Sulodexide reduces the inflammatory reaction and senescence of endothelial cells in conditions involving chronic venous disease

Tomasz URBANEK1 *, Zbigniew KRASINSKI2, Katarzyna SUMIŃSKA-JASIŃSKA3, Ewa BAUM4, Grażyna BOREJ-NOWICKA4, Beata BEGIER-KRASINSKA5, Andrzej BRĘBOROWICZ4,6

1Department of General and Vascular Surgery Medical University of Silesia, Katowice, Poland; 2Department of General and Vascular Surgery, Poznan University of Medical Sciences, Poznań, Poland; 3Alfa Wassermann Poland, Warsaw, Poland; 4Department of Pathophysiology, Poznan University of Medical Sciences, Poznan, Poland; 5Department of Cardiology and Hypertensiology, Poznan University of Medical Sciences, Poznan, Poland; 6Higher Vocational State School, Kalisz, Poznan

*Corresponding author: Tomasz Urbanek, Department of General and Vascular Surgery, Medical University of Silesia, Ziołowa 45/47, 40-635 Katowice, Poland. E-mail: urbanek.tom@interia.pl

ABSTRACT

BACKGROUND: According to previous studies, sulodexide suppresses intravascular inflammation when used in patients with chronic venous disease (CVD). In the current study, we tested the effect of prolonged in vitro exposure of human venous endothelial cells to the serum from patients with CVD, examining the function of these cells and how it is modified when these cells are simultaneously exposed to sulodexide.

METHODS: Human umbilical venous cells (HUVEC) were cultured in standard medium (control), in medium supplemented with 5% serum pooled from CVD patients (CVD-serum) or in medium from CVD patients who were treated with sulodexide (CVD-serum-SUL). The synthesis of interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and soluble intercellular adhesion molecule-1 (s-ICAM-1) were studied at the beginning of incubation and were measured after 9 and 15 days of exposure to the studied media. The concentration of IL-6 after cell stimulation by interleukin-1 (IL-1) was also measured. In a subsequent part of the experiment, the effect of the studied sera on the in vitro replicative ageing of HUVEC was evaluated. A total of 15 passages of the cell culture were performed and both the PDT (population doubling time) and the cell hypertrophy were assessed.

RESULTS: The concentrations of IL-6, MCP-1, and ICAM-1 gradually increased in the supernatants containing 5% CVD serum compared with the control medium. In the supernatants obtained after cell incubation with serum from sulodexide treated patients, the increase in concentrations of IL-6, MCP-1 and ICAM-1 was significantly less than the control. Release of IL-6 after stimulation with IL-1 (100 pg/mL) was the highest in the CVD-serum group: 3540±670 pg/10^5 cells vs. 1850±540 pg/10^5 cells in the control (P<0.01 vs. CVD-serum) and 2320±430 pg/10^5 cells in CVD-serum-SUL (P<0.02 vs. CVD-serum). PDT was significantly longer in the cells incubated with CVD serum compared with the control group, and PDT was reduced when serum from sulodexide treated patients was used. The cells became senescent in the presence of CVD serum, but the cells obtained from patients at the end of 8 weeks of treatment with sulodexide showed a much weaker inflammatory phenotype than the CVD group.

CONCLUSIONS: Chronic in vitro exposure of HUVEC to medium supplemented with CVD patient serum induces an inflammatory phenotype. Sulodexide treatment significantly reduces that effect and slows HUVEC senescence in the milieu of CVD serum.


Key words: Glucuronyl glucosamine glycan sulfate - Inflammation – Glycosaminoglycans.

