Jun N-Terminal Kinase in Rheumatoid Arthritis

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ABSTRACT

Potential mechanisms of joint destruction in rheumatoid arthritis (RA) were examined by studying the regulation of mitogen-activated protein kinases and collagenase gene expression in fibroblast-like synoviocytes (FLS). The three main mitogen-activated protein kinase families (p38, Jun N-terminal kinase (JNK), and extracellular signal-regulated kinases (ERKs)) were constitutively expressed in RA and osteoarthritis (OA) FLS. p38 and ERK1/2 were readily phosphorylated in both RA and OA FLS after interleukin-1 (IL-1) stimulation. JNK was phosphorylated in RA FLS but not OA FLS after IL-1 stimulation. Reverse transcription-polymerase chain reaction studies suggested that JNK2 is the major isof orm of the JNK family expressed by FLS. Northern blot analysis of collagenase gene expression demonstrated that RA FLS contained significantly more collagenase mRNA than OA FLS after IL-1 stimulation. The roles of JNK and p38 kinase were evaluated with the p38/JNK inhibitor SB 203580. Low concentrations of SB 203580 (1 μM, a concentration that only inhibits p38) had no significant effect on IL-1-induced collagenase expression in RA FLS whereas 25 μM (which inhibits p38, JNK2, and c-raf) blocked collagenase mRNA accumulation. IL-1-stimulated AP-1 binding was also inhibited by 25 μM SB 203580 in RA FLS. These studies suggest that OA and RA FLS have a different pattern of JNK phosphorylation, which might lead to enhanced collagenase gene expression in RA.

Rheumatoid arthritis (RA) is a chronic inflammatory arthritis marked by synovial hyperplasia with local invasion of bone and cartilage. Accumulating evidence suggests that RA fibroblast-like synoviocytes (FLS), which form the leading destructive front of rheumatoid synovium, possess unique characteristics. For instance, the rheumatoid cells proliferate in an anchorage-independent manner, lack contact inhibition, and constitutively produce increased amounts of growth factors (Kumkumian et al., 1989; Bucala et al., 1991). Also, RA FLS that have been implanted into SCID mice autonomously invade cartilage explants, whereas osteoarthritis (OA) FLS do not (Muller-Ladner et al., 1996). These studies suggest that RA synoviocytes are permanently imprinted or altered by the rheumatoid environment.

To explore the potential mechanisms of joint destruction, we evaluated the signal transduction and transcription factor pathways involved in collagenase gene expression by cultured FLS. Induction and activation of the transcription factor activator protein-1 (AP-1) were of particular interest (Karin et al., 1997; Firestein and Manning, 1999). AP-1 can be activated by protein kinases that phosphorylate specific amino acid residues, especially members of the mitogen-activated protein kinase (MAPK) family. Three major MAPK families have been identified: Jun N-terminal kinases (JNKs), extracellular signal-regulated kinases (ERKs), and the p38 kinases (Seger and Krebs, 1995). JNK is particularly important due to its ability to phosphorylate c-Jun, a key AP-1 component (Minden and Karin, 1997). In this study, the role of MAPK phosphorylation with subsequent AP-1 activation and collagenase gene expression was evaluated in interleukin-1 (IL-1)-stimulated FLS. Our data suggest that IL-1-induced JNK phosphorylation is increased in RA and that this pathway regulates collagenase gene expression. Hence, JNK phosphorylation in RA is a potential mechanism for excessive extracellular matrix destruction.

Materials and Methods

Patient Selection and Cell Preparation. FLS were isolated from RA and OA synovial tissues obtained at joint replacement surgery as described previously (Alvaro-Gracia et al., 1990). The diagnosis of RA conformed to the 1987 revised American College of Rheumatology criteria (Arnett et al., 1988). Briefly, the tissues were minced and incubated with 1 mg/ml collagenase in serum-free Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY).

