Direct Hepatotoxic Effect of KC Chemokine in the Liver Without Infiltration of Neutrophils

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KC is a mouse homolog of human chemokine gro-α (CXCL1), expression of which is increased in liver diseases. We show that activated, but not quiescent, hepatic stellate cells (HSCs) express KC. Hepatic stellate cells constitutively express the KC receptor, CXCR2. Addition of recombinant KC to HSCs undergoing activation in culture increases secretion and processing of Type I collagen. Overexpression of endogenous KC in the mouse liver could be achieved by an intraperitoneal injection of CCl_4, followed after 24 hrs by an injection of recombinant KC into circulation. This protocol resulted in about a 14-fold increase in concentration of KC protein in the liver. Overexpression of KC was associated with upregulation of the mRNA for CXCR2 and MIP-2 and with necrosis and increased synthesis of Type I collagen. This suggests that KC has a direct hepatotoxic effect, which led to a massive liver necrosis after 48 hrs. No accumulation of neutrophils was seen in the livers as judged by histology and reverse transcriptase-polymerase chain reaction analysis of myeloperoxidase mRNA. Autostimulation of KC and CXCR2 expression by recombinant KC protein in the mice with preexisting liver injury indicates a positive feedback regulation. Such regulation and direct hepatotoxicity of KC with increased collagen synthesis represent novel findings about the role of KC/gro-α in liver pathology. Exp Biol Med 230:573–586, 2005

Key words: KC; chemokine; liver; fibrosis

Introduction

Liver fibrosis is characterized by the accumulation of extracellular matrix proteins in the liver, including Type I collagen (1, 2). Hepatic stellate cells (HSCs; also called Ito cells, lipocytes, or fat-storing cells) are the major cell type responsible for collagen synthesis in the fibrotic liver (3). In normal liver, quiescent HSCs store vitamin A, but only express trace amounts of Type I collagen. On fibrogenic stimulus, HSCs become activated, a process in which they lose retinoid droplets, proliferate, change morphologically into myofibroblasts, and increase their synthesis of extracellular matrix proteins (4–6). The most common causative agents of liver fibrosis induce chronic inflammation, and persistent inflammation leads to fibrosis (7, 8). Therefore, one of the goals of antifibrotic therapy is to control chronic inflammation. The molecular events linking inflammation and fibrosis are still poorly understood. Profibrogenic agents in the liver cause destruction of hepatocytes and activation of Kupffer cells. Damaged hepatocytes and activated Kupffer cells secrete proinflammatory cytokines and chemokines, which recruit immune cells and increase inflammation (9–13). Initially, this response is aimed to heal the damaged tissue, but after a prolonged inflammation, fibrosis becomes persistent and progressive, resulting in loss of the liver function. Activated Kupffer cells produce interleukin (IL)-1, IL-6, tumor necrosis factor α (TNFα), and transforming growth factor β (TGFβ) (14–16). Hepatocytes (17, 18) and endothelial cells (19, 20) can also be a source of IL-1, IL-6, and TNFα in the injured liver.

Gro-α chemokine (CXCL1) was first described as a growth factor for human melanoma cell line 294T and was termed melanoma growth–stimulating activity (MGSA) (21). Subsequent studies have shown that a physiological role of gro-α is chemotaxis of neutrophils to the site of tissue injury (22, 23). Two studies have shown that gro-α can also be chemotactic to monocytes and T lymphocytes (24, 25). No other functions of this chemokine have been described. Gro-α is expressed by most cell types; in the liver, this includes endothelial cells, (26) Kupffer cells, (27) HSCs (28), and hepatocytes (18). There are three genes for gro proteins in humans; *gro-α*, *gro-β*, and *gro-γ*, encoding proteins that share 85% identity at the amino acid level (29). Gro-α belongs to the CXC family of chemokines, with the best studied member of this family being IL-8 (30, 31). Functional differences have been described between IL-8 and gro-α, although they share 44% identity including the ELR motif at

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the amino terminus followed by the CXC motif (where X can be any amino acid) (32). Both IL-8 and gro-α bind with high affinity to the same receptor, CXCR2; however IL-8 also binds with high affinity to the CXCR1 receptor, whereas binding of gro-α to CXCR1 is of low affinity (33, 34). The CXCR2 receptor is expressed on neutrophils, monocytes, and T cells, but in contrast to the CXCR1 receptor, also on Purkinje cells, neurons, and astrocytes (35–37). There is only one *gro* gene in mouse encoding for the protein named KC. KC is 68% identical to human gro-α and 88% identical to rat cytokine-induced neutrophil attractant (CINC1) protein (38). Two more CXC chemokines have been described in rats, CINC2 and MIP-2, whereas mice have only MIP-2 (39–41). These chemokines share about 60%–65% identity to KC and about 40% identity to IL-8.

Liver diseases that are associated with increased infiltration of neutrophils include alcoholic hepatitis (42), ischemia/reperfusion after liver transplantation or resection (43), and endotoxemia (44). Maltby *et al.* (45) have described increased gro-α levels in liver homogenates of patients with alcoholic hepatitis, which correlated with infiltration of neutrophils in the liver. However, IL-8 was similarly increased in these samples, so it was not possible to address which chemokine was the main chemotactic factor. Another study has confirmed increased IL-8 in livers of patients with alcoholic hepatitis (46). Mice overexpressing human IL-8 did not have extravasation of neutrophils into the liver parenchyma (47), although mouse neutrophils show chemotaxis to human IL-8 *in vitro* (33). Other animal studies have suggested a role of KC in liver injury. When mice were injected with acetaminophen, KC mRNA was induced severalfold in their livers. The increase was evident 12 hrs after the acetaminophen injection (48). Lipopolysaccharide (LPS) is implicated in liver injury caused by alcohol in humans (49, 50). Perfusion of rat livers with LPS resulted in a 5- to 10-fold increase in CINC1 protein in the perfusate at 150 mins after the start of perfusion (51). Thus, these experiments have shown that liver injury is associated with increased production of KC.

In this paper we show that KC is upregulated in acute CCl_4-mediated liver injury and that recombinant KC has a profibrogenic effect on isolated HSCs. Overexpression of KC had a proinflamatory and prefibrotic effect on the liver *in vivo*, which resulted in greatly increased acute liver injury. Increased infiltration of neutrophils into the liver, however, was not observed.

Materials and Methods

Isolation of HSCs. Hepatic stellate cells were isolated from normal rat livers by perfusion with collagenase and pronase, followed by centrifugation over stractan gradient, as described (5), and RNA was extracted from freshly isolated quiescent HSCs. For *in vitro* activation, HSCs were cultured in uncoated plastic dishes in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Two days after isolation, recombinant KC (Cell Sciences, Canton, MA) was added at 50 ng/ml. From Day 3 to Day 8, medium was changed every day, and fresh recombinant KC was added. Cells were collected for RNA and protein extraction after the indicated time-points, and the cell medium was collected for protein analysis. For treatment with cycloheximide and puromycin, activated rat HSCs (8 days in culture) were incubated with 50 μg/ml of cycloheximide or 20 μg/ml of puromycin for 3 hrs, when total RNA was extracted for analysis.

Injection of CCl_4 and Recombinant KC into Mice. BALB/c male mice (25 g) were injected intraperitoneally with a single dose of 2 μl/g of CCl_4 in 50% mineral oil. Twenty four hours after the CCl_4 injection, 200 μl of recombinant KC (1395-KC/CF; R&D Systems, Minneapolis, MN) at 5 μg/ml was injected through the tail vein (total dose, 40 ng/g). Control animals received saline instead of KC. Twenty four and 48 hrs after the KC or saline injections, the livers were harvested for histology (hematoxylin and eosin staining) and RNA and protein analysis. For the experiment shown in Figure 1, mice received only CCl_4 or mineral oil, and livers were harvested after 3 days.

