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Heparan sulfate expression is affected by inflammatory stimuli in primary human endothelial cells

Trine M. Reine · Marion Kusche-Gullberg · Almir Feta · Trond Jenssen · Svein O. Kolset

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Abstract In diabetes the endothelium is either chronically or transiently exposed to hyperglycemic conditions. In addition, endothelial dysfunction in diabetes is related to changes in the inflammatory response and the turnover of extracellular matrix. This study was undertaken to study the effects of inflammatory stimuli on one particular matrix component, the heparan sulfate (HS) proteoglycans (PGs) synthesized by primary human umbilical cord vein endothelial cells (HUVEC). Such cells were cultured *in vitro* in 5 mM and 25 mM glucose. The latter concentration was used to mimic hyperglycemic conditions in short-term experiments. HUVEC were also cultured in the presence of the inflammatory agents tumor necrosis factor α (TNF-α), interleukin 1α (IL-1α), interleukin 1β (IL-1β) and transforming growth factor β (TGF-β). The cells were labeled with ^{35}S-sulfate and ^{35}S-PGs were recovered for further analyses. The major part of the ^{35}S-PGs was secreted to the medium, irrespective of type of stimuli. Secreted ^{35}S-PGs were therefore isolated and subjected to further analyses. TNF-α and IL-1α slightly increased the release of ^{35}S-PGs to the culture medium, whereas IL-1β treatment gave a significant increase. The different treatments neither changed the ratio of ^{35}S-HS and ^{35}S-chondroitin sulfate (CS) nor the macromolecular properties of the ^{35}S-PGs. However, the ^{35}S-HS chains were slightly increased in size after TNF-α treatment, and slightly decreased after TGF-β treatment, but not affected by the other treatments. Compositional analysis of labeled disaccharides showed changes in the amount of 6-*O*-sulfated glucosamine residues after treatment with TNF-α, IL-1α and IL-1β. Western immunoblotting showed that major HSPGs recovered from these cells were collagen XVIII, perlecan and agrin, and that secretion of these distinct PGs was increased after IL-1β stimulation. Hence, short term inflammatory stimuli increased the release of HSPGs in HUVEC and affected both the size and sulfation pattern of HS, depending on type of stimuli.

Keywords Endothelial cells · Heparan sulfate · Hyperglycemia · Inflammation · Cytokines · 6-O sulfation

Abbreviations

AGE	Advanced glycation end product
CS	Chondroitin sulfate
aMan	Anhydromannitol
DS	Dermatan sulfate
ECM	Extracellular matrix
FCS	Fetal calf serum
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GlcA	Glucuronic acid
GlcN	Glucosamine
HG	High glucose
HS	Heparan sulfate

T. M. Reine · S. O. Kolset (✉)
Department of Nutrition, Institute of Basic Medical Sciences,
University of Oslo,
Box 1046, Blindern,
0316 Oslo, Norway
e-mail: s.o.kolset@medisin.uio.no

M. Kusche-Gullberg · A. Feta
Department of Biomedicine, University of Bergen,
5020 Bergen, Norway

T. Jenssen
Kidney Section, Department of Organ Transplantation,
Oslo University Hospital – Rikshospitalet,
0027 Oslo, Norway

T. Jenssen
Department of Clinical Medicine, University of Tromsø,
9037 Tromsø, Norway

HUVEC	Human umbilical cord vein endothelial cell
IdoA	Iduronic acid
IL-1α	Interleukin 1α
IL-1β	Interleukin 1β
LG	Low glucose
PG	Proteoglycan
TGFβ	Transforming growth factor β
TNFα	Tumor necrosis factor α
VEGF	Vascular endothelial growth factor
Qsulf	Quail sulfatase
6-OST	6-*O*-sulfotransferase

Introduction

The endothelium constitutes an important part of the human body, comprising approximately of 10^{13} cells and weighing around 1 kg [1]. These cells are continuously exposed to circulating blood and their turnover and functions affected, both in short term and long term perspectives, by changes in the composition of circulating cells, lipids and proteins. In diabetes, the glucose levels can fluctuate to a large extent, which can lead to generation of advanced glycation end products (AGE) [2]. In both type 1 and type 2 diabetes the endothelial cells are affected, and endothelial dysfunction related to vascular complications of these diseases has been the focus of increasing attention, both by clinical and basic researchers [3].

Endothelial dysfunction in relation to diabetes is a complex phenomenon, including both increased risk for atheroma formation, changes in vascular flexibility, increased expression of cytokines and chemokines and changes in the turnover of extracellular matrix (ECM) components and enzymes involved in extracellular matrix (ECM) turnover [4]. Several studies have shown that endothelial cells increase the release of inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin 1α (IL-1α) and interleukin 1β (IL-1β) under hyperglycemic conditions, and that the release of cell adhesion molecules also is elevated [5]. Clearly, this leads to micro- and macrovascular changes affecting several organs, but also to changes locally in the endothelium as a separate entity.

