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Original article

Distribution and role of tenascin-C in human osteoarthritic cartilage

Yutaka Nakoshi[1], Masahiro Hasegawa[1], Koji Akeda[1], Takahiro Iino[1], Akihiro Sudo[1], Toshimichi Yoshida[2], and Atsumasa Uchida[1]

[1] Department of Orthopedic Surgery, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu City, Mie 514-8507, Japan
[2] Department of Pathology and Matrix Biology, Mie University Graduate School of Medicine, Tsu City, Mie, Japan

Abstract

Background. Tenascin-C (TN-C) is expressed in the cartilage of osteoarthritis (OA). We examined whether TN-C was involved in cartilage repair of the diseased joints. Human articular cartilage samples were obtained from patients with OA and those with normal joints.

Methods. Immunohistochemistry testing of TN-C, chondroitin sulfate (CS), and proliferating cell nuclear antigen (PCNA) was performed. Chondrocytes were isolated from human cartilage and cultured. After treatment with TN-C, chondrocyte proliferation s was analyzed by bromodeoxyuridine (BrdU) incorporation assay using an enzyme-linked immunosorbent assay kit. Glycosaminoglycan content was determined by dimethylmethylene blue (DMMB) assay. The mRNA expression of aggrecan was also analyzed, by quantitative real-time polymerase chain reaction (PCR).

Results. In osteoarthritic cartilage, increased TN-C staining was observed with the degeneration of articular cartilage in comparison with normal cartilage. TN-C staining was shown in the cartilage surface overlying CS-positive areas. In addition, the expression of PCNA in the positive areas for TN-C was significantly higher than that in the negative areas. Treatment of human articular chondrocytes with 10 μg/ml TN-C accelerated chondrocyte proliferation, increased the proteoglycan amount in culture, and increased the expression of aggrecan mRNA.

Conclusions. Our findings indicate that the distribution of TN-C is related to CS production and chondrocyte proliferation in osteoarthritic cartilage and that TN-C has effects on DNA synthesis, proteoglycan content, and aggrecan mRNA expression in vitro. TN-C may be responsible for repair in human osteoarthritic cartilage.

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Introduction

Osteoarthritis (OA) is the most prevalent musculoskeletal disease and is characterized by the degradation of cartilage.[1] Recent studies have demonstrated that some growth factors, including basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), insulin-like growth factor-I, and bone morphogenetic protein, are upregulated in OA cartilage and that they could stimulate chondrocyte proliferation and/or production of the cartilage matrix.[2] These molecules may promote cartilage repair in OA and suppress the disease progression.

Tenascin-C (TN-C) is an extracellular matrix glycoprotein composed of six similar subunits linked in their amino-terminal domain by disulfide bonds.[3] TN-C is predominantly expressed during embryogenesis.[4] Although TN-C expression is restricted in normal adult tissues, it reappears in association with wound healing, inflammatory processes, or neoplasia in a number of tissues.[5] In the neoplastic lesions, TN-C promotes the proliferation and migration of epithelial and mesenchymal cells.[6,7] TN-C has been found to be a constituent of developing articular cartilage, but the expression of TN-C was markedly decreased in adult normal articular cartilage.[8,9] In diseased joints, including those with OA, TN-C was highly re-expressed in human cartilage.[10] A previous study has shown that the TN-C concentration was elevated in synovial fluid obtained with cartilage lesions of the knee joint.[11] We have also demonstrated that levels of TN-C in joint fluids seem to parallel the radiographic progression of OA.[12] However, it is still unclear whether the TN-C expressed in the diseased joints prevents the cartilage damage or accelerates it.

In this study, we hypothesized that TN-C may contribute to articular cartilage repair in OA. Immunohistochemically, we observed the distribution of TN-C expressed in OA cartilage and examined its correlation

with the proliferation of chondrocytes, using proliferating cell nuclear antigen (PCNA) and the deposition of sulfated glucosaminoglycans in the joint cartilage. Furthermore, the effects of TN-C on chondrocyte proliferation, glycosaminoglycan content, and the mRNA level of aggrecan were investigated in vitro. We investigated whether TN-C was involved in cartilage repair of diseased joints.

