TRANSLATIONAL PHYSIOLOGY

Differential requirement of members of the MAPK family for CCL2 expression by hepatic stellate cells

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Marra, Fabio, Wanda Delogu, Ilaria Petrai, Sabrina Pasta-caldi, Andrea Bonacchi, Eva Efsen, Sara Aleffi, Cristiana Bertolani, Massimo Pinzani, and Paolo Gentilini. Differential requirement of members of the MAPK family for CCL2 expression by hepatic stellate cells. *Am J Physiol Gastrointest Liver Physiol* 287: G18–G26, 2004. First published March 11, 2004; 10.1152/ajpgi.00336. 2003.—Hepatic stellate cells (HSC) coordinate the liver wound-healing response through secretion of several cytokines and chemokines, including CCL2 (formerly known as monocyte chemoattractant protein-1). In this study, we evaluated the role of different proteins of the MAPK family (ERK, p38^{MAPK}, and JNK) in the regulation of CCL2 expression by HSC, as an index of their proinflammatory activity. Several mediators activated all three MAPK, including TNF, IL-1, and PDGF. To assess the relative role of the different MAPKs, specific pharmacological inhibitors were used; namely, SB203580 (p38^{MAPK}), SP600125 (JNK), and PD98059 (MEK/ERK). The efficacy and specificity of the different inhibitors in our cellular system were verified analyzing the enzymatic activity of the different MAPKs using in vitro kinase assays and/or testing the inhibition of phosphorylation of downstream substrates. SB203580 and SP600125 dose-dependently inhibited CCL2 secretion and gene expression induced by IL-1 or TNF. In contrast, inhibition of ERK did not affect the upregulation of CCL2 induced by the two cytokines. Finally, activin A was also found to stimulate CCL2 expression and to activate ERK, JNK, p38, and their downstream targets. Unlike in cells exposed to proinflammatory cytokines, all three MAPKs were required to induce CCL2 secretion in response to activin. We conclude that members of the MAPK family differentially regulate cytokine-induced chemokine expression in human HSC.

activin; chemokines; fibrosis; platelet-derived growth factor

STUDIES CONDUCTED IN DIFFERENT laboratories have highlighted the importance of hepatic stellate cells (HSC) in the pathophysiology of the liver response to injury (30). HSC are not only the major matrix-producing cells during chronic liver injury, but they contribute to the modulation of the liver "wound-healing" response through several biological actions. A critical aspect of acute and chronic tissue damage is represented by the recruitment of inflammatory cells, and HSC have been shown to modulate the inflammatory response via secretion of several soluble mediators that regulate the recruitment and activation of leukocytes (reviewed in Ref. 20). In general, low expression of these mediators in quiescent HSC becomes dramatically upregulated on activation, suggesting that the modulation of inflammation occurs in conditions associated with tissue injury and the transition of HSC to a myofibroblast-like phenotype.

The chemokine family of cytokines is a large group of proteins capable of regulating migration of target cells via activation of specific membrane receptors (1). Chemokine receptors were initially identified on leukocytes, and several studies (25) have investigated the role of chemokines in the regulation of inflammatory cell recruitment in conditions of liver injury. However, chemokine receptor expression is not confined to leukocytes, and these proteins are involved in a large number of pathophysiological conditions, including fibrosis, immune response, and development of angiogenesis and cancer (1). CCL2 (formerly known as monocyte chemoattractant protein-1 or MCP-1) is a CC chemokine that recruits monocytes and lymphocytes in several inflammatory conditions, including liver injury. CCL2 is the major chemoattractant for monocytes produced by activated HSC, because >80% of the chemotactic activity of the conditioned medium is blocked by neutralizing antibodies against this chemokine (25). Thus identifying the mechanisms of regulation of CCL2 expression in HSC is of particular relevance for the biology of these cells.

MAPKs are a group of intracellular signaling proteins activated by multiple signals and are critically involved in the transmission of information from outside the cell to the nucleus, where they regulate transcription. ERK, JNK, and p38^{MAPK} are three widely expressed MAPKs that regulate different cellular functions (14). All these molecules share a common mechanism of activation, based on a kinase cascade initiated by a small G protein of the Ras superfamily. Downstream activation of an MAPK kinase kinase (e.g., Raf-1 for the ERK cascade) leads to phosphorylation of a dual specificity kinase (MAPK kinase, e.g., MEK-1), which results in phosphorylation of the actual MAPK on serine/threonine and tyrosine residues. This event is associated with activation of the MAPK and its translocation to the nucleus, where it phosphorylates downstream targets (14). In this study, we investigated the role of these three major members of the MAPK family in mediating the expression of CCL2 in HSC exposed to different cytokines. The results reported herein indicate that members of the MAPK family differentially regulate chemokine expression in human HSC exposed to soluble mediators.

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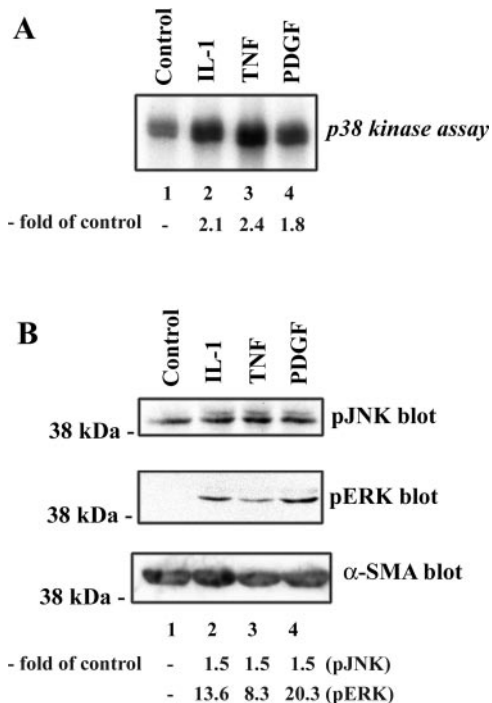
Fig. 1. Activation of MAPKs by different cytokines in human hepatic stellate cells (HSC). Subconfluent, cultured human HSC were serum deprived and exposed to IL-1α (20 ng/ml), TNF-α (100 ng/ml), or PDGF-BB (10 ng/ml) for 15 min. Cell lysates were used for analysis of p38^{MAPK} kinase activity (*A*) or analyzed by immunoblotting for the phosphorylated forms of ERK and JNK (*B*). The membrane was reblotted for α-smooth muscle actin (α-SMA) to show equal protein loading.

