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Regulation of p38 MAPK by MAPK Kinases 3 and 6 in Fibroblast-Like Synoviocytes

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The p38 MAPK signal transduction pathway is a key regulator of IL-1 and TNF-α production in rheumatoid arthritis. Previous studies demonstrated that upstream MAPK kinases (MKK3 and MKK6) that regulate p38 are activated in rheumatoid arthritis synovium. However, their functional relevance in fibroblast-like synoviocytes (FLS) has not been determined. To investigate the relative contribution of MKK3 and MKK6 to p38 activation, the effect of dominant-negative (DN) MKK3 and MKK6 constructs on cultured FLS was evaluated. Cultured FLS were stimulated with medium or IL-1β, and immunoblotting was performed. In some experiments, cells were lysed and immunoprecipitated with anti-p38 Ab, followed by in vitro kinase assay with [γ-32P]ATP and GST-activating transcription factor-2 as substrate. IL-1β rapidly induced p38 phosphorylation in cells transfected with empty vector (pcDNA3.1), but was inhibited by 25% in cells expressing DN MKK3 or DN MKK6. Cotransfection with both DN plasmids Asp-Asp-Lys-C.

Materials and Methods

Fibroblast-like synoviocytes

FLS were isolated from synovial tissues obtained from patients with RA at the time of joint replacement, as described previously (18). The diagnosis of RA conformed to the American College of Rheumatology 1987 revised criteria (19). Synovial tissues were minced and incubated with 1 mg/ml collagenase in serum-free DMEM (Life Technologies) for 2 h at 37°C, filtered through a nylon mesh, extensively washed, and cultured in DMEM supplemented with 10% FCS (endotoxin content <0.006 ng/ml; Invitrogen...
Life Technologies), penicillin, streptomycin, and l-glutamine in a humidified atmosphere containing 5% CO2. After overnight culture, nonadherent cells were removed, and adherent cells were trypsinized, split at a 1:3 ratio, and cultured in medium. Synovocytes were used from passage 4 through 9 in these experiments, when they are a homogeneous population of FLS (<1% CD11b, <1% phagocytic, and <1% FcRy II positive).

Abs and reagents

Affinity-purified rabbit polyclonal anti-MKK3 Abs, goat polyclonal anti-MKK6 Abs, and secondary Abs were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-phospho-MKK3/6, anti-phospho-p38 MAPK (Thr180/Tyr182), and anti-p38 MAPK Abs and GST-ATF-2 were purchased from Cell Signaling Technology. Human rhl-1β (rhl-1β) was purchased from R&D Systems. The p38 inhibitor, SB203580, was purchased from Calbiochem. Previous studies in our laboratory showed that the concentrations used in these experiments completely block p38, but do not significantly inhibit JNK in synovocytes (20). DN MKK3- and MKK6-expressing N-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-C (FLAG) tags were constructed by replacing Ser180 and Thr193 with Ala in MKK3 and Ser207 and Thr211 with Ala in MKK6, as previously described (12). The constructs were subcloned into the expression vector pcDNA3.1 and were kindly provided by Dr. R. Davis (University of Massachusetts, Worcester, MA) (Invitrogen Life Technologies), and empty vector was used as a negative control. The accession numbers of the parent sequences are: NM_0002756 (MKK3) and NM_0002758 (MKK6).

FLS transfection

Using the Amaxa Human Dermal Fibroblast Nucleofector kit (NHDF-adult) with program U-23, 2–10 × 10^5 cells were transfected with 20 μg of pcDNA3.1, 10 μg of pcDNA3.1 plus 10 μg of FLAG-DN MKK3, 10 μg of pcDNA plus 10 μg of FLAG-DN MKK6, or 10 μg of FLAG-DN MKK3 plus 10 μg of FLAG-DN MKK6, according to the manufacturer’s protocol. Briefly, FLS suspended in 100 μl of Amaxa Nucleofector solution were added to the DNA solution, and the mixture was transfected into the electroporation cuvette. Immediately after electroporation, the cells were suspended in 500 μl of cell culture medium and transferred to culture dishes or plates. Cell viability after transfection was 55–80% by trypan blue dye exclusion, and cell growth was not affected by transfection for at least 48 h.

