Glycosaminoglycan mimetics inhibit SDF-1/CXCL12-mediated migration and invasion of human hepatoma cells

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We have recently reported that the CXC-chemokine stromal cell-derived factor-1 (SDF-1)/CXCL12 induces proliferation, migration, and invasion of the Hep7 human hepatoma cells through its G-protein-coupled receptor CXCR4 and that glycosaminoglycans (GAGs) are involved in these events. Here, we demonstrate by surface plasmon resonance that the chemokine binds to GAG mimetics obtained by grafting carboxylate, sulfate or acetate groups onto a dextran backbone. We also demonstrate that chemically modified dextrans inhibit SDF-1/CXCL12-mediated in vitro chemotaxis and anchorage-independent cell growth in a dose-dependent manner. The binding of GAG mimetics to the chemokine and their effects in modulating the SDF-1/CXCL12 biological activities are mainly related to the presence of sulfate groups. Furthermore, the mRNA expression of enzymes involved in heparan sulfate biosynthesis, such as exostosin-1 and -2 or N-deacetylase/N-sulfotransferase enzymes remained unchanged, but heparanase mRNA and protein expressions in Hep7 cells were decreased upon GAG mimetic treatment. Moreover, decreasing heparanase-1 mRNA levels by RNA interference were decreased upon GAG mimetic treatment. Moreover, decreasing heparanase-1 mRNA levels by RNA interference significantly reduced SDF-1/CXCL2-mediated extracellular signal-regulated kinase 1/2 (ERK 1/2) phosphorylation. Therefore, we suggest that GAG mimetic effects on SDF-1/CXCL12-mediated in vitro chemotaxis and GAG mimetics on decreased heparanase expression, which impairs SDF-1/CXCL12-mediated hepatoma cell chemotaxis may rely on decreased heparanase expression, which impairs SDF-1/CXCL12's signaling. Altogether, these data suggest that GAG mimetics may compete with cellular heparan sulfate chains for the binding to SDF-1/CXCL12 and may affect heparanase expression, leading to reduced SDF-1/CXCL12-mediated in vitro chemotaxis and growth of hepatoma cells.

Keywords: chemokine/glycosaminoglycan/glycosaminoglycan mimetics/hepatocellular carcinoma/SDF-1/CXCL12

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of physiopathological functions, including embryonic development, mobilization of stem cells, HIV-infection, angiogenesis, and cancer progression, such as breast cancer or glioblastoma (Geminder et al. 2001; Kryczek et al. 2005; Burger and Kipps 2006).

Hepatocellular carcinoma (HCC) ranges among the most frequent cancer entities worldwide with an incidence of more than 500,000 cases per year and raising incidence rates in Western countries. HCC is associated with liver cirrhosis commonly resulting from inflammatory liver diseases, such as chronic hepatitis B or C, and also from nonviral diseases, such as chronic alcohol intake (Llovet and Beaugrand 2003). Tumor growth and metastasis dissemination result from intricate deregulated molecular machinery leading to diverse phenomena in tumor cells, such as resistance to the induction of apoptosis, as well as invasion and migration capabilities (Liotta and Kohn 2001).

Recently, SDF-1/CXCL12 has been shown to be directly involved in HCC progression. A strong expression of CXCR4 in the liver of HCC patients has been linked to tumor dissemination and poor prognosis (Schimanski et al. 2006). We have recently demonstrated that SDF-1/CXCL12 induces the growth, migration, and invasion of human hepatoma cells in vitro (Sutton, Friand, Brule-Donneger, et al. 2007). These biological effects depend on CXCR4. Interestingly, these data also demonstrated that endogenous GAGs participate in cellular functions. Indeed, hepatoma cell growth, migration, and invasion induced by SDF-1/CXCL12 were strongly reduced by the pre-incubation of the chemokine with exogenous excess of heparin or by the treatment of the cells with the pre-incubation of the chemokine with exogenous excess of heparin (Llovet and Beaugrand 2003). Tumor growth and metastasis dissemination result from intricate deregulated molecular machinery leading to diverse phenomena in tumor cells, such as resistance to the induction of apoptosis, as well as invasion and migration capabilities (Liotta and Kohn 2001).

The purpose of our study is (1) to inhibit the pro-tumoral effects of SDF-1/CXCL12 on human hepatoma cell line in vitro by the use of sulfated GAG mimetics developed under the name of RGTA (ReGeneraTing Agent), (2) to evaluate the influence of differences in the degree of substitution of sulfate or acetate residues on the anti-tumoral effects induced by RGTA, (3) to elucidate the molecular mechanisms of RGTA activities.

**Results**

**Binding of SDF-1/CXCL12 to GAG mimetics**

The GAG mimetics employed in this study are carboxymethyl-dextran sulfate that differ in the degree of substitution of sulfate or acetate residues on the anti-tumoral effects induced by RGTA, (3) to elucidate the molecular mechanisms of RGTA activities.

<table>
<thead>
<tr>
<th>Table I. Chemical characteristics of the GAG mimetics</th>
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<td>D40CM0.5</td>
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<td>D40CM0.5S0.5</td>
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The GAG mimetics are carboxymethyl-dextran sulfate with a degree of substitution of 0.5 for carboxymethyl residues (dsC). The degree of substitution ranges over 0.5 up to 1.5 for sulfate residues (dsS). The degree of substitution is 0.2 for acetate residues (dsAc) in D40CM0.5S1.2Ac0.2. All GAG mimetics are composed of the same number of glycosidic units.

The binding of mutated SDF-1 3/6 was much weaker with signals apparent at a concentration of at least 128 nM chemokine (Figure 1A). These findings indicate that the Lys24, His25, and Lys27 cluster is critical for the physical interaction of SDF-1/CXCL12 with the GAG mimetic D40CM0.5. Finally, we calculated that each D40CM0.5 molecule bound to about 80–90 SDF-1/CXCL12 molecules (66 RU of Bmax value obtained for a concentration of SDF-1/CXCL12 of 128 nM was 700 RU. When SDF-1/CXCL12 was flowed over control surfaces (containing streptavidin only), no significant signal was observed (not shown).

To investigate whether basic amino acids known to interact with heparin could also be involved in the binding of SDF-1/CXCL12 to D40CM0.5, we tested a SDF-1/CXCL12 derivative in which the basic residues Lys24, His25, and Lys27 were substituted by Ser (SDF-1 3/6) as described (Amara et al. 1999). The binding of mutated SDF-1 3/6 was much weaker with signals apparent at a concentration of at least 128 nM chemokine (Figure 1A). These findings indicate that the Lys24, His25, and Lys27 cluster is critical for the physical interaction of SDF-1/CXCL12 with the GAG mimetic D40CM0.5. Finally, we calculated that each D40CM0.5 molecule bound to about 80–90 SDF-1/CXCL12 molecules (66 RU of Bmax value obtained for a concentration of SDF-1/CXCL12 of 128 nM was 700 RU. When SDF-1/CXCL12 was flowed over control surfaces (containing streptavidin only), no significant signal was observed (not shown).

