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Original Article

Heparan sulfate on activated glomerular endothelial cells and exogenous heparinoids influence the rolling and adhesion of leucocytes

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Abstract

Background. Proliferative glomerulonephritides are characterized by the influx of leucocytes. Heparan sulfate (HS) plays an important role in the recruitment, rolling and firm adhesion of leucocytes to activated endothelium. Recently, we have shown the importance of HS on activated mouse glomerular endothelial cells (mGEnC-1) for the firm adhesion of leucocytes in a static adhesion assay.

In the present study, we evaluated the role of HS on glomerular endothelial cells and the effect of adding heparinoids on the leucocyte–glomerular endothelium interaction under dynamic flow conditions.

Methods. The number of rolling and firmly adhering leucocytes, and the rolling velocity of leucocytes was determined on a monolayer of unactivated or TNF-α-activated mGEnC-1 under dynamic flow conditions using physiological relevant shear stress rates in a flow chamber system. Furthermore, the effects of removal of HS on TNF-α-activated mGEnC-1 by heparinase III treatment, and of different concentrations of heparin, tinzaparin and HS, on the rolling and adhesion of leucocytes were evaluated.

Results. At the calculated physiological shear stress rate of 0.8 dynes/cm^2 the number of rolling and firmly adhering leucocytes to mGEnC-1 increased 2-fold after activation with TNF-α, whereas the rolling velocity of the leucocytes decreased 2-fold. Addition of heparin, tinzaparin or HS, and the removal of HS on mGEnC-1 reduced the number of leucocytes rolling and adhering to activated mGEnC-1 about 2–3-fold, while the rolling velocity increased more than 2-fold.

Conclusions. HS on activated glomerular endothelial cells is important for the interaction with leucocytes under flow conditions, while exogenous heparinoids interfere with this interaction. These results suggest that supplementary treatment of proliferative glomerulonephritides with heparinoids is an interesting option to pursue.

Keywords: adhesion of leucocytes; glomerular endothelial cells; glomerulonephritis; heparin; low molecular weight heparin; rolling of leucocytes

Introduction

Inflammatory lesions are manifested in a range of glomerular diseases and are characterized by the influx of leucocytes. The endothelial trafficking of leucocytes occurs in a sequential four-step process, i.e. tethering to, rolling over, firm adhesion to, and transmigration through the activated endothelium [1] (Figure 1). In all these steps, heparan sulfate (HS) plays an important role by binding chemokines, cytokines, selectins, integrins and adhesion molecules [2]. HS belongs to the family of strong negatively charged glycosaminoglycans that also includes heparin, which contains more sulfate groups and iduronic acid residues than HS (Figure 2).

The adherence of leucocytes to the vessel wall depends on the strength of the interaction between leucocytes and the endothelium, which is determined by receptor–ligand interactions and affected by shear forces imposed by the fluid flow in the circulation. Wall shear stress plays a role in maintaining vascular haemodynamics and haemostasis, and also affects heparan sulfate proteoglycan synthesis and fine structure [3]. An increased synthesis of glycosaminoglycan has been observed for endothelial cells that were subjected to a shear stress rate of 15–40 dynes/cm^2 for 24 h [4], while an inhibition of glycosaminoglycan synthesis has been observed for endothelial cells that
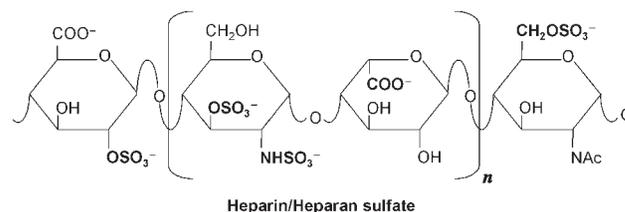
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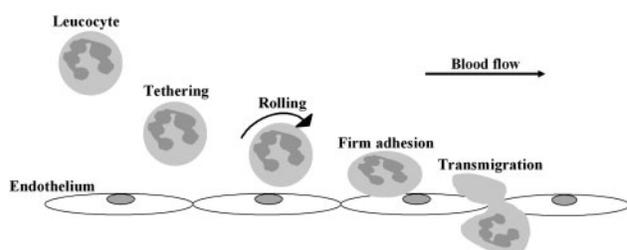
Fig. 1. The sequential steps of leucocyte trafficking on the activated endothelium. Circulating leucocytes are tethered to the activated endothelium. Subsequently, leucocytes are slowing down by rolling over the activated endothelium, which is facilitated by interaction of selectins and selectin ligands. During this process locally produced chemokines/cytokines bound to endothelial HS further activate integrin on leucocytes leading to stronger leucocyte–endothelium interactions. Thereafter, firmer adhesion of leucocytes to the activated endothelium takes place, which is mediated by integrins and integrin ligands. Finally, leucocytes transmigrate/cross the endothelial monolayer. Notably, in all steps in leucocyte trafficking over activated endothelium heparan sulfate is involved [26,27].