We have previously established that exposure of HUVEC to AGEs or hyperglycemia leads to changes in both proteoglycan (PG) expression [6] and secretion of matrix metalloproteinases [7]. We have also shown that the PG expression of kidney epithelial cells is modified by hyperglycemia or AGEs [8]. In general, several reports have documented changes in PG expression in diabetes, both in clinical research using biopsies and in experimental systems using cultured cells. One major focus has been on the sulfation of heparan sulfate (HS). Early reports [9] showed that decreased sulfation of HS is contributing to proteinuria. This is still an issue of some debate [10], primarily on the functional importance of HS changes seen in the diabetic kidney.

Several human cell lines have been used to study the effects of hyperglycemia on ECM changes. Some studies have focused on human mesangial cells [11], whereas others have made use of endothelial cells lines, either from aorta or umbilical cord. A limited number of studies have made use of primary human endothelial cells. In the present study we wanted to make use of primary cells from umbilical cord to investigate in more detail the effects of hyperglycemia and inflammatory conditions on HS expression. Results presented show that HS secretion, macromolecular properties and disaccharide sulfation was affected differently, depending on the inflammatory agent used. Our studies suggest that inflammatory conditions affect HS expression in human endothelial cells.

Materials and methods

Cell culture

Human umbilical cord vein endothelial cells (HUVEC) were isolated from umbilical cords as described [12]. Cells were cultured in 75 cm^2 cell culture flasks 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 used for experiments within three passages.

For experiments, confluent cells were subjected to high glucose (25 mM), IL-1α (2 ng/ml), TNF-α (7 ng/ml) [13], transforming growth factor β (TGF-β, 10 ng/ml) [14] or IL-1β (0.5 ng/ml)[15], all agents from R&D, in medium with 2% FCS for 24 h. Medium with low glucose (5 mM) was used as control in all experiments.

Metabolic labeling of glycosaminoglycans

HUVEC were metabolically labeled with 0.1–0.2 mCi/ml ^{35}S-sulfate (Hartmann Analytic) in RPMI-1640 sulfate free medium (GIBCO Invitrogen) containing 5 mM L-glutamine (Sigma) and with FCS reduced from 7 to 2% to increase labeling efficiency.

After labeling for 24 h under normal, hyperglycemic or inflammatory conditions, the culture medium was collected and cell debris removed by centrifugation. The cells were washed in PBS and harvested in lysis buffer (4.0 M guanidine-HCl, 0.1 M acetate buffer pH 6.5, 2% Triton X-

100). In order to remove unincorporated ^{35}S-sulfate and to change the buffer, samples of 1 ml were 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 smaller molecules remained associated with the column. The amount of ^{35}S-sulfate incorporated in newly synthesized ^{35}S -macromolecules was determined by scintillation counting. Protein content of the cell lysate was determined using Uptima BC Assay protein quantization kit (BioRad).

SDS-PAGE

Media-samples containing equal amount of radiolabeled material, approximately 10–15000 cpm, were subjected to SDS-PAGE (4–20% Tris–HCl gradient gels) before and after treatment with chondroitin ABC lyase (cABC, E. C.4.2.2.4, Sigma) or nitrous acid (HNO_2), and separated at 110 V for 2 h. CS (chondroitin sulfate) and DS (dermatan sulfate) glycosaminoglycan (GAG) chains were degraded by incubation at 37°C overnight with 0.01–0.02 units of cABC in 0.05 M Tris–HCl pH 8.0 containing 0.05 M sodium acetate and 0.02% BSA. In parallel samples, HS was depolymerized by HNO_2 deamination at pH 1.5, cleaving the polysaccharide at N-sulfated glucosamine (GlcN) units as described [16]. Briefly, equal volumes of cold 0.5 M Ba $(NO_2)_2$ and 0.5 M H_2SO_4 were mixed and centrifuged to remove precipitated $BaSO_4$. Equal volumes of the resulting HNO_2 and samples were mixed and incubated for 10 min at room temperature. The reaction was stopped by the addition of 2 M Na_2CO_3 to obtain neutral pH.

Prior to loading, the samples were incubated at room temperature for 15 min in sample buffer containing SDS and mercaptoethanol as reducing agent. After completed electrophoresis the gel was treated with fixing solution (isopropanol 25%, glacial acetic acid 10%) and Amplify (Amersham). Thereafter the gels were dried and subjected to fluorography using Amersham Hyperfilm™ ECL for approximately 4 days at −80°C. The intensity of the bands was quantified using the quantization software Scion Image (Scion Corp.) or Quantity One (BioRad).

Purification of ^{35}S-HS

After cABC depolymerization, the obtained HSPGs were subjected to alkali β-elimination to release intact ^{35}S-HS-chains from their core proteins. To the samples were added 5 M NaOH to a final concentration of 0.5 M and incubated at 4°C over night. The reaction was stopped by neutralization with 10 M HCl. The resultant ^{35}S-HS chains were recovered by DEAE ion-exchange chromatography. The samples were applied to individual DEAE-Sephacel polyprep-columns equilibrated in 0.15 M Tris–HCl buffer, pH 8.0 with 0.15 M NaCl, washed in the same buffer, thereafter with the same buffer with 0.3 M NaCl before bound material was eluted in Tris–HCl-buffer with 2.0 M NaCl. The eluate was desalted and concentrated using Vivaspin ultrafiltration devices (MW cut-off 3 kDa, GE Healthcare).

