

# Glycosaminoglycans and their synthetic mimetics inhibit RANTES-induced migration and invasion of human hepatoma cells

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## Abstract

The CC-chemokine regulated on activation, normal T-cell expressed, and presumably secreted (RANTES)/CCL5 mediates its biological activities through activation of G protein-coupled receptors, CCR1, CCR3, or CCR5, and binds to glycosaminoglycans. This study was undertaken to investigate whether this chemokine is involved in hepatoma cell migration or invasion and to modulate these effects *in vitro* by the use of glycosaminoglycan mimetics. We show that the human hepatoma Huh7 and Hep3B cells express RANTES/CCL5 G protein-coupled receptor CCR1 but not CCR3 nor CCR5. RANTES/CCL5 binding to these cells depends on CCR1 and glycosaminoglycans. Moreover, RANTES/CCL5 strongly stimulates the migration and the invasion of Huh7 cells and to a lesser extent that of Hep3B cells. RANTES/CCL5 also stimulates the tyrosine phosphorylation of focal adhesion kinase and activates matrix metalloproteinase-9 in Huh7 hepatoma cells, resulting in increased invasion of these cells. The fact that RANTES/CCL5-induced migration and invasion of Huh7 cells are both strongly inhibited by anti-CCR1

antibodies and heparin, as well as by  $\beta$ -D-xyloside treatment of the cells, suggests that CCR1 and glycosaminoglycans are involved in these events. We then show by surface plasmon resonance that synthetic glycosaminoglycan mimetics, OTR4120 or OTR4131, directly bind to RANTES/CCL5. The preincubation of the chemokine with each of these mimetics strongly inhibited RANTES-induced migration and invasion of Huh7 cells. Therefore, targeting the RANTES-glycosaminoglycan interaction could be a new therapeutic approach for human hepatocellular carcinoma. [Mol Cancer Ther 2007;6(11):2948–58]

## Introduction

Chemokines are chemoattractant cytokines for leukocytes and their receptors belong to a family of specific G protein-coupled receptors (GPCR). They recruit various types of leukocytes, including monocytes/macrophages, lymphocytes, and dendritic cells, thereby modulating host responses to tumors (1). Moreover, several chemokines can control angiogenesis, a process essential for tumor growth, or guide the growth and the mobility of tumor cells, thereby affecting the process of tumor progression (2, 3). Among them, the CC-chemokine regulated on activation, normal T-cell expressed, and presumably secreted (RANTES)/CCL5 is a powerful chemoattractant of monocytes, eosinophils, and activated CD4 T cells (1). It binds to the specific GPCRs, CCR1, CCR3, and CCR5. RANTES/CCL5, like other chemokines, also binds to glycosaminoglycans, which are long, linear, and heterogeneous sulfated polysaccharides (4). RANTES/CCL5 exhibits its selectivity in glycosaminoglycan binding with the highest affinity (nanomolar range) for heparin (5, 6). Glycosaminoglycans exist in covalent linkage to a protein core as proteoglycans. Both glycosaminoglycans and proteoglycans play major roles in multiple cancer-related processes (7). We previously showed that RANTES/CCL5 associates not only with its GPCRs but also with heparan sulfate proteoglycans belonging to the syndecan family, syndecan-1 and syndecan-4, in addition to CD44 on HeLa cells and macrophage-derived monocytes (8, 9). Moreover, soluble heparin can inhibit the biological activity of chemokines as shown *in vitro* (10) and *in vivo* (11).

Hepatocellular carcinoma (HCC) is becoming the most feared complication of cirrhosis. Inflammation is part of the liver wound-healing response that in chronic conditions leads to the development of fibrosis and cirrhosis (12). In the liver, chemokines are usually regarded as selective chemoattractants of leukocyte populations in different conditions of injury inflammation (13, 14). Chemokines also stimulate key biological processes in human stellate

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cells, such as activation, proliferation, and migration, leading to fibrogenesis (13, 15). Moreover, several lines of evidence suggest the potential involvement of numerous chemokines in the development of HCC. We recently showed that the CXC-chemokine stromal cell-derived factor-1/CXCL12 induces hepatoma cell proliferation, migration, and invasion through its GPCR, CXCR4, and that glycosaminoglycans play major roles in these events (16). In addition, recent observations suggest the contribution of the CCR1/CCL3 axis to HCC progression (17, 18).

The purpose of this study was to determine whether RANTES/CCL5 induces hepatoma cell migration and invasion, to elucidate some of the molecular underlying events including the involvement of GPCRs and glycosaminoglycans, and finally to modulate *in vitro* these effects by the use of glycosaminoglycan mimetics.

## Materials and Methods

### Materials

The glycosaminoglycan mimetics used in this study, OTR4120 and OTR4131, were obtained from OTR3 Sarl. These molecules are synthetic derivatives of dextran T40 composed of ~200 glucosidic units linked by  $\alpha$ 1-6 bonds (19–21). These two compounds are equally characterized by the presence of carboxylate and sulfate groups in extents similar to those found in heparin. They differ from each other by the presence of acetate groups introduced only in the product OTR4131 (Fig. 1). Low molecular weight heparin (H3149) was purchased from Sigma-Aldrich. RANTES/CCL5, RANTES/CCL5 biotinylated at residue 1 (B1-RANTES), and 3Ala-RANTES were synthesized by L. Martin and C. Vita (CEA Saclay, Gif-sur-Yvette, France).

### Cell Culture

Huh7 and Hep3B human hepatoma cell lines were grown as described (16, 22). For proteoglycan biosynthesis inhibition, cells were incubated with 1 mmol/L 4-methylumbelliferyl- $\beta$ -D-xyloside ( $\beta$ DX; Sigma-Aldrich) for 72 h as described (23).

### Flow Cytometry Analysis

Cells were incubated with B1-RANTES (0, 20, and 40 nmol/L), synthesized as described previously (5). It was previously tested that such biotinylation does not modify RANTES-binding properties to various cells (24). In parallel, cells were preincubated for 1 h at 37°C with anti-CCR1 antibody (specific for an epitope located at the amino acids 7–24, polyclonal rabbit IgG, VWR) or with B1-RANTES preincubated for 2 h at 20°C with heparin (100  $\mu$ g/mL). After washing, cells were labeled for 30 min at +4°C with streptavidin-Alexa Fluor 488 complex (1:100; Molecular Probes, Invitrogen). CCR1, CCR3, and CCR5 immunostaining for flow cytometry analysis was done using anti-CCR1 monoclonal antibody (mAb; mouse IgG2b; R&D Systems), anti-CCR3 mAb (mouse IgG2a, 10  $\mu$ g/mL; Santa Cruz Biotechnology, Inc.), or anti-CCR5 mAb (clone 2D7, IgG2a, 10  $\mu$ g/mL; BD Bioscience Pharmingen), respectively. The efficiency of  $\beta$ DX treatment was assessed

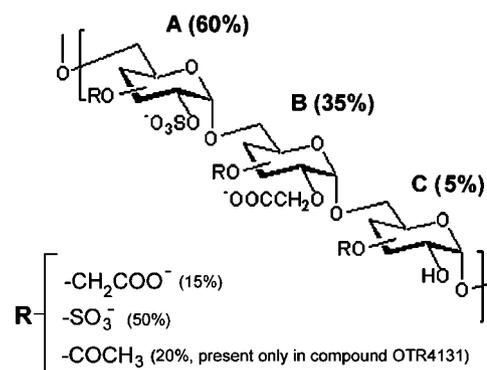
by immunofluorescence using a heparan sulfate mAb (clone 10E4; Seikagaku Corp.) as described (16).

