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ORIGINAL ARTICLE

Effect of hypoxia and interleukin-1β on expression of tenascin-C in temporomandibular joint

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OBJECTIVE: The expression of tenascin-C in the synovial membrane of the internal derangement (ID) of the temporomandibular joint (TMJ) has been reported. Hypoxia of the synovial membrane in TMJ is considered to be a cause for the pathophysiology of ID. In this study, we clarify the contribution of hypoxia and interleukin-1β in the expression of tenascin-C in ID of TMJ.

MATERIALS AND METHODS: Synovial fibroblasts and disk cells obtained from ID of TMJs were cultured and treated with interleukin-1β under normoxia and hypoxia. A Western blot analysis and reverse transcription-polymerase chain reaction analysis were used to identify tenascin-C in cultured synovial fibroblasts and disk cells. In addition, the immunohistochemical staining of tenascin-C was carried out for the specimens of ID of TMJs and normal.

RESULTS: The combination of hypoxia and interleukin-1β caused a significant increase in tenascin-C protein and mRNA of synovial fibroblasts. In contrast, the combination caused no increase in tenascin-C in disk cells. However, the immunohistochemical staining demonstrated tenascin-C to be significantly detected in both the synovial tissue and disks in ID of TMJ.

CONCLUSIONS: These results indicate that hypoxic conditions with inflammation modulate the tenascin-C expression in synovial fibroblasts, but not in disk cells.
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Keywords: tenascin-C; hypoxia; Western blot; RT-PCR; TMJ

Introduction

Concerning the cellular and molecular basis of the pathophysiology of internal derangement (ID) of the temporomandibular joint (TMJ), three mechanisms including direct mechanical injury, hypoxia-reperfusion injury, and neurogenic inflammation have so far been considered (Milam and Schmitz, 1995).

Hypoxia-reperfusion injury could be caused by a transient overcoming of hydorostatic pressures of intracapsular space in the TMJ against end-capillary perfusion pressure of intracapsular tissues during pathological mechanical stress (e.g., with clenching). The hydrostatic pressures of superior joint space in some human subjects were measured and found to approach 200 mmHg with clenching (Nitzan, 1994). Therefore, when the intracapsular hydrostatic pressure exceeds the end-capillary perfusion pressure, the blood flows in intracapsular tissues could be transiently disrupted, thus resulting in tissue hypoxia (Milam and Schmitz, 1995).

Tenascin-C (TN-C) is highly expressed in embryonic tissue during morphogenesis and is sparsely expressed in adults, but it reappears during wound healing, regeneration, or cancer (Chiquet-Ehrismann *et al*, 1986). In human TMJs with ID, an increased expression of TN-C, an extracellular matrix glycoprotein, at the protein and mRNA levels were demonstrated in the stroma of the hypertrophic synovial membranes, particularly on the surface of severely hypertrophic synovial membranes with inflammation, proliferation, irregular lining structures, and new capillary growth (Yoshida *et al*, 1996, 1997, 1999a, 2002). The localization patterns of matrix metalloprotease-3 (MMP-3) and transforming growth factor-β (TGF-β) resemble that of TN-C on the surface of the severely hypertrophic synovial membrane (Yoshida *et al*, 1999b). Interleukin-1 (IL-1), a major mediator of tissue injury in inflammatory synovitis, also induces TN-C expression and deposition in primary synovial fibroblast cultures (McCachren and Lightner, 1992). In addition, in human normal and osteoarthritic cartilage explants, the presence and the distribution of TN-C are influenced by IL-1β (Chevalier *et al*, 1996). IL-1 is elevated in the synovial fluid of patients with rheumatoid arthritis and plays an important role in amplifying and perpetuating both inflammation and joint destruction (Mochan *et al*, 1989; Chin *et al*, 1990). Several studies have recently shown that IL-1β concentrations

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were elevated in the synovial fluid samples obtained from TMJ patients with osteoarthritis (Kubota *et al*, 1997; Alstergren *et al*, 1998; Takahashi *et al*, 1998). IL-1β in synovial fluid is associated with pain and hyperalgesia in the TMJ (Alstergren *et al*, 1998). However, little is still known regarding how hypoxia and IL-1β are involved with the expression of TN-C in synovial tissue and disk tissue specimens.