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ABBREVIATIONS: RA, rheumatoid arthritis; FLS, fibroblast-like synoviocytes; MAPK, mitogen-activated protein kinase; OA, osteoarthritis; IL-1, interleukin-1; AP-1, activator protein-1; JNK, Jun N-terminal kinase; ERK, extracellular signal-regulated kinases; DMEM, Dulbecco’s modified Eagle’s medium; PMA, phorbol myristate acetate; TNF, tumor necrosis factor; RT, reverse transcriptase; PCR, polymerase chain reaction; SSPE, saline-sodium phosphate-EDTA; EMSA, electrophoretic mobility shift assay; MMP, matrix metalloproteinase.
for 2 h at 37°C, filtered through a nylon mesh, extensively washed, and cultured in DMEM supplemented with 10% fetal calf serum (Gibco, endotoxin content <0.006 ng/ml), penicillin, streptomycin, and L-glutamine in a humidified 5% CO₂ atmosphere. After overnight culture, nonadherent cells were removed, and adherent cells were cultivated in DMEM plus 10% fetal calf serum. At confluence, cells were trypsinized, split at a 1:3 ratio, and recultured in medium. Synoviocytes were used from passages 3 through 9 in these experiments, during which time they were a homogeneous population of FLS (<1% CD11b, <1% phagocytic, and <1% Fc-gamma RII receptor-positive).

**Western Blot Analysis.** Cells (5 × 10⁶) were incubated with medium, IL-1 (2 ng/ml), phorbol myristate acetate (PMA, 20 ng/ml; Sigma, St. Louis, MO) or tumor necrosis factor (TNF)-α medium, IL-1 (2 ng/ml), phorbol myristate acetate (PMA, 20 ng/ml; Boehringer Mannheim, Indianapolis, IN) for 15 min. Protein samples (25 μg/lane) from FLS were run on a 10% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane at 140 mA in 25 mM Tris-HCl pH 8.3, 192 mM glycine, 50% methanol. Western blot analysis was performed using a stress-activated protein kinase/JNK assay kit, a PhosphoPlus MAPK antibody kit, and a PhosphoPlus p38 MAPK (Thr180/Tyr182) antibody kit (New England BioLabs, Inc., Beverly, MA) according to the manufacturer’s instructions. Briefly, filters were blocked with Tris-buffered saline plus 0.1% Tween 20 and 5% dry milk for 1 to 3 h. This was followed by incubation with the appropriate antibody at 4°C overnight. The membrane was washed 3 times and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The proteins were visualized by chemiluminescence using hydrogen peroxide and luminol as a substrate using Kodak X-AR film.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Detection of JNK Isoforms.** Total RNA (5 μg) was used for RT reactions with random hexamer primers (GeneAmp RNA PCR Kit; Perkin Elmer, NJ) after PCR according to the Perkin-Elmer protocol. PCR products were visualized on 10% high-melting agarose gel electrophoresis and transferred onto a nitrocellulose membrane at 80°C. Membranes were then probed with medium, IL-1 (2 ng/ml), or IL-1 plus SB 203580 (25–50 μM) for 1 h. The cells were then lysed with 1 ml buffer A (10 mM HEPES pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol, 1 mM PMSF, and 0.1% NP-40), incubated on ice for 15 min, and centrifuged at 8500g at 4°C. The supernatants were discarded and the pellets resuspended in 4 ml of buffer A without NP-40. The samples were centrifuged again and the supernatant was discarded. Buffer C (100 μl; 25% glycerol, 20 mM HEPES pH 7.9, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA pH 8.0, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) was added to the samples and the samples were rocked at 4°C for 30 min. Particulate matter was pelleted for 30 min at 4°C in a microfuge and the supernatants were aliquoted and stored at −80°C.

