κB and tenascin-C expression in protein and mRNA levels were examined in cultured OA chondrocytes.

MATERIALS AND METHODS

Cartilage specimens. Human OA cartilage specimens were obtained from femoral condyles of 15 patients ages 63–87 years (average 72.1 yrs) who were undergoing total knee joint replacement for treatment of OA. Non-OA cartilage samples were obtained from femoral condyles of 3 patients ages 19–33 years (average 25.4 yrs) with no history of joint disease and evidence of macroscopic articular degeneration at the time of amputation for tumor resection. The specimens were immediately fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) at room temperature overnight, decalcified in treated K-CX (Falma, Tokyo, Japan), and embedded in paraffin. The sections were cut at 5-μm thickness and placed on silane-coated glass slides (Matsunami, Osaka, Japan).

All patients gave their informed consent, and this study was approved by the local ethics committee.

Immunofluorescence for cartilage specimens. For double-immunofluorescence staining of tenascin-C and active RelA subunits, after antigen retrieval was performed with 0.01 M citrate buffer at 97°C for 30 min, sections were incubated with normal goat serum (Dako, Carpinteria, CA, USA) at room temperature for 30 min. Then they were treated with primary antibodies, mouse monoclonal antibody against the active form of RelA (Chemicon, Temecula, CA, USA), and rabbit polyclonal anti-tenascin-C antibody (IBL, Takasaki, Gunma, Japan), at room temperature overnight. After 3 washes with PBS, the sections were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 546-goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) at room temperature for 3 h. Slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Negative controls were incubated with isotype-matched control instead of the primary antibodies. All slides were viewed through an epifluorescence microscope equipped with appropriate filters and photographed at equivalent exposures.

Chondrocyte isolation and culture. Chondrocytes were isolated from human articular cartilage during knee replacements under sterile conditions. Cartilage fragments were sharply curetted from the femoral condyles and the tibial plateaus of knee joints. Fragments were incubated in 0.8% pronase solution (Calbiochem, Darmstadt, Germany) dissolved in Dulbecco's modified Eagle's medium/Ham F12 (DMEM/F12; Gibco, Grand Island, NY, USA) for 30 min at 37°C with continuing agitation in an atmosphere of 5% CO_2. After they were washed in DMEM/F12, cartilage pieces were incubated with 0.4% collagenase (Roche, Penzberg, Germany) in DMEM/F12 for 90 min at 37°C with orbital mixing. The cell suspension was filtered using a 70-μm pore-size nylon filter (BD Biosciences, Bedford, MA, USA) to remove the tissue debris. The filtrate was centrifuged for 5 min at 1200 rpm. The cells were washed in DMEM/F12 with 10% fetal bovine serum (FBS) 3 times and plated at 1×10^5 cells/well on 6-well tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in DMEM/F12 supplemented with 10% FBS, 10 μg/ml gentamicin (Gibco), and 25 μg/ml ascorbic acid (Sigma, St. Louis, MO, USA). The purity of cells was checked by immunofluorescent staining of chondroitin sulfate (Seikagaku Corp., Tokyo, Japan) and type II collagen (Daiichi Fine Chemical, Toyama, Japan). The positive cells formed over 85% in both cases (data not shown). Chondrocytes were grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, and the medium was changed every 2 days. All experiments were performed using the cells of primary or secondary cultures isolated from 10 different patients with OA joints.

Immunofluorescence for cultured chondrocytes. Chondrocytes were cultured on culture slides (BD Biosciences) and incubated in fresh serum-free medium with 0.1% bovine serum albumin (BSA) for 24 h, and then 100 ng/ml TNF-α (PeproTech, London, UK) and 100 ng/ml TNF-α (PeproTech) with 100 μg/ml SN50 peptide (Calbiochem) were added to the medium. SN50 peptide has been shown to be a specific inhibitor of NF-κB activation[35-37]. After incubation, the cells were fixed and treated with 0.1% Triton X-100 to permeabilize nuclear membranes. The slides were treated with mouse monoclonal antibody against activated RelA subunit (Chemicon) and then with Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen).

To determine the expression of tenascin-C protein, after serum-free conditioning with 0.1% BSA for 24 h, chondrocytes were treated with 100 ng/ml TNF-α (PeproTech). They were incubated with 1 μM monensin (Sigma) for 5 h before fixation to accumulate secretory proteins in the cytoplasm by blocking intracytoplasmic transport[38]. The chondrocytes were incubated with mouse monoclonal anti-tenascin-C antibody (IBL) at 4°C and Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) for 3 h at room temperature. Nuclei were counterstained with Hoechst 33342 (Sigma). Negative controls were incubated with isotype-matched mouse control instead of the primary antibodies.

Western blot analysis. When the cells were 80% to 90% confluent, after 24 h of incubation with or without 100 ng/ml TNF-α, cultured chondrocytes were washed 3 times with ice-cold PBS and solubilized in a solution [10 mM Tris-HCl, 150 mM NaCl, 1 mM ethylene diamine tetraacetic acid (EDTA), 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate] containing protease inhibitor cocktail (Sigma), pH 7.4. The lysates were centrifuged for 15 min at 4°C at 14,000 g. The protein amounts of the samples were adjusted by measurement of protein concentrations using a BCA protein assay kit (Pierce, Rockford, IL, USA). The samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride microporous membrane (Millipore, Bedford, MA, USA) by a semi-dry transblot system (Atto, Tokyo, Japan). The membrane was blocked with 5% skim milk and 50 mM Tris-HCl/150 mM NaCl (pH 7.6) containing 0.1% Tween (TBS-T) at room temperature for 1 h and then incubated with mouse monoclonal anti-tenascin-C antibody (IBL) overnight at 4°C. After washing 3 times, the membrane was incubated with the appropriate horseradish peroxidase-labeled secondary antibody (Amersham Biosciences, Buckinghamshire, UK) for 1 h. The signal was visualized using ECL detection reagents (Amersham Biosciences) by the chemiluminescence method. In order to ensure that equal amounts of total proteins were charged, signals were normalized against ß-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

RNA extraction and cDNA synthesis for quantitative real-time polymerase chain reaction (PCR). After the cells were 80% to 90% confluent, chondrocytes were treated with different concentrations of TNF-α in the absence or presence of 100 μg/ml SN50 peptide under a serum-free condition with 0.1% BSA. Total RNA was isolated using Isogen (NipponGene, Toyama, Japan) according to the manufacturer's instructions. Complementary DNA (cDNA) synthesis was performed by oligo$(dT)_{15}$ priming from 1 μg of total RNA using a cDNA synthesis kit (Roche) according to the manufacturer's protocols. TaqMan gene expression assay primer-probe pairs were ordered for detection of tenascin-C (assay no. Hs00233648-m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; assay no. Hs99999905-m1). Quantitative analysis of the cDNA was performed using the ABI Prism 7000 Sequence Detector System (Applied Biosystems, Foster City, CA, USA) and TaqMan Universal PCR Master Mix (Roche). The thermal cycling conditions consisted of 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. GAPDH was used as the housekeeping gene for internal control. Tenascin-C mRNA levels were normalized by GAPDH levels of each sample. The levels were expressed as an x-fold induction compared with untreated cells.

