Selective Activation of the Mitogen-activated Protein Kinase Subgroups c-Jun NH_2 Terminal Kinase and p38 by IL-1 and TNF in Human Articular Chondrocytes

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Abstract

Previous studies suggested that tyrosine kinase activation is an important signal transduction event in the IL-1 response of chondrocytes. The present study identifies the mitogen-activated protein (MAP) kinases extracellular signal-regulated kinase (ERK)-1 and ERK-2 as major tyrosine phosphorylated proteins in IL-1 stimulated chondrocytes. Kinase assays on immunoprecipitates with myelin basic protein as substrate showed that ERK-1 and ERK-2 activation was detectable within 5 min after IL-1 stimulation and decreased to baseline within 60 min. Analysis of other members of the MAP kinase family showed that chondrocytes also express c-Jun NH_2 terminal kinase (JNK)-1, JNK-2, and p38 proteins. These kinases were time-dependently activated by IL-1. Among other chondrocyte activators tested, only TNF activated all three of the MAP kinase subgroups. JNK and p38 were not activated by any of the other cytokines and growth factors tested. However, ERK was also activated by PDGF, IGF-1, and IL-6. Phorbol 12-myristate 13-acetate, calcium ionophore, and cAMP analogues only increased ERK activity but had no significant effects on JNK or p38.

These results suggest differential activation of MAP kinase subgroups by extracellular stimuli. ERK is activated in response to qualitatively diverse extracellular stimuli and various second messenger agonists. In contrast, JNK and p38 are only activated by IL-1 or TNF, suggesting that these kinases participate in the induction of the catabolic program in cartilage. (*J. Clin. Invest.* 1996. 98:2425–2430.) Key words: Interleukin-1 • cartilage • MAP kinase • chondrocytes.

Introduction

IL-1 is one of the most potent cytokines that promotes cartilage catabolism. It induces the production of matrix metalloproteinases (1), suppresses type II collagen and aggrecan production (2–5), inhibits chondrocyte proliferation (6–8), and induces proinflammatory mediators including cytokines, prostaglandins, and nitric oxide (for review see reference 9). IL-1 injections into rabbit, rat, or mouse joints induced synovial fluid leukocytosis and proteoglycan loss from articular cartilage (10–13). Within one day after injection, a substantial reduction in cartilage proteoglycan synthesis occurred and persisted for at least 2 wk (12).

IL-1 induced signal transduction occurs via the 213–amino acid long cytoplasmic domain of IL-1RI. A diverse spectrum of intracellular signals can be activated by IL-1. Guanylate cyclase is activated by IL-1 in nitric oxide-dependent and nitric oxide-independent pathways (14–16). IL-1 activates phospholipase A2 (17) and induces expression of the inducible cyclooxygenase (18), resulting in increased levels of PGE2. The role of protein kinase C in IL-1 activation of chondrocytes is unclear (5, 19–24). IL-1 shares with TNF the ability to induce the ceramide pathway by activating sphingomyelinase (25, 26). Evidence for tyrosine kinase activation by IL-1 has been provided in chondrocytes and other cell types (27–29). In human articular chondrocytes, pharmacologic inhibitors of tyrosine kinases interfered with the induction of most IL-1 responsive genes including inducible nitric oxide synthase and cyclooxygenase, collagenases, stromelysin, and several cytokines (30, 31).

Activation of mitogen-activated protein (MAP)[1] kinases has been implicated in IL-1 signaling. Three subgroups of the MAP kinase family have been identified (32–34). These kinases are structurally related and all are phosphorylated on tyrosine and threonine and activated by upstream kinases, the MAP kinase kinases. The various members of the MAP kinase families differ in their substrate specificity and they are also activated by distinct upstream regulators and extracellular stimuli. The MAP kinases originally isolated from mammalian cells are the p44 and p42 MAP serine threonine kinases (also termed extracellular signal regulated kinase, ERK1, and ERK2) (35, 36).

The Jun NH_2-terminal kinases (JNK) were identified as protein kinases which bind and phosphorylate the c-Jun NH_2 terminal domain (33, 37). The JNKs include the 46 kD JNK1 (33) and 55 kD JNK2 (38) forms, which are closely related in their primary structures and are identically regulated (38). Three isoforms of stress activated protein kinase (SAPK) were identified (39). SAPKγ is identical to JNK1 and has a molecular mass of 46 kD, and SAPKα is identical to JNK2 with a molecular mass of 54 kD (33).

