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Interaction of RANTES with syndecan-1 and syndecan-4 expressed by human primary macrophages

Hocine Slimani[a], Nathalie Charnaux[a], Elisabeth Mbemba[a], Line Saffar[a], Roger Vassy[b], Claudio Vita[c], Liliane Gattegno[a,*]

[a] *Laboratoire de Biologie Cellulaire, Biothérapies Bénéfices et Risques, UPRES 3410, UFR-SMBH, Université Paris XIII, 74, rue Marcel Cachin, 93017, Bobigny, France, Hôpital Jean Verdier, 93017, Bondy*
[b] *Laboratoire de Ciblage Fonctionnel des Tumeurs Solides, UPRES 2360, UFR-SMBH, Université Paris XIII, 74, rue Marcel Cachin, 93017, Bobigny, France, Hôpital Jean Verdier, 93017, Bondy*
[c] *CEA, Saclay, Département d'Ingénierie et d'Etudes des Protéines, 91191 Gif-sur-Yvette, France*

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Abstract

Interaction of RANTES with its membrane ligands or receptors transduces multiple intracellular signals. Whether RANTES uses proteoglycans (PGs) belonging to the syndecan family to attach to primary cells expressing RANTES G-protein-coupled receptors (GPCRs) was investigated. We demonstrate that RANTES specifically binds to high and low affinity binding sites on human monocyte-derived macrophages (MDM). We show by co-immunoprecipitation experiments that RANTES is associated on these cells with syndecan-1 and syndecan-4, but neither with syndecan-2 nor with betaglycan, in addition to CD44 and its GPCRs, CCR5 and CCR1. Glycosaminidases pre-treatment of the monocyte derived-macrophages strongly decreases the binding of RANTES to syndecan-1 and syndecan-4 and also to CCR5, and abolishes RANTES binding to CD44. This suggests that glycosaminoglycans (GAGs) are involved in RANTES binding to the PGs and that such bindings facilitate the subsequent interaction of RANTES with CCR5, on the MDM, characterized by low membrane expression of CCR5. The role of these interactions in the pathophysiology of RANTES deserves further study.

Keywords: RANTES; CCR5; Syndecan; HIV; Macrophage

1. Introduction

Macrophages are thought to be one of the first type of cells in the body to be infected during the early stage of HIV transmission [1–5]. Moreover, macrophages together with naive and memory T cells [4,6,7] represent a major HIV reservoir in HIV infected individuals. Therefore, macrophages are key players in HIV-1 pathogenesis. CD4 is the HIV-1 primary receptor [8], but chemokine receptors have been identified as HIV coreceptors, especially CCR5 for R5 HIV strains and CXCR4 for X4 HIV strains [9–13]. We, in accordance with others, have previously reported that infection of human monocyte-derived macrophages (MDM) and peripheral blood lymphocytes (PBL) by HIV-1 R5 strains, but not PBL by X4 strain, is inhibited by β-chemokines such as RANTES or macrophage inflammatory protein (MIP)-1α [14,15], two physiological CCR5 ligands [9]. However, opposite effects of RANTES on HIV-1 infection of macrophages have also been described [16–18]. Chemokines mediate their biological activity through activation of G protein coupled receptors (GPCRs) [19], but most chemokines, including RANTES, are also able to bind to glycosaminoglycans (GAGs) [20–23]. Virtually, all GAGs exist in covalent linkage to a protein core as proteoglycans (PGs). RANTES exhibits selectivity in GAGs binding with the highest affinity (nanomolar range) for heparin [24,25]. It has been suggested that cell surface heparan sulfate (HS)

Abbreviations: MDM, monocyte-derived macrophages; PBL, peripheral blood lymphocytes; PGs, proteoglycans; SD, syndecan; MIP, macrophage inflammatory protein; MCP-1, monocyte chemoattractant protein-1; RANTES, regulated on activation normal T cell expressed and secreted; GAGs, glycosaminoglycans; SDF, stromal-cell derived factor; GPCRs, G-protein-coupled receptors; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DTT, dithiothreitol; TBS, Tris-buffered saline supplemented; FCS, fetal calf serum; IL-8, interleukin-8; FGF-2, fibroblast growth factor-2; hbEGF, heparin-binding epidermal growth factor

* Corresponding author. Tel.: +33-1-4838-7752; fax: +33-1-4802-6503.
E-mail address: liliane.gattegno@jvr.ap-hop-paris.fr (L. Gattegno).

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proteoglycans (HSPGs) act as necessary HIV attachment receptors on specific target cells. It has also been shown that soluble polyanions inhibit HIV infection [26–28]. Moreover, a recent study has suggested that a single family of HSPGs, the syndecans, efficiently mediates HIV-1 attachment and represent an abundant class of HIV attachment receptors on human primary macrophages [29]. Cells surface HSPGs are anchored in the cell membrane either via a transmembrane domain (the syndecans) or by glycosylphosphoinositol linkage (glypicans) [29]. In this study, we have evaluated whether RANTES binds to syndecans expressed on the plasma membrane of MDM.