**Electrophoretic Mobility Shift Assay (EMSA).** The Bandshift kit (Promega, Madison, WI) was used according to the manufacturer’s instructions. Consensus and control oligonucleotides (Santa Cruz Biotechnology) were labeled by polynucleotide kinase incorporation of [γ-³²P]ATP. Oligonucleotides sequences included the AP-1 consensus (5’ to 3’) (CGCTTGAAGCTTGGCCGGAA) or mutant binding sequence (CGCTTGAAGCTTGGCAGGA) and the NF-κB consensus (AGTTGAGGGACTTTCCAGGC) or mutant sequence (AGTTGAGGGACATTTCCAGGC). After the oligonucleotides were radioabeled, the nuclear extracts (4 μg of protein in 2 μl of nuclear extract) were mixed with 20 pmol of the appropriate ³²P-labeled consensus or mutant oligonucleotide in a total volume of 20 μl for 30 min at room temperature. The samples were then resolved on a 4% polyacrylamide gel. The gel was transferred to Whatman paper, dried, and visualized by autoradiography. Controls were performed in each case with mutant oligonucleotides or cold oligonucleotides to compete with labeled sequences.

**Statistical Analysis.** Statistics were performed by paired or unpaired Student’s t test unless otherwise stated. A comparison was considered statistically significant if p < .05.

**Results**

**Time Course of JNK, ERK, and p38 Phosphorylation in RA FLS.** Preliminary studies were performed to evaluate MAPK regulation in IL-1-activated FLS. Cells were stimulated with 2 ng/ml of IL-1 from 5 min to 6 h, and total and phospho-specific kinase levels were evaluated by Western blot analysis.

**Fig. 1.** Time course for MAPK phosphorylation in RA FLS. Cells were exposed to IL-1 (2 ng/ml) for various amounts of time (15–720 min) and assayed for total and phospho-JNK, -ERK, and -p38 as described in Materials and Methods. Maximal phosphorylation occurred at 15 to 30 min.
analysis. Figure 1 shows that 15 min was the optimal time point for evaluating MAPK phosphorylation under these experimental conditions. A dose response experiment with IL-1 (0.1–5 ng/ml) at that time point demonstrated maximal phosphorylation at 0.5 to 1 ng/ml (data not shown). Unless specified, cells were stimulated with 2 ng/ml of IL-1 for 15 min in subsequent studies.

**Phosphorylation Patterns of JNK, p38, and ERK in FLS.** RA and OA FLS were subsequently incubated with 2 ng/ml of IL-1 for 15 min and MAPK phosphorylation was determined by Western blot analysis. Figure 2 shows that each of the MAPKs was expressed and that this short incubation had no significant effect on the total amount of immunoreactive ERK, JNK, or p38 protein. The total amount of ERK, JNK, and p38 protein in RA FLS was not significantly greater than in OA FLS (n = 3 OA and RA FLS for ERK and p38; n = 7 for RA and n = 6 OA for JNK, p > .10; see also Fig. 3 for additional JNK examples). Using phospho-MAPK-specific antibodies, phosphorylated ERK1/2 and p38 were detected after IL-1 stimulation in RA and OA FLS (n = 3 OA and RA FLS for ERK and p38; n = 7 for RA and n = 8 OA for JNK). However, JNK phosphorylation was detected in RA (7/7 different cell lines) but not OA FLS (1/8 cell lines; p < .01 compared with RA by Fisher's exact test). In subsequent time course studies, JNK phosphorylation in OA FLS was not detected up to 60 min after IL-1 stimulation (data not shown).

To determine the specificity of these findings to IL-1, similar studies were performed using TNF-α (100 ng/ml) or PMA (20 ng/ml) as the activation signal. As with IL-1, PMA rapidly induced JNK phosphorylation in RA FLS (n = 3 each). One OA cell line contained small amounts of immunoreactive phospho-JNK after PMA stimulation (see Fig. 3A). Incubation of two additional OA FLS lines and one RA FLS line with anisomycin (50–100 μM)-induced JNK phosphorylation, confirming that the protein had the potential for activation under some conditions in OA (see Fig. 3B). TNF-α induced a small amount of phospho-JNK (the 46-kD isoform) in RA FLS but not in OA cells.