Western blot analysis

After transfection, FLS (5 × 10^5 cells/dish) were cultured in DMEM with 20% FCS in 60-mm dishes for 24 h, and were synchronized in DMEM with 0.1% FCS for 48 h. FLS were then treated with medium or rhIL-1β (2 ng/ml) for 24 h. Predeveloped sequence detection reagents for phospho-p38 were used. The extracts were then incubated with anti-FLAG Ab, and immunoprecipitates were washed three times with 1% Triton X-100 buffer and lysed in modified radioimmunoprecipitation assay buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM MgCl2, 1.5 mM ethylenediamine-tetra-acetic acid (EDTA, pH 8.0), 20 mM β-glycerophosphate, 50 mM NaF, 1 mM Na3VO4, 10 μg/ml aprotinin, 1 μM pepstatin A, and 1 mM PMSF). The protein concentrations in the extracts were determined using the micro bicinchoninic acid protein assay reagent. Then lysates were incubated with 2.5 μg of anti-p38 mAb for 4 h, followed by additional incubation with protein G-Sepharose overnight. The immunoprecipitates were washed three times with Tris-glycine-buffered 12% SDS-PAGE and transferred to nitrocellulose membrane (PerkinElmer Life Sciences). The membranes were blocked with TBS and 0.1% Tween 20 (TBST) containing 5% nonfat milk for 1 h at room temperature, followed by incubation with Ab to MKK3, phospho-MKK3/6, p38, phospho-p38, FLAG, or actin at 4°C overnight. After washing three times with TBST, the membrane was incubated with HRP-conjugated secondary Ab for 1 h at room temperature. Immunoreactive protein was detected with chemiluminescence and autoradiography (Eastman Kodak).

Immunoprecipitation and kinase assays

To measure the kinase activity of p38, FLS (5 × 10^5 cells/dish) were cultured in DMEM with 20% FCS in 60-mm dishes for 24 h after transfection and subsequently serum starved (0.1% FCS) for 48 h. FLS were then treated with either medium or rhIL-1β for 15 min. Cells were washed twice with ice-cold PBS and lysed in modified radioimmunoprecipitation assay buffer. Lysates were centrifuged at 15,000 × g for 10 min. Protein concentrations in the supernatant were determined using the micro bicinchoninic acid protein assay reagent. Then lysates were incubated with 2.5 μg of anti-p38 mAb for 4 h, followed by additional incubation with protein G-Sepharose overnight. The immunoprecipitates were washed three times with Tris-glycine-buffered 12% SDS-PAGE and transferred to nitrocellulose membrane (PerkinElmer Life Sciences). The membranes were blocked with TBS and 0.1% Tween 20 (TBST) containing 5% nonfat milk for 1 h at room temperature, followed by incubation with Ab to MKK3, phospho-MKK3/6, p38, phospho-p38, FLAG, or actin at 4°C overnight. After washing three times with TBST, the membrane was incubated with HRP-conjugated secondary Ab for 1 h at room temperature. Immunoreactive protein was detected with chemiluminescence and autoradiography (Eastman Kodak).

Results

Transfection efficiency of plasmids into FLS

Initial studies were performed after optimizing transfection protocols to confirm the transfection efficiency (22). FLAG-DN MKK3 and -DN MKK6 as well as empty vector (pcDNA3.1) were transfected into RA FLS, as described in Materials and Methods. FLS were then incubated with anti-FLAG Ab, and immunohistochemistry was performed. Fig. 1 shows a representative experiment using control cells (pcDNA3.1) or various combinations of DN MKK3 or DN MKK6 plasmids. The MKK transgenes were detected in ~90% of cells. This high degree of transfection efficiency allowed us to examine the effect of DN gene expression on FLS function in subsequent studies.

