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The possible role of *c-fos* expression in rheumatoid joint destruction

Abstract At present, although the etiology of rheumatoid arthritis (RA) remains unknown, most investigators believe that it is primarily an inflammatory disease of the synovial membrane of the joints. However, we have recently focused on the pathology of the early changes in articular cartilage and subchondral bone in RA patients, and have shown that RA involves articular cartilage and subchondral bone, not synovia. This research direction may lead to the development of a new specific treatment for the disease.

Key words Autoimmunity · *c-fos* · Rheumatoid arthritis · TRAP-positive multinucleated cell · Type II collagen

Introduction

It is generally accepted that the initial events leading to articular damage in rheumatoid arthritis (RA) are a proliferation of synovial cells together with inflammation and vascular neoformation in the synovial tissue stroma.[1,2] As the disease progresses, the proliferating synovial tissue extends over the articular cartilage and erodes from the joint surface down to the subchondral bone.

Recently, we have focused on the following clinical facts. First, although the administration of nonsteroidal anti-inflammatory drugs is recognized as fundamental in the conservative treatment of RA, these agents cannot completely suppress synovitis in the affected joints. Even after surgical synovectomy, i.e., removal of the inflammatory synovial tissue, most cases develop varying degrees of recurrent synovitis with time, and the progression of joint deterioration and deformity cannot be prevented.[1–3] Second, synovitis progression tends to decrease gradually when articular cartilage and bone are severely destroyed in the advanced stages of the disease.[4] Third, active synovitis diminishes considerably after the excision of articular cartilage and subchondral bone during prosthetic joint replacement even if the hypertrophied and inflamed synovia remains in situ.[5,6] This body of evidence indicates that synovitis is a secondary event caused by a preceding lesion in the articular cartilage and/or subchondral bone, and coincides with our previous observation that a high incidence of anti-type II collagen IgG antibodies appear during the early phase of RA.[7–9] The major antigenic determinants recognized by RA sera reside in the region represented by cyanogen bromide (CNBr) peptide-11 and -8 (CB-11, CB-8) of the human type II collagen molecule.[8,9] Anti-type II collagen antibody is all negative in sera from patients with gout, osteoarthritis (OA), and nonarthritic diseases.[7–9]

Different mechanisms of joint destruction in RA and OA

In an attempt to understand the pathogenesis of RA, we performed histological and immunohistochemical studies on arthritic joints from RA patients. Articular cartilage and adjacent subchondral bone samples obtained during surgery (synovectomy or prosthetic joint replacement) were macroscopically normal, apart from the bare area, and were not invaded by synovial pannus. Human osteoarthritic and rheumatoid arthritic articular cartilages were examined for type II collagen degradation using antibodies against type II collagen CNBr-derived peptides. Although histologically the surface of RA cartilage appeared smooth and undamaged, immunohistochemical analysis showed less staining for type II collagen and intense staining for type II collagen CNBr-derived peptides in the deep zone matrix. In contrast, the deep zone of articular cartilage from OA patients stained more intensively with antibody against type II collagen. Wholly different mechanisms may be the bases of joint destruction in RA and OA. Type II collagen breakdown peptides in the deep zone of rheumatoid arthritic cartilage may become epitopes, as noted previously.[10]

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c-fos expression in rheumatoid cartilage destruction

c-fos is one of the representative nuclear oncogenes, and is an immediate early response gene. Oncogenes have been found to encode normal or mutated forms of growth factors, growth factor receptors, and proteins involved in signal transduction. They are induced and transiently transcribed without prior protein synthesis in quiescent cells that are stimulated by serum growth factors or exposed to transforming oncogenes.[11] Recent experiments have demonstrated that c-Fos, the product of *c-fos*, represents the transcription factor AP-1 (activating protein-1) together with c-Jun or its related proteins (JunB or JunD), and may act as an intracellular messenger that converts short-term signals generated by extracellular stimuli into long-term cell phenotype changes by regulating the expression of downstream genes possessing the AP-1-binding site.[12] AP-1 binding sequences have been found in promoters of many genes, including human collagenase, MMP-3, interleukin-1, interleukin-6, and tumor necrosis factor α, which are essentially important in the pathogenesis of rheumatoid arthritis.[12–18]

