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Glycosaminoglycans are important mediators of neutrophilic inflammation *in vivo*

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ABSTRACT

The pro-inflammatory chemokine interleukin-8 (CXCL8) exerts its function by establishing a chemotactic gradient in infected or damaged tissues to guide neutrophil granulocytes to the site of inflammation via its G protein-coupled receptors (GPCRs) CXCR1 and CXCR2 located on neutrophils. Endothelial glycosaminoglycans (GAGs) have been proposed to support the chemotactic gradient formation and thus the inflammatory response by presenting the chemokine to approaching leukocytes. In this study, we show that neutrophil transmigration *in vitro* can be reduced by adding soluble GAGs and that this process is specific with respect to the nature of the glycan. To further investigate the GAG influence on neutrophil migration, we have used an engineered CXCL8 mutant protein (termed PA401) which exhibits a much higher affinity towards GAGs and an impaired GPCR activity. This dominant-negative mutant chemokine showed anti-inflammatory activity in various animal models of neutrophil-driven inflammation, i.e. in urinary tract infection, bleomycin-induced lung fibrosis, and experimental autoimmune uveitis. In all cases, treatment with PA401 resulted in a strong reduction of transmigrated inflammatory cells which became evident from histology sections and bronchoalveolar lavage. Since our CXCL8-based decoy targets GAGs and not GPCRs, our results show for the first time the crucial involvement of this glycan class in CXCL8/neutrophil-mediated inflammation and will thus pave the way to novel approaches of anti-inflammatory treatment.

1. Introduction

1.1. Interleukin-8

The chemokine interleukin-8 (IL-8; CXCL8) is a member of the CXC chemokine family and can be classified as pro-inflammatory chemokine due to its secretion at sites of inflammation by cytokine-activated endothelial cells and its chemotactic properties towards several types of inflammatory cells [1]. CXCL8 is involved in several pathologic processes such as lung diseases (chronic obstructive pulmonary disease, cystic fibrosis and asthma), autoimmune diseases (rheumatoid arthritis, psoriasis) and early tissue injury [2,3]. It exerts its pro-inflammatory effects by triggering neutrophil granulocytes via its specific seven-transmembrane spanning G protein-coupled receptors (GPCRs), CXCR1 and CXCR2, as well as via binding to its proposed co-receptors, the endothelially presented proteoglycans [4].

Abbreviations: BAL, bronchoalveolar lavage; BID, [bis in die] twice a day; CS, chondroitin sulphate; CXCL8, , C-X-C motif chemokine 8/interleukin-8; CXCR1/2, chemokine (C-X-C motif) receptor 1/2; EAU, experimental autoimmune uveitis; ELICO, ELISA-like competition assay; ELR, glutamic acid-leucine-arginine; FI, fluorescence intensity; GAG, glycosaminoglycan; GPCR, G protein-coupled receptor; HBSS−/−, Hank's Balanced Salt Solution (without calcium and magnesium); HMWH, high molecular weight heparin; HS, heparan sulphate; i.t., intratracheally; IPF, idiopathic pulmonary fibrosis; KC, murine CXCL1; LMWH, low molecular weight heparin; MIP-2, murine CXCL2; PA401, dominant negative interleukin-8 mutant; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; PDSAg peptide, pathogenic peptide derived from retinal S-antigen (S-Ag); s.c., subcutaneously; UPEC, uropathogenic *Escherichia coli*; UTI, urinary tract infection.

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1.2. Proteoglycans/glycosaminoglycans

Proteoglycans, which are located on the cell surface of eukaryotic cells, consist of a core protein on which glycosaminoglycan (GAG)-chains are covalently attached. GAGs, e.g. heparan sulphate (HS), heparin, chondroitin sulphate (CS), are highly sulphated, unbranched, O-linked heteropolysaccharides that are composed of repeating disaccharide units. Their molecular size and their sulphation patterns depend on the type and condition of the cells on which they are expressed [5].

1.3. Interleukin-8 GAG interaction

In the course of inflammation chemokines such as CXCL8 are presented by GAGs on the surface of endothelial cells as well as forming a chemotactic gradient in the infected or damaged tissue, which are crucial steps in the recruitment of leukocytes *in vivo* [6,7]. Binding of leukocytes to the chemokines presented by the endothelium leads to leukocyte activation, firm adhesion, transmigration across the endothelial barrier and accumulation of leukocytes at the centre of inflammation, where they further promote the immune response [7].

The interaction of CXCL8 and GAGs is driven by strong ionic forces between the sulphate groups of the carbohydrates and the basic residues of the protein. Specificity however is introduced through weak forces like hydrogen bonding and van der Waals forces [7]. The residues of CXCL8 involved in GAG-binding have been identified to include Arg^{60}, Lys^{64}, Lys^{67}, Arg^{68} in the C-terminal alpha-helix as well as His^{18} and Lys^{20} located in the proximal loop [8]. The residues of CXCL8 contributing to the binding and activation of CXCR1 and CXCR2 are the N-terminal ELR (glutamic acid-leucine-arginine) motif as well as a hydrophobic pocket in the proximal N-loop comprising of Ile^{10}, Tyr^{13}, Phe^{17}, Phe^{21} and Ile^{22} [9].