Macromolecular properties of ^{35}S-HSPGs and ^{35}S-HS

The approximate chain length of ^{35}S-labeled HS was determined by gel chromatography on a Sepharose CL-6B column (48x1 cm) run in 0.05 M Tris–HCl pH 8 with 0.2 M NaCl. Fractions of 400 or 800 μl were collected and analyzed for radioactivity by scintillation counting. The elution profiles were determined relative to the elution of the V_0 marker blue dextran (2500 kDa) and the V_t marker phenol red (0.36 kDa).

In some experiments, intact ^{35}S-HSPGs were subjected to Sepharose CL-4B gel chromatography, using the same buffers and elution markers.

Anionic properties of ^{35}S-HS

The polyanionic properties of ^{35}S-HS were analyzed by DEAE ion-exchange chromatography. The labeled molecules bound to the DEAE were eluted with a salt gradient ranging from 0.15 to 2.0 M NaCl in 0.05 M Tris–HCl pH 8.0. Aliquots of each fraction were subjected to scintillation counting and determination of the elution position of the internal CS-6 standard (Sigma) by the 1,9-dimethyl-methylene blue (DMMB) assay.

Structural analysis of disaccharides from ^{35}S-HS

For compositional analyses, samples containing approximately 150 000–250 000 cpm ^{35}S-HS was depolymerized by nitrous acid and the generated products reduced with $NaBH_4$ at 4°C over night. The reaction was terminated by addition of 4 M HAc and neutralized with NaOH.

The resulting di- and oligo-saccharides were separated by gel chromatography on a Sephadex G-15 (1.5×170 cm) column run in 0.2 M NH_4HCO_3. Internal standards, blue dextran (V_0) and glucuronolactone (Monosaccharides, Sigma) were applied with each sample. Presence of glucuronolactone in the eluted fractions was determined by the carbazole assay [17]. Aliquots of each eluted fraction were subjected to scintillation counting, and the fractions containing the radiolabeled disaccharides were pooled and desalted by lyophilisation.

Labeled disaccharides were analyzed by anion-exchange HPLC using a Whatman Partisil 10-SAX column (Whatman Inc.) eluted with a stepwise salt gradient ranging from 0.028 to 0.15 M KH_2PO_4. The elution profiles were compared to

those of the standards: GlcA-aMan_R6S (GMS), IdoA-aMan_R6S (IMS), IdoA2S- aMan_R (ISM), IdoA2S- aMan_R6S (ISMS), corresponding to GlcA-GlcNS6S, IdoA-GlcNS6S, IdoA2S-GlcNS and IdoA2S-GlcNS6S in the intact HS chain.

Western blotting

HUVEC were stimulated in MCDB-131 medium as described but with serum reduced to 2%. After 24 h the medium was collected and cell debris removed by centrifugation. Cells were washed in cold PBS and lysed in 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). PGs were purified by DEAE ion-exchange chromatography, and aliquots were either untreated or subjected to cABC or HNO_2 depolymerization of the GAG chains. The volumes of the media-samples were adjusted according to protein content in the corresponding cell fractions, before subjection to SDS-PAGE on 4–20% gradient gels and electroblotting onto PVDF membranes (Millipore) using the Criterion™gel system (BioRad). Primary antibodies polyclonal rabbit anti-human perlecan (kindly provided by prof. R.V. Iozzo, Thomas Jeffersons University, USA, 1:500 dilution), polyclonal rabbit anti-human collagen XVIII (H-140, Santa Cruz, 1:200) and monoclonal mouse anti-human agrin (Calbiochem, 1:1000) were used. The secondary antibodies used were HRP-linked donkey anti-rabbit IgG (1:50000) and HRP-linked sheep anti-mouse (1:5000), both from GE Healthcare. The membranes were developed using ECL Western Blotting Detection Reagents (GE Healthcare) and finally exposed to films.

Results

^{35}S-PG expression in HUVEC

To evaluate the influence of hyperglycemia and inflammatory mediators on endothelial PGs, HUVEC were cultured under normoglycemic conditions with 5 mM glucose (LG), in hyperglycemic medium with 25 mM glucose (HG) or in the presence of the inflammatory agents TNF-α, IL-1α, TGF-β and IL-1β. Cells were metabolically labeled with ^{35}S-sulfate and labeled macromolecules were recovered from the cell layer and the medium by gel chromatography and the amount of ^{35}S-macromolecules determined. Approximately 90% of the ^{35}S-macromolecules were secreted to the medium, as shown in Fig. 1A. Only a minor part of *de novo* synthesized macromolecules were found in the cell fraction. We therefore chose to focus on secreted PGs in this study. When cells were cultured in HG medium or in the presence of the inflammatory agent TGF-β, we did not observe any effect on the secretion of ^{35}S-macromolecules. In contrast, cells cultured in the presence of TNF-α, IL-1β and IL-1α showed a tendency to increased secretion. However, there was a statistically significant increase of approximately 40% for IL-1β as can be seen in Fig. 1B,

