Increased CD40 Expression on Articular Chondrocytes from Patients with Rheumatoid Arthritis: Contribution to Production of Cytokines and Matrix Metalloproteinases

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ABSTRACT. ***Objective.*** To examine expression of CD40 on articular chondrocytes derived from patients with rheumatoid arthritis (RA) and osteoarthritis (OA) and to investigate roles of CD40 on articular chondrocytes in production of inflammatory mediators associated with degradation of articular cartilage.

Methods. Articular cartilage samples were obtained from patients with RA and OA at total knee arthroplasty. Expression of CD40 on chondrocytes was examined using immunohistochemistry, reverse transcriptase-polymerase chain reaction (RT-PCR), and flow cytometry. Tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), matrix metalloproteinase (MMP)-3, and tissue inhibitor of matrix metalloproteinase (TIMP)-1 in culture supernatants of chondrocytes were measured by ELISA.

Results. Immunohistochemical staining revealed that CD40 was expressed on RA chondrocytes *in vivo*, but not in OA chondrocytes. RT-PCR and flow cytometry showed that cultured articular chondrocytes from RA patients, but not from OA patients, constitutively expressed CD40, and that interferon-γ (IFN-γ) enhanced the expression of CD40 on chondrocytes from both RA and OA patients. Membrane fraction of mouse myeloma Ag8 cells that was transfected with human CD154 (Ag8-hCD154) induced production of TNF-α, IL-6, and MMP-3 in RA chondrocytes, and pretreatment of RA chondrocytes with IFN-γ enhanced the response. OA chondrocytes did not respond to stimulation with the membrane fraction of Ag8-hCD154 cells alone, but the cells did produce TNF-α, IL-6, and MMP-3 after pretreatment with IFN-γ.

Conclusion. CD40-CD154 interaction augments the expression of inflammatory cytokines and MMP in chondrocytes and contributes to an intrinsic process of cartilage degradation in RA. (J Rheumatol 2004;31:1506–12)

Key Indexing Terms:
ARTICULAR CHONDROCYTES CD40 CYTOKINES
MATRIX METALLOPROTEINASE RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by hyperplasia of synovium and an excess of inflammatory cells, leading to progressive destruction of affected joints. Various cytokines, such as interleukin 1 (IL-1), IL-6, IL-12, IL-17, IL-18, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), are involved in most aspects of articular inflammation and destruction[1-5]. Within articular joints, synovial cells such as macrophages and fibroblasts have the potential to produce cytokines. Among the cytokines, IL-1, IL-6, and TNF-α are considered the most pivotal ones in the pathogenesis of RA because biological agents against them have been applied successfully for the treatment of RA[6-8].

Total activities of cartilage-degrading enzymes were reportedly increased in RA as a result of stimulation with the proinflammatory cytokines[1-3,9,10]. Those cartilage-degrading capacities might depend upon the balance between matrix metalloproteinase (MMP) and tissue inhibitor of matrix metalloproteinase (TIMP), which are synthesized by both synovial cells and articular chondrocytes[11]. Cultured chondrocytes are able to produce proinflammatory cytokines by themselves, which in turn stimulates the production of MMP, resulting in the degradation of cartilage in RA[12].

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However, the mechanisms by which articular chondrocytes produce excessive cytokines in RA remain unclear.

CD40 is a cell-surface molecule originally identified on B cells and some epithelial carcinomas[13,14], and it is also expressed on nonlymphoid cells such as fibroblasts[15], epithelial cells[16], myocytes[17], endothelial cells[18], and synovial cells[19]. In these cells, CD40 ligation induces several cell-surface molecules and cytokines[18,20]. Regarding RA, we and other investigators demonstrated the involvement of CD40-CD154 interaction in immunopathogenesis of the disease[19,21,22]. Freshly isolated synovial cells from RA patients express CD40, while synovial T cells express CD154, and the ligation of CD40 on synovial cells enhances the expression of IL-6, IL-12, CD54, CD106, stromal cell derived factor, and vascular endothelial growth factor[23-26]. *In vivo*, treatment with monoclonal antibody against CD154 prevents the development of type II collagen induced arthritis in mice[27]. These data suggest the relevance of the CD40-CD154 interaction to the pathogenesis of RA. However, to our knowledge no study has investigated the relationship between articular chondrocytes and CD40.

To explore whether CD40-CD154 interaction on chondrocytes could contribute to cartilage degradation, we compared expression of CD40 on articular chondrocytes from patients with RA and osteoarthritis (OA). In addition, we investigated whether ligation of CD40 could induce TNF-α, IL-6, MMP-3, and TIMP-1 in cultured articular chondrocytes from patients with RA.