Using immunohistochemical techniques, Trabandt et al.[11] found a positive correlation between the immunoreactivities for c-Fos and fibroblast-type collagenase in the fibroblast-like synovial cells derived from a RA joint. Moreover, Kuroki et al.[19] reported that *c-fos* supports the active growth of human synovial cells by facilitating synovial dendritic cell transformation into fibroblastic cells. It is known that these fibroblastic cells are a major source of cytokines, including interleukin-1, interleukin-6, and TNFα.[16,17] However, not only synovial cells but also articular chondrocytes themselves have been implicated in the progressive destruction of the articular cartilage matrix by producing a number of matrix-degrading enzymes and cytokines.

c-fos expression and the cartilage-matrix metabolism

Proteoglycan and type II collagen, the major components of cartilage matrix, may serve as important markers of the chondrocyte metabolism. To obtain more precise evidence for the role of *c-fos* in rheumatoid cartilage destruction, we examined changes in collagen and proteoglycan metabolisms by transfecting cultured human chondrocytes with human *c-fos*.

[^{35}S] sulfate and [^{3}H] proline incorporation measurements were used to monitor proteoglycan and collagen synthesis, respectively, and proteoglycan relative hydrodynamic size was analyzed by gel chromatography on Sepharose CL-2B. *c-fos* overexpression in human chondrocytes resulted in a decreased synthesis of both proteoglycan and collagen. Chondrocytes usually produce two proteoglycan populations with different sizes: a large proteoglycan, aggrecan, characteristic of the cartilage matrix, and small proteoglycans found in both nonchondrogenic and chondrogenic cells. The reduction of proteoglycan synthesis by *c-fos* expression was greater in the large proteoglycan fraction. Moreover, changes in

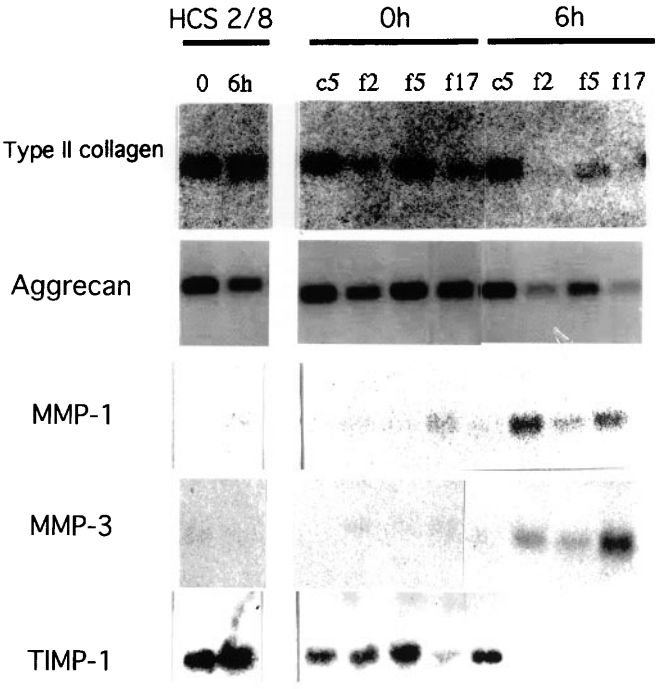

Fig. 1. Northern blot analysis of aggrecan, type II collagen, MMP-1, and TIMP-1 mRNAs in *c-fos* overexpressed human chondrocytes. Introduction of exogenous *c-fos* together with human metallothionein promoter (human MT2a) into human chondrocytes decreased the endogenous transcription of aggrecan, type II collagen, and TIMP-1, and increased that of MMP-1 after 6 h cadmium induction. HCS 2/8, untransfected HCS 2/8 cells, c5, pSV2*neo*-MT transfectant; (f2, 5, and 17), pSV2*neo*-MT-*hc-fos* transfectants

mRNA expression analyzed by Northern blot hybridization showed that exogenous *c-fos* introduction into human chondrocytes decreased the endogenous transcription of aggrecan, type II collagen, and TIMP-1, and increased transcription of MMP-1 and MMP-3 (Fig. 1).[20,21]