2. Materials and methods

2.1. Cells and cell culture

Peripheral blood mononuclear cells from cytapheresis from healthy HIV-seronegative donors (Etablissement Français du Sang, Paris, France) were cultured for 5 days as previously described [14,23]. Non-adherent cells were removed. Adherent cells were cultured as described [14,23] for at least 48 h. No contamination by T cells was observed [14,23]. K562 cells were cultured in RPMI 1640 (Invitrogen Corporation) supplemented with 10% fetal calf serum (FCS).

2.2. FACScan analysis of the cells

Aliquots of 5×10^5 scraped MDM were incubated for 30 min at 4 °C in 100-μl phosphate-buffered saline (PBS), supplemented with 0.05% bovine serum albumin (BSA, Sigma-Aldrich, Saint Quentin Fallavier, France) and with 1–2.5 μg of anti-CD4 mAb (murine IgG1; clone Q4120; Sigma-Aldrich), anti-CD14 mAb (murine IgG2b; Becton Dickinson, Pont de Claix, France), anti-CCR5 mAb 2D7 or anti-CXCR4 mAb 12G5 (mouse IgG2a; both from Pharmingen, Pont de Claix, France), anti-CCR1 mAb (mouse IgG2b; clone 55504.111; R&D systems, Abigdon, UK), anti-CD44 mAb (mouse IgG2a; clone F10-44-2; Serotec, Oxford, UK) or the isotypes (Pharmingen). After washing, cells were incubated for 30 min at 4 °C with FITC-labeled goat anti-mouse Ig antibodies (1/20; Pharmingen), fixed in 1% paraformaldehyde (PFA) (Sigma-Aldrich) in PBS (PBS–PFA) and analyzed on a FACScan (Becton Dickinson). Alternatively, adherent MDM were cultured in 24-well flat-bottom plates (at about 5×10^5 cells per well) in 1-ml culture medium. After three washes with PBS, MDM were incubated for 30 min at 4 °C in 300 μl of PBS–BSA supplemented either with anti-syndecan-1 mAb DL-101 (10 μg/ml; mouse IgG-1; clone DL-101, specific for an epitope corresponding to the ectodomain of human syndecan-1, Santa Cruz Biotechnology, California, USA), anti-syndecan-4 mAb (10 μg/ml; mouse IgG2a; clone 5G9, specific for an epitope corresponding to the ectodomain of human syndecan-4; Santa Cruz Biotechnology), anti-betaglycan Abs (10 μg/ml; goat IgG; R&D systems) or their isotypes (Pharmingen). In parallel, aliquots of these adherent MDM (5×10^5 cells per well) were permeabilized in the presence of 300-μl RPMI supplemented with 2% FCS and 0.3% saponin (Sigma-Aldrich), and then incubated in this medium for 30 min at +4 °C with anti-syndecan-2 goat Ab (10 μg/ml; goat IgG, specific for an epitope corresponding to the C-terminal domain of human syndecan-2; Santa Cruz Biotechnology) or its isotype. After washing, MDM were incubated for 30 min at 4 °C in 300-μl PBS–BSA supplemented with FITC-labeled goat anti-mouse or mouse anti-goat Ig antibodies (1/20; Pharmingen), fixed in 1% paraformaldehyde (PFA) (Sigma-Aldrich) in PBS (PBS–PFA), scrapped and analyzed by flow cytometry.

2.3. Immunofluorescence staining and microscope analysis of the cells

Adherent MDM, cultured on glass coverslips (at about 5×10^5 MDM per well), were incubated for 1 h at room temperature in 500-μl PBS supplemented with anti-syndecan-1 mAb DL-101 (10 μg/ml), anti-syndecan-4 mAb 5G9 (10 μg/ml), anti-betaglycan Ab (10 μg/ml) or their isotypes (10 μg/ml). Cells were then incubated for 30 min at room temperature, in the darkness, with a Cy-3 conjugated donkey anti-mouse antibody (1:400; Jackson Immunoresearch, Laboratories, Inc., Baltimore, Pennsylvania, USA), or Alexa fluor 488-labeled donkey anti-goat antibody (Molecular Probes, Eugene, OR), fixed in PBS–PFA and mounted in fluorescent mounting medium (Dako, Glostrop, Denmark). In parallel, MDM were fixed with methanol, air-dried, rehydratated with PBS and incubated for 1 h at room temperature with anti-syndecan-2 goat Ab (10 μg/ml) or its isotype. Bound antibodies were revealed with Alexa Fluor 488 labeled-donkey anti-goat secondary antibody and observed using an Olympus fluorescence microscope.

2.4. RANTES binding to MDM

^{125}I-RANTES (81 TBq/mmol) was from Perkin Elmer Life Sciences (Boston, MA, USA). For displacement binding assays, aliquots of adherent MDM (all at 5×10^5), were cultured in 24-well plates (Falcon, Strasbourg, France). After 48-h incubation in serum-free medium and three washings with ice-cold binding buffer (PBS/0.1% BSA), cells were incubated for 2 h at 4 °C in 0.3-ml binding buffer containing ^{125}I-RANTES (7.5 pM; Perkin Elmer) in the presence or absence of unlabeled RANTES (up to 50 nM). Incubation was terminated by removing the medium and washing the cells. After cell lysis in 5% NaOH, bound radioactivity was measured using a γ-counter (LKB 1261 Multigamma). Data were analyzed by fitting to a logistic curve or according to Scatchard. Results are means ± S.E. of three independent assays, each performed in triplicate. Alternatively, ^{125}I-RANTES was incubated for 1 h at 37 °C

with heparin-albumin, albumin or dextran (up to 330 μg/ml; Sigma-Aldrich) and the mixture was added to the cells.