RNA Analysis. Total RNA was extracted from HSCs or mouse livers according to standard protocol (52). Poly-A+ RNA was isolated from HSCs or whole livers using Qiagen Oligotex mRNA kit. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the rTth kit from Perkin Elmer (Foster City, CA) in the presence of 32P-dCTP, as described (53). One hundred nanograms of total RNA and 20 ng of poly-A+ RNA were used in the RT-PCR reactions. The following primers were employed: KC, 5′-TCGCCAATGAGCTGCGCTGTC and 3′-GCTTCAGGGTCAAGGCAAGCC, which amplify the 160-nt product; CXCR2,

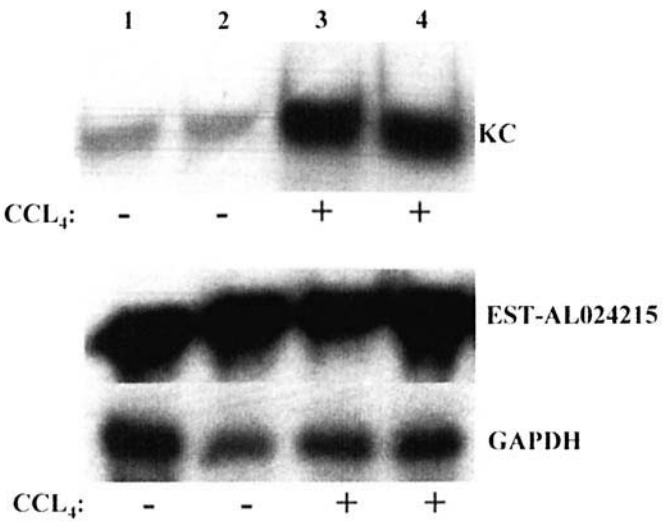


Figure 1. Upregulation of KC mRNA in acute liver injury. Total liver RNA was isolated 3 days after a single injection of CCl_4 (2 μl/g) into mice (lanes 3 and 4). Control mice received mineral oil (lanes 1 and 2). RNA was analyzed by RT-PCR with primers specific for KC mRNA (top panel) and for EST-AL024215 mRNA and GAPDH mRNA (controls; bottom panels). Migration of specific PCR products is indicated.

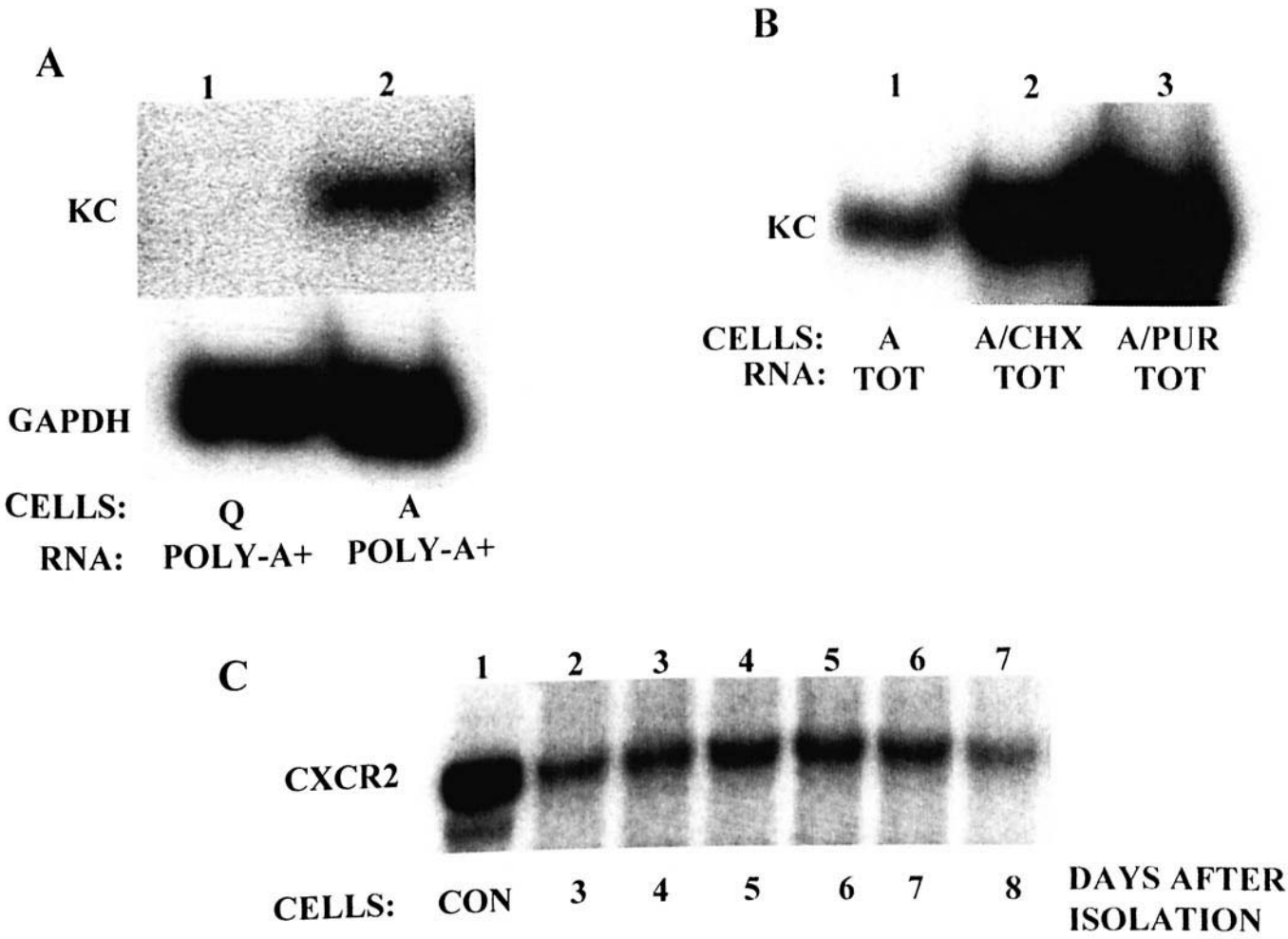


Figure 2. Expression of KC mRNA and CXCR2 receptor mRNA in isolated HSCs. (A) Expression of KC mRNA in HSCs. Poly-A+ RNA was extracted from freshly isolated rat HSCs (Q, lane 1) or HSCs cultured for 8 days (A, lane 2) and analyzed by RT-PCR specific for KC mRNA (top panel) or GAPDH mRNA (bottom panel) as an internal control. (B) Induction of KC mRNA by inhibitors of protein synthesis in HSCs. Total RNA from activated rat HSCs (A, lane 1) or activated HSCs treated with cycloheximide for 3 hrs (A/CHX, lane 2) or puromycin for 3 hrs (A/PUR, lane 3) was analyzed by RT-PCR with primers specific for KC. Migration of the specific PCR product is indicated. (C) Expression of CXCR2 mRNA in HSCs. Rat HSCs were cultured for the indicated time-points when total RNA was extracted and analyzed by RT-PCR with primers specific for CXCR2 mRNA (lanes 2–7). Lane 1 is total RNA from rat spleen analyzed with the same primers as a positive control.

5′-GCCTGTCTGGGCTGCATCTA and 3′-GTGTCTC-TTCTGGATCAGTG, which amplify the 180-nt product; EST-AL024215, 5′-ACAAGCCATGAAGCATGTGG and 3′-GGCTGCTATATAAGGAGATC, which amplify the 226-nt product; myeloperoxidase, 5′-CCGAATGACAAG-TATCGCAC and 3′-GGCACCTTGAAGCCATTGCG, which amplify the 170-nt product; IL-1α, 5′-GCAACGG-GAAGATTCTGAAG and 3′-TGACAAACTTCTGCCT-GACG, which amplify the 177-nt product; IL-1β, 5′-GCCCATCCTCTGTGACTCAT and 3′-AGGCCACAGG-TATTTTGTCG, which amplify the 230-nt product; TNFα, 5′-CGTCAGCCGATTTGCTATCT and 3′-CGGACTCCG-CAAAGTCTAAG, which amplify the 206-nt product; and MIP2, 5′-AAGTTTGCCTTGACCCTGAA and 3′-AGG-CACATCAGGTACGATCC, which amplify the 180-nt product. Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described before (53). Annealing and extension temperature was 50°C for all primers, and number of cycles was 20 for GAPDH, 30 for KC, IL-1α, IL-1β, TNFα, EST-AL024215, MIP2, and CXCR2, and 50 for myeloperoxidase. When poly-A+ RNA was used, 25 cycles were employed for determination of KC expression and 18 cycles for GAPDH expression. Polymerase chain reaction products were resolved on sequencing gels and visualized by autoradiography. The identity of PCR products was confirmed by sequencing.

RNase protection assays were done with templates for mouse collagen and GAPDH riboprobes as described (54). Templates for riboprobes for mouse cytokines and cytokine receptors were from Pharmingen (mCK-3b and mCR-6, respectively; San Diego, CA).

