Chondroitin sulfate A released from platelets blocks RANTES presentation on cell surfaces and RANTES-dependent firm adhesion of leukocytes

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The sequestration of chemokines on the surface of microvascular endothelium is an early event in the selective recruitment of leukocytes. The sequestration and presentation of chemokines must be tightly controlled to confine the extravasation of leukocytes and to prevent uncontrolled inflammation. We investigated whether soluble molecules released under physiological conditions could control chemokine immobilization on cell surfaces and function as regulatory chemokine binding molecules. We determined that human serum contains a molecule that suppresses RANTES (CCL5) binding to endothelial cells, PBMC and CHO cells. Using platelet-rich and platelet-free plasma, serum from patients with thrombocytopenia, and purified platelets, we identified platelets as the source of the chemokine-binding molecule and further identified it as chondroitin sulfate A. In contrast to platelet-derived fully-sulfated chondroitin sulfate A, low-sulfated chondroitin sulfate A present in plasma was almost inactive. Under physiological flow conditions chondroitin sulfate A was found to block RANTES-mediated firm adhesion of monocytes to endothelial cells. It also prevented RANTES-mediated influx of calcium in CCR5-transfected CHO cells while internalization of CCR5 was only marginally reduced. Taken together, chondroitin sulfate A released from platelets appears to act as an important regulatory molecule for cellular responses to chemokines.

Key words: Platelet / Glycosaminoglycan / Chemokine / Inflammation

1 Introduction

Chemokines play an important role in the specific and site-directed recruitment of leukocytes. The process of leukocyte emigration can be divided into a series of steps: rolling of leukocytes on the endothelium, firm adhesion, spreading, diapedesis and migration within the tissue [1]. Chemokines are critically involved at several levels. First, they induce firm adhesion to the endothelium through activation of integrins on rolling leukocytes [2]. Secondly, a chemokine gradient mediates diapedesis and extravasation of leukocytes and directs their migration within the tissue. It has been suggested that more than one chemokine and/or chemokine receptor is involved in these steps. For example the interaction of RANTES (CCL5) with CCR1 is responsible for the induced firm adhesion of monocytes, while CCR5 contributes to leukocyte spreading [3].

Received 30/11/01
Accepted 16/1/02

The induced firm adhesion of rolling leukocytes requires the sequestration and presentation of chemokines on the luminal surface of blood vessels [4–6]. Unbound chemokines would be rapidly diluted out in the blood stream. Chemokines are presented on the cell surface by membrane bound glycosaminoglycans [4, 7–9]. The expression of glycosaminoglycans on endothelial cells is under the control of pro-inflammatory cytokines. CD44, an anchor for hyaluronan [10], is up-regulated by IL-15, TNF-α and IL-1β [11, 12]. In patients with rheumatoid arthritis heparan sulfate proteoglycan is highly expressed on the endothelium [13]. Apart from cell surfaces [14], glycosaminoglycans are abundantly present in the extracellular matrix [15]. Thereby glycosaminoglycans may allow the formation of haptotactic gradients of immobilized chemokines that direct the migration of leukocytes within tissue.

The immobilization of chemokines also contributes to the inhibition of HIV by RANTES, as the enzymatic removal of surface glycosaminoglycans reduces the ability of RANTES to suppress HIV [16]. Surface-bound heparin sulfate is the binding partner of RANTES on PM1
cells, a human T cell line, while chondroitin sulfate binds RANTES on monocytes. The immobilization of chemokines by surface glycosaminoglycans is thought to increase the local concentration of chemokines and thereby enhance their interaction with the specific chemokine receptors [7, 17, 18].

