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

YUTAKA NAKOSHI, MASAHIRO HASEGAWA, AKIHIRO SUDO, TOSHIMICHI YOSHIDA, and ATSUMASA UCHIDA

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].

From the Department of Orthopedic Surgery and Department of Pathology and Matrix Biology, Mie University Graduate School of Medicine, Mie, Japan.

Y. Nakoshi, MD; M. Hasegawa MD, PhD; A. Sudo, MD, PhD, Department of Orthopedic Surgery; T. Yoshida, MD, PhD, Department of Pathology and Matrix Biology; A. Uchida, MD, PhD, Department of Orthopedic Surgery.

Address reprint requests to Dr. M. Hasegawa, Department of Orthopedic Surgery, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu City, Mie 514-8507, Japan. E-mail: masahase@clin.medic.mie-u.ac.jp

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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-ml) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; assay no. Hs99999905-ml). 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

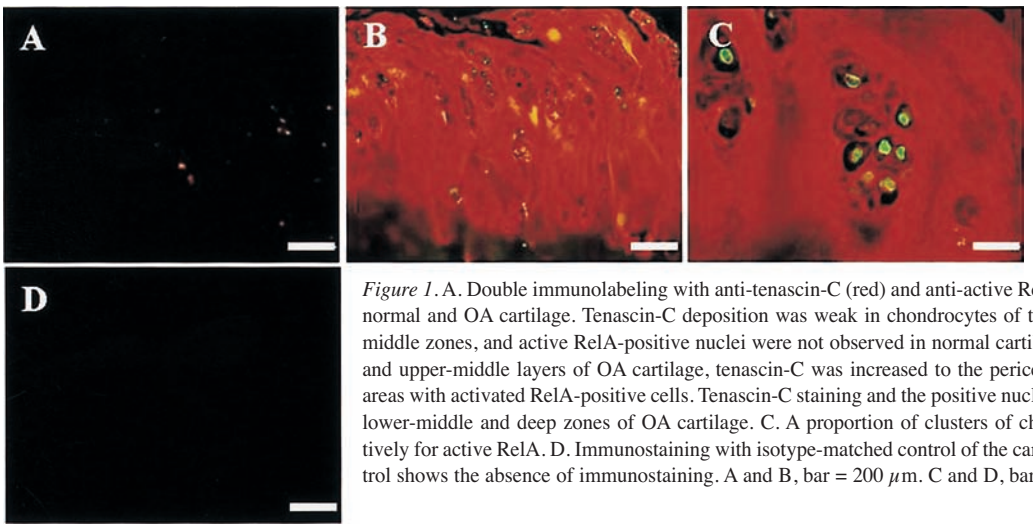

Figure 1. A. Double immunolabeling with anti-tenascin-C (red) and anti-active RelA (green) antibodies for normal and OA cartilage. Tenascin-C deposition was weak in chondrocytes of the superficial and upper-middle zones, and active RelA-positive nuclei were not observed in normal cartilage. B. In the 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.