MATERIALS AND METHODS

Materials

Recombinant, human IL-1α, TNF-α, and PDGF-BB were purchased form Peprotech (Rocky Hill, NJ). Recombinant human activin-A was kindly donated by Dr. Albert F. Parlow (National Hormone and Peptide Program, Harbor-University of California at Los Angeles Medical Center, Torrance, CA) and by Dr. Yuzuro Eto (Ajinomoto, Tokyo, Japan). Monoclonal antibodies against α-smooth muscle actin (clone 1A4) were purchased from Sigma (St. Louis, MO). Recombinant ATF-2 and polyclonal antibodies against the phosphorylated form of ERK, JNK, p38^{MAPK}, heat-shade protein-27 (HSP27), and c-Jun were from New England BioLabs (Beverly, MA). Polyclonal antibodies against JNK and p38^{MAPK}, and anti-ERK antibodies used for Western blot analysis were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-ERK antibodies used for immune complex kinase assay were from Upstate Biotechnology (Lake Placid, NY). Rabbit antibodies directed against baboon CCL2 (100% cross-reacting with human CCL2) were a kind gift of Dr. Anthony J. Valente (University of Texas, Health Science Center at San Antonio, San Antonio, TX). The plasmid encoding for the fusion protein glutathione *S*-transferase (GST)-Jun was kindly provided by Dr. Michael Karin (University of California, San Diego, CA). Radionuclides were purchased from ICN (Costa Mesa, CA). All other reagents were of analytical grade.

Cell Culture

Human HSC were isolated from wedge sections of normal liver tissue unsuitable for transplantation by collagenase/pronase digestion and centrifugation on stractan gradients. The cells were cultured in Iscove's modified Dulbecco's medium supplemented with 17% fetal

calf serum and characterized as described previously (6). After isolation, cells were plated on uncoated plastic dishes and grown until confluent. HSC used in these experiments were between *passages 3* and *6* showing an "activated" or "myofibroblast-like" phenotype. For all experiments, confluent cells were deprived of serum for 24 h before addition of the appropriate stimuli.

Analysis of CCL2 Secretion

Confluent HSC cultures in 24-well plates were serum deprived for 24 h, and after replacement of the medium with fresh serum-free medium, they were exposed to MAPK inhibitors for 15 min and then to cytokines for 24 h. At the end of the incubation, the medium was collected and stored at −20°C until assayed. CCL2 secretion in the conditioned medium was measured using Western blot analysis, as previously described (23). Briefly, 50–100 µl of conditioned medium were dried, separated by 15% SDS-PAGE, and electroblotted on a polyvinylidene difluoride membrane. The membranes were blocked overnight at 4°C with 2% bovine serum albumin in 0.1% PBS-Tween and then sequentially incubated at room temperature with an antiserum against baboon CCL2 (1:1,000) and with a horseradish peroxidase-conjugated secondary antibody. Detection was carried out using chemiluminescence according to the manufacturer's protocol (Amersham, Arlington Heights, IL). This technique has been shown to detect as little as 1 ng of human CCL2 and has been validated using a commercially available ELISA kit (23). Moreover, in selected experiments, the results obtained by Western blot analysis were confirmed using an ELISA kit (Biosource, Camarillo, CA).
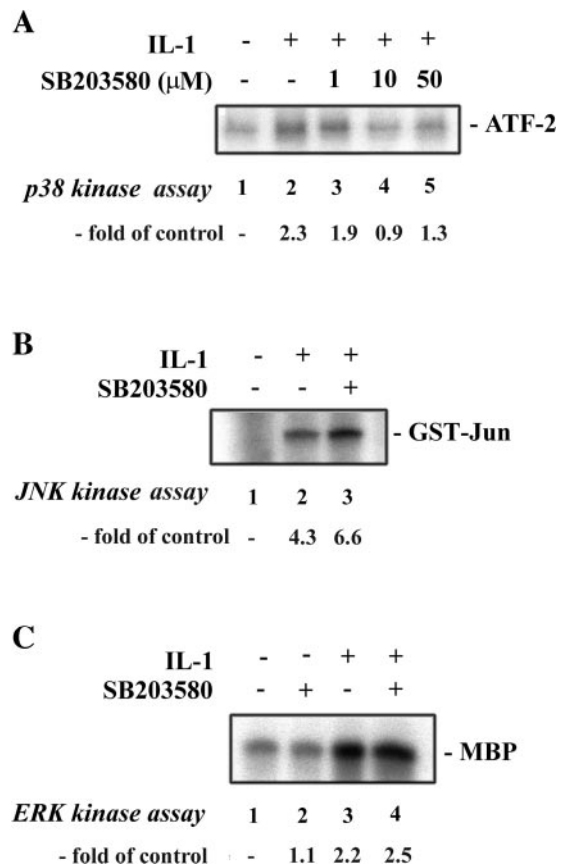

Fig. 2. SB203580 specifically inhibits p38^{MAPK}. HSC were serum deprived for 24 h and incubated with the indicated concentrations (*A*) or with 50 µM (*B* and *C*) SB203580 for 15 min, before exposure to 20 ng/ml IL-1α for an additional 15 min. Cell lysates were used for analysis of the enzymatic activity of p38^{MAPK} (*A*), JNK (*B*), or ERK (*C*) as described in MATERIALS AND METHODS. MBP, myelin basic protein, GST, glutathione *S*-transferase.