Western Blots. Western blots were performed using anticollagen antibody from Rockland (600-401-103; Gilbertsville, PA), as described (55). This antibody has been characterized in a previous paper (55). Fifty micrograms of total protein from HSCs and 200 μg of total liver proteins were loaded on the gel. As control, the samples were reprobed with anti-α

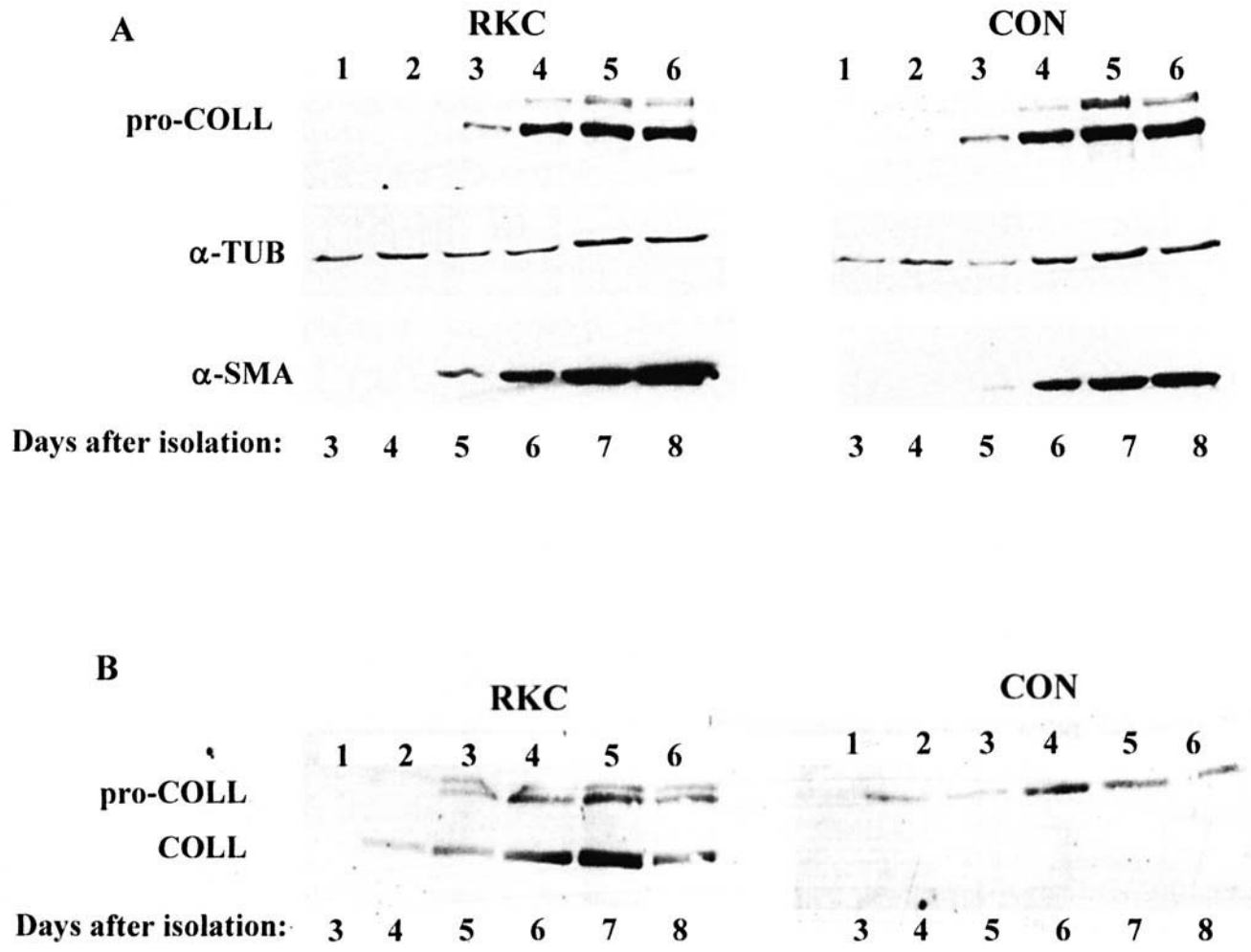


Figure 3 Effect of recombinant KC on HSCs activated in culture. (A) Effect of KC on cellular levels of procollagen α1(I) (pro-COLL) and α smooth muscle actin (α-SMA). KC was added at 50 ng/ml to HSCs after 2 days in culture, and cellular proteins were analyzed by Western blot at the indicated time-points (left panel). Right panel are the same cells without KC. Expression of α-tubulin (α-TUB) was used as internal control. Migration of the specific bands is indicated to the right. (B) Effects of KC on procollagen secreted into cell medium. Cell medium from the cells in A was analyzed for accumulation of procollagen by Western blot. Migration of 180-kda procollagen α1(I) (pro-COLL) and proteolitically processed 120-kDa collagen α1(I) (COLL) is indicated to the right.

tubulin antibody (Zymed, San Francisco, CA). For analysis of the cell medium of HSCs, 40 μl of medium, representing the equivalent number of cells, was loaded without previous concentration.

Liver Enzymes Measurement. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined in sera of three animals per group by the standard UV method using commercial reagents (Stanbio Laboratory, Boerne, TX). Results were expressed as units per liter, and the mean ± SEM is indicated.

KC Measurement by ELISA. Concentration of KC protein in sera and liver extracts was determined by ELISA (R&D Systems), according to manufacturer's protocol. Liver extracts were prepared by homogenizing liver in phosphate-buffered saline, following by removal of debris by centrifugation. Total protein concentration was estimated in each sample, and 200–500 μg was used for assay. Results for liver extracts were normalized per gram of protein and calculated per gram of liver tissue assuming that proteins represent 28% of wet liver weight (56, 57). Each sample was assayed in duplicate, and the average is shown in Figure 4C.

Results

KC mRNA Is Induced in Acute Liver Injury. To assess if KC expression is induced in acute liver injury, we injected mice with a single dose of CCl_4 (2 μl/g), and after 3 days, analyzed expression of KC mRNA in total liver by RT-PCR. As shown in Figure 1, top panel, there was a several-fold increase in the steady-state level of KC mRNA in two livers treated with CCl_4 (lanes 3 and 4) compared with control livers (lanes 1 and 2). A similar increase was seen in multiple experiments (see Fig. 4). As a control for RNA integrity, we amplified GAPDH mRNA and transcripts representing the expressed sequence tag AL024215. The latter encodes for a novel protein (designated as RIKEN cDNA 1810055D05). Both mRNAs were unchanged with CCl_4 treatment (Fig. 1, bottom panels). From these experiments, we concluded that KC mRNA is upregulated in acute liver necrosis.

