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Tumor necrosis factor-α and matrix metalloproteinase-3 production in rheumatoid arthritis synovial tissue is inhibited by blocking gap junction communication

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Abstract

Objective: *Our aim was to establish the existence of intercellular communication through gap junctions (ICGJ) in synovial tissues and to show that gap junction communications in synovial tissue plays a role in the pathogenesis of synovitis in patients with rheumatoid arthritis (RA).*

Material and methods: *Synovial tissues were obtained from patients with RA and osteoarthritis (OA) at the time of total knee arthroplasty. Immunohistochemistry was performed to determine the expression of Connexin 43 (Cx43) in the synovial tissues. The synovial tissues were cultured for 48 hours with various concentrations of a gap junction communication blocker (heptanol). The concentrations of TNF-α and metaloproteinases-3 (MMP-3) in the supernatants were measured using the enzyme immunoassay system (ELISA). In the preliminary stage, we had used 10 synovial tissues to determine the experimental conditions.*

Results: *The concentrations of TNF-α and MMP-3 in supernatants decreased by adding gap junction blocker (heptanol). The expression of Cx43 was positive in the synovial tissues from the RA patients; conversely, the synovial tissues from the OA patients exhibited weak staining.*

Conclusions: *Increased ICGP may contribute to the pathogenesis of synovitis in patients with RA because the use of a gap junction blocker inhibited the production of TNF-α and MMP-3. Thus, our findings suggest a functional role for gap junction communication in RA synovitis. It also suggests that the control of gap junction communication is a rational therapeutic target of RA synovitis.*

Key words: *connexin, gap junction, heptanol, arthritis, synovium.*

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Introduction

The gap junctions are membrane-spanning channels that facilitate intercellular communication by allowing small signaling molecules (up to 1 kDa), such as calcium ions, inositol phosphatase and cyclic nucleotides, to pass from cells to cells. Each gap junction pore is formed by the juxtaposition of two hemichannels in adjacent cells. A gap junction hemichannel is composed of six gap junction protein subunits (connexin, Cx) that dock to a corresponding structure in neighboring cells [1].

More than a dozen connexins have been identified, including at least seven human isoforms. Cx43 is widely expressed in different tissues, including the brain, heart, kidney, smooth muscle, ovary, some epithelia and osteoblasts [2]. It is the most abundant of the connexins. Immunologically, relevant peptides, normally between eight and ten amino acids in length, can be transferred through Cx43 gap junction [3]. Cross-presentation by intercellular peptide transfer through gap junction is also important for the function of the immune system and the infection system [4]. Gap-junctional intercellular communication also contributes to the regulation of osteoclastogenesis [1, 5-8]. The blocking of gap junction communication has been studied with, several inhibitors, such as heptanol [5], 182-glycyrrhetinic acid and deamide [9].

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Rheumatoid arthritis (RA) is a chronic and systemic inflammatory disease that is characterized by synovitis which leads to the destruction of articular cartilage and bone. Although the etiology of RA is not still fully understood, it is clear that both tumor necrosis factor-α (TNF-α) and matrix metalloproteinase 3 (MMP-3) play a pivotal role in inflammation and bone distraction.

In the current study, we investigated that the role of gap junctions in RA. We studied the effect of heptanol, an inhibitor of gap junctional communication, on the synovitis in RA. We showed that Cx43 was positive in RA synovial tissues. In addition heptanol decreased the production of both TNF-α and MMP-3 from synovial tissues.

Material and methods

Tissue samples

Synovial tissues were obtained from patients with RA and OA at the time of TKA. Informed consent was obtained before the TKA. For the immunochemical studies, synovial tissues were mounted in OCT (Lab-Tek Products, Napervill, IL, USA) oriented on cork bases, immediately snap-frozen in isopentane/liquidN_2 and stored at –80°C until use.

