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The shedding of syndecan-4 and syndecan-1 from HeLa cells and human primary macrophages is accelerated by SDF-1/CXCL12 and mediated by the matrix metalloproteinase-9

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We recently demonstrated that stromal cell-derived factor-1 (SDF-1/CXCL12) forms complexes with CXCR4, but also with syndecan-4 expressed by human primary lymphocytes and macrophages, and HeLa cells. We also suggested that syndecan-4 behaves as a SDF-1-signaling molecule. Here, we demonstrate that SDF-1 strongly accelerates the shedding of syndecan-4 ectodomains and to a lesser extent that of syndecan-1 from HeLa cells. The fact that this acceleration was not inhibited by the CXCR4 antagonist AMD3100, anti-CXCR4 mAb 12G5, and CXCR4 gene silencing suggests its CXCR4-independence. Pre-treating the cells with heparitinases I, III, or with the protein kinase C (PKC) inhibitor, bisindolylmaleimide, significantly inhibited this accelerated shedding, which suggests the involvement of both cell-surface heparan sulfate and PKC transduction pathway. In contrast, Map Kinase or NF-κB pathway inhibitors had no effect. Moreover, SDF-1 increases the matrix metalloproteinase-9 (MMP-9) mRNA level as well as MMP-9 activity in HeLa cells, and MMP-9 silencing by RNA interference strongly decreases the syndecan-1 and -4 ectodomain shedding accelerated by SDF-1. Finally, SDF-1 also accelerates in a CXCR4-independent manner, the shedding of syndecan-1 and -4 from human primary macrophages, which is significantly inhibited by anti-MMP-9 antibodies. This strongly indicates the role of MMP-9 in these events occurring in both a tumoral cell line and in human primary macrophages. Because MMP-9 plays a crucial role in extracellular matrix degradation during cancer cell metastasis and invasion, and shed ectodomains of syndecans may likely be involved in tumor cell proliferation, these data further indicate the multiplicity of the roles played by SDF-1 on tumor cell biology.

Key words: chemokine/CXCR4/glycosaminoglycan/proteoglycan/shedding

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Introduction

Chemokines are chemotactic cytokines that govern multiple aspects of host defense and inflammation such as hematopoiesis, leukocyte trafficking, and angiogenesis (Baggiolini *et al*., 1997). However, besides their classical role as leukocyte chemoattractants, chemokines have been shown to play important roles in tumor biology: angiogenesis, chemoattraction of neoplastic cells, and proliferation of these cells (Strieter, 2001; Burger and Kipps, 2006). Depending on the structure of a conserved cysteine-containing motif in the amino-terminal region of the molecule, four subgroups have been characterized and named C, CC, CXC, or CX_3C according to the number and spacing of these cysteine residues. Stromal cell-derived factor-1 (SDF-1), recently renamed CXCL12, is a CXC chemokine that is constitutively expressed in a wide variety of tissues and binds to the seven transmembrane G-protein-coupled receptor (GPCR) CXCR4 (Oberlin *et al*., 1996; Bleul *et al*., 1997). Recent interest in chemokines has increased substantially as a result of their emerging role in immune and inflammatory responses, hematopoeisis, HIV infection (Baggiolini *et al*., 1997; Ward *et al*., 1998), and tumor biology (Strieter, 2001). Recent studies strongly indicate that the SDF-1/CXCR4 axis is involved in angiogenesis (Burger and Kipps, 2006), invasiveness, migration of cancer cells (Brand *et al*., 2005), and tumor cell proliferation (Barbero *et al*., 2003). CXCR4 also functions as a coreceptor for T-cell-tropic HIV-1 isolates, and SDF-1 blocks cellular entry of T-cell-tropic HIV-1 in vitro (Oberlin *et al*., 1996; Bleul *et al*., 1997). Moreover, optimal inhibition of entry into the cells of X4 HIV isolates by SDF-1 requires interaction with cell-surface heparan sulfate (HS) (Valenzuela-Fernandez *et al*., 2001). In addition, heparan sulfate proteoglycans (HSPGs), the syndecans, were identified as HIV attachment receptors (Saphire *et al*., 2001).

The syndecans are a family of proteoglycans (PGs), which, together with the lipid-linked glypicans, are the major source of HS at cell surfaces (Bernfield *et al*., 1999; Zimmermann and David, 1999). By way of their HS chains, syndecans bind a wide variety of soluble and insoluble ligands, such as extracellular matrix components, cell adhesion molecules, growth factors, cytokines, and proteinases (Carey, 1997; Bernfield *et al*., 1999). As the HS chains of the cell-surface and shed syndecans can bind the same ligands, syndecan ectodomain shedding is a mechanism for producing soluble HSPG effectors that may often compete for the same ligands as their cell counterparts (Kainulainen *et al*., 1998).

Shed soluble syndecan ectodomains are found in inflammatory fluids where they maintain proteolytic and growth factor balance (Subramanian *et al*., 1997). Tissue injury is accompanied by cellular stress, accumulation of leukocyte-derived proteases (thrombin, plasmin, elastase etc.), and release of growth factors, each of which accelerate syndecan shedding (Subramanian *et al*., 1997). Shed syndecans ectodomains are newly defined mediators of inflammation (Fitzgerald *et al*., 2000). They may also induce the proliferation of cancer cells (Maeda *et al*., 2004). Matrix metalloproteinases (MMPs) are a family of over 20 highly homologous zinc proteinases. They are secreted as latent enzymes and require proteolytic cleavage for activation (Visse and Nagase, 2003). Chemokine stimulation of cell MMPs have already been described by several groups (Azenshtein *et al*., 2002; Yu *et al*., 2003; Bartolome *et al*., 2004). For instance, it has previously been demonstrated that SDF-1 stimulates the production of MMP-9 by murine RAW cells (Yu *et al*., 2003).

Until recently, while specific interaction between HS and SDF-1/CXCL12 has been demonstrated (Valenzuela-Fernandez *et al*., 2001), the possible relationship between SDF-1/CXCL12 and syndecans was not investigated. In this context, we previously showed that SDF-1 forms complexes on the tumoral HeLa cell line, which constitutively expresses CXCR4 (Hamon *et al*., 2004), and on human primary lymphocytes and macrophages. These complexes comprise CXCR4 as expected, and syndecan-4 but neither syndecan-1, CD44 nor betaglycan (Hamon *et al*., 2004). This suggested that SDF-1 may bind both syndecan-4 and its GPCR, CXCR4. Moreover, our recent data demonstrate that SDF-1 directly binds, in a glycosaminoglycans-(GAGs)-dependent manner, syndecan-4 expressed by the human primary lymphocytes and the HeLa cell line. In addition, we also showed that syndecan-4 behaves in HeLa cells as a specific SDF-1 receptor, involved in some SDF-1-induced transduction pathways (Charnaux, Brule, Hamon *et al*., 2005).

This study was undertaken (1) to investigate whether SDF-1 accelerates in a significant manner the shedding of PGs ectodomains from a tumoral cell line which constitutively expresses CXCR4, the HeLa cell line, (2) to elucidate which transduction pathway(s) and protease(s) may be involved in these processes, and (3) to determine whether such SDF-1-induced events also occur on human primary macrophages which express CXCR4.

Results

SDF-1 binds to HeLa cells through CXCR4 and glycosaminoglycan chains

We further observe, in accordance with our previous data, that HeLa cells constitutively express CXCR4, syndecan-4, syndecan-1, and CD44 as assessed by indirect immunofluorescence followed by flow cytometry analysis (data not shown).

In the present study, to analyze whether CXCR4 is involved in some SDF-1-induced effects, we have used the anti-CXCR4 mAb 12G5 (Bleul *et al*., 1997) and a specific antagonist of CXCR4, AMD3100, that inhibits the binding and function of SDF-1α with high affinity and potency (Schols *et al*., 1997). It acts through disruption of the SDF-1/CXCR4 axis. Therefore, AMD3100 also blocks signaling induced by SDF-1 (Hatse *et al*., 2002; Rubin *et al*., 2003) and migration of monocytic cells toward CXCL12 (Schols *et al*., 1997). AMD3100 is a symmetric bicyclam. It interacts mainly with three acidic anchor point residues of CXCR4: Asp 171, Asp 262, and Glu 288, two of which are located at one end and the third at the opposite end of the main ligand-binding pocket of CXCR4 (Rosenkilde *et al*., 2004). We observed that the binding of an anti-CXCR4 mAb 12G5 to the CXCR4 receptor expressed by HeLa cells was strongly inhibited, as expected (Hamon *et al*., 2004), by AMD3100 (89% ± 5% of inhibition; $p < 0.05$, $n = 3$ compared with untreated cells). In addition, AMD3100 also partly, but significantly, decreased the binding of biotinylated SDF-1α to these cells (42% ± 3.5%; $n = 3$; $p < 0.05$ compared with untreated cells) (Figure 1).

We previously showed that SDF-1α directly binds, besides CXCR4, to syndecan-4 expressed at the HeLa cells' surface and that its binding to the PG was inhibited if the cells were pre-treated by glycosaminidases, heparitinases I and III, and chondroitinase ABC (Hamon *et al*., 2004).

Together, our previous and present data strongly suggest that SDF-1 binds to HeLa cells through its GPCR, CXCR4, and glycosaminoglycans.