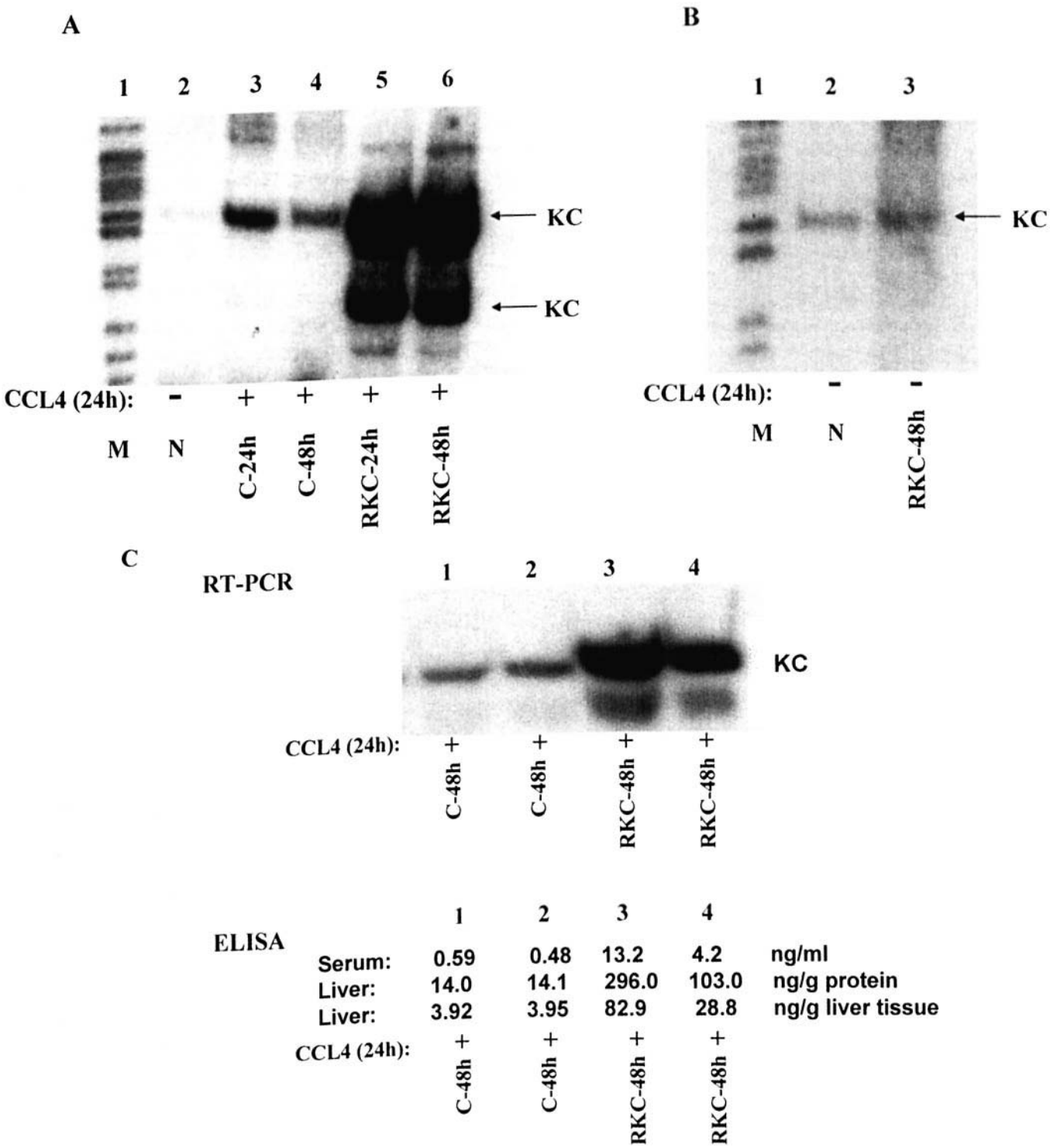


Figure 4. Autostimulation of KC expression in injured liver by recombinant KC. (A) Injection of KC protein stimulates expression of KC mRNA in CCl_4-treated mouse liver. Mice were injected with a single dose of CCl_4, and after 24 hrs, were injected with recombinant KC (RKC, lanes 5 and 6) or saline (C, lanes 3 and 4). Livers were harvested at 24 hrs after KC or saline injections (lanes 5 and 3) or at 48 hrs after KC or saline injections (lanes 4 and 6). Expression of KC was analyzed by RT-PCR using poly-A+ RNA, as in Fig. 2A. GAPDH signal was equal in all lanes (data not shown). Lane 2 is normal liver (N) and lane 1 is size marker (M). (B) Recombinant KC does not upregulate its mRNA in the absence of liver injury. Mice received only an injection of recombinant KC, and after 48 hrs, expression of KC mRNA was analyzed in the liver, as in A (lane 3). Lane 2 is normal liver (N) and lane 1 is size marker (M). (C) Expression of KC protein in the liver correlates with expression of its mRNA. Two mice were injected with CCl_4 for 24 hrs, followed by saline (C-48h; lanes 1 and 2), and two mice were injected with CCl_4 followed by recombinant KC (RKC-48h; lanes 3 and 4). Forty-eight hours after the saline or KC injections, KC mRNA in the liver was determined by RT-PCR (top panel), and KC protein was determined in sera and livers by ELISA (bottom panel). KC concentration in the liver was expressed as nanograms per gram of total protein and as nanograms per gram of liver tissue, assuming that liver proteins correspond to 28% of wet liver weight (56, 57). ELISA determinations were done in duplicate, and the average value is shown.

Activated HSCs Express KC mRNA. Liver injury is associated with activation of HSCs, and activation of HSCs can be reproduced *in vitro* by culturing freshly isolated HSCs on plastic (5). To investigate if expression of KC increases during culture activation of HSCs, we analyzed the level of KC mRNA in freshly isolated rat HSCs (quiescent) and in HSCs cultured for 8 days, when they were fully activated. We used the primers for mouse KC, which are 100% homologous to the rat CINC1 sequence, and therefore designated the PCR product as KC. There was an increase in expression of KC mRNA in activated HSCs (Fig. 2A, lane 2), compared to quiescent HSCs (Fig. 2A, lane 1). GAPDH mRNA was equally expressed in these samples, suggesting a comparable quality of both RNA preparations. Based on these results, we concluded that there is no expression of KC in quiescent HSCs, whereas activated HSCs express KC chemokine. A similar finding was previously published (28).

KC mRNA contains multiple AUUUA motifs in its 3′ untranslated region (UTR), which are often found in the 3′ UTRs of transcripts of immediate early genes and which regulate their stability. One of the characteristics of this class of mRNAs is that they can be superinduced by inhibitors of protein synthesis (58). To investigate if KC mRNA can be regulated by a similar mechanism, we treated activated HSCs with two inhibitors of protein synthesis and estimated the steady-state level of KC mRNA (Fig. 2B). Both cycloheximide (lane 2) and puromycin (lane 3) induced KC mRNA above the levels seen in untreated activated HSCs (lane 1) after 3 hrs. Thus, KC mRNA can be superinduced by inhibitors of protein synthesis in HSCs, suggesting that in HSCs, KC mRNA may be regulated at the level of mRNA stability.

The only known receptor that binds KC with high affinity is CXCR2 (34). Thus far, there has been no report about expression of this receptor in HSCs. To see if CXCR2 is expressed during activation of HSCs, we cultured rat HSCs and extracted RNA every day starting from Day 3 until Day 8 after isolation. These samples were analyzed for expression of CXCR2 mRNA by RT-PCR (Fig. 2C). The mRNA of CXCR2 was expressed in HSCs at Day 3 after isolation, while the cells still have the quiescent phenotype (Fig. 3A), and remained unchanged even when the HSCs became fully activated (Day 8). From these experiments, we concluded that HSCs constitutively express the CXCR2 receptor and therefore may respond to KC stimulation.

KC Increases Procollagen Secretion by HSCs. Having established that HSCs express the KC receptor even in the quiescent state, we wanted to assess the effect of KC chemokine on rat HSCs activated in culture. To this goal we added recombinant KC to rat HSCs that had been cultured for only 2 days. At this time-point, HSCs still have the quiescent phenotype and do not express Type I collagen or α-smooth muscle actin (α-SMA) as markers of activation (Fig. 3). Fresh medium supplemented with 50 ng/ml of KC was added daily until Day 7. Cellular proteins and cell medium were collected every day starting from Day 3 until Day 8 after isolation. The samples were analyzed for procollagen α1(I) protein expression by Western blot (Fig. 3). Expression of procollagen α1(I) was first detected in cellular extracts of HSCs at Day 5 in culture (Fig. 3A, right panel, lane 3). Addition of KC did not change the temporal appearance of cellular procollagen α1(I) (Fig. 3A, left panel, lane 3). Cellular level of procollagen α1(I) increased until Day 8, but was unaffected by treatment with KC (Fig. 3A, lanes 4, 5 and 6). Another marker of HSC activation, α-SMA, become detectable at Day 6 in control cells and at day 5 in KC-stimulated cells. Analysis of the cellular medium revealed that KC induced a dramatic increase in collagen α1(I) secretion from HSCs (Fig. 3B). In control cells, accumulation of procollagen α1(I) in the cellular medium started at Day 4 (Fig 3B, right panel, lane 2), and procollagen α1(I) accumulated at low levels at the subsequent time-points. The only detected molecular form was the unprocessed α1(I) procollagen chain of 180 kDa. Hepatic stellate cells stimulated with KC started secretion of procollagen α1(I) also at Day 4 (Fig. 3B, left panel, lane 2), but accumulated a much larger total amount of α1(I) chain in the medium at the subsequent time-points (lanes 4, 5, and 6). Most of the collagen was in the form of mature collagen α1(I) polypeptide of 120 kDa, with lesser amounts of the procollagen α1(I) form of 180 kDa (Fig. 3B, left panel, lanes 4 and 5). Appearance of the mature collagen α1(I) chain suggested not only that KC stimulated secretion, but also the activation of Type I collagen processing enzymes. From these experiments, we concluded that KC stimulates HSCs to secrete and process Type I collagen *in vitro*.