The purpose of this study was to investigate whether hypoxia and IL-1β modify the synthesis of TN-C in synovial fibroblasts and disk cells from human TMJ. In this study, we examined the expression of TN-C protein and the mRNA in cultured human synovial fibroblasts and disk cells treated with hypoxia and IL-1β. In addition, an immunohistochemical study of TN-C in ID and normal specimens of human TMJ was performed and compared with the *in vitro* data.

Patients and methods

Specimens of TMJ were obtained from 19 patients aged 20–72 years during therapeutic surgery and autopsy. All patients gave their informed consent and approval from the Ethics Committee of Wakayama Medical University was obtained.

Cell cultures of synovial tissues (three cases) and disk tissues (two cases) of ID were performed. After immediately removing the synovial tissue specimens from the TMJ, using sterile instruments and working in a sterile field, the synovial tissue specimens were washed in phosphate-buffered saline (PBS; GIBCO/BRL, Gland Island, NY, USA), and placed in Dulbecco's modified Eagle's medium (D-MEM; GIBCO/BRL) with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin and streptomycin in a 60 × 15 mm culture dish (Falcon; Becton Dickinson and Company, Franklin Lakes, NJ, USA). The tissue samples were incubated at 37°C in a humidified 5% CO_2 atmosphere for 7–14 days and finally when they reached 80% confluent conditions, they were passaged. The culture was continued until the cells increased to 20 dishes (100 × 20 mm). The primary cultures of cells from the synovium contained adherent macrophages but these were removed by successive passaging. The purity of the synovial fibroblast population was established by the absence of the macrophage marker CD68 (data not shown). The TMJ disks were minced and digested in 0.25% trypsin, 0.02 mM EDTA (GIBCO BRL; Life Technologies Inc., Grand Island NY, USA) and 4% collagenase (Roche Diagnostics, Mannheim, Germany) for 3 h. The disk cells were washed and resuspended in D-MEM with 10% heat-inactivated FBS, 1% penicillin and streptomycin. The cells were grown at 37°C in a humidified 5% CO_2 atmosphere for 7–14 days, and then were passaged and expanded to up to 20 dishes (100 × 20 mm). All experiments were carried out on cells between passages 6 and 13. The cells were plated at a density of 50 000/well in 12-well plates (IWAKI, Asahi Techno Glass, Funabashi, Japan) in a medium containing 10% FBS. The next day, the medium was changed to a serum-free medium and incubated for 24 h. The culture plates were rinsed with PBS, and 0.5 ml of fresh serum-free medium with or without 0–5 ng ml^{-1} of recombinant human IL-1β (PeproTech, London, UK) was added. The experiment was performed for 6, 12, 24 ,and 72 h in a humidified atmosphere of normoxic conditions (20% O_2, 5% CO_2, and 75% N_2), or hypoxic conditions (2% O_2, 5% CO_2, and 93% N_2). Three independent experiments involving separate cell capture were performed.

After the culture medium was removed and the cell culture plates were rinsed with PBS, an aliquot of 10% trichloroacetic acid was added to each well and then the plates were rocked gently for 30 min at 4°C. Adherent cells were scraped with a cell scraper. The scraped cells were transferred to microcentrifuge tubes and centrifuged at 15 000 × *g* for 10 min at 4°C. After the supernatant fluids were discarded, the pellets were resuspended with lysis buffer (9 M urea, 2% Triton X-100, 1% β-mercaptoethanol, 10% lithium dodecyl sulfate, 1 M Tris) and homogenized. The concentration of the extracted protein was determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were electrophoresed and separated on 6% SDS-polyacrylamide gels. The proteins were transferred electrophoretically onto a nitrocellulose membrane (Trans-Blot® Transfer Medium; Bio-Rad Laboratories) and treated with PBS containing 3% skimmed milk and 0.01% Tween-20 for 3 h. The membrane was then reacted with rabbit anti-human TN-C antibody (1:2000 dilution; Telios Pharmaceuticals, San Diego, CA, USA) and rabbit anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:500 dilution; Santa Cruz, CA, USA) at 4°C overnight. After washing in PBS containing 0.01% Tween-20, the reacted bands were then detected with horseradish peroxidase labeled anti-rabbit IgG antibody (Dako, Carpinteria, CA, USA) for 60 min at room temperature. The immunoreactive protein was visualized by using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). The membrane images were captured and the bands were quantified by NIH Image software to measure the relative integral optical density. GAPDH was used as an internal control. All experiments were repeated thrice.