SDF-1 accelerates the shedding of syndecan-4 and syndecan-1 from HeLa cells and human primary macrophages

We have then investigated whether SDF-1 accelerates in a significant manner the shedding of the ectodomains of its interacting PG, syndecan-4, and whether it also accelerates that of others PGs, such as syndecan-1 and CD44, expressed on the plasma membrane of HeLa cells and human primary macrophages. To this end, we first investigated whether syndecan-1, syndecan-4, and CD44 are constitutively shed from HeLa cells and human monocyte-derived macrophages (MDMs). We then analyzed, in parallel, as positive control, the effects of PMA on this shedding. Finally, we investigated the effects of SDF-1 stimulation of these cells on PG shedding. To detect shed PGs, the cell culture supernatants were dotted on cationic nylon membranes, and their respective immunoreactivity against anti-syndecan-1, anti-syndecan-4,

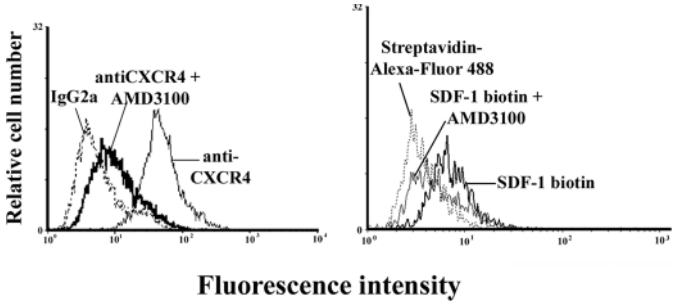

Fig. 1. SDF-1 binding to HeLa cells involves CXCR4. HeLa cells, pre-treated or not by AMD3100 (1.2 μM), were stained for FACS analysis either with anti-CXCR4 12G5 mAb or with biotinylated SDF-1α. Reactivity was compared respectively to an isotype-matched control monoclonal Ab or to streptavidin–Alexa Fluor 488. Data are representative of three individual experiments.

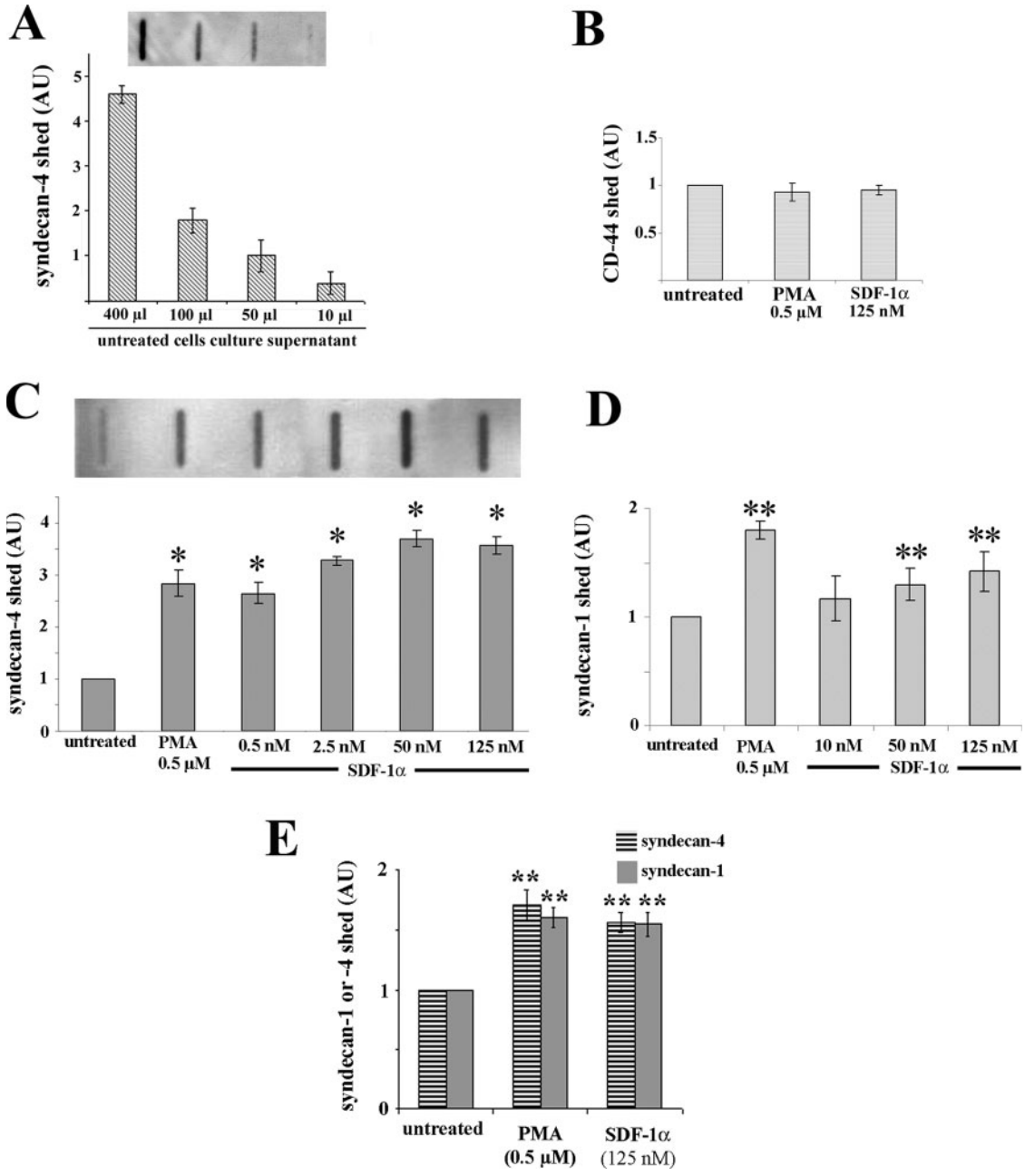

Fig. 2. SDF-1 accelerates the shedding of syndecan-4 and to a lesser extent that of syndecan-1 from HeLa cells. (**A**) Untreated HeLa cells culture supernatant (10, 50, 100, or 400 µl) were harvested and proteoglycans, partially purified by application to cationic membranes, were analyzed by dot blot. Syndecan-4 was detected by enhanced chemoluminescence detection, using anti-syndecan-4 5G9 mAb. The data are expressed as the amount of syndecan shed in absorbance units (AU), measured by densitometric scanning and analyzed with an Image software. An arbitrary value of 1 is attributed to the signal obtained for 50 µl of conditioned medium. Each point represents the mean ± SE of triplicate determinations. One of three individual experiments is shown. (**B**–**D**) HeLa cells were incubated or not for 18 h with PMA (0.5 µM) or SDF-1α at 0.5, 2.5, 10, 50, and 125 nM. (**E**) MDMs were incubated or not for 2 h with PMA (0.5 µM) or SDF-1α (125 nM). CD44 (**B**), syndecan-4 (**C** and **E**), and syndecan-1 (**D** and **E**) were detected using respectively anti-CD44, anti-syndecan-4 5G9, and anti-syndecan-1 BB4 mAbs. The data are expressed as the amount of syndecan shed, in relative absorbance units to the value given by the untreated cells. Each point represents the mean ± SE of triplicate determinations. One of three individual experiments is shown (significantly different from untreated control: $*p < 0.001$; $**p < 0.01$).

and anti-CD44 mAbs were analyzed. The results were quantified by scanning the exposed X-ray film with an Agfa scanner and using area measurement from Scion Imager software. In these experimental conditions, using anti-syndecan-4 or anti-syndecan-1 mAbs, increased amounts of HeLa cell- or MDM-conditioned media showed a linear increase of absorbance units (Figure 2A; data not shown).

As previously reported (Charnaux, Brule, Chaigneau *et al.*, 2005), we observed here that HeLa cell-conditioned medium is characterized by the presence of proteins, respectively immunoreactive with anti-syndecan-4 5G9, anti-syndecan-1 BB4, and anti-CD44 mAbs (Figure 2). Moreover, such proteins were also detected in MDM-conditioned medium (Figure 2). Such immunoreactive proteins were not detected

if the culture media were not previously incubated with the cells (data not shown). Therefore, syndecan-1, syndecan-4, and CD44 are constitutively shed from HeLa cells and MDM.

As expected (Charnaux, Brule, Chaigneau *et al*., 2005), syndecan-1 and syndecan-4 shedding from these cells was significantly increased, in three independent experiments, by incubating the cells for 2 or 18 h with the protein kinase C (PKC) activator (Fitzgerald *et al*., 2000), PMA (at 0.5 μM) ($p < 0.01$ and $p < 0.001$ respectively, $n = 3$; Student's *t*-test) (Figure 2C–E; data not shown). In contrast, PMA had no significant effect on the constitutive shedding of CD44 ($p = 0.8$, $n = 3$, Student's *t*-test) (Figure 2B; data not shown). Stimulating HeLa cells for 18 h at 37°C with different nanomolar concentrations of SDF-1α significantly accelerated, in a concentration dependent manner, in three independent experiments, syndecan-1 and syndecan-4 shedding, compared with the data observed in SDF-1- or PMA-free medium (Figure 2C and D). Indeed, SDF-1α, at concentration ranging from 0.5 to 125 nM, significantly and strongly accelerated the shedding of syndecan-4 from these cells ($p < 0.001$ compared with untreated cells, $n = 3$, for each concentration tested). Moreover, this increase in syndecan-4-shedding level, induced by 0.5 nM SDF-1α was similar to that induced by 0.5 μM PMA. This accelerated shedding was observed if 0.5 nM SDF-1α was applied to HeLa cells, then increased if 2.5 nM SDF-1α was used, and finally reached a maximum at 50 nM SDF-1α (Figure 2C). In contrast, syndecan-1 shedding was slightly, but significantly, accelerated if 50 nM SDF-1α or 125 nM SDF-1α was used ($p < 0.01$), while no increased shedding was observed if 10 nM SDF-1α was applied (Figure 2D). The fact that similar results were observed if SDF-1α was incubated for 2 h at 37°C with HeLa cells (data not shown) suggests that the increase in the medium content of syndecan-1 and syndecan-4, induced by SDF-1, may be due to proteolytic cleavage of PG ectodomains rather than increased synthesis of syndecans which would not be membrane inserted. In the same conditions, incubating the MDM for 2 h at 37°C with 25, 50, or 125 nM SDF-1 slightly, but significantly, accelerated the shedding of syndecan-1 and syndecan-4 from MDM, while 3 and 10 nM had no effect (Figure 2E; data not shown). In these experiments, syndecan-1 shedding, accelerated by SDF-1, was similar if HeLa cells or MDMs were used. In contrast, syndecan-4 shedding, accelerated by the chemokine, was about twofold lower if MDMs were used compared with HeLa cells.