The third subgroup of MAP kinase is p38, which was originally identified as a protein kinase from murine macrophages, which is tyrosine phosphorylated and activated in response to LPS (32). p38 was also discovered as an intracellular target for molecules that act as cytokine suppressive antiinflammatory drugs (40).

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1. *Abbreviations used in this paper:* ATF2, activating transcription factor 2; ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; JNK, c-Jun NH_2 terminal kinase; MAP, mitogen-activated protein; MBP, myelin basic protein; MKK, MAP kinase kinases; SAPK, stress activated protein kinase.

The present study analyzes the regulation of MAP kinase subgroups in chondrocytes and shows that ERK activation occurs in response to several qualitatively diverse extracellular stimuli, while activation of JNK and p38 is only seen in response to IL-1 or TNF, suggesting that these kinases are associated with chondrocyte responses that lead to cartilage degradation.

Methods

Chondrocyte isolation and culture. Cartilage was obtained at autopsy from donors without known history of joint disease. For all experiments reported here, cartilage from the femoral condyles and tibial plateaus of the knee joints was used. Chondrocytes were isolated by collagenase digestion of cartilage and cultured as previously described (41). All experiments were performed with primary or passage 1 cells.

Preparation of cell lysates. For kinase assays, 3×10^6 chondrocytes were cultured in 100 mm petri dishes and serum-starved for 24 h before stimulation. After washing with PBS containg Na_3VO_4, cells were lysed in lysis buffer (50 mM NaCl, 50 mM Tris pH 7.4, 0.5% NP–40, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM Na-F, 1 μg/ml aprotinin, 1 μg/ml leupeptin). 100 μg of lysates was used for JNK and p38 assay and 300 μg for ERK. For immunoblot assay, chondrocytes were lysed in RIPA lysis buffer (50 mM NaCl, 50 mM Tris pH 7.4, 0.5% DOC, 0.1% SDS, 1% NP-40, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM Na-F, 1 μg/ml aprotinin, 1 μg/ml leupeptin). 50 μg of whole cell lysate or precipitates of 200 μg of lysate with antibodies or GST fusion proteins were separated on 10–12% polyacrylamide gels.

Immunoblot assay. Whole cell lysates or precipitates obtained with specific antibodies against ERK, JNK, or p38 or with GST fusion proteins were separated by SDS PAGE. Blots were stained as described (42) with monoclonal antibodies to phosphotyrosine and ERK1+2, polyclonal antibodies for JNK1, JNK2, and p38 and horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). The stained blots were developed with enhanced chemiluminescence (Amersham Life Science Inc., Arlington Heights, IL) and exposed to Kodak film for 10–30 s.

In-gel kinase assay. Cell lysates were immunoprecipited at 4°C for 1 h with 4 μg of monoclonal antibody to ERK1 and ERK2 and agarose-conjugated goat anti-mouse IgG. The immune complexes were washed 3 times with lysis buffer, boiled in Laemmli buffer and separated on 10% SDS-PAGE containing 0.5 μg/ml of myelin basic protein (MBP) as substrate. The gel was washed 3 times for 1 h with washing buffer (20% 2-propanol, 50 mM Tris pH 8.0), 1 h with denaturation buffer (6 M guanidine–HCl, 50 mM Tris pH 8.0, 5 mM 2-mercaptoethanol). The proteins were renatured in 0.04% Tween-40, 50 mM Tris pH 8.0, 5 mM 2-mercaptoethanol for 16 h at 4°C with several buffer changes. The gels were washed for 30 min in kinase buffer (40 mM Hepes pH 8.0, 2 mM DTT, 0.1 mM EGTA, 5 mM MgAc) and incubated for 1 h in kinase buffer containing 20 μM ATP and 50 μCi [γ^{32}P]ATP. After washing with 5% TCA, 1% NaPP, phosphorylated MBP was visualized by autoradiography.