**Relative Expression of JNK Isoforms in RA FLS.** The expression of the three known JNK isoforms (JNK1, JNK2, and JNK3) was measured to determine whether differential MAPK gene expression accounted for the differences between OA and RA FLS. As noted above, the relative levels of JNK protein were similar in RA and OA. This was confirmed at the mRNA level and JNK2 was also identified the major isoform expressed (see Fig. 4). Little or no JNK1 or JNK3 mRNA was detected under these conditions.

**JNK Phosphorylation in RA and OA Synovium.** Our studies suggested that JNK is activated by IL-1 in RA FLS and that it might be a major pathway for AP-1 and collagenase gene regulation. To determine whether this process occurs in vivo, JNK phosphorylation was determined in extracts of intact RA and OA synovium. Figure 5 shows that phospho-JNK was detected in the synovium of all RA synovium tested (n = 3) but not in the OA tissue (n = 3).
Effect of SB 203580 on Collagenase Gene Expression.

Increased JNK activation in RA FLS suggested that expression of genes regulated by c-jun and AP-1 activation might be increased in RA. Therefore, Northern blot analysis was performed to compare collagenase gene expression in OA and RA FLS. Collagenase mRNA accumulation after IL-1 stimulation was significantly higher in RA FLS compared with OA FLS (G3PDH-normalized absorbance = 0.967 ± 0.163 for RA (n = 3) and 0.456 ± 0.110 for OA (n = 4); p < .05). RA FLS were then incubated in the presence of increasing concentrations of SB 203580. This compound, which is a potent inhibitor of p38 (IC50 = 70 nM; Cuenda et al., 1995) also inhibits β1 and β2 isoforms of JNK2 (Whitmarsh et al., 1997) as well as c-Raf (de Laszlo et al., 1998) at higher concentrations (10–25 μM). When SB 203580 was added at a concentration that completely inhibits p38 (1 μM), there was no significant effect on IL-1-induced collagenase gene expression in RA FLS (see Fig. 6) (p > .10; n = 3 RA cell lines). However, higher concentrations of SB 203580 that also inhibit JNK2 and c-raf suppressed IL-1-induced collagenase mRNA accumulation in RA FLS (p < .05; n = 3). The MEK1/2 inhibitor PD 98059 (100 μM; Dudley et al., 1995), which blocks ERK activation, also modestly decreased IL-1-induced collagenase gene expression in FLS (absorbance for IL-1 = 0.499 ± 0.135; absorbance for IL-1+PD 98059 = 0.283 ± 0.49; n = 3 RA and 3 OA).

Effects of SB 203580 on IL-1-Induced AP-1 Activation. Because of the effects of SB 203580 on collagenase gene expression in FLS, its ability to regulate AP-1 binding activity was determined in RA FLS by EMSA (n = 4). RA FLS were incubated with IL-1 (2 ng/ml), IL-1 plus SB 203580 (25–50 μM), or medium alone for 1 h. Nuclear extracts were isolated and evaluated for AP-1 binding activity. Figure 7 shows representative experiments in which a small amount of AP-1 was constitutively expressed by FLS and subsequently induced by IL-1. The IL-1-mediated increase was

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**Fig. 4.** JNK mRNA expression in FLS. RT-PCR was performed to determine relative levels of JNK1, 2, and 3 in FLS. JNK2 was the major isoform identified. Jurkat cells were used as positive controls. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was also assayed to confirm the relative amounts of RNA in each sample.

**Fig. 5.** Detection of phospho-JNK in synovial tissue. OA and RA synovial tissue extracts were assayed by Western blot analysis for phospho-JNK as described in Materials and Methods. Phospho-JNK was detected only in the RA samples.