Finally, SDF-1α had no effect on CD44 shedding from the HeLa cells or MDM ($p = 1$, $n = 3$; Student's *t*-test) (Figure 2B; data not shown).

Moreover, similar results were observed whether the cell culture medium was supplemented or not with serum. This rules out the involvement of proteases such as plasmin or thrombin, sensitive to seric antiproteases in these events (Subramanian *et al*., 1997).

Incubation of HeLa cells for 2 h at 37°C with SDF-1α (125 nM) also significantly decreased their plasma membrane expression of syndecan-4 and syndecan-1 compared with those observed on the corresponding untreated cells, as assessed by cytofluorimetry analysis after indirect immunofluorescence labeling (data not shown). In addition, a significant decrease in syndecan-4 membrane expression of MDM was observed if these cells were stimulated with SDF-1 compared with unstimulated MDM; in contrast, no change in their syndecan-1 membrane expression was detected in these conditions (data not shown); this may be related with differences in the respective turnover of the PGs at the MDM cell surface.

The shedding of syndecan-4 and syndecan-1 accelerated by SDF-1α on HeLa cells depends on PKC transduction pathways

We then investigated which transduction pathways may be involved in the SDF-1-accelerated shedding of syndecan-1 and syndecan-4 from HeLa cells. As SDF-1-accelerated shedding of syndecan-4 from these cells was about twofold higher than that observed for MDM, HeLa cells were used in the following experiments.

The compound U0126 inhibits two MAP kinase, MEK-1 and MEK-2; this inhibition is selective, as U0126 has little, if any, effect on the kinase activities of PKC and others kinases (Favata *et al*., 1998). Moreover, signaling through GPCR can activate the p42/p44 MAPKs through both G alpha-dependent (Ptx sensitive) and G alpha-independent (Ptx insensitive) mechanisms (Luttrell *et al*., 1999). In the present study, we observe, in accordance with others (Ganju *et al*., 1998), that SDF-1α activates the phosphorylation of a member of the MAPK family: indeed, incubation of the HeLa cells with SDF-1α induces the phosphorylation of the p42/p44 MAP kinases, and addition of 10 μM U0126 to the incubation medium inhibits this enhanced phosphorylation (data not shown).

However, pre-incubating the HeLa cells for 2 h at 37°C with SDF-1α (at 125 nM) and also with MEK inhibitor U0126 had no significant effect on the accelerated shedding of syndecan-4 or syndecan-1 induced by SDF-1α (respectively, $p = 1$ and $p = 0.8$, $n = 3$, Student's *t*-test). In the same conditions, U0126 had, as expected (Charnaux, Brule, Chaigneau *et al*., 2005), no significant effect on the shedding of the ectodomains of these PGs, induced by PMA, which involves PKC ($p = 0.8$ and $p = 0.7$, $n = 3$, Student's *t*-test) (Figure 3). However, in contrast, U0126 significantly reduced the MAP-kinase-dependent accelerated shedding of syndecan-1 and syndecan-4 induced by another chemokine, RANTES (data not shown). In addition, no inhibition of the SDF-1-induced shedding of syndecan-4 and syndecan-1 was observed using a specific MEK inhibitor, PD98059, strengthening the MAP-kinase independence of the syndecan-1 and syndecan-4 shedding, induced by SDF-1/CXCL12 (data not shown).

We then asked whether the shedding of syndecan-4 and that of syndecan-1 accelerated by SDF-1 involves PKC activity. For this purpose, we tested whether bisindolylmaleimide I, a potent and selective inhibitor of this pathway (Toullec *et al*., 1991), affected this shedding. While bisindolylmaleimide prevented in a significant manner the shedding of syndecan-1 and syndecan-4 accelerated by PMA, as expected (Fitzgerald *et al*., 2000), this compound also significantly prevented the shedding of syndecan-4 as well as that of syndecan-1 accelerated by SDF-1α (respectively, $p < 0.001$ and $p < 0.01$, $n = 5$, Student's *t*-test, compared with untreated cells) (Figure 3). In each assay, we tested the

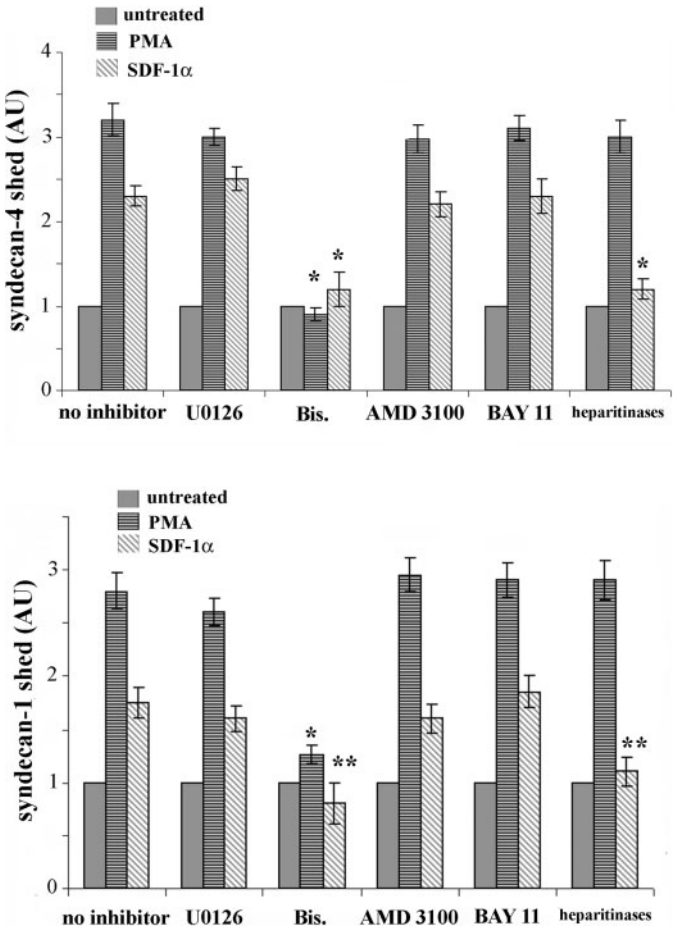

Fig. 3. The shedding of syndecan-1 and syndecan-4 induced by SDF-1 depends on cell-surface HS chains and on protein kinase C activity. HeLa cells were incubated for 2 h with PMA (0.5 μM), SDF-1α (125 nM), or in SDF-1 and PMA-free medium (untreated) in the absence (no inhibitor) or the presence of MEK1/2 inhibitor, U0126 (10 μM) or of a selective inhibitor of PKC, bisindolylmaleimide 1 (Bis, 1 μM), or of a NF-κB pathway inhibitor (Bay-11, 20 μM) or of a soluble CXCR4 inhibitor, AMD3100 (1.2 μM). In some experiments, HeLa cells were pre-treated with heparitinases I and III. The data are expressed as the amount of syndecan-4 or syndecan-1 shed in absorbance units, measured by densitometric scanning and analyzed with an Image software. Each point represents the mean ± SE of triplicate determinations. One of three individual experiments is shown. (*p* compared respectively to PMA- or SDF-1- treated cells incubated in the absence of inhibitor [no inhibitor], $*p < 0.001$; $**p < 0.01$).

effect of the MAP kinase or PKC inhibitor alone. In no case did the inhibitor affect the level of shedding compared with the untreated control (data not shown).

We next addressed the question of the involvement of the NF-κB pathway in the shedding of syndecan-4 and syndecan-1 induced by SDF-1. For that purpose, cells were pre-incubated with Bay-11 (20 μM), a specific NF-κB inhibitor, which is known to inhibit rapidly and irreversibly the activity of NF-κB (Najjar *et al.*, 2005). In these conditions, the SDF-1-accelerated shedding of syndecan-4 and that of syndecan-1 was not significantly decreased which indicates that the NF-κB transduction pathway may not be involved in these events (Figure 3).

Together, these data suggest that SDF-1-accelerated shedding of syndecan-4 and syndecan-1 from HeLa cells depends on PKC transduction pathways and indicate that MEK1/2 and NF-κB transduction pathways may not be involved in these events.