In a second set of experiments, we tested GAG mimetic variants, in which the degree of substitution for sulfate or acetate residues differs, for the binding to immobilized SDF-1/CXCL12. For that purpose, biotinylated SDF-1/CXCL12 carrying a biotin group on the C-terminal residue (Lys68) was immobilized on a streptavidin-bound sensor chip. Various GAG mimetics, D40CM0.5S0.5, D40CM0.5S0.8, D40CM0.5S1.2, D40CM0.5S1.5, D40CM0.5S1.2Ac0.2, were used (Table I). Increasing concentrations of each GAG mimetic were injected over SDF-1/CXCL12-immobilized or negative control surfaces (Figure 1B and data not shown). Under these experimental conditions, the dissociation phase was extremely slow (Figure 1B versus A). Global fitting of each binding curve gave kinetic values summarized in Table II. GAG mimetics with a high degree of substitution for sulfate residues (dsS) (D40CM0.5S1.2, D40CM0.5S1.2Ac0.2) exhibited a higher affinity for SDF-1/CXCL12 than GAG mimetics with a low dsS (D40CM0.5S0.5, D40CM0.5S0.8). We also confirmed that heparin (but not native dextran) binds to SDF-1/CXCL12 (Table II and data not shown). When GAG mimetics were flowed over control surfaces (containing streptavidin only), no significant signal was observed (data not shown).
Fig. 1. SPR analysis of SDF-1/CXCL12-D40CM0.5S1.2 interactions. (A) SDF-1/CXCL12 or SDF-1 3/6 was injected over flow cells of a BIAcore sensor chip containing streptavidin plus 66 RU of biotinylated D40CM0.5S1.2 at a flow rate of 20 µL/min for 10 min followed by running buffer alone. Each set of sensorgrams was obtained by injecting either SDF-1/CXCL12 or SDF-1 3/6 at (from top to bottom) 128 nM, 64 nM, 32 nM, or 16 nM. The response in RU was recorded as a function of time. (B) Sensorgram overlay of D40CM0.5S1.2 binding to immobilized SDF-1/CXCL12. D40CM0.5S1.2 was injected over SDF-1/CXCL12-immobilized sensor chip (surface density 1800 RU) at 0, 50, 100, 200, 300, 400, 600, and 1000 ng/mL. (C) GAG mimetics inhibited SDF-1/CXCL12 binding to hepatoma cells. Biotinylated SDF-1/CXCL12 (40 nM) alone (left) or pre-incubated with D40CM0.5S1.2Ac0.2 (middle) or D40CM0.5S1.2 (right) (both at 10 ng/mL) was added to the cells. SDF-1/CXCL12 binding to the cells was analyzed by flow cytometry using streptavidin Alexa fluor-488 (AF-488). Reactivity was compared to streptavidin AF-488 alone. Data shown are representative of three independent experiments.

We then investigated whether GAG mimetics could inhibit the binding of SDF-1/CXCL12 to immobilized heparin. For that purpose, SDF-1/CXCL12 (56 nM) was co-incubated with GAG mimetics at increasing concentrations and then injected over a biotinylated heparin sensor chip. GAG mimetics such as D40CM0.5S1.2, D40CM0.5S1.2Ac0.2, and D40CM0.5S1.5 strongly decrease (better than soluble heparin) the binding of SDF-1/CXCL12 to immobilized heparin in a dose-dependent manner, conversely to the less sulfated GAG mimetic variants D40CM0.5S0.5 or D40CM0.5S0.8 (Table II). Dextran had no effect.

Inhibition of SDF-1/CXCL12 binding to Huh7 hepatoma cells by GAG mimetics

In agreement with our previous studies (Sutton, Friand, Brule-Donneger, et al. 2007), we observed that biotinylated SDF-1/CXCL12 binds to Huh7 hepatoma cells (Figure 1C),
manner (Figure 2). At 10 ng/mL, D40CM0hibit Huh7 cell invasion and migration in a dose-dependent assay. Cells in the upper chamber were allowed to migrate to a streptavidin-bound sensor chip at 1800 RU. As the pre-incubation of the chemokine with 100 ng/mL heparin or 100 ng/mL HS decreased cell migration and cell invasion toward SDF-1/CXCL12. The pre-incubation of Huh7 cells with 100 ng/mL heparin (43 ± 3% or 52 ± 10% inhibition for cell migration or invasion, respectively).

Upper part of the table (Lines 1–6): biotinylated SDF-1/CXCL12 was immobilized to a streptavidin-bound sensor chip at 1800 RU. As the dissociation phase was very slow enabling the k on) values.

Lower part of the table (Lines 7–13): inhibition of SDF-1/CXCL12-heparin interaction induced by SDF-1/CXCL12 for 10 ng/mL D40CM0 inhibit Huh7 cells toward SDF-1/CXCL12 by 88 ± 6% and 48 ± 8%, respectively. Similar inhibitory effects were observed when the chemokine was pre-incubated with 100 ng/mL heparin (43 ± 3% or 52 ± 10% inhibition for cell migration or invasion, respectively).

Together, these data demonstrated that GAG mimetics inhibit SDF-1/CXCL12-induced chemotaxis in a dose-dependent manner and that the sulfate residues play a major role in the biological effects of GAG mimetics. To address the question whether carboxymethyl residues could also play a role in the inhibitory effects of GAG mimetics on SDF-1/CXCL12's biological activities, hepatoma cell chemotactic assays were performed when SDF-1/CXCL12 was pre-incubated with 10 ng/mL D40CM0. Strikingly cell migration and invasion were also moderately but significantly reduced (23 ± 8% and 26 ± 8%, respectively). That suggests that carboxymethyl residues are also involved in GAG mimetic activities.