Fig. 2. Structural features of heparin/heparan sulfate. The possible modifications, i.e. N-deacetylation and N-sulfation of N-acetyl-glucosamine, C-5 epimerization of glucuronate to iduronate and O-sulfation at various positions (C-2, C-3 and C-6), are indicated in bold. Heparin contains a higher iduronate/glucuronate ratio and a higher percentage of sulfated domains than heparan sulfate. Represented by n are 50–150 disaccharide units of glucosamine and uronic acid.

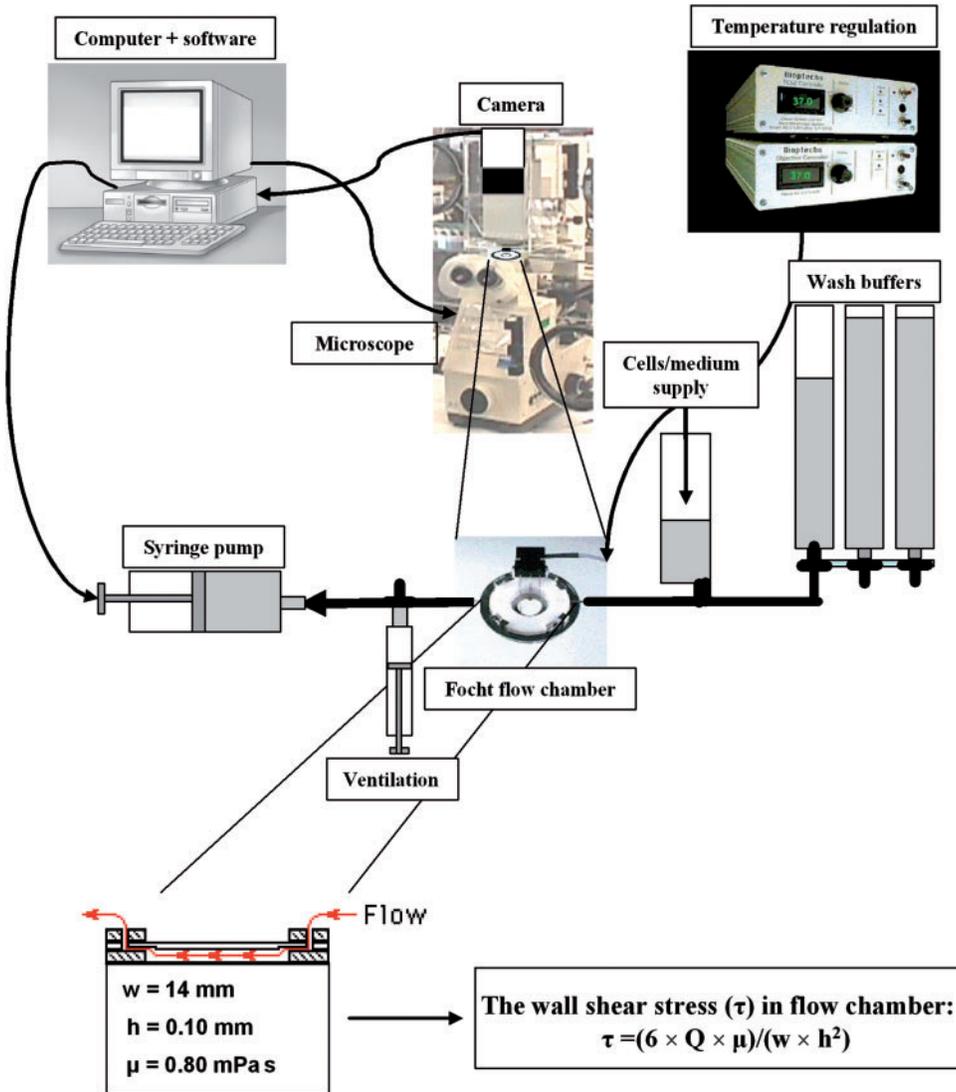## Subjects and methods

Cell cultures

The conditionally immortalized mouse glomerular endothelial cell line (mGEnC-1) with all features of primary glomerular endothelial cells was cultured as described [9]. The mGEnC-1 was grown on coverslips (40 mm) (Bioptechs, Inc., Butler, PA, USA) pre-coated with 1% gelatin and fibronectin (1 μg/cm^2) (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands) in normal growth medium at 37°C, until they were 100% confluent. Where indicated, cells were activated by incubation with TNF-α (10 ng/ml; Peprotech, Rocky Hill, NJ, USA) for 18 h. The murine granulocyte cell line 32Dcl3 [10,11], which lacks cell surface HS [12], but highly expresses L-selectin, Mac-1 and the granulocyte marker (NIMP-R14) [13], was maintained in RPMI-1640 (Invitrogen Life Technologies, Breda, the Netherlands), supplemented with 10% WEHI-3B-conditioned medium as a source of murine interleukin-3 (DSMZ, Braunschweig, Germany), 1% pyruvate, 1% gluta-max, 10% foetal calf serum and 1% penicillin/streptomycin (Invitrogen Life Technologies).