The shedding of syndecan-4 and syndecan-1, accelerated by SDF-1 from HeLa cells and MDM, is CXCR4 independent

We then asked whether the shedding of syndecan-4 and that of syndecan-1 accelerated by SDF-1α on HeLa cells involves CXCR4. For this purpose, we first tested whether AMD3100, a specific antagonist for CXCR4, affected this accelerated shedding. While this compound decreased, as described above, the binding of anti-CXCR4 mAb 12G5 to CXCR4 (89% inhibition) and that of biotinylated SDF-1α to HeLa cells (42% inhibition), this compound did not significantly prevent neither the shedding of syndecan-4 nor that of syndecan-1 accelerated by SDF-1α ($p = 0.9$ and $p = 0.7$, $n = 3$, Student's *t*-test, compared with the data observed in SDF-1-free medium) (Figure 3). In addition, the fact that no inhibition of this SDF-1-induced shedding was observed if HeLa cells were pre-incubated with the monoclonal anti-human CXCR4 antibody, clone 12G5 (data not shown) further rules out the involvement of CXCR4 in these events. To verify the CXCR4 independence of the SDF-1-accelerated shedding of syndecan-4 and syndecan-1 from HeLa cells, we used specific small interfering RNA (siRNA) to down-regulate CXCR4. CXCR4 expression of these cells was determined by the use of anti-CXCR4 mAb 12G5 and FACS analysis. Specific CXCR4 siRNA duplexes down-regulated CXCR4 protein expression (65% inhibition), whereas a negative control siRNA (sncRNA) did not (data not shown). Moreover, there was no effect of either CXCR4 siRNA or control siRNA on CCR5 protein expression. Nevertheless, in those conditions, the knockdown of CXCR4 did not affect SDF-1-accelerated shedding of syndecan-4 and syndecan-1 from HeLa cells (data not shown).

Moreover, AMD3100 had no effect on the shedding of syndecan-1 and syndecan-4 accelerated by SDF-1 from MDM (data not shown).

Together, these data strongly indicate the CXCR4 independence of the shedding of syndecan-1 and syndecan-4 accelerated by SDF-1 from HeLa cells and MDM.

The shedding of syndecan-4 and syndecan-1, accelerated by SDF-1 from HeLa cells, depends on cell-surface HS chains

In contrast, pre-treating HeLa cells with heparitinases I and III prior to their incubation with the chemokine induced a strong and significant decrease in the SDF-1-accelerated shedding of syndecan-4 and in that of syndecan-1 from HeLa cells (respectively, $p < 0.001$ and $p < 0.01$, $n = 3$) (Figure 3). However, this treatment had no effect on the constitutive shedding of syndecan-1 and syndecan-4 from these cells (data not shown). This enzymatic treatment also induces a significant decrease in the labeling of the cells by anti-HS mAb 10 E4 (data not shown), which indicates that the amount of HS chains present at the cell plasma membrane was decreased. In contrast, pre-treatment of the cells with heparitinases did not induce loss of cell-surface syndecan-1 and syndecan-4 labeling. In these conditions, using specific antibodies

and FACS analysis, we observed that the amount of cell-surface syndecan-1 and syndecan-4 remains unchanged if the HeLa cells were stimulated with SDF-1 compared to the unstimulated ones. This rules out that a significant decrease of these PGs occurs if these SDF-1-stimulated cells are pre-treated with the heparitinases mixture (data not shown).

Together, these data strongly suggest that HS chains are involved in the SDF-1-accelerated shedding of syndecan-4 and in that of syndecan-1 from HeLa cells.

SDF-1 does not bind to the ectodomains of syndecan-4, nor to those of syndecan-1 shed from HeLa cells

We then investigated whether SDF-1 forms complexes with the shed ectodomains of syndecan-4 and with those of syndecan-1, present in the supernatant culture medium of unstimulated HeLa cells. For this purpose, the enriched preparations of the shed PGs were incubated for 18 h with SDF-1α (at 50 or 125 nM) and then with anti-SDF-1-coated protein-G beads. Neither proteins immunoreactive with anti-syndecan-4 5G9, anti-syndecan-1 DL-101, nor with anti-CD44 mAbs were detected (Figure 4, lanes 1 and 2; data not shown). In these experiments, we have investigated in parallel, as positive control, whether RANTES forms complexes with the shed PGs. For this purpose, HeLa cells were stimulated for 18 h in the presence of RANTES (at 50 or 125 nM). Thereafter, the immunoprecipitates (IPs) performed with anti-RANTES-coated beads were characterized by the presence of proteins respectively immunoreactive with anti-syndecan-1 DL-101 or B-B4, anti-syndecan-4 5G9, and anti-CD44 mAbs, but not with the respective isotypes (data not shown). Moreover, as second positive control we have also characterized SDF-1 targets on HeLa cells. For this purpose, the cells were incubated with the chemokine and lysed. The SDF-1-bound ligands were collected on anti-SDF-1-coated beads. As previously described by our group (Hamon *et al*., 2004), immunoblotting SDF-1-bound ligands with anti-CXCR4 mAb 12G5 revealed, as expected, 48-kDa immunoreactive proteins (Figure 4, lane 3). Moreover, immunoreactive proteins with anti-syndecan-4 mAb 5G9 migrating as a smear of apparent molecular mass >250 kDa were observed (Figure 4, lane 5). No immunoreactivity with the tested antibodies occurred when the cells were incubated in SDF-1α-free buffer (Figure 4, lanes 4 and 6). These data demonstrate that SDF-1 binds a syndecan-4/CXCR4 complex expressed on HeLa cells.

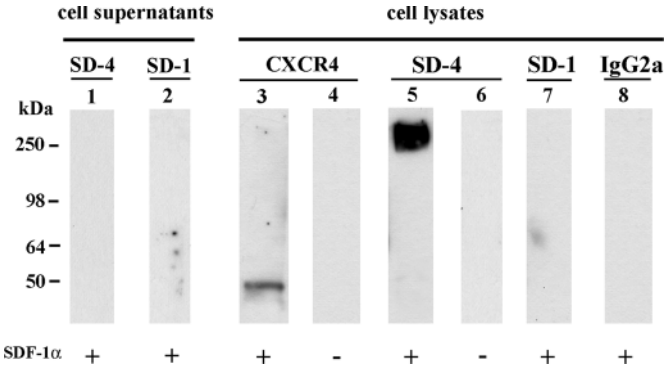

Fig. 4. SDF-1 does not bind to the ectodomains of shed syndecans. Shed PG ectodomains partially purified from HeLa cell culture supernatants, or HeLa cells were incubated with SDF-1α. Cells were lysed. As negative control, cells were incubated in SDF-1α-free buffer. The SDF-1-interacting proteins were collected on anti-SDF-1-coated beads, electroblotted, and revealed with anti-SD-4 mAb 5G9 (lanes 1, 5, and 6), anti-SD-1 mAb DL-101 (lanes 2 and 7), anti-CXCR4 mAb 12G5 (lanes 3 and 4), or the isotype IgG2a (lane 8).

Together, our data also suggest that, while RANTES forms complexes with the shed ectodomains of syndecan-1, syndecan-4, and CD44, as previously demonstrated by our group (Charnaux, Brule, Chaigneau *et al*., 2005), SDF-1 does not bind to the shed ectodomains of syndecan-1 and syndecan-4.

Regulation of gelatinase A (MMP-2) and gelatinase B (MMP-9) by SDF-1

Semi-quantitative RT–PCR analysis revealed that the mRNAs of several MMPs are expressed in HeLa cells: those of MMP-2, MMP-9, and MMP-14 were slightly detected, while those of MMP-7 or A disintegrin and metalloproteinase-17 (ADAM-17) were much more strongly expressed. However, the transcripts of MMP-2 and MMP-9 were strongly and significantly up-regulated by treatment of the cells for 8 or 16 h with PMA or SDF-1α ($p < 0.01$, $n = 3$ for both incubation time tested and for both MMPs, compared with the data observed in PMA- and SDF-1-free medium) (Figure 5A; data not shown). In the same conditions, the levels of MMP-7, MMP-14, and ADAM-17 mRNAs were not affected by the stimulation of the cells with SDF-1α (Figure 5A; data not shown).

According to these data, the gelatinase activities of both MMP-9 and MMP-2 from HeLa cells supernatants were analyzed using zymography performed with equal amounts of protein loaded.

In untreated cells, the 92-kDa activity, which represents pro-MMP-9, was clearly detected, while the 82-kDa activity, which corresponds to activated MMP-9, was faintly detected (Figure 5B, lane 1). Preincubation of the cell supernatants with amino-phenyl mercuric acetate (APMA), which activates the MMP proform to the activated form, resulted as expected in the conversion of most pro-MMP-9 (92 kDa) to an active form of 82-kDa size (Figure 5B, lane 2). In these experiments, the bands corresponding to the pro-MMP-9 and active MMP-9 forms were quantified by densitometric analysis. It is to note that parallel treatment of the cells with PMA (at 0.5 μM) or SDF-1α (at 50 nM) induced in three independent experiments significant increases in both the pro-MMP-9 (92 kDa) and the active MMP-9 forms (82 kDa) ($p < 0.01$ and $p < 0.001$, $n = 3$ for both activations and both forms of MMP-9 compared with the data observed for the untreated cells) (Figure 5B, lanes 3 and 4). The SDF-1α-stimulated secretion of both MMP-9 forms depends on chemokine concentration and on time of incubation (data not shown).