Statistical analysis. All data were expressed as mean ± standard deviation (SD). Numeric data were statistically evaluated by the Mann-Whitney U-test using Stat-View software (Abacus Concepts, Berkeley, CA, USA). A p value less than 0.05 was considered statistically significant.

RESULTS

Immunofluorescence for cartilage specimens. Double-immunolabeling of tenascin-C and the activated RelA subunit of NF-κB was performed in tissue specimens of OA and normal cartilages. In normal articular cartilage, tenascin-C staining was rarely observed in the superficial and upper-middle zones, and nuclear staining of active RelA was not found (Figure 1A). Conversely, tenascin-C labeling was strong in the pericellular and interterritorial areas in the superficial and upper-middle zones of OA specimens. Chondrocytes with active RelA-positive nuclei were dominant in the area of the tenascin-C-positive cartilage matrix and were often clustered in OA specimens (Figures 1B, 1C). Negative control slides incubated with isotype-matched control for the normal and OA cartilage showed complete absence of immunostaining (Figure 1D).

Immunofluorescence for cultured chondrocytes. We examined NF-κB signaling after TNF-α treatment in cultured chondrocytes. While only weak nuclear staining was seen in untreated cells, treatment of the cells with 100 ng/ml TNF-α resulted in

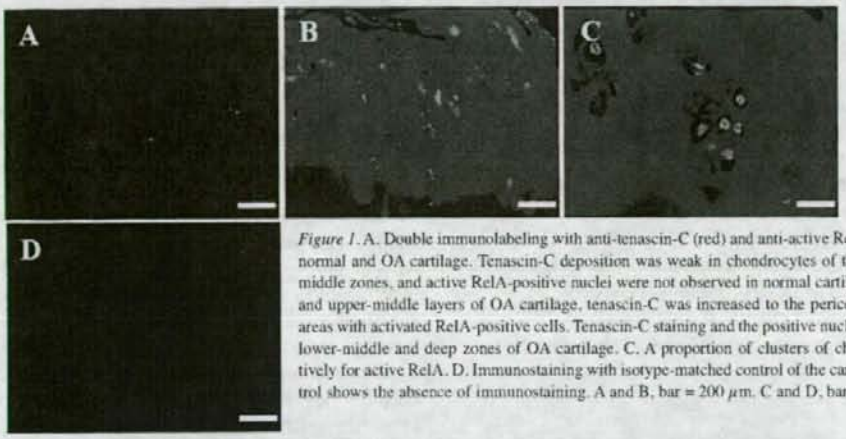

Figure 1. A. Double immunolabeling with anti-tenascin-C (red) and anti-active RelA (green) antibodies for normal and OA cartilage. Tenascin-C deposition was weak in chondrocytes of the superficial and upper-middle zones, and active RelA-positive nuclei were not observed in normal cartilage. B. In the superficial and upper-middle layers of OA cartilage, tenascin-C was increased to the pericellular and interterritorial areas with activated RelA-positive cells. Tenascin-C staining and the positive nuclei were diminished in the lower-middle and deep zones of OA cartilage. C. A proportion of clusters of chondrocytes stained positively for active RelA. D. Immunostaining with isotype-matched control of the cartilages as a negative control shows the absence of immunostaining. A and B, bar = 200 μm. C and D, bar = 50 μm.

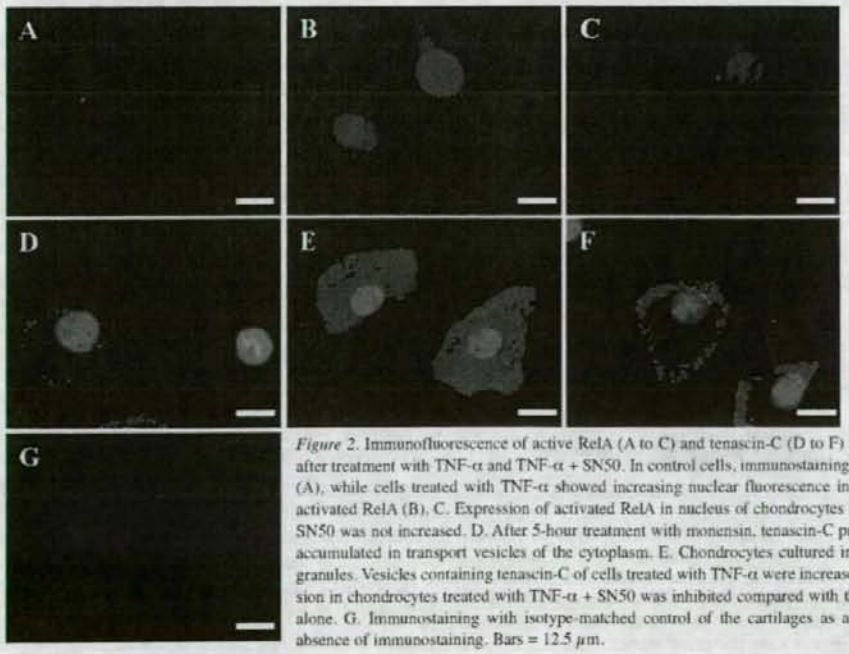

Figure 2. Immunofluorescence of active RelA (A to C) and tenascin-C (D to F) in cultured chondrocytes after treatment with TNF-α and TNF-α + SN50. In control cells, immunostaining of active RelA was faint (A), while cells treated with TNF-α showed increasing nuclear fluorescence indicating translocation of activated RelA (B). C. Expression of activated RelA in nucleus of chondrocytes treated with TNF-α with SN50 was not increased. D. After 5-hour treatment with monensin, tenascin-C produced by chondrocytes accumulated in transport vesicles of the cytoplasm. E. Chondrocytes cultured in 0.1% BSA show a few granules. Vesicles containing tenascin-C of cells treated with TNF-α were increased. F. Tenascin-C expression in chondrocytes treated with TNF-α + SN50 was inhibited compared with those treated with TNF-α alone. G. Immunostaining with isotype-matched control of the cartilages as a negative control shows absence of immunostaining. Bars = 12.5 μm.

apparent nuclear staining of active RelA in chondrocytes (Figures 2A, 2B). And the results showed that SN50 inhibited expression of activated RelA in nucleus (Figure 2C). When chondrocytes were treated with TNF-α, more abundant vesicles containing tenascin-C protein were observed in the cytoplasm than in the cells without TNF-α treatment as controls, indicating increased production of tenascin-C proteins in chondrocytes (Figures 2D, 2E). Moreover, SN50 inhibited the increases of tenascin-C expression (Figure 2F). Negative control slides incubated with isotype-matched controls for the cells showed complete absence of immunostaining (Figure 2G).