Materials and methods

Cartilage specimens

Human OA cartilage specimens were obtained from the femoral condyles of 36 patients, aged 63–87 years (average, 72.9 years) who were undergoing total knee joint replacement for the treatment of OA. Non-OA cartilage specimens were obtained from the femoral condyles of 4 patients aged 19–33 years (average, 24.4 years) with no history of joint disease and no evidence of macroscopic articular degeneration at the time of amputation for tumor resection. All patients gave their informed consent and this study was approved by the local ethics committee. The specimens were immediately fixed in 4% paraformaldehyde in 0.1% phosphate-buffered saline (PBS; pH 7.4) at room temperature (RT) 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). Serial sections were stained with safranin O, and we assessed cartilage destruction in each area using Mankin's scoring system, a 14-point scoring system for histological findings.[13] Based on the sum of the scores, each section was ranked as one of four histological grades: normal, 0–2; mild, 3–6; moderate, 7–10; and severe, 11–14.

Chondrocyte isolation and culture

Chondrocytes were isolated from human articular cartilage during knee replacements for advanced OA under sterile conditions. Cartilage fragments, damaged by fibrillation resulting from OA change, were removed from the femoral condyles of knee joints with a sharp curette. The cartilage fragments were incubated in 0.8% Pronase solution (Calbiochem, Darmstadt, Germany) and dissolved in Dulbecco's modified Eagle's medium/Ham F12 (DMEM/F12) (GIBCO, Grand Island, NY, USA) for 30 min at 37°C with continuous agitation in an atmosphere of 5% CO_2. After washing with DMEM/F12, the cartilage pieces were incubated with 0.4% collagenase (Roche Diagnostics, 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 with DMEM/F12 containing 10% fetal bovine serum (FBS) three times and plated on 96-, 12-. or 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 the cells was checked by immunofluorescence staining with chondroitin sulfate (CS) (Seikagaku, Tokyo, Japan) and type II collagen (Daiichi Fine Chemical, Toyama, Japan). More than 85% of the cells were positive in both cases (data not shown). Chondrocytes were grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, with the medium changed every 2 days. Each of the experiments was performed using the cells of primary culture isolated from ten different patients (age 70–78 years, average 73.4 years).

Immunohistochemistry

Immunofluorescence staining of OA cartilage specimens for TN-C and CS, after antigen retrieval, was performed with 0.01 M citrate buffer at 97°C for 30 min, and sections were incubated with normal goat serum (Dako, Carpinteria, CA, USA) at RT for 30 min. Then they were treated with primary antibodies, mouse monoclonal anti-CS antibody reacted to 4- and 6-sulfates (Seikagaku Corporation, Tokyo, Japan) or rabbit polyclonal anti-TN-C antibody (IBL, Gunma, Japan) at RT overnight. After washes with TBS, the sections were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG or Alexa Fluor 546-goat anti-rabbit IgG at RT for 3 h. Slides were mounted with Vectashield (Vector Laboratories). All slides were viewed through an epifluorescence microscope equipped with appropriate filters.

Double immunohistochemistry of TN-C and CS was performed. The sections were exposed to the monoclonal anti-CS antibody reacted to 4- and 6-sulfates (Seikagaku) overnight at 4°C and treated with alkaline phosphatase (ALP)-labeled goat anti-mouse IgG Fab′ (Nichirei Biosciences, Tokyo, Japan) for 30 min. The color reaction was achieved with a Fuchsin+ solution (Dako). The immune complexes were removed by treatment with 0.01 N HCl. Then the sections were incubated in 0.04% Proteinase K (Sigma) for 10 min at RT to retrieve the antigens, followed by incubation with monoclonal anti-TN-C antibody (4F10TT; IBL), which is specific to the epidermal growth factor (EGF)-like domain, and reacted with all TN-C variants, overnight at 4°C. The sections were then treated with ALP-labeled goat anti-mouse IgG Fab′ (Nichirei Biosciences) and developed with BCIP/NBT solution (Dako).