The production of MMP-2 slightly, but significantly, increased by SDF-1α treatment ($p < 0.05$, $n = 3$), whereas PMA had no effect (Figure 5B). However, the fact that only

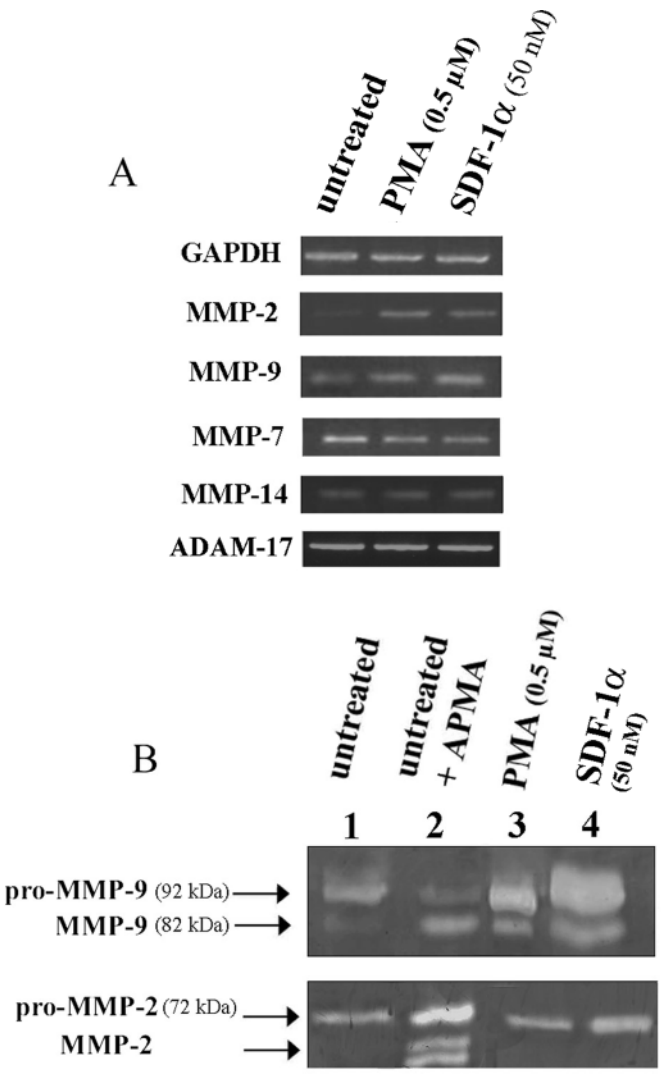

Fig. 5. SDF-1 induces MMP-9 activation. (**A**) HeLa cells were incubated or not for 8 h with PMA (0.5 μM) or SDF-1α (50 nM). The effect on MMP-2, MMP-9, MMP-7, MMP-14, and ADAM-17 mRNAs synthesis was studied by RT–PCR. PCR products were analyzed on agarose gel stained with ethidium bromide. Primers and conditions are specified in the *Materials and Methods* section. PCR for housekeeping gene GAPDH was used as a control for equal loading of RNA into the RTPCR reaction tubes. Obtaining bands of consistent intensity for GAPDH allowed comparaison of the amount of PCR products between different samples. The size of the PCR products were 496 bp for MMP-2, 325 bp for MMP-7, 255 bp for MMP-9, 180 bp for MMP-14, 440 bp for ADAM-17, and 983 bp for GAPDH. (**B**) Analysis of gelatinolytic activity of both MMP-2 and MMP-9 in the culture media of Hela cells. HeLa cells were either untreated (lane 1) or incubated with PMA (0.5 μM) (lane 3) or SDF-1α (50 nM) (lane 4) in serum-free media for 24 h at 37°C. Conditioned media were collected and analyzed by gelatin zymography, performed with equal amounts of protein loaded. Gelanolytic activities are detected as 'clear bands' in the gel. In some experiments, supernatants of untreated cells was incubated with APMA for 1 h at 37°C, before loading (lane 2). One of three individual experiments is shown.

pro-MMP-2 was detected suggests that SDF-1 was devoid of effect on this enzyme activity.

Together, these data demonstrate that SDF-1α stimulates strongly the production and activation of MMP-9 proteins, while no activation of other MMPs proteins in HeLa cells was observed under these conditions.

Role of MMP-9 on the SDF-1α-accelerated shedding of syndecan-4 and syndecan-1 from HeLa cells and MDM

We then used an RNA interference (RNAi) method to silence MMP-9 in HeLa cells in order to test whether this MMP may be involved in the shedding of syndecan-4 and in that of syndecan-1 accelerated by SDF-1. As described (Sanceau *et al.*, 2003), the construct encoded an RNA that targets the MMP-9 mRNA, and the sequence had no homology with other members of the MMP family. The MMP-9 double-strand RNA (MMP-9 dsRNA) and a negative control siRNA (sncRNA) were each tested for their ability to suppress MMP-9 specifically.

Transfection of HeLa cells with MMP-9 dsRNA resulted in an MMP-9 mRNA down-regulation reaching 70% reduction on day 3, compared with the mock-transfected cells ($p < 0.001$, $n = 3$) (Figure 6A). When measuring by zymography, protein expression in the HeLa cell supernatants of the SDF-1α-treated cells, we found a down-regulation of pro-MMP-9 and active MMP-9 protein expressions reaching each 50% compared with the corresponding mock-transfected cells ($p < 0.001$ for both forms of MMP-9 compared with the data observed for the mock-treated cells, $n = 3$) (Figure 6B; data not shown). The sncRNA construct caused no significant reduction of MMP-9 mRNA and protein expression ($p = 0.6$) (Figure 6A and B).

Together, these findings indicate that RNAi efficiently and specifically inhibits endogenous MMP-9 gene expression in HeLa cells.

We then repeatedly observed that the shedding of syndecan-4 and that of syndecan-1 accelerated by SDF-1α was significantly reduced after knockdown of MMP-9, as compared with the data respectively observed in mock-transfected cells or in cells transfected with sncRNA (Figure 6C) ($p < 0.001$). These data strongly indicate that MMP-9 is involved in the molecular events leading to SDF-1-accelerated shedding of syndecan-1 and syndecan-4 from HeLa cells.

Interestingly, pre-treating the MDM with anti-MMP-9 Abs also decreased in a significant manner the shedding of syndecan-1 and syndecan-4 induced by SDF-1 on these cells (Figure 6D). In the same conditions, the specific isotype was devoid of effect.

Discussion

Chemokine and PGs may play important role in many aspects of inflammation and tumor biology such as angiogenesis, cell migration, and cell proliferation (Strieter, 2001; Götte, 2003; Burger and Kipps, 2006; Taylor and Gallo, 2006). The remodeling of tumor cell surface by proteases may likely be involved in these puzzled pathological events. However, whether a chemokine like SDF-1/CXCL12 may modify in a dynamic manner a tumor cell surface and its environment has not been extensively investigated.

Ectodomain shedding of PGs is an important regulatory mechanism, as it rapidly changes the surface phenotype of affected cells and generates soluble ectodomains that can function as paracrine or autocrine effectors. A growing body of evidence indicates that these molecular and cellular