Removal of endothelial heparan sulfate

HS on TNF-α-activated mGEnC-1 was removed by treatment with 0.25 U/ml heparinase III (Sigma-Aldrich, Zwijndrecht, the Netherlands) in cell culture medium (pH 7.5) for 1 h at 37°C. The terminal 4,5-unsaturated uronate residues that arise after heparinase III treatment are recognized by antibody 3G10 [14], which was used to evaluate enzyme activity.

Adhesion experiments under flow conditions

Adhesion of 32Dcl3 granulocyte cells to mGEnC-1 was measured under flow conditions in a parallel plate Focht flow perfusion chamber with well-defined characteristics, essentially as depicted in Figure 3. A coverslip with a monolayer of mGEnC-1 was assembled in the flow chamber, which was mounted on an inverted phase-contrast microscope stage (Axiovert 35M, Zeiss, Germany) that was connected to a camera (VarioCam, PCO computer Optics GmbH, Kelheim, Germany) and controlled by a computer. The x, y and z positions of the microscope table were regulated by a computer-driven x, y, z stage controller. Then, 32Dcl3 granulocyte cells were resuspended in plain

were subjected to a shear stress rate of 1 dyne/cm^2 for 2 h [5]. Furthermore, endothelial HS participates in mechanosensing that mediates nitric oxide production in response to shear stress [6], while vascular smooth muscle cell HS promotes contractile responses to increases in shear stress [7]. The concerted action of adhesion molecules, chemokines and endothelial HS during inflammation is dependent on the shear stress in a specific microvascular bed. The *in vivo* conditions of dynamic blood flow can be mimicked *in vitro* by flow chambers (Figure 3), which are frequently used to evaluate the rolling and firm adhesion of leucocytes to cultured endothelium [8]. In a flow chamber the shear stress rate (τ) (in dynes/cm^2) can be calculated by the Navier–Stokes equation and set by adjusting the volumetric flow rate (Figure 3).

Recently, we have shown the importance of HS on activated mouse glomerular endothelial cells (mGEnC-1) [9] for the firm adhesion of leucocytes by using an *in vitro* static adhesion assay (A. Rops *et al.*, unpublished data). Heparin and low molecular weight heparin are frequently clinically applied as anti-coagulants, but could also be considered for supplementary treatment of glomerulonephritis by interfering with the interaction between leucocytes and glomerular endothelium. Currently, there are no data on whether the HS on activated glomerular endothelium plays a role in the interaction of leucocytes under dynamic flow conditions. Furthermore, it is not known whether heparinoids are able to interfere with the interaction of leucocytes with the activated glomerular endothelium under dynamic flow conditions. Therefore, we evaluated *in vitro* the number of rolling and firmly adhering leucocytes and the rolling velocity of leucocytes on glomerular endothelial cells under dynamic flow conditions after removal of HS on glomerular endothelial cells and in the presence or absence of heparin, tinzaparin or HS.

Fig. 3. *In vitro* dynamic flow chamber system. The shear stress rate (τ) expressed in dynes/cm^2 in the flow chamber can be calculated and set according to the Navier–Stokes equation, $\tau = (6Q\mu)/(wh^2)$, in which Q is the volumetric flow rate, μ is the viscosity of the fluid (in mPa s), w is the slide width and h is the height of the flow chamber, respectively.

DMEM-HAM F12 medium (Invitrogen Life Technologies) at a concentration of 0.5×10^6 cells/ml and pre-warmed to 37°C. The 32Dcl3 granulocyte cells were aspirated from a reservoir through the perfusion chamber with a withdrawal syringe pump (Harvard apparatus, Holliston, MA, USA; ANTEC, Leiden, The Netherlands). The complete system was present in a 37°C incubator. Temperature fine-tuning was performed by the Bioptechs FCS2 controller.