Control of the above-mentioned turnover of the matrix components can be achieved by the establishment of an intricate balance between synthesis and degradation of the type II collagen-associated molecules such as MMPs and TIMPs. It is therefore reasonable to hypothesize that the coordinated expression of both MMPs and TIMPs is necessary for cartilage homeostasis, and that loss of this control mechanism leads to an imbalance between degradative proteinases over protective TIMPs. Previous findings demonstrated that MMP-1 and MMP-3 can be regulated at multiple points of transcription. Although the maximal induction of MMP-1 and MMP-3 requires functional cooperation between AP-1 and a neighboring regulatory sequence in the vicinity of their promoters,[14,22,23] the AP-1 binding site in their promoter sequences plays a prominent role. Regarding the regulation of TIMPs, factors affecting their expression have not yet been clarified.[24–26] On the basis of these observations, we attempted to examine the mechanisms of AP-1 binding and their influence on the regulation of MMP-1 and TIMP-1 expressions in the chondrocytes. It is known that c-Fos, the product of *c-fos*, together with c-Jun protein or its related proteins (JunB or JunD) represent

the transcription factor AP-1 (activating protein-1), whose binding sequences are found in promoters of many genes, including human MMP-1, MMP-3, and TIMP-1. CAT promoter-containing plasmids, MMP-1 promoter-CAT, or TIMP-1 promoter-CAT were transfected into human articular chondrocytes with and without expression vectors for c-Jun, JunB, JunD, c-Fos, Fra-1, or Fra-2. Then, to monitor the activities of MMP-1 and TIMP-1 promoters, CAT activity was analyzed. The MMP-1 promoter was strongly activated by Jun-related proteins as well as the Fos/Jun-related protein heterocomplex. On the other hand, c-Fos combined with any of the Jun-related proteins failed to stimulate the TIMP-1 promoter, although the latter was activated by the Fra-1 or Fra-2/Jun-related protein heterocomplex. Furthermore, c-Fos inhibited activation of the TIMP-1 promoter by JunD. Therefore, a potential explanation that MMP-1 (and most probably MMP-3) is induced by AP-1 could be plausible, although some controversy might exist concerning the AP-1 stimulating effect on TIMP-1.[21]

In vivo expression of *hc-fos* mRNA, histological localization of type II collagen, and identification of cells producing MMP-1 and TIMP-1 in rheumatoid arthritic cartilage

Recently, *c-fos* and the transcription factor NF-κB, described as regulators of the immunoglobulin κ light-chain, have been found to play important roles in the activation of numerous genes involved in the immune and inflammatory response.[11,14,15,27] Although previous observations point to the presence of *c-fos* and NF-κB in RA synovitis,[11,19,28–34] changes in expression of the mRNAs of these molecules in the cartilage have not yet been determined. In order to substantiate the in vitro findings in situ, paraffin sections of articular cartilage from normal, RA, and OA patients were subjected to in situ hybridization for the *c-fos* gene. Since the specific biological significance of *c-fos* has not yet been evaluated in vivo, a combination of in situ hybridization for *c-fos* and immunohistochemical analysis for MMP-1 and TIMP-1 was used to elucidate the regulatory role of *c-fos* in the inflammation process.

We found that in the cartilage matrix of the RA patients, staining for type II collagen was lost and this was accompanied by an increase in MMP-1 staining intensity. This finding was more significant in severely damaged RA cartilage. Less severely and mildly damaged RA cartilage appeared to retain the normal matrix structure, although changes in chondrocytes together with type II collagen degradation and an increase in MMP-1 staining intensity had already started from the middle and deep layers of the cartilage. Interestingly, the expression level of *c-fos* mRNA was significant in the chondrocytes of RA patients, and was detected mainly in the chondrocytes in the middle and deep layers of the articular cartilage of early-stage RA (Fig. 2). On the other hand, although a decrease in the type II collagen and an increase in the MMP-1 staining intensities could be observed in specimens from highly inflammed OA cartilage, the expression of *c-fos* could be detected in only a few chondrocytes, and exclusively in the superficial layer of the OA cartilage.[21]

The factors affecting TIMP-1 expression in cartilage seem to operate in a more intricate way. Although TIMP-1 could be detected in cartilage from patients with either RA or OA, MMP-1 expression in RA exceeded TIMP-1 expression; in OA cartilage the opposite was found to be true.[21] Our results were similar to those reported by McCachren,[35] who studied synovial changes in RA: interstitial collagenase and stromelysin were expressed by synovial lining cells in patients with active RA, but TIMP-1 expression appeared