Binding of RANTES and other chemokines to the cell surface can be inhibited by preincubation with soluble glycosaminoglycans, indicating that soluble glycosaminoglycans compete with cell bound glycosaminoglycans for binding to chemokines [4, 8, 19–21]. In the case of RANTES the order of binding was heparin > heparin sulfate > chondroitin sulfate > dermatan sulfate [19]. By site-directed mutagenesis and N-terminal truncations of RANTES and MIP-1α the interaction sites with glycosaminoglycans were mapped [22–24]. In addition, soluble glycosaminoglycans may also influence the interaction of chemokines with their specific receptors. Soluble glycosaminoglycans inhibit binding of IL-8 (CXCL8) to CXCR1 and CXCR2 and binding of MIP-1α (CCL3) to CCR1 [19]. Interestingly, heparin has been shown to block the influx of calcium in CCR5-positive cells stimulated with RANTES, but does not block binding of RANTES to CCR5 [25]. Moreover, complexes of RANTES and heparin inhibit HIV infection more efficiently than RANTES alone [25, 26]. In addition to glycosaminoglycans, the viral chemokine-binding protein M-T1 interrupts the binding of CC chemokines with their cognate chemokine receptors and modulates the interaction of chemokines with glycosaminoglycans [27].

Sequestration of chemokines on the surface of microvascular endothelium is an early event in the selective recruitment of leukocytes. We hypothesized that an in vivo control mechanism may help to regulate this critical stage in leukocyte emigration. An excessive sequestration of chemokines could enhance tissue damage or cause unwanted recruitment. We investigated whether soluble chemokine-binding molecules released under physiological conditions may add an additional level of regulation by buffering chemokine binding to cell surfaces and designed a series of experiments to study the relevance of such molecules.

2 Results

2.1 RANTES binding to the surface of endothelial cells, PBMC and CHO cells

Using FACS analysis and monoclonal antibodies directed against human RANTES we demonstrated that RANTES can bind to the surface of several cell types including human microvascular endothelial cells, PBMC and CHO cells (Fig. 1). The cells were incubated on ice with various concentrations of RANTES and then stained with a monoclonal antibody specific for RANTES (VL-1). The binding of RANTES was detectable at concentrations below 1 μg/ml. Comparing four different monoclonal antibodies against RANTES (VL-1, VL-2, VL-3, VL-4) we obtained the following results: VL-1 = VL-2 ≥ VL-3 = VL-4. Interestingly, the VL-3 and VL-4 antibodies pre-incubated with RANTES prevented binding of RANTES to cell surfaces, while pre-incubation with VL-1 or VL-2 had no such influence, suggesting that VL-3 and VL-4 recognize glycosaminoglycan binding epitopes (Fig. 2).

The efficient binding of RANTES to cell surfaces prompted us to look for natural mediators that could regulate RANTES binding to cell surfaces under physiological conditions. Human plasma or serum appeared as promising candidates as chemokines are produced during inflammation and may be released into the circulation.

2.2 Human serum prevents binding of RANTES to cell surfaces

To identify potential binding molecules for RANTES, we pre-incubated RANTES protein with human serum and then analyzed binding of RANTES to CHO cells. As shown in Fig. 3 a pre-incubation of RANTES (3.3 μg/ml) with serum reduced the mean value of cell bound RANTES from 478 to 19. Similar results were obtained for endothelial cells and PBMC (data not shown). Serum was diluted with PBS to estimate its capacity to prevent the surface binding of RANTES. The binding of RANTES (10 μg/ml) was reduced by 97%, 54% and 31% when
The monoclonal antibodies VL-3 and VL-4 prevent the immobilization of RANTES. RANTES (3 μg/ml) was pre-incubated for 30 min with the RANTES antibodies VL-1 to VL-4 (20 μg/ml) or medium as control. RANTES binding to CHO cells was measured by flow cytometry using the antibody VL-1. One out of three representative experiments is shown.

In the fraction of serum was of 90%, 30% and 10%, respectively (data not shown).

To exclude the possibility that serum components could prevent detection of cell-bound RANTES with the antibody VL-1, CHO cells were first loaded with RANTES and then incubated with serum. When serum was added after binding of RANTES, it did not prevent detection of RANTES by VL-1. We also excluded the possibility that molecules in the serum (e.g. chemokines other than RANTES) compete with RANTES for binding to cell surfaces. For that purpose the cells were pre-incubated with serum, washed and then loaded with RANTES. Pre-incubation of CHO cells with serum did not prevent the subsequent binding of RANTES (data not shown).