JNK assay. Hypotonic detergent cellular extracts were prepared and the solid-state JNK assay was performed as described (37). The extracts were mixed with 10 μl of GSH-agarose suspension (Sigma Chemical Co., St. Louis, MO) to which GST–c-Jun (1-223) was bound. The mixture was rotated at 4°C for 3 h in a microfuge tube, pelleted and then washed 3 times in lysis buffer. The beads were then resuspended in 30 μl kinase buffer (20 mM Hepes pH 7.6, 20 mM $MgCl_2$, 10 mM β-glycerolphosphate, 0.1 mM Na_3VO_4, 10 mM DTT) containing 20 μM ATP and 5 μCi [γ^{32}P]ATP. After 20 min at 30°C, the reaction was terminated by washing with lysis buffer. Phosphorylated proteins were eluted with Laemmli buffer and resolved on 10% SDS-PAGE, followed by autoradiography.

p38 kinase assay. Cell lysates were incubated overnight at 4°C with 4 μg of rabbit antibody which recognizes human and murine p38/40 (Santa Cruz Biotechnology, Santa Cruz, CA). This was followed by the addition of 40 μl goat anti-rabbit IgG1 coupled to agarose and incubation for 1 h at 4°C. The precipitates were washed 4 times with 100 μl lysis buffer, 1 time with kinase buffer without $MgCl_2$ and ATP and resuspended in 15 μl kinase buffer (20 mM Hepes pH 7.6, 20 mM β glycerolphosphate, 0.1 mM Na_3VO_4, 10 mM DTT, 10 mM $MgCl_2$, and 20 μM ATP). The samples were incubated with GST-ATF–2 fusion protein and 1 μl of 10 μCi of [γ-^{32}P]ATP for 10 min at 30°C. The reaction was terminated by the addition of 20 μl 2 × Laemmli sample buffer before analysis by SDS-PAGE and autoradiography.

Reagents. IL-1β, TNFα, LPS, PDGF-AA, IGF-I, IFNγ, IL-6, TGFβ1 were purchased from R&D Systems (Minneapolis, MN); monoclonal antibody for $p44^{MAPK}$ and $p42^{MAPK}$ from Zymed Laboratories Inc. (San Francisco, CA); polyclonal antibodies specific for ERK1, ERK2, JNK1, JNK2 or p38 from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phosphotyrosine antibody 4G10 from Upstate Biotechnology Inc. (Lake Placid, NY). Rabbit myelin basic protein (MBP) was purchased from Sigma Chemical Co. GST-c-Jun (1-223) and GST-ATF2 were gifts of Drs. Michael Karin (University of California, San Diego) and Richard Ulevitch (The Scripps Research Institute).

Results

ERKs are major tyrosine-phosphorylated proteins in IL-1β stimulated chondrocytes. Previous studies had demonstrated that pharmacologic protein tyrosine kinase inhibitors prevented the expression of most IL-1β inducible proteins in human articular chondrocytes. The first set of experiments in this study analyzed tyrosine phosphorylation of proteins in IL-1β stimulated chondrocytes. Western blotting of whole cell lysates identified several proteins that are tyrosine-phosphorylated after IL-1β stimulation at ~ 210, 150, and 65 kD. The most prominent changes in tyrosine phosphorylation occurred at 42–44 kD (Fig. 1 *A*). This is the molecular mass of MAP kinases ERK-1 (44 kD) and ERK-2 (42 kD). Western blotting of the same membranes with antibody to ERK showed that chondrocytes express ERK and that protein levels did not detectably change after IL-1β treatment (Fig. 1 *B*).

To determine which forms of ERK are expressed in chondrocytes and activated in response to IL-1β, cell lysates were

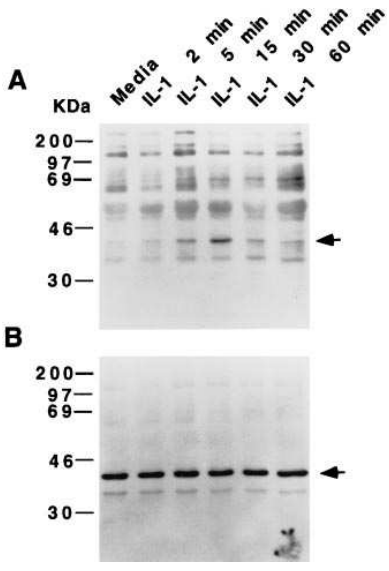

Figure 1. ERK are major tyrosine-phosphorylated proteins in IL-1 stimulated chondrocytes. Chondrocyte lysates were prepared at the indicated time points after stimulation with 1 ng/ml IL-1β. Whole cell lysates were subjected to Western blotting with antibody (4G10) to phosphotyrosine (*A*) or antibody that recognizes both, ERK-1 and ERK-2 (*B*).