Modulation of intracellular signaling in RA FLS by the DN MKK3 and DN MKK6 transfection

To analyze the relative contribution of MKK3 and MKK6 to IL-1β-stimulated p38 activation in RA FLS, the expression of phospho-p38, phospho-MKK3, and phospho-MKK6 as well as the transduced protein and β-actin were determined by Western blot analysis. As shown in Fig. 2A, immunoreactive MKK3 and MKK6 protein expression were markedly greater in cells transfected with DN plasmids. The identity of the bands as the respective DN MKK proteins was confirmed using anti-FLAG Ab. Fig. 2 also shows that p38 phosphorylation is induced in IL-1-stimulated cells. The
ELISA. As shown in Fig. 4, IL-1 culture supernatants were collected after 24 h and assayed by cytokine mediators, cells were transfected and then activated with IL-1. We examined whether these kinases also regulate production of inflammatory cytokines. Cultured FLS were transfected with FLAG-DN MKK3 and -DN MKK6 plasmids, as described in Materials and Methods. The presence of the transgene was assessed by immunohistochemistry for FLAG, and the percentage of positive cells is indicated on the figure. A, Empty vector (pcDNA3.1, 20 μg)-transfected cells. B, DN MKK3 (empty vector, 10 μg; 3DN, 10 μg)-transfected cells. C, DN MKK6 (empty vector, 10 μg; 6DN, 10 μg)-transfected cells. D, Cotransfection with both DN MKK3- and DN MKK6 (DN MKK3, 10 μg; DN MKK6, 10 μg)-transfected cells. Transfection efficiency is shown as mean percentage ± SE.

DN MKK3 and DN MKK6 clones each modestly decreased phospho-p38 levels by 24.7 ± 1.4% and 24.7 ± 12.7%, respectively, in the cells exposed to cytokine (Fig. 2, A and B, for normalized results). However, cotransfection with both DN constructs was synergistic with respect to p38 inhibition (72.3 ± 6.1% inhibition; p < 0.01 compared with control and p < 0.05 compared with individual DN MKKs) (Fig. 2, A and B). Of interest, a similar pattern of inhibition was observed for phospho-MKK3/6 in the cells transfected with the DN clones (Fig. 2C), suggesting the pathway involves either autocrine activation or autophosphorylation of the upstream kinases.

**Inhibition of in vitro p38 MAPK activity by MKK3 and MKK6**

To further investigate the functional profile of MKK3 and MKK6, in vitro p38 kinase assays were performed using GST-ATF-2 as substrate. As shown in Fig. 3 and Table I, kinase activity was significantly increased in anti-p38 Ab immunoprecipitates from IL-1-stimulated FLS. Kinase activity was decreased by DN MKK3, DN MKK6, or the combination of both DN constructs compared with empty vector-transfected cells (32.3, 53.2, or 63.1% inhibition, respectively). In contrast to the Western blot studies, the suppressive effect of DN MKK6 was greater than DN MKK3 (p < 0.05). Similar suppression was observed if a saturating concentration of the p38 inhibitor SB203580 (3 μM) was added to the kinase reaction as a positive control (56.2% inhibition).

**Regulation of MMP-3, IL-6, and IL-8 protein by MKK3 and MKK6**

The preceding studies suggest that both MKK3 and MKK6 can contribute to p38 phosphorylation and kinase activity. To determine whether these kinases also regulate production of inflammatory mediators, cells were transfected and then activated with IL-1. Culture supernatants were collected after 24 h and assayed by ELISA. As shown in Fig. 4, IL-1β induced MMP-3, IL-6, and IL-8 protein production, but this activity was suppressed in DN MKK3-, DN MKK6-, and DN MKK3 + DN MKK6-transfected cells. Production was most effectively suppressed in combination DN-cotransfected cells, but the effect of DN MKK3 was greater than DN MKK6 (p < 0.05). Exogenous SB203580 was comparable to MKK blockade, indicating that the p38 was effectively blocked. DN MKK3 and the combination of DN MKK3 and DN MKK6 also inhibited IL-6 production by FLS stimulated with TNF-α (10 ng/ml) as much as SB203580 (n = 2; data not shown).