Recombinant KC Increases Expression of Its mRNA in Injured Liver. Because KC stimulated secretion of collagen α1(I) from HSCs, we wanted to see if KC was fibrogenic in an animal model of liver injury. We first induced acute liver injury by injecting a single dose of CCl_4 into mice. Twenty four hours after CCl_4 injection, the mice were injected through the tail vein with recombinant KC or saline, as a control. As another control, some mice were injected with mineral oil, followed by recombinant KC after 24 hrs. Recombinant KC was an active form of KC containing amino acids 29–96 and was free of carriers and endotoxins. The livers were harvested 24 or 48 hrs after the KC or saline injections and analyzed. Figure 4 shows expression of KC mRNA in these livers. Injection of CCl_4 followed by saline injection resulted in a several-fold upregulation of KC mRNA at 24 and 48 hrs after the saline injection (Fig. 4A, lanes 3 and 4) compared with normal liver (Fig. 4A, lane 2). This increase was similar to that shown in Figure 1. However, injection of CCl_4 followed by injection of recombinant KC resulted in about a 100-fold overexpression of KC mRNA, which was sustained at 24 and 48 hrs after KC administration (lanes 5 and 6). Both RT-PCR products resolved in these lanes (indicated by arrows) were sequenced, and they both represent KC. They appeared because of denaturation of the abundant double-stranded RT-PCR product in the sequencing gel used and different migration of the two DNA strands. Therefore, total

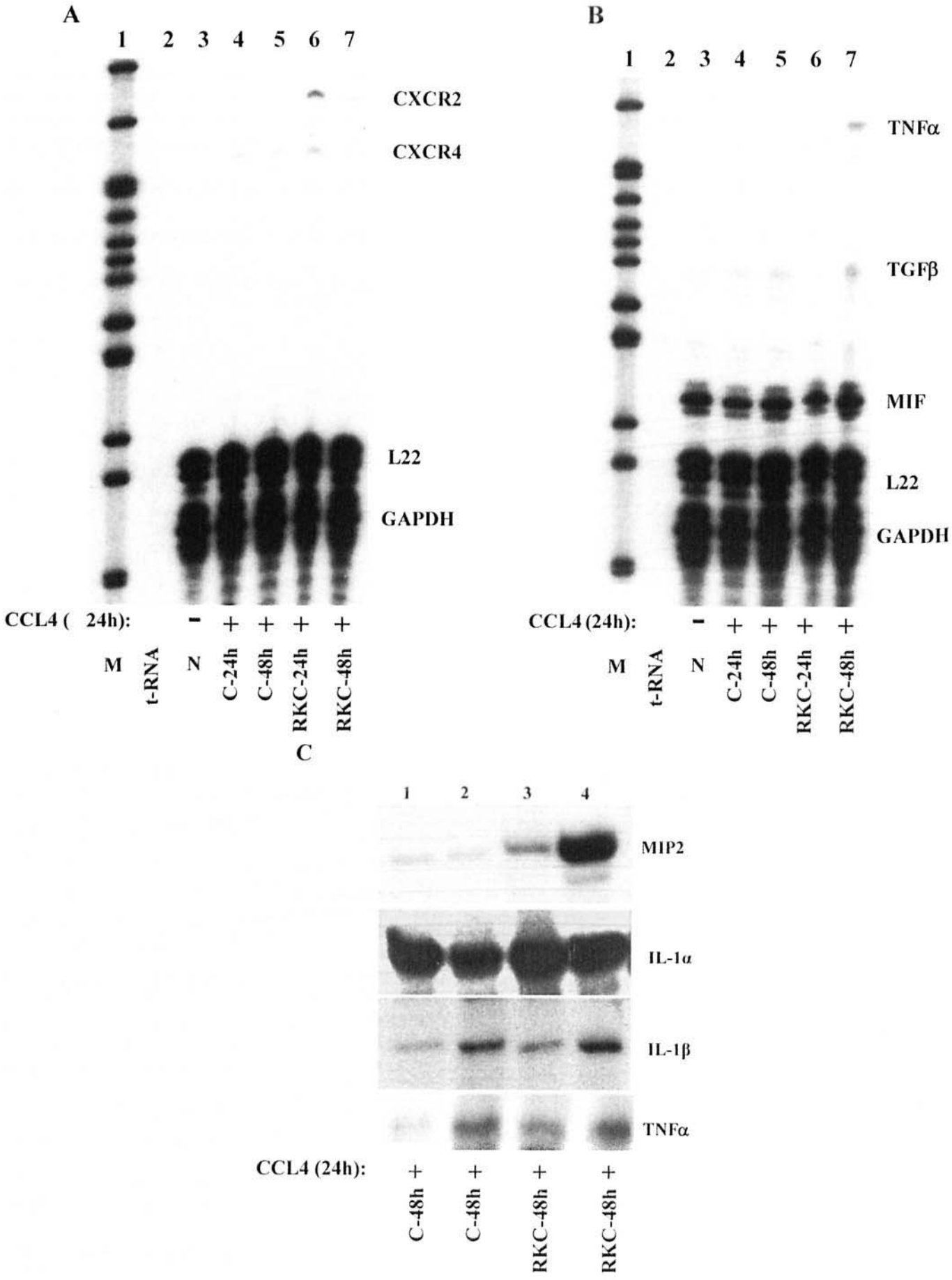
KC expression is the sum of these bands. Injection of oil followed by KC did not produce a high induction of KC mRNA (Fig. 4B, lane 3), suggesting that preexisting liver damage is a prerequisite for autostimulation of KC mRNA expression by the KC protein. This control also excludes the possibility that KC preparation was contaminated with endotoxin, a known inducer of chemokine mRNA (50). The experiment was repeated (Fig. 4C), with two additional animals receiving CCl_4 followed by saline (C-48h) and two receiving CCL_4 followed by recombinant KC (RKC-48h), and the samples were collected 48 hrs after the saline or KC injections. We analyzed expression of KC mRNA by RT-PCR (top panel) and KC protein by ELISA in sera and liver homogenates (bottom panel). The concentration in the liver was expressed as nanograms per gram of liver protein and as nanograms per gram of liver tissue, which was calculated assuming that proteins represent 28% of wet liver weight (56, 57). There was a 14-fold increase in KC protein in the livers of test animals, which correlated well with the expression of the mRNA. There was also increase in KC concentration in the sera of test animals, but the levels in the sera (nanograms per milliliter) remained about 6-fold lower that the levels in the liver (nanograms per gram; Fig. 4C). From these results, we concluded that a single injection of KC protein in the circulation potentates expression of KC gene in the liver, but only if there is preexisting acute necrosis.

KC Induces Expression of MIP2 in the Liver. The high degree of induction of KC gene expression by recombinant KC in the injured liver prompted us to investigate what other genes are affected by overexpression of KC. To this goal, we analyzed expression of cytokines and chemokine receptors by RNase protection assay. Figure 5A shows expression of three chemokine receptors, CXCR2, CXCR4, and CXCR5, mRNA. We could detect increased expression of CXCR2 receptor mRNA, but only in the livers of mice that received CCl_4 for 24 hrs followed by recombinant KC (lanes 6 and 7). This receptor was not induced by CCl_4 treatment alone (lanes 3 and 4) nor was it expressed in normal liver (lane 2). The CXCR4 receptor was expressed at a low level in all the livers that received CCl_4, whereas the CXCR5 receptor could not be detected in any sample. From this we concluded that overexpression of KC induces expression of its receptor (CXCR2) in the liver.

Expression of various cytokines is shown in Figure 5B. Overexpression of KC resulted in minimal up-regulation of mRNA for TNFα and TGFβ1. This was seen only at 48 hrs after KC administration (lane 7). No expression was detected in the other samples. Expression of MIF was unchanged in all samples compared with normal liver, and we did not detect expression of lymphotoxin (LT) α, LTβ, IL-6, interferon (INF) γ, INFβ, TGFβ2, and TGFβ3 by RNase protection assay (RPA). To further analyze changes in expression of proinflammatory genes, we employed a more sensitive RT-PCR assay and assessed the expression of MIP2, IL-1α, IL-1β, and TNFα in two additional test and control animals (Fig. 5C). Expression of MIP2 was strongly induced by KC, whereas there was no significant change in expression of IL-1α, IL-1β, and TNFα. Thus, slight upregulation of TNFα seen in the RPA assay (Fig. 5B) was probably caused by variation among the animals, and we concluded that only MIP2 was strongly induced by KC.