Chondroitin sulfate immunoreactivity was red, whereas TN-C immunoreactivity was bluish-violet. Each step was followed by extensive washing in Tris-buffered saline (TBS; pH 7.6). For negative controls, normal mouse IgG was used instead of the first and second primary antibodies.

For double immunohistochemistry of TN-C and PCNA, the sections were treated with a monoclonal anti-PCNA antibody (Dako) and goat anti-mouse IgG Fab′ labeled with peroxidase.[14] The color development was achieved with diaminobenzidine (DAB)/H_2O_2 solution. After the immune complexes were removed by treatment with 0.01 N HCl, they were incubated with 4F10TT antibody and with ALP-labeled goat anti-mouse IgG Fab′, followed by color development with BCIP/NBT. Immunoreactivity of PCNA was brown and that of TN-C was bluish-violet.

To examine the correlation between TN-C staining and chondrocyte proliferation in OA cartilage, three random fields (237 μm^2/field) of each sample from ten articular cartilage samples were observed. Numbers of PCNA strong-positive nuclei and the total number of nuclei were counted in TN-C strong-positive or -negative areas and the labeling indices were provided by determining the percentages of positive nuclei. For negative controls, normal mouse IgG was used instead of the first and second primary antibodies.

Purification of TN-C and its recombinant fragments

TN-C was purified from conditioned medium of U-251 MG human glioma cells by ammonium sulfate precipitation, Sephacryl S-500 gel filtration, and Mono Q ion-exchange chromatography (GE Healthare Bio-Sciences Corp., NJ, USA) as previously described.[6,7]

Cell proliferation assay

Cell proliferation was determined using a colorimetric immunoassay based on the measurement of bromodeoxyuridine (BrdU) incorporated by DNA synthesis. The cells were seeded at 1×10^4 cells/well on 96-well plates (Becton Dickinson Labware). After incubation in a serum-free medium with 0.1% bovine serum albumin (BSA) for 24 h, the cells were treated with 1 μg/ml or 10 μg/ml of TN-C and further cultured for 12 h before the proliferation assay. The BrdU solution was added to a final concentration of 100 μM and the cells were incubated at 37°C for an additional 2 h. The assay was performed using a cell proliferation enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics) according to the manufacturer's instructions. The value obtained from control cells without TN-C treatment was regarded as 100% and the effects of TN-C on cell proliferation were expressed by the relative values.

Proteoglycan content assay

Chondrocytes were cultured at a density of 4×10^4 cells/well in 12-well plates (Becton Dickinson Labware). The cells were cultured at serum starvation with 0.1% BSA for 24 h after chondrocytes reached confluence, and 1 μg/ml or 10 μg/ml of TN-C was added to the wells. After 24 h, the glycosaminoglycan content of the chondrocytes in culture was determined by the dimethylmethylene blue (DMMB ; Polysciences , Warrington, PA, USA) method.[15] In brief, the samples were digested with 20 μg/ml papain in a buffer of 0.1 M sodium acetate, 50 mM ethylenediaminetetraacetic acid (EDTA), and 5 mM L-cysteine at pH 5.53, for 12 h at 60°C, then 200 μl of 16 μg/ml DMMB solution was added to the papain digest of a 150-μl aliquot. The optical densities at 595 and 530 nm were immediately monitored with a spectrophotometer. The ratio of 530/595 nm could determine the glycosaminoglycan amounts. A calibration curve was provided using bovine chondroitin sulfate (ICN Biomedicals, Aurora, OH, USA) as a standard and the amount was normalized by DNA content measured using Hoechst 33258 dye (Polysciences).