A second set of chemotactic experiments were carried out to investigate whether the inhibitory effects of RGTA on SDF-1/CXCL12-induced chemotactic activities were solely related to a direct interaction between the mimetics and the chemokine or could result from additional specific effects of these chemically modified dextrans on Huh7 cells. Therefore, the cells were pre-treated with D40CM0.S1,2 or D40CM0.5.S1,2, then added to the upper chamber and allowed to migrate toward SDF-1/CXCL12 through fibroconnectin or Matrigel-coated polycarbonate membranes. Under these conditions, the migration or the invasion of Huh7 cells toward SDF-1/CXCL12 was also strongly inhibited when the cells were pre-treated with D40CM0.S1,2 or D40CM0.5.S1,2 at 10 ng/mL (Figure 3A and data not shown). The pre-incubation of the hepatoma cells with 10 ng/mL D40CM0.S1,2 or D40CM0.5.S1,2 inhibits their migration by 90 ± 4% or 97 ± 2% and their invasion by 95 ± 9% or 93 ± 4%, respectively (n = 3, P < 0.05) (Figure 3A and data not shown). Similar results were also observed when Huh7 cells pre-incubated with RGTA were washed prior to the chemotaxis assay, suggesting that the inhibitory effects of GAG mimetics were not related to their diffusion through the filter pores. When cells were pre-incubated with RGTA, their migration and invasion toward serum-free control medium tend to increase as compared to untreated cells (P = 0.10).

As a negative control, the pre-incubation of Huh7 cells with dextran did not affect Huh7 cell migration toward SDF-1/CXCL12. The pre-incubation of Huh7 cells with 100 ng/mL heparin or 100 ng/mL HS inhibits their invasion toward SDF-1/CXCL12 by 88 ± 6% and by 67 ± 3%, respectively (n = 3, P < 0.05). Cell migration toward SDF-1/CXCL12 was also reduced by 55 ± 9% or by 53 ± 2% when pre-incubating cells with heparin or HS, respectively. HS or heparin (at 100 ng/mL), incubated with cells, does not significantly influence basal cell migration or invasion toward serum-free control medium (data not shown).

To address the question whether the inhibitory effects of GAG mimetics, incubated with cells, could be related to an
altered cell adhesion or cell spreading, we explored the effects of D_{40}CM_{0.5}S_{1.2} on basal cell adhesion and spreading. Huh7 cell adhesion was not significantly affected by GAG mimetics up to 1 µg/mL (data not shown). Huh7 cell spreading remained unchanged when cells were treated with 10 or 100 ng/mL but slightly and significantly decreased upon 1 µg/mL D_{40}CM_{0.5}S_{1.2} treatment (Figure 3B).

**GAG mimetic D_{40}CM_{0.5}S_{1.2} impedes SDF-1/CXCL12-mediated hepatoma cell anchorage-independent growth in vitro**

Cell growth in an anchorage-independent in vitro cell assay was used as a surrogate in vitro tumorigenicity assay. SDF-1/CXCL12 induced colony formation when the cells were grown in soft agar. After 3 weeks, the colony number of 3 nM SDF-1/CXCL12-treated cells (6.4 ± 0.5 colonies/field) or 40 nM SDF-1/CXCL12-treated cells (8.2 ± 0.6 colonies/field) was significantly higher than in untreated control cells (4.1 ± 0.5 colonies/field, P < 0.05) (Figure 3C). Therefore, SDF-1/CXCL12 enhanced the anchorage-independent growth of Huh7 cells. Interestingly, this effect can be inhibited by the pre-incubation of SDF-1/CXCL12 with 10 ng/mL D_{40}CM_{0.5}S_{1.2} since the colony formation after pre-incubation was reduced by 50% as compared to SDF-1/CXCL12-treated cells (3.3 ± 0.7 colonies/field for 3 nM SDF-1 and 4.1 ± 0.6 colonies/field for 40 nM SDF-1/CXCL12; P < 0.01). D_{40}CM_{0.5}S_{1.2} alone tends to increase the colony formation as compared to untreated cells (5.2 ± 0.6 versus 4.1 ± 0.5 colonies/field; P = 0.10). Strikingly, the colony number of cells treated with SDF-1/CXCL12 and D_{40}CM_{0.5}S_{1.2} (3.3 ± 0.7 or 4.1 ± 0.6 colonies/field for 3 nM or 40 nM SDF-1/CXCL12 and D_{40}CM_{0.5}S_{1.2}) was lower than that of cells treated with D_{40}CM_{0.5}S_{1.2} alone (5.2 ± 0.6 colonies/field) (P < 0.05 or P = 0.09, respectively) (Figure 3C).
GAG mimetics bind to Huh7 cells
To analyze the molecular mechanisms involved in these inhibitory effects, we investigated whether GAG mimetics could bind to Huh7 cells. As shown in Figure 3D, biotinylated D40CM0.5S1.2 binds to hepatoma cells. The binding is maximal after 15 min incubation and occurs even if the cells incubated with the biotinylated GAG mimic were washed before the addition of streptavidin. To explore whether GAG mimic binding could occur when cells are first stripped of molecules attached to endogenous GAGs, the binding of GAG mimetics to Huh7 cells...
was assessed by flow cytometry after cell treatment with heparitinases. Under these experimental conditions, GAG mimetic binding to the Huh7 cells remained unchanged suggesting that the stripping of molecules attached to endogenous GAGs does not influence the GAG mimetic binding (data not shown).

This binding was not affected when the cells were pre-incubated with anti-CXCR4 antibodies, suggesting that D40CM0.5S1.2 cell binding may be CXCR4 independent (Figure 3D).

We therefore explored the putative changes in the expression of CXCR4, upon cell treatment with GAG mimetics. As assessed by semi-quantitative RT-PCR and by flow cytometry analysis, the pre-treatment of Huh7 cells with D40CM0.5S1.2 or D40CM0.5S1.2Ac0.2 did not affect the expression of CXCR4 at both transcriptional and translational levels (Figure 3E and data not shown).

We have previously demonstrated that SDC-4 is a membrane HS proteoglycan ligand for SDF-1/CXCL12 (Hamon et al. 2004; Charnaux et al. 2005; Sutton, Friand, Brule-Donneger, et al. 2007). SDC-4 mRNA and protein levels remained unchanged in GAG mimetic-treated cells (Figure 3E and F). In addition, HS proteoglycans such as syndecan-1 (SDC-1), syndecan-2 (SDC-2), glypicanc-3 (GPC-3), and glypicanc-6 (GPC-6) were strongly expressed in Huh7 cells at the transcriptional level, whereas mRNAs encoding for glypicanc-1 (GPC-1), glypicanc-2 (GPC-2), glypicanc-4 (GPC-4), or glypicanc-5 (GPC-5) were barely or not detected. Hepatoma cell incubation with GAG mimetics did not change the levels of mRNAs encoding for these membrane HS proteoglycans (Figure 3E).