The experiment started with a continuous flow of plain DMEM-HAM F12 medium of 37°C for 2 min over the monolayer of mGEnC-1. Subsequently, 32Dcl3 granulocyte cells were perfused for 60 s at shear stress rates in the physiological range of 0.4–1.6 dynes/cm^2, i.e. with a volumetric flow rate Q of 1.2–4.7 µl/s, respectively. Video recordings of one microscopic field (0.14 mm^2) were read continuously (1 image/s) and analysed afterwards with an in-house developed software program [15]. Individual 32Dcl3 granulocyte cells received a unique ID and could be tracked and analysed in time, which allowed the determination

of the average number of rolling (defined at a movement of 4–100 µm/s) and firmly adhering (defined at a movement of 0–4 µm/s) cells, as well as the average rolling velocity. The effect of 10, 50 or 250 µg/ml heparin (Sigma-Aldrich), tinzaparin (Innohep®) (Brocacef Holding NV, Maarssen, the Netherlands) or HS from bovine kidney (Seikagaku, Tokyo, Japan) [16] on the average number of rolling and firmly adhering 32Dcl3 granulocyte cells, and on the average velocity of rolling 32Dcl3 granulocyte cells was evaluated on TNF-α-activated mGEnC-1 at a shear stress rate of 0.8 dynes/cm^2 for 60 s with a recording of 10 images/s. All perfusions were performed in triplicate on at least three separate experiments.

Statistical analysis

All values are expressed as means ± SEM and significance was determined by Mann–Whitney U-testing using

GraphPad Prism, version 4.0 software (GraphPad Software, Inc., San Diego, CA).

Results

Rolling and adhesion of granulocytes to unstimulated and TNF-α-activated mouse glomerular endothelial cells at different shear stress rates

We calculated an approximate shear stress rate in mouse glomerular capillaries of about 0.8 dynes/cm² using Hagen–Poiseuille's law. However, the actual value of the shear stress rate in glomerular capillaries may be different from 0.8 dynes/cm², since Hagen–Poiseuille's law is only suitable for Newtonian fluids, while in addition the viscosity and volumetric flow rate of the blood in the glomerular capillaries, and the diameter of the glomerular capillaries may dynamically change. Therefore, we evaluated the number of rolling and firmly adhering 32Dcl3 granulocyte cells on both unstimulated and TNF-α-activated mGEnC-1 at shear stress rates of 0.4, 0.8 and 1.6 dynes/cm², respectively. In addition, the average rolling velocity of 32Dcl3 granulocyte cells at different shear stress rates was determined. It appeared that at a shear stress rate of 0.4 dynes/cm², the results were not discriminating, since the numbers of rolling and firmly adhering 32Dcl3 granulocyte cells, and the average rolling velocity of 32Dcl3 granulocyte cells were not significantly different between unstimulated mGEnC-1 and TNF-α-activated mGEnC-1 (data not shown). In contrast, at a shear stress rate of 0.8 dynes/cm² the numbers of rolling and firmly adhering 32Dcl3 granulocyte cells to mGEnC-1 increased about 2-fold after activation with TNF-α (Figure 4A and B), while the average rolling velocity decreased about 2-fold (Figure 4C). At a shear stress rate of 1.6 dynes/cm² the numbers of rolling and firmly adhering 32Dcl3 granulocyte cells, and the average rolling velocity were already reduced several folds on unactivated mGEnC-1 when compared to the shear stress rate of 0.8 dynes/cm² (Figure 4A and B). However, despite these lower numbers, also at a shear stress rate of 1.6 dynes/cm² the numbers of rolling and firmly adhering 32Dcl3 granulocyte cells to mGEnC-1 significantly increased after activation with TNF-α (Figure 4A and B). In contrast to the shear stress rate of 0.8 dynes/cm², the average rolling velocity of 32Dcl3 granulocyte cells at a shear stress rate of 1.6 dynes/cm² was not significantly affected by activation of mGEnC-1 (Figure 4C). These results indicate that both rolling and firm adhesion of 32Dcl3 granulocyte cells as well as the average rolling velocity are affected by the activation status of mGEnC-1 and by different shear stress rates. Since the calculated shear stress rate in glomerular capillaries is 0.8 dynes/cm², this shear stress is the most suitable rate to test leucocyte trafficking in our *in vitro* flow system.
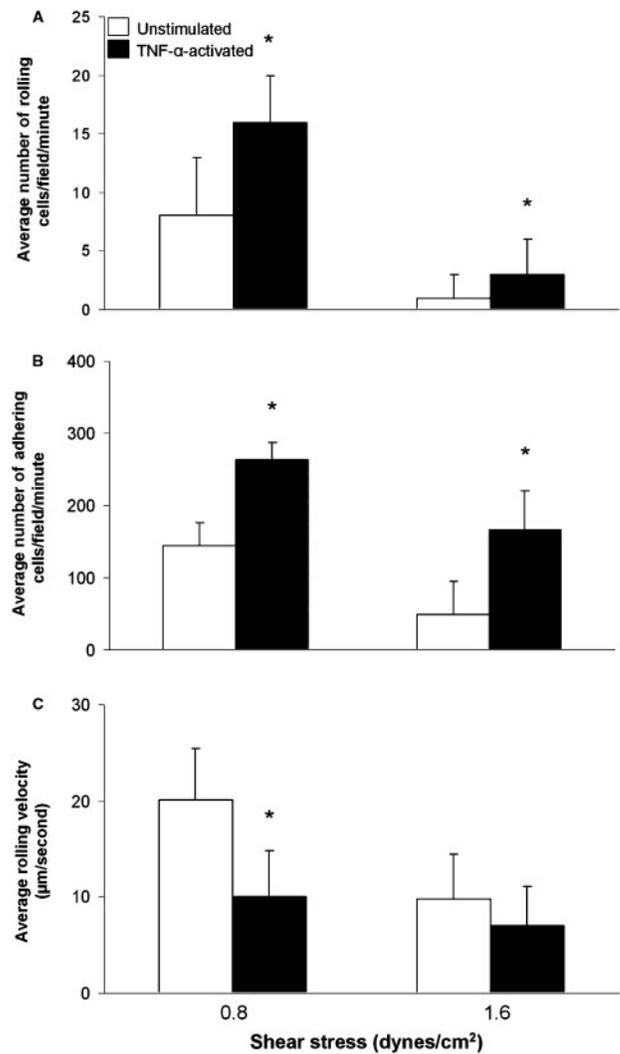
Fig. 4. Rolling and adhesion of 32Dcl3 granulocyte cells on unstimulated and TNF-α-activated mouse glomerular endothelial cells at different shear stress rates. A monolayer of mGEnC-1 was either unstimulated or stimulated with TNF-α for 18 h. Then, 32Dcl3 granulocyte cells were perfused at shear stress rates of 0.8 or 1.6 dynes/cm². The average number ± SEM of rolling cells (**A**), the average number ± SEM of firmly adhering cells (**B**) and the average ± SEM rolling velocity (**C**) is shown from three independent experiments. *$P < 0.05$ compared with unstimulated mGEnC-1.