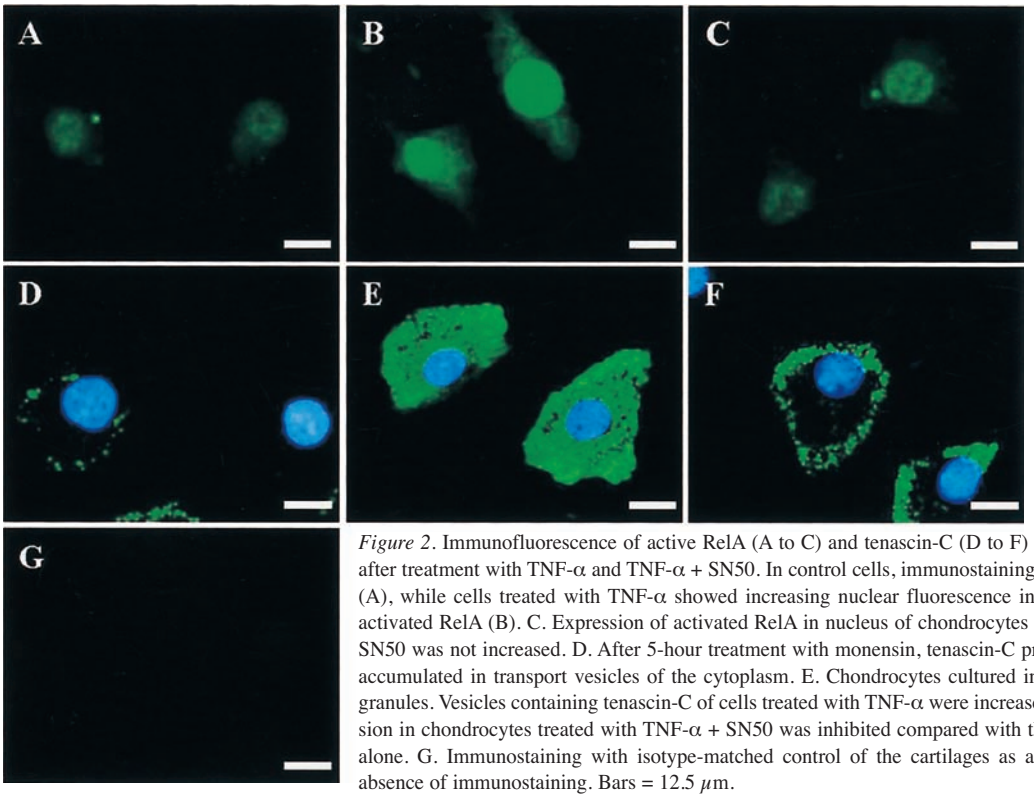

Figure 2. Immunofluorescence of active RelA (A to C) and tenascin-C (D to F) in cultured chondrocytes after treatment with TNF-α and TNF-α + SN50. In control cells, immunostaining of active RelA was faint (A), while cells treated with TNF-α showed increasing nuclear fluorescence indicating translocation 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.

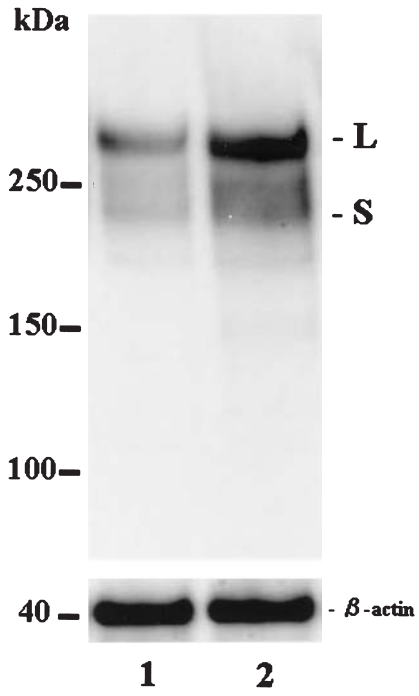

Figure 3. Western blot analysis of lysates from cultured chondrocytes incubated with or without TNF-α. After TNF-α treatment, bands for tenascin-C, particularly the large variants, were denser and thicker (lane 2) compared with no treatment (lane 1). Positions of the largest (L) and smallest (S) bands 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

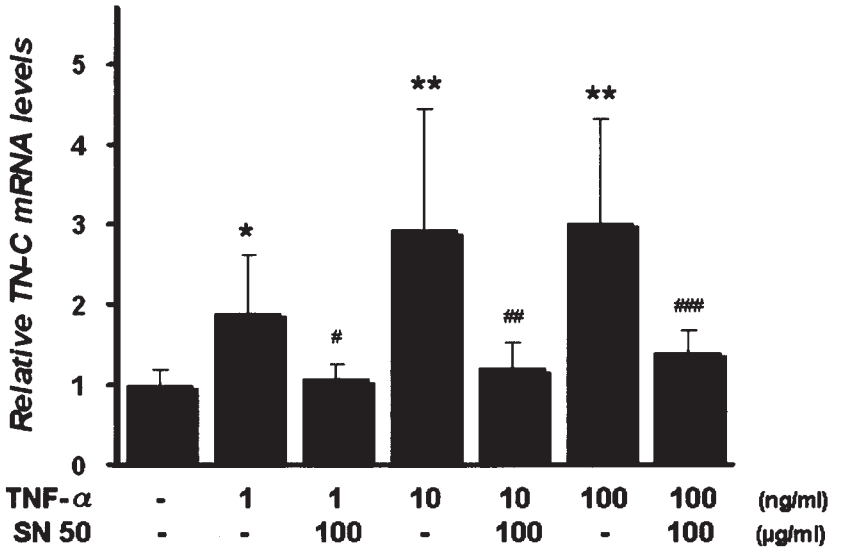

Figure 4. Regulation of tenascin-C mRNA after treatment with TNF-α and TNF-α + SN50. Expression of tenascin-C mRNA was increased by addition of 1 ng/ml TNF-α. Significant stimulatory effects of 10 ng/ml TNF-α were observed and were similar to those of 100 ng/ml TNF-α. Expression of tenascin-C mRNA stimulated 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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