Total RNA of cells was extracted by using RNeasy® RNA extraction Mini kit (Qiagen, Valencia, CA, USA) from cultured cells. First-strand cDNA was synthesized from total RNA using SuperScript™ (First-Strand Synthesis System for RT-PCR; Invitrogen Life Technologies, Carlsbad, CA, USA). Reverse transcription-polymerase chain reaction (RT-PCR) was carried out using *Taq* DNA polymerase (Ampli Taq Gold®; Applied Biosystems, Foster City, CA, USA) and the specific primers. The sequence of the used TN-C primers are sense 5′-TTCAATCTCTCCTGGATGGCTACCG-3′, antisense 5′-ATAGGTAATCCGGAAGCTCTCCA CTTG-3′, PCR was performed for 28 cycles and the expected size of the products was 875 bp. As a control 18S ribosomal RNA (18S rRNA) was amplified using primers of 18S rRNA: sense 5′-GTTGGTGGAGC-GATTTGTCT-3′ and antisense 5′-GGCCTCAC-TAAACCATCCAA-3′. PCR was performed for 25

cycles. The samples were incubated in a PCR thermal cycler (GeneAmp®PCR System; Applied Biosystems) at 61°C (TN-C) or 60°C (18S rRNA), annealing temperature. The amplified PCR products were analyzed in 2% agarose gel electrophoresis and stained with ethidium bromide. The gel images were captured and the bands were quantified by NIH Image software to measure relative integral optical density. Each product was normalized with that of 18S rRNA from the same template and shown as a ratio. All experiments were repeated thrice.

The following specimens of TMJ were used for immunohistochemical studies: twelve specimens of ID and two specimens of normal TMJ of oral cancer patients. The indirect enzyme immunostaining method was performed with the above mentioned materials. Five-μm thick sections were cut from 14 paraffin-embedded blocks and were placed onto silan-coated glass slides. The sections were de-waxed by xylene and then were rehydrated in serial alcohol. The endogenous peroxidase was blocked by immersing the sections in 0.3% H_2O_2 in methanol for 20 min at room temperature. After blocking with 3% skimmed milk (0.01% Tween PBS) for 60 min at room temperature, they were treated with a rabbit polyclonal antibody against human TN-C (Telios) at 4°C for 12 h. After washes with PBS, they were treated with horseradish peroxidase labeled anti-rabbit IgG antibody (DAKO) for 60 min at room temperature. The color was developed by 3,3′-diaminobenzidine/H_2O_2 solution followed by counterstaining with Mayer's hematoxylin. The negative controls were subjected to the same protocol except for the omission of the primary antibody. Each slide was analyzed by two independent observers. The results were evaluated semi-quantitatively on four scales: −, no expression; +/−, indistinct expression; +, clear expression; ++, high level expression. The TN-C staining scales were analyzed on the synovial membrane and disk of each specimen.

Values were expressed as mean ± standard deviation. We analyzed the Western blotting and RT-PCR data to identify any statistical differences using ANOVA and multiple-comparison tests.

Results

According to the Western immunoblot analysis, TN-C protein was detected in larger quantity in synovial fibroblasts than in disk cells (Figure 1). The expression of TN-C protein was found to increase considerably by hypoxia and IL-1β (1 ng ml^{-1}) (H + IL) in comparison with normoxia (N) or IL-1β only (N + IL) or hypoxia only (H) in synovial fibroblasts after 24 h treatment