Previous studies have shown that ^{35}S-macromolecules secreted from HUVEC were almost exclusively PGs [6]. To investigate if the different experimental conditions employed in this study affected the types of ^{35}S-PGs synthesized and secreted by HUVEC, equal amounts of ^{35}S-macromolecules recovered from the medium were divided into three aliquots. The different aliquots were analyzed directly by SDS-PAGE, or after depolymerization with HNO_2 treatment or digestion with cABC, an enzyme that depolymerizes CS and DS, prior to SDS-PAGE analyses. From Fig. 2 it is evident that HUVEC secreted three major components that are ^{35}S-PGs. The high molecular weight ^{35}S-PGs were of HS nature, as they were depolymerized with HNO_2 (labeled H in the figure). The two other components were PGs of the CS/DS type, evident by their complete degradation with cABC (labeled C in the figure). This was also confirmed by CL-4B gel chromatography (results not shown). Based on quantization of the different bands the proportion of HS was 26±5% for all samples. From these analyses it is also evident that the relative amounts of HS and CS/DS did not change after the different treatments. Hence, the increase in ^{35}S-PG secretion shown in Fig. 1b after stimuli with TNF-α, IL-1α and IL-1β most likely reflect an increased secretion of both types of PGs.

Macromolecular properties of ^{35}S-HSPGs and ^{35}S-HS

To investigate if the observed increase in ^{35}S-PG secretion (Fig. 1B) was due to changes in the molecular size, the ^{35}S -PGs were subjected to Sepharose CL-4B gel chromatography. The elution patterns were almost identical for all six samples analyzed; suggesting that the molecular size of the secreted PGs were not affected (result not shown). However, to analyze this in more detail, ^{35}S-PGs were treated with cABC to degrade CS/DS chains, and then subjected to alkali treatment to release intact HS chains from the PGs. Comparative analyses of the released ^{35}S- HS chains by Sepharose CL-6B chromatography, shown in Fig. 3, revealed similar elution profiles with two exceptions. The ^{35}S-HS chains from HUVEC treated with TGF-β were slightly shorter than those from control (LG) cells and corresponding chains from TNF-α treated cells were somewhat longer than control. The changes were minor but reproducible. From these studies we conclude that some inflammatory stimulators used affect the macromolecular properties of HS expressed by HUVEC, although in different ways. ^{35}S-CS chains were also analyzed by Sepharose CL-6B gel chromatography and no difference in elution patterns was

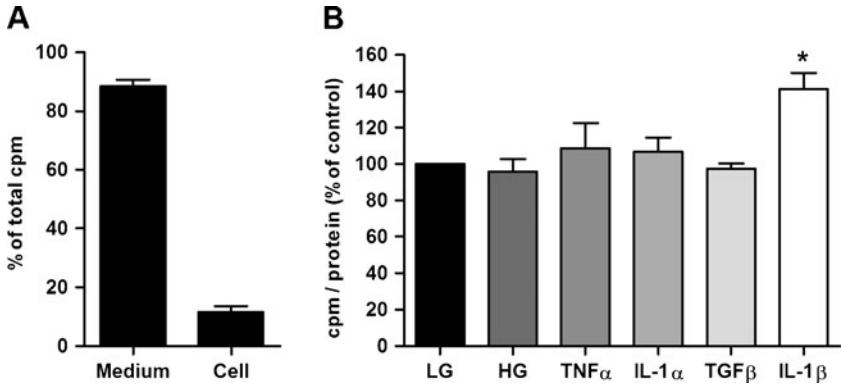

Fig. 1 ^{35}S-macromolecules in HUVEC. HUVEC were metabolically labeled with ^{35}S-sulfate and de novo synthesized ^{35}S-PGs were harvested, purified by gel filtration and quantified by scintillation counting. **a** Percentage of secreted (medium) versus cellular (cell) ^{35}S-PGs synthesized by HUVEC under normoglycemic conditions. The results are based on HUVEC from five different individuals, quantified by scintillation counting and presented as mean % of total radioactivity (cpm) ± S.D. **b** ^{35}S-PGs secreted from HUVEC subjected to normoglycemia (LG), hyperglycemia (HG) or various inflammatory conditions (TNF-α, IL-1α, TGF-β, IL-1β). The results are based on data from 4 or 7 (IL-1β) individuals. Ratios obtained from scintillation counting (cpm/mg protein) are presented as percent of control (LG) ± S.E.M. Statistically significant differences were identified using students *t* tests ($P<0.01$) and denoted by asterisks

observed for material obtained from HUVEC subjected to the different treatments (not shown).

Sulfation of ^{35}S-HS from HUVEC

The increase in ^{35}S-PG synthesis shown in Fig. 1B could possibly be due to differences in sulfation density of the HS chains expressed. To investigate this further, the anionic properties of ^{35}S-HS chains were analyzed by DEAE ion-exchange chromatography with CS as internal standard. The elution profiles were almost identical for all the samples analyzed. In Fig. 4 samples from control cells (LG) and from cells exposed to IL-1β are shown. No difference in the peak elution compared to the position of the internal standard could be observed, however, the material from IL-1β treated cells displayed a less heterogeneous profile than the control material.