Northern Blot Analysis and RNAse Protection Assay

Isolation of total RNA and Northern blot analysis was performed using a previously described methodology (11). Total RNA (10–15 μg) was used in each experiment. Before the transfer, the ethidium bromide-stained gel was photographed to check for equal RNA loading. For RNAse protection assay, 2–10 μg of total RNA were used together with a commercially available kit from BD Pharmingen (Palo Alto, CA). ^{32}P-labeled cRNA was transcribed from MultiProbe templates (BD Pharmingen), according to the manufacturer's instructions. After hybridization, protected fragments were separated on a sequencing gel and autoradiographed.

Western Blot Analysis of Cellular Proteins

Confluent, serum-starved HSC were treated with the appropriate conditions, quickly placed on ice, and washed with ice-cold PBS. The monolayer was lysed in RIPA buffer [20 mM Tris·HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM phenyl methyl sulfonyl fluoride, 0.05% (wt/vol) aprotinin] and transferred to microcentrifuge tubes. Insoluble proteins were discarded by centrifugation at 12,000 rpm at 4°C. Protein concentration in the supernatant was measured in triplicate using a commercially available assay (Pierce, Rockford, IL). Equal amounts of total cellular proteins were separated by SDS-PAGE and analyzed by Western blot using the specified antibodies.

Immune Complex Kinase Assays

ERK assay. ERK was immunoprecipitated from 25 μg of total cell lysate using polyclonal anti-ERK antibodies and protein A sepharose. After being washed, the immunobeads were incubated in a buffer containing 10 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.5 mM dithiotreitol, 0.5 mM Na_3VO_4, 25 μM ATP, 1 μCi [γ-^{32}P]ATP, and 0.4 mg/ml myelin basic protein for 30 min at 30°C. At the end of the incubation, the reaction was stopped by the addition of Laemmli buffer and run on 15% SDS-PAGE. After electrophoresis, the gel was dried and autoradiographed.

JNK assay. This assay was performed as described in detail previously (28). Briefly, proteins were immunoprecipitated with polyclonal anti-JNK antibodies (Santa Cruz Biotechnology). After being washed, immunobeads were incubated in reaction buffer (20 mM HEPES, pH 7.6, 20 mM $MgCl_2$, 2 mM DTT, 25 mM β-glycerophosphate, and 0.1 mM sodium orthovanadate) containing 20 mM ATP, 5 μCi [γ-^{32}P]ATP, and 1 μg recombinant ATF-2 (Santa Cruz Biotechnology) or GST-Jun for 30 min at 37°C. The reaction was stopped by the addition of Laemmli buffer and was then subjected to 10% SDS-PAGE.

Fig. 3. Specificity of the 3 different MAPK inhibitors in HSC. Serum-deprived HSC were preincubated with the indicated concentrations of SB203580, PD98059, or SP600125 and then exposed to 100 ng/ml TNF-α for 15 min. Total cell lysates were used for immunoblotting against the phosphorylated form of heat-shock protein-27 (HSP27; *A* and *B*), assay of JNK activity (*C*), or immunoblotting against the phosphorylated form of ERK (*D*). In *A*, *B*, and *D*, the membranes were reblotted for total p38^{MAPK} or ERK to show equal protein loading.
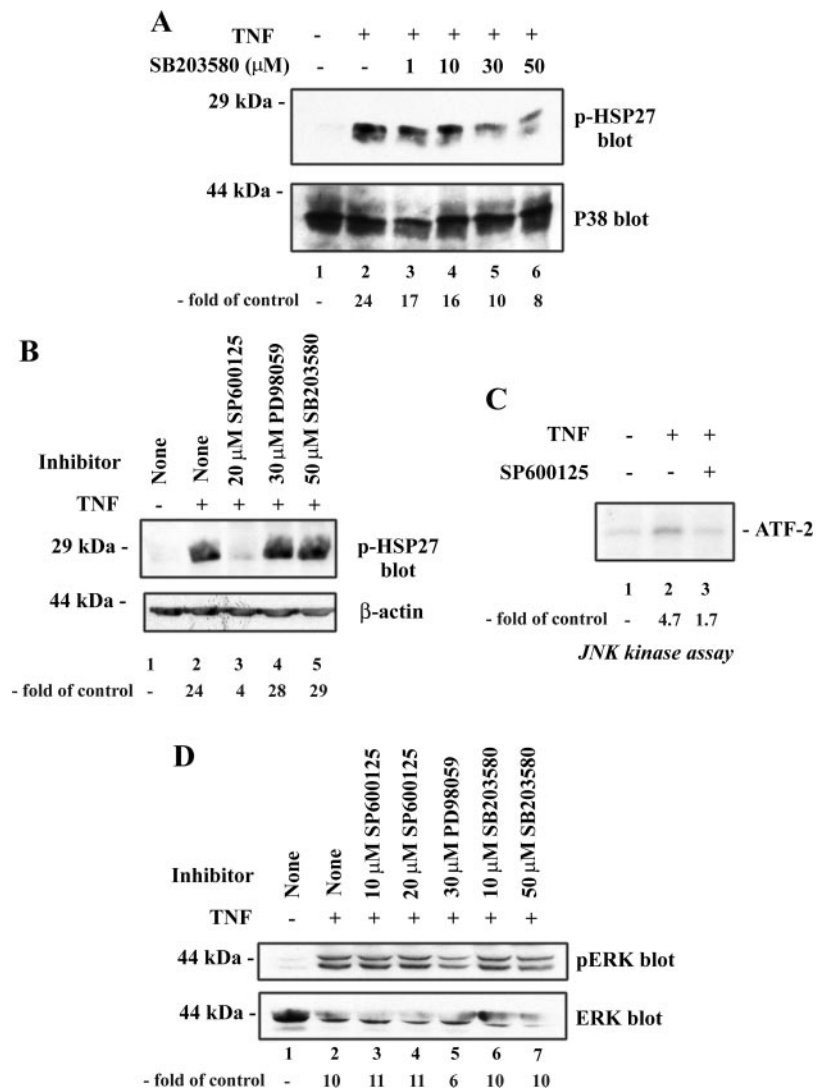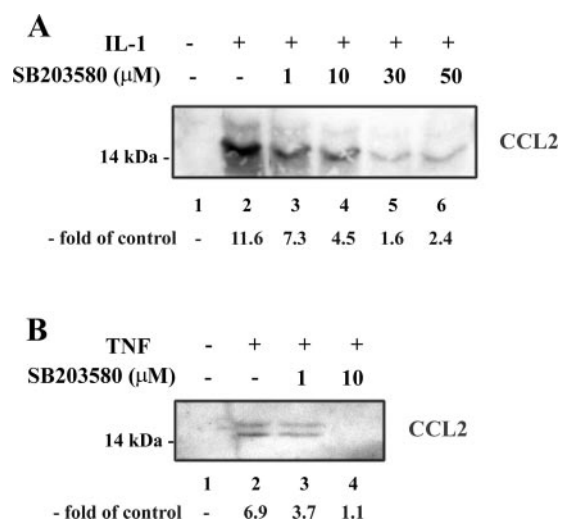
Fig. 4. Inhibition of p38^{MAPK} blocks cytokine-induced secretion of CCL2. Serum-deprived HSC were preincubated with the indicated concentrations of SB203580 and then exposed to IL-1 (20 ng/ml; *A*) or TNF (100 ng/ml; *B*) for 24 h. Cell-free supernatants were analyzed by Western blot analysis using antibodies against CCL2, as described in MATERIALS AND METHODS.

