Original Article

Shiga-toxin-induced firm adhesion of human leukocytes to endothelium is in part mediated by heparan sulfate

Joyce Geelen1, Federica Valsecchi2, Thea van der Velden1, Lambertus van den Heuvel1, Leo Monnens3 and Marina Morigi2

1Department of Paediatric Nephrology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, 2Unit of Nephrology and Dialysis, Mario Negri Institute for Pharmacological Research, Bergamo, Italy and 3Department of Physiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Abstract

Background. Shiga toxin (Stx) is the main pathogenic factor in the haemolytic–uraemic syndrome (HUS). Stx damages the renal endothelium, which leads to inflammation and coagulation. Endothelial heparan sulfate proteoglycans (HSPG), and heparan sulfate in particular, play an important role in the inflammatory process by acting as a ligand for L-selectin. Furthermore, leukocytes are able to interact with chemokines bound to HSPG (examples are IL-8, RANTES and MCP-1). This leads to an activation of integrins on leukocytes and results in more stable leukocyte-endothelial wall adhesion. In this study, we have evaluated the effect of a subtoxic dose of Stx1 and Stx2 on the HSPG and its role in adhesion of leukocytes.

Methods. Primary human umbilical venous endothelial cells (HUVEC) and primary human glomerular microvascular endothelial cells (GMVEC) were incubated for 24 h with a subtoxic dose of Stx1 or Stx2. Then, cells were treated with heparan sulfate-degrading enzyme heparitinase I or left untreated, followed by determination of binding leukocytes to endothelial cells in a parallel plate flow chamber.

Results. In both cell types, Stx increased the amount of firmly adherent leukocytes. After removal of endothelial heparan sulfate, the number of adhering leukocytes decreased.

Conclusions. HSPG have a distinctive role in adhesion of leukocytes to endothelial cells stimulated by a subtoxic dose of Stx.

Keywords: endothelial cells; haemolytic-uraemic syndrome; heparan sulfate proteoglycans; leukocytes; Shiga toxin

Introduction

Haemolytic–uraemic syndrome (HUS) is a form of acute renal failure in children, combined with thrombocytopenia and haemolytic anaemia. Although new insight has been gained in the pathogenesis of genetic variants of HUS, the underlying mechanism in infection-related HUS is still not understood [1]. The most important pathogen in infection-related HUS is Escherichia coli O157:H7, which produces Shiga toxin (Stx) [2]. There are two different variants, Stx1 and Stx2, which share 50% homology and have a similar biological effect. This toxin has a cytotoxic effect on the renal endothelium. It internalizes after binding to a specific receptor (CD77; Gb3) and disables the ribosome [1]. This undermines the protein synthesis of the cells and will ultimately lead to cell death. Because of this damage, two different biological cascades are initiated: coagulation and inflammation. Renal biopsies of patients with HUS show occluded glomerular arterioles and deposition of inflammatory cells [1].

During the last decade it has become evident that heparan sulfate proteoglycans (HSPG) are crucial for leukocyte-endothelium trafficking [3,4]. These negatively charged structures are ubiquitously expressed on cell surfaces, blood cells and in the extracellular matrix [3,4]. HSPG is a macromolecular structure consisting of a protein backbone covalently carrying heparan sulfate (HS) chains. HS belongs to the family of glycosaminoglycans that comprises the highly sulfated heparin and non-sulfated hyaluronan. The HS chain shows a high diversity in the structure, which is dictated by combinations of possible modifications such as sulfation patterns. This allows HSPG to interact with many different proteins like cytokines, chemokines and cell-adhesion molecules. HSPG on endothelial cells play an important role in tethering, rolling, firm binding and transmigration of leukocytes to the extracellular matrix [3,4]. After an inflammatory insult, endothelial cells will express more cell adhesion molecules (E- and P-selectin), which will bind to leukocytes that express ligands such as P-selectin glycoprotein ligand-1. During the rolling phase, endothelial HS
will serve as a ligand for L-selectin on leukocytes. They will roll over the endothelial cells and get into contact with endothelial HS-bound chemokines, which in their turn activates integrins on leukocytes like MAC-1. Subsequently, leukocytes will adhere more firmly, which is in part mediated by the HS-MAC-1 interaction. Finally, the leukocytes will transmigrate through the endothelium to the extracellular matrix.

Recently, Wang et al. described a reduced neutrophil infiltration in an experimental peritonitis in genetically engineered mice with low sulfation of endothelial HSPG [5]. Furthermore, Rops et al. have demonstrated that HS on mouse glomerular endothelium activated by TNFα is crucial for the number of rolling and firmly adhering leukocytes [6].

In this study, we focused on the inflammatory process induced by Stx in HUS. The performed experiments allow us to evaluate the effect of Stx on firm leukocyte adhesion under dynamic flow conditions and whether HSPG are involved. Since application of shear stress changes the phenotypic appearance of endothelial cells, these experiments approach the in vivo situation [7]. Endothelial cells from umbilical origin and glomerular endothelial cells were used. The latter were selected as being the primary target of Stx.

