Biochimica et Biophysica Acta xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Serglycin secretion is part of the inflammatory response in activated primary human endothelial cells in vitro ☆

Trine M. Reine [a,1], Tram Thu Vuong [a,1], Trond G. Jenssen [b,c], Svein O. Kolset [a,*]

[a] Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Box 1046, Blindern, 0316 Oslo, Norway
[b] Department of Transplant Medicine, Section of Nephrology, Oslo University Hospital, Rikshospitalet, Oslo, Norway
[c] Institute of Clinical Medicine, Faculty of Health Science, University of Tromsø, Tromsø, Norway

ARTICLE INFO

Article history:
Received 26 November 2013
Received in revised form 20 January 2014
Accepted 1 February 2014
Available online xxxx

Keywords:
Serglycin
Chemokine
Primary human endothelial cell
Inflammation
Lipopolysaccharide
Interleukin-1 beta

ABSTRACT

Background: Endothelial cells have important functions in e.g. regulating blood pressure, coagulation and host defense reactions. Serglycin is highly expressed by endothelial cells, but there is limited data on the roles of this proteoglycan in immune reactions.
Methods: Cultured primary human endothelial cells were exposed to proinflammatory agents lipopolysaccharide (LPS) and interleukin 1β (IL-1β). The response in serglycin synthesis, secretion and intracellular localization and effect on the proteoglycan binding chemokines CXCL-1 and CXCL-8 were determined by qRT-PCR, Western blotting, immunocytochemistry, ELISA and serglycin knockdown experiments.
Results: Both LPS and IL-1β increased the synthesis and secretion of serglycin, while only IL-1β increased serglycin mRNA expression. Stimulation increased the number of serglycin containing vesicles, with a greater portion of large vesicles after LPS treatment. Also, increased intracellular and secreted levels of CXCL-1 and CXCL-8 were observed. The increase in CXCL-8 secretion was unchanged in serglycin knockdown cells. However, the increase in CXCL-1 secretion from IL-1β stimulation was reduced 27% in serglycin knockdown cells; while the LPS-induced secretion was not affected. In serglycin expressing cells CXCL-1 positive vesicles were evenly distributed throughout the cytoplasm, while confided to the Golgi region in serglycin knockdown cells. This was the case only for IL-1β stimulated cells. LPS-induced CXCL-1 distribution was unaffected by serglycin expression.
Conclusions: These results suggest that different signaling pathways are involved in regulating secretion of serglycin and partner molecules in activated endothelial cells.
General significance: This knowledge increases our understanding of the roles of serglycin in immune reactions. This article is part of a Special Issue entitled: Matrix-mediated cell behavior and properties.

1. Introduction

Endothelial cells have important functions in regulation of blood coagulation, blood pressure, wound healing, filtering of urine in kidneys, as barriers for bacteria and regulation of inflammatory responses in circulation [1]. To participate in such a variety of important functions endothelial cells express cell surface molecules important for cell–cell and cell–matrix interactions and for recognition of pathogens, growth factors and signaling molecules such as cytokines and chemokines. Endothelial cells also express proteoglycans (PGs), including syndecans and glypicans to be exposed on the cell surface, and perlecan and biglycan to be part of the extracellular matrix [2–4]. Endothelial cells also express serglycin, regarded as the major intracellular PG [5]. Serglycin has been shown to be one of the major PGs in cultured human umbilical vein endothelial cells (HUVECs) [6]. In this study it was also shown that serglycin was mainly secreted to apical medium of HUVEC cultured on semipermeable filters to induce polarization. Serglycin was furthermore shown to co-localize to a certain extent with CXCL-1 (GROα) and participate in the regulation of secretion of this chemokine, suggesting that serglycin can participate in inflammatory reactions mediated by endothelial cells. Many of the functions of serglycin studied so far suggest that this particular PG is involved in the intracellular storage, transport, secretion, protection and regulation of several bioactive molecules. These include a wide range of molecules ranging from histamine, chymases, carboxypeptidases, and cytolytic enzymes to cytokines and chemokines [5].