Immunochemistry

The sections were cut to a thickness of 6 γm with a cryostat (Bright Instruments Huntington, UK) and air dried for overnight. The sections were immersed in cold acetone (4°C) for 5 min, followed by incubation with 0.03% hydrogen peroxide for 10 min to block endogenous peroxidase activities. The tissues were stained with Cx43 antibodies raised against Cx43 (antimouse Cx43 monoclonal antibody, DACO Co); the antibodies were used as a 1 : 100 dilution in phosphate-buffered saline (PBS) for 2 hours at 37°C. The tissues were fixed with 3% PFA and 2% sucrose in PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes on ice. The staining was carried out with rhodamineconjugated rabbit anti-mouse immunoglobulin secondary antibodies diluted to a strength of 1 : 100 in PBS for 30 minutes at 37°C.

Quantification of cell death

Cells death was quantified by trypan blue uptake.

Cell cultures

Synovial tissues were obtained at the time of TKA. A part of each tissue was dissected into small pieces (1 g) for organ culture and rinsed well with PBS (pH 7.4). Each piece of the synovial tissue was cultured in 1 ml of RPMI (GIBCO BRL, Gaithersburg, Maryland, USA) with various concentrations (0-3 mM) of a gap junction communication blocker (heptanol) for 48 hours in 5% CO_2 in air at 37°C. Ethanol was used as a control. Culture supernatants were collected and kept frozen at –80°C until they were used for the measurement of the concentration of the MMP-3, TNF-α and IL-1β. The determination of MMP-3 used a one-step sandwich enzyme immunoassay system (Fuji Chemical Industries, Toyama, Japan) [10]. Two sets of monoclonal antibodies that recognize different epitopes on the human MMP-3 molecule were used in the assay system. The concentrations of TNF-α were also measured by the sandwich enzyme assay (high sensitivity TNF-α kit, Funakoshi Industries, Tokyo, Japan).

Statistical analysis

Correlation was estimated by Spearman's rank correlation coefficient, and Wilcoxon test. All statistical analyses, including correlation coefficients and *p* values, were carried out using the Stat-view statistical package (Abacus Concepts, Berkeley, CA). Differences were considered significant if *p* was less than 0.05 by the Mann-Whitney U test.

Results

Immunochemistry

The Cx43 was positive in lining cells, sublining cells and along the lymphoid nodule area in synovial tissues from the RA patients (Fig. 1). Conversely, synovial tissues from the OA patients exhibited weak staining (Fig. 1).

Quantification of cell death

Cell death was quantified by trypan blue uptake. There was no trypan blue uptake by cells in either the heptanol treatment cultures or the ethanol treatment cultures (Fig. 2).

The concentrations of TNF-α, MMP-3 and IL-1β

The concentrations of TNF-α, MMP-3 and IL-1β in supernatants decreased by adding heptanol dose-dependently (Fig. 3A-E), compared to the control. Heptanol, which is a gap-junction inhibitor, significantly reduced the amount of constitutive secretion of MMP-3 ($p = 0.018$) and TNF-α ($p = 0.04$) (Fig. 3F, G). We performed the same study five times and obtained similar results each time.

Discussion

In this study, we demonstrated that the concentrations of TNF-α and MMP-3 in supernatants in patients with RA decreased by adding a gap junction blocker (heptanol). In addition, Cx43 was positive in synovial tissues from the RA patients. Conversely, synovial tissues from the OA patients exhibited weak staining of Cx43. Thus, increased intercellular communication through gap junctions may contribute to the pathogenesis of synovitis in patients with RA.

We have reported that irsogladine (2,4-diamino-6-(2,5-dichlorophenly)-s-triazine maleate), a drug that reinforces