### Reverse Transcription-PCR

CCR1, CCR3, CCR5, matrix metalloproteinase-9 (MMP-9), and glyceraldehyde-3-phosphate dehydrogenase mRNAs were amplified by reverse transcription-PCR (25). Specific primers were designed as follows: CCR1, 5'-CTCTTCCTGTTACGCTTCC-3' (forward) and 5'-CCAAATGTCTGCTCTGCTCA-3' (reverse); CCR3, 5'-TCTTCCTCGTCACCTTCCA-3' (forward) and 5'-ATCACCTCTGTACCAGCAT-3' (reverse); and CCR5, 5'-GCTGAGACATCCGTCCCTA-3' (forward) and 5'-GGTGAATGAAGACCTCCTTTITG-5' (reverse). MMP-9 and glyceraldehyde-3-phosphate dehydrogenase primers were designed as described (16). In some experiments, optimum semiquantitative reverse transcription-PCR conditions were established to remain in the linear phase of amplification curve.

### Cell Migration and Invasion Assays

Cell migration or invasion was done using BioCoat cell migration chambers (Becton Dickinson) as described (16). Briefly, inserts containing 8- $\mu$ m pore size filters were coated with fibronectin (100  $\mu$ g/mL; Santa Cruz Biotechnology) for migration or Matrigel (320  $\mu$ g/mL; BD Bioscience Pharmingen) for invasion assay. The chemokine RANTES/CCL5 was added to 500  $\mu$ L DMEM supplemented with 10% FCS in the lower chamber. Hepatocyte growth factor (HGF; 20 ng/mL) was used as a positive control (16). After 24 h, cells that had migrated through the filter pores were fixed with methanol, stained with Mayer's hemalum, and counted. In parallel, cells were preincubated for 2 h at 37°C with the following inhibitors: anti-CCR1 antibody (5  $\mu$ g/mL, rabbit polyclonal IgG; Santa Cruz Biotechnology) or anti-MMP-9 mAb (5  $\mu$ g/mL, IgG1; Santa Cruz Biotechnology). Alternatively, cells were treated with



**Figure 1.** Schematic representation of glycosaminoglycan mimetics OTR4120 and OTR4131. The global degree of substitution (SD) of carboxymethyl ( $SD_{CM}$ ) and sulfate ( $SD_S$ ) groups is  $SD_{CM} = 0.5$  and  $SD_S = 1.2$ , which are equal on both products. The presence of acetate groups ( $SD_{Ac} = 0.2$ ) characterizes the product OTR4131. The **A**, **B**, and **C** percentages were calculated from the relative content of each group at the C-2 position determined by  $^1H$  nuclear magnetic resonance spectrometry. In addition, **R** represents the proportion of each substituted group at both the C-3 and C-4 positions. For a simplified representation, the substituted glucosidic units **A**, **B**, and **C** are arranged in an arbitrary combination.

1 mmol/L  $\beta$ DX for 48 h, and for each insert,  $2.5 \times 10^5$  cells in 0.1% bovine serum albumin/DMEM were further incubated with 1 mmol/L  $\beta$ DX for 24-h migration or invasion assay. Alternatively, RANTES/CCL5 was preincubated for 2 h at 20°C with heparin. Heparin alone or RANTES/CCL5 preincubated with heparin was added to the lower chamber of culture.

#### Phosphotyrosine Residue Immunostaining

Huh7 cells were serum deprived for 24 h, incubated for 20 min at 37°C in 10% FCS-DMEM supplemented or not with RANTES (3 nmol/L), fixed with paraformaldehyde (1%), and permeabilized in 0.05% Triton X-100 (Sigma-Aldrich). Cells were immunostained on phosphotyrosine residues using Tyr(P) mAb (4G10, 10  $\mu$ g/mL; Cell Signaling) and Alexa Fluor 488 goat anti-mouse IgG (1:400).

#### Activation of Focal Adhesion Components by RANTES/CCL5

Huh7 cells were cultured for 48 h in 0.1% FCS-DMEM and incubated at 37°C for 15 min with RANTES/CCL5 (3 and 50 nmol/L). Phosphorylated focal adhesion kinase (FAK) was revealed using polyclonal anti-FAK-(P)-Tyr<sup>577</sup> antibodies (Cell Signaling). Parallel immunoblotting with anti-total FAK polyclonal antibodies (Cell Signaling) was done to confirm equal loading of samples.

#### Gelatin Zymography

Gelatin zymography was done as described (26). Briefly, Huh7 cells were incubated for 24 h in serum-free medium supplemented or not with RANTES/CCL5 (3 nmol/L) or 3Aa-RANTES (3 nmol/L). Conditioned media were resolved on 10% SDS-PAGE, 0.1% gelatin (Sigma-Aldrich), with equal amounts of proteins loaded. After SDS extraction, gelatinolytic activity was developed as described (16, 26).

#### Surface Plasmon Resonance

Optical biosensor experiments were done with a BIAcore 3000 optical biosensor (BIAcore). Biotinylated RANTES (at position 1 or 66) was coupled to the surface of a SensorChip SA (carboxymethylated dextran with immobilized streptavidin for capture of biotinylated ligand). Biotinylated RANTES [20  $\mu$ L of 5  $\mu$ g/mL in HEPES-buffered saline—50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 3 mmol/L EDTA, 0.005% surfactant P-20] was then injected, at 20  $\mu$ L/min flow rate, to two channels of the streptavidin-coated sensorship to a resonance unit value of 1,500 (a third channel was not loaded and used as negative control). In a typical analysis, OTR4120 or OTR4131 at different concentrations (0, 10, 30, 100, 300, and 1,000 ng/mL) was flowed onto the biotinylated RANTES-coated surface at a rate of 30  $\mu$ L/min for a 10-min association time, after which the channels were rinsed with the running buffer [10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 3.4 mmol/L EDTA, 0.005% Tween 20] to analyze the dissociation phase. After each experiment, the biosensor surface was regenerated with 3 mol/L NaCl (10  $\mu$ L). Flow cell, temperature, flow rate, sample volume, and mixing were selected with the BIAcore control software (BIAcore). Affinities were determined by analysis of the kinetic of the association assuming a 1:1 Langmuir model using BIAevaluation software.

#### Statistical Analysis

For the determination of statistical significance, an ANOVA test was done with the StatView software. A *P* value of <0.05 was used as the criterion of statistical significance.