Removal of HS on TNF-α-activated mouse glomerular endothelial cells and the addition of heparin, tinzaparin or HS interfere with the rolling and adhesion of 32Dcl3 granulocyte cells

Previously, we showed that heparin, the low molecular weight heparin tinzaparin and HS reduced the firm adhesion of 32Dcl3 granulocyte cells to TNF-α-activated mGEnC-1 under static conditions in an *in vitro* adhesion assay (Table 1; A. Rops *et al.*, unpublished data). Here, we evaluated the effect of removal of HS on TNF-α-activated mGEnC-1 by heparinase III treatment, and the addition of heparin, tinzaparin and HS on the number of rolling and firmly adhering 32Dcl3 granulocyte cells, and on the rolling velocity of 32Dcl3 granulocyte cells

Table 1. Percentage inhibition and the concentrations giving 25% inhibition of firm adhesion of granulocytes to activated mGEnC-1 by heparin, tinzaparin and heparan sulfate in a static adhesion system

| Heparinoid/GAG	Inhibition of adhesion at 250 µg/ml (%)	IC$_{25}$ (µg/ml)
Heparin	44.7 ± 1.7	18
Tinzaparin (Innohep®)	54.1 ± 0.9	11
Heparan sulfate	42.1 ± 0.5	36

The percentage inhibition of adhesion by 250 µg/ml heparin, tinzaparin or heparan sulfate is expressed as the mean ± SEM of six experiments. The concentrations giving 25% inhibition (IC$_{25}$) in µg/ml are indicated. GAG, glycosaminoglycan.

on activated mGEnC-1 cells at the shear stress rate of 0.8 dynes/cm^2. It appeared that removal of HS on mGEnC-1 and the addition of 250 µg/ml heparin, tinzaparin or HS significantly reduced about 2–4-fold the number of rolling and firmly adhering 32Dcl3 granulocyte cells on TNF-α-activated mGEnC-1 (Figure 5A and B), while the rolling velocity of 32Dcl3 granulocyte cells increased more than 2-fold (Figure 5C). We also tested the effect of two lower concentrations of heparin and tinzaparin, i.e. 10 and 50 µg/ml. Notably, the concentration of 10 µg/ml approximates the concentrations that can be found in the circulation of patients that have received heparin or tinzaparin intravenously. It appeared that even the addition of 10 µg/ml heparin (Figure 6A) or tinzaparin (Figure 6B) reduced the number of rolling 32Dcl3 granulocyte cells, while these effects were even more pronounced for the number of firmly adhering 32Dcl3 granulocyte cells (Figure 6C and D) and the average rolling velocity (Figure 6E and F). In summary, our data show that HS on activated glomerular endothelium is crucial for the interaction with leucocytes under dynamic flow conditions, while heparin(oids) interfere with the interaction of leucocytes with activated glomerular endothelium.

