Human hepatic stellate cells express CCR5 and RANTES to induce proliferation and migration

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Schwabe, Robert F., Ramón Bataller, and David A. Brenner. Human hepatic stellate cells express CCR5 and RANTES to induce proliferation and migration. Am J Physiol Gastrointest Liver Physiol 285: G949–G958, 2003. First published June 26, 2003; 10.1152/ajpgi.00215.2003.—Activated hepatic stellate cells (HSCs) are the main producers of extracellular matrix in the fibrotic liver and are involved in the regulation of hepatic inflammation. The aim of this study was to characterize the role of regulated on activation, normal T-cell expressed, and presumably secreted (RANTES) in activated HSCs. RANTES mRNA and protein secretion were strongly induced after stimulating HSCs with TNF-α, IL-1β, or CD40L. RANTES production was NF-κB dependent, because inhibitor-κB (IkB) superrepressor and dominant-negative IkB kinase-2 almost completely blocked RANTES expression. NF-κB activation was sufficient to drive RANTES expression as demonstrated by the strong induction of RANTES in HSCs expressing NF-κB-inducing kinase. The JNK/activator protein-1 pathway also contributed to RANTES expression as demonstrated by the blocking effects of the JNK inhibitor SP600125. HSCs responded to stimulation with recombinant human (rh)RANTES with an increase in intracellular calcium concentration and a rapid increase in free radical formation. Furthermore, rhRANTES induced ERK phosphorylation, ERK-dependent [3H]thymidine incorporation, and HSC proliferation. Additionally, rhRANTES induced focal adhesion kinase phosphorylation and a substantial increase in HSC migration. HSCs functionally expressed chemokine receptor-5 (CCR5), as shown by flow-cytometric analysis and RT-PCR, and the inhibitory effects of a blocking CCR5 antibody on rhRANTES-induced ERK activation, proliferation, and migration. Diphenylene iodonium and N-acetylcysteine inhibited rhRANTES-induced ERK activation and HSC proliferation, indicating that NADPH oxidase-dependent production of reactive oxygen species was required. In conclusion, RANTES and CCR5 represent potential mediators of 1) HSC migration and proliferation and 2) a cross-talk between HSCs and leukocytes during fibrogenesis.

HEPATIC STELLATE CELLS (HSCs) play a key role in the development of liver fibrosis and are the major producers of extracellular matrix in the liver after undergoing an activation process that results in a phenotypic change from retinoid-storing quiescent cells to activated HSCs with a myofibroblast phenotype (15, 26). This activation process is closely mimicked by culturing HSCs on plastic or type I collagen and serves as a model to study fibrogenic responses under defined conditions (33). In the injured liver, HSCs accumulate at the site of injury where they produce extracellular matrix leading to characteristic patterns of collagen deposition. Migration of HSCs is believed to be critical for the accumulation of HSCs at the site of injury. Besides their role in the production of extracellular matrix, HSCs are also involved in the regulation of inflammatory processes during liver injury. HSCs produce a variety of chemokines including monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant/IL-8 (27, 33, 42, 45), and express chemokine receptors rendering them responsive to chemokines. It has been suggested that chemokines induce the migration of HSCs to the site of injury and attract a leukocytic infiltrate to the site of injury (31, 32), another characteristic histological feature of the fibrotic liver (14, 29). This concept is supported by a study that demonstrates the expression of MCP-1 in fibrous septa of patients with hepatitis C and a positive correlation of MCP-1 expression with mononuclear infiltration (31). The leukocytic infiltrate itself is an abundant source of chemokines and growth factors that may, in turn, promote HSC activation and proliferation (25). Therefore, chemokines secreted by HSCs may be part of a cytokine network within the liver that regulates the interaction of resident and nonresident cells during the hepatic wound-healing response. This hypothesis is supported by the fact that HSCs express the molecular machinery to interact with infiltrating leukocytes such as ICAM, VCAM, and CD40 and are able to present antigen and to stimulate the proliferation of allogenic lymphocytes (5, 18, 20, 42).