KC Increases Collagen Synthesis in Injured Liver. Transforming growth factor-β induces Type I collagen synthesis in the liver (59). Because TGFβ1 was induced by KC, we wanted to know if this was also associated with increased Type I collagen expression. We analyzed expression of collagen α1(I) mRNA by RPA (Fig. 6A) and collagen protein expression by Western blot (Fig. 6B). Injection of a single dose of CCl_4 followed by saline resulted in upregulation of collagen α1(I) mRNA starting 2 days after the CCl_4 injection (lane 4), followed by a severalfold increase at day 3 (lane 5). Injection of CCl_4 followed by KC resulted in a delayed upregulation of collagen α1(I) mRNA. No mRNA was detected at Day 2 after CCl_4 injection (lane 6), but an increased level was seen at Day 3 (lane 7). We could not take additional time-points because the animals had to be killed because of failing livers. Analysis of collagen content in the liver by Western blot revealed that injection of CCl_4 increased procollagen α1(I) deposition in the liver at Day 2 (Fig. 6B, lane 2), with a subsequent increase by Day 3 (lane 3). This was in parallel with increase of collagen α1(I) mRNA in these samples (Fig. 6A, lanes 4 and 5). When animals received CCl_4 followed by recombinant KC, we could not detect procollagen α1(I) at Day 2 after CCl_4 (lane 4). However, 1 day later, there was a dramatic increase in procollagen α1(I) deposition in the liver (Fig. 6B, lane 5). This was concomitant with the increase in collagen α1(I) mRNA (Fig. 6A, lane 7). The predominant form was 120-kDa mature collagen α1(I) chain, and the total level was several-fold higher than in livers of mice without KC injection (cf. lanes 5 and 3). Appearance of the mature collagen α1(I) chain is of significance, because the terminal peptides must be cleaved off from procollagen for fibrils to polymerize. Therefore, appearance of the 120-kDa mature collagen α1(I) chain may indicate the initiation of fibrillogenesis. We concluded from these experiments that overexpression of KC increases the prefibrotic response in the liver, consistent with the results with culture-activated HSCs.

KC Increases Necrosis of the Liver Without Infiltration of Neutrophils. The only known function of KC is chemotaxis of neutrophils to the site of tissue injury (22, 23, 60). To assess if proinflamatory changes (Fig. 5) and increased collagen synthesis (Fig. 6) were associated with accumulation of neutrophils in the liver, we analyzed liver sections by histology (Fig. 7A). In the two representative control animals, a single injection of CCl_4 resulted in pericentral necrosis after 72 hrs, which was limited to discrete areas covering about 40%–50% of the sections (top panels). The mice that received KC after CCl_4 showed larger areas of necrosis (covering about 80% of the sections), with the remaining parenchyma showing prene-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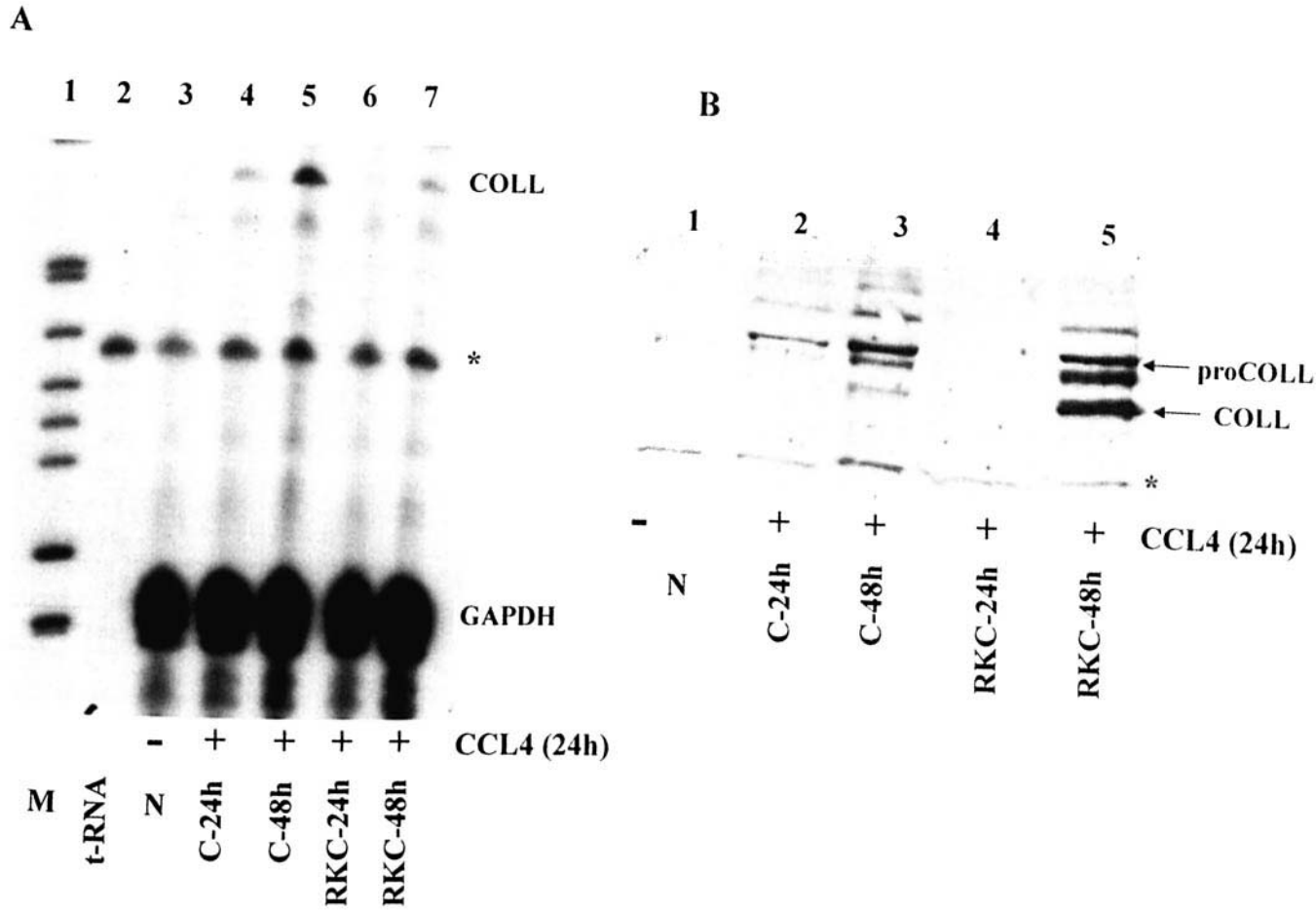

Figure 6. Effects of KC on expression of collagen α1(I) mRNA and protein in injured liver. (A) Expression of collagen α1(I) mRNA. RNase protection assay with the samples described in Figure 5A. GAPDH served as an internal control, and migration of a nonspecific band is indicated by asterisk. (B) Expression of collagen α1(I) protein. Western blot with total liver proteins extracted from samples described in Figure 5A. Migration of 180-kDa pro-collagen α1(I) and mature 120-kDa collagen α1(I) chain is indicated. Asterisk indicates a nonspecific band, which can serve as an internal standard.

crotic changes (bottom panels). Serum aminotransferase levels measured in three CCl_4 + saline– or three CCl_4 + KC–treated animals are shown in Figure 7B. Injection of CCl_4 + recombinant KC increased serum AST and ALT levels by 3- to 5-fold compared with animals injected with CCl_4 alone. From these results, we concluded that KC greatly enhanced liver injury.

Careful inspection of the independent liver histology samples by pathologists did not reveal infiltration of neutrophils. To confirm the absence of neutrophils found in the histological examination, we analyzed expression of myeloperoxidase mRNA as a marker of myeloid cells (61, 62). We employed sensitive RT-PCR (Fig. 8A), but despite using 50 cycles, which clearly detected myeloperoxidase mRNA in total RNA of blood cells of a normal mouse (Fig. 8A, lane 6), no expression of myeloperoxidase mRNA could be detected in the liver 24 hrs after KC (lane 4), with barely detectable expression after 48 hrs (lane 5). Myeloperoxidase mRNA also could not be detected in animals treated with CCl_4 only or in a normal liver (lanes 1, 2, and 3). GAPDH expression, as determined by RT-PCR, was similar in all samples (data not shown). We repeated measurement of myeloperoxidase mRNA in two additional control and test animals at 48 hrs after saline or KC injections and did not see a difference in expression (Fig. 8B), despite a 14-fold higher KC concentration in the livers of KC-injected animals compared with control animals (Fig. 4C). We concluded that overexpression of KC did not result in significant accumulation of neutrophils within the liver and that the catastrophic liver failure

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Figure 5. Effects of KC on expression of chemokine receptors and cytokines in injured liver. (A) Expression of chemokine receptor mRNA. RNase protection assay for expression of CXCR2, CXCR4, and CXCR5 mRNAs in normal liver (N, lane 3) and in livers of mice 24 hrs after injection of CCl_4 that was followed by saline for 24 (C-24h; lane 4) or 48 hrs (C-48h; lane 5) or by recombinant KC for 24 (RKC-24h; lane 6) or 48 hrs (RKC-48h; lane 7). Lane 2 is tRNA control (tRNA) and lane 1 is size marker (M). Expression of ribosomal protein L22 and GAPDH served as an internal control. Migration of specific bands is indicated. (B) Expression of cytokine mRNA. The same experiment as in A except a multi-riboprobe set specific for MIF, LTα, LTβ, IL-6, INFγ, INFβ, TGFβ1, TGFβ2, TGFβ3, and TNFα was used. (C) Expression of MIP2 and IL-1 mRNA. Two additional livers, described in Figure 4C, were analyzed by RT-PCR for expression of MIP2, IL-1α, IL-1β, and TNFα.