To further investigate possible changes in the sulfation of HS, ^{35}S-HS obtained from the different HUVEC cultures were analyzed for disaccharide composition by strong anionic-exchange chromatography, as shown in Fig 5A. The proportion of ^{35}S recovered as disaccharides after Sephadex G-15 separation was approximately 90%. The disaccharide elution profiles were compared with those of characterized HS disaccharide standards, and the amount of the distinct ^{35}S-labeled, O-sulfated disaccharides were calculated. As can be seen from Fig. 5B, treatment with TNF-α, IL-1α and IL-1β resulted in a small but reproducible increase in the 2-*O*-sulfated disaccharide IdoA2S-GlcNS. No obvious effect was observed in ^{35}S-HS from HUVEC

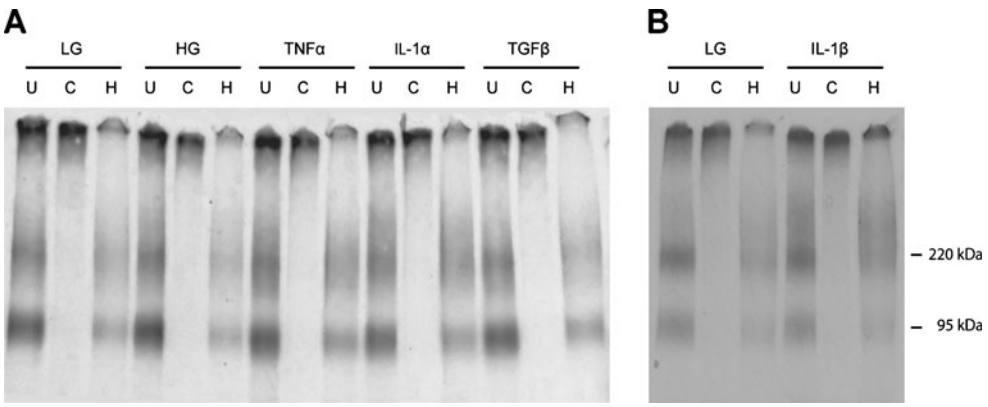

Fig. 2 SDS-PAGE of ^{35}S-PGs secreted from HUVEC. HUVEC were metabolically labeled with ^{35}S-sulfate and exposed to the indicated inflammatory or hyperglycemic conditions. Secreted ^{35}S-PGs were subjected to SDS-PAGE before and after HS depolymerization with HNO_2 or CS/DS digestion with cABC. Samples containing equal amounts of cpm were loaded to each well. **a** SDS-PAGE of ^{35}S-PGs secreted from cells exposed to high glucose (HG), TNF-α, IL-1α and TGF-β compared to control (LG). **b** SDS-PAGE of ^{35}S-PGs secreted from cells exposed to IL-1β compared to control (LG). The migration positions of molecular weight markers (in kDa) are shown on the right side of the panel. The letters above each lane indicate the treatment of the sample, U: Untreated, C: cABC digested, H: HNO_2 treated