**GAG mimetics reduced heparanase expression**

In order to analyze the possible effects of GAG mimetics on GAG metabolism, we determined the expression of enzymes involved in HS biosynthesis, namely exostosin-1 and -2 (EXT-1 and -2) and N-deacetylase N-sulfotransferases (NDST-1, -2 and -3), in Huh7 cells pre-treated with GAG mimetics or with heparin using semi-quantitative RT-PCR. In the presence of 10 ng/mL D40CM0.5S1.2 or D40CM0.5S1.2Ac0.2, the transcriptional levels of EXT-1, EXT-2, and NDSTs were not modified as compared to untreated cells (Figure 4A). Similar results were observed with 100 ng/mL heparin (data not shown). We also measured the expression of heparanase, involved in HS degradation, at the mRNA level. Strikingly, the heparanase-1 mRNA level was strongly reduced in GAG mimetic-treated cells conversely to untreated control cells as assessed by semi-quantitative RT-PCR (Figure 4A) \( (n = 3; P < 0.05) \). A 4-fold decrease in the heparanase mRNA level measured by real-time PCR was observed in GAG mimetic-treated cells as compared to untreated cells (Figure 4B). A lower but significant decrease in the heparanase mRNA level was also observed in heparin-treated cells (data not shown).

Additionally, we demonstrated that 10 ng/mL D40CM0.5S1.2 or D40CM0.5S1.2Ac0.2 decreased the expression of heparanase (50 kDa) as assessed by Western blot analysis (Figure 4C).

It was previously demonstrated that RGTA inhibit heparanase degradation of the sulfated-labeled extracellular matrix (Rouet et al. 2006). In the present study, we studied heparanase activity in Huh7 cells, treated or not with 10 ng/mL D40CM0.5S1.2 or D40CM0.5S1.2Ac0.2, by the use of a colorimetric assay. Heparanase activity in untreated hepatoma cells was 0.4 ± 0.05 U/mL whereas it was 0.3 ± 0.07 U/mL or 0.3 ± 0.09 U/mL in cells treated with D40CM0.5S1.2 or D40CM0.5S1.2Ac0.2, respectively. Therefore, GAG mimetic treatment did not significantly modify heparanase activity in human hepatoma cells, even if a slight decrease was observed.

**Heparanase silencing by RNA interference decreased SDF-1/CXCL12-mediated chemotaxis**

We then explored the expression of HS chains on Huh7 cells in which the level of mRNA encoding for heparanase was decreased by RNA interference. The heparanase mRNA level was strongly reduced in cells transfected with specific heparanase dsRNA as compared to cells transfected with a negative control small interfering RNA (SNC dsRNA) or mock-transfected cells (data not shown). In these heparanase dsRNA-transfected cells, slightly more of the HS-specific epitope 10E4 was detected by the commercial anti-HS antibody 10E4 (Seikagaku) as compared to mock- or SNC-transfected cells (Figure 5A). The 10E4 antibody recognizes mixed HS domains containing both N-acetylated and N-sulfated disaccharide units (Van den Born et al. 1995) which may harbor heparanase cleavage sites (Gong et al. 2003). We then explored hepatoma Huh7 cell adhesion in cells transfected with specific heparanase siRNA. No modulation of cell adhesion or spreading was observed in cells in which heparanase expression was decreased by RNA interference as compared to mock- or SNC-transfected cells (data not shown).

In contrast, SDF-1/CXCL12-induced migration and invasion of heparanase dsRNA-transfected cells were significantly reduced as compared to mock-transfected cells or to SNC-transfected cells (Figure 5B and data not shown). Heparanase silencing had no significant effect on Huh7 hepatoma cell migration or invasion toward serum-free control medium. Furthermore, heparanase silencing or cell treatment with 1 ng/mL D40CM0.5S1.2 decreased Huh7 cell invasion toward SDF-1/CXCL12 by 82 ± 5% or 81 ± 3%, respectively whereas the combination of both abolished it.

Finally, we explored the relationship between decreased heparanase expression induced by GAG mimetics and the reduction in cell migration and invasion toward SDF-1/CXCL12. We have previously demonstrated that SDF-1/CXCL12-induced ERK 1/2 activation (by phosphorylation) and that SDF-1/CXCL12-induced chemotaxis of Huh7 cells depends on ERK 1/2 signaling (Sutton, Friand, Brule-Donneger, et al. 2007). Furthermore, it was also demonstrated by others that heparanase augmented the SDF-1/CXCL12-triggered phosphorylation in ERK-2 in T cells and increased the SDF-1/CXCL12-induced T-cell chemotaxis across fibroconnectin (Sotnikov et al. 2004). Therefore, we investigated whether heparanase silencing by RNA interference could reduce SDF-1/CXCL12-induced ERK 1/2 activation.

As shown in Figure 5C, specific heparanase RNA interference reduced ERK 1/2 phosphorylation induced by the chemokine. This suggests that heparanase may influence the MAPK activation induced by SDF-1/CXCL12 leading to a decrease in SDF-1/CXCL12-induced Huh7 cell chemotaxis. Heparanase silencing had no effect on basal ERK 1/2 activation in Huh7 cells (Figure 5C). Finally, we explored the effects of SDF-1/CXCL12 on the heparanase mRNA level and demonstrated that SDF-1/CXCL12 did not modify heparanase at the transcriptional level (data not shown).
Fig. 4. (A) Analysis of enzymes involved in GAG metabolism upon GAG mimetic treatment by RT-PCR. Huh7 cells were pre-incubated or not with D40CM0.5S12 (10 ng/mL) or D40CM0.5S12Ac0.2 (10 ng/mL) for 16 h at 37°C. EXT-1-, EXT-2-, NDST-1- to -3-, or heparanase mRNA expression was analyzed by semi-quantitative RT-PCR. Values were normalized using the GAPDH gene. (B) qPCR analysis of heparanase downregulation in cells treated with D40CM0.5S12 or D40CM0.5S12Ac0.2 at 10 ng/mL. Values were normalized using the GAPDH gene. *P < 0.05 as compared to untreated control cells. (C) Heparanase protein expression was decreased in Huh7 cells incubated with D40CM0.5S12 or D40CM0.5S12Ac0.2 at 10 ng/mL as assessed by Western blot assay. β-Actin was used as an internal control.