As part of the immune defense endothelial cells respond to lipopolysaccharide (LPS), a structure present in the cell wall of gram-negative bacteria. LPS is a classical pathogen-associated molecular pattern (PAMP) recognized by Toll-like receptor (TLR) 4, activating intracellular signaling proteins and the transcription factors nuclear factor κB (NFκB), activating protein-1 (AP-1) and interferon regulatory factor (IRF) 5 and IRF3 [7,8]. This leads to changes in gene expression, resulting in increased expression of effector-molecules such as cytokines and

☆ This article is part of a Special Issue entitled: Matrix-mediated cell behavior and properties.

* Corresponding author. Tel.: +47 22851383.
E-mail address: s.o.kolset@medisin.uio.no (S.O. Kolset).

[1] These authors contributed equally to this work.

0304-4165/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbagen.2014.02.002

chemokines. TLR4 signaling can be divided into the MyD88-dependent pathway, responsible for the expression of proinflammatory cytokines, and the MyD88 independent/TRIF dependent pathway mediating the activation of type 1 interferon genes [7,8].

Endothelial cells also respond to several cytokines released systemically during inflammatory reactions, including the classical proinflammatory IL-1 cytokines. IL-1β signals via IL-1 receptor type 1 (IL-1R1), also resulting in activation of transcription factors including NFκB, inducing the mRNA expression of a series of genes [9]. Several adaptor proteins, including MyD88 and IRAKs, mediate both TLR4 and IL-1R signaling, and both LPS signaling and IL-1β signaling activate the same intracellular signaling pathways [10]. However, there are also differences in the signaling systems being activated by these two activators relevant for inflammation. Specific molecules for TLR4 signaling include the MyD88 independent/TRIF-dependent pathway [7,10].

To further explore the possible importance and implications of serglycin in endothelial cells in inflammatory reactions we cultured primary human endothelial cells in the presence of LPS or IL-1β and studied the expression, secretion and intracellular localization of serglycin. Results presented suggest that both agents increase serglycin expression and secretion, but that the interactions of serglycin with partner molecules differ depending on type of stimuli. Serglycin secretion is, accordingly, regulated differently by two distinct types of inflammatory stimuli, and is clearly part of the inflammatory response in human endothelial cells.

2. Materials & methods

2.1. Cell culture

Human umbilical cord vein endothelial cells (HUVECs) were isolated from umbilical cords as described [11]. Cells were cultured at 37 °C and 5.0% CO_2 in MCDB-131 medium (Sigma) containing 5 mM glucose and supplemented with 7% heat-inactivated fetal calf serum (FCS, Sigma), basic fibroblast growth factor (bFGF, 1 ng/ml, R&D), hydrocortisone (1 μg/ml, Sigma), epidermal growth factor (EGF, 10 ng/ml, R&D), gentamicine (50 μg/ml, GIBCO Invitrogen) and fungizone (250 ng/ml, GIBCO Invitrogen). The medium was replaced three times a week and cells were used for experiments within three passages. The purity of the endothelial cell cultures was verified by microscopic observations of each culture as well as regular staining for the endothelial cell marker von Willebrand factor (vWF). For experiments, cells were seeded in wells or chamber slides and grown to confluence. Incubations with IL-1β (0.5 ng/ml, R&D) or LPS (1 μg/ml, Sigma) were performed for 24 h in a medium containing 2% FCS.

2.2. Gene expression analysis

HUVEC was cultured as described above. Total RNA was isolated using the E.Z.N.A. Total RNA kit 1 (R6834-02, Omega Bio-Tek) and lysis buffer containing β-mercaptoethanol, according to the manufacturer's instructions. RNA quantity measurements were performed using the ND1000 Spectrophotometer (Saveen Werner) and RNA was stored at −80 °C until further analysis. A quantity of 250 ng RNA was reverse transcribed in a total volume of 20 μl using the "High capacity RNA-to-cDNA kit" (4387406, Applied Biosystems). Quantitative Real-time PCR (qRT-PCR) was performed on an ABI Prism 7900HT (Applied Biosystems) using TaqMan Gene Expression Master Mix (4369016, Applied Biosystems) in a total volume of 20 μl. For gene expression analysis of serglycin (*SRGN*), CXCL-1 (*CXCL1*), CXCL-8 (*CXCL8*) and the endogenous control 60S ribosomal protein L30 (*RPL30*) [12], we used inventoried TaqMan gene expression assay (ID: Hs01004159_m1, Hs00236937_m1, Hs00174103_m1 and Hs00265497_m1). The relative mRNA level for each transcript was calculated by the ΔΔCt method [13,14]. Briefly, the cycle threshold (Ct) values for *SRGN*, *CXCL1* and *CXCL8* were normalized against the Ct values for the housekeeping gene *RPL30* (=ΔCt). For comparison of gene expression in stimulated versus control cells, ΔΔCt was calculated as ΔCt in stimulated cells subtracted the ΔCt for control cells. The fold change in mRNA expression was calculated as $2^{-\Delta\Delta Ct}$. $2^{-\Delta Ct}$ values were calculated for gene expression in response to LPS or IL-1β in siSRGN compared to scramble transfected controls.