RNA extraction and cDNA synthesis

The cells were seeded at 1×10^5 cells/well on 6-well plates (Becton Dickinson Labware). After the cells reached confluence, chondrocytes were treated with different concentrations of TN-C (1 μg/ml or 10 μg/ml) under serum-free conditions with 0.1% BSA. After 12 h, total RNA was isolated using ISOGEN (Nippon-Gene, Toyama, Japan) according to the manufacturer's instructions. Complementary DNA (cDNA) synthesis was performed by oligo $(dT)_{15}$ priming of 1 μg of total RNA using a cDNA synthesis kit (Roche Diagnostics) according to the manufacturer's protocols. TaqMan gene expression assay primer-probe pairs were ordered for the detection of aggrecan (Assay ID. Hs00153936_ml) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Assay ID. Hs99999905_ml; Applied Biosystems, Foster City, CA, USA). Quantitative analysis of the cDNA was performed using the ABI Prism 7000 Sequence Detector System (Applied Biosystems) and Taqman Universal polymerase chain reaction (PCR) Master Mix (Roche Diagnosis). The thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. GAPDH was used as the housekeeping gene for internal control. Aggrecan 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 means ± standard deviation (SD). Numeric data were statistically evaluated by the Kruskal-Wallis test and the Mann-Whitney *U*-test, using StatView software (Abacus Concepts, Berkeley, CA, USA). A *P* value less than 0.05 was considered to be statistically significant.

Results

Immunofluorescence for OA cartilage specimens

Immunolabeling of CS and TN-C was performed in tissue specimens of OA cartilage. The intensity of CS staining was decreased in the superficial zones and increased in the middle zones of the OA specimens (Fig. 1A). TN-C staining was observed in the superficial and middle zones (Fig. 1B). TN-C labeling was detected in matrices surrounding chondrocytes overlying CS-stained area.

Immunohistochemistry of chondroitin sulfate and TN-C in articular cartilage

Normal articular cartilage classified with no evidence of OA, having a total score of 0 to 2 using Mankin's scoring system, was obtained from the femoral condyles of knee joints. A uniform distribution of CS in the pericellular and interterritorial matrices of the middle and deep zones particularly was found. TN-C staining was barely observed in the superficial and upper-middle zones of normal articular cartilage (Fig. 2A, B, C). Sections with total scores of 3 to 6 were classified as mild OA cartilage. An enhancement of TN-C staining was observed at the damaged surface and in the upper-middle zones. In contrast, the intensity of CS staining was decreased in the superficial zones but maintained in TN-C-positive areas of the upper-middle zone (Fig. 2D, E, F). Cartilage samples with total scores from 7 to 10 were ranked as moderate OA. Clefts in the deep zone, some clusters of chondrocytes, and loss of the surface and middle zones were observed. In this stage, CS staining became weaker and marked TN-C staining appeared in the pericellular matrix of chondrocytes in the lower-middle zones. CS staining was partly restricted to the TN-C-positive area (Fig. 2G, H, I). In samples of severe OA, with total scores from 11 to 14, CS staining was found only in the deep zone. TN-C was detected in matrices surrounding the chondrocytes of deep zones overlying CS staining (Fig. 2J, K, L). The control sections showed no staining (data not shown).

TN-C expression and chondrocyte proliferation in articular cartilage

In normal cartilage, TN-C staining and PCNA-positive nuclei were hardly observed (Fig. 3A). In the TN-C-positive area of OA cartilage, PCNA-labeled nuclei were significantly more frequent than in the negative area (Fig. 3B, C). Labeling indices of PCNA in TN-C-positive areas were significantly higher than those in the -negative areas ($p < 0.01$) (Table 1). The control slides showed no staining (data not shown).

Proliferation of cultured chondrocytes after TN-C treatment

Compared with the control cells without TN-C, BrdU incorporation of the cells treated with 10 μg/ml TN-C was significantly increased ($P < 0.01$), while that of the cells treated with 1 μg/ml of TN-C was not (Table 2).