## Results

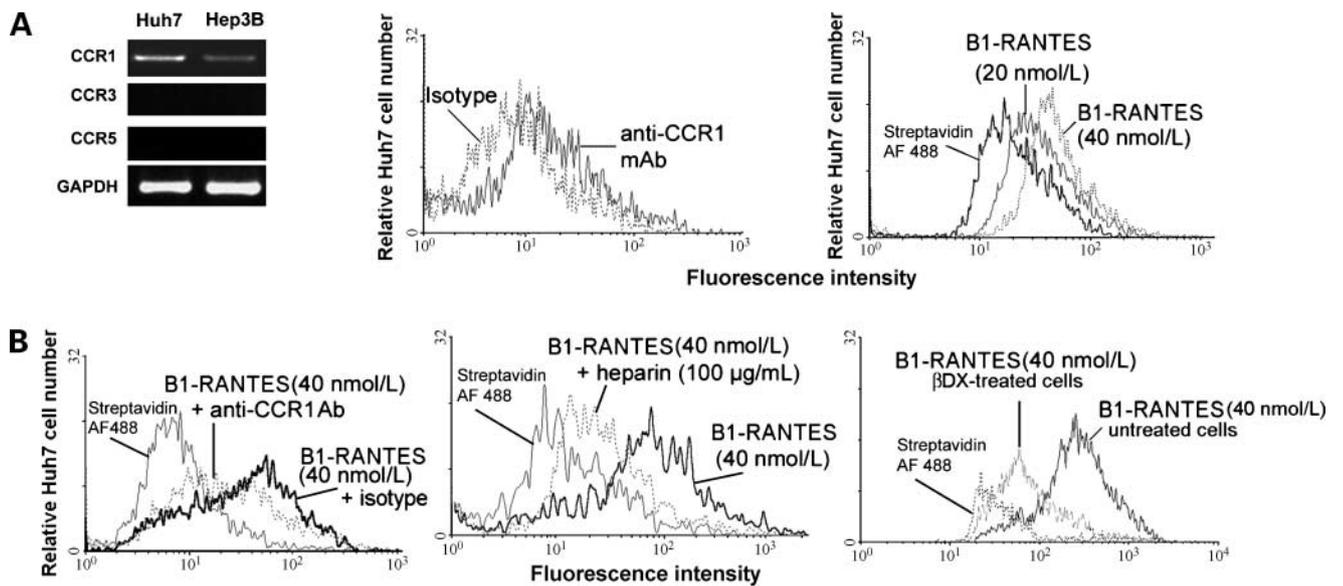
#### Binding of RANTES/CCL5 to Human Hepatoma Cells

To determine whether human hepatoma cells may be a target for RANTES/CCL5, we first examined Huh7 and Hep3B cell expression of RANTES GPCRs, CCR1, CCR3, and CCR5, through which RANTES/CCL5 exerts many of its biological effects. CCR1 mRNA was strongly expressed in Huh7 but faintly in Hep3B cells as determined by reverse transcription-PCR, whereas CCR5 and CCR3 expression was not detectable in both cell types (Fig. 2A). We then analyzed the expression of CCR1 on the cell plasma membrane by flow cytometry. Incubation with anti-CCR1 mAb resulted in a fluorescent staining of Huh7 and Hep3B cells compared with incubation with an isotype-matched control antibody (Fig. 2A; data not shown), showing that these hepatoma cells express CCR1 proteins. By contrast, CCR5 and CCR3 were not detected (data not shown).

Biotinylated RANTES/CCL5 clearly bound, in a dose-dependent manner (0, 20, and 40 nmol/L), to Huh7 cells (Fig. 2A). The fact that at a concentration of 125 nmol/L RANTES/CCL5 the binding of the chemokine to the cells was barely detectable (data not shown) may be related to RANTES/CCL5 aggregation, which is generally observed at this high concentration (5). RANTES/CCL5 binding was significantly inhibited when the Huh7 cells were preincubated with anti-CCR1 antibody ( $43 \pm 17\%$  inhibition;  $n = 4$ ;  $P < 0.05$ ) compared with an isotype-matched control antibody, suggesting that the chemokine binds to Huh7 cells at least partly through CCR1 (Fig. 2B).

Interestingly, RANTES/CCL5 binding to these cells was strongly inhibited when the chemokine was preincubated for 2 h with heparin at 100  $\mu$ g/mL ( $81 \pm 19\%$  inhibition;  $n = 3$ ;  $P < 0.05$ ; Fig. 2B). To strengthen the role played by glycosaminoglycans expressed on the Huh7 cell surface in the binding of RANTES/CCL5, the cells were treated with  $\beta$ DX, used here as alternative acceptors for the assembly of glycosaminoglycan chains. Flow cytometry analysis revealed as expected (23) that heparan sulfate-specific immunofluorescence staining was decreased by  $80 \pm 8\%$  ( $n = 3$ ) when Huh7 cells were treated with  $\beta$ DX compared with untreated control cells (data not shown). In these conditions, RANTES/CCL5 binding to the cells was strongly reduced (Fig. 2B). If the flow cytometry-binding assay was done in the presence of both anti-CCR1 and heparin simultaneously, the binding of B1-RANTES to the cells was abolished (data not shown), suggesting that CCR1 and glycosaminoglycans are the two predominant RANTES/CCL5 ligands on hepatoma cells.

Moreover, RANTES/CCL5 also binds to another hepatoma cell line, Hep3B cells, and this binding was also significantly and partly inhibited by anti-CCR1 antibody and strongly by heparin (100  $\mu$ g/mL; data not shown).



**Figure 2.** RANTES/CCL5 binding to Huh7 cells. **A**, *left*, total RNA was extracted from Huh7 and Hep3B cell lines and mRNA expression was analyzed by reverse transcription-PCR using each primer as indicated. *Middle*, Huh7 cells were incubated with anti-CCR1 mAb or its isotype and then with Alexa Fluor 488-labeled secondary antibodies. Immunolabeling was analyzed by flow cytometry. *Right*, Huh7 cells were incubated with B1-RANTES at 0, 20, or 40 nmol/L. Binding was analyzed by flow cytometry using streptavidin-Alexa Fluor 488. Reactivity was compared with streptavidin-Alexa Fluor 488 (AF 488). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **B**, *left*, Huh7 cells were preincubated or not with anti-CCR1 antibody or with its isotype before the addition of B1-RANTES (40 nmol/L). *Middle*, B1-RANTES (40 nmol/L) was preincubated or not with heparin (100 µg/mL) and the suspension was added to the cells. *Right*, cells were pretreated with βDX and then assayed for B1-RANTES binding. Data are representative of five independent experiments.

### RANTES/CCL5-Induced Chemotactic Effects on Human Hepatoma Cells

**RANTES/CCL5 Stimulates Hepatoma Cell Migration.** As hepatoma cell migration is an important determinant in the progression of HCC, we investigated whether RANTES/CCL5 stimulates Huh7 and Hep3B cell migration. As shown in Fig. 3A, RANTES/CCL5 (0, 0.1, 1, 3, 30, and 50 nmol/L) induced a dose-dependent Huh7 cell migration: The maximum migration was observed when 50 nmol/L RANTES/CCL5 was applied in the assay conditions (Fig. 3A). This chemotactic effect depended on CCR1 because anti-CCR1 antibodies strongly reduced it ( $95 \pm 9\%$  inhibition;  $n = 4$ ;  $P < 0.05$ ; Fig. 3B). Moreover, this RANTES-induced cell migration was also strongly decreased by preincubating the chemokine with heparin (100 ng/mL;  $90 \pm 12\%$  inhibition;  $n = 4$ ;  $P < 0.05$ ), which suggests that glycosaminoglycans are also involved in these events (Fig. 3B). When the cells were pretreated with βDX, Huh7 cell chemotaxis toward RANTES/CCL5 was strongly inhibited ( $86 \pm 10\%$ ;  $n = 3$ ;  $P < 0.05$ ), as well as cell migration toward HGF, which is known to bind to heparan sulfate chains and syndecan-1 ( $62 \pm 4\%$ ;  $P < 0.05$ ;  $n = 3$ ; Fig. 3B; refs. 27, 28). This suggests that glycosaminoglycan chains, carried by membrane proteoglycans, are involved in RANTES/CCL5-induced Huh7 cell migration (Fig. 3B). None of these compounds significantly affected the basal migration (data not shown). To strengthen the role played by glycosaminoglycans in the mitogenic effects of RANTES/CCL5, we used a glycosaminoglycan binding-