Fig. 5. Huh7 cells were transfected with heparanase dsRNA or SNC dsRNA or were mock transfected. (A) Heparan sulfate expression on Huh7 cell surface was increased in heparanase (Hep′) dsRNA transfected cells as compared to mock-transfected cells, as assessed by flow cytometry analysis. (B) SDF-1/CXCL12-induced Huh7 cell invasion was reduced in heparanase dsRNA-transfected cells as compared to mock- or SNC-transfected cells. Invasion of the mock-transfected cells induced by SDF-1/CXCL12 was set to 100%. **P < 0.01 versus mock-transfected cells. (C) Heparanase silencing decreased ERK 1/2 phosphorylation induced by SDF-1/CXCL12. Cells were transfected with heparanase dsRNA or SNC dsRNA. Seventy-two hours after transfection, cells were incubated with or without SDF-1/CXCL12 for 15 min and then assessed for the activation of ERK 1/2 by Western blot analysis.
Discussion

Blockade of the SDF-1/CXCR4 signaling by targeting either the ligand or the receptor has been previously carried out by traditional methods using small molecule receptor antagonists, receptor blocking antibodies, and modified chemokines (Tamamura et al. 2003; Faber et al. 2007). However, the specificity of SDF-1/CXCL12-GAG interactions suggests an alternative (Harvey et al. 2007). In the development of HCC, this new interfering strategy is supported by observations showing that soluble GAGs such as heparin inhibit the SDF-1/CXCL12-induced in vitro chemotaxis of human hepatoma cells (Sutton, Friand, Brule-Donneger, et al. 2007). Furthermore, treatment of human hepatoma cells with β-D-xylolides, used as alternative acceptors for the assembly of GAG chains, leads to a decreased cell expression of GAGs and consequently to reduced cell migration and invasion toward SDF-1/CXCL12 (Sutton, Friand, Brule-Donneger, et al. 2007).

In the current study, we hypothesized that synthetic GAG mimetics could modulate these SDF-1/CXCL12-induced biological effects in vitro. These polymers are engineered to mimic the properties of GAGs toward various heparin-binding proteins. This family of compounds is obtained by grafting carbohydrates, acetyl, and sulfate groups onto a dextran backbone. These molecules stimulate tissue repair when applied to the site of injury or systematically (Meddahi et al. 1996; Meddahi et al. 2002; Alexakis et al. 2004) and have at least a 10-fold lower anticoagulant activity than heparin. In human hepatoma cell lines, these chemically modified dextrans inhibit the pro-tumoral effects of the chemokine RANTES/CCL5, such as cell invasion or migration (Sutton, Friand, Papy-Garcia, et al. 2007).

In the study herein, SPR experiments reported two different molecular interactions between SDF-1/CXCL12 and GAG mimetics. Indeed, two different sets of $K_d$s indicated in Table II are not describing the same molecular interaction. The $K_d$ for binding of carbohydrates to immobilized SDF-1/CXCL12 is approximately 10-fold stronger than in the reverse set-up (when GAG mimetics are immobilized). Such a discrepancy reflects the huge difference in the dissociation phase ($k_{off}$).

Indeed, when GAG mimetics are immobilized, each SDF-1/CXCL12 molecule may interact monovalently as an independent molecule and may dissociate easily from GAG mimetic chains. These carbohydrate chains may behave as very flexible and extended polymers and exist, in solution, as an expandable coil (as suggested by their capacity to cross dialysis membranes of pore size that retains 1000 Da proteins (data not shown)).

Conversely, one chain of immobilized D$_{40}$CM$_{0.5}$S$_{1.2}$ (with a molecular weight of 59200 Da) could bind about 80–90 of SDF-1/CXCL12 molecules (with a molecular weight of 7831 Da). Therefore, for the polyvalent interaction of carbohydrates to immobilized SDF-1/CXCL12, we observed a very slow dissociation phase, which may reflect the extremely low probability that the all (up to 80) SDF-1/CXCL12 binding sites of a molecule of GAG mimetic are simultaneously dissociated.

The same basic residues, amino acids Lys$^{24}$, His$^{25}$, and Lys$^{27}$, which are located in the BBXB motif involved in the binding to heparin (Amara et al. 1999; Murphy et al. 2007), are also involved in the binding of SDF-1/CXCL12 to GAGs and GAG mimetics as demonstrated by reduced binding of the mutated SDF-1 3/6. The fact that SDF-1 3/6 still binds to GAG mimetics but only at high SDF-1/CXCL12 concentration may be explained by the existence of other basic residues apart from Lys$^{24}$, His$^{25}$, and Lys$^{27}$ cluster that may contribute to form the binding site, as described by others for heparin (Amara et al. 1999). Indeed, other amino acids (Lys$^{43}$ and Arg$^{43}$) have been shown to be involved in the binding of SDF-1/CXCL12 to heparin (Sadir et al. 2004).

We then postulated that GAG mimetics competitively inhibit the binding of the chemokine to heparin. This hypothesis was strengthened by the fact that D$_{40}$CM$_{0.5}$S$_{1.2}$, D$_{40}$CM$_{0.5}$S$_{1.2}$Ac$_{0.2}$, D$_{40}$CM$_{0.5}$S$_{1.5}$ inhibits the binding of SDF-1/CXCL12 to immobilized heparin more than does heparin itself, as assessed by SPR experiments. D$_{40}$CM$_{0.5}$S$_{0.5}$ and D$_{40}$CM$_{0.5}$S$_{0.8}$, which have the lowest degree of substitution for sulfate, exhibit a lower affinity for SDF-1/CXCL12 than other GAG mimetics (D$_{40}$CM$_{0.5}$S$_{1.2}$ or D$_{40}$CM$_{0.5}$S$_{1.2}$Ac$_{0.2}$ or D$_{40}$CM$_{0.5}$S$_{1.3}$), the most sulfated GAG mimetic binds to SDF-1/CXCL12 with an affinity similar to that observed for D$_{40}$CM$_{0.5}$S$_{1.2}$ or D$_{40}$CM$_{0.5}$S$_{1.2}$Ac$_{0.2}$. It is interesting to note that the binding of D$_{40}$CM$_{0.5}$S$_{1.2}$ and D$_{40}$CM$_{0.5}$S$_{1.2}$Ac$_{0.2}$ to SDF-1/CXCL12 displays similar kinetic characteristics ($K_d$, $B_{max}$) although the presence of acetate groups in D$_{40}$CM$_{0.5}$S$_{1.2}$Ac$_{0.2}$ could confer some hydrophobicity.