1.4. Dominant negative engineering of interleukin-8

We have recently engineered a dominant-negative CXCL8 mutant, termed PA401, with knocked-out GPCR binding and increased GAG binding affinity [10]. This mutant showed good anti-inflammatory activity in LPS- and tobacco-induced lung inflammation [11]. Here we have used this CXCL8 mutant to further prove the involvement of GAGs in diverse CXCL8-mediated, neutrophil-related disease models based on the strong, GPCR-independent, anti-inflammatory activity of PA401.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless stated otherwise. Dominant negative (dn) CXCL8 decoy (PA401) was designed and produced by ProtAffin Biotechnologie AG (Graz, Austria) as described in [10,12].

2.2. Transendothelial migration assay

Membranes of the transwell plates (HTS Transwell-96 Well Plate, PC membrane with 5 μM pore size, Corning, USA) were coated with collagen (from rat tail) using a final concentration of 1 μg/25 μL collagen in 0.1% acetic acid per well in the upper compartment and an incubation time of 2 h at 37 °C, 5% CO_2. After the incubation time, wells were washed with HBSS−/− (100 μL/well), air-dried, and cells were seeded and grown in the wells for three days before the assay. EA.hy926 (CRL-1730, ATCC, USA) endothelial cells were trypsinated from their cultivation flask and diluted with full growth medium (consisting of DMEM (Gibco, USA), 10% FBS (GE Healthcare, USA), 4 mM L-Glutamine (GE Healthcare, USA) and 1% Pen/Strep 1:100 (GE Healthcare, USA)) to a final concentration of 7000 cells/75 μL/well. Cells were filled into the upper well and 220 μL cultivation medium was added to the lower transwell compartment.

On the day of the assay human neutrophils were isolated from whole blood of healthy voluntary donors via density gradient centrifugation using Ficoll paque plus (GE Healthcare, USA) followed by a dextran gradient separation. The suspension cells were labelled using Calcein-AM.

200,000 cells were needed per well, so the respective number of cells was harvested by centrifugation and resuspended in 10 mL HBSS−/− (PAA Laboratories GmbH, Linz/Austria). 10 μL of 2 mM Calcein-AM were added to the cells and incubated for 30 min at 37 °C and 5% CO_2. After labelling, suspension cells were harvested by centrifugation, washed with 10 mL HBSS−/−, divided in the number of treatment schemes in different tubes, again harvested by centrifugation, resuspended in the decoy protein/GAG dilutions and pipetted in the allocated well. 10 μg/mL PA401 (=1,3 μM for the monomeric protein) and 10 μg/mL GAG concentrations were used for inhibition of chemotaxis, with 10 nM CXCL8 as chemoattractant in the lower compartment.

The transmigration assay was run for 2 h at 37 °C and 5% CO_2. Detection was performed by measuring the fluorescence intensity (FI) of migrated cells in the lower wells at 485 nm (excitation)/535 nm (emission) using the SpectraMax M3 Plate Reader (Molecular Devices, USA).

2.3. ELICO-chemokine displacement fingerprint

The ELICO measurements were conducted as described in a recently published procedure [13]. Different types of chemokines, being CCL2, CCL3, CCL5, CCL11, CXCL4, CXCL8, CXCL10, CXCL11, CXCL12, were incubated (250 nM) on a HS coated plate and PA401 (100 μM to 6 nM) was used as competitor for HS-binding.

2.4. Animal experiments

Animal care and handling procedures including providing of food and water *ad libitum*, 12-h light and dark cycle and keeping in ventilated cages were performed in accordance with the European guidelines (and Brazilian for the bleomycin model) and all the experiments were conducted under conditions previously approved by the local animal ethics committees.

2.5. Murine model of urinary tract infection (UTI)

Female C3H/HeN wild type mice were obtained from Charles River, Germany. Mice were divided in 2 groups of 10 mice each, that received a single intraperitoneal injection of either 1 μg of the CXCL8-based decoy (PA401) in 100 μL saline or saline only (control group) 10 min before induction of UTI. UTI was induced by intravesical inoculation of 4×10^8 CFU of the uropathogenic *Escherichia coli* (UPEC type CFT073, ATCC, USA) in 20 μL PBS. Liquid collodion (MMD0014, Mavidon, At Erica, The Netherlands) was placed in the urethra for 3 h for urethral obstruction and mice were sacrificed 36 h after infection. Bladders and kidneys were removed and fixed in 10% buffered formalin and embedded in paraffin as previously described [14]. Periodic-acid Schiff stained sections were analysed for the number of renal microabscesses and to score inflammation in the urinary bladder as follows: 0 = no submucosal edema and neutrophilic infiltrates, 1 = little, 2 = moderate, 3 = strong submucosal edema and neutrophilic infiltrates. Histopathological evaluation was performed by an observer