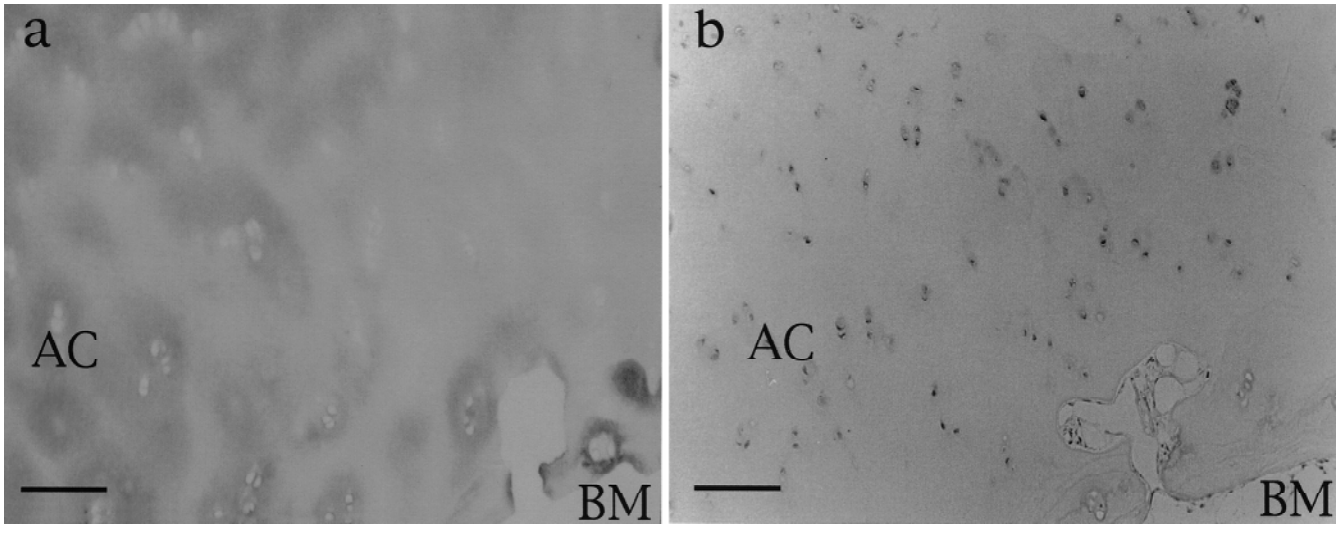

Fig. 2. In situ localization of *hc-fos* mRNA in less damaged articular cartilage from a RA patient. Although staining for type II collagen remained mainly in the interterritorial matrix of the superficial, middle and deep layers, and in the territorial matrix of the deep layer of the cartilage (**a**), markedly increased expression of *hc-fos* mRNA could already be found in the chondrocytes of the middle and deep layers (**b**). *BM*, bone marrow; *AC*, articular cartilage. *Bar* 50 μm

to be less intense than the expression of proteases. When considering these findings, it should be kept in mind that during arthritic cartilage destruction many MMP and TIMP protein products are involved in a complicated network of interactions. Yet among these MMPs and TIMPs, MMP-1 and TIMP-1 have been known to play crucial roles and could be regarded as the two main representatives of their enzyme families. In regard to this, the above results may indicate significant differences between RA and OA in the MMP-1 and TIMP-1 production during articular cartilage degradation, and that such differences may be correlated with the different *c-fos* expression patterns observed in RA and OA.

Pathological changes in cartilage and subchondral bone marrow in patients with rheumatoid arthritis

Another striking result came from histological and immunohistological studies of articular cartilage and subchondral bone samples obtained from patients with RA. This was the considerable formation of islands that invaded into the deep zone of articular cartilage through the calcified cartilage from below, where the subchondral bone was located (Fig. 3). These islands did not contact the articular margins and contained significant amount of TRAP-positive multinucleated cells,[10] and most of these cells expressed *c-fos*. Inflammation was confirmed in the underlying subchondral bone. CD68-positive mononuclear cells, MT-1 positive cells, and HLA-DR-positive cells were also detected. We think that the presence of T cells (MT-1-positive cells) may be crucial for anti-type II collagen IgG antibody production in this region. These pathological changes in the subchondral area may not only produce subchondral cysts, but may also trigger destruction of the RA joint.[10] However, the precise mechanisms of TRAP-positive cell generation and activation in RA have not been fully elucidated. A histological study of early and advanced arthritic lesions was carried out on decalcified joints from the hind limbs of collagen-induced arthritis (CIA) rats. In CIA rodents,[35] antibody against autologous type II collagen is a critical factor involved in the pathogenesis of CIA,[36,37] and L3T4$_+$ T cells are required for immunity against type II collagen.[38] Interestingly, we observed identical pathological changes in human subchondral bone where RA preceded considerable inflammation in the synovial membrane. In the early phase of CIA, TRAP-positive multinucleated cells could be already detected in the subchondral bare area and the cartilage erosive front. Although expression of *c-fos* was found only in a few lining synovial cells near the bare area, its level was already high in the chondrocytes of the deep layer and in TRAP-positive multinucleated cells in the subchondral bare area (M. Tsuji 2001, unpublished data). These TRAP-positive multinucleated cells also expressed a receptor for calcitonin, a reliable marker of the osteoclast phenotype. However, since not all of these cells expressed RANK (receptor activator of nuclear factor kB), we could not definitively identify them all as osteoclasts (M. Tsuji 2001, unpublished data).