Discussion

In this study, we describe the rolling and adhesion of leucocytes to unstimulated or TNF-α-activated mouse glomerular endothelial cells (mGEnC-1) at shear stress rates that approach the shear stress rate in mouse glomerular capillaries. Furthermore, we evaluate the effects of clinically applied heparin, the low molecular weight heparin tinzaparin and HS, on leucocyte trafficking using an *in vitro* flow system.

At the shear stress rates of 0.4 and 1.6 dynes/cm^2 no or minor differences could be observed in the rolling, adhesion and velocity of 32Dcl3 granulocyte cells on unactivated mGEnC-1 *vs* activated mGEnC-1, which may be explained by too low and too high volumetric flow rates, respectively. The lower rolling velocity of 32Dcl3 granulocyte cells at 1.6 dynes/cm^2 compared with 0.8 dynes/cm^2 may be explained by the fact that
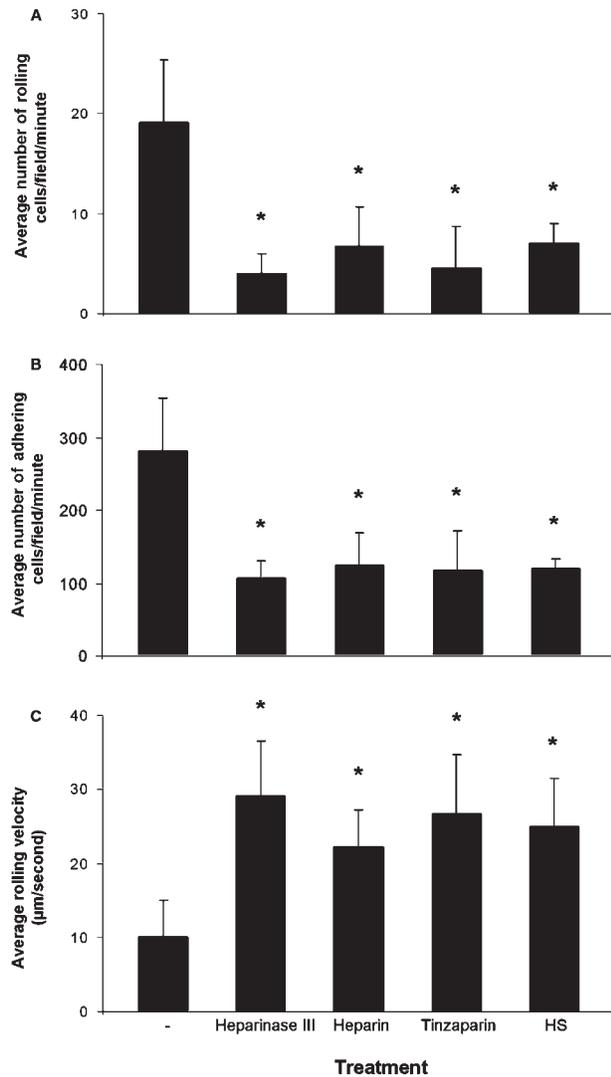
Fig. 5. Removal of HS on TNF-α-activated mouse glomerular endothelial cells and the addition of heparin, tinzaparin or HS, interfere with the rolling and adhesion of 32Dcl3 granulocyte cells. A monolayer of mGEnC-1 was stimulated with TNF-α for 18 h. Activated mGEnC-1 was incubated with 32Dcl3 granulocyte cells without further addition, after removal of HS on mGEnC-1 with heparinase III, or co-incubated with 250 µg/ml heparin, tinzaparin or HS. The 32Dcl3 granulocyte cells were perfused at a shear stress rate of 0.8 dynes/cm^2. The average number ± SEM of rolling cells (A), the average number ± SEM of firmly adhering cells (B) and the average ± SEM rolling velocity (C) is shown from three independent experiments. *$P < 0.05$ compared with untreated cells. HS, heparan sulfate.