p38^{MAPK} assay. This assay was carried out as described for JNK assay, except for the use of anti-p38 polyclonal antibodies for immunoprecipitation and the constant use of ATF-2 as a substrate.

Data Reproducibility

All autoradiograms and autoluminograms are representative of at least three experiments with comparable results.

RESULTS

Activation of MAPK represents a common signaling pathway for a number of cytokine receptors. We analyzed the ability of two proinflammatory cytokines, TNF and IL-1, to induce activation of three major members of the MAPK family in human HSC (Fig. 1). Both cytokines were able to induce activation of the three kinases, as indicated by immune complex kinase assays of p38^{MAPK} activity and by increased phosphorylation of ERK and JNK, as an index of activation. Interestingly, a similar pattern of activation was observed when HSC were exposed to PDGF, a polypeptide mitogen whose biological actions differ from those of TNF and IL-1 (29). In fact, the three cytokines similarly activated MAPK members, although PDGF was more effective in activating ERK, whereas proinflammatory cytokines more potently induced JNK and p38^{MAPK} (Fig. 1 and data not shown).

The three pathways comprising the MAPK family are activated via different signal cascades that result in activation of each individual MAPK. We first analyzed the ability of widely used phamacological inhibitors of the different MAPK pathways to specifically inhibit these systems in human HSC. At the concentrations used in these studies, no toxic effects on cultured HSC were observed analyzing trypan blue exclusion after incubation with the different drugs for as long as 24 h. SB203580 inhibits the catalytic activity of p38^{MAPK} by competitive binding in the ATP pocket. (18). We assessed the specificity of inhibition of SB203580 in our system by testing the effects of this compound on the enzymatic activity of the different MAPK. SB203580 clearly inhibited the activation of

p38^{MAPK} induced by IL-1 (Fig. 2*A*), with complete inhibition between 10 and 50 μM, as previously reported (8). At 50 μm, SB203580 did not exert any inhibitory action on the other two MAPK, ERK and JNK, as indicated by immune complex kinase assay experiments (Fig. 2, *B* and *C*).

To test the contribution of the other members of the MAPK family, we employed two other phamacological inhibitors developed to achieve specific inhibition of these pathways. PD98059 inhibits MEK-mediated phosphorylation of ERK, thereby blocking the activation of this latter molecule (10). On the other hand, the recently developed inhibitor of JNK, SP600125, blocks the enzymatic activity of the kinase by binding to the ATP-binding site (2). To confirm their specificity of action in our cell system, we tested the ability of the three drugs to inhibit phosphorylation of target molecules located downstream of MAPK. Thus SB203580 blocked TNF-induced phosphorylation of HSP27 (Fig. 3*A*), a member of the HSP family that has been shown to be a direct target of p38^{MAPK} kinase activity (8). Furthermore, when TNF-stimulated cells were preincubated with the inhibitors or ERK or JNK, no effects on HSP27 phosphorylation were observed, thus showing that neither PD98059 nor SP600125 significantly inhibits
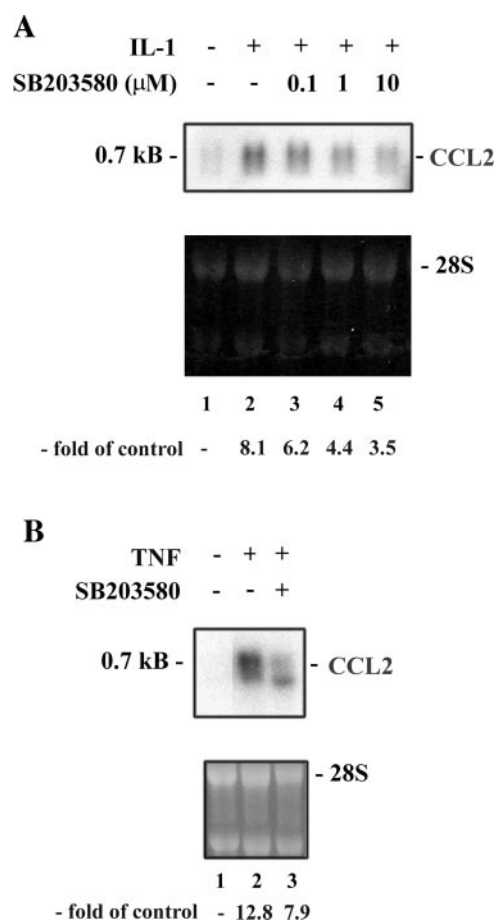