We then demonstrated that GAG mimetics inhibit SDF-1/CXCL12-induced chemotactic effects and anchorage-independent cell growth of human hepatoma cells. Using cell migration chambers, the pre-incubation of the chemokine SDF-1/CXCL12 with various GAG mimetics inhibits the Huh7 human hepatoma cell migration through fibronectin or cell invasion through a reconstructed extracellular matrix in a dose-dependent manner. The inhibitory effects of GAG mimetics occur at very low concentrations since D$_{40}$CM$_{0.5}$S$_{1.2}$, D$_{40}$CM$_{0.5}$S$_{1.2}$Ac$_{0.2}$, D$_{40}$CM$_{0.5}$S$_{1.5}$ nearly abolished cell migration or invasion at 10 ng/mL. Given the kinetic characteristics of the binding of GAG mimetics to the chemokine, it was not surprising that D$_{40}$CM$_{0.5}$S$_{0.5}$ and D$_{40}$CM$_{0.5}$S$_{0.8}$ were the less efficient GAG mimetics in inhibiting the effects of SDF-1/CXCL12 on human hepatoma cells. Therefore, one of the mechanisms underlying the inhibitory effects of GAG mimetics on SDF-1/CXCL12-mediated hepatoma cell chemotaxis may be related to the fact that the polymers could occupy the heparin-binding sites of the chemokine.

Furthermore, these data suggest that the inhibitory effects of the GAG mimetics on SDF-1/CXCL12-mediated Huh7 cell chemotaxis is related mainly to the presence of sulfate motifs in the polymers and that the acetate residues may not play a major role. Nevertheless, the role of the carboxymethyl residues is also demonstrated at a lesser extent since 10 ng/mL D$_{40}$CM$_{0.5}$ slightly but significantly inhibits SDF-1/CXCL12-mediated Huh7 cell chemotaxis. Furthermore, it has been shown that nonsulfated dextran destabilizes the secondary structure and inhibits the activity of some heparin-binding growth factors (Bitoun et al. 1999; Hamma-Kourbeli et al. 2001). The relationship between the biological activity of dextran derivatives and their chemical composition is therefore complex and needs further investigations.

Besides the binding of GAG mimetics to the heparin-binding site of SDF-1/CXCL12 leading to a sequestration of the chemokine, experiments involving the pretreatment of cells with GAG mimetics suggest that the inhibitory effect of
carbohydrates on SDF-1/CXCL12-induced migration not only relies on a direct interaction between GAG mimetics and the chemokine but also on specific cellular changes induced by the GAG mimetics.

Our data demonstrated that GAG mimetics bind to hepatoma cells. Nevertheless, the treatment of hepatoma cells with GAG mimetics does not change the expression of SDF-1/CXCL12 cellular membrane ligands such as CXCR4 or syndecan-4, its proteoglycan co-receptor, at both translational and transcriptional levels. It was previously demonstrated that sulfated polysaccharides, such as low-molecular-weight fucoidan-modulated vascular endothelial growth factor (VEGF) by binding to both VEGF and VEGF receptors (Lake et al. 2006). In the same way, heparin saccharides play an essential role in binding to both fibroblast growth factors and their receptors (Goodger et al. 2008). To our knowledge, the binding of heparin or HS chains or of any polyanionic structures to CXCR4 has never been demonstrated. In the present study, we suggest that GAG mimetics may interact with SDF-1/CXCL12 but probably not with its specific membrane G-protein-coupled receptor CXCR4 since the binding of GAG mimetics to cells is CXCR4-independent. Moreover, the cell binding of GAG mimetics still occurs when the cells are first stripped of molecules attached to endogenous GAGs, suggesting that these carbohydrates may bind cells through specific binding sites on cell surface. Further extensive studies are needed to identify these GAG mimetic binding sites.

GAG mimetics did not affect cell adhesion and spreading at low concentrations (10 ng/mL and 100 ng/mL). However, GAG mimetics slightly but significantly reduced cell spreading at 1 µg/mL. Inhibitory effects of GAG mimetics on hepatoma cell chemotaxis occur at very low concentration (10 ng/mL). Furthermore, cell migration or invasion toward serum-free control medium was not significantly affected by GAG mimetics. Therefore, we suggest that a reduced cell spreading may be marginally involved in the inhibitory effects of GAG mimetics on SDF-1/CXCL12-mediated cell chemotaxis.

Our data demonstrated that the expression of heparanase, which hydrolyzes internal glycosidic linkages of HS chains, was reduced, at both transcriptional and translational levels, when hepatoma cells were incubated with GAG mimetics. These results agreed also with those showing that unfraccionated or low-molecular-weight heparin decreased the heparanase expression (Takahashi et al. 2005). Similarly, it has been demonstrated that heparanase mRNA upregulation in high glucose-treated endothelial cells was prevented by heparin (Han et al. 2007). The precise mechanisms of downregulation of heparanase transcripts in response to the polymers or heparin are still unknown and deserve further extensive studies. Furthermore, it has been demonstrated that chemically modified, nonanti-coagulant species of heparin or sulfated oligosaccharides such as maltotetraose polysulfate (MHS) or phosphomannopentaose polysulfate (PI-88) inhibited heparanase activity and reduced tumor growth and angiogenesis (Lapiere et al. 1996; Parish et al. 1999; Joyce et al. 2005; Ilan et al. 2006). Previously, it was demonstrated using a radioisotope-based assay that RGTA inhibits heparanase activity (Rouet et al. 2006). In the present study, by the use of a colorimetric assay, we did not observe any significant reduced heparanase activity in Huh7 cells treated with GAG mimetics. Such an unexpected discrepancy may be related to a possible lower sensitivity of the colorimetric assay.

In addition to the well-studied catalytic feature of the enzyme, heparanase was noted to exert biological functions apparently independent of its enzymatic activity, such as enhanced cell adhesion and spreading (Goldshmidt et al. 2003). Furthermore, heparanase was shown to interact with cell membrane HS, resulting in clustering of SDC-1 and SDC-4 (Levy-Adam et al. 2008). In the present study, heparanase silencing increased cell surface HS quantity but strikingly did not affect basal Huh7 cell adhesion or spreading. However, heparanase silencing strongly reduced SDF-1/CXCL12-mediated chemotaxis. An increased chemotactic migration toward SDF-1 was observed in heparanase-overexpressing human glioma cells (Hong et al. 2008). Furthermore, it has been demonstrated that heparanase, which had no chemotactic effect on its own, increased the SDF-1-triggered phosphorylation of Pyk-2 and extracellular signal-regulated kinase-2 and the SDF-1-induced T-cell chemotaxis across fibronectin (Sotnikov et al. 2004). Our present data also demonstrated that heparanase silencing decreased SDF-1/CXCL12-induced ERK 1/2 phosphorylation. As we have previously reported that heparanase cell chemotaxis induced by SDF-1/CXCL12 depended on extracellular signal-regulated kinases (Sutton, Friand, Brule-Donneger, et al. 2007), we therefore could hypothesize that the decrease in heparanase activity could impair SDF-1/CXCL12-triggered signaling pathways resulting in impaired SDF-1/CXCL12 chemotactic activities. Additionally, we could propose that the changes in heparanase function may also affect penetration of the matrigel matrix, which includes basement membrane HSPGs functioning as a migration barrier. Strikingly, our data demonstrated that heparanase silencing by RNA interference did not modify cell invasion toward the serum-free control medium. We then suggest that such a mechanism may be marginally involved in the GAG mimetic inhibitory effects on SDF-1/CXCL12-mediated chemotaxis.