The CC chemokine regulated on activation, normal T cell expressed, and presumably secreted (RANTES) is a powerful chemoattractant of monocytes, eosinophils, and activated CD4 T cells (19, 40). The potential role of RANTES in hepatic fibrogenic and inflammatory responses is not known. In normal liver, RANTES expression is low, whereas RANTES is elevated in...
patients with alcoholic hepatitis or chronic hepatitis C (1, 21, 28). In this study, we analyze the potential role of RANTES and its receptor chemokine receptor-5 (CCR5) in culture-activated HSCs. HSC secrete RANTES after stimulation with TNF-α, IL-1β, and CD40L in an inhibitor-xB (IxB) kinase (IKKα/β NF-xB) and JNK-dependent manner. Activated HSCs express CCR5 and respond to recombinant human (rh)RANTES stimulation with an increase in proliferation and migration in a CCR5-dependent manner. RANTES and CCR5 may be involved in regulating HSC migration to sites of injury and may act as mediators of HSC-leukocyte interaction.

MATERIALS AND METHODS

Isolation and culture of primary human HSCs. The isolation of HSCs was performed by a two-step collagenase perfusion from surgical specimens of normal human liver as previously described (42). All tissues were obtained through qualified medical staff, with donor consent and the approval of the University of North Carolina Ethics Committee. Cells were seeded on uncoated plastic tissue culture dishes and cultured in DMEM (GIBCO-BRL, Grand Island, NY) supplemented with 10% FCS and standard antibiotics in a 95% air-5% CO2 humidified atmosphere at 37°C. The purity after 4 days in culture was ~98%, as estimated by autofluorescence. Cells between passages 3 and 9 were used for experiments. No noticeable differences were detected among the four independent cell preparations used for this study.

Adenoviral infection. Adenoviruses expressing the catalytically inactive mutants IKKα K44M dominant-negative (AdIKKαdn) and IKKβ K44M (AdIKKβdn), the mutated IxB S32A/S36A superrepressor (AdIxBer), green fluorescent protein (AdGFP), CD40L (AdCD40L), and an NF-xB driven luciferase reporter (AdNF-xB) have been previously described (39, 41, 42). The adenovirus expressing NF-xB-inducing kinase (AdNIK) (a gift from Dr. Christian Jobin, University of North Carolina, Chapel Hill, NC) was created by the Cre-Lox recombination method by using a previously described wild-type NIK plasmid (34). HSCs were infected at 500 multiplicity of infection (MOI) resulting in a transduction rate >80% for 5 h in DMEM containing 2% FBS. After 24 h, the media were changed and HSCs were cultured for an additional 24 h and then stimulated as indicated. AdGFP was included as a control to ensure adequate adenoviral gene transfer in each experiment.

ELISA for RANTES. HSCs were cultured in six-well plates at a density of 4 × 10^4 cells/well for 24 h. After adenoviral infection, HSC were serum starved for 24 h and supernatants were collected after 24 h of treatment with rhTNF-α at 10 ng/ml, IL-1β at 5 ng/ml, or IFN-γ at 1,000 U/ml (all R&D Systems, Minneapolis, MN). For some experiments, HSCs were infected with AdCD40L or AdGFP for 24 h, and supernatants were collected after an additional 24 h. For the experiment involving AdNIK, HSCs were infected with AdNIK or AdGFP for 24 h and supernatants were collected after an additional 24 h. A sandwich ELISA for RANTES (R&D Systems) was performed according to the manufacturer’s instructions. Each experiment was performed in two separate wells per treatment, and experiments were repeated at least twice.