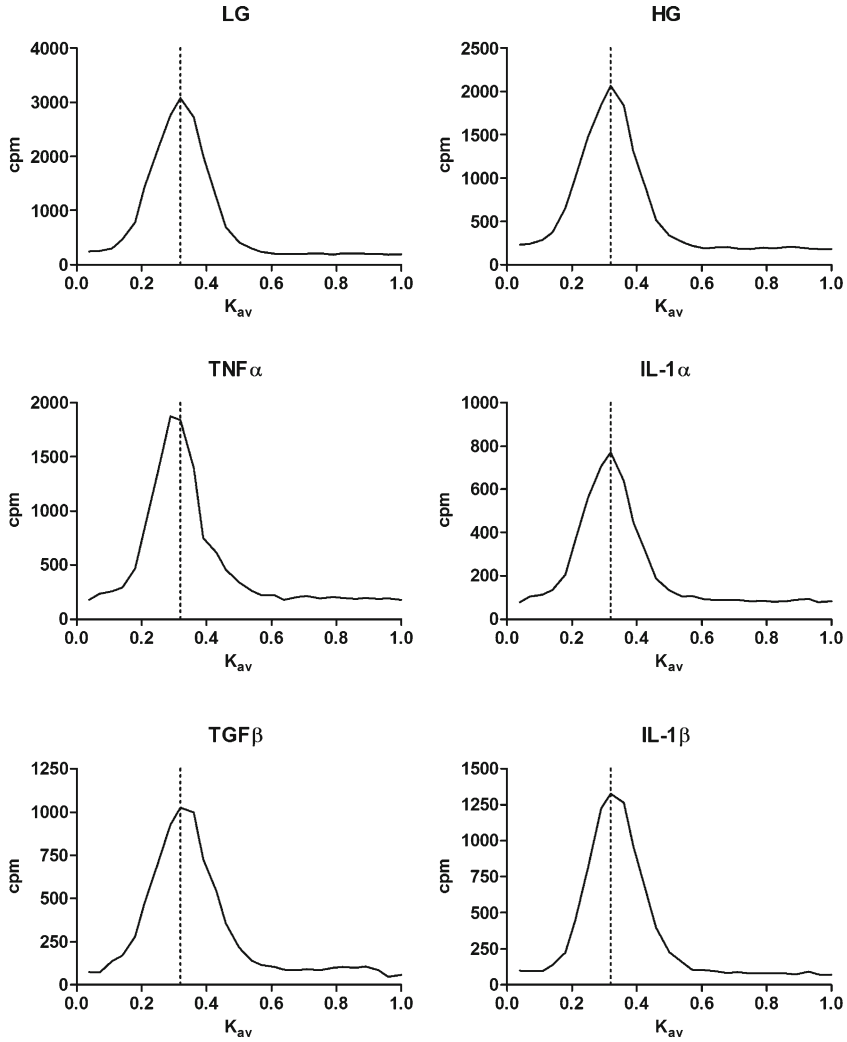

Fig. 3 Effect of hyperglycemia and inflammatory agents on ^{35}S-HS chain length. Cultured HUVEC were exposed to the indicated treatments and metabolically labeled with ^{35}S-sulfate. ^{35}S-HS chains were isolated and analyzed by Sepharose CL-6B gel chromatography. The column was eluted with 0.15 M NaCl, 0.05 M Tris–HCl pH 8. Shown is one representative chromatogram out of 4 independent labeling experiments. The dotted line in each graph indicates the peak K_{av} value of the control (LG)

treated with TGF-β or exposed to hyperglycemia. The overall relation between 2-*O*-sulfated and 6-*O*-sulfated disaccharide units was essentially unchanged in HUVEC treated with HG and IL-1β (Table 1). Treatment with TNF-α and TGFβ, on the other hand, resulted in a small decrease in the total amount of 6-*O*-sulfated glucosamine residues whereas IL-1α treatment had the opposite effect. In summary, our analyses suggest that stimulation of HUVEC with the inflammatory agents used in this study may result in minor, but possibly important, changes in HS sulfation. In contrast, both control cells and IL- 1β stimulated cells released CS disaccharides where approximately 75% was of the chondroitin 4-*O*-sulfate type (unpublished data).

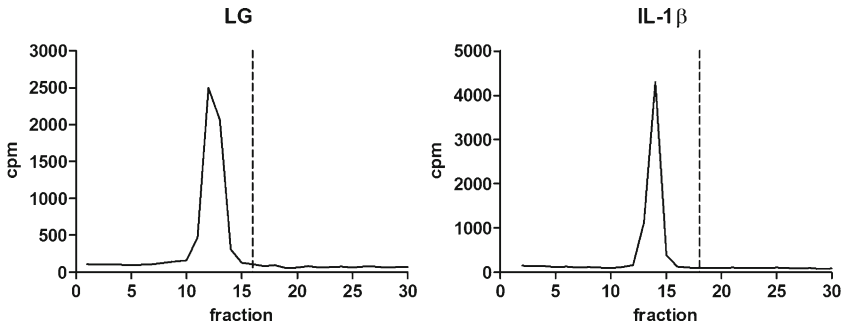

Fig. 4 Effect of IL-1β on ^{35}S-HS polyanionic properties. HUVEC were metabolically labeled with ^{35}S-sulfate in the absence (LG) or presence (IL-1β) of IL-1β for 24 h. Isolated ^{35}S-HS chains were analyzed by DEAE ion-exchange chromatography with a salt gradient ranging from 0.15 to 2 M NaCl. The peak fraction of the internal standard, CS-6, detected by the DMMB assay, is indicated by the dotted line