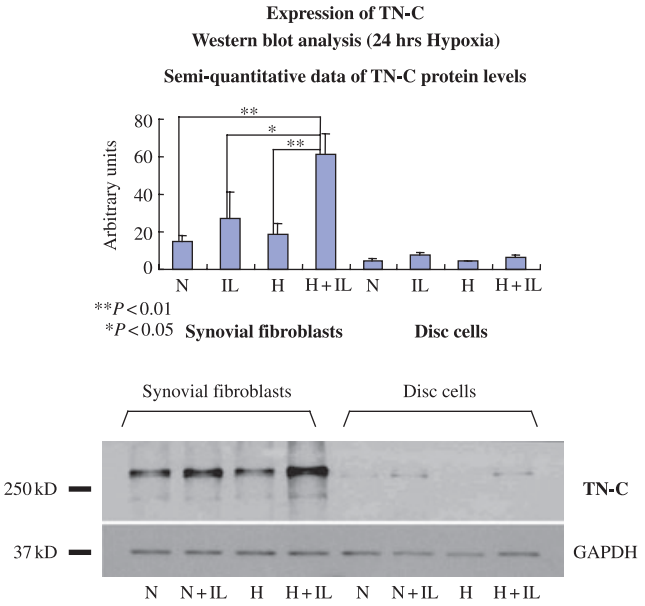

Figure 1 A Western blot analysis of the expression of tenascin-C protein in cultured synovial fibroblasts (three samples) and disk cells (two samples) incubated with or without IL-1β (1 ng ml^{-1}) for 24 h in normoxia or hypoxia (2% O_2) condition. Normoxia (N), normoxia + IL-1β (N + IL), hypoxia (H), hypoxia + IL-1β (H + IL). Asterisks denote significant differences (**P < 0.01, *P < 0.05)

Table 1 Clinical and immunohistochemical data of the patients

Sample no.	*Diagnosis*	*Displacement of TMJ disk*	*Perforation of TMJ disk*	*Tenascin-C detection in the synovial membranes*	*Tenascin-C detection in the disks*
1	Internal derangement	+		+	−
2	Internal derangement	+		++	+
3	Internal derangement	+	+	++	++
4	Internal derangement	+		++	++
5	Internal derangement	+	+	++	++
6	Internal derangement	+		+	+
7	Internal derangement	+		++	++
8	Internal derangement			+/−	+
9	Internal derangement	+		++	++
10	Internal derangement	+	+	++	++
11	Internal derangement	+		+/−	++
12	Internal derangement	+		+	−
13	Autopsy sample (control)			+	+
14	Autopsy sample (control)			+	+

The semi-quantitative values for tenascin-C expression in the human TMJ synovial membrane and disk. −, no expression; +/−, indistinct expression; +, clear expression; ++, high level expression.

($P < 0.01$). Under hypoxia conditions for 12 and 72 h, the expression of TN-C protein was lower than at 24 h with or without IL-1β in synovial fibroblasts ($P < 0.05$). In the synovial fibroblasts, the effects of IL-1β on TN-C expression depended on its concentration. The TN-C expression of synovial fibroblasts was induced more intensely in the IL-1β concentration below 1 ng ml^{-1} in hypoxia than in normoxia. Although in normoxia the expression of TN-C protein of synovial fibroblasts treated with the IL-1β (5 ng ml^{-1}) was strongly induced, in hypoxia the expression decreased dramatically. Similarly, in the disk cells the effects of IL-1β on TN-C expression depended on its concentration, but the expression of TN-C protein was small.

In synovial fibroblasts TN-C mRNA was significantly induced with IL-1β (1 ng ml^{-1}) in hypoxia condition (H + IL) for 6 h ($P < 0.01$) (Figure 2). In only normoxia (N) or hypoxia (H) condition TN-C was not significantly induced in synovial fibroblasts. Under hypoxia conditions for 24 h, TN-C mRNA expression was lower than at 6 h with IL-1β in synovial fibroblasts. In the disk cells IL-1β upregulated TN-C mRNA, but no significant difference was observed between normoxia + IL-1β (N + IL) and hypoxia + IL-1β (H + IL) (Figure 2).

The immunohistochemical localizations of TN-C were examined in the synovial membrane and disk of each specimen (Table 1). Immunolabeled TN-C was present at low levels in normal healthy synovial membranes and disks, while it was upregulated in the inflamed synovial membranes and deformed disks of ID (Figure 3). TN-C showed intense diffusion within the stroma of the sublining layer in the hypertrophic synovial membrane (Figure 3, a-1). In the deformed disks of ID, TN-C was colocalized in the abnormally arranged collagen fibers (Figure 3, b-1). In addition, in the disks with perforation, immunostaining of TN-C was specifically seen at the stroma of the perforated area.