RT-PCR. RNA isolation and RT-PCR were performed as previously described (42). Specific primers were designed as follows: RANTES 5'-TCAGCTGGGAGGGCTAATGAGCCG-GAA-3' sense and 5'-GAGGAGTTTGAACCGGCCAAGC-3' antisense; CCR5 5'-TGACTCCTGGAATCTCTGAAAAT-3' sense and 5'-TTCTGAAGTCTCCGGGACAA-3' antisense; β-actin 5'-CACCAGGGGCTACAGAGTGC-3' sense and 5'-GATTTCAAGGTTGATGCTCT-3' antisense. PCR for RANTES and β-actin were run for 28 cycles, and the PCR for CCR5 was run for 35 cycles.

Flow-cytometric analysis. The expression of CCR5 was assessed by flow cytometric analysis. After detachment by PBS containing 2 mM EDTA, cells were stained either with anti-CCR5 (Pharminingen, San Diego, CA) or an isotype-matched control antibody (Pharminingen) at 5 μg/ml for 1 h on ice. After cells were extensively washed, they were incubated with FITC-labeled goat-anti-mouse-IgG. Viable cells were gated, and 5,000 cells were analyzed by using a FACScan instrument (Becton-Dickinson, Franklin Lakes, NJ).

Western blot analysis. Whole cell extracts (25 μg) were loaded onto 10% SDS-acrylamide gels and blotted onto nitrocellulose membranes. After membranes were blocked, they were incubated with anti-phospho-ERK (1:1,000; New England Biolabs, Beverly, MA) for 12 h at 4°C followed by incubation with horseradish peroxidase-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:1,000 for 45 min and chemiluminescent detection (Amersham, Arlington Heights, IL). To determine focal adhesion kinase (FAK) phosphorylation, 500 μg protein extract were immunoprecipitated with anti-FAK (Santa Cruz Biotechnology) for 2 h and blots were probed with anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) as suggested by the manufacturer.

Kinase assays. ERK activity assays were performed similarly as described previously (9). Briefly, cells were lysed in Triton lysis buffer containing protease and phosphatase inhibitors. Fifty micrograms of protein were immunoprecipitated with 2 μl anti-ERK-2 (Santa Cruz Biotechnology) for 2 h followed by 20 μl protein A/G agarose (Santa Cruz Biotechnology) for 1 h. The kinase reaction was performed in the presence of [γ-32P]ATP for 30 min at 30°C by using myelinic basic protein (Sigma, St. Louis, MO) as substrate. Supernatants from the kinase reactions were analyzed on 10% SDS-acrylamide gels. Equal substrate loading was confirmed by Coomassie blue staining, and dried gels were exposed to a Biomax film (Kodak, Rochester, NY). NF-xB reporter gene assay. HSCs were infected with AdNIK (500 MOI) or AdGFP (500 MOI) for 12 h followed by infection with AdNF-xB-Blue (100 MOI) for an additional 12 h. NF-xB-dependent gene transcription was analyzed 12 h later on a 2010 Monolight luminometer (Becton Dickinson) and adjusted for protein content.

Measurement of free radical formation. HSCs plated in 24-wells were loaded with the redox-sensitive dye 2',7'-dichlorofluorescein diacetate (H2DCFDA; 10 μM; Molecular Probes, Eugene, OR) for 20 min at 37°C, washed, and stimulated with rhRANTES (25 ng/ml). Reactive oxygen species formation was measured by using a multiwell fluorescence scanner (CytoFluor 2300; Millipore, Bedford, MA). In a second approach, free radical formation was measured by using a confocal laser-scanning microscope (model LSM-410; Carl Zeiss, Oberkochen, Germany). HSCs (1 × 10^6) that had been plated on 40-mm diameter glass coverslips were loaded with 5 μM H2DCFDA for 20 min at 37°C. Excitation was 488 nm using an argon laser, and fluorescence was divided by 560-nm band-pass and 590-nm long-pass barrier filters by using a ×63 objective lens with pinholes set to 4 in both channels. Subsequently, either rhRANTES (25 ng/ml) or buffer were added to the medium, and images were collected at given time points.
Cells were loaded with fluo-4 (10 μM; Molecular Probes) for 20 min at 37°C, rinsed with DMEM, and stimulated with rhRANTES (25 ng/ml). Changes in intracellular Ca²⁺ were measured with a CytoFluor 2300 multwell fluorescence scanner by using 485- and 535-nm filters (20-nm bandwidth) for excitation and emission, respectively.