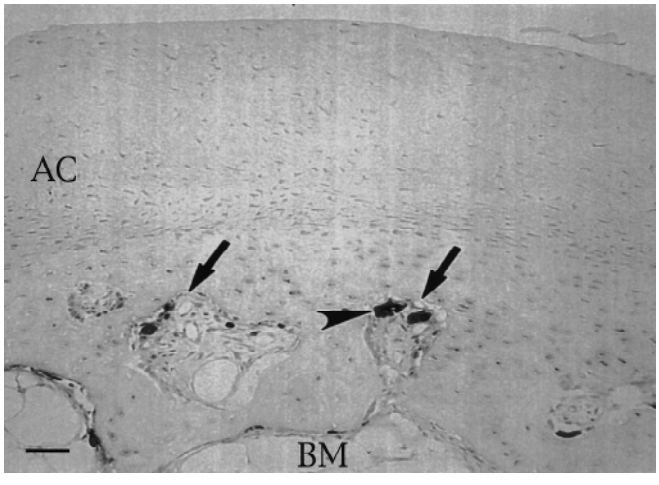

Fig. 3. TRAP staining of sample tissues from RA patients. There is considerable formation of islands (*arrows*) that invade the deep zone of the articular cartilage (*AC*) through the calcified cartilage from below, where the subchondral bone (*SB*) is located. These islands contain a significant amount of TRAP-positive multinucleated cells (arrowhead). *Bar* 100 μm

Osteoclast formation is a contact-dependent process controlled by osteoblastic cells.[39] Osteoblasts provide two essential signals that program osteoclast progenitors to differentiate into osteoclasts: one is mediated by the macrophage colony-stimulating factor (M-CSF), and the other by a novel ligand member of the tumor necrosis factor (TNF), termed RANKL (receptor activator of nuclear factor kB ligand). M-CSF and RANKL together are essential for osteoclast production and promote the multinucleation of pre-fusion osteoclasts and the survival of nascent osteoclasts.[39,40] Disruption of *c-fos* gives rise to severe osteopetrotic bone disorders caused by defects in osteoclast progenitors.[41,42] Osteoclasts are derived from cells of the monocyte/macrophage lineage, and *c-fos* causes a lineage shift between the osteoclast and macrophage cells.[43] Osteoclast progenitors can enter and leave the circulation, but the mechanism of this translocation is not fully understood. A more detailed study is needed to clarify the precise mechanisms of TRAP-positive multinucleated cell generation and activation, since these cells, positive for both TRAP and *c-fos*, may act as potential effector cells in RA joint destruction.

In summary, we think that by continuously concentrating on the study of synovitis, the actual key event in the etiology of RA may be overlooked. Our findings indicate that not only synovial cells but also chondrocytes and TRAP-positive multinucleated cells are potential effector cells in rheumatic joint destruction. We propose the possibility that initiation of the rheumatoid process is triggered by the interaction between articular cartilage and subchondral bone marrow cells. It also seems possible that synovial inflammation may play a pivotal role in amplifying the earlier changes that appear in the subchondral area. Since the expression level of the *c-fos* gene in RA patients is increased in the chondrocytes and TRAP-positive multinucleated cells in the subchondral area, *c-fos* may be the prime poten-

tial candidate for the main pathologic factor in the pathogenesis of RA joint destruction. Thus, further studies examining modulation of the *c-fos* expression should provide an effective approach to preventing RA joint destruction.

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