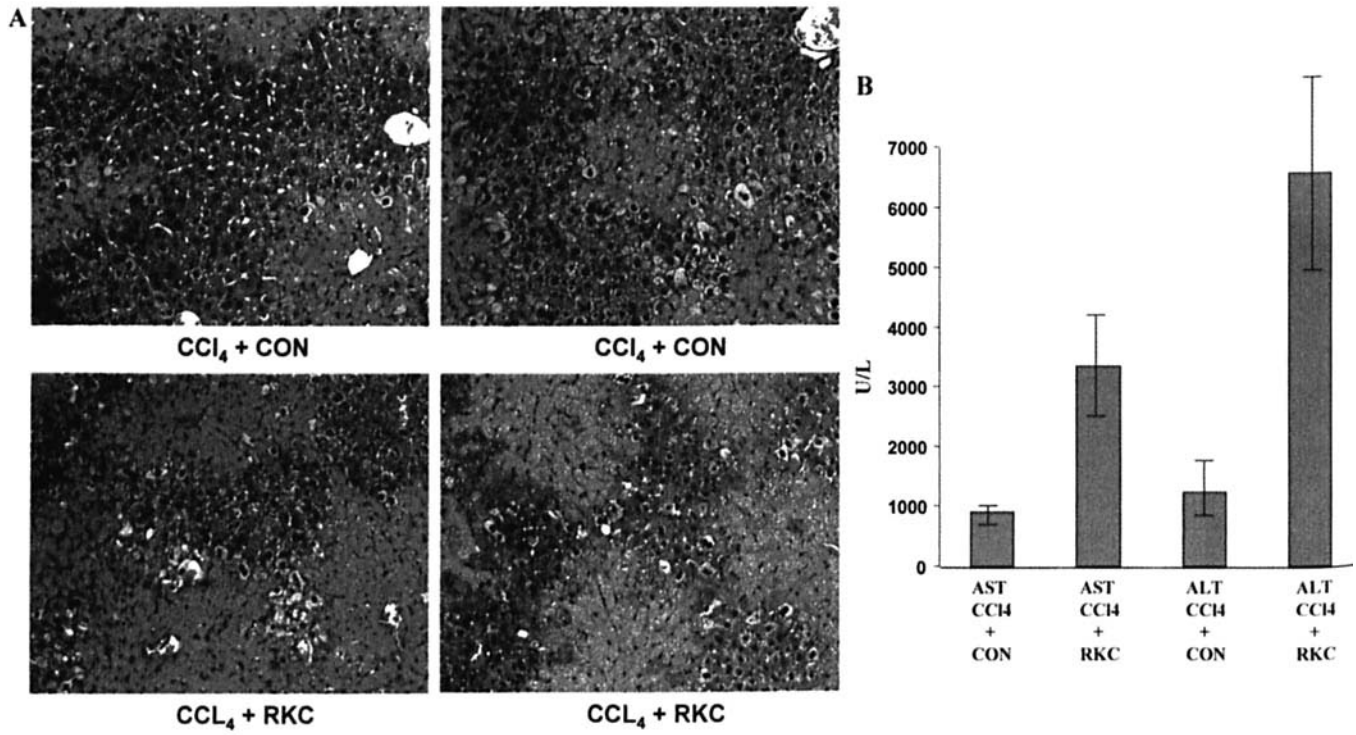

Figure 7. Enhanced necrosis of the liver after overexpression of KC. (A) Histology of liver samples. (Top panels) Mice were injected with a single dose of CCl_4 followed by saline injection 24 hrs later (CCl_4 + CON). The livers were harvested 48 hrs after the saline injection. (Bottom panels) Mice were injected with a single dose of CCl_4 followed by KC injection 24 hrs later (CCl_4 + RKC). The livers were harvested 48 hrs after the KC injection. Liver section were stained with hematoxylin/eosin, and images were taken under ×10 magnification. Expression of KC in these livers is shown in Figure 4C. (B) Serum aminotransferase levels. Serum AST and ALT levels were measured in three mice treated with CCl_4 followed by saline (CCl_4 + CON) or with CCl_4 followed by recombinant KC (CCl_4 + RKC). The blood samples were taken 48 hrs after the saline or RKC injections. The results are shown as units per liter, and the mean ± SEM is indicated.

associated with overexpression of KC must have been a result of direct hepatotoxicity of KC and MIP2.

Discussion

KC, the mouse homolog of human gro-α, belongs to the CXC family of chemokines. The only well-documented function of this group of chemokines is chemotaxis and extravasation of neutrophils (30, 31). We now describe some novel effects of KC on isolated HSCs and whole liver. To specifically overexpress endogenous KC in the liver, we first induced acute liver injury by CCl_4. Twenty-four hours after the CCl_4, we intravenously injected a single dose of recombinant KC. This combination resulted in about a 14-fold increase in KC expression within the liver, which was sustained for at least 2 days (Fig. 4C). Because injected chemokines are cleared form the circulation within 2 hrs (63), a single KC injection most likely only served to activate the KC gene expression in the preinjured liver. Injection of KC without preexisting liver injury did not result in activation of endogenous KC gene expression (Fig. 4B). Autostimulation of KC gene expression by the KC protein was previously shown in endothelial cells (64) and in melanoma cells (65). Autocrine stimulation may be a common feature of CXC chemokines, because expression of the *IL-8* gene can also be induced in monocytes by IL-8 protein (66). In our system, KC also induced expression of another CXC chemokine, MIP2 (Fig. 5C).

Using this approach, we showed that (1) KC expression is upregulated in acute liver injury; (2) HSCs express the receptor for KC, CXCR2, suggesting that they can be stimulated by KC; (3) addition of KC to HSCs undergoing activation in culture results in increased secretion of Type I collagen, indicating that KC may act as prefibrotic cytokine; (4) injection of KC into circulation of mice with preexisting acute liver necrosis results in dramatic upregulation of KC mRNA and protein; thus, KC may perpetuate its own synthesis in an injured liver by a positive feedback mechanism; (5) KC induces expression of its receptor, CXCR2, which may further enhance the downstream effects; and (6) overexpression of KC in the injured liver greatly augments liver damage without infiltration of neutrophils. Increased hepatotoxicity associated with high expression of KC was probably mediated by direct effects of KC on liver cells.

Activated HSCs express mRNA for KC at a low level (Fig. 2B, lane 1); however, hepatocytes are the main source of KC in the liver (67–70). Hepatocytes also express CXCR2 (71). Increased levels of gro-α and IL-8 in the liver are associated with increased neutrophil sequestration within the liver in alcoholic hepatitis (45, 46, 72) and in a rat model of alcohol-induced liver injury (73). These studies