Proliferation assays. DNA synthesis was estimated as the amount of [³H]thymidine (ICN, Irvine, CA) incorporated into TCA-precipitable material. Cells were plated in 24-well dishes at a density of 2 × 10⁴ cells/well and serum starved for 24 h followed by the incubation with either rhRANTES at 25 ng/ml or PDGF-BB (Sigma) at 5 ng/ml in the presence or absence of N-acetylcyesteine (NAC) (Sigma) at 10⁻⁴ M, the ERK-specific inhibitor PD-98059 (Calbiochem, La Jolla, CA) at 5 × 10⁻⁶ M, NADPH oxidase inhibitor diphenylene iodonium (Sigma) at 10⁻⁶ M, or the calcium chelating agent BAPTA-AM (Calbiochem) at 10 μM. Twenty hours later, cells were pulsed with 1 μCi/ml [³H]thymidine for 4 h, followed by precipitation and lysis. For the cell growth assay, HSCs were plated in six-well dishes at a density of 5 × 10⁴ cells/well followed by serum starvation for 24 h and the addition of agonists in fresh serum-free medium at days 0, 2, and 4. Cells were trypsinized at days 0, 2, and 4 and cell density was determined by using a hemacytometer (Brand, Blaubrand, Germany). [³H]thymidine incorporation and proliferation experiments were performed in triplicate wells for each condition, and results are expressed as percent increase over the cells incubated with serum-free medium alone.

Migration assays. Filters (5-μm pore size; Whatman, Clifton, NJ) were coated with 20 μg/ml collagen I (BD Biosciences, Bedford, MA) at 37°C for 60 min and placed between the upper and lower chambers of the Boyden system (Neumofrère, Gaithersburg, MD). The lower chamber was filled with serum-free medium (205 μl) containing agonists. Serum-starved HSCs were trypsinized and placed into the upper chamber (10⁶ cells/ml). After 6 h of incubation at 37°C, cells adhering to the upper side of the filter were removed and fixed in 96% methanol for 2 min and stained with Harris' hematoxylin solution. Cells migrated to the lower side of the filter were measured by a light microscope at 6–10 randomly chosen high-power fields. For the in vitro wound healing assay, HSCs were grown to confluence on 40-mm-diameter glass coverslips coated with 20 μg/ml collagen I. Cells were serum-starved for 24 h, and the cell monolayer was disrupted by a pipette tip to create a 2-mm linear cell-free zone. Cells were washed with serum-free medium and incubated with agonists for 20 h.

Statistical analysis. For the determination of statistical significance, a paired Student’s t-test was performed. A P value of <0.05 was used as the criterion of statistical significance.

RESULTS

Human HSCs express RANTES. To assess whether cultured human HSCs express RANTES, we first ana-

![Fig. 1](http://ajpgi.physiology.org/)