MATERIALS AND METHODS

Subjects. Cartilage specimens were obtained from 8 patients with RA and 6 patients with OA who had undergone total knee joint replacement. RA was diagnosed according to 1987 criteria of the American College of Rheumatology[28] (ACR). Knee OA was diagnosed from clinical and radiological evaluations, based on the ACR criteria[29].

Selection of cartilage. Each specimen of cartilage (femoral condyles and tibial plateaus) obtained from patients with RA and OA under aseptic conditions was carefully dissected from the underlying bone. These pieces of cartilage were used for the following experiments. Areas of fibrocartilage were identified and excluded.

Articular chondrocyte culture. Cartilage was minced and digested sequentially with 4 mg/ml actinase E (Kaken Pharmaceutical Co., Tokyo, Japan) in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) for 1 h at 37°C, followed by 0.25 mg/ml of collagenase-P (*Clostridium histolyticum*, Roche Diagnostics, Mannheim, Germany) in DMEM containing 10% fetal calf serum (FCS) (Sigma, St. Louis, MO, USA) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin; Gibco) for 12–16 h at 37°C. The cells were collected by passing them through nylon mesh and washed 3 times with DMEM-10% FCS. Freshly isolated chondrocytes were seeded into monolayer culture at a cell density of 2×10^5 cells/well in 24-well culture plates (Costar, Cambridge, MA, USA) and cultured in 1 ml DMEM-10% FCS and ascorbic acid (25 µg/ml) at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Immunohistochemistry. Specimens of articular cartilage were derived from 3 patients with RA and 3 patients with OA. Cartilage was immediately frozen in OCT compound using liquid nitrogen. Sections 4 µm thick were air-dried and fixed in cold acetone for 10 min at –20°C, and internal peroxidase activity was blocked by incubating in 30% H_2O_2-phosphate buffered saline (PBS) for 10 min at room temperature. Immunohistochemical staining was performed using a commercial kit (Vectastain Universal Quick Kit; Vector, Burlingame, CA, USA). Briefly, all sections were incubated with 2.5% normal horse serum for 30 min, followed by 10 µg/ml of the first antibody recognizing CD40 (Mab89; Coulter, Hialeah, FL, USA) for 30 min at room temperature. Mouse IgG1 (Dako, Glostrup, Denmark) was used as a control. After extensive washing with PBS, the sections were incubated 15 min with the horse biotin-conjugated antibody, which commonly recognizes mouse IgG. After extensive washing with PBS, all sections were exposed to a streptavidin/peroxidase-preformed complex for 10 min and then covered with DAB tetrahydrochloride for 2 min. All sections were counterstained with hematoxylin.

Reverse transcription-polymerase chain reaction (RT-PCR). Total cellular RNA was isolated from cultured articular chondrocytes with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed to cDNA in 20 µl of RT solution including SuperScript II (Invitrogen). For PCR, 2 µl of RT products were used in a total volume of 50 µl containing the following reagents: 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.2 mM dNTPs, 1 U of AmpliTaq polymerase (Roche Diagnostics, Basel, Switzerland), and a pair of primers (500 nM each). The thermocycle conditions were 35 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. The PCR products were electrophoresed in a 2% agarose gel and visualized with ethidium bromide. The sequences of the primers were: CD40 forward: 5'-ATGGTTCGTCTGCCTCTGCAG-3', CD40 reverse: 5'-CTGGGCAGGGCTCGCAGATGG-3', ß-actin forward: 5'-AAGAGAGGCATCCTCACCCT-3', ß-actin reverse: 5'-TACATGGCTGGGGTGTTGAA-3'.

Flow cytometry. Chondrocytes were detached from culture flasks by trypsinization. In all experiments, 1×10^6 cells were treated with 10 µg/ml goat IgG to block nonspecific binding. Cells were then reacted with 5 µg/ml anti-CD40 antibody (Mab89) or control mouse IgG1 for 30 min at 4°C, followed by incubation with fluorescein-conjugated $F(ab')_2$ goat anti-mouse IgG (Becton Dickinson, San Jose, CA, USA) for 30 min at 4°C. The cells were washed and fixed with 2% paraformaldehyde, and the fluorescein intensity was measured with Cytoron Absolute (Ortho-Clinical Diagnostics, Tokyo, Japan).