**Fig. 6.** Effect of SB 203580 on IL-1-induced collagenase gene expression in RA FLS. Cells were exposed to IL-1 (2 ng/ml) in the presence of various concentrations of SB 203580 for 18 h. Collagenase mRNA levels were assayed by Northern blot and normalized to G3PDH expression. n = 3 for RA; *p < .01 for 10 and 25 μM SB 203580 compared with IL-1 alone.
The MAPKs are a family of kinases that respond to diverse stimuli and are composed of parallel protein kinase cascades. In addition to the JNKs (also called stress-activated protein kinases), there are two other well-defined pathways: ERK1 and ERK2, also referred to as p42/p44 MAPKs, and the p38 kinases (Karin, 1998). Activation of certain cytokine receptors, growth factor receptor tyrosine kinases, and G protein-coupled receptors activate the ERKs. p38 protein kinases are induced by lipopolysaccharide, proinflammatory cytokines, and cellular stresses like osmotic shock. The JNK proteins, which include three different isoforms and two to four splice variants each (Casanova et al., 1996) are activated by a variety of stimuli, including UV irradiation, protein synthesis inhibitors, and cytokines.

The MAPK families regulate a number of transcription factors, with subsequent activation of matrix metalloproteinase (MMP) and cytokine gene expression (Lee et al., 1994). In light of the key role of these inflammatory mediators in RA, we surmised that MAPKs are likely important regulatory proteins in FLS. AP-1 is of particular interest due to the promiscuous distribution of its binding site within the upstream regulatory regions of MMP genes (including collagenase-1 and collagenase-3; Rutter et al., 1997; Pendas et al., 1997). AP-1 is a protein complex, including c-Jun and c-Fos, held together by a leucine “zipper”, in which residues of leucine interact with the homologous region on its partner.

AP-1 activation is due to both transcriptional and post-translational regulation. For instance, cytokine stimulation induces mRNA accumulation for both the c-jun and c-fos genes in cultured FLS (Boyle et al., 1997). In addition, AP-1 proteins can be activated by protein kinases that phosphorylate specific amino acid residues (Minden et al., 1994; Kallunki et al., 1994). c-Jun is phosphorylated at two N-terminal serines (amino acids 63 and 73) by two closely related c-Jun amino-terminal kinases (JNK1 and JNK2). Recently, a third JNK (JNK3), which is primarily expressed in neural cells, has also been identified (Gupta et al., 1996). JNK2 binds c-Jun with at least 25-fold higher affinity than JNK1 and may be the more physiologically relevant activator of AP-1 in FLS (Kallunki et al., 1994). The N-terminal phosphorylation of c-Jun by JNKs enhances the transcriptional activity 30-fold. Notably, JNK1 appears to be a key regulator of Th1 differentiation in mice (Dong et al., 1998).

To evaluate the potential role of MAPKs in RA, Western blot analysis was performed using specific antibodies for ERK, JNK, and p38. All three MAPK families were expressed by OA and RA FLS to a similar extent (both at the protein and mRNA levels). IL-1 induced rapid phosphorylation of ERK and p38 in both OA and RA FLS. Surprisingly, immunoreactive phospho-JNK was detected only in the IL-1-induced RA FLS even though OA and RA synoviocytes expressed similar amounts of JNK mRNA and protein. This suggests that the mechanism resides in a more proximal site in the MAPK cascade. The explanation is not likely due to variability in IL-1 receptor density because IL-1 readily induced phosphorylation of p38 and ERK pathways.

Like IL-1, PMA and TNF-α induced JNK phosphorylation in RA FLS whereas less phosphorylated product was detected in OA FLS. In some OA cell lines, PMA and anisomycin increased JNK phosphorylation, indicating that the protein can be activated under some culture conditions. The mechanism by which phorbol esters activate JNK is distinct from IL-1 and involves activation of PKC-ε in conjunction with calcineurin (Werlen et al., 1998). The lower activity of TNF-α compared with IL-1 in RA cells is consistent with the observation that TNF-α is a much weaker inducer of collagenase than IL-1 in synoviocytes (Firestein and Paine, 1992) and that TNF-α primarily activates JNK1 in other types of fibroblasts (Westwick et al., 1994). The differences in JNK phosphorylation were also observed in synovial tissue, because phospo-JNK was detected in RA but not OA synovium. Our observation of increased JNK phosphorylation in RA is consistent with a recent study implicating JNK and AP-1 activation in Pas-mediated apoptosis in RA but not OA synoviocytes (Okamoto et al., 1997). Taken together, these data suggest that RA synoviocytes preferentially utilize the JNK pathway in response to a variety of stimuli (including IL-1, TNF-α, Fas ligand, and phorbol esters) compared with OA FLS.