Fig. 1. Activated human hepatic stellate cells (HSCs) express regulated on activation, normal T cell expressed, and secreted (RANTES) after TNF-α, IL-1β treatment, or CD40L and NF-κB-inducing kinase (NIK) overexpression. A: activated HSCs (passage 3–6) were serum starved for 24 h. Cells were either left untreated (“Un”) or infected with adeno-virus-expressing CD40L (AdCD40L) or green fluorescent protein (AdGFP) (both 500 multiplicity of infection (MOI)) for 24 h followed by 30-h serum starvation or treated with TNF-α (10 ng/ml) or IL-1β (5 ng/ml) for 5 h. RANTES mRNA and β-actin levels were analyzed by RT-PCR. B: HSCs were serum starved for 24 h. Cells were either left untreated (“Un”) or infected with AdGFP or AdCD40L (500 MOI) for 24 h followed by 48-h serum starvation or treated with TNF-α (10 ng/ml) or IL-1β (5 ng/ml) for 24 h. RANTES levels were measured in undiluted cell culture supernatants by ELISA. Shown is the mean of 4 independent experiments performed in duplicate. C: HSCs were infected AdGFP or AdNIK (both 500 MOI) for 12 h followed by infection with NF-κB-Bluc (100 MOI) for 12 h. After 12 h of serum starvation, luciferase activity was measured in cellular extracts and adjusted to protein content to demonstrate NF-κB activation by AdNIK. Shown is one representative experiment. D–E: HSCs were infected with AdGFP or AdNIK (both 500 MOI). The media were exchanged after 24 h. After an additional 24 h, RNA was extracted to analyze RANTES levels by RT-PCR (D). Cell culture supernatants were collected to measure RANTES secretion by ELISA (E). Shown is 1 representative experiment performed in duplicate.
lyzed RANTES mRNA levels in activated HSCs under different culture conditions. Unstimulated HSCs expressed only trace amounts of RANTES mRNA, but RANTES mRNA was strongly induced by proinflammatory cytokines including TNF-α, IL-1β, and adenovirally expressed CD40L (Fig. 1A). We then explored the secretion of RANTES by HSCs by measuring RANTES in cell culture supernatants by ELISA. Only small amounts of RANTES were detected in the supernatants of unstimulated HSCs (Fig. 1B), but RANTES secretion was strongly induced by TNF-α (29.1-fold vs. untreated, P < 0.01), IL-1β (14.8-fold vs. untreated, P < 0.01), and CD40L (5.0-fold vs. GFP, P < 0.01). Infection with a control virus expressing GFP induced the secretion of RANTES only weakly (2.2-fold vs. untreated, P = 0.08, not significant) (Fig. 1B). RANTES was also induced by prolonged treatment with IFN-γ, but other treatments including ethanol and 20% FCS did not increase RANTES secretion over baseline (data not shown).

**Secretion of RANTES depends on the IKK/NF-κB and JNK pathways.** Expression of NIK in HSCs induced a 16.1-fold induction of NF-κB-dependent reporter gene transcription (Fig. 1C) and strongly up-regulated RANTES mRNA and protein expression (Fig. 1, D and E), indicating that the activation of NF-κB alone was sufficient for the secretion of RANTES in HSCs. To further assess the role of NF-κB in cytokine-induced RANTES expression, HSCs were infected with adenoviruses containing IKK1dn, IKK2dn, and IkBsr and then stimulated with TNF-α and IL-1β. Whereas IKK1dn and the GFP control virus did not significantly alter TNF-α- and IL-1β-induced RANTES expression at both the mRNA and the protein levels, IKK2dn and IkBsr almost completely inhibited TNF-α- and IL-1β-induced RANTES mRNA and protein expression (Fig. 2, A–D). These data show that the IKK2 → NF-κB pathway is crucial for the regulation of RANTES that is consistent with the predominant role of IKK2 in mediating NF-κB activation in many cell types including HSCs (42). To explore the involvement of the JNK/activator protein-1 (JNK/AP-1) pathway in RANTES secretion, HSCs were treated with the small molecule JNK inhibitor SP600125, which efficiently blocked JNK activity as demonstrated by its inhibitory effect on the phosphorylation of the JNK target c-Jun (Fig. 3A). SP600125 treatment reduced the induction of RANTES mRNA and protein expression after TNF-α, IL-1β (Fig. 3, B and C), and CD40L (data not shown), suggesting an additional role for the JNK/AP-1 pathway in the transcriptional control of RANTES in HSCs.

**HSCs express CCR5.** To determine whether HSCs may be a target for RANTES, we analyzed the expression of the CCR5 through which RANTES exerts many of its biological effects. CCR5 mRNA was expressed in activated HSCs as determined by RT-PCR (Fig. 4A). To further confirm these findings, we analyzed the expression CCR5 on the cell surface by flow cytometry. Incubation with an antibody against CCR5 resulted in considerable fluorescent staining of HSCs compared with incubation with an isotype-matched control antibody (Fig. 4B).