Table 1. Labeling index of PCNA in TN-C expression

	TN-C staining	
	(–)	(+)
Labeling index (%)	4.7 ± 4.4	41.0 ± 10.5*

* $P < 0.01$ compared with TN-C negative areas
Results are means ± standard deviation
PCNA, proliferating cell nuclear antigen; TN-C, tenascin-C

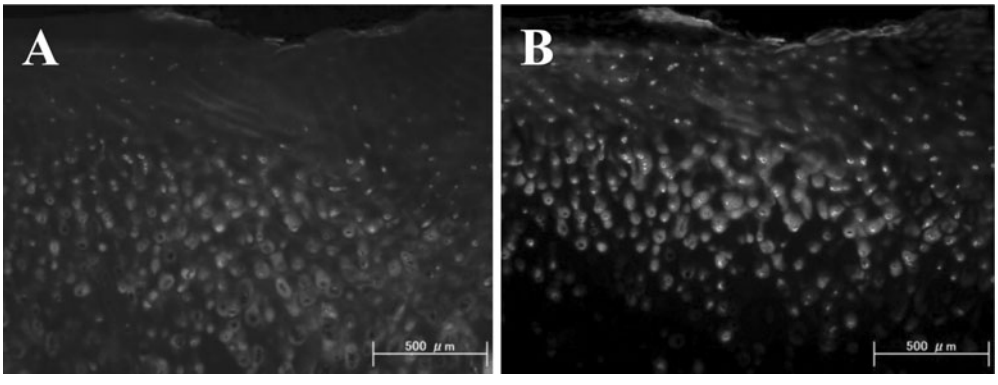

Fig. 1A,B. Photomicrographs are representative of osteoarthritis (OA) articular cartilage showing immunofluorescence staining for chondroitin sulfate (CS, **A**) and tenascin-C (TN-C, **B**). *Bars*, 500 μm for **A** and **B**