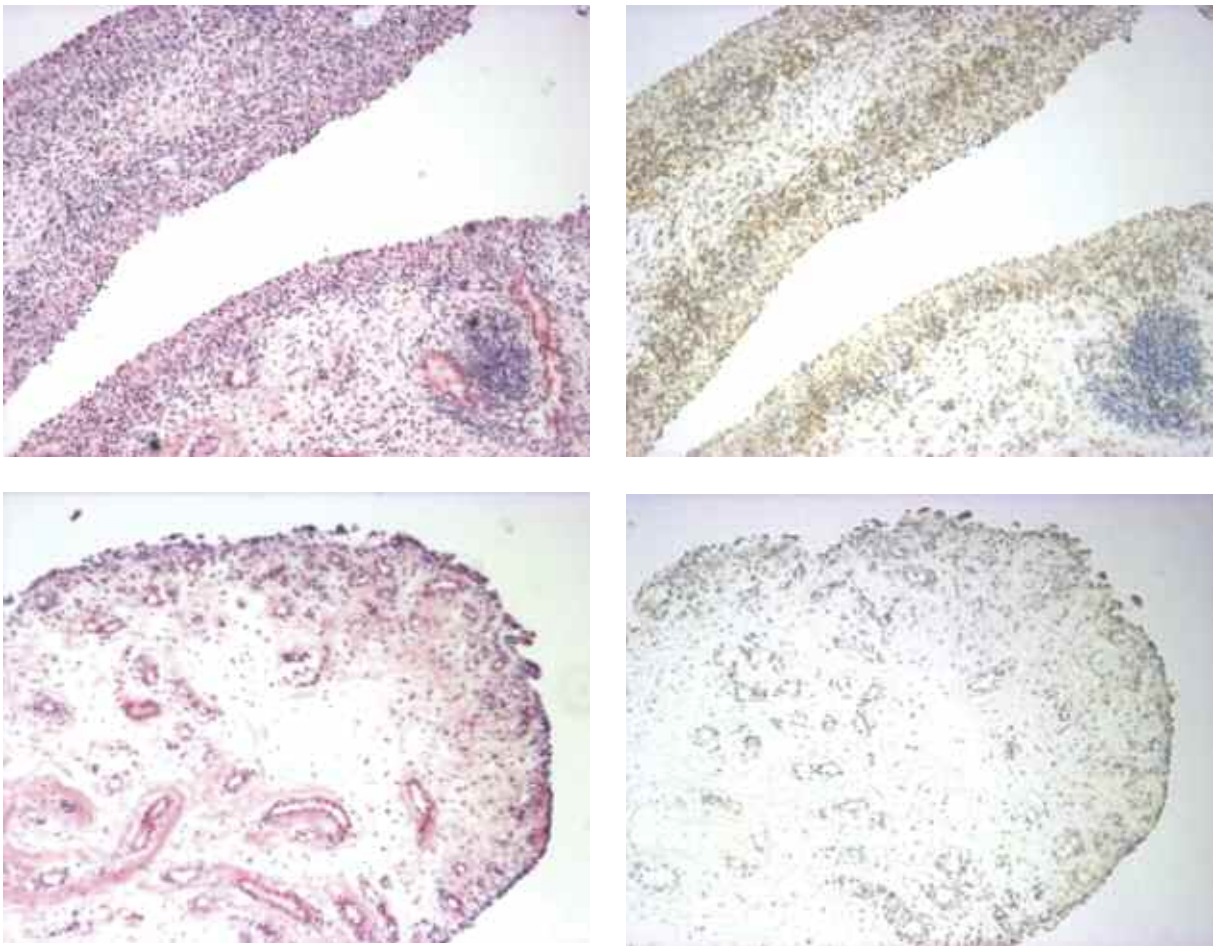

Fig. 1. Upper left: synovium from RA, stained by HE (40×). Upper right: synovium from RA, stained by Cx43 (40×). Upper left: synovium from OA, stained by HE (40×). Upper right: synovium from OA, stained by Cx43 (40×)