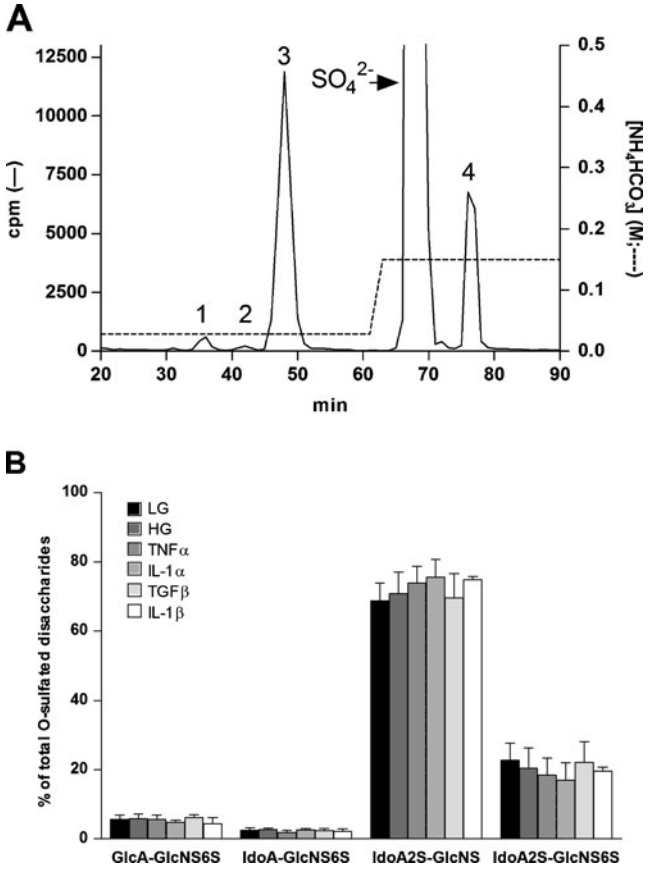

Fig. 5 HPLC analysis of disaccharides derived from ^{35}S-HS secreted from HUVEC. HUVEC were metabolically labeled with ^{35}S-sulfate in the absence or presence of the indicated treatments. Isolated ^{35}S-disaccharides were analyzed by anion-exchange HPLC SAX chromatography. **a** One elution profile from control material (LG) is shown. Isolated disaccharides were analyzed on a Partisil-10 SAX column eluted at a rate of 1 ml/min with KH_2PO_4 solutions of stepwise increasing concentration (as indicated by the broken line). Mono-O-sulfated disaccharides were eluted with 0.028 M and di-O-sulfated disaccharides with 0.15 M KH_2PO_4. The numbered arrows indicate the elution position of ^{3}H-labeled disaccharide standards and represent the following disaccharide units in the intact HS chain; *1*, GlcA-GlcNS6S; *2*, IdoA-GlcNS6S; *3*, IdoA2S-GlcNS; *4*, IdoA2S-GlcNS6S; SO_4^{2-}, represents inorganic sulfate derived from labeled N-sulfate groups in the intact polysaccharide. **b** The identity of the disaccharides was verified by comparing with the elution of characterized standards. The results are based on data from 4 different individuals (with exception of IL-1β, which is based on a duplicate from one individual) and presented as % of total O-sulfated disaccharides ± S.E.M. Statistically significant differences were investigated using students t tests ($P<0.01$). No significant differences were observed

HSPGs expressed by HUVEC

Finally, to investigate the effect of inflammatory conditions on the secretion of major extracellular matrix HSPGs expressed by primary endothelial cells, medium was harvested from control cells and cells exposed to IL-1β. IL-1β treated cells were chosen as they showed the largest increase in ^{35}S-PG secretion (see Fig. 1B). Samples were subjected to Western blotting using antibodies against perlecan, agrin and collagen XVIII, both before and after treatment with cABC or HNO_2. From Fig. 6 it is evident that HUVEC express and secrete the HSPGs perlecan, agrin and collagen XVIII. Furthermore, the secretion of all these three distinct PGs increased after IL-1β stimulation.

Table 1 % of O-^{35}S-sulfated disaccharides after the indicated treatments

Treatment	Total 2-O-^{35}S containing disaccharides	Total 6-O-^{35}S containing disaccharides
LG	92.8±1.5	32.2±4.9
HG	92.5±2.0	32.2±7.7
TNFα	92.1±2.0	27.3±6.2
IL-1α	91.6±2.1	34.5±8.0
TGFβ	93.5±1.6	28.4±6.1
IL-1β	92.5±2.5	32.5±0.5

Discussion

In this study we used primary human endothelial cells to investigate the effects of short term exposure to hyperglycemia and inflammatory conditions induced by relevant cytokines. The target for our analyzes has been the PGs because these highly diverse and polyanionic macromolecules are part of the glycocalyx [18], important for rolling and homing of lymphocytes [19, 20] and an integral part of the endothelial basement membrane [21]. Changes in PG biosynthesis and turnover may therefore affect several aspects of endothelial functions. In both type 1 and type 2 diabetes, the endothelium is affected and endothelial dysfunction in relation to these conditions is receiving increasing attention [22].

The major part of the PGs was secreted from HUVEC and was therefore the focus of these studies. We have previously established that HUVEC secreted low amounts of syndecan-1, probably as a result of shedding. Furthermore, hyperglycemia reduced PG secretion in a previous study, most likely due to culturing conditions different from than those used here. Those cells were cultured for three days in hyperglycemia, with a different medium and higher serum concentration. In addition, the cells were labeled under serum-free conditions [6]. The data presented show that HSPGs are not generally affected by inflammatory stimuli, but do, to some extent, respond differently depending upon the agent used. PG secretion was increased slightly with TNF-α, IL-1α and significantly after IL-1β exposure, but not with hyperglycemic conditions, nor with TGF-β treatment. Using Western immunoblotting we were able to show that IL-1β treatment indeed resulted in increased

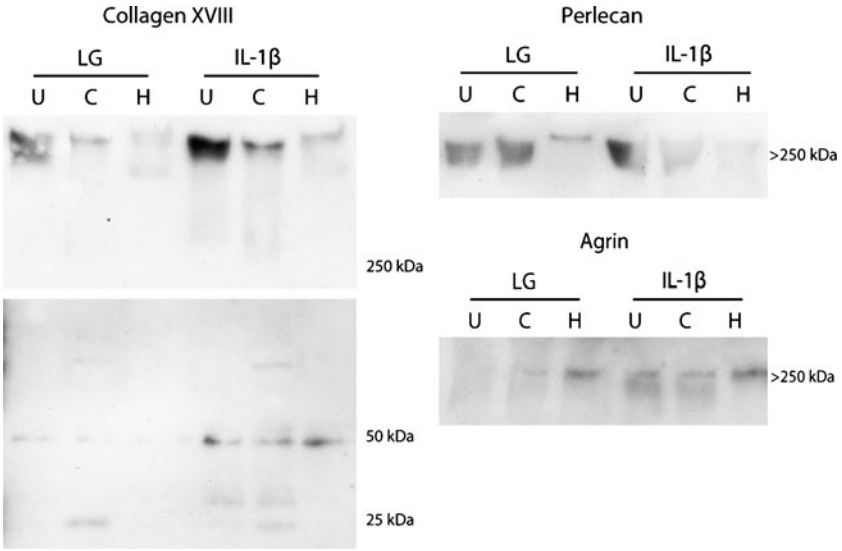

Fig. 6 Expression of HSPGs in HUVEC. Secreted PGs obtained from HUVEC cultured without (LG) and with IL-1β (IL1β) were subjected to Western blotting using antibodies against perlecan, agrin and collagen XVIII. Prior to loading, the samples were either subjected to HS depolymerization with HNO_2 or CS/DS digestion with cABC or no treatment. The lower part of the collagen XVIII immunoblot was exposed for a longer time to detect lower molecular weight fragments. The migration positions of molecular weight markers (in kDa) are shown on the right side of the panel. The letters above the lanes indicate the treatment of the sample, U: Untreated, C: cABC digested, H: HNO_2 treated