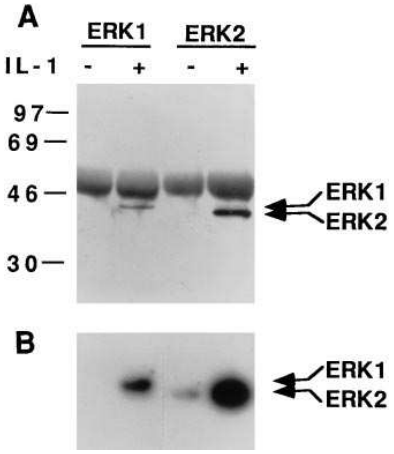

Figure 2. Tyrosine phosphorylation and activation of ERK-1 and ERK-2. Chondrocytes were cultured in media or stimulated with IL-1β (1 ng/ml) for 15 min. Cell lysates were immunoprecipitated with antibodies specific for ERK-1 or ERK-2. (*A*) The precipitates were analyzed by Western blotting with antibody to phosphotyrosine (4G10). (*B*) The precipitates were analyzed by in gel kinase assay using MBP as substrate.

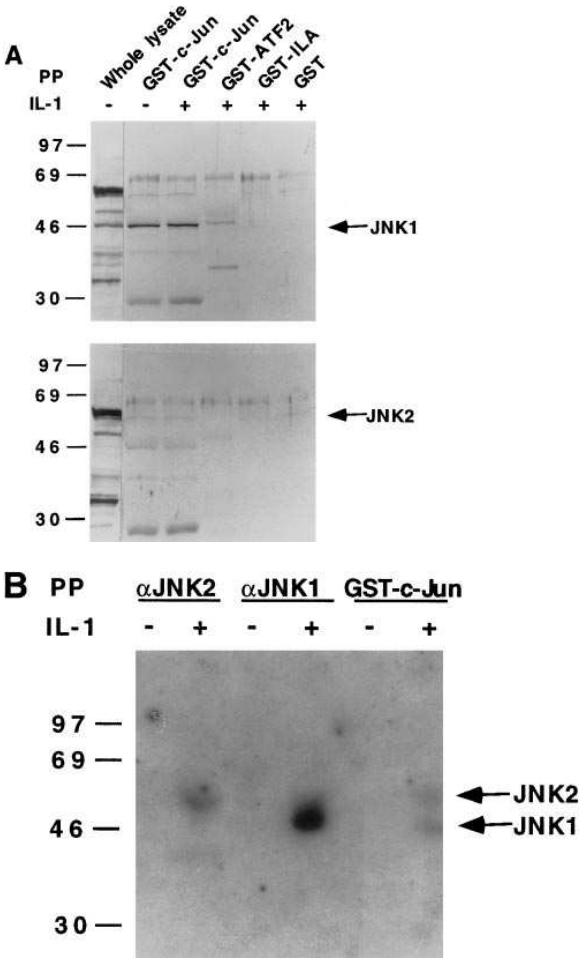

Figure 3. Expression and activation of JNK. Chondrocytes were unstimulated or treated with IL-1 for 20 min. Whole cell lysates from unstimulated cells or precipitates of lysates with GST-c-Jun, GST-ATF2, GST-ILA, or GST were subjected to Western blotting with antibody to JNK1, which has weak cross-reactivity with JNK2 (*A*, *top*) or antibody specific for JNK2 (*A*, *bottom*). Lysates from unstimulated or IL-1 stimulated cells were precipitated with antibodies to JNK1 or JNK2 or GST-c-Jun and then subjected to in gel kinase assay with GST-c-Jun as substrate (*B*).

immunoprecipitated with antibodies specific for ERK-1 or ERK-2. The precipitates were analyzed by Western blotting with antibody to phosphotyrosine (4G10). Both ERK-1 and ERK-2 were tyrosine phosphorylated after IL-1β stimulation (Fig. 2 *A*). The precipitates were further analyzed by in-gel kinase assay for ERK activity using myelin basic protein (MBP) as substrate. ERK-1 activity was not detected in unstimulated cells but was induced by IL-1β. Low levels of ERK-2 activity were present in unstimulated cells and this increased markedly in response to IL-1β (Fig. 2 *B*).

These results indicate that ERK-1 and ERK-2 are expressed in chondrocytes. These kinases are tyrosine phosphorylated and activated after stimulation with IL-1β.