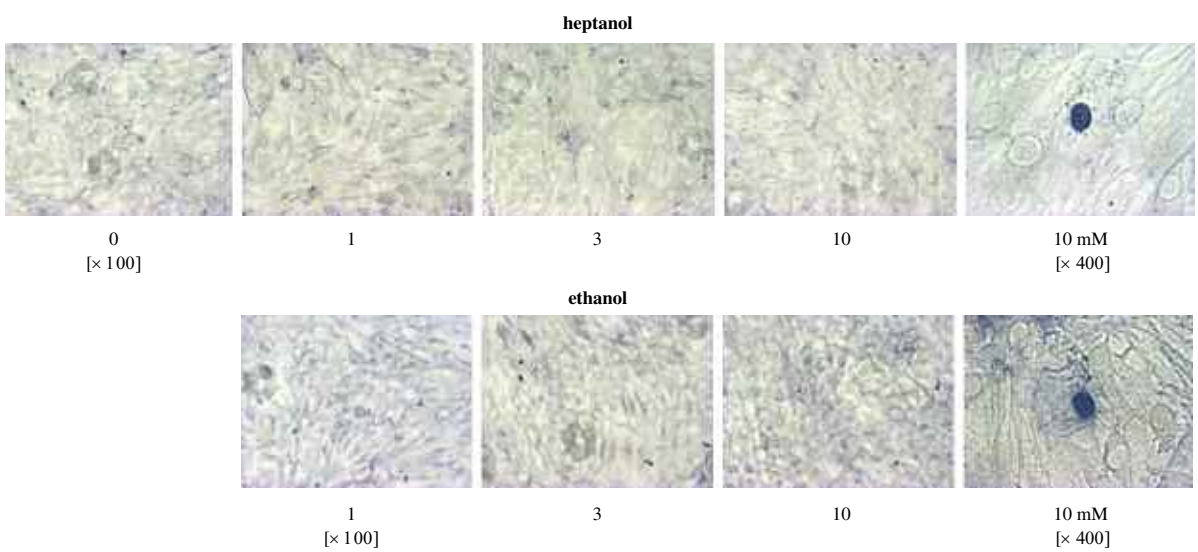

Fig. 2. Upper: heptanol treatment cultures by trypan blue staining. Lower: ethanol treatment cultures by trypan blue staining

gap-junctional intracellular communication and is used for the treatment of gastritis and peptic ulcers, reduces aphthous stomatitis/oral ulcers in patients with Behcet's disease. The improvement of gap-junctional intercellular communication may contribute to the treatment of aphthous stomatitis in patients with BD [11]. Thus, controlling the gap junction communication may be a rational therapeutic target of some diseases.

Synovial cells are connected to each other by an extensive networks of gap junctions, and it may mediate some cellular processes [12, 13]. Kolomytkin *et al.* reported the existence of ICGP in synovial lining cells and in primary and passed cultures of human synovial cells. The formation of gap-junction channels capable of mediating ionic and molecular communication was a regular feature of human synovial cells. Kolomytkin *et al*. also [14] reported that IL-1β produced a dose-dependent increase in MMP activity that was blocked by exposure to the gap junction inhibitors. The communication through gap junctions early in IL-1β signal transduction is critical to the process of cytokine-regulated secretion of MMPs by synovial cells in rabbit.