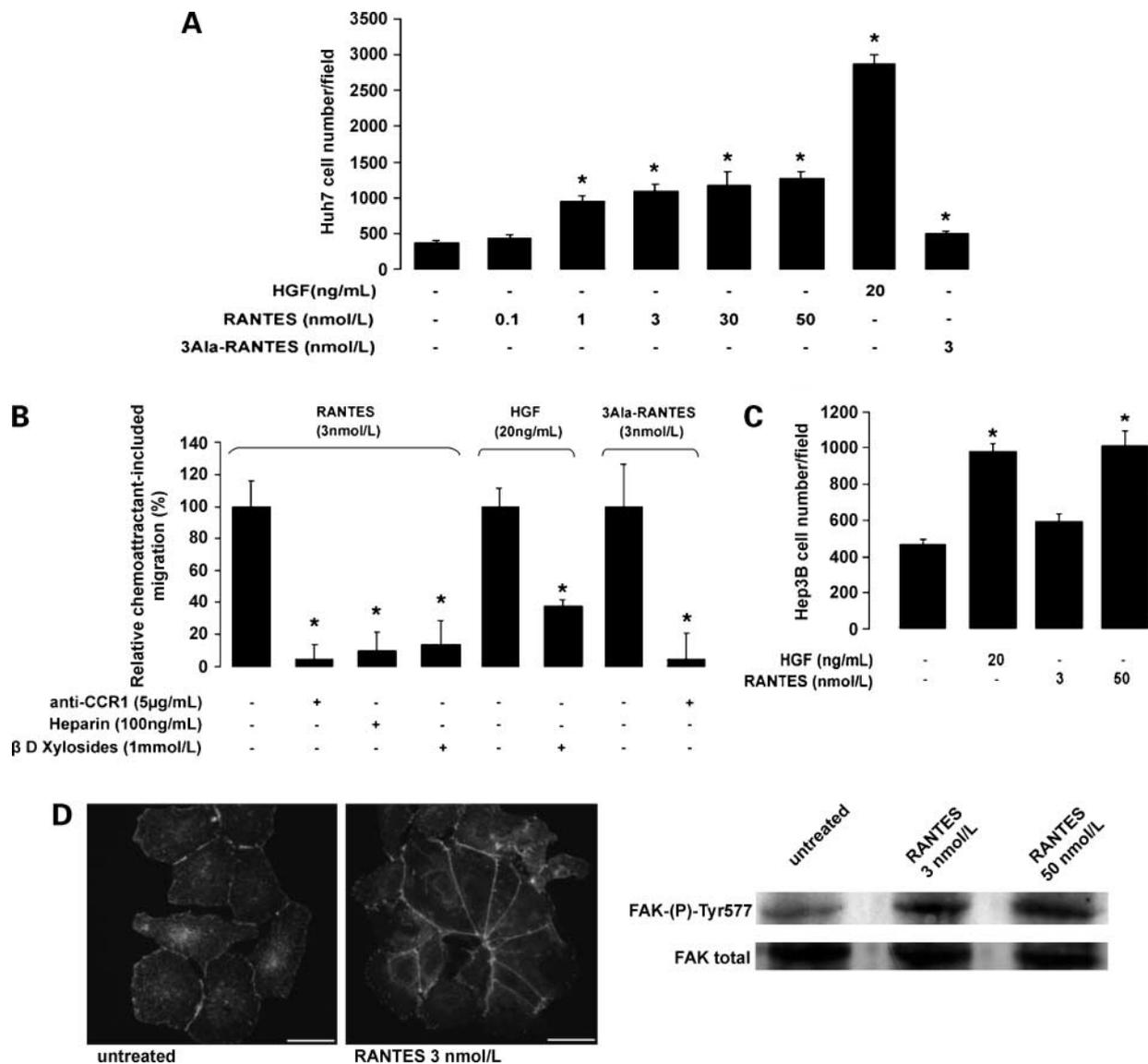
deficient RANTES/CCL5 mutant, 3Ala-RANTES, which has positively charged groups in the  $^{44}\text{RKNR}^{47}$  glycosaminoglycan-binding domain replaced by neutral alanine. 3Ala-RANTES (3 nmol/L) did promote a slight but significant chemotaxis relative to control in the absence of RANTES (Fig. 3A), which was strongly reduced when the cells were pretreated with anti-CCR1 antibodies ( $90 \pm 5\%$  inhibition;  $n = 3$ ;  $P < 0.01$ ; Fig. 3B). However, Huh7 cell migration induced by 3 nmol/L 3Ala-RANTES was decreased by  $70 \pm 5\%$  compared with that induced by 3 nmol/L RANTES/CCL5 ( $n = 3$ ;  $P < 0.05$ ; Fig. 3A).

RANTES/CCL5 also stimulated the migration of another hepatoma cell line, the Hep3B cells. However, the effects of RANTES/CCL5 on Hep3B cells were less important because a 50 nmol/L RANTES/CCL5 concentration was required to increase significantly the migration of these cells, whereas 3 nmol/L RANTES/CCL5 had no effect ( $n = 3$ ;  $P < 0.05$ ; Fig. 3C).

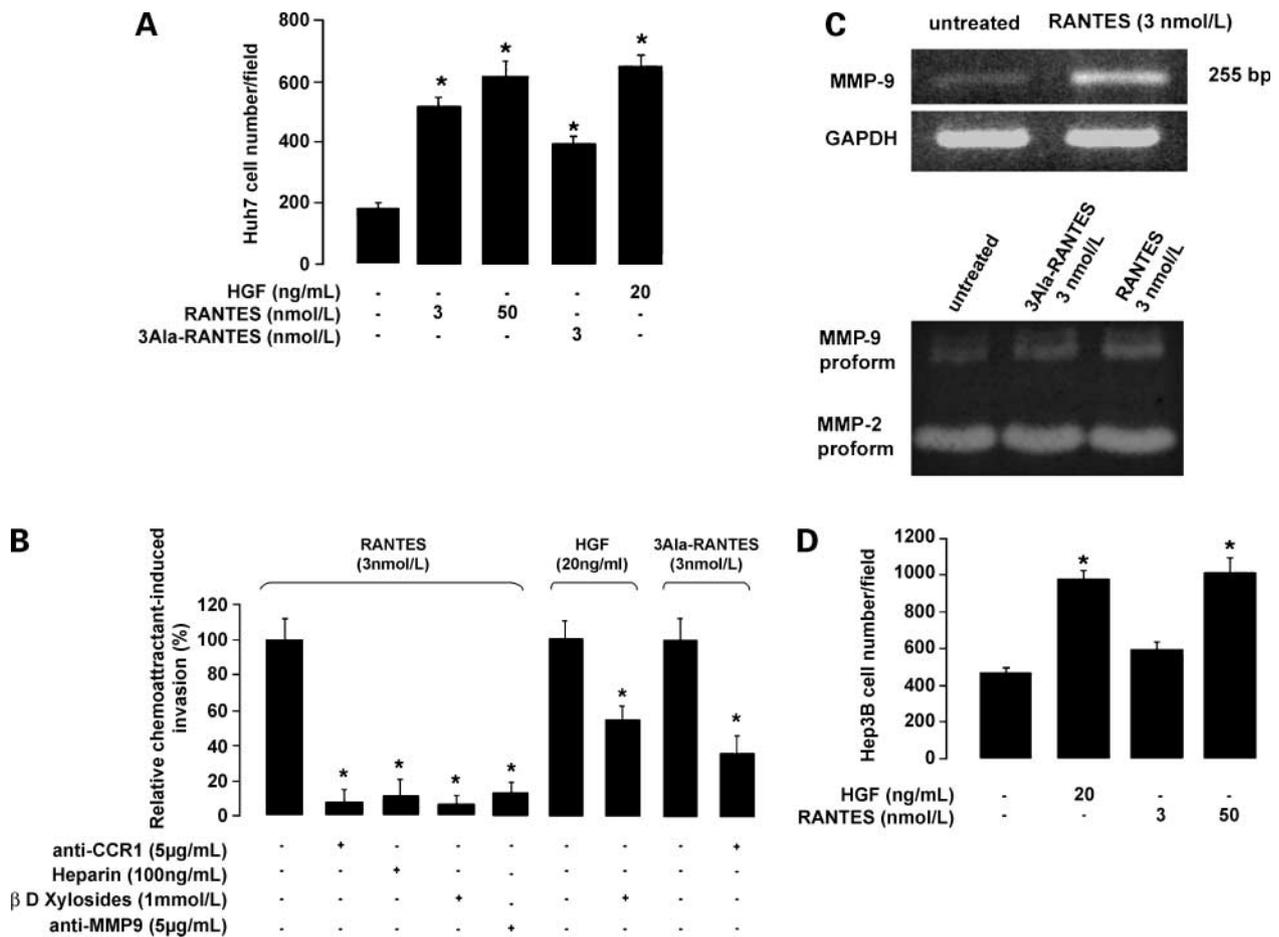
As focal adhesion molecules are key molecules in cell migration (29), we studied the effect of RANTES on the activation of focal adhesion components in Huh7 cells. Subconfluent cells were exposed to a 15-min incubation with RANTES (3 nmol/L), fixed, and examined by indirect immunostaining for phosphotyrosine residues using a Tyr(P) (4G10) antibody. Membranous staining was more intense in RANTES-stimulated Huh7 cells compared with control cells, suggesting that the chemokine induced the phosphorylation of focal adhesion components (Fig. 3D). Furthermore, the FAK was immunoblotted from lysates of