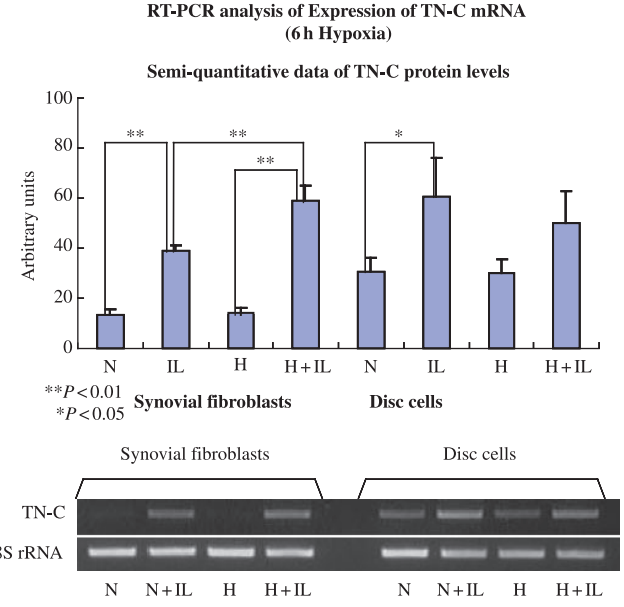

Figure 2 A semi-quantitative RT-PCR analysis of the expression of tenascin-C mRNA in cultured synovial fibroblasts (three samples) and disk cells (two samples) incubated with IL-1β (1 ng ml^{-1}) for 6 h in normoxia or hypoxia (2% O_2) condition. 18S rRNA is internal control. Normoxia (N), normoxia + IL-1β (N + IL), hypoxia (H), hypoxia + IL-1β (H + IL). Asterisks denote significant differences (**$P < 0.01$, *$P < 0.05$)

Discussion

Interleukin-1β induced TN-C expression and deposition in primary cultures of synovial fibroblasts from large joints (McCachren and Lightner, 1992). The findings of this study confirmed that IL-1β enhances TN-C expression at both the protein and mRNA levels in synovial fibroblasts and disks of TMJ. Hypoxia is thus reported to increase IL-1 in the human alveolar macrophage and human mononuclear cells (Ghezzi *et al*, 1991; Hempel *et al*, 1996). The repeated hypoxia condition is caused by excessive mechanical stress in a diseased TMJ. Macrophage-like cells (A cells in synovial tissues) are a plausible source of IL-1β. In addition, hypoxia/reoxygenation induces the activation of NF-κB synovial fibroblasts. The activation of NF-κB is involved in the induction of gene expression, which encodes inflammatory cytokines including TNF-α, IL-1β, and IL-8 (Han *et al*, 2003). Therefore, A cells (macrophage-like cells) may produce IL-1 by hypoxia, and the produced IL-1 may induce TN-C in B cells (synovial fibroblasts) in TMJ with ID. In this study, we surprisingly found that hypoxia dramatically enhanced the expression of TN-C induced by Il-1β in synovial fibroblasts with a low concentration of IL-1β (1 ng ml^{-1}). In the synovial fluid from arthritic TMJ, the IL-1β concentration was <1 ng ml^{-1} (Alstergren *et al*, 1995, 1998, 1999, 2003). IL-1β at a concentration below 1 ng ml^{-1} was found to treat effectively the TN-C expression of synovial fibroblasts under hypoxia. As a result, TN-C could thus be upregulated by a low dose of IL-1β induced by hypoxia/reoxygenation, which is caused by repeated mechanical stress.

Immunohistochemistry using specimens from patients with ID also showed diffuse and intense TN-C staining in the stroma of a sublining layer, containing synovial fibroblasts, in the hypertorophic synovial membranes, and these findings were compatible with the *in vitro* findings. Deformed disks, especially at abnormally arranged collagen fibers were also strongly labeled with the anti-TN-C antibody. However, the treatment of IL-1β and hypoxia did not alter the expression level of TN-C in cultured disk cells. As cartilage is an avascular tissue, it is well known that chondrocytes adapt well to hypoxia (Rajpurohit *et al*, 1996) and chondrocytes can survive in oxygen at a level as low as $<0.1\%$. Therefore, disk cells could be lower responders against hypoxic treatment, and TN-C protein in the diseased disks may thus originate mainly from synovial fibroblasts. Previous studies on TN-C expression have also demonstrated that mechanical stress and loading directly upregulate TN-C expression in myotendious junction, bone, and muscle (Webb *et al*, 1997; Fluck *et al*, 2000; Jarvinen *et al*, 2003). TN-C labeling in the myotendious junction suggests that TN-C provides elasticity to the