Expression of JNK and p38 proteins in chondrocytes. The next set of experiments analyzed expression of other MAP kinase subgroups in chondrocytes. Whole chondrocyte lysates or precipitates with GST-c-Jun, or a GST fusion protein containing ATF2, also a specific target of JNK (43) were prepared. These were submitted to Western blotting with antibody to JNK1 which has weak cross-reactivity with JNK2 (Fig. 3 *A*, top) and antibody specific for JNK2 (Fig. 3 *A*, bottom). This showed that human chondrocytes contain JNK1 with a molecular mass of ~ 46 kD and JNK2 with a molecular mass of ~ 54 kD. A fusion protein with GST and the intracellular domain of ILA, a member of the human TNF receptor family (44) and GST were used as specificity controls. These proteins did not precipitate JNK1 or JNK2. GST-ATF2 precipitated lower levels of JNK1 but not JNK2.

In a different experimental approach lysates from unstimulated and IL-1 stimulated chondrocytes were immunoprecipitated with antibodies that recognize JNK1 and JNK2 or antibody specific for JNK2 or precipitated with GST–c-Jun and then subjected to in gel kinase assay with GST–c-Jun as substrate. This showed activation of JNK1 and JNK2 by IL-1 (Fig. 3 *B*).

The expression of p38 was demonstrated with two different antibodies recognizing either the COOH terminus or the NH_2 terminus of the protein. p38 was detectable in whole cell lysates and in immunoprecipitates (Fig. 4).

Kinetics of ERK, JNK and p38 activation. Chondrocytes were stimulated with IL-1β and harvested at several time points between 2 and 60 min. For comparative analysis, aliquots of the same chondrocyte lysates were immunoprecipitated with antibody to ERK or p38 and tested for phosphorylation of MBP or ATF-2 as substrates. JNK was analyzed by a solid phase assay where the cell lysates were incubated with a GST–c-Jun fusion protein. This was precipitated with glutathione–agarose beads and used for the kinase assay. The phosphorylated substrates were separated on SDS PAGE and visualized by autoradiography. Unstimulated chondrocytes did not contain detectable JNK or p38 activity and only low levels of ERK activity. Within 15 min of IL-1β stimulation there was a strong increase in the activation of the kinases. ERK and p38 activation were detectable after 5 min and JNK after 15 min. Kinase activities returned to basal values by 60 min (Fig. 5).

Extracellular stimuli of MAP kinase subgroups. To address stimulus specificity of the activation of the MAP kinase subgroups, cytokines and growth factors which represent major chondrocyte regulators were analyzed. Three separate experi-

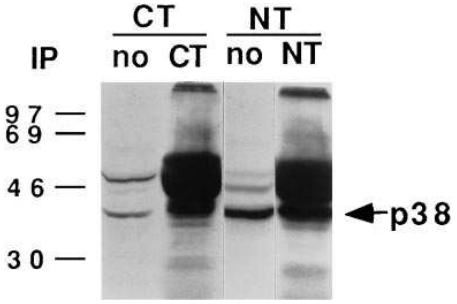

Figure 4. Expression of p38 in chondrocytes. Lysates from unstimulated chondrocytes were subjected to Western blotting without immunoprecipitation (no IP) or after immunoprecipitation with antibody to COOH terminus (CT) or NH_2 terminus (NT) of p38. Western blotting was performed with antibody to COOH terminus (CT, first 2 lanes) or with antibody to NH_2 terminus (NT), last 2 lanes.

ments showed that IL-1β was the strongest activator of ERK. TNF, IGF, IL-6, PDGF, and LPS also activated ERK but were less potent than IL-1. IFNγ and TGFβ did not detectably increase ERK activity (Fig. 6). IGF-1, IL-6, IFNγ, and TGFβ were also evaluated at earlier (2 min) and later (120 min) time points and did not show activation of JNK (not shown). These results suggest that of the stimuli tested, only IL-1 and TNF activate p38 and JNK. ERK is activated by a broad spectrum of qualitatively diverse extracellular stimuli.

Second messenger agonists of MAP kinase activation. Second messenger agonists were tested to determine signaling pathways that can lead to the activation of MAP kinases. Increases in ERK activity were observed in response to db-cAMP, PMA, and A23187 (Fig. 7). The combination of PMA and A23187 did not show synergistic effects. All of the second messenger agonists were much weaker inducers of ERK activation as compared to IL-1β. JNK was not activated by any of these second messenger agonists and p38 was increased only in response to high concentrations of PMA (not shown).