Cell line and preparation of membrane fraction. Mouse myeloma Ag8 cells were transfected with the expression plasmid for human CD154 (Ag8-hCD154) provided by Drs. J. Inoue and T. Ishida (University of Tokyo, Tokyo, Japan). Parental Ag8 cells and Ag8-hCD154 cells were maintained in RPMI-1640-10% FBS. Ag8-hCD154 cells that strongly expressed hCD154 were positively selected using anti-human CD154 Mab and the MACS system (Miltenyi Biotec, Auburn, CA, USA) every 4 weeks. Membrane fractions of these Ag8-hCD154 cells and the parental Ag8 cells were prepared according to the methods of Rezzonico, *et al*[30] and used in the following experiments.

Measurements of TNF-α, IL-6, MMP-3, and TIMP-1. Concentrations of TNF-α, IL-6, MMP-3, and TIMP-1 in the culture supernatants were measured using commercial ELISA kits (TNF-α and IL-6: BioSource, Camarillo, CA, USA; MMP-3 and TIMP-1: Fuji Chemical, Takaoka, Japan) according to the manufacturers' instructions. The sensitivity of these ELISA kits was 0.5 pg/ml for TNF-α, 7.8 pg/ml for IL-6, 12.5 ng/ml for MMP-3, and 51 ng/ml for TIMP-1.

Statistical analysis. All data are presented as mean ± SEM. Wilcoxon signed-ranks test was used for statistical analysis. Results were considered statistically significant at $p < 0.05$.

RESULTS

Expression of CD40 in frozen sections of articular cartilage. Positive immunohistochemical staining for CD40 was detected in articular cartilage derived from all 3 RA patients examined, but not in the 3 OA patients. CD40-positive

chondrocytes were detected in the superficial and deep zones of RA cartilage. Representative results were shown in Figure 1.

Expression of CD40 mRNA on cultured articular chondrocytes. RT-PCR using specific primers for CD40 was performed to detect mRNA expression in cultured chondrocytes. Messenger RNA of CD40 was constitutively expressed in RA chondrocytes, but not in OA chondrocytes. IFN-γ induced the expression of CD40 mRNA in both RA and OA chondrocytes (Figure 2).

Analysis of CD40 expression on articular chondrocytes using flow cytometry. To investigate cell-surface expression of CD40, chondrocytes were cultured with or without 200 U/ml of IFN-γ for 48 h, stained with anti-CD40 antibody or control mouse IgG1, and analyzed by flow cytometry (Figure 3). RA chondrocytes expressed CD40 without stimulation (3.4–6.1%, n = 3), and IFN-γ increased the percentage of CD40-positive cells (29.4–33.1%). Conversely, OA chondrocytes marginally expressed CD40 (0.3–2.2%, n = 3), and induction of CD40 by IFN-γ was relatively weak (1.9–4.5%).

Effect of CD40 ligation on production of TNF-α, IL-6, MMP-3, and TIMP-1 by chondrocytes. To analyze biological function of CD40 on chondrocytes, the culture supernatants were collected after chondrocytes (2×10^5 cells/ml) were cultured with or without IFN-γ (200 U/ml) for 48 h and then stimulated with the membrane fraction of Ag8-hCD154 or Ag8 for 48 h. We confirmed that the membrane fraction of Ag8-hCD154 induced TNF-α production of normal peripheral blood monocytes in the presence of IFN-γ, and this stimulatory activity was almost completely neutralized by anti-CD154 Mab (data not shown). Ligation of CD40 on RA chondrocytes significantly elevated TNF-α and IL-6 production, but not on OA chondrocytes (Figure 4). The ligation of CD40 did induce TNF-α and IL-6 production in OA chondrocytes when the cells were precultured with IFN-γ (Figure 4). Pretreatment of RA chondrocytes with IFN-γ enhanced the production of TNF-α and IL-6 by CD40 liga-