2.3 Identification of the RANTES-binding molecule as chondroitin sulfate A released from platelets

The first suggestion as to the source of the RANTES-binding molecule came from the observation that plasma anti-coagulated with EDTA did not prevent binding of RANTES (Fig. 3 b). We therefore investigated whether the RANTES-binding molecule could be generated by coagulation of EDTA-plasma with CaCl₂. To distinguish between molecules generated by the coagulation cascade, and molecules released from platelets, we removed the platelets from the plasma by centrifugation for 15 min at 2000 × g. After coagulation with CaCl₂, only the platelet-rich plasma, but not by the platelet-free plasma was able to prevent RANTES binding to CHO cells indicating that the RANTES binding molecule is released from platelets (Fig. 4 a). We also examined sera from patients with thrombocytopenia (platelets below 50,000/μl). These sera show a significantly lower capacity to block RANTES-binding to CHO cells than sera from individuals with platelet counts above 250,000/μl (Fig. 4 b).

To directly demonstrate that platelets release the RANTES-binding molecule, we purified platelets from healthy donors and activated them (1 × 10⁶/μl) with thrombin or collagen. The cell-free supernatant of non-activated platelets, and control medium containing thrombin or collagen, were unable to prevent RANTES binding, while the supernatant of activated platelets almost completely inhibited binding of RANTES (10 μg/ml) to cell surfaces (Fig. 5). When the supernatant of activated platelets was diluted to 30%, 10% and 3% corresponding to platelet counts of 300,000/μl (comparable to serum), 100,000/μl and 33,000/μl, the inhibition was 97%, 54% and 30%, respectively (data not shown). This shows that activated purified platelets have the same capacity as serum to prevent RANTES binding. We could demonstrate that the platelet-derived RANTES-binding molecule has a molecular weight above 100 kDa and readily binds to DEAE dextran at pH...
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Fig. 4. Activation of platelets is required for the ability of serum to block RANTES binding. (a) Platelet-rich plasma generated by centrifugation of EDTA blood at 200 × g for 15 min and platelet-free plasma generated by further centrifugation at 2,000 × g for 15 min were brought to coagulation by addition of 5 mM CaCl₂. Pre-incubation of RANTES (10⁻⁶ g/ml) with coagulated platelet-rich but not platelet-free plasma prevents the immobilization on cell surfaces. One out of three representative experiments is shown. (b) Sera from patients with thrombocytopenia (platelet count given as × 10³/µl) have a reduced ability to prevent the immobilization of RANTES.

7.0 (data not shown). We therefore assumed that negatively charged macromolecules like glycosaminoglycans are the responsible factors. As platelets are known to release chondroitin sulfate A after activation [28], we examined if chondroitin sulfate A might be the responsible factor and digested the supernatant of activated platelets with chondroinitase ABC [29] or heparinase I as control (Fig. 6). Following digestion with chondroinitase the ability of the platelet supernatant to prevent binding of RANTES was completely abolished, while digestion with heparinase had no influence. These results indicate that chondroitin sulfate A is the platelet-derived binding molecule that blocks RANTES binding.

2.4 Chondroitin sulfate A prevents the immobilization of RANTES on cell surfaces and blocks functional responses of RANTES

RANTES was preincubated for 1 h with various concentrations of chondroitin sulfate A and further incubated for 1 h with CHO cells. As shown in Fig. 7a, chondroitin sulfate A (Sigma) reduced binding of RANTES to CHO cells with an IC₅₀ of 4.1 µg/ml and achieved more than 90% suppression at 100 µg/ml. Chondroitin sulfate A (Sigma) also reduced the binding of RANTES to lymphocytes, monocytes and endothelial cells by almost two orders of magnitude (Fig. 7b). The binding of chemokines to cell surfaces is thought to result in higher local concentrations and may thereby increase functional responses via specific chemokine receptors. Using Ca-flux and internalization assays we studied the effect of chondroitin sulfate A on RANTES-mediated activation of CCR5. Pre-incubation of RANTES with chondroitin sulfate A inhibited the influx of calcium into CCR5-transfected CHO cells without affecting their response to ionomycin (Fig. 8). Interestingly, RANTES-induced internalization of CCR5 was only marginally influenced by chondroitin sul-
Fig. 7. Effects of chondroitin sulfate A on RANTES binding to cell surfaces. (a) Chondroitin sulfate A prevents the binding of RANTES (10 μg/ml) to CHO cells with an IC50 of 4.1 μg/ml. (b) Inhibition of RANTES binding to lymphocytes, monocytes, IL-1β activated and non-activated human microvascular endothelial cells (HMVEC) by pre-incubation of RANTES with 100 μg/ml chondroitin sulfate A. One out of two representative experiments is shown.