fast rolling cells weakly interacted with activated mGEnC-1 and lost their interaction at 1.6 dynes/cm^2, while the slowly rolling cells interacted more strongly with activated mGEnC-1 and resisted the shear stress rate of 1.6 dynes/cm^2, which also has been found by others [17]. The calculated shear stress rate of about 0.8 dynes/cm^2 in glomerular capillaries *in vivo* appeared to be the most suitable shear stress rate in our *in vitro* flow system. Activation of mGEnC-1 induced significantly more rolling and adhesion of 32Dcl3 granulocyte cells, which was accompanied with a reduced
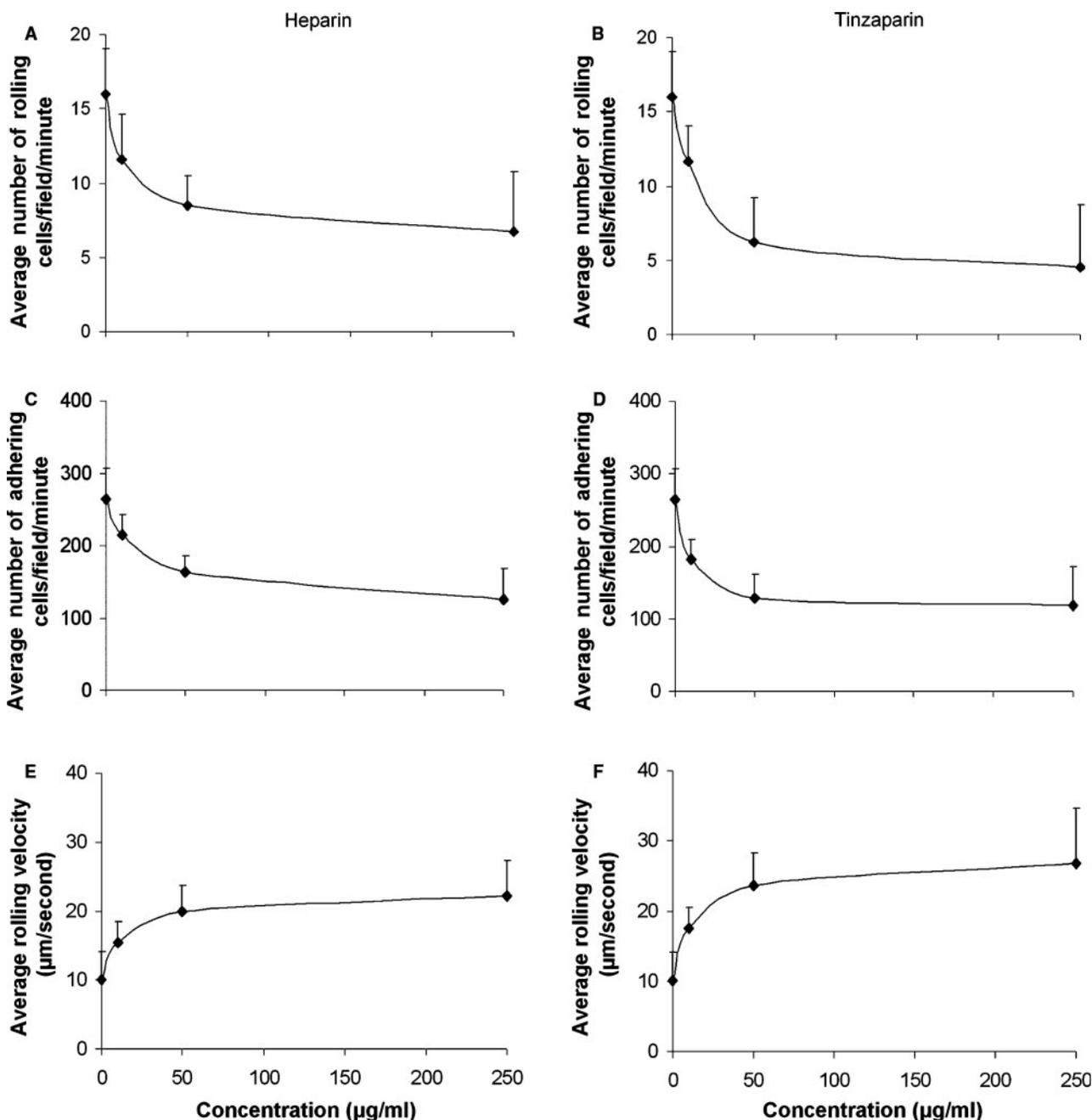
Fig. 6. Dose-response effect of heparin and tinzaparin on the rolling and adhesion of 32Dcl3 granulocyte cells on TNF-α-activated mouse glomerular endothelial cells. A monolayer of mGEnC-1 was stimulated with TNF-α for 18 h. Activated mGEnC-1 was incubated with 32Dcl3 granulocyte cells without further addition, or together with 10, 50 or 250 μg/ml heparin or tinzaparin, respectively. The 32Dcl3 granulocyte cells were perfused at a shear stress rate of 0.8 dynes/cm². The average number ± SEM of rolling cells (**A** and **B**), the average number ± SEM of firmly adhering cells (**C** and **D**) and the average ± SEM rolling velocity (**E** and **F**) are shown from three independent experiments.