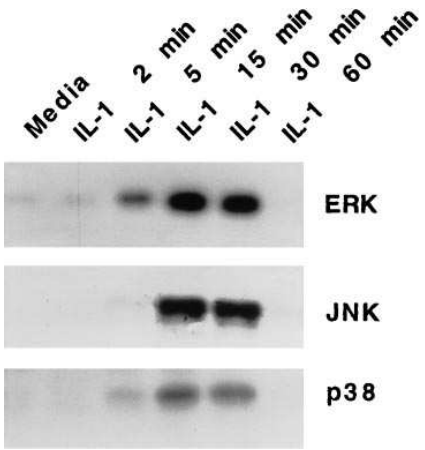

Figure 5. Time course of IL-1 activation of MAP kinase subgroups. Chondrocytes were cultured in media or stimulated with IL-1β (1 ng/ml) for the time periods indicated. Cell lysates were precipitated with antibody recognizing both ERK-1 and ERK-2, GST-c-Jun beads or antibody to p38. The precipitates were assayed for kinase activity with MBP as substrate for ERK, c-Jun for JNK and ATF-2 for p38.

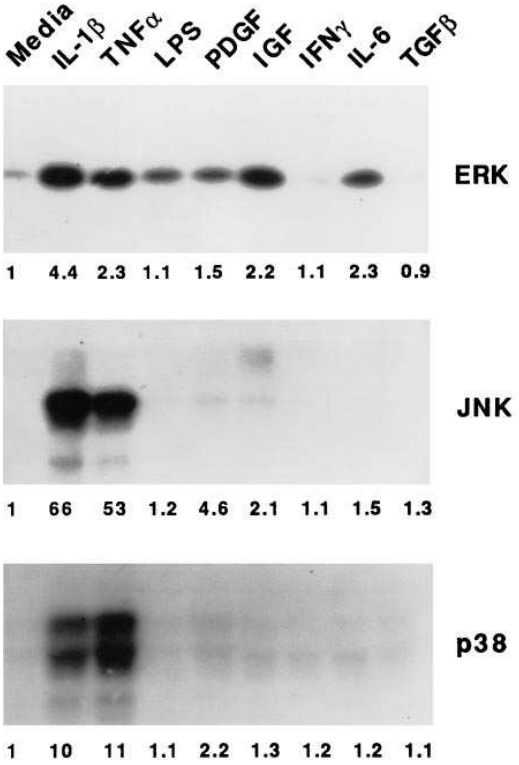

Figure 6. Extracellular stimuli of MAP kinase activation. Chondrocytes were stimulated with IL-1β (1 ng/ml), TNFα (10 ng/ml), LPS (1 μg/ml), PDGF-AA (20 ng/ml), IGF-I (50 ng/ml), IFNγ (10 ng/ml), IL-6 (10 ng/ml) or TGFβ1 (10 ng/ml) for 20 min. ERK, JNK, and p38 were analyzed as described in legend to Fig. 5. The figure shows one experiment representative of three separate experiments. The densitometry values are the mean values from these three experiments. Control lanes were assigned a value of 1.

Discussion

This study was based on previous observations that protein tyrosine kinase activation was required for the expression of a broad spectrum of IL-1 inducible genes in human articular chondrocytes (30, 31). The first series of experiments in the present study showed that IL-1 causes an increase in tyrosine phosphorylation of several proteins. Analysis of whole chondrocyte lysates showed IL-1-induced tyrosine phosphorylation at ~ 210, 150, 65, and 42–44 kD. The tyrosine phosphorylated proteins between 42 and 44 kD were identified as MAP kinases ERK-1 and ERK-2. These proteins are constitutively expressed in chondrocytes and short-term incubation (5–60 min) with IL-1 did not alter their levels. However, associated with IL-1 induced tyrosine phosphorylation of ERK-1 and ERK-2 was stimulation of kinase activity. Immunoprecipitation with ERK-1 or ERK-2 specific antibodies showed that IL-1 activated both isoforms.