Western blot analysis. Tenascin-C protein produced by cultured chondrocytes was also analyzed by Western blotting. An anti-tenascin-C antibody, 4F10TT, which is specific to the extracellular growth factor-like domain, reacted with all tenascin-C variants with molecular weights of 350 to 210 kDa. In chondrocyte lysates, the major band was seen at 350 kDa, comigrating with the large variants of human glioma tenascin-C (Figure 3: L). The smallest variant (Figure 3: S), which lacks the alternatively spliced FN III repeats and has a molecular weight of 210 kDa, was weakly labeled. Treatment of chondrocytes with TNF-α increased significantly in expression of tenascin-C, especially the large variants (Figure 3).

Quantitative real-time PCR. We determined tenascin-C upregulation in OA chondrocytes stimulated by TNF-α and TNF-α with SN50 at the mRNA level (Figure 4). Quantitative real-time PCR revealed that the tenascin-C mRNA level was increased in response to 1 ng/ml TNF-α (1.90 ± 0.73; $p < 0.05$) in comparison with the level in untreated cells. The levels were also significantly upregulated by TNF-α treatment of 10 ng/ml TNF-α (2.92 ± 1.51; $p < 0.01$) and 100 ng/ml (3.02 ± 1.30; $p < 0.01$) in a dose-dependent manner. In addition, SN50 suppressed the tenascin-C expression stimulated by TNF-α of 1 ng/ml (1.06 ± 0.20; $p < 0.05$), 10 ng/ml (1.20 ± 0.32; $p < 0.01$), and 100 ng/ml (1.40 ± 0.29; $p < 0.05$), respectively.

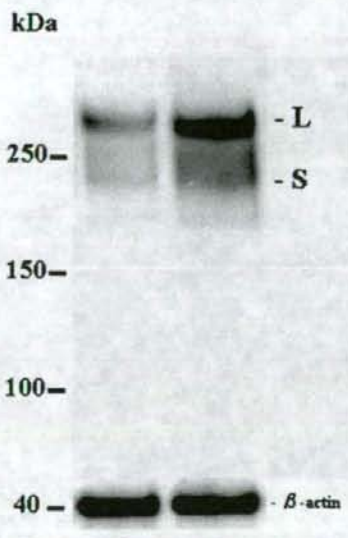

Figure 3. Western blot analysis of lysates from cultured chondrocytes incubated with or without TNF-α. After TNF-α treatment, bands for tenascin-C, particularly the large variants, were denser and thicker (lane 2) compared with no treatment (lane 1). Positions of the largest (L) and smallest (S) bands of human glioma tenascin-C, which comigrated in the gel, are indicated. Molecular weights of standard proteins are indicated on the left.

DISCUSSION

We noted strong tenascin-C immunostaining, mainly in the pericellular and interterritorial matrix of chondrocytes, as well as fibrillated cartilage, as described in previous studies[15-18]. We also demonstrated that enhanced tenascin-C labeling was associated with clusters of chondrocytes showing nuclear staining of active RelA subunit. Previous studies demonstrated that treatment of explants from OA cartilage with IL-1ß shows an enhanced tenascin-C staining in both the pericellular and interterritorial zones[32], and that IL-1ß stimulation induces activation of RelA subunit in human osteoarthritic chondrocytes[34]. Strong tenascin-C staining in the OA cartilage was considered to be induced by these proinflammatory cytokines through NF-κB signaling. It has been reported that chondrocytes isolated from OA express proinflammatory cytokines and their receptors more highly than normal cells[39]. Indeed, IL-1ß stimulates expression of tenascin-C mRNA in cultured chondrocytes *in vitro*[16].

To examine whether TNF-α also stimulates tenascin-C synthesis, we examined tenascin-C expression in cultured chondrocytes isolated from OA cartilage and observed that TNF-α induces nuclear translocation of active RelA; moreover, our results showed that SN50 inhibited activated RelA expression in nucleus of chondrocytes[36,37]. We observed that TNF-α stimulated the expression of tenascin-C on protein and mRNA levels, using immunofluorescence and quantitative real-time PCR. We also found that SN50 inhibited the immunostaining and mRNA expression of tenascin-C stimulated by TNF-α. Western blotting showed dominant secretion of large tenascin-C variants in human articular chondrocytes. Thus, our findings revealed that TNF-α could stimulate tenascin-C production, through NF-κB signaling with RelA activation in chondrocytes.

It has been considered that TNF-α stimulates different pathways of life and death through activating the transcription factor NF-κB, and that hyperactivation of NF-κB promotes cell survival and/or cell proliferation in most cell types[40-42]. The common combination in NF-κB complex is a p50-RelA heterodimer that is combined with IκB protein, which inhibits the translocation of the NF-κB complex into the nucleus. Stimuli such as TNF-α and IL-1ß dissociate the NF-κB complex from IκB and translocate it into the nucleus. This active

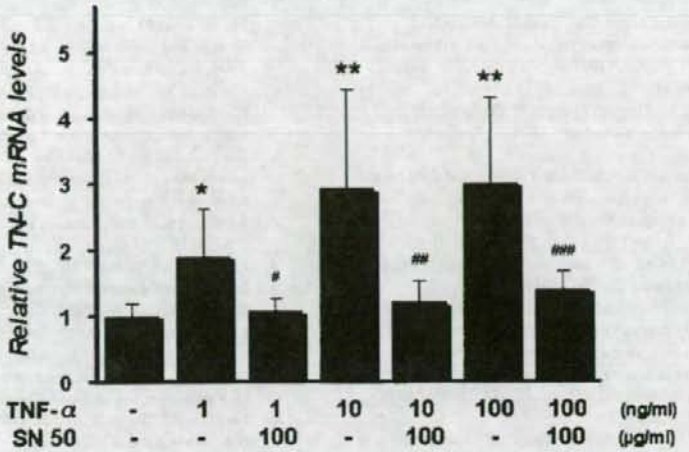

Figure 4. Regulation of tenascin-C mRNA after treatment with TNF-α and TNF-α + SN50. Expression of tenascin-C mRNA was increased by addition of 1 ng/ml TNF-α. Significant stimulatory effects of 10 ng/ml TNF-α were observed and were similar to those of 100 ng/ml TNF-α. Expression of tenascin-C mRNA stimulated at each concentration of TNF-α was inhibited by SN50. Tenascin-C mRNA levels were normalized by the GAPDH level of each sample. Data are expressed as values relative to levels in the control cells without TNF-α and SN50 treatment. Values are mean ± standard deviation. *$p < 0.05$; **$p < 0.01$ versus control cells. #$p < 0.05$ versus 1 ng/ml TNF-α-stimulated cells; ##$p < 0.01$ versus 10 ng/ml TNF-α-stimulated cells; ###$p < 0.05$ versus 100 ng/ml TNF-α-stimulated cells.