Fig. 8. Pre-incubation of RANTES with chondroitin sulfate A blocks RANTES-induced calcium influx into CCR5 transfected CHO cells. Cells were loaded with Fura-3AM and influx of calcium was induced with 1 μg/ml RANTES (black curve) or complexes of RANTES (1 μg/ml) and chondroitin sulfate A (100 μg/ml) (gray curve). Ionomycin was added at 200 s as positive control and MnCl₂ (5 mM) at 300 s to quench the fluorescence. One out of two representative experiments is shown.

Fig. 9. Chondroitin sulfate A prevents the RANTES mediated firm adhesion of MonoMac 6 cells on activated human microvascular endothelial cells. RANTES (0.5 μg/ml) was pre-incubated with chondroitin sulfate A (CSA) (100 μg/ml) and added to IL-1β activated endothelial cells. RANTES alone served as positive control while chondroitin sulfate A and medium alone served as negative control. One out of three representative experiments is shown.

3 Discussion

The presentation of chemokines on endothelial surfaces is essential for the selective recruitment of leukocytes. During inflammation, tissue damage or thrombosis, chemokines are produced and potentially released into the circulation. Uncontrolled accumulation of surface-bound chemokine could hinder the sensitivity of a required response, or even promote an overly aggressive inflam-
matory response. We hypothesized that endogenous chemokine binding molecules may act to remove chemokines from the circulation and function as chemokine “buffers”. Using RANTES as a probe, we demonstrate that chondroitin sulfate A released from platelets efficiently modulates binding of RANTES to endothelial cells, PBMC and CHO cells. Experiments with platelet-free, and platelet-rich plasma, serum from patients with thrombocytopenia, and purified platelets, showed that platelets are the source of RANTES-binding molecules in human serum. Upon activation with thrombin or collagen, platelets release a surplus of chondroitin sulfate A that not only blocks the surface binding of platelet-derived RANTES [30, but also efficiently prevents the immobilization of exogenous RANTES added in concentrations up to 10 μg/ml. When the supernatant of activated platelets was digested with chondroitinase ABC, the immobilization of RANTES could no longer be blocked, demonstrating that chondroitin sulfate is the responsible factor. Dilution of serum with PBS showed that RANTES presentation could be reduced by over 97% and 54% if the serum fraction was 90% and 30%, respectively. Almost indentical results were obtained with purified platelets used at a concentration of 300,000/μl and 100,000/μl. Okayama et al. [28] calculated the amount of chondroitin sulfate A released from platelets as approx. 1.5 μg hexuronate/ml under the assumption of 300,000 platelets/μl. They also describe that chondroitin sulfate A released from platelets is fully sulfated, while chondroitin sulfate A present in plasma (5.8 μg hexuronate/ml) is hardly sulfated. As plasma was unable to reduce RANTES binding, low-sulfated chondroitin-sulfate A has little activity to suppress the surface binding of RANTES. Commercially available chondroitin sulfate A (Sigma) blocked RANTES presentation with an IC_{50} or 4.1 μg/ml and is therefore almost tenfold less active than fully sulfated platelet-derived chondroitin sulfate A. It is conceivable that also under in vivo conditions at sites of tissue damage with accumulation and activation of platelets, sufficient concentrations of chondroitin sulfate A are released to affect the presentation of chemokines.