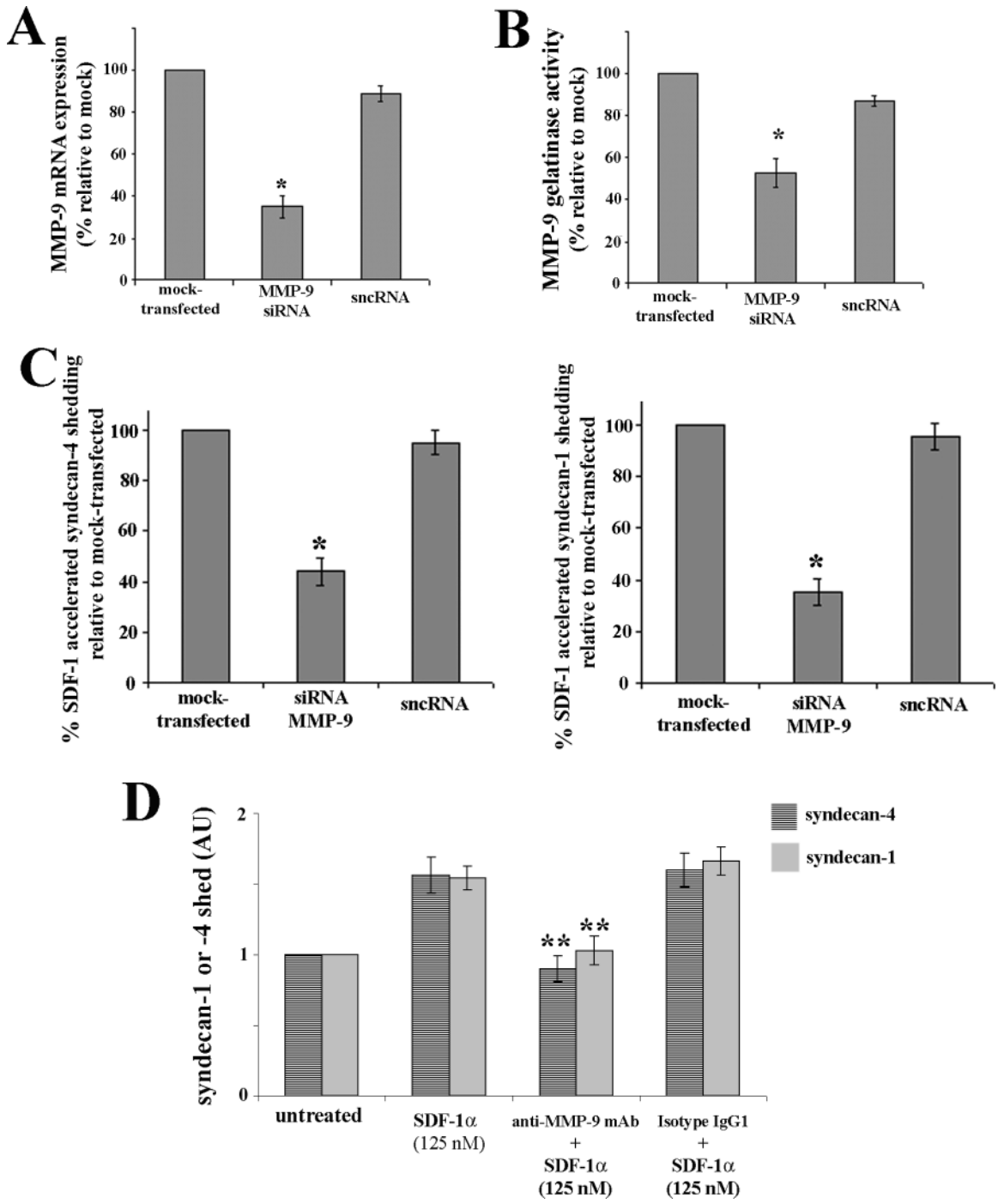

Fig. 6. MMP-9 silencing by RNA interference prevented the SDF-1α-accelerated shedding of syndecan-1 and syndecan-4 from HeLa cells. (**A**) HeLa cells were analyzed 3 days post-transfection with MMP-9 dsRNA, sncRNA, or mock-treated cells for MMP-9 mRNA. Cellular levels of MMP-9-specific mRNA (relative to GAPDH mRNA) were measured by semi-quantitative RT–PCR. MMP-9-specific mRNA are depicted relative to mock-transfected control. (**B**) HeLa cells, mock-, dsRNA-, or sncRNA-transfected, were treated with 50 nM SDF-1α for 24 h. MMP-9 protein expression was monitored by zymography analysis, and gelatinolytic intensity was expressed as percent, relative to mock-transfected cells. Only proform MMP-9 quantification is shown. Similar results were obtained for the active MMP-9 form. Each point represents the mean ± SE of triplicate determinations. One of three individual experiments is shown. (**C**) HeLa cells, 3 days post-transfection with MMP-9 dsRNAs or sncRNA or mock transfected, were treated or not for 2 h with 50 nM SDF-1α. Conditioned media were harvested and proteoglycans, partially purified by application to cationic membranes, were analyzed by dot blot. The amount of syndecan-1 or syndecan-4 shed is expressed in absorbance units, measured by densitometric scanning. Results are expressed as the percentage of SDF-1-accelerated shedding of syndecan-1 and syndecan-4 relative to mock-transfected cells. Each point represents the mean ± SE of triplicate determinations. One of three individual experiments is shown. (p compared to the corresponding mock-transfected cells. *$p < 0.001$). (**D**) MDMs were pre-incubated with anti-MMP-9 mAb or its isotype IgG1 for 30 min and then stimulated or not with SDF-1α for 2 h. Harvested conditioned media were analyzed by dot blot. The data are expressed as the amount of syndecan shed, in relative absorbance units to the value given by the untreated cells. Each point represents the mean ± SE of triplicate determinations. One of three individual experiments is shown. **$p < 0.01$ compared to SDF-1-stimulated cells, pre-incubated with IgG1.

features enable ectodomain shedding to regulate many pathophysiological processes, such as inflammation, tissue repair, and cancer cell proliferation (Maeda *et al.*, 2004). Such shedding then generates C-terminal fragment (CTF) membrane bound of syndecan (Fitzgerald *et al.*, 2000). These syndecan CTFs are then further cleaved upon the transmembrane region by presenilin-dependent γ secretase upon ectodomain shedding (Schutz *et al.*, 2003). Together, these data strongly indicate that the role of syndecans may be highly dynamic and complex.

We have previously shown that SDF-1/CXCL12 directly binds, in a glycosaminoglycan-dependent manner, to syndecan-4 (Charnaux, Brule, Hamon *et al.*, 2005). We have also shown that, upon SDF-1/CXCL12 stimulation of HeLa cells, syndecan-4, but not syndecan-1, syndecan-2, CD44, or betaglycan, undergoes tyrosine phosphorylation. Finally, by reducing syndecan-4 expression using RNAi or by pre-treating HeLa cells with heparitinases I and III, we previously strongly suggested the involvement of syndecan-4 and HS in p42/44 MAPK and Jun/SAP kinases activations by CXCL12 in HeLa cells.

In accordance with previous studies (Reiland *et al.*, 1996; Subramanian *et al.*, 1997; Fitzgerald *et al.*, 2000; Day *et al.*, 2003), we observed here that syndecan-1, syndecan-4, and CD44 are constitutively shed from the HeLa cells and from human primary monocyte-derived macrophages. We then showed that SDF-1/CXCL12 at nM physiological concentrations (Baggiolini *et al.*, 1997) significantly and strongly accelerates the shedding of the ectodomains of syndecan-4 from HeLa cells and exerts a less enhancing effect on the shedding of syndecan-1. In contrast, CD44 shedding was not affected by the chemokine. This chemokine also accelerates the shedding of syndecan-1 and syndecan-4 from MDM.

As SDF-1 was shown to form a cell-surface multimolecular complex which comprises, as expected, CXCR4 (Hamon *et al.*, 2004), we asked whether this GPCR is implicated in the SDF-1-accelerated shedding of PGs from HeLa cells. For this purpose, HeLa cells were pre-incubated with AMD3100, a CXCR4 inhibitor, or with the monoclonal anti-human CXCR4-neutralizing antibody, clone 12G5, and then incubated with CXCL12. Alternatively, HeLa cell CXCR4 expression was strongly decreased by the use of RNAi. In these experimental conditions, the syndecan-4 or syndecan-1 shedding accelerated by the chemokine from HeLa cells was not affected, suggesting a CXCR4-independent mechanism. Moreover, AMD3100 had no effect on the shedding of syndecan-1 and -4, accelerated by SDF-1 from MDM, which further argues for the CXCR4 independence of these events. Pre-treating HeLa cells with heparitinases prior to their incubation with SDF-1 resulted in a strong decrease (1) in their labeling by anti-HS mAb 10E4, which demonstrates the decrease in plasma membrane HS, and (2) in the SDF-1-accelerated shedding of syndecan-1 and -4. In addition, we observed that cell-surface syndecan-1 and -4 expression was unchanged in the SDF-1-stimulated cells pre-treated by heparitinases compared with the unstimulated ones. This argues for the lack of SDF-1-accelerated shedding of the PGs from these glycosaminidase-treated cells and suggests the involvement of cell-surface HS chains in SDF-1-induced shedding.

We investigated which downstream transduction pathway may be involved in the shedding induced by SDF-1 from HeLa cells. These cells were used in the following experiments because the SDF-1-accelerated shedding of syndecan-4 from these cells was twofold higher than that observed from MDM.

In each assay, we tested the effects of PKC, MAP kinase, or NF-κB inhibitors. No effect on PG shedding was observed if these inhibitors were applied alone. These data suggest that these inhibitors do not affect the synthesis, glycosylation, or transport of syndecans to the cell surface during the assay periods. However, MAP kinases or NF-κB inhibitors did not affect SDF-1-accelerated shedding of syndecan-1 and syndecan-4. In contrast, bisindolylmaleimide I, a potent and selective inhibitor of PKC, strongly prevented this shedding, suggesting the involvement of this transduction pathway in these events.

Moreover, while RANTES forms GAGs-dependent complexes with the shed ectodomains of syndecan-1, syndecan-4, and CD44, as recently described by our group (Charnaux, Brule, Chaigneau *et al.*, 2005), we show that SDF-1 does not bind to syndecan-4 ectodomains but binds to membrane-associated syndecan-4, as previously demonstrated (Hamon *et al.*, 2004). It could be that a proper conformation of PG and GAG chains on the cell surface is crucial for interaction with SDF-1. Consistent with our results, Netelenbos *et al.* (2003) demonstrated that PGs on bone marrow endothelial cells bind and present SDF-1 toward hematopoietic progenitor cells, whereas isolated endothelial PGs do not bind SDF-1, suggesting the necessity of membrane association. Zhang *et al.* (2001) reported that membrane-associated HSPGs, but not similar soluble HSPGs, induced fibroblast growth factor receptor signaling by fibroblast growth factor-2. They suggested that membrane association of HSPG is critical for interaction with the protein and that high density and cell-surface association of PGs could impose particular chain orientation of GAGs resulting in optimal protein binding. Our previous data also demonstrated that CXCR4 and syndecan-4 associated at the cell surface, even in the absence of SDF-1 (Hamon *et al.*, 2004). Such association could impose particular syndecan-4 protein conformation, resulting in SDF-1 binding. Furthermore, it has been demonstrated that soluble syndecan ectodomains can be modified by heparanase, which is an endoglycosidase that degrades HS to lower molecular weight fragments (Kato *et al.*, 1998). Whether such mechanisms could be involved in the lack of association between soluble syndecan ectodomains and SDF-1 remained unknown.

The role of syndecan shedding in the biological effect of SDF-1 may then be related with changes in the target cell-surface phenotype rather than with a competitive inhibition in the binding of the chemokine to the target cell surface.

Interestingly, we recently demonstrated that RANTES/CCL5 (1) induces a syndecan-1 and -4 shedding from HeLa cells, which depends on CCR5 and on both Erk1/2 MAPK and PKC transduction pathways, and (2) forms GAGs-dependent complexes with the shed ectodomains of these PGs (Charnaux, Brule, Chaigneau *et al.*, 2005). The respective molecular events involved in the PG shedding induced by RANTES or SDF-1 differ according to the chemokine.

Moreover, the binding capability of both chemokines to the shed syndecans ectodomains also differs. Indeed, RANTES and SDF-1, respectively, CC and CXC chemokines, are quite different: they show different GAG-binding epitopes and different quaternary structure (dimeric and oligomeric states) (Lortat-Jacob *et al*., 2002; Handel *et al*., 2005). They also exhibit different expression patterns. RANTES is preferentially secreted during inflammatory process, whereas SDF-1 is constitutively expressed in various cell types and may play a 'homeostatic' role. Therefore, syndecan-1 and -4 shedding induced by RANTES could be expected to occur during inflammatory and wound repair processes, whereas PGs shedding accelerated by SDF-1 could occur even in the absence of any tissue injury.