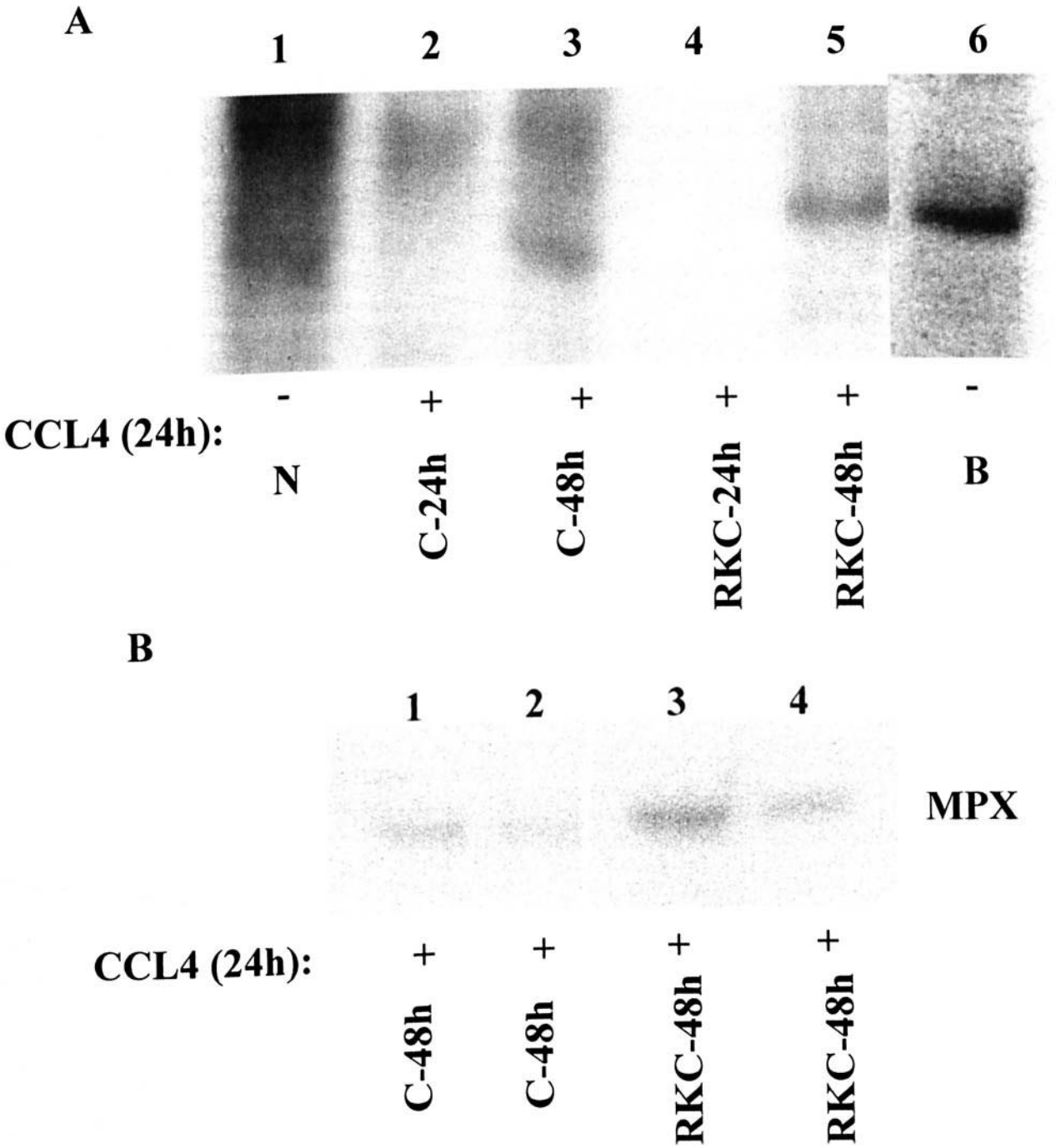

Figure 8. Expression of myeloperoxidase mRNA. (A) Samples described in Figure 5A were analyzed by RT-PCR with primers specific for myeloperoxidase mRNA. B in lane 6 is total RNA extracted from blood cells that served as a positive control. Migration of the specific band is shown. (B) Two additional livers, described in Figure 4C, were analyzed by RT-PCR for expression of myeloperoxidase mRNA by RT-PCR.

suggested that CXC chemokines are sufficient for chemotaxis of neutrophils into the liver. However, several studies have shown no correlation between high level of chemokines and infiltration of neutrophils. For example, in alcoholic cirrhosis, there is a marked increase in IL-8 expression in the liver without sequestration of neutrophils (72). Thyroid glands affected with Graves disease express gro-α at a high level, but there is no infiltration of neutrophils in the glands (74). Overexpression of human IL-8 in the liver of transgenic mice did not result in accumulation of neutrophils within the liver (47), although human IL-8 can cause chemotaxis of mouse neutrophils *in vitro* (33). Likewise, injection of recombinant KC into circulation of mice did not result in recruitment of neutrophils into the liver (75). In our model, despite about a 14-fold increased amount of KC in the liver (Fig. 4C), we could not see significant accumulation of neutrophils either by histology or by expression of myeloperoxidase mRNA (61, 62). The concentration of KC in the liver, expressed as nanograms per gram, was about 6-fold higher than

concentration in plasma, expressed as nanograms per milliliter (Fig. 4C). Because chemokines injected into circulation have a half-life of 2 hrs (63) and high KC expression was sustained in the liver for 2 days, it is not likely that a lack of concentration gradient of KC between the liver and the plasma was responsible for the absence of chemotaxis (76). A more likely explanation is that chemotaxis of neutrophils into the liver is a complex process that requires, in addition to KC, some other factors. In a wound healing model, elevated levels of gro-α in the wound persisted for 2 weeks after the number of neutrophils in the wound has returned to normal, suggesting that an increased level of gro-α was not sufficient to maintain high number neutrophils at the site of injury (60). The rat homolog of KC, CINC1, overexpressed in rat livers by adenovirus delivery, increased neutrophil accumulation within the liver 4 days after viral injections (77). However, the number of neutrophils in the liver overexpressing CINC1 was only twice that of the control liver. At the same time, the overexpression of CINC1 caused liver damage, which was in excess of what could be attributed to the increased number of neutrophils. In fact, depletion of rat neutrophils did not have any influence on liver damage and fibrosis in a bile duct ligation model of liver fibrosis (78). Therefore, the authors concluded that CINC1 must have a direct hepatotoxic effect (77). Although we cannot exclude that KC contributes to chemotaxis of neutrophils into the liver, it is clear that direct effects of KC (and its rat homolog CINC1) on the liver are far more prominent than chemotaxis of neutrophils. These effects include massive hepatic necrosis (Fig. 7) and (77) and induction of expression of MIP2 and TGFβ1. Bautista *et al.* (79) have reported that MIP2 is cytotoxic in alcoholic hepatitis, additional evidence that CXC chemokines have a direct hepatotoxic effect. Up-regulation of the CXCR2 receptor by KC suggests a positive feedback loop, which may have accelerated the liver failure. Involvement of other proinflammatory cytokines in this process could not be confirmed, because we did not see an increase in expression of TNFα, IL-1α, IL-1β, LTα, LTβ, IL-6, INFγ, and INFβ (Fig. 5). We are currently studying the change in expression of other genes resulting from high KC expression in the liver.

Activated HSCs express KC mRNA, whereas this mRNA could not be detected in quiescent HSCs (Fig. 2). A similar result was reported for rat CINC1 (28). KC mRNA can be induced in HSCs by inhibitors of protein synthesis (Fig. 2B), suggesting that KC mRNA may be regulated in HSCs at the level of mRNA stability, as reported for the other cell types (38). However, this is the first report that HSCs can express the receptor for KC, CXCR2 (Fig. 2). The fact that HSCs constitutively express CXCR2 suggests that quiescent HSCs can be stimulated by KC released by hepatocytes, endothelial cells, Kupffer cells, or by activated HSCs. Addition of KC to HSCs undergoing activation in culture resulted in increased extracellular accumulation of Type I collagen (Fig. 3). Nonstimulated HSCs secreted smaller amounts of procollagen α(I) chain of 180 kDa, whereas the KC-stimulated cells secreted much larger amounts of mature α1(I) chain of 120 kd (Fig. 3B). Because accumulation of 120-kDa collagen α1(I) chain indicates processing of procollagen and fibrillogenesis (80), the effect of KC on HSCs *in vitro* is prefibrotic. The cellular levels of procollagen protein were unchanged with KC treatment (Fig. 3A), suggesting that the prefibrotic effect is to increase the efficiency of export and processing of Type I collagen and/or to inhibit its degradation. A prefibrotic effect of KC was observed *in vivo*, as well, where overexpression of KC was associated with increased accumulation of 120-kDa collagen α1(I) chain in the liver (Fig. 6B). Expression of Type I collagen is regulated at the translational level by the 5′ stem-loop RNA element (53). Binding of 5′ stem-loop RNA binding proteins is required for synthesis of triple helical Type I collagen (55); therefore, the small increase in mRNA level may result in large increase in protein level (Fig. 6) because of activation of translation by increased activity of the 5′ stem-loop binding proteins.

In summary, overexpression of KC in the liver greatly augments acute liver necrosis. KC also increases expression of MIP2. Together, these chemokines may exhibit direct hepatotoxic effect (77, 79). This effect is not associated with chemotaxis of neutrophils. KC acts as a prefibrotic cytokine on isolated HSCs; in the whole liver, it triggers massive necrosis that is associated with up-regulation of prefibrotic genes. Autostimulation of KC gene expression by the KC protein, which is also associated with upregulation of its receptor, indicates a positive feedback regulation. Such regulation indicates that any uncontrolled expression of KC may rapidly lead to catastrophic liver failure. Positive feedback regulation of KC and its role in direct liver damage is an important novel finding for liver pathology.

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