To investigate the influence of chondroitin sulfate A on RANTES-mediated firm adhesion of leukocytes we performed flow assays with monocytes on activated human microvascular endothelial cells. Chondroitin sulfate A efficiently reduced the RANTES-mediated adhesion of monocytes to control levels. The blockade of monocyte adhesion by chondroitin sulfate A could result from reduced presentation of RANTES on the endothelium or reduced activation of chemokine receptors. It has been shown that complexes of RANTES and glycosaminoglycans are able to bind CCR5 but do not elicit Ca flux [25]. Chondroitin sulfate A released from platelets may therefore have a dual effect by inhibiting the presentation of chemokines, and by altering the interactions of chemokines with their specific receptors. We therefore examined the influence of chondroitin sulfate A on RANTES induced calcium influx and internalization of CCR5 and found that chondroitin sulfate A reduces calcium influx but only marginally reduces the down-modulation of CCR5. Interestingly, in patients with thrombocytopenia a low number of platelets correlates with a low capacity of serum to block the presentation of RANTES. One could speculate that thrombocytopenia observed in patients with certain connective tissue disorders may contribute to the severity of the autoimmune disease.

The immobilization of chemokines on cell surfaces occurs primarily via glycosaminoglycans expressed on the cell surface, as the enzymatic removal of glycosaminoglycans reduces chemokine binding [7, 16]. Soluble glycosaminoglycans would compete with cell-bound glycosaminoglycans for the binding of chemokines. We demonstrated that chondroitin sulfate A directly binds to RANTES and excluded the possibility that serum or supernatant from activated platelets contain factors (e.g. other chemokines) that compete with the binding of RANTES to cell surfaces. Glycosaminoglycans are found ubiquitously at the cell surface [14] and in the extracellular matrix [15]. Accordingly, we found that under in vitro conditions RANTES binds to a large variety of cells including lymphocytes, monocytes, CHO cells and endothelial cells. However, in vivo, the immobilization of RANTES may be much more restricted as the majority of RANTES will be present in a complex with glycosaminoglycans. Platelets will release RANTES from α-granules together with chondroitin sulfate A and a similar observation has been described for T cells [26]. The complex of RANTES and chondroitin sulfate A is not able to bind to surface glycosaminoglycans but may still be specifically immobilized by receptors for chondroitin sulfate A. At least two receptors for chondroitin sulfate A have been described to date. These include the v6 and v7 isoforms of the glycoprotein CD44 [31] and the macrophage mannose receptor [32] that can both be expressed on endothelial cells. Expression of CD44 and its splice variants is under the control of inflammatory cytokines, such as IL-1α, IL-15 and TNF-α [11, 12, 33, 34]. The expression of receptors for glycosaminoglycans on endothelial cells may allow the specific binding of complexes consisting of chemokines and chondroitin sulfate A and contribute to the localization of chemokines at sites of inflammation. The release of chondroitin sulfate A appears as important mechanism to control the recruitment of leukocytes at a very early stage.
4 Materials and methods

4.1 Detection of surface-bound RANTES by flow cytometry

Binding of RANTES was measured on CHO cells, human PBMC obtained by Ficoll density centrifugation from buffy coats of healthy donors and primary human microvascular endothelial cells (Promocell, Heidelberg, Germany) stimulated for 18 h with 5 ng/ml IL-1β where indicated. Cells were incubated for 1 h on ice with various concentrations of RANTES diluted in RPMI 1640. After three washing steps with PBS the cells were stained with one of the monoclonal antibodies VL-1, VL-2, VL-3 or VL-4 (10 μg/ml) directed against human RANTES [35] or IgG2b isotype control antibodies (Sigma) followed by a PE-conjugated rabbit anti-mouse F(ab)_2 fragment (Dako). Where indicated, RANTES was pre-incubated for 1 h with one of several reagents: RANTES antibodies (10 μg/ml) diluted in RPMI 1640 (Life Technologies), human serum, human plasma anti-coagulated with EDTA, platelet-rich or platelet-free plasma coagulated with 5 mM CaCl₂, supernatant of purified platelets and purified chondroitin sulfate A (Sigma) diluted in RPMI 1640. Where indicated platelet supernatant was digested with 1.3 U/ml chondroinase ABC (Sigma) [29] for 3 h at 37 °C or 33 U/ml heparinase (Sigma) for 3 h at 25 °C. Cells were analyzed by flow cytometry on a FACScalibur (Becton Dickinson) using CellQuest analysis software.