NF-κB complex binds to the NF-κB binding site of responsive genes and induces their transcription. Studies have suggested that the signaling in RelA activation particularly affects cell proliferation[43-45]. Knockout mice missing RelA were reported to have died before birth from liver cell apoptosis[43]. NF-κB activation may play an important role in resistance to the cytostatic effect of TNF-α. Tenascin-C is also known to promote proliferation in various cells[9-13]. In our study, in areas of dense tenascin-C deposition, chondrocyte clusters with nuclear RelA staining could often be observed in OA cartilages. These findings suggest that deposited tenascin-C can promote chondrocyte proliferation through NF-κB signaling in OA cartilage rather than cell death. In addition, our recent studies using tenascin-C-deficient mice demonstrated that activation of NF-κB in the lung tissues of asthmatic mice is decreased compared with their wild-type counterparts, and that TNF-α expression is diminished in mice with concanavalin A-induced hepatitis[46,47]. The expression and function of these molecules may be reciprocally regulated in inflammatory tissues.

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REFERENCES

1. Chiquet-Ehrismann R. Tenascins, a growing family of extracellular matrix proteins. Experientia 1995;51:853-62.
2. Whitby DJ, Longaker MT, Harrison MR, Adzick NS, Ferguson MW. Rapid epithelialization of fetal wounds is associated with the early deposition of tenascin. J Cell Sci 1991;99:583-6.
3. Mackie EJ, Halfter W, Liverani D. Induction of tenascin in healing wounds. J Cell Biol 1988;107:2757-67.
4. Li T-F, Konttinen YT, Xu J-W, et al. Tenascin expression in some normal and pathological conditions of the musculoskeletal system. Biomed Rev 1996;6:83-94.
5. Mackie EJ, Chiquet-Erishmann R, Pearson CA, et al. Tenascin is a stromal marker for epithelial malignancy in the mammary gland. Proc Natl Acad Sci USA 1987;84:4621-5.
6. Yoshida T, Matsumoto E, Hanamura N, et al. Co-expression of tenascin and fibronectin in epithelial and stromal cells of benign lesions and ductal carcinomas in the human breast. J Pathol 1997;182:421-8.
7. Yoshida T, Yoshimura E, Numata H, Sakakura Y, Sakakura T. Involvement of tenascin-C in proliferation and migration of laryngeal carcinoma cells. Virchows Arch 1999;435:496-500.
8. Yoshimura E, Majima A, Sakakura Y, Sakakura T, Yoshida T. Expression of tenascin-C and the integrin alpha 9 subunit in regeneration of rat nasal mucosa after chemical injury: involvement in migration and proliferation of epithelial cells. Histochem Cell Biol 1999;111:259-64.
9. Tsunoda T, Inada H, Kalembeyi I, et al. Involvement of large tenascin-C splice variants in breast cancer progression. Am J Pathol 2003;162:1857-67.
10. Tamaoki M, Imanaka-Yoshida K, Yokoyama K, et al. Tenascin-C regulates recruitment of myofibroblasts during tissue repair after mycordial injury. Am J Pathol 2005;167:71-80.
11. Fujinaga K, Onoda K, Yamamoto K, et al. Locally applied cilostazol suppresses neointimal hyperplasia by inhibiting tenascin-C synthesis and smooth muscle cell proliferation in free artery grafts. J Thorac Cardiovasc Surg 2004;128:357-63.
12. Pacifici M, Iwamoto M, Golden EB, Leatherman JL, Lee YS, Chuong CM. Tenascin is associated with articular cartilage

development. Dev Dyn 1993;198:123-34.
13. Murphy LI, Fischer D, Chiquet-Ehrismann R, Mackie EJ. Tenascin-C induced stimulation of chondrogenesis is dependent on the presence of the C-terminal fibrinogen-like globular domain. FEBS Lett 2000;480:189-92.
14. Mackie EJ, Murphy AI. The role of tenascin-C and related glycoproteins in early chondrogenesis. Microsc Res Tech 1998;43:102-10.
15. Pfander D, Rahmanzadeh R, Scheller E. Presence and distribution of collagen II, collagen I, fibronectin, and tenascin in rabbit normal and osteoarthritic cartilage. J Rheumatol 1999;26:386-94.
16. Pfander D, Heinz N, Rothe P, Carl H-D, Swoboda B. Tenascin and aggrecan expression by articular chondrocytes is influenced by interleukin 1B. Ann Rheum Dis 2004;63:240-4.
17. Salter DM. Tenascin is increased in cartilage and synovium from arthritic knees. Br J Rheumatol 1993;32:780-6.
18. Chevalier X, Groult N, Larget-Piet B, Zardi L, Hornebeck W. Tenascin distribution in articular cartilage from normal subjects and from patients with osteoarthritis and rheumatoid arthritis. Arthritis Rheum 1994;37:1013-22.
19. Hasegawa M, Hirata H, Sudo A, et al. Tenascin-C concentration in synovial fluid correlates with radiographic progression of knee osteoarthritis. J Rheumatol 2004;31:2021-6.
20. Buckwalter JA, Martin JA. Osteoarthritis. Adv Drug Deliv Rev 2006;58:150-67.
21. Shinmei M, Masuda K, Kikuchi T, Shimomura Y, Okada Y. Production of cytokines by chondrocytes and its role in proteoglycan degradation. J Rheumatol 1991;18 Suppl 27:89-91.
22. Borden P, Solymar D, Sucharczuk A, Lindman B, Cannon P, Heller RA. Cytokine control of interstitial collagenase and collagenase-3 gene expression in human chondrocytes. J Biol Chem 1996;271:23577-81.
23. Goldring MB. The role of the chondrocyte in osteoarthritis. Arthritis Rheum 2000;43:1916-26.
24. Goldring SR, Golding MB. The role of cytokines in cartilage matrix degeneration in osteoarthritis. Clin Orthop Relat Res 2004;427:27-36.
25. Martel-Pelletier J, Alaaeddine N, Pelletier JP. Cytokines and their role in the pathophysiology of osteoarthritis. Front Biosci 1999;4:694-703.
26. Fernandes JC, Martel-Pelletier J, Pelletier JP. The role of cytokines in osteoarthritis pathophysiology. Biorheology 2002;39:237-46.
27. Ismaiel S, Atkins RM, Pearse MF, Dieppe PA, Elson CJ. Susceptibility of normal and arthritic human articular cartilage to degradative stimuli. Br J Rheumatol 1992;31:369-73.
28. Webb GR, Westacott CI, Elson CJ. Chondrocyte tumor necrosis factor receptors and focal loss of cartilage in osteoarthritis. Osteoarthritis Cartilage 1997;5:427-37.
29. McCachren SS, Lightner VA. Expression of human tenascin in synovitis and its regulation by interleukin-1. Arthritis Rheum 1992;35:1185-96.
30. Nakamura Y, Esnault S, Maeda T, Kelly EA, Malter JS, Jarjour NN. Ets-1 regulates TNF-alpha-induced matrix metalloproteinase-9 and tenascin expression in primary bronchial fibroblasts. J Immunol 2004;172:1945-52.
31. Harkonen E, Virtanen I, Linnala A, Laitinen LL, Kinnula VL. Modulation of fibronectin and tenascin production in human bronchial epithelial cells by inflammatory cytokines in vitro. Am J Respir Cell Mol Biol 1995;13:109-15.
32. Chevalier X, Claudepierre P, Groult N, Godeau GJ. Influence of interleukin 1 beta on tenascin distribution in human normal and osteoarthritic cartilage. Ann Rheum Dis 1996;55:772-5.
33. Ding GJ, Fischer PA, Boltz RC, et al. Characterization and quantitation of NF-kB nuclear translocation induced by interleukin-1 and tumor necrosis factor-a. Development and use of a high capacity fluorescence cytometric system. J Biol Chem 1998;273:28897-905.
34. Largo R, Aluarez-Soria MA, Diez-Ortego I, et al. Glucosamine inhibits IL-1B-induced NF-kB activation in human osteoarthritic chondrocytes. Osteoarthritis Cartilage 2003;11:290-8.
35. Lin YZ, Yao SY, Veach RA, Torgerson TR, Hawiger J. Inhibition of nuclear translocation of transcription factor NF-kB by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. J Biol Chem 1995;270:14255-8.
36. Fiorentini C, Guerra N, Facchetti M, et al. Nerve growth factor regulates dopamine D(2) receptor expression in prolactinoma cell lines via p75(NGFR)-mediated activation of nuclear factor-kB. Mol Endocrinol 2002;16:353-66.
37. Guizzetti M, Bordi F, Dieguez-Acuna FJ, et al. Nuclear factor kB activation by muscarinic receptors in astroglial cells: effect of ethanol. Neurosci 2003;120:941-50.
38. Noda N, Minoura H, Nishiura R, et al. Expression of tenascin-C in stromal cells of the murine uterus during early pregnancy: Induction by interleukin-1a, prostaglandin E(2), and prostaglandin F(2 a). Biol Reprod 2000;63:1713-20.
39. Tetlow LC, Adlam DJ, Woolley DE. Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. Arthritis Rheum 2001;44:585-94.
40. Ghosh S, Karin M. Missing pieces in the NF-kB puzzle. Cell 2002;109:81-96.
41. Karin M, Lin A. NF-kB at the crossroads of life and death. Nat Immunol 2002;3:221-7.
42. Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. Suppression of TNF-a-induced apoptosis by NF-kB. Science 1996;274:787-9.
43. Beg A, Sha WC, Bronson RT, Ghosh S, Baltimore D. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kB. Nature 1995;376:167-70.
44. Bargou RC, Emmerich F, Krappmann D, et al. Constitutive nuclear factor-kB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. J Clin Invest 1997;100:2961-9.
45. Beg AA, Baltimore D. An essential role for NF-kB in preventing TNF-a-induced cell death. Science 1996;274:782-4.
46. Nakahara H, Gabazza EC, Fujimoto H, et al. Deficiency of tenascin C attenuates allergen-induced bronchial asthma in the mouse. Eur J Immunol 2006;36:3334-45.
47. El-Karef A, Yoshida T, Gabazza E, et al. Deficiency of tenascin-C attenuates liver fibrosis in immune-mediated chronic hepatitis in mice. J Pathol 2007;211:86-94.