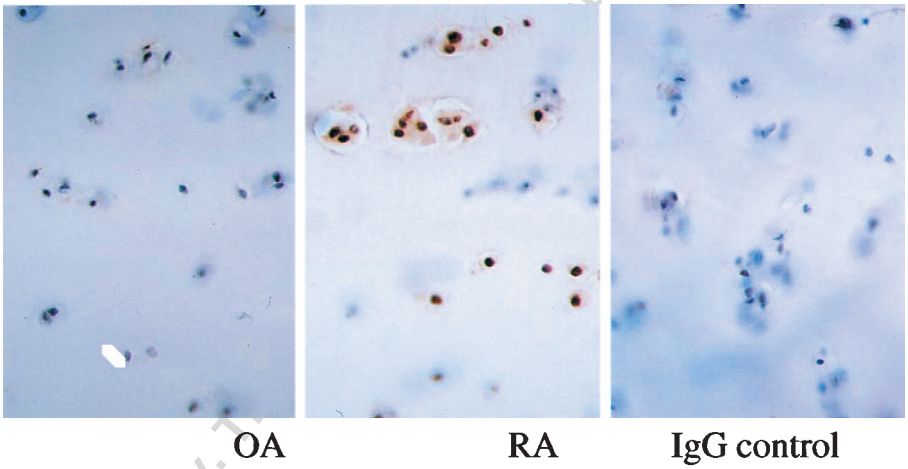

Figure 1. Immunohistological staining of CD40 of articular cartilage. Frozen sections of RA and OA articular cartilages were stained with anti-human CD40 antibody or mouse IgG as a control. Biotin-conjugated anti-mouse IgG was used as the second antibody, followed by horseradish peroxidase-labeled avidin/biotin complex. CD40-positive cells were visualized as a brown color by DAB tetrahydrochloride.

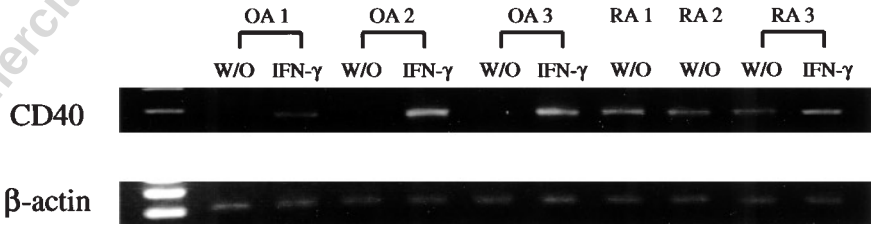

Figure 2. Expression of CD40 mRNA in cultured chondrocytes from articular cartilage. Articular chondrocytes were cultured with or without IFN-γ for 48 h and total RNA was extracted. CD40 mRNA was detected by RT-PCR. Messenger RNA of CD40 was constitutively expressed in RA chondrocytes, but not in OA chondrocytes. IFN-γ induced the expression of CD40 mRNA in both RA and OA chondrocytes. Amplification of ß-actin was performed using the same cDNA as an internal control.

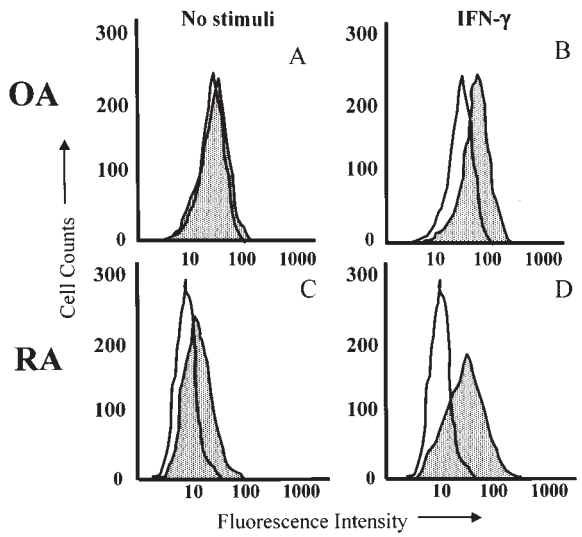

Figure 3. Analysis of CD40 expression on articular chondrocytes using flow cytometry. Articular chondrocytes from OA (A and B) and RA (C and D) were cultured in DMEM plus 10% FBS alone (A and C) or with IFN-γ (B and D) for 48 h. Cells were harvested and stained with anti-CD40 antibody (shaded area) or control mouse IgG (open area), followed by FITC-anti-mouse IgG. Positive cells were detected using flow cytometry.

tion (Figure 4). As shown in Table 1, MMP-3 levels in the culture supernatants were significantly elevated by CD40 ligation in RA chondrocytes, but not in OA chondrocytes. Although the CD40 ligation did not affect TIMP-1 levels in the same culture supernatants from both RA and OA chondrocytes, the MMP-3/TIMP-1 ratio in the culture supernatants was significantly elevated in RA, but not in OA.