The endothelium lining the blood vessels determines various aspects of intravascular homeostasis, such as vascular tone, thrombosis and fibrinolyis, inflammatory response, and interaction of the inflammatory cells with the vascular wall.1-3 Dysfunction of the endothelial cells is one of the main mechanisms responsible for initiating various pathological conditions both in the arteries and in the veins.3-7
In patients with chronic venous disease (CVD), blood flow shear stress disturbances and venous hypertension cause changes in endothelial cell functional properties, leading to the release of inflammatory cytokines and chemokines and the recruitment of leukocytes, which also contributes to intravascular and perivascular inflammation.\textsuperscript{2-3} It was shown previously that endothelial cells harvested from patients with CVD have an abnormal phenotype with a strong inflammatory pattern.\textsuperscript{6-8} Tisato \textit{et al.} demonstrated that such cells had higher baseline transcriptional NF-\textit{kB} activity and increased release of osteoprotegerin and vascular endothelial growth factor (VEGF), with the magnitude of these changes correlated with the intensity of CVD.\textsuperscript{6} Release of the platelet derived growth factor-BB (PDGF-BB) from the endothelial cells harvested from the CVD patients correlated with the reflex time values measured in the cells’ donors.\textsuperscript{9} Komarow \textit{et al.} clinically confirmed the endothelial cell dysfunction in CVD patients by means of the FMD (Flow Mediated Dilation) Test.\textsuperscript{10} Taka­kase documented the inflammatory reaction activation in a model of venous hypertension, including granulo­cyte, lymphocyte, monocyte and macrophage activation as well as the release of metalloproteinase 2, metallo­proteinase 9, P-selectin and ICAM.\textsuperscript{11,12} The significant role of the inflammatory process in the pathogenesis and progression of CVD explains why treatment with anti-inflammatory drugs that suppress the release of the inflammatory reaction mediators may be potentially beneficial in the pharmacological treatment of this disease.\textsuperscript{7,13,14} According to previous studies, one promising molecule to consider is sulodexide, a mixture of natural glycosaminoglycans that exhibits significant therapeutic potential in CVD patients.\textsuperscript{14,15} As previously documented, sulodexide suppresses intravascular inflammation through its effect on endo­thelial cells, leukocytes, and metalloproteinase expres­sion.\textsuperscript{14,16,17} The positive effect of sulodexide on vessel glyocalyx restoration has also been described.\textsuperscript{17} Recently, we demonstrated that CVD patients treated for 8 weeks with orally administered sulodexide exhibited reduced intravascular inflammation and increased protec­tion of the endothelial cells against extracellular matrix changes related to the metalloproteinase expression.\textsuperscript{18} In the present study, we tested the effect of prolonged \textit{in vitro} exposure of the human venous endothelial cells to the serum from the patients with CVD, studying the function of these cells and how their function is modified when simultaneously exposed to sulodexide.

\section*{Material and methods}

Experiments were performed on human umbilical vein endothelial cells (HUVEC; Cascade Biologics, Paisley, UK), which were maintained through \textit{in vitro} cell culture. The cells were grown in 200PRF medium supplemented with foetal bovine serum (2%), basic Fibroblast Growth Factor (1.5 µg/mL), human Epidermal Growth Factor (5 µg/mL), and hydrocortisone (1 µg/mL), all products that were purchased from Cascade Biologics, Paisley, UK. A culture of the cells was estab­lished in 75 cm\textsuperscript{2} culture flasks, and afterwards, the cells were reseeded into 6-well culture plates or 25 cm\textsuperscript{2} culture flasks for further experiments (Corning BV Life Sciences, Schiphil, Netherlands).

During the experiment, the long-term effect of serum on HUVEC function was evaluated in 8 of the patients with CVD (stage C5 according to CEAP classification). The mean age of the patients was 59.2±8.2 yrs., and the mean time from the venous ulcer healing was 5 months, with patient healing times ranging from 3 to 11 months. For all of the patients, the patient informed consent was obtained, and the protocol of the study was approved by the local Ethical Committee. To confirm the incom­petence of both the superficial and deep vein systems, all of the patients, except physical examination, the venous duplex Doppler ultrasound examination was performed. The study exclusion criteria were as follows: the presence of deep or superficial vein thrombo­sis or post-thrombotic changes, chronic leg ischaemia (ABI<0.95), diabetes, renal failure, the necessity of antiocoagulant, antiplatelet or non-steroid anti-inflamma­tory drug treatment, administration of statins, steroids or immunosuppressive therapy, the use of phlebotropic drugs during or within a month before the enrolment, known infection, and previous surgery (within 6 months before enrolment). All patients were routinely treated with class 2 compression therapy, and after the study enrolment, pharmacological treatment with sulodexide (twice daily via the oral route in a dose of 500 LSU for 8 weeks) was introduced. The serum from 8 patients with CVD was harvested before the start of treatment with sulodexide and after 8 weeks of therapy. In the control group, pooled serum from 5 healthy donors was used.
The following experimental groups were studied:
A. medium + 5% serum (Control);
B. medium + 5% serum from CVD patients collected before treatment with sulodexide (CVD);
C. medium + 5% serum from CVD patients collected after 8 weeks of treatment with sulodexide (CVD + SUL).