**RANTES induces Ca^{2+} increase and free radical production in HSCs through CCR5.** We then investigated whether RANTES exerts biological effects in HSCs. For this purpose, we stimulated cultured HSCs with rhRANTES and explored intracellular signaling events including Ca^{2+} increase and free radical production, which are induced by RANTES in other cell types (12, 37). Stimulation of fluo-4-loaded HSCs with RANTES resulted in Ca^{2+} increase consisting of a brief Ca^{2+} peak followed by a sustained phase (Fig. 5). Although the magnitude of Ca^{2+} increase was significantly lower that that observed after angiotensin II stimulation (data not shown), the pattern of the response was similar to that evoked by angiotensin II and other vasoactive substances in HSCs (2, 3). Import-
A formation (Fig. 6B). An increase in free radical formation resulted in a marked and rapid increase in free radical expression.

Fig. 4. Activated human HSCs express chemokine receptor-5 (CCR5). A: RNA was extracted from activated human HSCs (passage 4) or whole normal human. RT-PCR for CCR5 and β-actin was performed as described. B: Activated HSCs (passage 4) were detached with EDTA (2 mM) in PBS for 20 min followed by incubation with α-CCR5 (5 μg/ml) or isotype-matched isotype antibody (5 μg/ml) and fluorescein-conjugated secondary anti-mouse antibody at 4°C. Five thousand cells were analyzed by flow cytometry after gating viable cells. Shown is 1 representative experiment.

B

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**Treatment**

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**β-Actin**

- *p<0.05 compared to TNFα/vehicle
- **p<0.05 compared to IL-1β/vehicle

Fig. 3. JNK inhibition reduces TNF-α and IL-1β-induced RANTES expression.

A: To demonstrate the effects of the JNK inhibitor SP600125 (20 μM) on JNK activity, HSCs were pretreated with SP600125 or DMSO (0.1%) for 2 h followed by TNF-α or IL-1β treatment for 15 min. Phosphorylation of the JNK target c-Jun was analyzed by Western blotting. B: HSCs were pretreated with SP600125 (20 μM) or DMSO (0.1%) for 2 h. HSCs were incubated with TNF-α (10 ng/ml) or IL-1β (5 ng/ml) for 5 h and RANTES mRNA expression was analyzed by RT-PCR. C: HSCs were pretreated with SP600125 (20 μM) or DMSO (0.1%) for 2 h. HSCs were incubated with TNF-α (10 ng/ml) or IL-1β (5 ng/ml) for 24 h. RANTES secretion was measured by ELISA.

RANTES induces ERK activation and cell proliferation through CCR5. Because proliferation of activated HSCs is considered a key event in hepatic wound healing, we investigated the effects of RANTES on HSC proliferation and signaling pathways that mediate proliferation in HSCs. First, we explored the effect of RANTES on the phosphorylation of ERK, a member of MAPK family that mediates proliferation in HSCs (30, 46). RANTES caused an increase in both ERK phosphorylation and activity (Fig. 7A). This effect was substantially attenuated by a CCR5 blocking antibody, but not an isotype-matched control antibody, and by the antioxidant NAC (10−4 M). Accordingly, RANTES induced a moderate increase in DNA synthesis (twofold), as measured by the [3H]thymidine incorporation into the cells (Fig. 7B). This effect was less pronounced compared with that observed after stimulation of cells with 10 ng/ml PDGF-BB (8-fold, data not shown), which is among the most powerful mitogens for HSCs. In addition, incubation of cells with PD98059 (5×10−6 M), a selective MEK inhibitor, prevented RANTES-induced [3H]thymidine incorporation (Fig. 7B). The mitogenic effect of RANTES was also markedly blunted by incubation with a blocking antibody against CCR5 but not by an isotype-matched control antibody. Moreover, incubation of cells with the antioxidant NAC (10−4 M) or the NADPH oxidase inhibitor diphenylene iodonium (10−6 M) attenuated [3H]thymidine incorporation induced by rhRANTES (Fig. 7B). These data are consistent with studies from our laboratory demonstrating a crucial role for NADPH oxidase in the proliferation of HSCs (R. Bataller, unpublished observations). Intracellular calcium was required for rhRANTES- and PDGF-induced [3H]thymidine incorporation as demonstrated by the inhibitory action was found in the cytoplasm of the majority of the cells already after 2 min of rhRANTES stimulation (Fig. 6B). Again, CCR5 blockade markedly blunted RANTES-induced free radical formation (Fig. 6A), further demonstrating a role for this receptor in mediating RANTES effects in HSCs.