Fig. 5. Inhibition of p38^{MAPK} blocks cytokine-induced gene expression of CCL2. Serum-deprived HSC were preincubated with the indicated concentrations (*A*) or with 50 μM (*B*) SB203580 for 15 min and then exposed to 20 ng/ml IL-1 or 100 ng/ml TNF for 4 h. Total RNA was analyzed by Northern blot analysis using a radiolabeled cDNA probe encoding for CCL2. The bottom part of each panel shows a photograph of the ethidium bromide-stained gel before transfer, indicating equal RNA loading.

the activity of p38^{MAPK}. On the other hand, the inhibitory action of SP600125 on JNK was confirmed, demonstrating a reduction in kinase activity in a direct enzymatic assay (Fig. 3*C*). Finally, whereas PD98059 effectively reduced ERK phosphorylation, an index of activation, no effects on ERK activation were observed incubating the cells with the other inhibitors (Fig. 3*D*). In aggregate, these experiments indicate that the available phamacological inhibitors of the MAPK cascade are reliable tools for the assessment of the role of these pathways in the modulation of different biological activities in HSC.

We next evaluated the contribution of MAPK activation to the proinflammatory actions of activated HSC, analyzing the expression and secretion of the chemokine CCL2. Because p38^{MAPK} was originally identified as a molecule regulating cytokine production (19), we first analyzed the contribution of
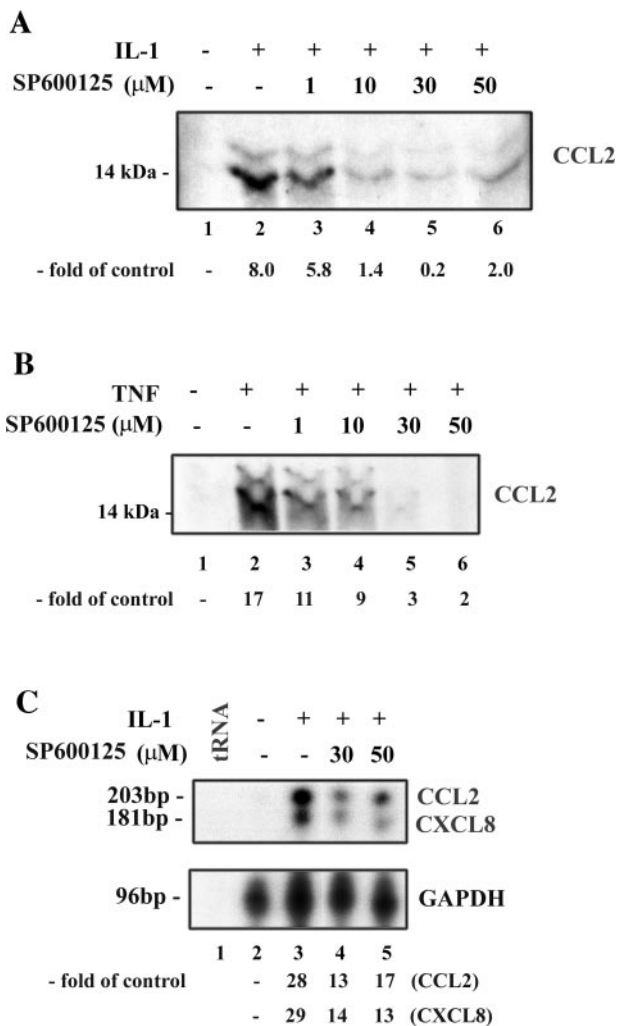

Fig. 6. Inhibition of JNK blocks cytokine-induced expression of CCL2. Serum-deprived HSC were preincubated with the indicated concentrations of SP600125 and then exposed to IL-1 (20 ng/ml; *A*) or TNF (100 ng/ml; *B*) for 24 h. Cell-free supernatants were analyzed by Western blot analysis using antibodies against CCL2, as described in MATERIALS AND METHODS. *C*: HSC were preincubated with the indicated concentrations of SP600125 for 15 min and then exposed to 20 ng/ml IL-1 for 4 h. Total RNA was analyzed by RNAse protection assay a radiolabeled cRNA probe encoding for CCL2, CXCL8, and GAPDH, as described in MATERIALS AND METHODS. The size of the protected fragments is indicated on the *left*. In *lane 1*, the radiolabeled probe was hybridized with tRNA as a negative control.
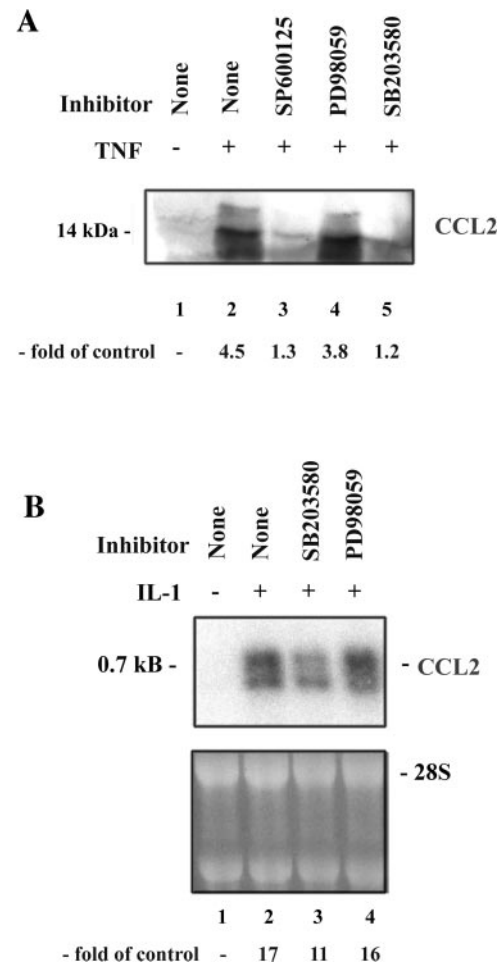