Chondrocytes also express two other MAP kinase subgroups, p38 and JNK. In other cell systems the three MAP kinase subgroups are coupled to different extracellular stimuli (45). In chondrocytes, IL-1 and TNF activated all three MAP kinase subgroups and these two cytokines were the only inducers of p38 and JNK. This is in contrast to ERK which was activated by several of the extracellular stimuli tested. Other cy-

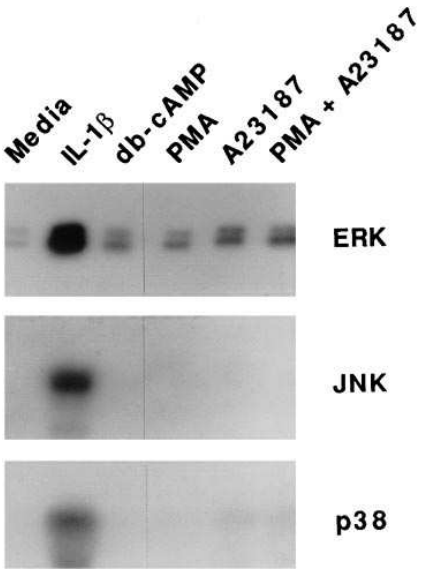

Figure 7. Second messenger agonists and MAP kinase activation. Chondrocytes were stimulated with IL-1β (1 ng/ml), db-cAMP (1 mM), PMA (10 ng/ml), A23187 (2 μM), or PMA plus A23187 for 30 min. Cell lysates were precipitated with antibody to ERK (recognizing ERK1 and ERK2) and tested in kinase assays with MBP as substrate, GST–c-Jun beads or antibody to p38 and assayed for kinase activity with ATF-2 as substrate. The doublet bands show the activities of ERK-1 and ERK-2 since immunoprecipitation was performed with antibodies that recognize both ERK-1 and ERK-2.

tokines and growth factors that are known chondrocyte activators had no detectable effect on p38 or JNK. TGFβ, IGF, and PDGF stimulate chondrocyte proliferation and extracellular matrix synthesis, thus having anabolic effects on cartilage. TGFβ did not activate any of the MAP kinases. IGF-1 and PDGF only activated ERK. IL-6 and other members of this cytokine family have qualitatively distinct effects on chondrocyte function. They induce the expression of protease inhibitors but are not significant inducers of metalloproteinases (46). IL-6 activated ERK but not p38 or JNK in chondrocytes. IFNγ is also distinct in its effects on chondrocytes. It inhibits matrix metalloproteinase synthesis and also collagen synthesis (47, 48). IFNγ did not activate any of the MAP kinase subgroups.

Collectively, these results on extracellular stimuli indicate that p38 and JNK are closely associated with chondrocyte activation by the catabolic stimuli IL-1 and TNF. ERK, however, is inducible by a broader spectrum of qualitatively diverse chondrocyte activators. The results from studies with second messenger agonists correlated with the inducibility of ERK by heterogeneous stimuli. Increases in ERK activity were observed in response to PMA, A23187, and cAMP. However, p38 and JNK were not or only weakly activated by these stimuli.

Several upstream kinases which are responsible for MAP kinase activation have recently been characterized and they show specificity for the three subfamilies of MAP kinase: MKK1 and MKK2 activate ERK, MKK3 and MKK4 activate p38, and MKK4 activates JNK (49). The murine homologue of MKK4 (termed SEK1) phosphorylates stress activated protein kinases/JNK, leading to c-Jun activation (45).

Upstream regulators of MAP kinases vary with the extracellular stimulus and the cell type analyzed. JNK was activated by EGF or NGF and this was dependent on H-Ras activation. In contrast, TNF-induced JNK was Ras independent (45). Ras activates Raf-1 and MKK. Raf-1 directly contributed to ERK activation but not to JNK activation (45).

Activation of ERK in response to extracellular stimuli is mediated by Ras proteins (for review see reference 50). Receptor activation results in the recruitment of the nucleotide exchange factor Sos to the plasma membrane where it catalyzes the exchange of GDP to GTP (51, 52). Ras × GTP activates Raf-1 (53, 54). Raf then activates MEKK which phosphorylate ERK (for review see references 50 and 55). The activation of ERK-1 and ERK-2 by the broad spectrum of extracellular stimuli is likely to be mediated by this pathway. However, the activation of JNK and p38 appears to require additional signal. This is consistent with observations that constitutively active HaRas leads only to partial activation of JNK but maximally activates ERK and HaRas was not required for JNK activation by TNF (45). Recently, the small GTPases Rac and Cdc42Hs were shown to participate in signal transduction through the activation of JNK and p38 but not ERK (56). In conclusion, the IL-1 or TNF activated signaling pathway that leads the induction of genes that are responsible for cartilage degradation includes activation of JNK and p38. Inhibition of these kinases may represent a novel therapeutic approach for arthritis.

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