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effects of the calcium chelator BAPTA-AM (Fig. 7C), suggesting that the RANTES-induced increase in intracellular calcium increases HSC proliferation. To confirm that the rhRANTES-induced increase in [3H]thymidine incorporation was associated with cell proliferation, cell count experiments were performed. The addition of rhRANTES to serum-free medium caused a mild but significant increase in cell number and was blocked by anti-CCR5, but not an isotype-matched control antibody (Fig. 7D). Taken together, RANTES exerts a mild mitogenic effect toward HSCs via CCR5 and subsequent ERK activation.

RANTES induces HSC migration through CCR5. To determine whether RANTES is a chemotactic factor for HSCs, the effects of RANTES on HSC migration were evaluated by a migration assay in a modified Boyden chamber. rhRANTES (25 ng/ml) stimulated the migration of cultured HSCs through an 8-μm pore-size membrane (Fig. 8A) as efficiently as PDGF-BB at 5 ng/ml (data not shown). This result was confirmed in an in vitro scratch wound healing assay. Compared with buffer, rhRANTES markedly induced HSC migration into the cell-free zone (Fig. 8B). The motogenic effect of rhRANTES was attenuated in both cell migration assays when HSCs were preincubated with a blocking antibody against CCR5, but not an isotype-matched control (Fig. 8, A and B). We then explored whether rhRANTES activates FAK, a key molecule in the regulation of HSC migration (10, 36). Treatment with rhRANTES induced a twofold increase in tyrosine-phosphorylated FAK after 10 min as determined by Western blot analysis for tyrosine-phosphorylation of immunoprecipitated FAK (Fig. 8C).

DISCUSSION

Chemokines stimulate key biological processes in HSCs such as activation, proliferation, and migration. These responses are required for the accumulation of activated HSCs at the sites of hepatic injury, a key feature in the hepatic wound healing response (8, 32). HSCs are not only a target, but also a source for chemokines and are believed to contribute to the attraction of leukocytes (15, 25, 27, 33, 45). Therefore, the chemokine system appears to affect fibrogenesis by regulating the cross-talk between HSCs and cells of the immune system to achieve a concerted cellular response during the hepatic wound healing process.

This study demonstrated that activated HSCs express the CC chemokine RANTES. RANTES is expressed at low levels in activated HSCs and is strongly upregulated by proinflammatory cytokines including TNF-α, IL-1β, and CD40L. In HSCs, RANTES gene expression is regulated in an NF-κB- and AP-1-dependent manner as seen by the blocking effects on RANTES mRNA expression and protein secretion by IκBsr, IKK2dn, and SP600125. Conversely, NF-κB activation is sufficient to drive RANTES expression as shown in HSCs transduced with NIK. These findings are consistent with previous studies (6, 16, 44) showing that
NF-κB and AP-1 play important roles in the transcription of the RANTES gene.