average rolling velocity of the 32Dcl3 granulocyte cells. To our knowledge, our data are the first to show leucocyte trafficking on a well-defined glomerular endothelial cell line using an *in vitro* flow system. Our data confirm the *in vitro* results of various other studies that show differences in the number of rolling and firmly adhering monocytes, eosinophils, leucocytes and granulocytes under different shear stress rates to activated human umbilical vein endothelial cells (HUVEC) [18,19]. Furthermore, an increased

number of rolling and firmly adhering neutrophils accompanied with a reduced rolling velocity on TNF-α-activated endothelium compared to unacti-vated endothelium *in vitro* has also been described for endothelium from other vascular beds [20].

Recently, we have shown that HS on activated glomerular endothelial cells was crucial for the firm adhesion of leucocytes using a static adhesion assay. Furthermore, heparin, HS and low molecular weight heparin preparations such as tinzaparin reduced the

firm adhesion of 32Dcl3 granulocyte cells to activated mGEnC-1 about 2-fold under static conditions (A. Rops *et al.*, unpublished data). In the current study, we demonstrated that removal of HS on activated mGEnC-1 and addition of heparin, tinzaparin and HS reduced the number of rolling and firmly adhering 32Dcl3 granulocyte cells several fold, which was accompanied with an increased rolling velocity. Rolling of leucocytes is mediated by L-selectin on the leucocyte and the L-selectin ligand, i.e. 6-*O*-sulfated HS, on activated (glomerular) endothelium [2]. Firm adhesion of leucocytes is mediated by integrins such as Mac-1 on the leucocyte and the Mac-1 ligand, i.e. *N*- and 6-*O*-sulfated HS, on activated (glomerular) endothelium [2]. The effects we observed after the removal of HS on glomerular endothelial cells directly prove that HS on activated glomerular endothelial cells is crucial for the rolling, firm adhesion and rolling velocity of leucocytes, most likely because of the loss of the L-selectin/Mac-1 HS ligands. Notably, the 32Dcl3 granulocyte cells lack cell surface HS, so we exclusively could evaluate the role of HS on glomerular endothelial cells in the interaction with leucocytes. The effects of the addition of heparin, tinzaparin and HS can be explained by the competitive binding of these compounds to L-selectin and/or Mac-1. Therefore, both the loss of endothelial HS and the competitive binding of heparinoids to L-selectin/Mac-1 on leucocytes lead to interference with the leucocyte–endothelium interaction, i.e. a lower number of rolling leucocytes, a higher rolling velocity of leucocytes and a lower number of firmly adhering leucocytes. Another possible explanation is that removal of endothelial HS or incubation with heparinoids leads to loss of bound chemokines that activate integrins on leucocytes required for the firm interaction with endothelial cells. However, this possibility is very unlikely, since our 32Dcl3 cells appear to be already fully activated and express high levels of L-selectin and Mac-1. Furthermore, we have pre-incubated activated mGEnC-1 with high concentrations of heparin in order to compete off HS-bound chemokines. However, this revealed that the adhesion of 32Dcl3 granulocyte cells was not affected (data not shown) as has been shown by others [16]. Other *in vitro* studies under flow conditions with different vascular beds also showed that the addition of heparinoids decreased the number of rolling and firmly adhering leucocytes to activated endothelium [21–23]. In a recent study, it was shown that in particular endothelial HS is important for the leucocyte trafficking over activated endothelium. Mice that were conditionally deficient in the HS biosynthetic enzyme N-deacetylase/N-sulfotransferase-1, i.e. in both endothelium and leucocytes, showed a 2–3-fold reduction of *N*-, 2-*O*- and 6-*O*-sulfated groups on the endothelium and a higher velocity of rolling leucocytes by reduced binding to L-selectin [24].

Treatment of proliferative forms of glomerulonephritis with immunosuppressive drugs like corticosteroids and cyclophosphamide is basically the same as 30 years ago. Therefore, the determination of the exact structure of HS domains on activated glomerular endothelium that serve as ligands for L-selectin, Mac-1 or other leucocyte receptors could lead to the development of well-defined HS-based therapeutics without anticoagulant activity for supplementary treatment of these forms of glomerulonephritis. However, determination of the fine structure, i.e. sequence, of these specific HS domains remains a major future challenge, which will require additional research, in which our glomerular endothelial cell line mGEnC-1 and the flow system used could be of value. Until these defined HS-based compounds become available, it is an interesting option to evaluate heparin and low molecular weight heparin preparations with a low antithrombotic activity as supplementary therapeutics in the treatment of proliferative glomerulonephritis [25].

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