4.2 Purification and activation of platelets

Human blood anti-coagulated with sodium citrate was centrifuged for 20 min at 180 x g. The plasma was carefully recovered and incubated for 15 min at 37 °C with 1 mM aspirin and 0.3 U/ml apyrase (Sigma). After addition of citric acid and EDTA at final concentrations of 9 mM and 5 mM, respectively, the plasma was centrifuged for 20 min at 800 x g and the pellet resuspended in buffer B at pH 6.2 (138 mM NaCl, 2.9 mM KCl, 1 mM MgCl₂, 0.36 mM NaH₂PO₄, 20 mM HEPES, 0.6 U/ml apyrase). After centrifugation the pellet was resuspended to a final concentration of 1 x 10⁶ platelets/ml in buffer B at pH 7.4 containing 5 mM glucose. Where indicated platelets were activated for 10 min with 1 U/ml thrombin (Sigma) or 100 μg/ml collagen (Nycemed, Ismaning, Germany) and cell-free supernatant generated by centrifugation for 15 min at 4000 x g.

4.3 Down-modulation of CCR5

Human PBMC were prepared from the buffy coats of healthy donors by Ficoll density gradient centrifugations. Cells (5 x 10⁵) were incubated in RPMI 1640 for 30 min at 37 °C with various concentrations of chemokines. After centrifugation the cells were stained for surface expression of CCR5 for 1 h on ice with the monoclonal antibody MC-1 (10 μg/ml) [36] followed by a PE-conjugated rabbit anti mouse F(ab)_2 fragment (Dako) and analyzed by flow cytometry as described above.

4.4 Measurement of intracellular Ca²⁺ response

Calcium flux experiments were performed with a fast filter spectrofluorometer measuring emission at 340 and 380 nm (LSB-50, Perkin-Elmer). CCR5-transfected CHO cells [36] were removed from the culture flasks with PBS containing 1.5 mM EDTA washed in Hanks’ balanced salt solution and incubated for 1 h at 37 °C with 10 μM FuraPE3-AM (Molecular Probes) in RPMI 1640 containing 2 mM glutamate, 1% FCS, 10 mM HEPES, 1 mM Na-pyruvate, 50 μM 2-mercaptoethanol and 0.2% Pluronic, pH 6.8. The cells were washed in Hanks’ balanced salt solution with 10 mM HEPES and further incubated for 20 min at 37 °C. Aliquots of 400 μl were added into a 0.5 ml micro-cuvette heated to 37 °C. After approx. 100 s RANTES or complexes of RANTES and chondroitin sulfate A (Sigma) were added under constant stirring in a volume of 100 μl followed by addition of 50 μM ionomycin at 200 s and 20 mM MnCl₂ at 300 s. The data were analyzed by FL-Winlab software.

4.5 Monocytic cell adhesion under laminar flow conditions

Confluent human microvascular endothelial cells activated with IL-1β (10 ng/ml) were pre-incubated with RANTES (0.5 μg/ml) or complexes consisting of RANTES (0.5 μg/ml) and chondroitin sulfate A (Sigma, 100 μg/ml). MonoMac 6 cells (1 x 10⁶/ml) were perfused at 1.5 dyne/cm² in assay buffer (Hanks’ balanced salt solution, 10 mM HEPES, 0.5 % bovine serum albumin, 1 mM MgCl₂/CaCl₂). After 5 min shear resistant cell arrest was analyzed in multiple high-power fields recorded by video microscopy and is given as adherent cells/mm².

Acknowledgements: We thank Prof. W. Siess, Institut für Prophylaxe der Kreislaufkrankheiten, for help with purification of platelets and Dr. A. Proudfoot and Dr. T. Wells, Serono Pharmaceutical Research Institute, for providing RANTES. This work was supported by a grant from Deutsche Forschungsgemeinschaft (MA2198/1-3).

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