DISCUSSION

We observed that CD40 is constitutively expressed on articular chondrocytes from patients with RA but not those from patients with OA. IFN-γ induced CD40 protein on the surface of OA chondrocytes and enhanced CD40 expression on RA chondrocytes. It is well documented that IFN-γ upregulates the expression of CD40 in various types of cells[21,22]. These observations are compatible with the fact that CD40 is inducible on the cell surface of cultured articular chondrocytes by IFN-γ, suggesting that articular chondrocytes derived from RA patients may be preactivated by some mediators *in vivo*.

Our study also showed that engagement of CD40 by CD154 activated articular chondrocytes derived from RA patients to produce TNF-α and IL-6. However, the molecular pathways of CD154-induced cytokine expression remain unknown. CD40 ligation is known to upregulate nuclear factor-κB (NF-κB) in leukocytes[31], and recently was reported to induce NF-κB, activator protein-1, and Egr-1 in endothelial cells[32]. Those transcription factors might be associated with the regulation of various cytokines (i.e., TNF-α, IL-6), although we did not confirm induction of transcription factors in cultured articular chondrocytes in this study. Because ligation of CD40 induced TNF-α and IL-6 production by articular chondrocytes as well as synovial cells, blocking the CD40-CD154 interaction may have therapeutic value for patients with RA. This possibility is supported by reports that anti-CD154 blocking antibody successfully prevented development of autoimmune diseases in animal models and acute rejection in allotransplantation models[27,33-35].

MMP are one of the key factors in cartilage degradation of RA[36,37]. In particular, MMP-3 is elevated in sera of RA patients and is correlated with the activity of arthritis[38,39]. In contrast, TIMP show protective effects against cartilage degradation by inhibiting the activity of MMP *in vivo*[40]. Our results revealed that ligation of CD40 on cultured articular chondrocytes derived from RA patients induced MMP-3 production and markedly elevated the ratio of MMP-3/TIMP-1. These observations suggest that the CD40-CD154 system can shift joint cartilage of patients with RA into destructive mode because of the matrix-degrading enzymes, in addition to inducing the production of inflammatory cytokines.

The clinical features of RA are characterized by joint destruction with inflammation and autoimmunity that is distinguished from the cartilage degradation in OA. Although many reports show that synovial cells play a central role in the joint destruction of RA through the production of various soluble mediators[1,3], little is known about the contribution of chondrocytes themselves to the development of inflammation and the degradation of articular cartilage. Our findings suggest the novel concept that articular chondrocytes may participate in inflammation and joint destruction through CD40-CD154 interaction in RA.

CD154 is primarily expressed on activated T cells[41], and it has been reported that a number of CD154+ T cells infiltrate the RA synovium. Ligation of CD40 on synovial cells in the primary culture enhances the production of TNF-α[19] and induces the proliferation of synovial fibroblasts[22], which may contribute to the development of synovitis in RA. These observations and results of our study suggest that CD40 on articular chondrocytes and synovial cells may be activated by CD154 expressed on infiltrating T cells in RA synovium.

In healthy articular cartilage, chondrocytes are surrounded by the hyaline matrix of cartilage, which prevents inflammatory cells such as lymphocytes, plasma cells, and macrophages from reaching chondrocytes. Thus, CD40 on chondrocytes does not appear to interact with CD154 on infiltrating T cells. However, Smeets, *et al* recently reported that T cells, B cells, macrophages, plasma cells, and mast cells were found at the chondrocyte-pannus junction (CPJ) in patients with early or middle-phase RA who did not require joint surgery, while T cells were rarely