Fig. 7. Inhibition of ERK does not affect cytokine-induced expression of CCL2. *A*: serum-deprived HSC were preincubated with 20 μM SP600125, 50 μM PD98059, or 50 μM SB203580 and then exposed to TNF (100 ng/ml) for 24 h. Cell-free supernatants were analyzed by Western blot analysis using antibodies against CCL2, as described in MATERIALS AND METHODS. *B*: HSC were preincubated with 50 μM SB203580 or 50 μM PD98059 for 15 min and then exposed to 20 ng/ml IL-1 for 4 h. Total RNA was analyzed by Northern blotting as described in Fig. 5.

this MAPK on CCL2 expression (Fig. 4). Both IL-1 and TNF markedly increased CCL2 secretion, which was visualized by Western blot analysis as two major bands due to different glycosylation, as previously reported in these and other cells (25). Preincubation of HSC with micromolar concentrations of the p38^{MAPK} inhibitor SB203580 resulted in a complete and dose-dependent inhibition of CCL2 secretion in the conditioned medium. Remarkably, the dose response of the inhibitory action of SB203580 on CCL2 secretion was closely resembling the one exerted on HSP27 phosphorylation. To test whether this effect was associated with reduced gene expression for this chemokine, we analyzed the effects of SB203580 on steady-state mRNA levels for CCL2 after stimulation with proinflammatory cytokines (Fig. 5). The inhibitor of p38^{MAPK} determined a marked reduction in CCL2 mRNA, showing that reduced gene expression contributes to the effects of p38^{MAPK} inhibition.

We next established the possible contribution of the two other members of the MAPK family to cytokine-stimulated CCL2 expression. When HSC were exposed to increasing

concentrations of SP600125, the ability of IL-1 or TNF to induce CCL2 secretion was markedly blocked (Fig. 6*B*). Similarly, IL-1-dependent stimulation of CCL2 gene expression was reduced by SP600125 (Fig. 6*C*). Interestingly, the induction of CXCL8 (IL-8) expression by IL-1 was also inhibited, thus showing that JNK activation is necessary for induction of other members of the chemokine family. Conversely, exposure of HSC to PD98059, an inhibitor of the ERK pathway, at concentrations effective in blocking ERK activation and PDGF's action on DNA synthesis (22) did not modify cytokine-induced secretion or gene expression of CCL2 (Fig. 7). Thus, despite the fact that both IL-1 and TNF effectively activate ERK, this pathway is dispensable to obtain activation of CCL2 expression in response to these agonists.

Proinflammatory cytokines represent a well-established stimulus to chemokine expression in different cell types, including HSC. To further characterize the role of MAPK activation in the modulation of CCL2 expression, we investigated the actions of an unrelated cytokine. Activin A is a protein of the transforming growth factor (TGF)-β superfamily that has been shown to increase procollagen expression in rodent HSC (36). We investigated the effects of activin A on CCL2 secretion by HSC (Fig. 8). Incubation with increasing concentrations of activin A resulted in a clear induction of CCL2 expression (Fig. 8*A*), although to a much lower extent than with TNF or IL-1. In fact, the increase in CCL2 secretion in response to activin A was only between 1.5- to 3-fold over basal levels. Interestingly, when stimulation with activin A was associated with submaximal concentrations of proinflammatory cytokines, an additive effect was found (Fig. 8*B*). Conversely, no effects on DNA synthesis or cell number were observed in response to activin A (data not shown). Because activin binds to a receptor of the TGF-β superfamily, we analyzed the pattern of MAPK activation in HSC exposed to activin A. Also in this case, all three MAPK were stimulated to some extent by activin A, as demonstrated by increased phos-
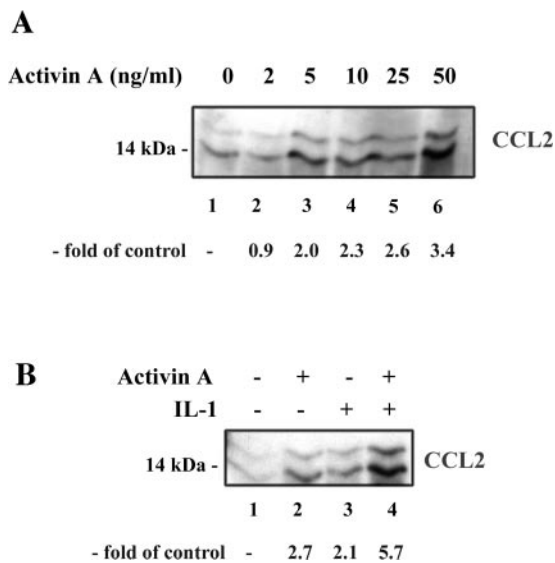
Fig. 8. Activin A induces CCL2 secretion. *A*: serum-deprived HSC were incubated with increasing concentrations of activin A, an indicated, for 24 h. Cell-free supernatants were analyzed by Western blot analysis using antibodies against CCL2. *B*: HSC were incubated with 50 ng/ml activin A and/or with 1 ng/ml IL-1 for 24 h, and CCL2 was analyzed by Western blot analysis.
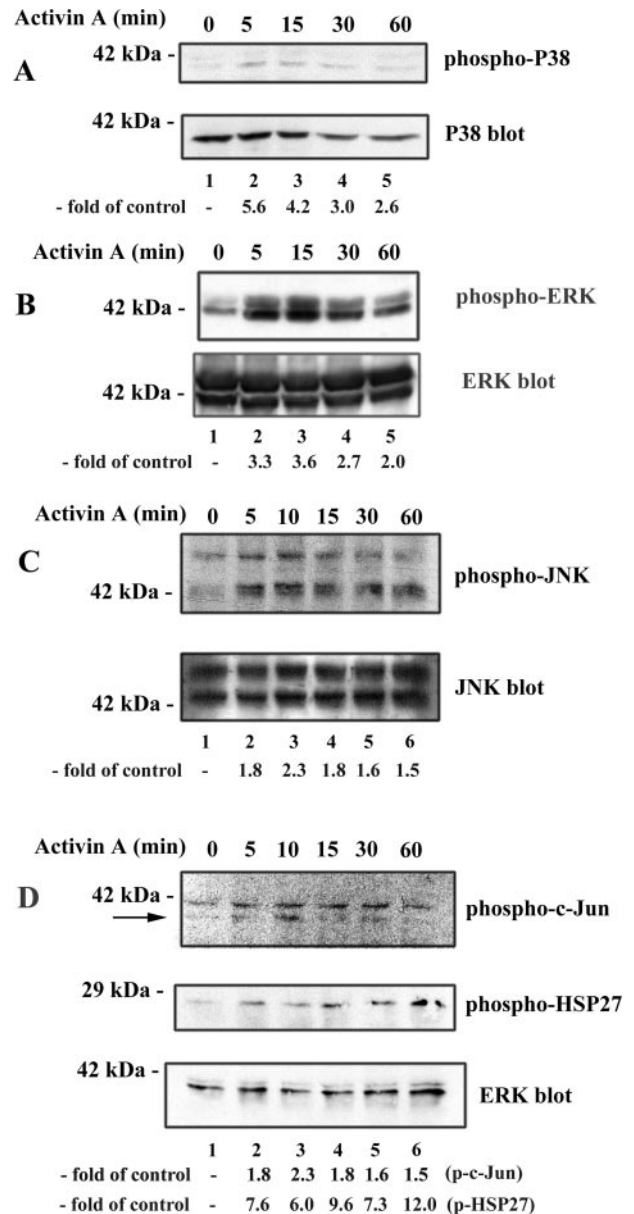
Fig. 9. Activin-mediated MAPK activation in HSC. Serum-deprived HSC were incubated with 50 ng/ml activin A for the indicated time points. Cell lysates were analyzed by immunoblotting with the indicated antibodies. See increased phosphorylation of ERK (*A*), p38^{MAPK} (*B*), and JNK (*C*). *D*: increased phosphorylation of HSP27 and c-Jun, targets of the kinase action of p38^{MAPK} and JNK, respectively.