RANTES/CCL5-stimulated or RANTES/CCL5-unstimulated control Huh7 cells with anti-FAK antibodies and with anti-FAK-(P)-Tyr<sup>577</sup> phosphospecific antibodies, respectively. The level of tyrosine phosphorylation at FAK-Tyr<sup>577</sup> from RANTES/CCL5-treated Huh7 cells was higher compared with that of RANTES/CCL5-untreated control cells (Fig. 3D).

*RANTES/CCL5 Stimulates Huh7 Cell Invasion into Matrigel.* RANTES/CCL5 (3 nmol/L) induced a significant increase in Huh7 cell invasion into Matrigel ( $n = 3$ ;  $P < 0.05$ ; Fig. 4A). This was strongly inhibited by the preincubation of the cells with anti-CCR1 antibody ( $93 \pm 5\%$  inhibition;  $n = 3$ ;  $P < 0.05$ ), suggesting the role of CCR1 in this RANTES/CCL5-induced invasion (Fig. 4B). This was also strongly



**Figure 3.** RANTES/CCL5 induced the migration of Huh7 or Hep3B cells. **A**, RANTES/CCL5 induced a dose-dependent Huh7 cell migration. RANTES at 0 to 50 nmol/L concentrations, as indicated, 20 ng/mL HGF, or 3 nmol/L 3Ala-RANTES/CCL5 was used for this experiment. Columns, mean of Huh7 cells counted by field for three independent experiments; bars, SE. \*,  $P < 0.05$  versus cells that migrated in the absence of chemoattractant. **B**, RANTES/CCL5-induced Huh7 cell migration was decreased by anti-CCR1 antibody. Preincubation of RANTES/CCL5 with heparin (100 ng/mL) or treatment of the cells with  $\beta$ DX significantly reduced RANTES/CCL5-induced cell migration. HGF-induced Huh7 cell migration was decreased by  $\beta$ DX cell pretreatment. 3Ala-RANTES-induced Huh7 cell migration was decreased by anti-CCR1 antibody. Migration induced by RANTES/CCL5 or HGF or 3Ala-RANTES minus background of unstimulated cells was set to 100% and represents the respective controls. RANTES/CCL5-induced, HGF-induced, or 3Ala-RANTES-induced migration in the presence of inhibitor is shown as a percentage of control. \*,  $P < 0.05$  versus chemoattracted cells in the absence of inhibitor. **C**, chemotactic migration of Hep3B cells was induced by addition of HGF (20 ng/mL) or RANTES/CCL5 (3 and 50 nmol/L) into the bottom chamber. \*,  $P < 0.05$  versus control cells. **D**, RANTES/CCL5 induced phosphorylation of focal adhesion components in Huh7 cells. *Left*, Huh7 cells, incubated with RANTES/CCL5 (3 nmol/L), were examined by indirect immunostaining for phosphotyrosine residues using anti-Tyr(p) mAbs (4G10). Bar, 5  $\mu$ m. *Right*, Western blot analysis of phosphorylated and total forms of FAK in Huh7 cells that were either untreated or stimulated with RANTES/CCL5 (3 or 50 nmol/L). Representative of three individual experiments.



**Figure 4.** RANTES/CCL5 induced the invasion of Huh7 or Hep3B cells. **A**, RANTES/CCL5 induced a dose-dependent Huh7 cell invasion into Matrigel. RANTES at 0 to 50 nmol/L concentrations, as indicated, 20 ng/mL HGF, or 3 nmol/L 3Ala-RANTES/CCL5 was used for this experiment. *Columns*, mean of Huh7 cells counted by field for three independent experiments; *bars*, SE. \*,  $P < 0.05$  versus cells that migrated in the absence of chemoattractant. **B**, RANTES/CCL5-induced Huh7 cell invasion was decreased by anti-CCR1 antibody or by anti-MMP-9 mAb. Preincubation of RANTES/CCL5 with heparin (100 ng/mL) or treatment of the cells with  $\beta$ DX significantly reduced RANTES/CCL5-induced cell invasion. HGF-induced Huh7 cell invasion was decreased by  $\beta$ DX cell pretreatment. 3Ala-RANTES-induced Huh7 cell invasion was decreased by anti-CCR1 antibodies. Invasion induced in the respective controls (RANTES/CCL5 or HGF or 3Ala-RANTES alone minus background of unstimulated cells) was set to 100%. RANTES/CCL5-induced, HGF-induced, or 3Ala-RANTES-induced invasion in the presence of inhibitor is shown as a percentage of control. \*,  $P < 0.05$  versus chemoattracted cells in the absence of inhibitor. **C**, *top*, RANTES/CCL5 increases MMP-9 expression. Huh7 cells were incubated or not for 16 h with RANTES/CCL5 (3 nmol/L). Its effect on MMP-9 mRNA synthesis was studied by reverse transcription-PCR. PCR products were analyzed on agarose gel stained with ethidium bromide. *Bottom*, analysis of gelatinolytic activity of MMP-9 in the conditioned medium of Huh7 cells. Cells were treated with RANTES/CCL5 (3 nmol/L) or 3Ala-RANTES (3 nmol/L) in serum-free medium. Conditioned media were collected and analyzed by gelatin zymography, done with equal amounts of protein loaded. Representative of three experiments. **D**, chemotactic migration of Hep3B cells was induced by addition of HGF (20 ng/mL) or RANTES/CCL5 (3 and 50 nmol/L) into the bottom chamber. \*,  $P < 0.05$  versus control cells.

inhibited by the preincubation of RANTES/CCL5 with heparin (100 ng/mL;  $90 \pm 11\%$  inhibition;  $n = 3$ ;  $P < 0.05$ ) or by treating the cells with  $\beta$ DX ( $95 \pm 5\%$  inhibition;  $n = 3$ ;  $P < 0.05$ ; Fig. 4B). As control, cell invasion induced by HGF was also reduced by  $\beta$ DX treatment ( $46 \pm 8\%$  inhibition;  $n = 3$ ;  $P < 0.05$ ; Fig. 4B). These data strongly indicate the role of glycosaminoglycans in this RANTES/CCL5-induced invasion of Huh7 cells. In these experiments, whereas  $\beta$ DX treatment of the cells did not affect their basal invasion, heparin itself increased it by  $50 \pm 10\%$  (data not shown). 3Ala-RANTES (3 nmol/L) did promote a significant cell invasion relative to control in the absence of RANTES/CCL5 (Fig. 4A), which was reduced when cells were

pretreated with anti-CCR1 antibodies ( $64 \pm 10\%$  inhibition;  $n = 3$ ;  $P < 0.05$ ; Fig. 4B). However, Huh7 cell invasion induced by 3 nmol/L 3Ala-RANTES was decreased by  $30 \pm 5\%$  compared with that induced by 3 nmol/L RANTES/CCL5 ( $n = 3$ ;  $P < 0.05$ ; Fig. 4A). Together, these data indicate that RANTES-glycosaminoglycan interactions are involved at least partly in this RANTES-induced cell invasion of hepatoma cells.