**Effect of DN MKK3 and DN MKK6 on p38 phosphorylation**

Cultured FLS were stimulated with medium or IL-1β (2 ng/ml) for 15 min. Total proteins were extracted and evaluated by Western blot analysis. A, Representative blot of independent experiments (n = 4). Note marked increase in MKK3 and MKK6 expression after transfection with the indicated construct. Coexpression of both proteins is readily demonstrated with the anti-FLAG Ab (higher m.w. band = MKK6). B, Actin-normalized phospho-p38 levels in transfected cells (n = 4). The combination of DN MKK3 and DN MKK6 markedly decreased p38 phosphorylation. Data are presented as percentage of IL-1-induced expression for each individual cell line. 3DN = DN MKK3; 6DN = DN MKK6; 3/6DN = cotransfection with DN MKK3 and DN MKK6. *p < 0.05 and **p < 0.01, respectively, compared with empty vector. C, Actin-normalized phospho-MKK3/6 levels in transfected cells (n = 3). The combination of DN MKK3 and DN MKK6 markedly decreased MKK3/6 phosphorylation. Data are presented as percentage of IL-1-induced expression for each individual cell line. 3DN = DN MKK3; 6DN = DN MKK6; 3/6DN = cotransfection with DN MKK3 and DN MKK6. *p < 0.05 and **p < 0.01, respectively, compared with empty vector.
SB203580 were not statistically significant (Fig. 5). These data indicate that cytokine and MMP regulation by p38 and MKKs is independent of mRNA levels and is most likely related to translational mechanisms.

Discussion

RA is a chronic inflammatory disease marked by synovial hyperplasia and local invasion into the extracellular matrix. Synovitis is regulated by cytokines such as IL-1 and TNF-α that activate a broad array of cell signaling mechanisms (23). MAPKs are especially important because they control the production of MMPs and cytokines that participate in the rheumatoid process. The three MAPK families (ERK, JNK, and p38) accomplish this by phosphorylating numerous key transcription factors, such as AP-1 and ATF-1/2 (1, 2). In addition to engaging transcription factors directly, some p38 functions are also regulated through downstream kinases such as MAPKAPK-2 (4, 6, 11).

Several MAPK members are activated in the rheumatoid synovium and have been implicated in the pathogenesis of RA. p38 is thought to be crucial because selective p38 inhibitors block joint inflammation and destruction in several animal models of arthritis. Four p38 isoforms (p38α, -β, -γ, and -δ) have been characterized. The best-studied subtype is p38α, which can be phosphorylated in many inflammatory cell lineages and regulates cytokine production by macrophages (3). The pyridinylimidazole compounds, exemplified by SB203580, were originally identified as cytokine synthesis inhibitors that subsequently were found to be selective inhibitors of p38. SB203580 specifically inhibits p38α and -β forms by competing for the ATP-binding pocket and blocking kinase activity (7, 10).

The present study was designed to evaluate the potential role of two main upstream kinases of p38, MKK3 and MKK6, on the activation of RA FLS. We previously demonstrated that both MKKs are phosphorylated in rheumatoid synovium and are constitutively expressed in cultured FLS (17). MKK3 and MKK6 each form stable complexes with p38 in FLS that can phosphorylate ATF-2 after cytokine exposure. Western blot studies using stimulated FLS suggested that MKK3 is preferentially phosphorylated. However, functional studies to assess the relative contribution of MKK3 and MKK6 were not possible due to difficulty transfecting primary synoviocytes and the lack of selective small molecule inhibitors. More recently, protocols have been developed that permit high transfection efficiency in FLS (~90%). These advances allowed us to study the functional effects of the MKK3 and/or MKK6 inhibition using well-characterized DN constructs (12).

Our studies are the first to assess MKK3 and MKK6 function in primary human cells and demonstrate that both MKK3 and MKK6 contribute to the phosphorylation of p38, induction of p38 kinase activity, and release of key inflammatory gene products. The DN MKK constructs also inhibited phosphorylation of MKK3 and MKK6, suggesting that either autophosphorylation or positive feedback loops from p38 contribute to MKK activation. A similar scenario has been observed with selective p38 inhibitors, which can decrease p38 phosphorylation even though the compounds do not block the phosphorylation sites on the kinase (24). We observed subtle differences in the relative contributions of the two kinases to the p38 pathway. For instance, MKK6 appears to be more active in the kinase assays, whereas MKK3 had a greater contribution to the release of cytokines and MMPs. Because production of inflammatory mediators is probably the most important aspect with respect to a therapeutic target, these data suggest that either a selective MKK3 or, more likely, a combined MKK3/6 inhibitor would be the most effective in a disease such as RA.