Ectodomain shedding is usually mediated by peptide hydroxamate-sensitive metalloproteinases which are collectively called sheddases or secretases (Schlöndorff and Blobel, 1999; Arribas and Borroto, 2002). Several ADAMs have been shown to function as cell-associated sheddases. Among these, TACE (TNFα-converting enzyme, ADAM-17) sheds a wide variety of surface proteins. But available data indicate that ectodomain shedding of CD44 (Shi *et al*., 2001), syndecan-1, and syndecan-4 (Fitzgerald *et al*., 2000) is TACE independent. Consistent with these findings, other cell-surface-associated and soluble sheddases have been described. Examples of other cell-surface-associated sheddases include MMP-14 (MT1-MMP) for CD44 shedding (Kajita *et al*., 2001). Among the soluble sheddases, MMP-7 has been found to shed syndecan-1 (Li *et al*., 2002). These data indicate that certain shed proteins are substrates of more than one sheddase and suggest that different sheddases act in a tissue-specific dependent manner, and also possibly in a disease-specific manner.

We demonstrate that SDF-1/CXCL12 increases the mRNAs of MMP-2 and MMP-9 in HeLa cells, while those of MMP-7, MMP-14, and ADAM-17 were not affected by the chemokine. However, only pro-MMP-2 was detected in SDF-1-stimulated HeLa cells supernatants, suggesting that the chemokine did not activate MMP-2. In contrast, pro-MMP-9 and active MMP-9 forms significantly increased in the supernatants of SDF-1-stimulated HeLa cells. Reducing MMP-9 mRNA and MMP-9 gelatinase activity by siRNA technology resulted in significant decrease of the SDF-1/CXCL12-accelerated shedding of syndecan-1 and syndecan-4. Interestingly, stimulating MDM by SDF-1 in the presence of anti-MMP9 Abs also prevented the shedding of syndecan-1 and -4. This strongly indicates the involvement of MMP-9 in these events which occur in HeLa cells and human primary macrophages. In the present study, similar inductions of syndecan-1 and -4 shedding by SDF-1 were observed if the cell culture medium was supplemented or not with serum. Therefore, that the SDF-1-induced shedding of syndecan-1 and syndecan-4 involves proteases such as plasmin or thrombin can be excluded because these proteases are inactivated by anti-proteases present if the culture medium is supplemented with serum (Subramanian *et al*., 1997). Moreover, no amino acid sequences susceptible for direct thrombin and plasmin cleavage are present in syndecan-1 (Schmidt *et al*., 2005).

Taken together, we demonstrate that the SDF-1-accelerated shedding of syndecan-1 and syndecan-4 depends on cell-surface HS chains and on PKC pathway. In contrast, direct interaction of SDF-1 with CXCR4 would not be necessary.

Syndecan-4 is known to be phosphorylated by growth factors, including CXCL12 (Oh *et al*., 1997; Horowitz and Simons, 1998; Hamon *et al*., 2004; Choi *et al*., 2005). A feature of syndecan-4 signaling is also activation of PKC (Oh *et al*., 1997; Lim *et al*., 2003). Co-distribution of clustered syndecan-4 with the alpha isoform of PKC in focal adhesion has been demonstrated (Oh *et al*., 1997; Couchman *et al*., 2002). Furthermore, Lim *et al*. (2003) have suggested a mechanism where syndecan-4 binds PKC alpha and localizes it to focal adhesions, whose assembly may be regulated by the kinase. These data suggest the possible role of syndecan-4 in cell adhesion, spreading, and stress fiber formation. Indeed, syndecan-4 can promote cell spreading in a beta(1) integrin-dependent fashion through PKC alpha and RhoA (Thodeti *et al*., 2003).

Our data could suggest that SDF-1 binds to syndecan-4 through its glycosaminoglycan chains, inducing then phosphorylation of the C-terminal region of this PG, which in turn activates the PKC pathway, leading to increase expression and activation of the MMP-9. Thereafter, this protease may cleave syndecan-4 and therefore induce the ectodomain shedding. Whether these phenomena have direct or indirect consequences on cell adhesion, spreading and stress fiber formation now needs to be investigated.

However, we also observed here a slight shedding of syndecan-1 induced by SDF-1 from HeLa cells and MDM. Our previous data have nevertheless suggested that (1) SDF-1 directly binds to syndecan-4, (2) only syndecan-4 can be considered as an SDF-1-signaling molecule, and (3) this chemokine does not interact with syndecan-1 (Hamon *et al*., 2004). One can therefore suppose that the shedding of PGs belonging to the same family may involve common sheddases, such as MMP-9, as demonstrated previously (Kainulainen *et al*., 1998). Moreover, whether the induction of syndecan-1 and -4 shedding and of MMP-9 activity by SDF-1 is a direct or an indirect mechanism, mediated through the effects of other endogeneous cytokines or chemokines, remains to be investigated.

In conclusion, our data suggest that (1) SDF-1 accelerates the shedding of syndecan-4 and, to a lesser extent, that of syndecan-1 from HeLa cells and (2) cell-surface HS chains, PKC transduction pathways, and MMP-9 may be involved in these puzzled events. Moreover, we also observe that SDF-1 slightly accelerates the shedding of syndecan-1 and syndecan-4 from human primary cells such as primary macrophages and strongly suggest (1) the CXCR4 independence of these events and (2) the involvement of MMP-9.

The role played by these events in physiopathological situations, such as cancer and inflammation, deserves further studies. Whether the syndecan-1 and syndecan-4 ectodomains modulate cellular effects of SDF-1 on tumor cells is presently under investigation in our laboratory.

Materials and Methods

Cell culture

HeLa cells were cultured in Dulbecco minimal essentiel medium (DMEM; Invitrogen Corporation, Paris, France) containing 10% fetal calf serum (FCS) (Biowhittaker, Paris, France) and 2 mM L-glutamine (Invitrogen Corporation) and split twice a week. Peripheral blood mononuclear cells were isolated from cytapheresis from heathly blood donors (Etablissement français du sang, Paris, France) and cultured in most experiments for 5 days as described (Hamon *et al.*, 2004). Non-adherent cells were then removed. Adherent cells, which comprised more than 95% monocyte-derived macrophages, were cultured for at least another 48 h.

Flow cytometry

Adherent HeLa cells or MDMs were cultured in 24-well flat-bottom plates (at 2×10^5 cells per well) in 1 ml of culture medium. After three washes with PBS supplemented with 0.05% BSA (Sigma-Aldrich, Saint Quentin Fallavier, France) (PBS–BSA), cells were incubated for 30 min at 4°C in 150 µl of PBS–BSA, supplemented with anti-CXCR4 mAb 12G5 (mouse IgG2a, Pharmingen, Pont-de Claix, France), anti-syndecan-1 mAb DL-101 (mouse IgG1), anti-syndecan-4 mAb 5G9 (mouse IgG2a), anti-CD44 (mouse IgG1) (all from Santa Cruz Biotechnology, Santa Cruz, CA) (all at 10 µg/ml), their isotypes (Pharmingen), or biotinylated SDF-1α (a gift from F. Baleux, Laboratoire de Chimie Organique, Institut Pasteur, Paris, France). After washing, cells were labeled for 30 min at 4°C with either FITC-labeled goat-anti-mouse Ig (1/25, Pharmingen) or streptavidin–Alexa Fluor 488 complex (1/100, Molecular probe Inc., Eugene, OR), fixed in 1% paraformaldehyde (Sigma-Aldrich) in PBS, and scraped. Five thousand cells were analyzed by using a FACScan instrument (Becton Dickinson, Le Pont de Claix, France). As control, biotinylated SDF-1α was omitted. In some experiments, cells were pre-incubated for 1 h at 37°C with AMD3100 (1.2 µM), a CXCR4 inhibitor (Sigma-Aldrich).

Shedding assays

Adherent HeLa cells or MDMs were cultured in 24-well tissue culture plates (at 2×10^5 cells per well). At the time of the assay, culture media were replaced with fresh culture medium supplemented or not by the indicated test agents: 0.5 µM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) and SDF-1α (a gift from F. Baleux, Laboratoire de Chimie Organique, at 0, 0.5, 2.5, 10, 50, and 125 nM) in the presence or absence of MEK1/2 inhibitor, U0126 (1,4-diamino-2,3-dicyano 1,4-bis[2-aminophenylthio]butadiene) (at 10 µM, Cell Signaling Technology, Ozyme, France), a potent and selective inhibitor of PKC, bisindolylmaleimide-1 (at 1 µM, Merck Biosciences, La Jolla, CA), a specific antagonist for CXCR4, AMD3100 (at 1.2 µM), anti-CXCR4 mAb 12G5 (at 10 µg/mL, Zymed laboratories Inc, Montrouge, France), or a NF-κB inhibitor, Bay11-7082 [E-3-(4-methylphenylsulfonyl)-2-propenenitrile; Bay11] (at 20 µM, Calbiochem, VWR International SAS, Fontenay-sous-Bois, France). Cells were incubated for 2 or 18 h at 37°C in the conditioned media. Aliquots of the cells were labeled with Trypan Blue exclusion dye and examined by phase microscopy for survival and morphology. Conditioned media were used for dot blot analysis, as described below. Cells were analyzed in parallel by flow cytometry after indirect immunofluorescence labeling or were lysed, and proteins were measured by BCA protein assay (Pierce Perbio Science, Brebières, France).

In some experiments, cells were pre-incubated for 2 h at 37°C with heparitinase III (EC 4.2.2.7., at 200 mUI/ml) and heparitinase I (EC 4.2.2.8., at 100 mUI/ml) (both from Sigma-Aldrich), which are eliminases that cleave HS into disaccharide or tetrasaccharide units (Ernst *et al.*, 1996). The efficiency of the enzymatic treatment was assayed by immunocytochemistry experiments using an anti-HS 10E4 mAb (data not shown). For that purpose, treated cells were stained with 10E4 mAb (10 µg/ml, mouse IgM, Seikagaku, Tokyo, Japan) revealed with FITC-labeled goat anti-mouse Ig (1:100, Pharmingen).