RA synoviocytes are known to produce increased amounts of several cytokines and growth factors compared with OA FLS (Firestein and Zvaifler, 1990). Our studies showed that collagenase gene expression is significantly greater in RA FLS, which is consistent with these previous observations. The MAPK inhibitor SB 203580 was then used to evaluate the mechanisms of enhanced collagenase expression. Al-
though primarily considered a p38 inhibitor, certain isofoms of JNK (namely, JNK2β1 and JNK2β2) as well as other kinases like c-Raf are also inhibited at higher concentrations. Low concentrations of SB 203580 that inhibit p38 (but do not block JNK) had little effect on IL-1-induced collagenase gene expression in RA FLS. However, concentrations that also inhibit JNK2 (10–25 μM) blocked collagenase gene expression. A role for c-Raf, which is also inhibited by SB 203580, has not been defined and additional studies to determine the precise role of JNK will require more selective inhibitors. ERKs also contribute somewhat to collagenase regulation, although inhibition of this pathway only modestly decreased MMP expression.

Previous reports suggest that p38 might play an important role in phorbol ester-induced type IV collagenase production by a squamous cell carcinoma cell line (Simon et al., 1998). Additional studies indicated that intact JNK1 and ERK signaling pathways are also required for maximal stimulation (Gum et al., 1997). Also, multiple MAPK families appear to regulate collagenase expression in some cultured fibroblasts (Reunanen et al., 1998). In contrast with our studies with FLS, induction of collagenase and stromelysin gene expression in cultured endothelial cells was almost completely blocked by ≤1 μM SB 203580 (Ridley et al., 1997). Hence, the function of the individual MAPK pathways in MMP gene activation appears to depend on the cell type, culture conditions, and specific MMP examined. Our data suggest that ERK can contribute to collagenase gene expression and that, while we can not rule out a combined role of p38 and JNK because SB 203580 also inhibits p38 at the concentrations used to inhibit the latter. However, p38 alone cannot account for collagenase induction by IL-1 in FLS.

The effects of the SB 203580 on collagenase led us to evaluate its effect on IL-1-induced AP-1 binding activity. As demonstrated previously, IL-1 markedly increased AP-1 activity in nuclear extracts of FLS (Minden et al., 1994). Concentrations of SB 203580 that inhibit JNK2 and c-Raf also suppressed AP-1 activation in IL-1 stimulated synoviocytes. Of course, the EMSA assays only demonstrate AP-1 binding activity and can not provide information on the phosphorylation status of this transcription factor. Nevertheless, these data suggest that JNK2 regulates AP-1 activation in RA cells. SB 203580 had no effect on NF-κB activation, indicating that it was not toxic to FLS at the concentrations tested.

Activation of AP-1 and MMPs likely plays an important role in RA, an inflammatory disease marked by synovial hyperplasia and progressive bone and cartilage destruction (Firestein, 1996). Nuclear extracts from RA synovium contain significantly more AP-1 binding activity than OA, and synovial AP-1 binding increases very early in the course of murine collagen-induced arthritis (Asahara et al., 1997; Han et al., 1998). The collagenase gene, which is regulated by AP-1, is overexpressed in the intimal lining of RA synovium compared with OA (McCachren et al., 1990; Firestein et al., 1991). FLS in the intimal lining are the major source of MMPs in RA, and IL-1 is among the most potent inducers of collagenase and AP-1 in these cells.

Increased activation of JNK in RA synoviocytes suggests this signal transduction pathway can participate in the pathogenesis of RA. The observation that JNK is activated in RA synovium suggests a potential mechanism for increased AP-1 and collagenase expression compared with OA. This process could contribute to the highly destructive nature of rheumatoid synovium. Therefore, a selective JNK inhibitor could be a useful therapeutic approach to RA.

References


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