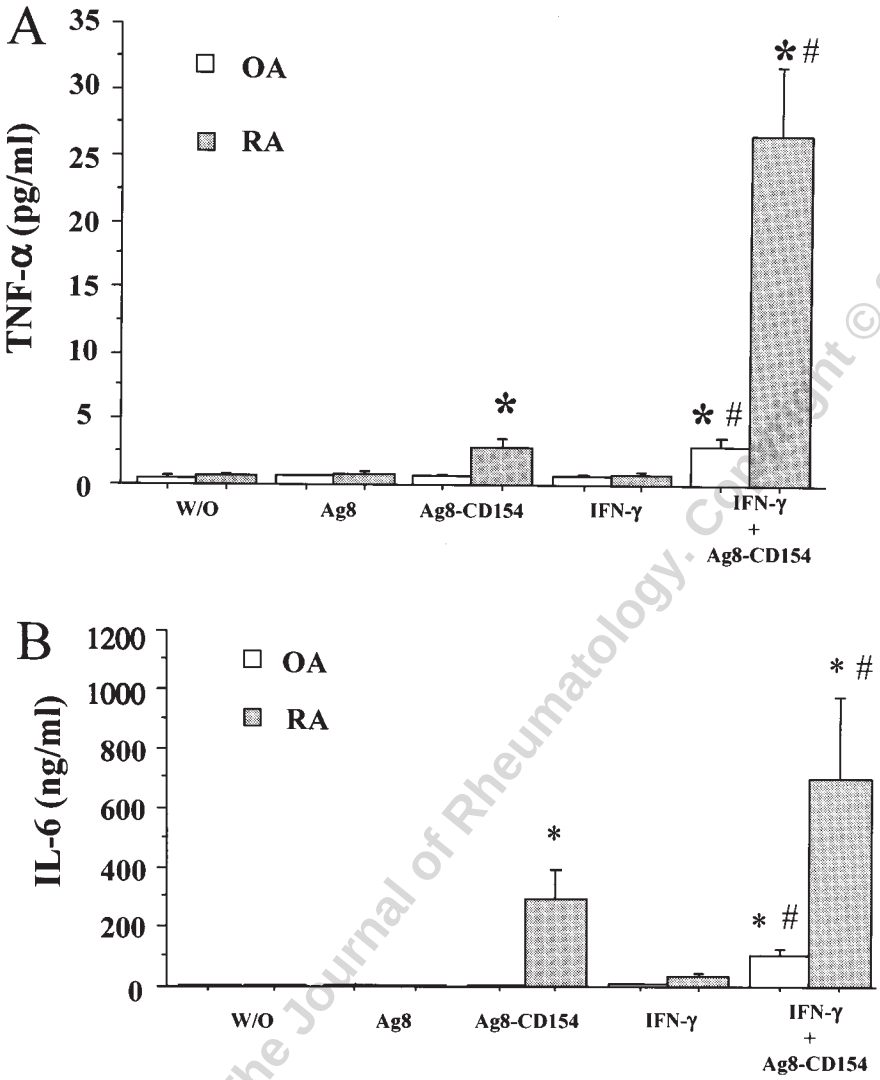

Figure 4. Effect of CD40 engagement on TNF-α and IL-6 production by cultured chondrocytes. RA and OA chondrocytes precultured with or without IFN-γ for 48 h were stimulated by Ag8-hCD154 for 48 h. Concentrations of TNF-α (A) and IL-6 (B) in the culture supernatants were determined by ELISA. Bars represent mean ± SEM for chondrocytes from 6 patients with OA (white bars) and 8 patients with RA (shaded bars). *$p < 0.05$ compared to without stimulation; $^{\#}p < 0.05$ compared to Ag8-hCD154 alone.

Table 1. Induction of MMP-3 and TIMP-1 by CD40-CD154 signaling on chondrocytes. Articular chondrocytes from rheumatoid arthritis (RA) and osteoarthritis (OA) were cultured in DMEM plus 10% FBS with Ag8-hCD154. Concentrations of MMP-3 and TIMP-1 in the supernatants were determined by ELISA.

	MMP-3, µg/ml		TIMP-1, ng/ml		MMP-3/TIMP-1	
	No Stimuli	CD154	No Stimuli	CD154	No Stimuli	CD154
OA, n = 6	3.8 ± 1.5	3.7 ± 1.6	335.6 ± 89.4	238.8 ± 63.2	15.3 ± 5.1	23.0 ± 7.8
RA, n = 8	5.1 ± 1.2	9.3 ± 1.8*	344.3 ± 70.8	287.9 ± 42.6	15.3 ± 3.2	37.2 ± 9.5*

* $p < 0.05$ compared to without stimuli.

found at the CPJ of samples obtained from joint surgery of patients with late-phase RA[42]. These findings suggest that CD40 on articular chondrocytes can interact with CD154 on synovial T cells at the CPJ in patients with early and middle-phase RA, and articular chondrocytes may produce excessive proinflammatory cytokines and MMP.

In addition, elevation of soluble CD154 (sCD154) was reported in sera of RA patients with vasculitis[43]. Soluble CD154 results from the shedding of CD154 on activated T cells and platelets[44,45] and elicits the agonistic property of the CD40 molecule. We detected sCD154 in synovial fluids of patients with active RA, but not in inactive RA or OA (data not shown). We speculate that an agonistic sCD154 may exist at the CPJ of RA joints and interact with CD40 on articular chondrocytes.

Our results support the concept that interaction between CD40 on articular chondrocytes and CD154 may play a significant role in the intrinsic potency for cartilage degradation. Blockage of CD40-CD154 interaction using humanized CD154 antibody may be an innovative strategy for the treatment of RA.

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