2.5. RANTES binding to ligands or receptors

To collect RANTES ligands, scraped MDM (2×10^7) were incubated in parallel for 2 h at +4 °C with or without RANTES (at 2.5 μg in 500 μl of PBS). Alternatively, MDM were preincubated for 2 h at 37 °C with heparitinase I (0.1 U/ml), heparitinase III (0.5 U/ml) and chondroitinase ABC (0.2 U/ml) (all from Sigma) mixture. In other experiments, MDM (2×10^7) were subsequently incubated in 500 μl of PBS with RANTES (2 μg), and after washing with anti-RANTES mAb in 500 μl of PBS (2 μg; mouse IgG1; clone 21445.1; R&D systems), for 2 h at +4 °C. MDM were then lysed at 4 °C in 500-μl buffer (150 mM NaCl, 20 mM Tris/HCl, pH 8.2, supplemented with 1% Brij 97 and 10 mM PMSF, 5 mM iodoacetamide, 25 mM phenanthrolin, 20 μg/ml aprotinin, all from Sigma-Aldrich). Lysates were cleared by centrifugation at 1000 × *g* for 30 min at 4 °C. Immunocomplexes were collected in the presence of 10 mM dithiothreitol (DTT) (Sigma-Aldrich), by incubation for 18 h at 4 °C with 100 μl of protein G-Sepharose beads (Pharmacia, Paris, France), precoated [30–32] or not with anti-RANTES mAb or its isotype (each at 2.5 μg). Weak reducing conditions during the collection of the immunocomplexes were used to eliminate cross-reactivity with nonspecific proteins [33]. In parallel, the same experiments were also performed with K562 cells.

Alternatively, the MDM were incubated with RANTES, as just described, and lysed in the presence of Brij 97. Aliquots of MDM lysates were then incubated with Protein G-Sepharose beads coupled either with anti-syndecan-1 mAb DL-101, anti-syndecan-4 mAb 5G9, anti-syndecan-2 or anti-betaglycan Abs (each at 2 μg). As negative control, the MDM were incubated in RANTES-free buffer. Immunocomplexes were then collected as just described.

To release bound ligands, beads were boiled for 10 min with 120 μl of 2× SDS-PAGE sample buffer and centrifuged (400 × *g*; 5 min; 15 °C). Lysates or eluted proteins were submitted to SDS-PAGE (12% polyacrylamide) under non-reducing conditions and transferred onto Immobilon strips which were then saturated for 18 h at 37 °C with PBS or Tris-buffered saline (TBS) supplemented with 5% BSA