The function of the cells in the 25 cm² HUVEC monolayers was evaluated after 15 days of chronic exposure to the studied media. The synthesis of interleukin-6 (IL-6), monocyte chemoattractant protein -1(MCP-1) and soluble intercellular adhesion molecule - 1 (s-ICAM-1) was studied at the beginning of incubation and after 9 and 15 days of exposure to the studied media. The concentrations of these molecules in the cell supernatants were measured with commercially available ELISA tests (R&D, Abingdon, UK).

At the end of incubation, the cells in the monolayers were stimulated with interleukin-1 (100 pg/mL), and the release of IL-6 was measured. Synthesis of the studied molecules was expressed per number of cells in the well, which were counted in a haemocytometer after harvesting with a trypsin 0.05%-EDTA 0.02% solution. In parallel wells, the intracellular generation of free radicals was measured using the probe 2′7′-dichlorodihydrofluorescent diacetate, which is converted to the fluorescent 2′7′-dichlorodihydrofluorescein in the presence of free radicals. The fluorescence of the cell lysates was measured in a microplate fluorescent reader (Victor-2, Perkin Elmer Life Sciences, Finland), with an excitation of 485 nm and an emission of 530 nm. The generation of free radicals was expressed per number of cells.

In the second part of the experiment, we evaluated the effect of the studied sera (5% control serum and 5% serum collected from CVD patients before and after 8 weeks sulodexide treatment) on the in vitro replicative ageing of HUVEC according to method used previously in our lab. ¹⁰

The cells were seeded into 25 cm² culture flasks at a density of 1.25 x 10⁶ cells/flask and exposed to the studied media described previously. Every three days, the cells were harvested from the flask, their confluence was counted using a haemocytometer, and the cells were again seeded into the flask at a density of 1.25 x 10⁶ cells/flask. Fifteen passages were performed in each experimental group. The population doubling time (PDT) of the cultured cells was calculated according to the following formula:

\[
PDT = \ln \frac{N}{N_0} / t
\]

Where \(N\): number of cells harvested from the flask; \(N_0\): number of cells seeded into the flask; \(T\): time of culture.

After the last passage, cells from each group were seeded onto 6-well plates and grown in monolayers in the presence of the studied media. Afterwards, the medium was replaced in all groups with control medium. After 24 hours incubation, the concentration of the inflammatory mediators (IL-6, MCP-1 and s-ICAM-1) was measured in the supernatants. Then, after harvesting with a trypsin 0.05%-EDTA 0.02% solution, the number of cells in the monolayers from each experimental group was counted using a haemocytometer. In a parallel well, the intracellular generation of free radicals was measured as described above. Afterwards, the cells were lysed with 0.1 N NaOH, and the concentration of protein in the lysate was measured using the Lowry method. ²⁰ Cell hypertrophy was calculated from the ratio of total cell protein in the cell lysate to the total number of cells counted in the corresponding well.

**Statistical analysis**

All results are presented as the mean±SD. Statistical analysis of the data was performed with either the Wilcoxon Test or ANOVA with a post-hoc Dunn Test. A P-value less than 0.05 was considered significant.