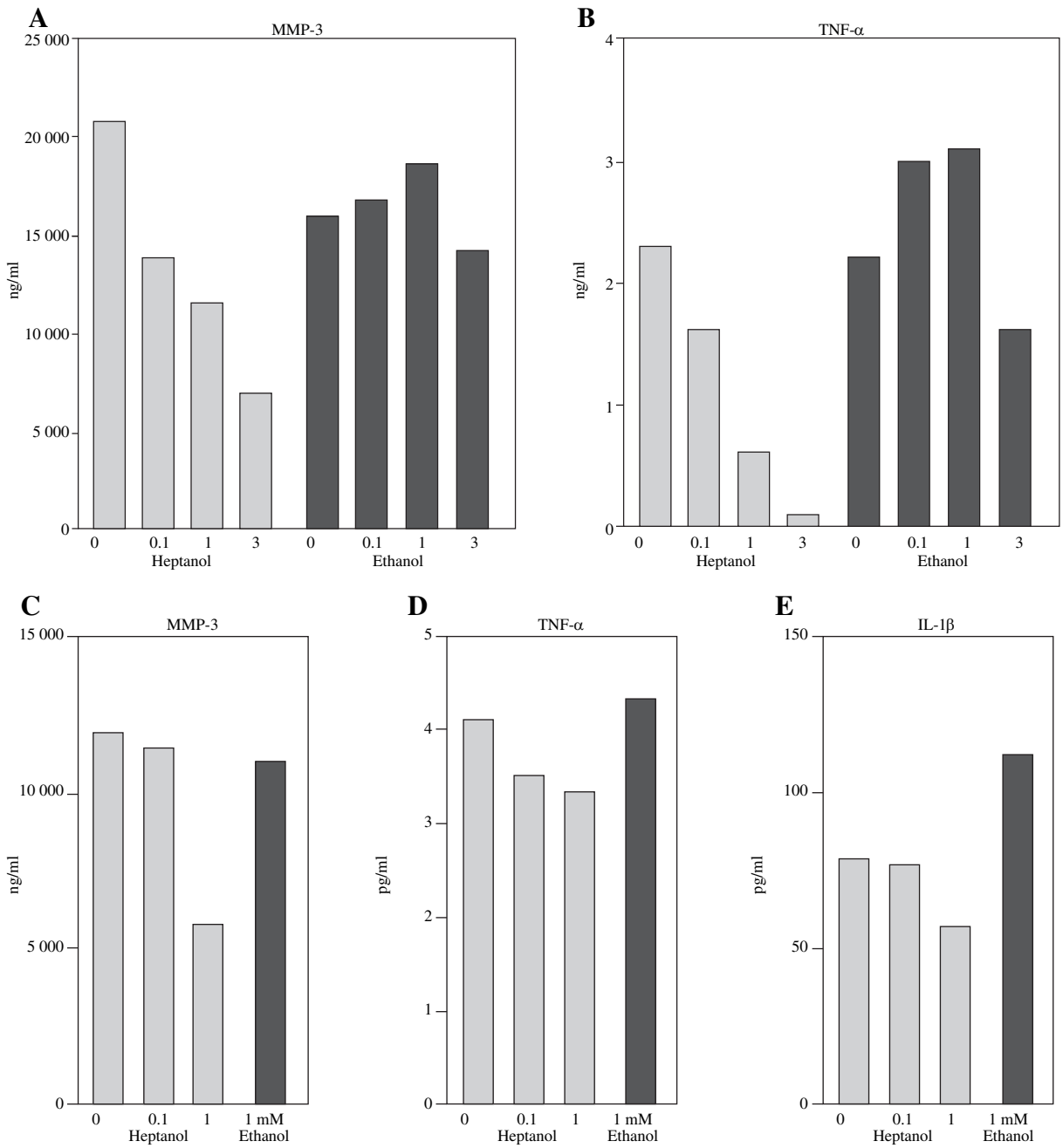

Fig. 3. The concentrations of TNF-α, MMP-3 and IL-1β in supernatants

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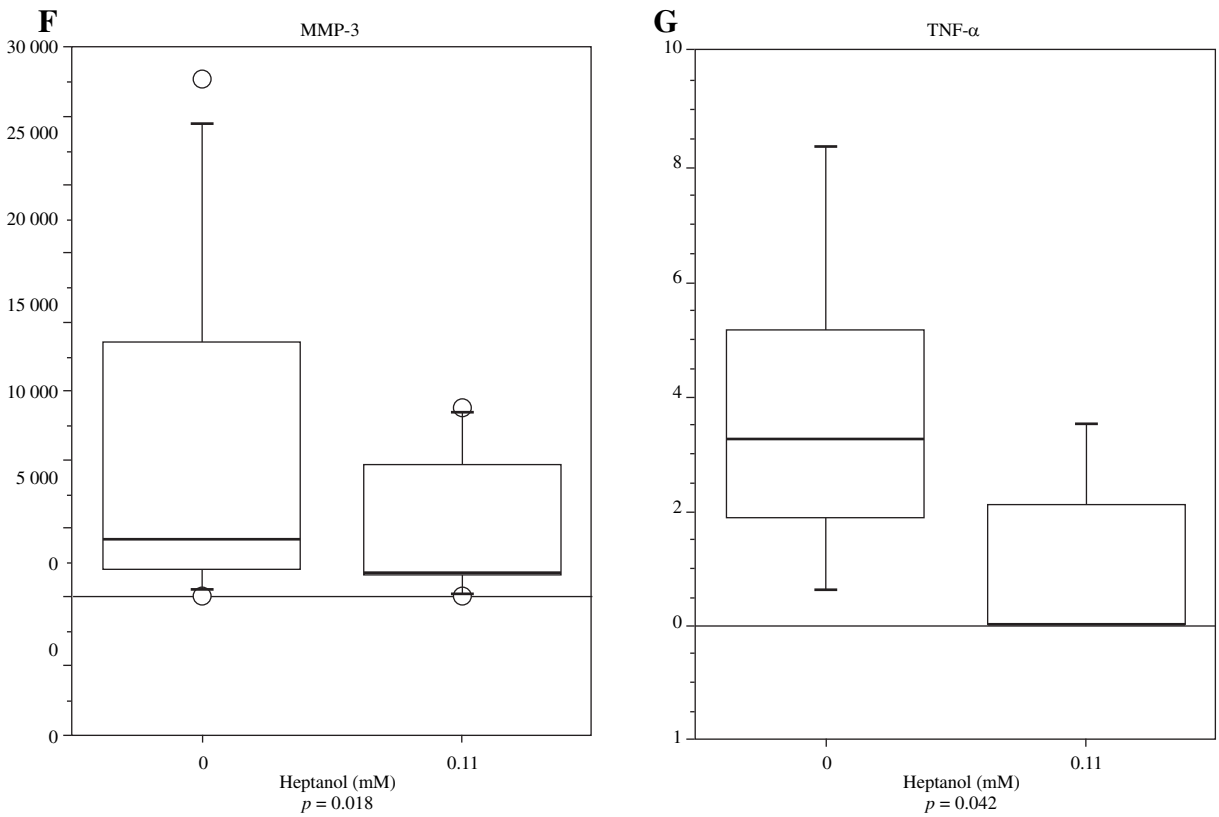

Fig. 3. The concentrations of TNF-α, MMP-3 and IL-1β in supernatants

Marino *et al*. reported that increased ICJP may contribute to the progression of osteoarthritis [15]. The synovial lining cells from patients with osteoarthritis (OA) produced MMP constitutively and in response to stimulation by IL-1β. ICJP is critical to the cell's ability to secrete MMP.

Osteoclasts play an important role in the bone destruction of RA. Ilvesaro *et al*. [5] reported that bone-resorbing osteoclasts contained gap-junctional connexin 43, and a heptanol, a gap-junctional inhibitor, dramatically inhibited bone resorption, which caused a decrease in the number and activity of osteoclasts. Heptanol also decreased the actin rings and in the total resorbed area. In addition, RANKL-induced osteoclast fusion is shown to be associated with the upregulation of Cx43 expression in mouse bone marrow cultures [9]. When a gap junction inhibitor is added to the bone marrow-derived cultures, the number of TRAP-positive multinucleated cells is reduced. Thus, the gap junctions are needed for the fusion of precursors to mature osteoclasts; gap-junctional Cx43 plays a functional role in osteoclasts.

Recently, it was reported that cross-presentation by intercellular peptide transfer through gap junctions is also important for the function of the immune system and the infection system. In 2005, Neijssen *et al.* [4] presented a mechanism of the antigen presentation system of two adjacent cells and is lost in most tumors: The mechanism is that gap-junction-mediated intercellular peptides are coupled for the presentation by bystander major histocompatibility complex (MHC) class I molecules and transferred to professional antigen presenting cells for cross-priming. They found that peptides up to ten amino acids long derived from viral or cellular proteins may be transferred from one cell to its neighbor through gap junctions. These peptides follow the classical MHC I pathway and are displayed on the surface for recognition by killer T cells.

In summary, increased ICGJ may contribute to the pathogenesis of synovitis and bone destruction in patients with RA, because a gap junction blocker inhibited the production of TNF-α and MMP-3. Thus, controlling the ICGJ may be a rational therapeutic target of synovitis and bone distraction of RA.

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