Strikingly, 3 nmol/L RANTES/CCL5 increased the level of mRNA encoding for MMP-9 and the MMP-9 proform (1.8-fold increase;  $n = 3$ ;  $P < 0.05$ ), as assessed by gelatin zymography, in Huh7 cells (Fig. 4C). 3Ala-RANTES (3 nmol/L) also increased the MMP-9 proform but less

than did 3 nmol/L RANTES (1.4-fold increase;  $n = 3$ ;  $P < 0.05$ ; Fig. 4C) Moreover, Huh7 cell preincubation with anti-MMP-9 mAb resulted in an  $82 \pm 8\%$  inhibition ( $n = 3$ ;  $P < 0.05$ ) of the RANTES/CCL5-dependent cell invasion (Fig. 4B).

Furthermore, RANTES/CCL5 also stimulated the invasion of Hep3B cells. However, the effects of RANTES/CCL5 on Hep3B cell were less important because 50 nmol/L but not 3 nmol/L RANTES/CCL5 induced a significant increase in this effect ( $n = 3$ ;  $P < 0.05$ ; Fig. 4D).

#### Effect of Synthetic Glycosaminoglycan Mimetics on RANTES Binding to the Cells

Together, the above data suggest that soluble glycosaminoglycans, such as heparin, may interfere with the migration and invasion induced by RANTES/CCL5 on human hepatoma cells. Therefore, we tested the ability of synthetic glycosaminoglycan mimetics in modulating these RANTES/CCL5 effects. Synthetic polymers, called RGTA (for regenerating agent; refs. 19, 20, 30), have been engineered to mimic the stabilizing properties of glycosaminoglycans toward heparin-binding growth factors. OTR4120 and OTR4131 were tested in our assays.

Surface plasmon resonance done on a BIAcore system was used to study whether a direct interaction between RANTES/CCL5 and OTR4120 or OTR4131, respectively, occurs (Fig. 5A). B1-RANTES was immobilized to a streptavidin-bound sensorship. Increasing concentrations of OTR4131 or OTR4120 (10, 30, 100, 300, and 1,000 ng/mL) were injected over both RANTES-immobilized or negative control surfaces. B1-RANTES was immobilized at 1,500 resonance units on sensorship. When a glycosaminoglycan mimetic solution was injected into biosensor flow cells and flowed over RANTES-immobilized surfaces for 10 min, a typical increase of the surface plasmon resonance response (in resonance unit) versus time was obtained, corresponding to the association phase, which was then followed (20 min) by a decrease of surface plasmon resonance response, the dissociation phase, when the chemokine was replaced by running buffer. The RANTES-glycosaminoglycan mimetic complexes dissociate at a rather slow rate, suggesting the presence of strong and complementary interactions in these complexes. The association and dissociation phases of the obtained sensorgrams could be fitted to the Langmuir ( $A + B = AB$ ) model and analyzed by BIAEVAI software. Global fitting of binding curves gave association rate constants ( $k_{on}$ ) and dissociation rate constants ( $k_{off}$ ), which are given in Table 1. Similar results were observed when RANTES biotinylated at residue 66 was immobilized to the streptavidin-bound sensorship (data not shown).

When OTR4131 or OTR4120, respectively, was flowed over control surfaces (containing streptavidin only), no significant signal (no binding) was observed (data not shown).

Taken together, these data strongly suggest that RANTES directly binds to the glycosaminoglycan mimetics OTR4131 or OTR4120.

We then tested whether these glycosaminoglycan mimetics modulate RANTES/CCL5 binding to Huh7 cells. We

observed that OTR4131 or OTR4120 at 1  $\mu\text{g}/\text{mL}$  strongly inhibited RANTES/CCL5 binding to these cells by  $68 \pm 3\%$  and  $77 \pm 17\%$ , respectively ( $P < 0.001$  and  $0.01$ , respectively;  $n = 3$ ; Fig. 5B).

#### Effect of Synthetic Glycosaminoglycan Mimetics on RANTES/CCL5-Induced Hepatoma Cell Migration and Invasion

The effect of RANTES/CCL5 on hepatoma cell migration and invasion was considerably stronger in Huh7 compared with Hep3B cells. Therefore, we tested the ability of glycosaminoglycan mimetics in modulating RANTES/CCL5-induced migration or invasion of Huh7 cells.