release of perlecan, agrin and collagen XVIII. Further, we observed that the macromolecular properties of secreted HSPGs were affected by TNF-α, as shown by the slight increase in the molecular size of the HS chains, and by TGF-β through a small decrease in the molecular size of HS chains. None of the other treatments, including hyperglycemia, resulted in any observed differences in chain length.

Finally, compositional analyses of ^{35}S-HS disaccharides generated by low pH nitrous acid deamination demonstrated reduced total 6-*O*-sulfation after treatment with TNF-α and TGF-β and a small increase with IL-1α. With the type of disaccharide analysis used in this study, it is not possible to determine the amount of the non-*O*-sulfated GlcA- or IdoA-containing disaccharides or the sulfation of the transition zones between N-sulfated and N-acetylated sequences [23]. Therefore, we can not exclude that, in addition to the observed changes in 6-*O*-sulfation, other small differences in disaccharide composition have occurred that are lost in our analysis. However, in this study we focused on the O-sulfation pattern of the N-sulfated domains as most, if not all, HS ligands require O-sulfation and bind to the N-sulfated domains of HS. Taken together, the data presented, using primary human endothelial cells, show that short term exposure to hyperglycemia does not affect HSPG expression, whereas several of the inflammatory agents used modulate the expression, in particular IL-1β.

Changes in PG expression in HUVEC following inflammatory stimuli are relevant for several issues related to endothelial dysfunction in diabetes. It has been shown that the 6-*O*-sulfate groups in heparin are anti-inflammatory by blocking the binding of L- and P-selectin to HS [24]. Therefore, although small, the changes in 6-*O*-sulfation in HS expressed by HUVEC might have impact on such interactions. Vascular endothelial growth factor (VEGF) is an HS binding growth factor, important for growth and formation of blood vessels. 6-*O*-sulfation of HS was shown to be of particular importance for binding to VEGF and promoting signaling [25]. Also, using endothelial cells from umbilical cords, it has been shown that VEGF induced tube formation was reduced when 6-*O*-sulfation of HS was decreased [26].

Furthermore, changes in 6-*O*-sulfation of HS have been shown to affect FGF-2 binding to the FGF receptor and decrease FGF-2 induced proliferation of target cells. In this particular study angiogenesis in chick embryos was inhibited by 6-de-*O*-sulfated heparin [27], suggesting that changes in 6-*O*-sulfation can have important implications for endothelial cell functions. Interestingly, most 6-*O*-sulfotransferase 1 (6-OST-1) knockout mice die at embryonic or perinatal stages, and mice who survive are small in size [28]. These studies therefore suggest that 6-*O*-sulfation is important for the biological activities of HS. Changes in HS expression in human endothelial cells exposed to other types of stimuli have also been reported [6, 29, 30].

Changes in 6-*O*-sulfation may be at the level of biosynthesis, through regulation of 6-OST-1 and the other 6-OST isoforms. Secondly, HS may be modified postranslationally, through the action of two 6-*O*-sulfatases, Sulf 1 and 2. Changes in HS sulfation induced by Quail sulfatase 1 (Qsulf1) have been shown to affect Wnt signaling [31]. Recently, deficiency in Sulf1 and Sulf2 in human and mouse systems were

demonstrated to affect VEGF mediated cross-talk between podocytes and endothelial cells in the kidneys, affecting filtration functions [32]. This latter study suggests a link between changes in 6-*O*-sulfation and proteinuria, highly relevant for studies on complications related to diabetes. Whether these sulfatases are upregulated in diabetes or during inflammation have not been studied in any detail and merits further investigation. Another enzyme capable of modifying HS, the heparanase, leading to generation of HS oligosaccharides, has been shown to increase in endothelial cells under hyperglycemic conditions, and possibly affecting the atherosclerotic process [33]. Consequently, changes in either molecular size or in sulfation of HS are relevant to diabetes and changes in the micro- and macrovasculature.

From these and other studies it is evident that the sulfation pattern of HS, including 6-*O*-sulfation, is important for the biological activities of HS. Results from the present study show that the 6-*O*-sulfation is changed when HUVEC were cultured with TNF-α, IL-1α or IL-1β for only 24 h. These changes in HS structures are likely to affect interactions with growth factors and possibly also chemokines. Inhibition of serglycin secretion from polarized Il-1β stimulated HUVEC was recently shown to affect secretion of the chemokine GRO-α [15]. Changes in HS sulfation in stimulated HUVEC are likely to have other biological effects relevant to diabetes and should be the subject of further studies.

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