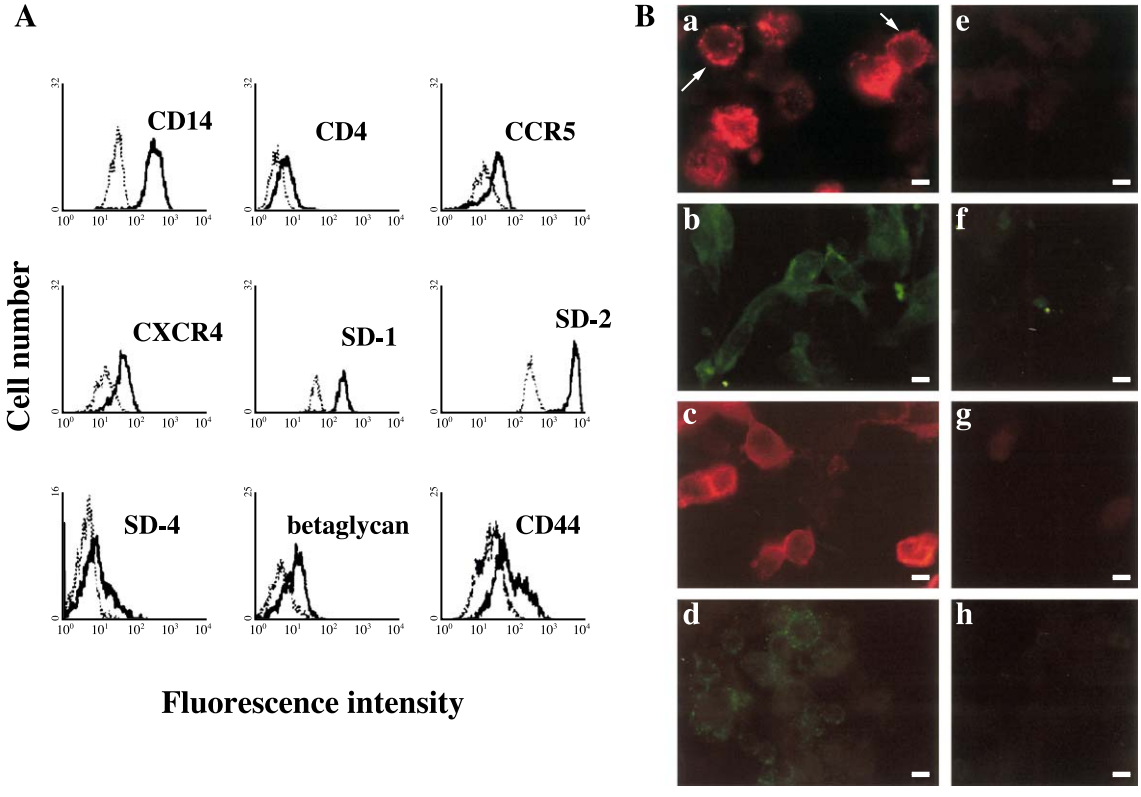

Fig. 1. Immunofluorescence analysis of MDM. (A) MDM membrane expression of CD14, CD4, CCR5, CXCR4, syndecan-1 (SD-1), syndecan-2 (SD-2), syndecan-4 (SD-4), betaglycan and CD44 by FACScan analysis. MDM were stained for FACS analysis, as described in Materials and methods, with anti-CD14 mAb, anti-CD4 mAb Q4120, anti-CCR5 mAb 2D7, anti-CXCR4 mAb 12G5, anti-syndecan-1 mAb DL-101, anti-syndecan-2 Ab, anti-syndecan-4 mAb 5G9, anti-betaglycan Ab or anti-CD44 mAb (thick lines). Reactivity was compared to an isotype-matched control monoclonal or polyclonal Ab (dotted lines). Data are representative of three to eight individual experiments. (B) Immunofluorescence microscopic analysis of syndecan-1, syndecan-2, syndecan-4 and betaglycan from MDM. Adherent MDM were immunostained, as described in Materials and methods, with anti-syndecan-1 mAb, DL-101 (a), goat anti-syndecan-2 Ab (b), anti-syndecan-4 mAb 5G9 (c), goat anti-betaglycan Ab (d), or with their isotypes, mouse IgG1 (e), mouse IgG2a (g) or goat IgG (f, h). Data are representative of three individual experiments. Bar: 5 μm.

(w/v). Excess BSA was washed out with PBS or TBS supplemented with 0.5% BSA and 0.2% Tween 20 (v/v) (Sigma-Aldrich). Strips were incubated for 1 h at room temperature with anti-CCR5 2D7 or 3A9 (Pharmingen), anti-syndecan-1 DL-101, anti-syndecan-2, anti-syndecan-4 5G9, anti-betaglycan, anti-CD44, anti-HS 10E4 (mouse IgM; Seikagaku) or 3G10 (mouse IgG2b; Seikagaku), anti-CXCR4 12G5 or G19 antibodies (G19 is a goat polyclonal antibody from Santa Cruz Biotechnology, raised against a peptide GYQKKLRSMTDKYRLHLSV, mimicking according to Brelot et al. [34] the first extracellular domain of CXCR4), or anti-RANTES mAb or polyclonal Abs (R&D systems) or their isotypes (all at 1/1000–1/5000). After three washes, strips were incubated with HRP-labeled anti-mouse or anti-goat IgG (at 1/5000-1/20000). After washing, strips were revealed by enhanced chemilumiscence reagent (Amersham Pharmacia Biotech, UK, or Supersignal West Dura Extended, Pierce). In parallel, in some experiments, lysates were submitted to the glycosaminidases mixture and then treated as just described.

3. Results and discussion

3.1. Immunofluorescence labeling of MDM

MDM expressed CD14, and low levels of CD4, CCR5, CXCR4 and CCR1, as assessed by indirect immunofluorescence assay and FACS analysis (Fig. 1A and data not shown). They also expressed PGs belonging to the syndecan family, syndecan-1, syndecan-2, syndecan-4 besides other PGs such as betaglycan and CD44 (Fig. 1A and B). Syndecan-1, syndecan-2, syndecan-4 and betaglycan decorated their plasma membrane, as revealed by microscopic analysis (Fig. 1B).

3.2. Specific binding of RANTES to MDM

We have previously reported that RANTES binding to MDM is inhibited by soluble heparin or chondroitin sulfate (respectively 42% and 30% inhibition), by anti CCR5 mAb 2D7 (47%) and by heparitinase, chondroitinase or neuraminidase treatment of these cells (22%, 25% and 48%) [23]. Therefore, this binding involves CCR5, and negatively charged glycans or GAGs. In the present study, we observe that ^{125}I-RANTES (7.5 pM) binds in a dose-dependent and saturable manner to MDM; this binding is significantly inhibited by increasing concentrations of unlabeled RANTES (Fig. 2A). Minimum ^{125}I-RANTES binding (B/T) to MDM, determined in the presence of 10.4 nM unlabeled RANTES, was 13 ± 0.9%. Scatchard analysis of the displacement curve (Fig. 2B), from three independent assays of specific ^{125}I-RANTES (7.5 pM) binding to MDM obtained from three different blood donors, in the presence of increasing concentrations of unlabeled RANTES (up to 10.4 nM), has revealed two classes of specific binding sites:

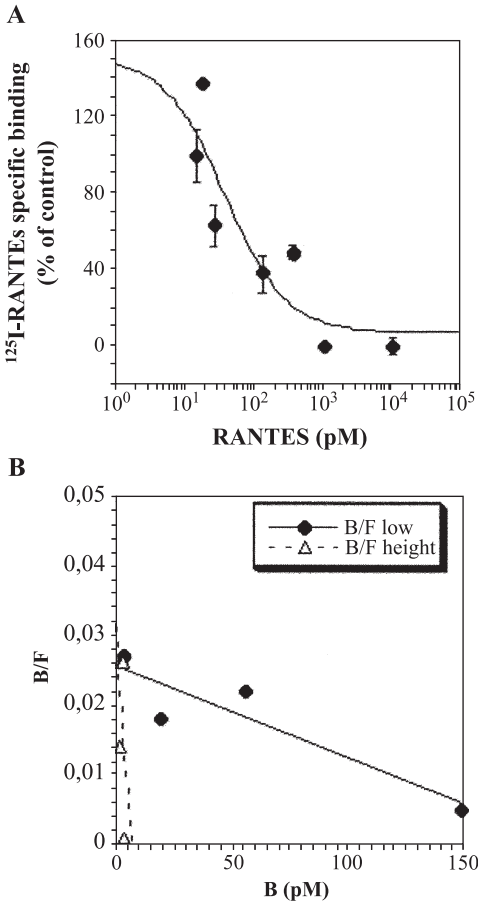

Fig. 2. Binding of RANTES to MDM. Binding (A) and Scatchard (B) plots were obtained by incubating unlabeled RANTES, at the indicated concentrations, and ^{125}I-RANTES, at 7.5 pM, with MDM (A, B) for 2 h at 4 °C. Results are the means ± S.E. (bars) of three separate experiments performed in triplicate.

one with 3600 ± 1560 binding sites per cell and a 0.260 ± 0.1 nM K_d and the other with 90 300 ± 26,000 binding sites per cell and a 11.2 ± 3 nM K_d (Fig. 2B). Moreover, ^{125}I-RANTES binding to MDM was inhibited by 330 μg/ml heparin-albumin (by 43 ± 12% inhibition: $n=4$; $P<0.05$), while albumin or dextran had no effect. Therefore, RANTES specifically binds to high and low affinity binding sites of MDM, and heparin presented on a protein carrier inhibits this binding. In these experiments, because RANTES aggregates at high micromolar concentrations, low nanomolar concentrations of unlabeled RANTES were used for the displacement binding assays. In these conditions, it cannot be excluded that higher concentrations on non-aggregated cold ligand may be needed to further displace the binding of ^{125}I-RANTES.

3.3. Binding of PGs to the complexes formed between RANTES and GPCRs on MDM

To determine whether RANTES binds to PGs expressed on the plasma membrane of MDM, different procedures

were used in order to collect the proteins interacting with RANTES: after incubation with RANTES and subsequently with anti-RANTES antibodies, the MDM were lysed and the immunocomplexes were collected on protein-G beads; alternatively, the MDM were lysed just after their incubation with RANTES and the RANTES interacting proteins were collected on anti-RANTES coated beads. Similar data were obtained in both experiments. Our previous studies [31,32] have demonstrated, in accordance with others [35], that the presence of the detergent BRIJ-97 in the cell solubilization buffer did not modify the molecular interactions which occur during the formation of multimolecular complexes between a ligand and its targets, expressed on the plasma membranes of living cells. Therefore, in the present study the cells were lysed in the presence of this detergent.

Western blot analysis of immunoprecipitates revealed 46–48-kDa proteins, immunoreactive with anti-CCR5 2D7 or 3A9 mAbs and anti-CCR1 mAb, but neither with anti-CXCR4 mAb 12G5 nor with anti-CXCR4 Ab G19 (Fig. 3, lanes 1, 12 and 13, and data not shown). Further analysis of these immunoprecipitates revealed mainly 40–60-kDa proteins and proteins migrating as smears of apparent molecular masses >250 kDa, all immunoreactive with anti-syndecan-4 5G9, and 50–52-kDa proteins immunoreactive with anti-syndecan-1 mAb DL-101. Moreover, proteins of 110 kDa, characterized by their immunoreactivity with anti-CD44 mAb, were also detected. In addition, 40–60-kDa proteins, 110-kDa proteins and proteins migrating as smears of >250 kDa were also immunoreactive with anti-HS 10E4 mAbs (Fig. 3, lanes 3, 5, 8, and 10). As it has been