**Material and methods**

**Culture of endothelial cells**

Human umbilical venous endothelial cells (HUVEC) were harvested after collagenase treatment (Worthington, NJ, USA) and cultured according to a previously described method [8]. HUVEC from five donors (passages 2–4) were used. Glomerular microvascular endothelial cells (GMVEC) were obtained from human kidneys and cultured as described previously [9]. GMVEC from three donors in passages 7–10 were used. Every donor was used once in the experiments. For the adhesion experiments, cells were seeded on plastic cover slips (Thermanox, Nunc Inc., Naperville, USA). The growth medium of both cell types was refreshed every 2 days.

**Isolation of leukocytes**

Leukocytes were isolated from fresh human venous blood collected from healthy volunteers after informed consent as described previously [11]. Briefly, after dilution of the blood with cold saline, it was centrifugated at 200 × g for 10 min at 4°C. Subsequently, the cell pellet was gently put on four volumes of Emagel (Boehringerwerke AG, Marburg, Germany) in order to sediment the erythrocytes. This was performed for 45 min at 4°C. The supernatant was removed and centrifugated at 4°C for 10 min at 500 × g and the pellet was washed twice by centrifugation with saline. Then ammonium chloride was added for lysis of the remaining erythrocytes. After new washing steps with saline, cells were resuspended in the culture medium at a concentration of 1 × 10⁶ cells per ml. For every experiment the leukocytes of one donor were used.

**Incubation and manipulation of endothelial cells**

Endothelial cells were incubated for 24 h with a subtoxic dose (50 pM) of Stx1 (kindly provided by Dr M. Bielaszewska, Munster, Germany). This dose does not exert a cytotoxic effect, which was confirmed with trypan blue exclusion. 50 pM of Stx1 was free of lipopolysaccharide (LPS) as assessed with a Limulus-assay (detection limit 0.006 ng/ml). Stx2 (Toxin Technology Inc., Sarasota, USA) was also used in a subtoxic dose (50 pM), since this toxin was able to increase the adhesion of leukocytes as demonstrated by the work of Zoja et al. [12]. As the experiments were performed in the same laboratory, the toxin was produced equally as described by Zoja et al. [12]. It contains 117 pg LPS/µg Stx2, which is 0.41 pg/ml LPS at a dose of 50 pM Stx2. This is below the detection limit of the Limulus assay. This indicates that LPS does not influence the final effects observed in the cells after stimulation with Stx1 and Stx2.

After incubation with Stx, HS was cleaved off by the HS-degrading enzyme heparitinase I (Seikagaku Co., Tokyo, Japan). This enzyme cleaves the alpha-N-acetyl-D-glucosaminidic linkage in heparan sulfate, producing disaccharides. Briefly, the enzyme was dissolved in 0.5 U/ml in a binding buffer (10 mmol/l HEPES, 137 mmol/l NaCl, 4 mmol/l KCl, 11 mmol/lD-glucose and 0.1% bovine serum albumin (BSA), pH 7.4) and incubated for 30 min at 37°C.

The efficacy of the enzyme was evaluated with immunofluorescence and FACS analysis with antibodies directed against heparan sulfate [primary antibody: mouse anti-human heparan sulfate 10E4 (US Biological, Swampscott, USA); secondary antibody: goat anti-mouse FITC conjugated (Jackson Immunoresearch, West Grove USA)]. For FACS analysis, cells were detached by using EDTA 10 mM.

**Adhesion assay under dynamic flow conditions**

A parallel-plate flow chamber was used to apply shear stress. This system is described in the article of Morigi et al. [11]. In short, cover slips with a monolayer of endothelial cells were placed in the flow chamber. First, a cell-free medium with 0.5% BSA was flowed over the cells during 2 min at 0.6 dynes/cm² for equilibration. Then, the leukocyte suspension was perfused through the chamber with 1.5 dynes/cm² during 10 min to approach the level of shear stress in glomerular arterioles. This was followed by again a cell-free medium at 3 dynes/cm² during 5 min. At this higher level of shear stress, the number of firmly adhering leukocytes can be analysed. The experiment was visualized and recorded with a video recording system connected to an inverted phase-contrast microscope. The number of firmly
adhering cells was calculated in 10 random equal fields by two observers.

**Statistical analysis**

Results are expressed as mean ± SD. Data were analysed with Student’s *t*-test. The statistical significance level was defined as *P* < 0.05.

**Results**

**Cleavage of HS from endothelial cells**

To evaluate the efficacy of the HS-cleaving enzyme, the amount of HS after treatment with heparitinase I was estimated with immunofluorescence and FACS analysis.