Taken together, our data demonstrate that GAG mimetics inhibit human hepatoma cell invasion or migration induced by SDF-1/CXCL12 in vitro. The mechanisms by which GAG mimetics alter the cell ability to migrate toward the chemokine are complex. On the one hand, GAG mimetics may likely compete with HS for the binding to the chemokine and occupy the heparin-binding sites of SDF-1/CXCL12. In vivo, this competition could lead to a sequestration mechanism that would reduce chemokine availability and prevent the binding of SDF-1/CXCL12 to its natural interaction partners (membrane-bound receptors and GAGs). On the other hand, our data suggest that GAG mimetics by themselves could also induce cellular modifications such as reduced expression of heparanase, leading to a decreased SDF-1/CXCL12-induced ERK 1/2 activation and SDF-1/CXCL12-mediated cell chemotaxis. Strikingly, in addition to their inhibitory effects on cell chemotaxis and anchorage-independent cell growth induced by SDF-1/CXCL12, GAG mimetics tend to increase basal cell migration as well as basal anchorage-independent cell growth. These data suggest that certain mechanisms underlying effects of RGTA on hepatoma cells remained unclear and are independent of SDF-1/CXCL12 activities. Therefore, our in vitro results must be confirmed by data obtained in vivo, in order to exclude any deleterious protumoral effects of GAG mimetics on HCC progression. Nevertheless, it can be proposed that targeting the SDF-1/CXCL12-GAG interaction by the use of well-defined sulfated polysaccharides of nonanimal origin could be a new therapeutic approach for HCC.
Material and methods

Materials

The GAG mimetics used in this study were provided from UMR CNRS 7149 and OTR3 (SAS Paris 75001, France): D$_{40}$CM$_{0.5}$, D$_{40}$CM$_{5.5}$S$_{1.2}$ (OTR4120), D$_{40}$CM$_{0.5}$S$_{1.5}$ (OTR4126), D$_{40}$CM$_{0.5}$s$_{1.2}$Ac$_{0.2}$ (OTR4131), D$_{40}$CM$_{0.5}$S$_{0.5}$ (OTR4135), and D$_{40}$CM$_{0.5}$S$_{0.8}$ (EP-38). These molecules are synthetic derivatives of dextran T40 composed of about 200 glucosidic units linked by α1–6 bonds. These products have been previously described (Alexakis et al. 2004; Barbosa et al. 2005; Papy-Garcia et al. 2005; Rouet et al. 2006; Sutton, Friand, Papy-Garcia, et al. 2007). The chemical characterization of all dextran derivatives was performed by calculating the global degree of substitution (ds) of each group per glucosidic unit. The ds values were determined by acidimetric titration for CH$_2$COONa and -SO$_3$Na contents and by elementary analysis for confirmation of the −OSO$_3$Na content. The global acetate ds was determined by 400 MHz $^1$H nuclear magnetic resonance (NMR) analysis performed in D$_2$O by comparing the integration of the acetate methyl group protons (3H) to the integration of the global anomic proton (1H). 600 MHz $^1$H and computational modeling were used to determine each group substitution in the specific sugar positions (C2, C3, and C4) as described (Papy-Garcia et al. 2005). Their chemical characteristics are summarized in Table I. The working concentrations of all used polysaccharide solutions were obtained by gradual dilutions of concentrated solutions prepared at the mg/mL range from pure dried powdered material. Low-molecular-weight heparin (H3149) and HS (H7640) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

For the biotinylation of polysaccharides, heparin and GAG mimetics were labeled by reaction of the polysaccharide terminal reducing group with biocytin hydrazide (BCH) as described previously (Ledoux et al. 2000). Briefly, 15 mg of polysaccharide was dissolved in 50 µL of formamide containing 50 mM of BCH and heated at 37°C for 24 h. Free BCH and formamide were removed by gel filtration chromatography, and homogeneity of the labeled polysaccharides was checked by gel permeation high performance chromatography (TSK gel G4000 PWXL) as previously described (Papy-Garcia et al. 2005).

Wild-type SDF-1/CXCL12 (amino acids 1–67), SDF-1/CXCL12-biotin (B-SDF-1, amino acids 1–68), and SDF-1 3/6, in which the basic residues Lys$^{24}$, His$^{25}$, and Lys$^{27}$ were replaced by Ser, were synthesized by the Merrifield solid phase method on a fully automated peptide synthesizer using Fmoc (N-(9-fluorenylmethyl)carbonyl) chemistry as described previously (Amara et al. 1999; Sadir et al. 2004). Selective biotinylation at the carboxy-terminal position was achieved by incorporating a lysine residue (Lys$^{68}$) bearing a 4,4-dimethyl-2, 6-dioxocyclohex-1-ethylenide (Dde) protective group on the side chain. Coupling of biotin was performed on the peptide resin after Dde deprotection. It was previously checked that biotinylation does not modify SDF-1/CXCL12-binding properties to cells (Sadir et al. 2004).

Cell culture

The human hepatoma Huh7 cell line was obtained from ATCC and was cultured as previously described (Sutton, Friand, Brule-Donneger, et al. 2007).

Cell migration and invasion assays

Cell migration or invasion was performed using Bio-coat cell migration chambers (Becton Dickinson, Pont-de-Claira, France) as described (Sutton, Friand, Brule-Donneger, et al. 2007). Briefly, inserts were coated with fibronectin (100 µg/mL, Santa Cruz Biotechnology) for migration or Matrigel (320 µg/mL, BD Bioscience Pharmingen, Pont-de-Claira, France) for invasion assay. SDF-1/CXCL12 (at 3 or 125 nM for invasion or migration assay, respectively) was preincubated or not with GAG mimetics for 2 h at 20°C, and the mixture was then added to 500 µL serum-free DMEM in the lower chamber. After 24 h, cells that had migrated through the filter pores were fixed with methanol, stained with Mayer’s hemalum, and counted.

Alternatively, cells were pre-incubated for 2 h at 37°C with GAG mimetics at the indicated concentrations and chemotrafficked toward SDF-1/CXCL12 placed in the lower chamber.