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Elevated Levels of Prothrombin Fragment 1 + 2 Indicate High Risk of Thrombosis

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Prothrombin fragment 1 + 2 (F1 + 2) is considered to be useful for diagnosis of thrombosis. However, the evidence for a diagnosis of thrombosis by F1 + 2 is still not well established. The plasma concentrations of F1 + 2, soluble fibrin, D-dimer, and thrombin-antithrombin complex were measured in 694 patients suspected of having thrombosis and then were correlated with thrombosis. Plasma concentrations of F1 + 2, soluble fibrin, D-dimer, and thrombin-antithrombin complex were significantly higher in patients with thrombosis, compared with patients without thrombosis. When cutoff values of more than 300 pmol/L for F1 + 2 were used for the diagnosis, more than 50% of the patients were thus found to have thrombosis. The findings showed that F1 + 2, soluble fibrin, D-dimer, and thrombin-antithrombin complex have similar diagnostic ability. The plasma concentration of F1 + 2 closely was well correlated with thrombin-antithrombin complex, soluble fibrin, and D-dimer. Finally, F1 + 2 is one of the most useful parameters for the diagnosis of thrombosis.

Keywords: thrombosis; F1 + 2; SF, D-dimer; TAT

The prothrombin fragment 1 + 2 (F1 + 2) is cleaved from the aminoterminal end of human prothrombin when this zymogen is activated by factor Xa to yield thrombin.[1] The determination of human F1 + 2 in plasma with an antibody against a synthetic peptide has been reported.[2] Monitoring of F1 + 2 in patients treated with oral anticoagulants[3,4] and elevated plasma levels of F1 + 2 in patients with disseminated intravascular coagulation (DIC)[5,6] have been reported. Increased plasma level of thrombin-antithrombin complex (TAT)[7] also reflected with thrombin generation such as enhanced F1 + 2 levels.

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Disseminated intravascular coagulation[8,9] is often observed in patients with leukemia, solid cancers, infections, gynecological conditions, and aneurysms, and it is also frequently associated with severe bleeding and organ failure. Because DIC is frequently a fatal condition,[10] it is important to diagnose DIC at an early stage using hemostatic molecular markers.[11] Pulmonary embolism (PE) is a common, frequently undiagnosed, and potentially fatal cause of several common symptoms, for example, dyspnoea and chest pain.[12-14] Because PE is often a fatal disease caused by deep-vein thrombosis (DVT), the early evaluation of DVT[15] and PE[16] is therefore considered to be clinically important.

Fibrin-related markers such as D-dimer, fibrin and fibrinogen degradation products, and soluble fibrin (SF) are considered to be useful for the diagnosis of thrombosis. These markers are reported to be elevated in DVT/PE,[17-19] DIC,[20-22] acute myocardial infarction (AMI),[23,24] and thrombotic thrombocytopenic purpura.[25] In this regard, D-dimer has been

Table 1. Clinical Characteristics of the Patients Included in the Study

Underlying Diseases	Total	Without Thrombosis	With Thrombosis	Rate (%)
Solid cancer	191	167	24	12.5
Orthopedic diseases	178	150	28	16.0
Cardiovascular diseases	60	53	7	12.0
Collagen diseases	36	33	3	8.0
Digestive diseases	36	34	2	6.0
Infectious diseases	29	18	11	38.0
Hematological diseases	26	20	6	23.1
Diabetes mellitus	16	11	5	31.3
Without underlying diseases	15	0	15	100
Aneurysm and varicose	12	5	7	58.3
Trauma and burn	11	5	6	54.5
Obstetric diseases	6	6	0	0
Thrombophilia	4	2	2	50.0
Other diseases	11	11	0	0
Total	633	517	116	18.3

reported to be a negative predictor for DVT and less than 0.5 μg/mL of D-dimer is considered to exclude DVT in the most commonly used D-dimer assays in Europe and North America.[15] The International Society of Thrombosis and Haemostasis (ISTH) established the diagnostic criteria for overt-DIC using fibrin-related markers.[26] D-dimer is widely used to diagnose thrombosis as DVT, but many of the commercially available D-dimer assay kits contain different monoclonal antibodies and standard substances, and are based on different assay systems. The issue of standardization of D-dimer assays remains to be resolved, and several studies[27,28] have reported the basic data for standardization of D-dimer.