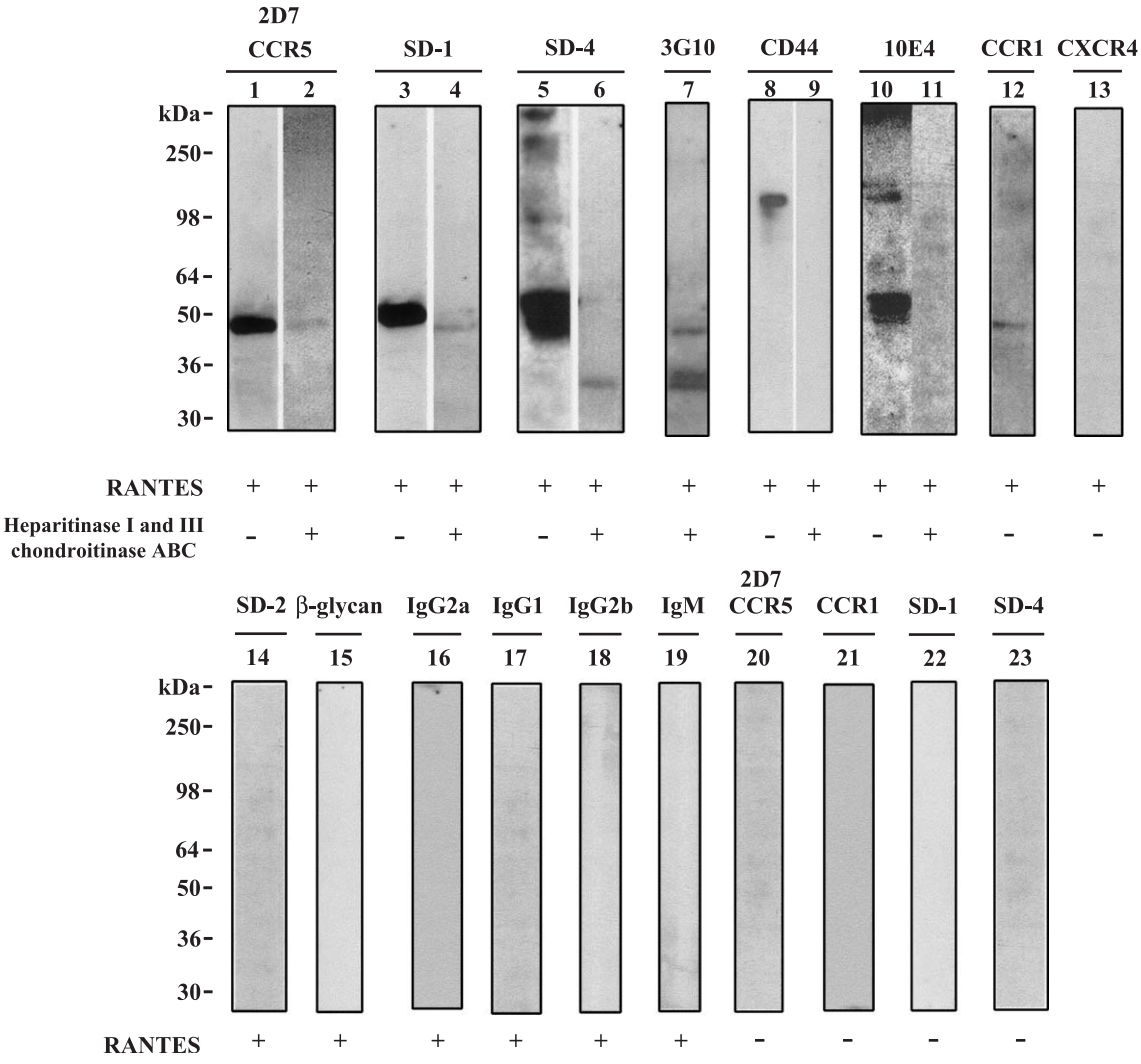

Fig. 3. Binding of syndecan-1 and syndecan-4 to the complexes formed by RANTES and GPCRs at the plasma membrane of MDM. MDM were pretreated (lanes 2, 4, 6, 7, 9, and 11) or not (lanes 1, 3, 5, 8, 10, and 12–23) with heparitinases I, III and chondroitinase ABC, and sequentially incubated (lanes 1–19) or not (lanes 20–23) with RANTES and then with anti-RANTES antibodies. Cells were lysed. Immunocomplexes were collected on protein-G beads, separated on SDS-12% PAGE gels, electroblotted onto Immobilon strips and revealed using anti-CCR5 2D7 (lanes 1, 2, and 20), anti-syndecan-1 DL-101 (lanes 3, 4, and 22), anti-syndecan-4 5G9 (lanes 5, 6, and 23), anti-CD44 (lanes 8 and 9), anti-heparan sulfate 10E4 (lanes 10 and 11), anti-CCR1 (lanes 12 and 21), anti-CXCR4 12G5 (13) mAbs, anti-syndecan-2 (lane 14), or anti-betaglycan (lane 15), Abs or their isotypes, IgG2a, IgG1, IgM or IgG2b (lanes 16–19). Data shown represent one of three individual experiments, using the MDM from different blood donors.

previously reported that mAb 10E4 reacts with an epitope that occurs in native heparan sulfate chains and is destroyed by N-desulfatation of the GAG [36], these data suggest that the PGs bound to RANTES on MDM have HS chains.

No immunoreactivity with anti-syndecan-2, anti-betaglycan Abs nor with the isotypes was detected. No immunoreactivity was observed with all tested antibodies when the MDM were incubated, as negative controls, in RANTES-free buffer (Fig. 3, lanes 14–23).

In the same conditions, we did not detect any RANTES ligands from the K562 cells (data not shown). As it has been previously reported that K562 cells express betaglycan and syndecan-3 [29], these data further rule out RANTES binding to betaglycan.