Because HSCs may not only be a source but also a target for chemokines (8, 27, 32, 33, 45), we determined whether HSCs respond to exogenous RANTES. We provide evidence that rhRANTES modulates key biological responses in HSCs: 1) stimulation with rhRANTES induces the release of calcium from intracellular storages and increases free radical formation, 2) RANTES induces the activation of ERK and stimulates [3H]thymidine incorporation and HSC proliferation in a redox- and intracellular calcium-dependent manner, and 3) RANTES potently stimulates the migration of HSCs and induces FAK phosphorylation. Intracellular calcium and free radical are important second messen-

whether HSCs respond to exogenous RANTES. We provide evidence that rhRANTES modulates key biological responses in HSCs: 1) stimulation with rhRANTES induces the release of calcium from intracellular storages and increases free radical formation, 2) RANTES induces the activation of ERK and stimulates [3H]thymidine incorporation and HSC proliferation in a redox- and intracellular calcium-dependent manner, and 3) RANTES potently stimulates the migration of HSCs and induces FAK phosphorylation. Intracellular calcium and free radical are important second messen-
tors required for activation, proliferation, and contraction in HSCs (4, 13, 35). Although inflammatory mediators such as TNF-α and IL-1β, which are required for RANTES expression in HSCs, are upregulated in patients with liver fibrosis (22, 23, 47), the expression of RANTES in vivo has not been investigated in our study, and therefore, we can only speculate on its functional role in vivo. Because proliferation and migration are key biological events during fibrogenesis in vivo leading to HSCs accumulation at sites of injury, RANTES may contribute to this accumulation during fibrogenesis by potently stimulating migration in HSCs and, to a lesser extent, cell proliferation. Our study also did not address whether RANTES secretion by HSCs may modulate the infiltration of inflammatory cells into the injured liver. The fact that liver-infiltrating lymphocytes express CCR5 support this concept (7, 43). Chemokines including RANTES have been shown to act as regulators of T cell differentiation (24) and RANTES has been associated with a T helper cell 1 response (11) indicating that RANTES expression by HSCs might affect the T helper cell cytokine balance within the liver and thereby modulate fibrogenesis.

Our study found that HSCs express CCR5 and that many of the biological effects of RANTES, e.g., ERK activation, proliferation, and migration, are largely mediated by CCR5. The expression of CCR5 makes HSCs a target for chemokines secreted by infiltrating leukocytes, hepatocytes, and biliary epithelial cells as well as HSCs themselves (38). Our study did not address whether HSC-derived RANTES may act on HSCs in an autocrine fashion. Because the most potent biological effect of RANTES in our study was cell migration, it seems that even under inflammatory conditions, RANTES would exert its biological effects in a paracrine rather than an autocrine fashion.

Because blocking of CCR5 did not completely abolish RANTES-induced responses, it is likely that other chemokine receptors such as CCR1 and CCR3, which are expressed in HSCs (data not shown), mediate some effects of RANTES in HSCs. However, CCR5 contributed to ~70% of rhRANTES-induced signaling events in our study and appears to be the major receptor for RANTES in HSCs. Also, it remains to be determined whether CCR5 renders HSCs responsive to other CCR5 ligands such as macrophage inflammatory protein-1α and -1β and the HIV virus that may cause rapid progression of liver fibrosis in patients coinfected with hepatitis C virus and human immunodeficiency virus.

The role of chemokines in human disease is not well defined and most data derived from animal models suggest that there may be a high degree of redundancy in the chemokine-CCR system (17). Although the results of our study suggest that RANTES and CCR5 stimulate HSC migration and proliferation and may act as potential mediators of a HSC-leukocyte cross talk, it is unclear how RANTES and CCR5 expression by HSCs affects liver injury and whether inhibition of RANTES-CCR5 interaction may be beneficial or can be compensated for by other chemokines and CCRs. On the one hand, it is possible that RANTES increases the attraction of leukocytes to stimulate ECM degradation and tissue remodeling. On the other hand, RANTES-CCR5 interaction may further enhance fibrogenesis by stimulating inflammation, HSC proliferation, and migration. Thus additional studies are needed to investigate the expression patterns of RANTES and CCR5 in vivo to further define their specific functions during hepatic injury and fibrogenesis.

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DISCLOSURES

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