The present study was designed to evaluate the cutoff values of F1 + 2 in the diagnosis of thrombosis such as DVT, DIC, cerebral thrombosis, and AMI prospectively and to compare the findings to those for TAT, D-dimer, and SF. For this purpose, we determined the plasma concentrations of these molecules in 694 patients suspected of having thrombosis and also in 67 healthy volunteers.

Materials and Methods

Subjects

From June 1, 2003 to September 31, 2004, 694 patients (age range = 57.7 ± 17.8 years; mean = ±SD 398 females and 296 males) were suspected to have thrombosis (DVT, DIC, cerebral thrombosis or acute myocardial infarction) at hospitals affiliated with Mie University Graduate School of Medicine. The plasma concentrations of F1 + 2, SF, TAT, and D-dimer were examined in these patients and then were correlated with thrombosis. The study protocol was approved by the Human Ethics Review Committees of participating institutions, and a signed informed consent form was obtained from each subject. Thirty-four patients within 3 days after operation (OPE) and 29 patients who had undergone liver transplantation (LT) were excluded from analysis of the cutoff value. However, of the remaining 631 patients, 515 patients did not have any thrombosis, whereas 116 patients had thrombotic diseases, 66 with DVT, 27 with DIC, 10 with cerebral vascular accidents due to thrombosis (CVA), 5 with AMI, 4 with portal vein thrombosis, and 4 with arteriosclerosis obliterans. Deep-vein thrombosis was diagnosed by either ultrasonography or venography. Disseminated intravascular coagulation was diagnosed based on the ISTH overt-DIC diagnostic criteria.[26] Cerebral vascular accidents due to thrombosis were diagnosed either by computed tomography or by magnetic resonance imaging, and AMI was diagnosed by electrocardiograms and clinicolaboratory data. The underlying diseases of these patients are shown in Table 1.

Citrated blood samples were obtained from the peripheral veins of healthy subjects (see below) and from patients under fasting conditions. The blood samples were then centrifuged for 20 minutes at 3000 rpm. The supernatants (plasma) were analyzed within 4 hours. The plasma concentrations of F1 + 2, SF, TAT, and D-dimer were measured in patients with thrombosis at the onset and those without thrombosis at first consultation. The same parameters were also measured in 67 healthy subjects (age range = 38.6 ±

17.7-years-old; 58 males and 9 females) who were free of any diseases including thrombotic disease or hyperlipidemia, as confirmed by annual medical checkup.

Measurement of Plasma Concentrations of F1 + 2, TAT, D-Dimer, and SF

The plasma levels of F1 + 2 were measured by a new enzyme-linked immunosorbent assay (ELISA) for the determination of F1 + 2 (Dade Behring Marburg GmbH, Marburg, Germany). Two different monoclonal antibodies in this kit recognize the terminal end of N fragment 2. The plasma levels of TAT were measured using the TAT test (Sysmex, Kobe, Japan) by ELISA. The plasma D-dimer levels were measured by LPIA-ACE D-dimer (Mitsubishi Kagaku Iatron Inc, Tokyo, Japan) using JIF23 monoclonal antibody. The JIF23 monoclonal antibody, which recognizes plasmin-digested N-terminus of the γ chain on the D region, was used for latex agglutination.[29] Soluble fibrin was also determined by the latex agglutination method using IATRO SF (Mitsubishi Kagaku Iatron Inc) containing monoclonal antibody IF-43, which recognizes a segment of the fibrin Aa chain (Aα-17-78) residue segment exposed in the E region of fibrin monomer (FM) when the FM molecule binds to the D region of another FM or fibrinogen. The antibody is coated for the SF assay.[30]

Statistical Analysis

The data are expressed as mean ± SD. Differences between groups were examined for statistical significance using the Mann-Whitney *U* test, whereas the correlation between the 2 variables was evaluated by Pearson's correlation analysis. A *P* value of less than .05 denoted the presence of a statistically significant difference. The usefulness of D-dimer and SF for the diagnosis of thrombosis, DVT, and DIC were examined by a receiver operating characteristic (ROC) analysis.[31] The cutoff values were determined by an ROC analysis. All statistical analyses were performed using the SPSS II software package (SPSS, Tokyo, Japan).

Results

The frequency of thrombotic diseases was high in patients with solid cancer, orthopedic diseases, hematological diseases, infectious diseases, cardiovascular diseases, diabetes mellitus, and aneurysm (Table 1). In healthy subjects, plasma concentrations of F1 + 2 were not distributed normally, with a maximum value of 214 pmol/L, minimum value 42 pmol/L, and median value of 121 pmol/L. The 95% confidence interval (95% CI) of F1 + 2 was from 56 to 213 pmol/L.

The plasma levels of F1 + 2, SF, D-dimer, and TAT (median; 25-75 percentile) were significantly higher in the patients with thrombosis (516 pmol/L; 349-709 pmol/L, 18.49 μg/mL; 8.68-35.24 μg/mL, 11.38 μg/mL; 6.58-19.06 μg/mL, and 15.87 ng/mL; 9.04-36.98 ng/mL, respectively), OPE (431 pmol/L; 331-520 pmol/L, 13.32 μg/mL; 8.17-22.10 μg/mL, 7.32 μg/mL; 3.04-12.78 μg/mL, and 17.38 ng/mL; 13.04-32.87ng/mL, respectively), and LT (590 pmol/L; 249-985 pmol/L, 16.03 μg/mL; 5.60-24.08 μg/mL, 6.54 μg/mL; 2.67-12.68 μg/mL, and 19.36 ng/mL; 12.69-32.51 ng/mL, respectively) than in those without thrombosis (192 pmol/L; 138-274 pmol/L, 2.79 μg/mL; 0.76-5.29 μg/mL, 0.88 μg/mL; 0.45-2.27 μg/mL, and 2.43 ng/mL; 1.58-5.50 ng/mL; $P < .01$, each; Figure 1). On other hand, the plasma concentrations of F1 + 2, SF, D-dimer, and TAT were significantly higher in patients without thrombosis than in healthy subjects ($P < .01$, each).

Figure 2 shows the positive predictive values (PPV) and negative predictive values (NPV) for several cutoff values of F1 + 2 in patients with thrombosis. When F1 + 2 levels of >300 pmol/L were used, more than 50% of the patients, excluding either those with LT or those who had undergone an operation, had some thrombosis and NPV for thrombosis was >95%. When the cutoff values were set at <100 pmol/L for F1 + 2, NPV for thrombosis was 100%, but PPV was <25%.

An ROC analysis showed the curves of F1 + 2, SF, D-dimer, and TAT to be similar and useful for the diagnosis of all thromboses (Figure 3). The area under the curve (AUC) of those markers was markedly high (Table 2). An ROC analysis provided adequate cutoff values of F1 + 2 (300 pmol/L), SF 6.8 (μg/mL), D-dimer (3.0 μg/mL), and TAT (7.8 ng/mL) for the diagnosis of all thromboses (Table 3). The sensitivity and specificity of F1 + 2 were 86.2% and 80.6%, respectively. These statistical values of F1 + 2 were similar to those of other molecular markers.