The effects of DN MKK3 and DN MKK6 have not been studied in primary human cells, but their combined or individual effects have been examined in tumor cells and rodent cell lines. These results differ in some respects from FLS. For instance, DN MKK3 rather than DN MKK6 blocks α1 integrin-mediated p38 activation in invasive breast cancer cells (25). Similarly, activin A and hepatocyte growth factor-mediated p38 activation is inhibited by DN MKK3, but not by DN MKK6 in pancreatic cancer cells (26). Hypoxia-induced endoglin expression in mouse endothelial cell line is also inhibited by DN MKK3 (27). In bovine capillary endothelium, fibroblast growth factor-2-induced p38 activation is decreased by both DN MKK3 and 6 (28). Finally, pervanadate-induced p38 phosphorylation is inhibited by DN MKK3 as well as DN MKK6 in rat vascular smooth muscle cells (29). MKK function has also been evaluated in genetically engineered mice, in which TNF-α-induced cytokine expression in fibroblasts is decreased in MKK3−/− animals (30). Defective IL-12 production has also been noted in macrophages and dendritic cells from MKK3−/− mice (31). Both MKK3 and MKK6 are essential for TNF-α-stimulated p38 activation in vivo (32).

In addition to cell lineage-specific hierarchy of MKKs, the types of cellular stresses that activate MKK3 compared with MKK6 also vary widely. Our data suggest that, unlike some forms of stimulation, both MKK3 and MKK6 are rapidly activated in FLS by IL-1, which is a key cytokine implicated in RA. However, MKK6 is the primary pathway to p38 in murine lymphoma cells and human epithelial cells after osmotic stress (33), while MKK3 is required for full activation of p38 in murine embryonic fibroblasts.
MKK6 is the principal activator of p38 in human epithelial cells stimulated with IL-1 (34), but MKK3 appears to be an important activator of p38 in LPS-stimulated murine peritoneal macrophages (31). In contrast, both the p38α and δ isoforms are activated by MKK3 in murine mesangial cells stimulated by TGF-β1 (35).

The mechanisms by which p38 regulates gene expression are equally complex. In cytokine-stimulated synoviocytes, p38 inhibition by DN MKK3/6 or SB203580 had little effect on inflammatory gene expression despite significant decreases in the encoded proteins. Previous studies have indicated that this method of suppression is related to MAPKAPK-2-mediated translational effects. For instance, inhibition of TNF-α and IL-1β protein synthesis by p38 blockade is not accompanied by a decrease in the corresponding steady state mRNA levels (36, 37). MAPKAPK-2 knockout mice exhibit impaired TNF-α protein synthesis, with no discernible change in TNF-α mRNA transcription or stability (38, 39). IL-6 and IL-8 mRNA translation is also regulated by p38, but extent of inhibition of protein production varies with cell type (40–42). These data are consistent with our studies on cytokine regulation by MKK3 and MKK6 in FLS, indicating that suppression of the p38 pathway is complex, but that downstream effects involve posttranslational mechanism.

The regulation of MMPs by p38 can involve several mechanisms. For example, wild-type MKK3 overexpression in human skin fibroblasts increases MMP-1 and MMP-3 mRNA expression, in part by stabilizing their respective RNA transcripts (4). The mRNA t1/2 appears to be prolonged due to decreased deadenylation of AU-rich tails (11). Our observation that DN MKK3 and DN MKK6 modestly decreased MMP-3 mRNA is consistent with an effect on gene transcription or mRNA stability. However, the changes in MMP-3 mRNA are relatively small compared with the decrease in MMP-3 protein levels. Hence, MMP regulation in FLS by p38 probably results from combination of mechanisms. The differences between our data and the dermal fibroblast study (4) are probably related to the distinct biology of wild-type overexpression compared with inhibition of the endogenous kinase.

In conclusion, our studies demonstrate that, unlike many tumor cells or physical stresses, MKK3 and MKK6 are key p38 regulators in cytokine-stimulated FLS. Both MKKs contribute to p38 regulation by MKK3 and MKK6 in FLS, indicating that suppression of the p38 pathway is complex, but that downstream effects involve posttranslational mechanism.

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activation, but MKK3 might be more important when attempting to suppress cytokine production. However, both must be blocked to interrupt p38 as effectively as small molecule p38 inhibitors in FLS. Because p38 pathway may participate in joint destruction and inflammation, selective MKK3, MKK6, or combined inhibitors have therapeutic potential for RA. Distinct activation pathways in different cell lineages suggests that the safety and efficacy profile might differ from competitive p38 inhibitors.

Disclosures

The authors have no financial conflict of interest.

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