2.3. Western blotting

HUVECs were grown in MCDB-131 medium as described, but with FCS reduced to 2%. After 24 h the conditioned medium was collected and cell debris removed by centrifugation. Samples of equal volumes were boiled in Laemmli buffer and subjected to SDS-PAGE on 4–20% gradient gels and electroblotted onto PVDF membranes (Millipore) using the Criterion™ gel system (BioRad). Incubation with primary antibody polyclonal rabbit anti-human serglycin (HPA000759, Atlas Antibodies, 1:100) was followed by secondary antibody HRP-linked goat anti-rabbit (NA934, GE-Healthcare,1:20 000). The membranes were developed using ECL Western Blotting Detection Reagents (GE Healthcare) and finally exposed to films (Amersham Hyperfilm™ ECL). The intensity of the bands was quantified by Carestream Molecular Imaging software.

2.4. Metabolic labeling

HUVEC cultures were metabolically labeled with 0.1 mCi/ml ^{35}S-sulfate (Hartmann Analytic) in RPMI-1640 sulfate free medium (GIBCO Invitrogen) added 5 mM L-glutamine (Sigma) and with FCS reduced from 7 to 2% to increase labeling efficiency. Labeling was performed in the absence (Ctr) or presence of IL-1β or LPS. After labeling for 24 h, the culture medium was collected. Cells were washed in PBS and harvested either in lysis buffer (4.0 M guanidine-HCl, 0.1 M acetate buffer pH 6.5, 2% Triton X-100) or RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% SDS, 1% Na-deoxycholate, 10 mM EDTA, 10 mM $Na_4P_2O_7$ and phosphatase inhibitor tablet freshly added). In order to remove unincorporated ^{35}S-sulfate, samples were diluted to 1 ml and applied to Sephadex G50 fine (GE Healthcare) gel chromatography columns, with a bed volume of 4 ml. The ^{35}S-macromolecules were eluted in the void volume with 1.5 ml buffer (0.05 M Tris–HCl, 0.05 M NaCl, pH 8), while lower molecular weight molecules remained associated with the column. The amount of ^{35}S-sulfate incorporated in newly synthesized ^{35}S-macromolecules was determined by scintillation counting in triplicates. ^{35}S-macromolecules in HUVEC almost exclusively comprise PGs [15]. Protein content of the cell lysate was determined using Uptima BC Assay protein quantization kit (BioRad), in RIPA-lysates prior to G50 fine gel chromatography or in guanidine-lysates after changing the buffer on the G50 fine column. The results are presented as mean cpm/mg cellular protein.

2.5. Immunoprecipitation

HUVECs were metabolically labeled with ^{35}S-sulfate as described; thereafter the medium was harvested and the cells were washed in PBS and lysed in RIPA buffer. The volumes of both medium and cell lysates were adjusted to the protein content of the cell fractions, and subjected to G50-fine gel chromatography as described above. Samples were incubated over night at agitation and 4 °C with polyclonal rabbit anti-human serglycin (HPA000759, Atlas Antibodies, 1:100) or monoclonal mouse anti-human serglycin (clone 1D8, AbNova, 1:500). In initial experiments medium from the monocytic cell line THP-1 [16] was included as positive control for serglycin, and as negative control unconditioned medium or concentration matched irregular IgG control (Mouse Gamma Globulin, Jackson ImmunoResearch, 015-000-002) was included. Further, 30 μl Protein A/G solution (sc-3003, Santa Cruz) was added and the incubation was continued for 2 h. The samples were then centrifuged and washed three times in 0.05 M Tris–HCl

Please cite this article as: T.M. Reine, et al., Serglycin secretion is part of the inflammatory response in activated primary human endothelial cells in vitro, Biochim. Biophys. Acta (2014), http://dx.doi.org/10.1016/j.bbagen.2014.02.002