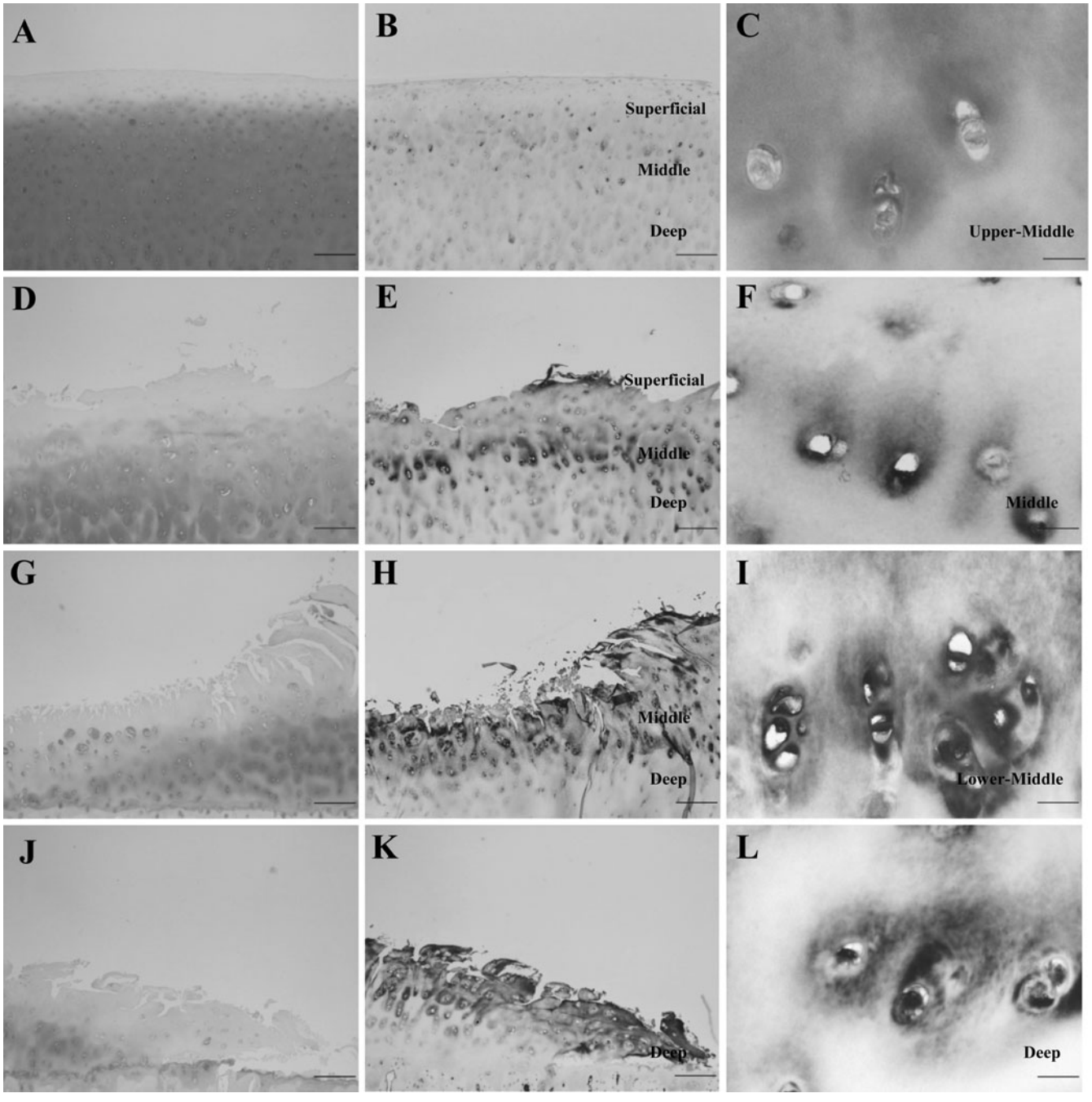

Fig. 2A–L. Photomicrographs are representative of articular cartilage stained with safranin O (**A**, **D**, **G**, and **J**), and double-immunostained with anti-TN-C and anti-CS antibodies (**B**, **C**, **E**, **F**, **H**, **I**, **K**, and **L**). *Bars*, 500 μm for **A**, **B**, **D**, **E**, **G**, **H**, **J**, and **K**; 50 μm for **C**, **F**, **I**, and **L**

Table 2. Proliferation, proteoglycan (PG) content, and aggrecan mRNA in cultured chondrocytes after TN-C treatment

TN-C (μg/ml)	0 (control)	1	10
BrdU incorporation (%)	100.0 ± 19.8	104.9 ± 46.1	159.8 ± 35.0*
PG content (μg/μg DNA)	4.2 ± 1.7	4.0 ± 1.6	6.0 ± 1.4**
Relative aggrecan mRNA levels	1.00 ± 0.18	1.35 ± 0.69	3.25 ± 1.32***

* $P < 0.01$ compared with control; ** $P < 0.05$ compared with control; *** $P < 0.01$ compared with control
Results are means ± standard deviation
BrdU, bromodeoxyuridine