phorylation of ERK (Fig. 9*A*), p38^{MAPK} (Fig. 9*B*), and JNK (Fig. 9*C*). Activation of these two latter pathways was also confirmed by the increased phosphorylation of HSP27 and c-Jun, targets of the kinase action of p38^{MAPK} and JNK, respectively (Fig. 9*D*).

To establish whether the role of the different MAPK was similar in activin-stimulated cell as in HSC exposed to proinflammatory cytokines, we evaluated the effects of the three inhibitors on CCL2 secretion. The increase in CCL2 secretion in the conditioned medium induced by activin A was completely blocked by the p38^{MAPK} inhibitor CCL2 secretion caused by this cytokine, as indicated by Western blot analysis and confirmed by ELISA assays (Fig. 10, *A* and *B*). Similarly,
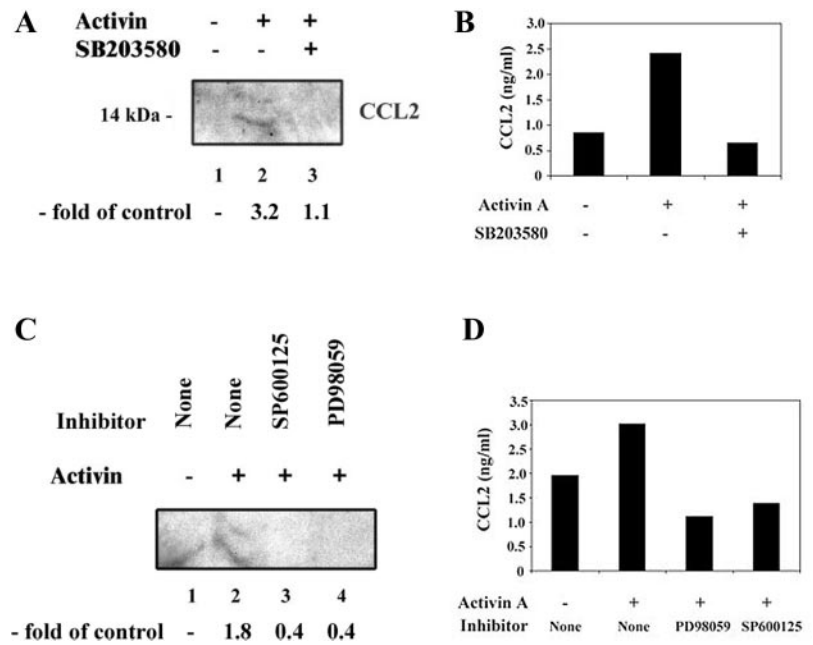
Fig. 10. Inhibition of p38^{MAPK} blocks cytokine-induced secretion of CCL2. *A*: HSC were preincubated with the 50 μM SB203580 and then exposed to activin A (100 ng/ml) for 24 h. Cell-free supernatants were analyzed by Western blot analysis using antibodies against CCL2. *B*: an alternative experiment with cells treated as in *A* was assayed using a specific ELISA on cell-conditioned medium. *C*: HSC were preincubated with the 30 μM SP600125 or 30 μM PD98059 and then exposed to activin A (100 ng/ml; *A*) for 24 h. Supernatants were analyzed by Western blot analysis using antibodies against CCL2. *D*: conditioned media collected from cells treated as in *C* were analyzed by ELISA (mean of 2 independent experiments).

preincubation of HSC with the inhibitors of JNK or MEK prevented the effects of Activin A (Fig. 10, *C* and *D*). Thus the molecular mechanisms underlying activin-mediated expression of CCL2 differ from those involved in cells exposed to proinflammatory cytokines, because all three members of the MAPK family are required when activin A is used as an agonist.