Table 4 shows the correlation between F1 + 2 and other hemostatic molecular markers. The plasma levels of F1 + 2 were closely correlated with SF, D-dimer, and TAT, and the correlation between F1 + 2 and TAT was the best.

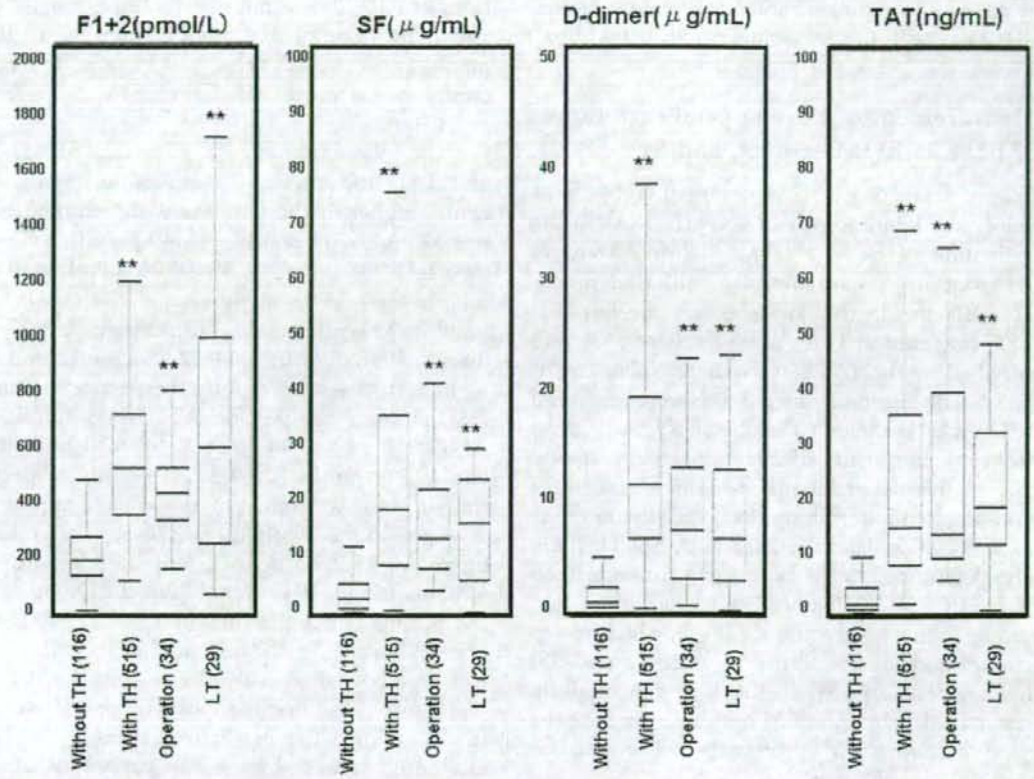

Figure 1. Plasma levels of F1 + 2, SF, D-dimer, and TAT in patients with or without thrombosis, those after the operation and those after LT. SF indicates soluble fibrin; TAT, thrombin-antithrombin complex; LT, liver transplantation. ** indicates $P < .01$ (comparison to without thrombosis). The box shows 25 percentile, median, and 75 percentile. Operation; patients within 3 days after operation, LT; patients after liver transplantation.

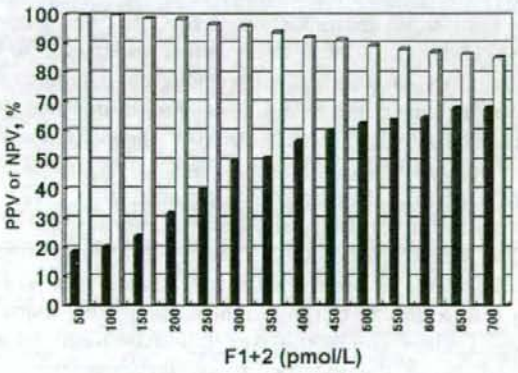

Figure 2. The PPV and NPV for plasma concentrations of F1 + 2 in thrombosis. PPV indicates positive predictive values; NPV, negative predictive values. Solid bar represents PPV and white bar represents NPV.

Discussion

The frequency of thrombotic diseases was high in patients with solid cancer, orthopedic diseases, hematological diseases, infectious diseases, cardiovascular diseases, diabetes mellitus, and aneurysm, suggesting that prevention of thrombosis will be important in these underlying diseases. Although the sample number was not ideal in this study, DVT was frequently associated with cancer and orthopaedic diseases, whereas DIC was frequently associated with cancer, infectious diseases, and aneurysm. Such frequencies were similar to those reported in previous studies.[9,12,13] Regarding the underlying diseases frequently associated with thrombosis (eg, DVT and DIC), the risk for thrombosis should be evaluated by a simple test. In the present study, we demonstrated the concentrations

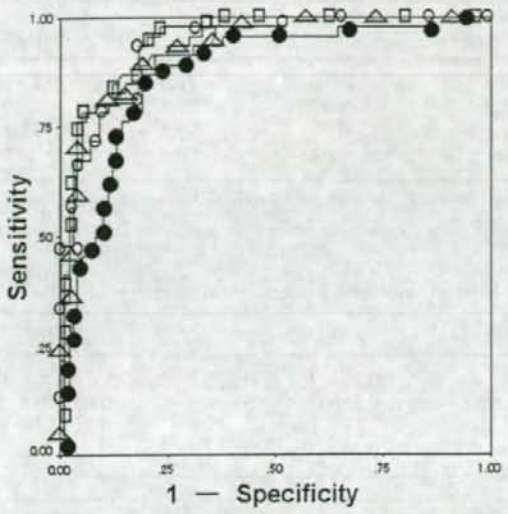

Figure 3. ROC analysis of F1 + 2, SF, D-dimer, and TAT for thrombosis. ROC indicates receiver operating characteristic; SF, soluble fibrin; TAT, thrombin-antithrombin complex. (□) TAT; (○) F1+2; (▯) SF; (•) D-dimer.

Table 2. Area Under the Curve of Receiver Operating Characteristic in F1 + 2, Soluble Fibrin, D-Dimer, and Thrombin-Antithrombin Complex

	AUC
F1 + 2	0.938
D-dimer	0.910
SF	0.901
TAT	0.940

NOTES: AUC = area under the curve; SF = soluble fibrin; TAT = thrombin-antithrombin complex.

of F1 + 2 to be significantly high in patients with thrombosis such as DIC, DVT, CVA, and AMI, and these findings were similar to those for SF, D-dimer, and TAT.