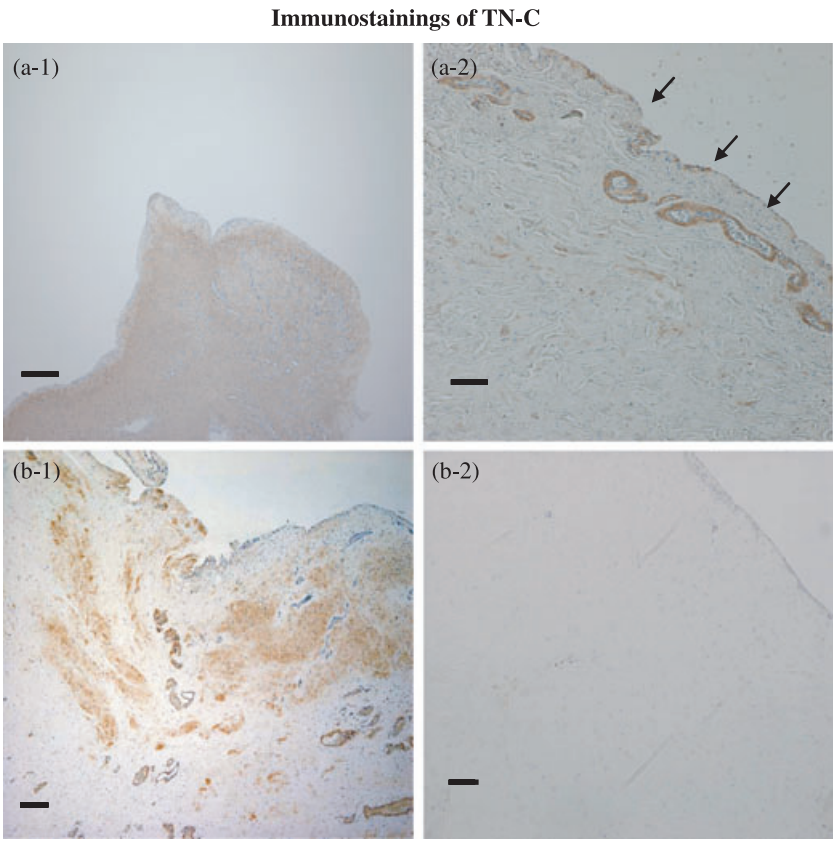

Figure 3 Immunohistochemical staining for tenascin-C (TN-C) protein. Color developed by DAB. (**a**-1): TN-C is intensely diffused within the stroma of the sublining layer in the hypertrophic synovial membrane of ID. (**a**-2): In normal synovial membrane (arrows), TN-C is stained at the surface of the synovial membrane and blood vessels. (**b**-1): In the deformed disks of ID, TN-C is localized at abnormally arranged collagen fibers. (**b**-2): In normal disk, TN-C is weakly diffused within the whole disk. Bar = 100 μm

mesenchymal tissue, which is subjected to heavy tensile loading (Alstergren *et al*, 1995). The expression in the bone and muscle is also regulated by mechanical loading (Webb *et al*, 1997; Fluck *et al*, 2000). In fact, in the promoter region of the TN-C genome, an element sensitive to mechanical stress has been found (Chiquet-Ehrismann *et al*, 1994). Therefore, the TN-C protein in the diseased disks may also be directly upregulated by mechanical stress.

Tenascin-C contributes to dynamic tissue remodeling after tissue injury (Imanaka-Yoshida *et al*, 2001, 2004). The expression in TMJ could therefore be also involved in the pathological remodeling of the synovial tissue, disk and condyle of the diseased joint as well as in the repair of such tissues. In this study, hypoxia and IL-1β synergistically enhanced the TN-C expression in cultured synovial fibroblast of TMJ. A strong expression of TN-C was therefore confirmed in the pathological phase of the synovial membrane of ID. These findings indicate that hypoxia of synovial membrane, via TN-C upregulation, could thus be a major factor for the progression of ID in the TMJ.

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