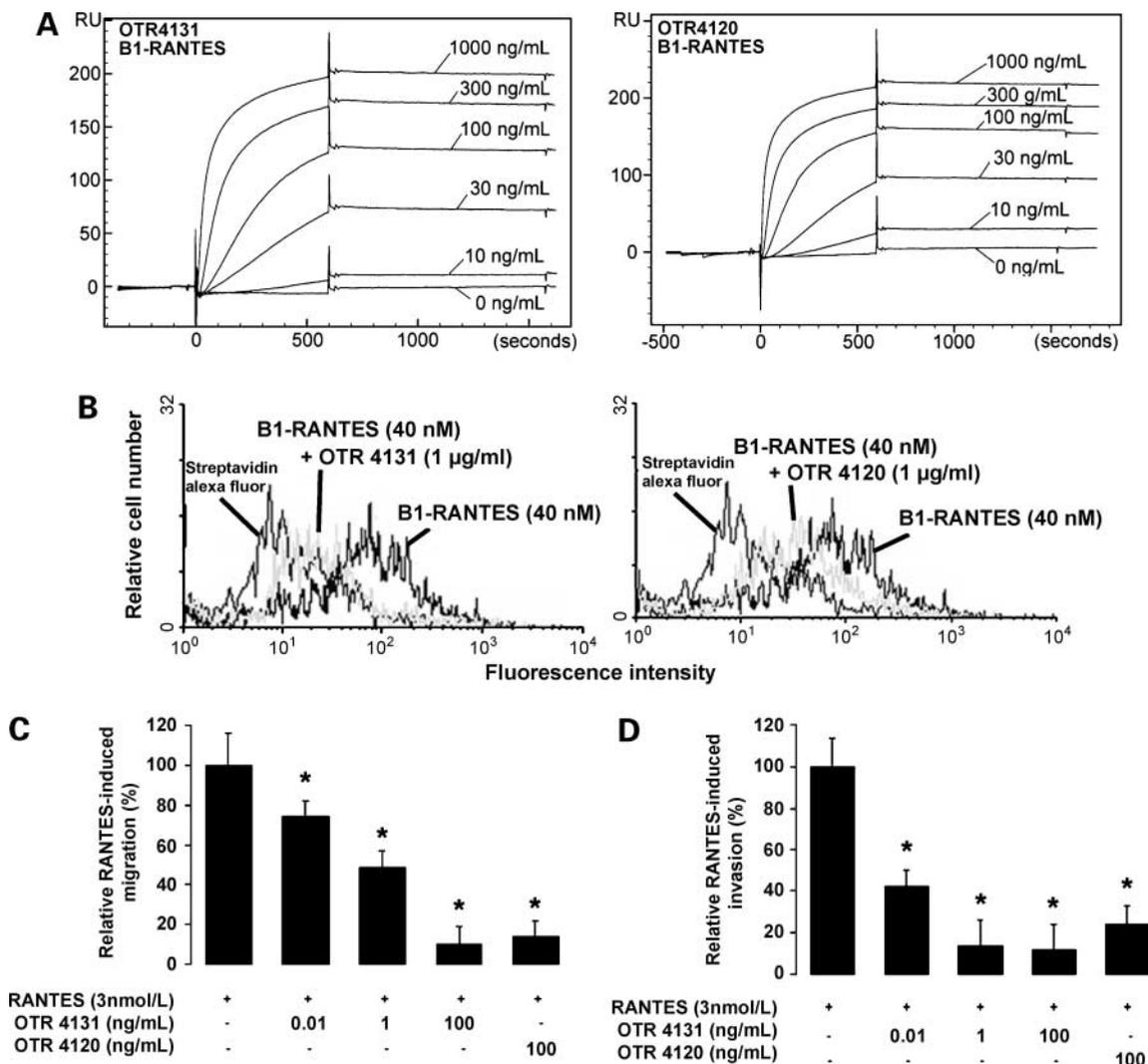
To find the optimum concentration of OTR4120 or OTR4131 decreasing RANTES/CCL5-induced migration or invasion, a range of OTR4131 or OTR4120 concentrations in the presence or absence of 3 nmol/L RANTES/CCL5 was tested for the ability to inhibit Huh7 cell migration or invasion in the Transwell assay (Fig. 5C; data not shown). The largest decrease in migration induced by 3 nmol/L RANTES/CCL5 was found for 100 ng/mL OTR4131. At this concentration, migration was decreased by  $90 \pm 9\%$  ( $n = 3$ ;  $P < 0.01$ ). As expected, lower decreases were observed for 1 and 0.01 ng/mL OTR4131 ( $51 \pm 8\%$  and  $26 \pm 8\%$ , respectively;  $n = 3$ ;  $P < 0.05$ ; Fig. 5C). Similar results were observed when using OTR4120 at 100, 1, and 0.01 ng/mL (Fig. 5C; data not shown). OTR4131 (100 ng/mL) also induced the largest decrease in hepatoma cell invasion toward 3 nmol/L RANTES/CCL5. At this concentration, invasion was decreased by  $88 \pm 12\%$  ( $P < 0.01$ ). Inhibitions ( $86 \pm 12\%$  and  $58 \pm 8\%$ ) in RANTES/CCL5-induced cell invasion were observed for 1 and 0.01 ng/mL OTR4131, respectively ( $P < 0.05$ ). Similar decreased cell invasion was obtained when using OTR4120 at 100, 1, and 0.01 ng/mL (Fig. 5D; data not shown).

Moreover, the addition of 1 ng/mL OTR431 decreased hepatoma cell migration or invasion induced by 3, 30, or 50 nmol/L RANTES/CCL5 by  $58 \pm 8\%$ ,  $50 \pm 9\%$ , and  $46 \pm 8\%$ , respectively ( $n = 3$ ), when compared with RANTES/CCL5 alone. Cell invasion induced by 3, 30, or 50 nmol/L RANTES/CCL5 was also reduced on addition of 1 ng/mL OTR4131 by  $88 \pm 7\%$ ,  $83 \pm 9\%$ , and  $80 \pm 5\%$ , respectively ( $n = 3$ ). Similar results were observed for 1 ng/mL OTR4120.

## Discussion

*In vivo*, CCR1 has been detected on human hepatoma cells and to a lesser extent on endothelial cells in hepatoma tissues but not in normal liver tissues (17). Recently, the role of CCR1 and one of its ligand, CCL3, was also shown in HCC progression (18). Indeed, in a model of *N*-nitrosodiethylamine-induced HCC, tumor foci number, sizes, and angiogenesis were markedly reduced in CCR1- and CCL3-deficient mice compared with wild-type mice (18). In accordance with others, we show that CCR1 is expressed in Huh7 and Hep3B cells (17), whereas CCR3 and CCR5 are not detected at their plasma membrane.

We show that RANTES/CCL5 binds to human hepatoma Huh7 or Hep3B cells and induces at a 3 nmol/L



**Figure 5.** Glycosaminoglycan mimetics inhibited RANTES/CCL5-induced hepatoma cell migration and invasion. **A**, surface plasmon resonance analysis of RANTES-glycosaminoglycan mimetic interaction. Sensorgram overlay of OTR4131 (*left*) or OTR4120 (*right*) binding to immobilized B1-RANTES. OTR4131 or OTR4120 was injected over RANTES-immobilized sensorship [surface density, 1,500 resonance units (RU)] at 0, 10, 30, 100, 300, and 1,000 ng/mL concentration for 10 min (0–600 s) at 30  $\mu$ L/min flow rate followed by running buffer for 20 min. The resonance unit response was recorded as a function of time and kinetic rates were calculated from both association and dissociation phases. **B**, glycosaminoglycan mimetics inhibited RANTES/CCL5 binding to Huh7 cells. B1-RANTES (40 nmol/L) was preincubated or not with OTR4131 or OTR4120 (both at 1  $\mu$ g/mL) and the suspension was added to the cells. RANTES/CCL5 binding to the cells was analyzed by flow cytometry using streptavidin-Alexa Fluor 488. Reactivity was compared with streptavidin-Alexa Fluor 488. Data are representative of five independent experiments. **C** and **D**, OTR4131 and OTR4120 inhibited RANTES/CCL5-induced chemotactic effects on Huh7 cells. The preincubation of RANTES/CCL5 with OTR4131 (0.01, 1, or 100 ng/mL) or OTR4120 (100 ng/mL) significantly reduced RANTES/CCL5-induced Huh7 cell migration (**C**) or invasion (**D**). Migration or invasion induced in the controls (RANTES/CCL5 alone minus background of unstimulated cells) was set to 100%. RANTES/CCL5-induced migration or invasion of the cells in the presence of inhibitor is shown as a percentage of control. \*,  $P < 0.05$  versus control cells.

physiologic concentration their migration and invasion *in vitro* at least partly through CCR1. These chemotactic and invasive effects may be related to the activation of the focal adhesion complex components because the chemokine increases the phosphorylation of FAK as described in other cell lines (15, 31).

These data have to be related to the importance of invasion and metastasis as major determinants in the progression of HCC. As overexpression of MMP-9 has been associated with capsular infiltration and growth of HCC

(32, 33), we explored the involvement of MMP-9 in RANTES/CCL5-induced invasive effects on hepatoma cells. We show that RANTES/CCL5 increases MMP-9 enzymatic activity in Huh7 cells as described previously in other cell lines (34, 35). Moreover, blocking the MMP-9 in these cells by mAb resulted in decreased RANTES-induced Huh7 cell invasion, suggesting the involvement of this MMP.