Alternatively, we found that RANTES can be co-immunoprecipitated with syndecan-1 or syndecan-4 but neither with syndecan-2 nor with betaglycan (data not shown). This further argues for the occurrence of some selectivity in RANTES binding to PGs expressed on MDM.

Taken together, these data indicate that beside its specific GPCRs, CCR1 and CCR5, RANTES binds PGs belonging to the syndecan family, syndecan-4, syndecan-1 and also other PGs such as CD44 expressed on MDM. Therefore, syndecan-1, syndecan-4 and CD44 molecules, but neither syndecan-2 nor betaglycan, are associated to the complex formed between RANTES and GPCRs on MDM.

In parallel, Western blot analysis of MDM lysates, prepared in the presence of BRIJ 97, revealed the presence of proteins migrating as smears: one characterized by apparent molecular masses ranging from about 40 to 80 kDa, and immunoreactive with anti-syndecan-1 mAb DL-101, and the other by masses ranging from 34 to 60 kDa, immunoreactive with anti-syndecan-4 mAb 5G9 (Fig. 4, lanes 1 and 4). No immunoreactivity with the respective isotypes was detected (Fig. 4, lanes 3 and 6). GAGs removal from the PGs, by glycosaminidase treatment of these lysates, induced a shift of the apparent molecular masses of these PGs, to 45 kDa for the PGs immunoreactive with the anti-syndecan-1 mAb DL-101, and to 31–33 kDa for those immunoreactive with anti-syndecan-4 mAb 5G9 (Fig. 4, lane 2 versus 1 and 5 versus 4).

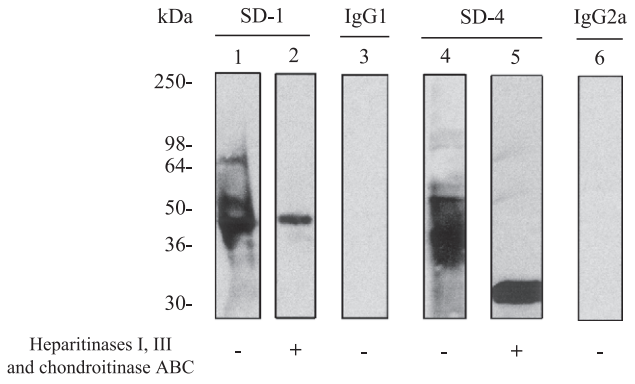

Fig. 4. Immunoblot analysis of syndecan-1 and syndecan-4 from MDM lysates MDM lysates, treated (lanes 2 and 5) or not (lanes 1, 3, 4 and 6) with heparitinases I, III and chondroitinase ABC, were submitted to SDS-PAGE, electroblotted and revealed with anti-syndecan-1 mAb DL 101 (lanes 1 and 2), anti-syndecan-4 mAb 5G9 (lanes 4 and 5) or their isotypes, mouse IgG1 (lane 3) or IgG2a (lane 6). Data shown are representative of three individual experiments, using the MDM from different blood donors.

Taken together, these data indicate that some of the syndecan-4 molecules bound by RANTES were further oligomerized in the presence of the chemokine, and suggest that the syndecan-1 and syndecan-4 molecules bound by the chemokine are glycanated.

3.4. GAGs dependence of RANTES binding to syndecan-1 and -4

If the MDM were pre-treated with heparitinases I and III, and chondroitinase ABC mixture, a decrease in the immunoreactivity of the RANTES-bound material with anti-syndecan-1 DL-101, anti-syndecan-4 5G9 and anti-CCR5 2D7 mAbs was observed (Fig. 3, lane 4 versus 3, lane 6 versus 5 and lane 2 versus 1). This suggests that GAGs removal from the cells by glycosaminidases treatment has decreased the binding of RANTES to syndecan-1, syndecan-4 and CCR5. Moreover, we also observed in these conditions a shift of the respective apparent molecular masses of the PGs bound by RANTES, from 50–52 kDa to 45 kDa for RANTES bound syndecan-1, and from more than 250 and 40–60 kDa, respectively, to 33 kDa for RANTES bound syndecan-4 (Fig. 3, lane 4 versus 3 and lane 6 versus 5).

The 45-kDa proteins were immunoreactive not only with the anti-syndecan-1 mAb DL-101 but also with the anti-stub 3G10 mAb, while the 33-kDa proteins were immunoreactive not only with anti-syndecan-4 mAb 5G9 but also with 3G10 mAb (Fig. 3, lanes 4, 6 and 7). Moreover, in these conditions, no RANTES bound CD44 was detected, which demonstrates that RANTES binding to CD44 expressed on MDM is GAGs-dependent (Fig. 3, lane 9).

These data indicate that GAGs-dependent binding of RANTES to syndecan-1, syndecan-4 and CD44 may facilitate RANTES subsequent interaction with CCR5 on MDM characterized by low membrane expression of CCR5. However, these data do not rule out that subsequent interactions of RANTES with the respective core proteins of syndecan-1 or syndecan-4 may also occur.