DISCUSSION

CCL2 is an "inflammatory" chemokine that recruits and activates monocytes and T cells. Increased expression of CCL2 has been reported in patients with different types of liver disease and in experimental models of acute and chronic liver injury (21). Modulation of CCL2 expression is associated with reduced inflammation in several conditions of liver injury, including carbon tetrachloride intoxication or parasitic liver injury. Nevertheless, the role of this chemokine in liver pathophysiology is likely to be more complex, because mice deficient for CCR2, the receptor for CCL2, show exacerbation of liver damage during acetaminophen intoxication (12). In addition, experimental evidence indicates that in different matrix-producing cells, CCL2 induces biological responses that favor tissue repair (34, 37). Thus this chemokine may be considered not just as a proinflammatory mediator, but also as a profibrogenic cytokine. HSC are an example of the bidirectional regulation played by the chemokine system, because exposure of cultured HSC to several chemokines, including CCL2, results in stimulation of cell migration and activation of intracellular signaling (3, 4, 24). Because CCL2 and other chemokines acting on HSC are expressed at high levels in areas of active fibrogenesis, this system may represent a molecular mechanism linking fibrosis and inflammation during chronic liver injury. Therefore, unraveling the molecular mechanisms underlying CCL2 expression is relevant to understanding the development of liver inflammation and fibrosis. The results of this study provide information on the role of three major members of the MAPK family, ERK, p38^{MAPK}, and JNK, in

the regulation of CCL2 expression in HSC. The MAPKs are activated by a wide group of stimuli, including soluble mediators, but the pattern of activation of the different members varies according to the cell type and the stimulus used. In HSC, proinflammatory cytokines that effectively induce CCL2 expression, such as IL-1 and TNF-α, resulted in activation of all three MAPK members. To dissect the relative contribution of each of these kinases, we used specific pharmacological inhibitors, and assessed the efficacy and specificity of their action in this cellular system. When tested on HSC, all three drugs effectively inhibited a specific pathway without affecting signals downstream of other members of the family. These data demonstrate that pharmacological inhibition is a reliable tool to analyze the role of signaling molecules in this setting. Using this approach, we found that activation of p38^{MAPK} and JNK is required for CCL2 secretion and gene expression, whereas inhibition of ERK had no effects. p38^{MAPK} and JNK belong to the subgroup of stress-activated protein kinases because they may be activated by conditions such as proinflammatory cytokines, ultraviolet light, DNA damage, and osmotic stress (17). p38^{MAPK} activation is necessary for secretion of cytokines and chemokines in many cellular systems, but the downstream actions of this signaling molecule have not been completely elucidated. Even without inhibiting the binding of NF-κB to target DNA elements, p38^{MAPK} may interfere with NF-κB-dependent transcription via modification of the transactivation domain of p65 (16, 38). In addition, the p38^{MAPK} pathway has been recently shown to regulate the accessibility to NF-κB of the promoters of some genes, including CCL2, via histone acetylation (31). Because NF-κB activation critically regulates chemokine expression in HSC stimulated with IL-1 or TNF (11), this mechanism is likely to mediate, at lest in part, the effects of p38^{MAPK}. In addition, p38^{MAPK} has been shown to upregulate the expression of CCL2 in human monocytes by inducing the formation of an AP-1 complex containing c-Jun and ATF-2 (5) and to be required for AP-1-dependent upregulation of CCL2 in endothelial cells (7). A further action of

p38^{MAPK} is to promote translation of mRNAs acting on the elongation factor 3eIFA through activation of MNK-1 and -2 (17). However, in the present study, we observed that SB203580 inhibits steady-state mRNA levels of CCL2, indicating that an action at the sole posttranscriptional level is not sufficient to explain the mechanism of inhibition in this cell type.

Activation of JNK leads to phosphorylation of c-Jun and increased transcription via formation of AP-1 complexes. Several AP-1 binding sites have been described in the CCL2 promoter, and interference with binding of this transcription factor results in a reduced ability to stimulate transcription of this chemokine (26). Interestingly, although the activation of ERK also leads to formation of the AP-1 complex (22), interfering with ERK did not result in inhibition of CCL2 expression when HSC were treated with the proinflammatory cytokines TNF or IL-1. A potentially relevant difference is that the regulation of AP-1 formation in response to ERK activation is mainly mediated by enhancement of expression of AP-1 components, whereas JNK and p38^{MAPK} can recruit AP-1 through the direct phosphorylation of AP-1 components such as c-Jun and ATF-2 (17). An alternative, and not mutually exclusive, hypothesis is that additional signals generated by JNK activation are necessary to enable the cells to increase CCL2 gene and protein expression in response to proinflammatory cytokines. Interestingly, Schwabe et al. (32, 33) have recently demonstrated that activation of JNK contributes to chemokine induction in response to CD40 ligation or activation of toll-like receptor-4 in HSC. These data confirm the relevance of JNK in the pathway leading to chemokine expression in this cell type.

An additional novel finding provided by the present study concerns the biological effects of activin A and its downstream signaling. Activins belong to the TGF-β superfamily, but their role in HSC biology is only partially understood. Activin A has been shown to upregulate collagen expression in rodent HSC, thus participating in liver fibrogenesis (9, 36). Data from this study indicate that the induction of proinflammatory cytokines, such as CCL2, is an additional effect of activin A that could contribute to modulation of the liver wound-healing response. Interestingly, activation of all three MAPKs, including ERK, was required to obtain an induction of CCL2 expression by activin A. Also, in other systems, ERK activation has been shown to be necessary to achieve full stimulation of the expression of CCL2 or other chemokines (15, 35). The requirement for ERK in activin-stimulated cells could be related to the fact that activin is a much weaker stimulus of CCL2 expression than proinflammatory cytokines, and therefore, only the convergence of several signaling pathways may result in effective stimulation. Alternatively, the contribution of other pathways interfering with ERK, such as the Smads, may be critical in activin-stimulated cells (27). Taken together, these data confirm that the molecular mechanisms regulating CCL2 expression are cell and agonist specific and identify the MAPKs as key regulators of chemokine expression in HSC. This group of signaling molecules may therefore represent a potential target for phamacological treatment of conditions associated with upregulation of chemokine expression and excessive inflammation. As drugs interfering with the activation of p38^{MAPK} and JNK are entering the clinical arena for the treatment of chronic inflammatory conditions (13), these in vitro data may help to better understand their possible action in chronic liver injury and to establish endpoints for clinical studies.

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