In healthy subjects, the plasma concentrations of F1 + 2 were not distributed normally, and the 95% CI of F1 + 2 ranged from 56 pmol/L to 213 pmol/L, thus indicating that the patients with more than 220 pmol/L of F1 + 2 may have a hypercoagulable state. In contrast, the plasma levels of F1 + 2 were significantly high in patients with thrombosis such as the plasma levels of SF, D-dimer, and TAT. As a result, high concentrations of SF, D-dimer, and TAT could thus be considered as markers of thrombosis, because these parameters were also reported to be elevated in DVT,[32,33] DIC,[20,34] and hyperlipidemia.[35] It should be noted, however, no prospective studies have previously evaluated the F1 + 2 assay including the cutoff value for the diagnosis of thrombosis. The plasma levels of F1 + 2 were significantly high in patients with all types of thrombosis although the levels were also high in some patients who were not found to have any thrombosis.

More than 50% of patients who had more than 300 pmol/L of F1 + 2 had some thrombosis, suggesting that these patients need anticoagulant therapy such as aspirin for atherosclerotic thrombosis or warfarin for venous thrombosis. It is considered that these patients with a high value of F1 + 2 have hypercoagulable state. D-dimer is also useful for the diagnosis of DVT, but the cutoff values of D-dimer should be mentioned in each measurement kit.[28]

An ROC analysis showed that the curves of F1 + 2, SF, D-dimer, and TAT to be similar. Because both AUC of these markers, especially F1 + 2 and TAT, were high in ROC analysis, we believe that these markers are useful for the diagnosis of either thrombosis or a hypercoagulable state. In particular, both F1 + 2 and TAT may be more useful than D-dimer and SF for the diagnosis of thrombosis by AUC. An ROC analysis provided adequate cutoff values of F1 + 2 (300 pmol/L), SF (6.8 μg/mL), D-dimer (3.0 μg/mL), and TAT (7.8 ng/mL) for the diagnosis of all types of thromboses. The sensitivity and specificity of F1 + 2 were 86.2% and 80.6%, respectively. These statistical values of F1 + 2 were similar to those observed for other molecular markers, thus suggesting that the ability to diagnose thrombosis is similar among F1 + 2, D-dimer, SF, and TAT. Soluble fibrin has also been reported to reflect the early phase of DVT/PE, whereas D-dimer reflects secondary fibrinolysis after clot formation and the measurements of both D-dimer and SF may be recommended.[36,37] Both F1 + 2 and TAT reflect an earlier phase of thrombosis. The plasma levels of F1 + 2 were closely correlated with those of SF, D-dimer, and TAT and the correlation between F1 + 2 and TAT was best, thus suggesting that both markers reflect thrombin formation. Specificity for thrombosis was better in F1 + 2 than in TAT, thus suggesting that F1 + 2 may be the most useful marker for the earlier phase of thrombosis.

In conclusion, our findings suggest that high concentrations of hemostatic molecular markers,

Table 3. Cutoff Values of F1 + 2, Soluble Fibrin, D-Dimer, and Thrombin-Antithrombin Complex

	Cutoff Values	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Odds Ratio
F1 + 2	300 pmol/L	86.2	80.6	50.0	96.3	26.07
SF	6.8 μg/mL	89.7	88.0	62.7	97.4	63.01
D-dimer	3.0 μg/mL	82.8	82.4	51.3	95.5	22.47
TAT	7.8 ng/mL	82.4	82.4	72.4	90.4	24.79

NOTE: PPV = positive predictive values; NPV = negative predictive values; SF = soluble fibrin; TAT = thrombin-antithrombin complex.

Table 4. Correlation Between F1 + 2 and Other Hemostatic Molecular Markers[a]

	F1 + 2	SF	D-Dimer	TAT
F1+2	1.0	0.543 ($P < .001$)	0.681 ($P < .001$)	0.760 ($P < .001$)
SF	0.543 ($P < .001$)	1.0	0.588 ($P < .001$)	0.691 ($P < .001$)
D-dimer	0.681 ($P < .001$)	0.588 ($P < .001$)	1.0	0.710 ($P < .001$)
TAT	0.760 ($P < .001$)	0.691 ($P < .001$)	0.710 ($P < .001$)	1.0

NOTE: SF = soluble fibrin; TAT = thrombin-antithrombin complex.
[a]The numbers show the correlation coefficients.

especially F1 + 2 which is also known as a marker for a hypercoagulable state, reflect a high risk for thrombosis.

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References

1. Aronson DL, Stevan L, Ball AL, Franza BR Jr, Finlayson JS. Generation of the combined prothrombin activation peptide (F1+2) during the clotting of blood and plasma. *J Clin Invest*. 1977;60:1410-1418.
2. Pelzer H, Schwarz A, Stüber W. Determination of human prothrombin activation fragment 1+2 in plasma with an antibody against a synthetic peptide. *Thromb Haemost*, 1991;65:153-159
3. Mannucci PM, Bottasso B, Tripodi A, Bonomi AB. Prothrombin fragment 1+2 and intensity of treatment with oral anticoagulants. *Thromb Haemost*. 1991;66:741.
4. Hafner G, Swars H, Erbel R, et al. Monitoring prothrombin fragment 1+2 during initiation of oral anticoagulant therapy after intracoronary stenting. *Ann Hematol*. 1992;65:83-87.
5. Takahashi H, Wada K, Niwano H, Shibata A. Comparison of prothrombin fragment 1+2 with thrombin-antithrombin III complex in plasma of patients with disseminated intravascular coagulation. *Blood Coag Fibr*. 1992;3:813-818.
6. Asakura H, Shiratori Y, Jokaji H, et al. Changes in plasma levels of prothrombin fragment F1+2 in cases of disseminated intravascular coagulation. *Acta Haematol*. 1993;89:22-25.
7. Asakura H, Wada H, Okamoto K, et al. Evaluation of hemostatic molecular markers for diagnosis of disseminated intravascular coagulation in patients with infections. *Thromb Haemost*. 2006;95:282-287.
8. Levi M, de Jonge E, van der Poll T, Ten Cate H. Disseminated intravascular coagulation. *Thromb Haemost*. 1999;82:695-705.
9. Wada H. Disseminated intravascular coagulation. *Clin Chim Acta*. 2004;344:13-21.
10. Okabayashi K, Wada H, Ohta S, Shiku H, Nobori T, Maruyama K. Hemostatic markers and the sepsis-related organ failure assessment score in patients with disseminated intravascular coagulation in an intensive care unit. *Am J Hematol*. 2004;76:225-229.
11. Wada H, Wakita Y, Nakase T, et al. Outcome of disseminated intravascular coagulation in relation to the score when treatment was begun. *Thromb Haemost*, 1995;74:848-852.
12. Heit JA, Silverstein MD, Mohr DN, et al. The epidemiology of venous thromboembolism in the community. *Thromb Haemost*. 2001;86:452-463.
13. Oger E. Incidence of venous thromboembolism: a community-based study in Western France. EPI-GETBP Study Group. Groupe d'Etude de la Thrombose de Bretagne Occidentale. *Thromb Haemost*. 2000;83:657-660.
14. Courtney DM, Kline JA. Identification of prearrest clinical factors associated with outpatient fatal pulmonary embolism. *Acad Emerg Med*. 2001;8:1136-1142.