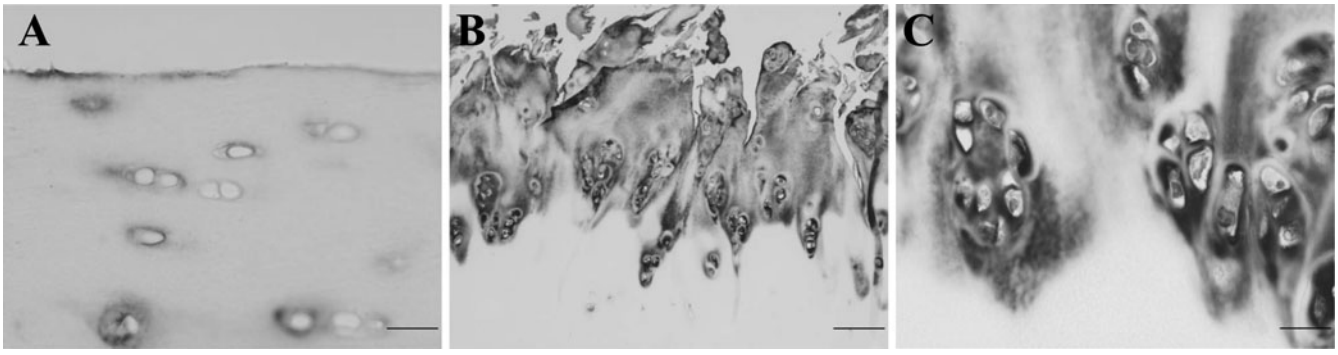

Fig. 3A,B. Photomicrographs are representative of articular cartilage double-immunostained for TN-C and proliferating cell nuclear antigen (PCNA). *Bars*, 50 μm for **A** and **C**, 200 μm for **B**

Glycosaminoglycan content of chondrocyte culture after TN-C treatment

Glycosaminoglycan content was corrected by the DNA content of each culture. Whereas the effect of treatment with 1 μg/ml TN-C showed no statistical significance, similar to that of the control cells, the treatment with 10 μg/ml TN-C significantly enhanced glycosaminoglycan accumulation in chondrocyte culture ($P < 0.05$; Table 2).

Aggrecan mRNA levels in cultured chondrocytes after TN-C treatment

Aggrecan transcripts were quantified by real-time PCR. Quantitative analysis by real-time PCR demonstrated approximately threefold upregulation of aggrecan mRNA levels in chondrocytes after 10 μg/ml TN-C treatment compared with untreated cell levels ($P < 0.01$), while treatment with 1 μg/ml TN-C showed slight upregulation, but there was no significant difference (Table 2).

Discussion

In this study, we noted the appearance of TN-C staining and its increase in the pericellular and interterritorial matrices of the middle and deep zones as well as fibrillated cartilage. Several studies have also shown increased TN-C labeling in osteoarthritic conditions compared with normal cartilage.[9,10] The increase of TN-C surrounding chondrocytes suggested that the cells themselves could produce TN-C in OA cartilage. The expression of TN-C, as well as aggrecan, in chondrocytes was regulated by interleukin (IL)-1β and was increased in OA cartilage.[9] In addition, mechanical loading is well known to regulate TN-C expression.[16] Physiologically relevant stress responses could be related to the expression of TN-C in wounds or around tumors.[17] We found enhanced TN-C labeling in parallel with decreased CS staining in damaged OA cartilage in some sections. CS is a sulfated glycosaminoglycan found in articular cartilage and is composed of repeating disaccharide units of glucuronic acid and galactosamine. Articular cartilage resists compressive force because of its capacity for binding water to the CS domains of aggrecan. Therefore, CS loss may result in increased mechanical loading of chondrocytes, followed by the upregulation of TN-C expression. We suggest that the regulation of TN-C increase and CS loss is performed by common mechanisms in cartilage. Furthermore, the present study also showed the colocalization of CS with TN-C staining in cartilage matrix around chondrocytes in part of the survival zones in the damaged cartilage.

In the middle to deep zones of articular cartilage in an OA joint, where chondrocyte cluster formation is shown, this being a major characteristic phenotype in the diseased cartilage, we found that the labeling indices of PCNA in chondrocytes were significantly higher in TN-C-positive areas than in -negative areas. It has also been found that TN-C-rich foci were associated with PCNA immunoreactivity in smooth muscle cells.[18] We also demonstrated a promoting effect of TN-C on the proliferation of cultured chondrocytes. TN-C may stimulate chondrocyte proliferation for repair in the pathogenesis of OA cartilage.