4. Conclusion

Syndecans are HSPGs involved in specific binding of growth factors and growth factors receptors. For instance, it has been previously demonstrated that syndecan-2, expressed on human MDM, selectively binds the macrophage-derived growth factors, fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor and heparin-binding epidermal growth factor (hbEGF), but not chemokines, such as MIP-1α, MIP-1β, monocyte chemoattractant

protein-1 (MCP-1), interleukin-8 (IL-8) or RANTES [37]. MDM express high levels of syndecan-1 and syndecan-4 and low levels of syndecan-3 [29]. In addition, MDM also express betaglycan, CD44 but not glypican-1 [29,38,39]. We have investigated here whether besides its GPCRs, RANTES uses HSPGs belonging to the syndecan family, to bind to human primary macrophages. We have confirmed by specific immunostaining the MDM membrane expression of syndecan-1, syndecan-2, syndecan-4, betaglycan and CD44. We have also observed low membrane MDM expression of CXR4, CCR5 and CCR1 [14,15,17,40]. We have previously reported that RANTES binding to MDM is inhibited by anti-CCR5 mAb 2D7 and by heparitinase pretreatment of the cells [23], suggesting the role of both HSPGs and GPCRs. To further investigate the role of these molecules in RANTES binding, we have analyzed here the binding characteristics of RANTES to human primary macrophages. Few studies have reported displacement binding assays to cells, of iodine-labeled RANTES by unlabeled RANTES [41]. Here, we show that RANTES specifically binds to MDM, in a dose-dependent and saturable manner with two classes of binding sites, high affinity ones characterized by a 0.26 nM K_d, and low affinity ones characterized by a 11.2 nM K_d. These K_d values are in agreement with those previously reported respectively for the binding of β-chemokines to CCR5 [42] or of RANTES to heparin [24]. Exogenous heparin-albumin also inhibits this binding. Because RANTES aggregates at high micromolar concentrations, low nanomolar concentrations of unlabeled RANTES were used for the displacement binding assays. Therefore, it cannot be excluded that higher concentrations of cold non-aggregated RANTES would be needed to further displace the binding of ^{125}I-RANTES.

The biological activity of chemokines has been shown to be influenced by their association with GAGs [37,41]. For example, the presence of GAGs attached to the cell surface has been reported to increase the binding affinity of RANTES to CCR1 and others GPCRs [41]. Enzymatic removal of GAGs from lymphocytes was reported to abrogate the ability of RANTES to elicit an intracellular Ca^{2+} signal [43]. However, in another work, no effect of cell surface GAGs was found on chemokine binding [24]. It was also demonstrated that removal of GAGs from the surface of a PM1 T cell line or from MDM reduces the antiviral effect of RANTES [15,28]. Moreover, enzymatic removal of GAGs from the surface of CCR5 expressing CHO cells by different glycosidases did not result in a reduction of the ability of RANTES to bind CCR5 or to induce a functional response [24]. Therefore, studies on the role of GAGs on RANTES binding and function are often conflicting.

Our data show that RANTES, added to the plasma membrane of intact MDM, induces the formation of complexes which comprise glycanated syndecans, syndecan-1 and syndecan-4, and also another PG, CD44, besides the specific RANTES GPCRs, CCR5 and CCR1, but neither syndecan-2 nor betaglycan. RANTES binding to CD44 expressed on HeLa cells has been recently demonstrated [38]. In the present study, pretreatment of the MDM with glycosaminidases decreases the binding of RANTES to syndecan-1, syndecan-4, and CCR5. Such pretreatment also abolishes the binding of RANTES to CD44. This indicates that GAGs-dependent binding of RANTES to syndecan-1, syndecan-4 and CD44 facilitates the subsequent interaction of this chemokine with CCR5 on MDM, which are characterized here by a low membrane expression of CCR5.

In the present study, we have not detected any RANTES GPCRs, syndecan-1, syndecan-2 or syndecan-4 molecules, neither on the plasma membrane of the human K562 leukemia cells nor in their lysates. Moreover, no RANTES ligands were detected on these cells. As it has been previously reported that K562 cells express betaglycan and syndecan-3 [29], these data further rule out RANTES binding to betaglycan. However, whether RANTES also binds to PGs belonging to the glypican family has to be investigated. The reason why such interactions of RANTES with syndecan-1, syndecan-4 and CD44 but neither with syndecan-2 nor betaglycan occurs has now to be investigated. Whether this may be related with differences in GAG composition (HS versus CS) of the PGs and/or degree of GAG side chains or higher sulfatation deserves further study.

In conclusion, the present data show that besides CD44, PGs belonging to the syndecan family, syndecan-1 and syndecan-4, but neither syndecan-2 nor betaglycan, serve as ligands for RANTES on human primary macrophages. The GAGs dependence of RANTES binding to syndecan-1 and syndecan-4 does not rule out that additional subsequent protein–protein interactions may take place. Whether these molecules belonging to the syndecan family also serve as signaling receptors involved in specific functions remains to be investigated. For instance, syndecan-4 possesses a phosphoinositol 4,5-biphosphate binding site in its cytoplasmic tail that allows it to bind and activate protein kinase Cα [44,45]. The role of the respective associations of RANTES with syndecan-1 and syndecan-4 in signal transduction, in the pathophysiology of chemokines or GPCRs, and in HIV infection deserves further study.

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