In some experiments, MDMs were pre-incubated for 30 min with anti-MMP-9 mAb at 10 µg/ml (clone 2C3, IgG1, Santa Cruz Biotechnology) or its respective isotype IgG1 and then stimulated by SDF-1α for 2 h.

Dot Immunoassays

HeLa cell- or MDM-conditioned media (50 µl) were applied to cationic nylon membrane (Amersham N+, Amersham Biosciences, UK) under mild vacuum in an immunoblot apparatus (Slot blot, Amersham Biosciences). This membrane was pre-treated by incubation with PBS supplemented with Tris (10 mM; Sigma-Aldrich). The membrane was then blocked by a 1-h incubation at 37°C in PBS supplemented with 0.5% BSA, 3% non-fat dry milk, and 0.5% Tween 20, washed twice with PBS/0.5% BSA supplemented with 0.5% Tween 20 (all from Sigma Aldrich) and then incubated overnight at 4°C, with anti-syndecan-1 mAb BB4 (mouse IgG1, specific for the ectodomain of syndecan-1 of human origin, Serotec, Oxford, UK), anti-syndecan-4 mAb, anti-CD44 mAb, or with their respective isotypes (mouse IgG1 or IgG2a both from Pharmingen) (all at 0.1 µg/ml). After washing, the membrane was incubated with a 5000-fold dilution of HRP-conjugated anti-mouse IgG (Amersham Biosciences). Detection was performed using the ECL system (Amersham Biosciences). Results were quantified by scanning the exposed X-ray film with an Agfa scanner and using area measurement from Scion Imager. Increased amounts of HeLa cell-conditioned media showed a linear increase of absorbance units. Results are expressed as the amount of PG shed, in relative absorbance units to the value given by the untreated cells (AU). The values are normalized for micrograms of cellular proteins. Each point represents the mean ± standard errors of triplicate determinations of individual experiments. Statistical significance between independent experiments was calculated using the Student's *t*-test.

Possible binding of SDF-1 to the shed ectodomains of PGs

The enriched ectodomains of PGs which were shed from the HeLa cells were prepared as previously described (Charnaux, Brule, Chaigneau *et al.*, 2005) and incubated for 18 h in the presence of SDF-1α (at 50 or 250 nM) or RANTES (at 50 or

250 nM, gift from C. Vita, CEA, Saclay, France) and protease inhibitors. Alternatively, HeLa cells (2×10^7) were incubated or not for 2 h with SDF-1α (50 nM) at 4°C. Cells were lysed by incubation in 500 µl of lysis buffer (150 nM NaCl, 20 mM Tris-HCl, pH 8.2, supplemented with 1% Brij 97 and with antiproteases inhibitors), as described (Hamon *et al.*, 2004). Cells lysates or enriched ectodomains of PGs were then incubated for 24 h with anti-SDF-1 or anti-RANTES mAbs coated to protein G sepharose (R&D systems, Lille, France), prepared as described (Hamon *et al.*, 2004). IP complexes were washed three times with PBS prior to separation by 12% SDS–PAGE. Following electrophoresis, proteins were transferred to PVDF membranes, blocked, and incubated with the appropriate primary antibodies or their respective isotypes, as described above. The immunoblotted proteins were visualized with HRP-linked secondary antibodies using ECL substrate.

RT-PCR

MMP-2 mRNA and MMP-9 mRNA, MMP-1, MMP-7, and ADAM-17 mRNAs, and to normalize for total RNA input, glyceraldehyde 3 phosphodehydrogenase mRNA were quantified by RT–PCR. Total cellular RNA was extracted from confluent monolayers of stimulated or unstimulated HeLa cells grown in a 6-well tissue culture, using a Qiagen RNA-DNA mini-kit (Qiagen, Courtaboeuf, France). Reverse transcription was performed using an advantage RT for PCR kit (BD Bioscience Clontech, Mountain View, CA). The following synthetic MMP-2 primers were used: upper primer 5′ CTC AGA TCC GTG GTG AGA TCT 3′ and lower primer 5′ CTT TGG TTC TCC AGC TTC AGG 3′. These primers were designed to amplify a 496-bp coding sequence of MMP-2. The following synthetic MMP-9 primers were used: upper primer 5′ AAG ATG CTG CTG TTC AGC GGG 3′ and lower primer 5′ GTC CTC AGG GCA CTG CAG GAT 3′. These primers were designed to amplify a 255-bp coding sequence of MMP-9. The following synthetic MMP-14 primers were used: upper primer 5′ TCG GCC CAA AGC AGC AGC TTC 3′ and lower primer 5′ CTT CAT GGT GTC TGC ATC AGC 3′. These primers were designed to amplify a 180-bp coding sequence of MMP-14. The following synthetic MMP-7 primers were used: upper primer 5′ GAG GAT GAA CGC TGG ACG GAT 3′ and lower primer 5′ GGG TGA CAT AAT TGC TAA ATG GA 3′. These primers were designed to amplify a 325-bp coding sequence of MMP-7. The following synthetic ADAM-17 primers were used: upper primer 5′ GCA CAG GTA ATA GCA GTG AGT GC 3′ and lower primer 5′ CAC ACA ATG GAC AAG AAT GCT G 3′. These primers were designed to amplify a 440-bp coding sequence of ADAM-17. Optimum semi-quantitative RT–PCR conditions were established to remain in the exponential phase of amplification curve. After amplification, 30 µl were electrophoresed in 2% agarose and analyzed.

Gelatin zymography

HeLa cells were incubated for 2, 4, 6, 8,18, or 24 h in serum-free medium supplemented or not with exogeneous SDF-1α (at 2.5, 10, 50, and 125 nM). Conditioned media were collected and mixed with non-reducing sample buffer (62.5 mM Tris HCl, 2% (w/v) SDS, 10% (w/v) glycerol) and subject to electrophoresis in a 10% SDS–PAGE gel containing 0.1% (w/v) gelatin (Sigma-Aldrich), with equal amounts of proteins loaded. In some experiments, supernatants of untreated cells were incubated with APMA (1 mM) for 1 h at 37°C before loading. Electrophoresis was carried out at 10°C. The SDS in the acrylamide gel was extracted by incubation with 2.5% Triton X100 solution. Gelatinolytic activities were developed in a buffer containing 50 mM Tris-HCl, pH 7.4, 5 mM $CaCl_2$, 200 mM NaCl, and 0.05% Brij 35 (all from Sigma-Aldrich) at 37°C for 24 h. The gel was stained with Coomassie Blue R-250, destained and processed using a Agfa Duoscan T 1200 scanner. The intensity of the bands in zymography was quantified using the Scion Imager software. In these experiments, the bands corresponding to the proform and active form of MMPs were quantified by densitometric analysis. To validate the quantitation of the gelatin zymography for MMP-9, we used the HT1080 cell line, which is known to express large amount of MMP-9 (Maquoi *et al.*, 2002). Increased amounts of HT1080 cell-conditioned media showed a linear increase of the intensity of the bands in zymography (data not shown). As quantified standards were not available, the values were analyzed as arbitrary units normalized for protein concentration.

siRNAs preparation and transfection

CXCR4 gene-specific sense and antisense 21-nt single-stranded RNAs with symmetric 2 nt-3′(2′-deoxy) thymidine overhangs were designed as described (Zhou *et al.*, 2004), chemically synthesized and HPLC purified (Eurogentec, Seraing, Belgium). RNA sequences corresponding to CXCR4 double-strand RNA (CXCR4 dsRNA) were: sense 5′GCA-UGA-CGG-ACA-AGU-ACA-GdTdT3′ and antisense 5′CUG-UAC-UUG-UCC-GUC-AUG-CdTdT3′. The siRNA sequence targeting human MMP-9 chosen in the study (from mRNA sequence; GenBank accession number NM-004994) corresponds to the coding region 377–403 relative to the first nucleotide of the start codon. Sense siRNA sequence was 5′CAUCACCUAUUGGAUCCAA dTdT3′. Antisense siRNA was 5′UUGGAUCCAAU AGGUGAUGdTdT3′. For RNAi experiments, dsRNAs were generated by mixing equimolar amounts (50 µM) of sense and anti-sense single-stranded RNAs in annealing buffer (50 mM Tris, pH 7.5–8.0, 100 mM NaCl in DEPC-treated water) for 1 min at 94°C, followed by 60 min incubation at room temperature. HeLa cells were transfected with 160 nM dsRNA in serum-free medium using Jetsi transfectant reagent (Eurogentec) following the manufacter's instructions. Mock cells were cultured in parallel and transfected with the transfection mixture lacking dsRNA. Cells transfected were used 3 days after transfection. In each experiment, a negative control sncRNA (Eurogentec) was used.

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Conflict of interest statement

None declared.

Abbreviations

ADAM, A disintegrin and metalloproteinase; AU, absorbance unit; dsRNA, double-strand RNA; GAGs, glycosaminoglycans; GPCR, G-protein-coupled receptor; HS, heparan sulfate; HSPGs, heparan sulfate proteoglycans; MAPKs, MAPKinases; MDM, monocyte-derived macrophages; MMP, matrix metalloproteinase; PGs, proteoglycans; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RANTES, regulated on activation normal T-cell expressed and secreted; RT–PCR, reverse transcription–polymerase chain reaction; SDF-1, stromal cell-derived factor-1; siRNA, small interfering RNA.

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