Our in vitro studies suggest that TN-C upregulates the mRNA expression of aggrecan in chondrocytes and increases the amount of glycosaminoglycan in the culture. Aggrecan consists of a long protein core to which more than 100 glycosaminoglycan chains are attached. Increases in aggrecan gene expression are correlated with increases in secreted proteoglycan synthesis. The components of proteoglycan are produced separately by the chondrocytes and extruded into the pericellular matrix as aggrecan. In OA cartilage, increased synthesis of aggrecan induced by TN-C stimulation could partly explain their colocalization. Chondrocytes are thought to be surrounded by a specialized

microenvironment known as a chondron. TN-C appears to be an important constituent of this microenvironment.[19] The chondron has been shown to represent a key site of degradative matrix changes in OA cartilage. Aggrecan is a major component of the chondron and an important factor in the retention of collagen within cartilage.[20] Day et al[21] showed that the aggrecan G3 domain could bind to TN-C and that alternative splicing of the domain influenced the binding interaction. The splice variant with the highest affinity to TN-C has been confirmed to be expressed by chondrocytes. From our immunohistochemical findings, showing that CS staining was decreased but partly maintained in TN-C-positive areas surrounding chondrocytes in OA cartilage, we speculate that the molecular interaction of TN-C could maintain the accumulation of aggrecan with a CS side chain in the chondron and modulate the anchoring of the chondrocytes to the matrix. Furthermore, in normal deep cartilage, previous studies have demonstrated that chondrocytes in deep zones are more active in glycosaminoglycan synthesis than superficial chondrocytes, and TN-C could be found in the deep areas of some normal cartilage.[9,22,23] It may be that the chondrocytes obtained from zones that maintained both CS and TN-C staining or those obtained from deep almost intact areas in human OA cartilage may have sensitized TN-C responsiveness.

It is known that TN-C is expressed during chondrogenesis within the embryo, and in vitro studies were devised to investigate the function of TN-C during chondrogenesis.[24] TN-C may be correlated to the development of permanent articular cartilage composed of specific matrix. Also, matrix protein such as aggrecan is required for repair in osteoarthritic cartilage, and our study, by the double-immunostaining and articular chondrocyte culture experiments, indicated that TN-C could produce cartilaginous matrix during OA disease. However, it was reported that transgenic mice without distributed TN-C gene production developed normally.[25] Other factors may replace or supplement TN-C action. A previous study has shown that fibronectin appears diminished in the repair of epithelial tissue in mice lacking TN-C.[26] We also observed that myocardial tissue repair was partly abnormal in TN-C-deficient mice, and it is not known whether cartilage repair occurs during the disease progression in TN-C knockout mice.[7]

A previous in vivo study showed that 400 ng/ml TN-C did not trigger adverse reactions within joint cavities.[27] The repair tissue formed within defects treated with TN-C had more fibrous components. It was surprising that TN-C failed to elicit chondrogenesis in that experiment, but the concentration of TN-C was lower than the 10 μg/ml used in our study to induce chondrocyte proliferation, glycosaminoglycan production, and aggrecan mRNA expression. The influence of 10 μg/ml TN-C requires further study in vivo. Furthermore, it is considered that TGF-β conducts cartilage repair in OA, and it has been well studied that these kinds of factors also induce TN-C expression in various kinds of cells.[28–30] The coordination of TN-C with these molecules may develop a microenvironment of chondrocytes for cartilage repair in vivo. The present study, using an expression pattern and gain-of-function type in vitro experiment, showed some association of TN-C with cartilage repair. To establish a cause-and-effect relationship, however, it is necessary to perform loss-of-function type experiments using small interfering (si)RNA for TN-C in vitro and using TN-C-knockout animals. In the future, TN-C might be used for repairing OA cartilage.

Conclusions

TN-C could be one of the key molecules for regulating chondrocyte proliferation and forming cartilage matrices in OA cartilage. Further investigation is needed to clarify the biological functions of TN-C in articular cartilage disease in vivo.

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