The immunofluorescence staining of endothelial cells by the anti-HS antibody 10E4 is shown in Figure 1A. Removal of HS by heparitinase I revealed a complete abolishment of staining (Figure 1B). To quantify these results, we repeated this analysis by FACS. This confirmed the immunofluorescence staining, since treatment of the cells with the enzyme strongly reduced the staining (Figure 2).

**Flow experiments**

After incubation of both endothelial cells with Stx1 and Stx2, there clearly was an increased number of firmly adhering leukocytes to HUVEC (range: Stx1 13–55; Stx2 38–70) (Figure 3) or GMVEC (range: Stx1 52–60; Stx2 51–62) (Figure 4). Removal of HS from endothelial cells that were not incubated with Stx did not have a significant effect on firm leukocyte adhesion. However, treatment of the Stx-incubated endothelial cells with heparitinase I clearly revealed a decreased number of firmly adhering leukocytes (range: HUVEC Stx1 18–35; HUVEC Stx2 28–63; GMVEC Stx1 34–37; GMVEC Stx2 32–39) (Figures 3 and 4). Taken together, removal of HS from Stx-incubated endothelial cells decreased the number of firmly adhering leukocytes.

**Discussion**

We showed that HS plays an important role in the adhesion of leukocytes to endothelial cells after treatment with a subtoxic dose of Stx. After removal of endothelial HS from Stx-incubated endothelial cells, the number of firmly adherent leukocytes is clearly decreased.

Since Stx is considered a cytotoxic toxin, our results are very interesting. The increase in adhesion of leukocytes indicates that there must also be a stimulatory effect, besides inhibition of the protein synthesis. Several mechanisms for our results can be suggested. One possibility is an increased expression of HSPG after incubation with a subtoxic dose of Stx. Another option is an increase in bound chemokines on endothelium–HSPG after treatment with Stx. This will result in more adhering leukocytes. The basal level of HSPG present on endothelial cells will be sufficient to bind additional released chemokines. Zoja et al. described an NF-κB-dependent up-regulation of mRNA from IL-8 and MCP-1 by Stx and inhibition of leukocyte adherence after the application of antibodies against IL-8.
Fig. 3. Number of firm adherent leukocytes on HUVEC. HUVEC (n = 5) was incubated for 24 h with Stx1 or Stx2. Furthermore, they were treated with heparitinase I (HEP) or remained untreated. The number of firmly adherent leukocytes was calculated. Stx increased the amount of firmly adhering leukocytes. Removal of HS decreased this effect. *P < 0.05; **P < 0.01.

Fig. 4. Number of firm adherent leukocytes on GMVEC. GMVEC (n = 3) was incubated for 24 h with Stx1 or Stx2. Furthermore, they were treated with heparitinase I (HEP) or remained untreated. The number of firmly adhering leukocytes was calculated. Stx increased the amount of adherent leukocytes. Removal of HS decreased this effect. *P < 0.05; **P < 0.01.

and MCP-1 [12]. The work of Matussek et al. confirmed the up-regulation of mRNA of pro-inflammatory proteins by Stx [13]. When released chemokines bind to HSPG on endothelial cells, they are protected from proteolysis and are able to rapidly induce activation of integrins on leukocytes [3].

By using the parallel-flow chamber, we have applied shear stress to the endothelial cells, which mimics the in vivo situation. It is possible that the combination of Stx exposure and shear stress will increase the biological effect of Stx on endothelial cells. Moreover, during the development of HUS in patients, leukocytes like neutrophils and monocytes will become activated [14,15]. This will lead to increased serum-levels of different cytokines. In our experiments, leukocytes from healthy donors in a serum-free medium were used. Possibly, in vivo there will be a much stronger effect of Stx in combination with cytokines from activated leukocytes.
The residual number of firmly adhering leukocytes after removal of HS from Stx-incubated endothelial cells indicates that also HS-independent factors are involved in the increased adhesion of leukocytes. Fractalkine could be such a factor, since Ramos et al. demonstrated a selective depletion of mononuclear leukocytes expressing the receptor for fractalkine [16]. Furthermore, fractalkine-receptor positive leukocytes were observed in renal biopsies of patients with HUS.

In conclusion, our data show that Stx can increase the number of firmly adhering leukocytes to endothelial cells, which can be partially blocked by removal of HS. These findings strongly suggest a role for endothelial HS in the inflammatory process in the renal vasculature during HUS. However, it cannot explain the preferential damage of glomerular endothelial cells observed in HUS patients.

As possible therapeutic options for future treatment, the use of N-desulfated heparin, which inhibits leukocyte adhesion and transmigration with low anticoagulant activity, or blocking of chemokine receptors can be considered [17,18].

Acknowledgements. This work was supported by a grant from the Dutch Kidney Foundation (PC 153) and ‘Stichting de 3 lichten’. The authors like to thank Dr M. Bielaszewska for her support. Furthermore, the authors would like to thank Dr J. van der Vlag and Dr A. Rops for their technical support.

Conflict of interest statement. None declared.

References


Received for publication: 19.11.07
Accepted in revised form: 8.4.08