Cell adhesion and spreading assays

For cell adhesion assay, Huh7 cells were incubated for 16 h at 37°C with GAG mimetics. Then, pretreated cells were plated on an 8-well Labtek pre-coated with fibronectin (100 µg/mL) for 2 h at 37°C. They were then washed with PBS, detached with trypsin, and counted. For cell spreading assay, Huh7 cells were incubated for 16 h at 37°C with GAG mimetics. Then, pretreated cells were plated on an 8-well Labtek pre-coated with fibronectin (100 µg/mL) for 2 h at 37°C, permeabilized in 0.05% Triton X-100 (Sigma-Aldrich) and fixed with paraformaldehyde (1%). Cells were stained with Alexa Fluor 568 phalloidin in order to visualize filamentous actin and observed with a fluorescence microscope (Zeiss, AXIOPHOT, France S.A.). Ten fields of stained cells were photographed for each treatment. Cell areas expressed in square inches were evaluated on 30 cells by treatment with the Scion Image software (Scion Image Software and National Institutes of Health, Release Beta 3b Software). Histograms represent mean of cell areas ± SEM of three different experiments.

Flow cytometry analysis

Cells were incubated for 1 h at 4°C with biotinylated SDF-1/CXCL12. After washing, cells were labeled for 30 min at 4°C with streptavidin-Alexa Fluor 488 complex (1/100, Molecular Probes, Invitrogen, Cergy-Pontoise, France). In some experiments, biotinylated SDF-1/CXCL12 was preincubated with GAG mimetics for 1 h at room temperature and the mixture was added to the cells. HS immunostaining for flow cytometry analysis was performed using an anti-HS mAb (clone 10E4, Seikagaku Corporation, Tokyo, Japan) as described (Sutton, Friand, Papy-Garcia, et al. 2007). SDC-4 immunostaining for flow cytometry analysis was carried out using a syndecan-4 mAb (clone 5G9; mouse IgG2a; Santa Cruz Biotechnology) for migration or Matrigel (320 µg/mL, BD Bioscience Pharmingen, 10 µg/mL) or with its isotype IgG2a (BD Bioscience Pharmingen) prior to the addition of biotinylated D$_{40}$CM$_{0.5}$S$_{1.2}$. To explore whether GAG mimetics binding could occur when cells are first stripped of molecules attached to endogenous GAGs, Huh7 cells were treated with heparitinases I
PCR conditions were established to remain in the linear phase
of amplification (Wegrowski et al. 2006; Sutton, Friand, Brule-Donneger, et al. 2007). Specific primers were designed as follows:

- CXCR4, (forward) 5′-ACCACAGCCGAGCTGA ACCTTTGGTG-3′, (reverse) 5′-ACAGGGAATTGTCGGTCT GTCCGC-3′; NDST-2 (NM_003635), (forward) 5′-TGTTCC TTCAATGCGACG-3′, (reverse) 5′-GCTTGTGGCCA ATCCA-3′; NDST-3 (NM_004784), (forward) 5′-TGTCGGCAG GGTCTTGATGTT-3′, (reverse) 5′-CACTATGGCAACAG AAGCAC-3′; EXT-1 (NM_001207), (forward) 5′-CAGGC GGAGAATGCCTGACTGG-3′, (reverse) 5′-CTCCTGCT GTGGTCTTGATGCC-3′; EXT-2 (NM_000401), (forward) 5′-GACATTCCATCCAGACCCG-3′, (reverse) 5′-GGAG GAACAAGACGACAG-3′; heparanase-1 (NM_006665), (forward) 5′-TTCGATCCCAAGAGGAATCAAC-3′, (reverse) 5′-GTAGTATGCCATGACTGATC-3′.

Cyclers R

Huh7 cells (2 × 10⁶) were incubated at 37°C for 16 h with GAG mimetics, washed with PBS, and scraped in the NP40 lysis buffer (phosphate-buffered saline supplemented with 1% NP-40, 10 mM PMSF, 5 mM iodoacetamide, 25 mM o-phenanthroline, and 20 µg/mL aprotinin). Lysates were obtained by centrifugation at 10,000 × g for 15 min at 4°C and protein concentration was determined using the BCA protein assay kit (Pierce, Thermo Fisher Scientific, Brébières, France). Twenty micrograms of total protein was subjected to SDS–PAGE, transferred to the nitrocellulose membrane, and probed with anti-heparanase rabbit polyclonal antibodies (HPA-1, clone H-80, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by HRP-conjugated secondary antibodies. Heparanase active form was detected using the HRP-mediated chemiluminescent substrate. β-Actin was used as the internal protein control.

Heparanase expression

Huh7 cells (2 × 10⁶) were incubated at 37°C for 16 h with GAG mimetics, washed with PBS, and scraped in the NP40 lysis buffer (phosphate-buffered saline supplemented with 1% NP-40, 10 mM PMSF, 5 mM iodoacetamide, 25 mM o-phenanthroline, and 20 µg/mL aprotinin). Lysates were obtained by centrifugation at 10,000 × g for 15 min at 4°C, and supernatants were taken as samples. Heparanase activity was determined with a standard curve (prepared with unlabeled HS). The absorbance was read by a microplate reader at the wavelength of 450 nm.

Heparanase activity

Heparanase activities were assayed in cell lysates by using a heparan-degrading enzyme assay kit (TakaRa Bio Inc., Saint-Germain-en-Laye, France). Huh7 cells (1 × 10⁶ cells) were lysed with 150 µL extraction buffer and centrifuged at 10,000 × g for 5 min at 4°C, and supernatants were taken as samples. Heparanase activity was determined with a standard curve (performed by using unlabeled HS). The absorbance was read by a microplate reader at the wavelength of 450 nm.

When 200 µm dsRNA in serum-free medium using JetSI transfectant reagent (Eurogentec) following the manufacturer’s instructions. Mock cells were cultured in parallel and transfected with the transfection mixture lacking dsRNA. In each experiment, an SNC-RNA (Eurogentec) was used as a negative control. Cells transfected with heparanase dsRNA or mock-transfected were used 3 days post-transfection for further analysis.

Soft-agar growth assay

Anchorag­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­­…
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 using BIAEvaluation software.

**Western blot analysis of ERK 1/2 phosphorylation**

Huh7 cells (2 × 10⁵) were either transfected with SNC dsRNA or with heparanase dsRNA. Seventy-two hours after transfection, cells were incubated or not with SDF-1/CXCL12 (3 nM) for 15 min and the activity (phosphorylation) of ERK 1/2 (p44/p42) [Thr 202/Tyr 204] or for their total counterparts (all from Cell Signaling, Bagnols-sur-Céze, France) as described (Sutton, Friand, Brule-Donneger, et al. 2007).

**Statistical analysis**

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

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