Interestingly, RANTES/CCL5 binding to Huh7 and Hep3B cells also depends on glycosaminoglycans because

the preincubation of the chemokine with heparin had a strong inhibitory effect on RANTES/CCL5 binding. RANTES/CCL5 has been shown to bind to glycosaminoglycans, such as heparan sulfate chains (5). Such protein-glycosaminoglycan interaction is believed to be dominated by electrostatic interactions of the side chains of protein basic residues with sulfate and carboxylate functions of glycosaminoglycans. RANTES is high positively charged with a net charge of +5 (5 Lys, 5 Arg, 1 Asp, and 4 Glu) at neutral pH. The highly positive charge density of RANTES may contribute to strengthen the chemokine interaction with negatively charged glycosaminoglycans. The glycosaminoglycan-binding sites of RANTES/CCL5, a BBXB motif, involving R44, K45, and R47 and defined as the 40s loop, is only marginally involved in CCR5 binding and activation but largely overlaps the CCR1 and CCR3 binding and activation domain in RANTES (5, 6, 36). Furthermore, we previously showed that RANTES binds to membrane heparan sulfate proteoglycans belonging to the syndecan family, syndecan-1 and syndecan-4, at the plasma membrane of primary lymphocytes, monocyte-derived macrophages, and CCR5-transfected HeLa cells (8, 9) and confirmed that RANTES/CCL5 binds to CD44 in accordance with others (37).

In the present study, we suggest that RANTES-glycosaminoglycan interactions play major roles in mediating RANTES-induced migration and invasion of human hepatoma cells. Indeed, blocking the heparin-binding site of RANTES with soluble glycosaminoglycans, such as heparin, or decreasing the cellular glycosaminoglycan level by  $\beta$ DX strongly reduced hepatoma cell migration or invasion induced by RANTES. Therefore, glycosaminoglycans may likely interfere with the mitogenic effects of RANTES/CCL5 on human hepatoma cells.

We then hypothesize that synthetic glycosaminoglycan mimetics modulate these RANTES-induced effects *in vitro*. This family of compounds are obtained by grafting specific amounts of carboxylate and sulfate groups onto a dextran backbone. Some of these glycosaminoglycan mimetics were shown to enhance tissue repair in various *in vivo* models, including skin (38), bone (39), or colon (40). They have at least 10-fold less anticoagulant activity than does heparin.

This family of products interacts with various heparin-binding growth factors, such as fibroblast growth factor, transforming growth factor- $\beta$ , and vascular endothelial growth factor (20, 41). In the present study, we used the synthetic polymers OTR4120 and OTR4131. Our results showed that their preincubation with RANTES/CCL5 inhibits the chemokine binding to human Huh7 hepatoma cells. Importantly, these synthetic glycosaminoglycan mimetics also strongly inhibit in a dose-dependent manner RANTES-induced Huh7 human hepatoma cell mobility across fibronectin or through reconstituted extracellular matrix *in vitro*. One of the molecular mechanisms of such an effect probably relies on a direct binding of glycosaminoglycan mimetics to RANTES/CCL5, which could result in an inhibition of the chemokine binding to glycosaminoglycans carried by proteoglycans expressed at the cell surface. However, it has been shown that the glycosaminoglycan mimetic cell treatment induced the changes in glycosaminoglycan amount, composition, and localization in C2.7 myoblast cultures undergoing differentiation (19). Therefore, it cannot be excluded that, in the present study, OTR4120 or OTR4131 could also interfere by this way with RANTES binding to the cells, resulting in the decreased chemotactic effects. It is interesting to note that non-differential activity was found with the two glycosaminoglycan mimetics used in our study, showing that the presence of acetate groups, which confers in some extent some hydrophobicity to the mimetic OTR4131, does not modify its inhibitory effect. This suggests that the inhibition is related mainly to the presence of charged motifs in the polymers and that acetate groups in heparan sulfate chains may not play an important role for RANTES interaction.

Furthermore, it has been shown that nonsulfated dextran derivatives destabilize the secondary structure and inhibit the activity of some heparin-binding growth factors (42, 43). The relationship between the biological activity of dextran derivatives and their chemical composition is therefore complex and needs further investigations.

In our experiments, we tested a RANTES variant, 3Ala-RANTES, which has positively charged groups in the <sup>44</sup>RKNR<sup>47</sup> glycosaminoglycan-binding domain replaced by neutral alanines. It has been previously shown by others that the variant <sup>44</sup>AANA<sup>47</sup> exhibited an 80% reduction in its capacity to bind to heparin (6). Moreover, it showed an 80-fold reduction in affinity for CCR1, despite normal binding to CCR5, and had a significant reduced ability to recruit CCR1<sup>+</sup> monocytes (6). Our data show that 3Ala-RANTES is less efficient than RANTES/CCL5 in promoting chemotaxis, which suggests a role for glycosaminoglycans in RANTES/CCL5-mediated migration and invasion of hepatoma cells. 3Ala-RANTES does, nonetheless, promote a slight but significant increase in migration and a large increase in invasion relative to control in the absence of RANTES/CCL5. Both were strongly reduced by preincubating the hepatoma cells with anti-CCR1 antibodies, suggesting that these biological effects induced by 3Ala-RANTES depend on CCR1. Nevertheless, we cannot exclude residual glycosaminoglycan-binding activity of

**Table 1. Summary of binding and functional variables of OTR4131 or OTR4120 on RANTES**

	B1-RANTES		
	$k_{on}$ ( $10^5 \text{ mol/M}^{-1} \text{ s}^{-1}$ )	$k_{off}$ ( $10^{-5} \text{ s}^{-1}$ )	$K_d$ (pmol/L)
OTR4131	19	9.0	48
OTR4120	18	3.2	17

NOTE: B1-RANTES was immobilized to a streptavidin-bound sensorship. The kinetic rate constants,  $k_{on}$  and  $k_{off}$ , and dissociation constants,  $K_d = k_{off} / k_{on}$ , of OTR4120 and OTR4131 were obtained by global analysis of association and dissociation phases in optical biosensor experiments at 10 to 1,000 ng/mL.

3Ala-RANTES. In fact, whereas the <sup>44</sup>RKNR<sup>47</sup> glycosaminoglycan-binding domain is the principal motif for heparin binding (6), other amino acids (Ser<sup>31</sup> to Lys<sup>33</sup> in the 30s region and Tyr<sup>3</sup> in the NH<sub>2</sub>-terminal region) have been involved in heparin binding (44).

Strikingly, 3Ala-RANTES induces a stronger increase in cell invasion than in cell migration. Different mechanisms that underlie the migration of cells across fibronectin and the invasion of cells through Matrigel, a reconstituted basement membrane, might account for this differential activity in movement. On one hand, heparan sulfate chains carried by proteoglycans, such as syndecan-4, are highly involved in cell adherence to fibronectin and in cell mobility across fibronectin (45, 46). Moreover, we previously showed that RANTES/CCL5 associates with syndecan-4 and syndecan-1 in a glycosaminoglycan-dependent manner on HeLa and macrophage cell surfaces (8, 9). On the other hand, the relative invasion of basement membrane by metastatic cells often correlates with the levels of proteolytic enzyme expression. We showed here that 3Ala-RANTES still increases the enzymatic activity of MMP-9, which is involved in hepatoma cell invasion. Nevertheless, *in vivo* migration and invasion assays should be done to ensure that these differences observed *in vitro* are still detected.

In summary, our results indicate that RANTES/CCL5 exerts its chemotactic effects, migration and invasion, through CCR1 on human hepatoma cells and that glycosaminoglycan chains expressed at the human hepatoma cell plasma membrane play major roles in these events. We also show, *in vitro*, that synthetic glycosaminoglycan mimetics or RANTES variant can interfere with these RANTES-induced deleterious functions. Thus, additional *in vivo* studies are needed to further address whether synthetic glycosaminoglycan mimetics or RANTES/CCL5 mutants could be of major interest in HCC therapy.

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# Molecular Cancer Therapeutics

## Glycosaminoglycans and their synthetic mimetics inhibit RANTES-induced migration and invasion of human hepatoma cells

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