**Results**

Chronic exposure of the endothelial monolayers to media that was supplemented with serum from CVD patients gradually stimulated the release of inflammatory mediators during the 15 days of treatment compared to levels observed in the control group. The concentration of IL-6 at the end of incubation was 55% higher in the CVD group than the control (P<0.01), but the CVD-SUL group IL-6 concentration was only 18% higher than the control (P<0.05). Similarly, the concentration of MCP-1 in the CVD group was 26% higher than the control (P<0.02), whereas the MCP-1 concentration for the CVD-SUL group was only 7% higher than the control (P<0.05). The CVD group exhibited higher levels of s-ICAM release from the endothelial monolayers (64%) compared with the control (P<0.01), but these
release levels were only 16% higher in the CVD-SUL group (P<0.02) compared with the control (Figure 1).

In cells that were exposed during the 15 days with medium supplemented with CVD serum, oxidative stress was 68% higher (P<0.02) than in the control cells, whereas in the cells treated with CVD-SUL serum, the intracellular generation of free radicals was higher only by 11% (P<0.05) compared with the control. Cells treated with CVD serum released more IL-6 than the control cells following stimulation with interleukin-1 (+91%; P<0.01), and the level of IL-6 released from cells exposed to CVD-SUL serum was only 25% higher (P<0.05) than in the control (Figure 2).

In the endothelial cells undergoing replicative ageing, PDT was prolonged from 108±22 hours to 192±29 hours for the control cells. For cells cultured in medium supplemented with CVD serum, the PDT at the end of the experiment was 275±29 hours (P<0.01 vs. control), and for cells cultured in the presence of CVD-SUL serum, PDT at the end of the experiment was 225±15 hours (P<0.05 vs. control and CVD group). The senescent endothelial cells demonstrated hypertrophy based on the following:

Figure 1.—Synthesis of IL-6 (A), MCP-1 (B) and s-ICAM1(C) in endothelial cells exposed during 15 days to control medium (C), medium with CVD serum (CVD) or CVD serum with sulodexide (CVD-SUL) (N=6).
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Figure 2.—Intracellular generation of free radicals (A) and IL-6 (B) release from cells after stimulation with interleukin-1 (100 pg/ml) in endothelial cells cultured for 15 days in control medium (CON) or medium with CVD serum (CVD) or CVD serum with sulodexide (CVD+SUL) (N.=6).

Figure 3.—Oxidative stress (A), synthesis of IL-6 (B), MCP-1 (C) and ICAM (D) in senescent control cells (CON) and senescent cells after replicative aging in presence of CVD serum (CVD) or CVD serum during treatment with sulodexide (CVD+SUL) (N.=7).

on a measurement of 405±26 µg protein/10^5 cells in the control group compared with 258±31 µg protein/10^5 cells at the start of the experiment. This hypertrophy was even more visible in the CVD group, which measured 523±36 µg protein/10^5 cells (P<0.01 vs. control) and was less intense than the CVD-SUL group measured at 443 µg protein/10^5 cells (P<0.05 vs. CVD group).

The senescent endothelial cells, which were cultured in the presence of CVD serum, demonstrated the strongest pro-inflammatory profile as reflected by strong intracellular oxidative stress and the unstimulated release of inflammatory mediators, compared with the control cells (Figure 3). The cells became senescent in the presence of CVD serum, but the senescent cells cultured...
with the serum obtained from the patients at the end of 8 weeks of treatment with sulodexide, demonstrating a much weaker inflammatory phenotype than the CVD group (Figure 3).

Discussion

Dysfunction of the endothelial cells is one of the main factors signalling the progression of CVD. Under in vivo conditions, abnormal function of the endothelial cell lining in the veins is related to altered shear stress, increased intravascular pressure, and interactions of activated leukocytes with the endothelium and various inflammatory mediators present in blood. To discover new targets for pharmacological therapy, potential pharmacological modification of the inflammatory reaction activation and its progression is proposed. In studies concerning the beneficial effects of MPFF (micronized purified flavonoid fraction), aside from the clinical improvements observed in the in vivo studies (RELIEF study), the influence of this molecule on leucocyte recruitment, including their activation and adhesion to the endothelial cells, was also documented in in vitro studies. Another molecule potentially influencing the inflammatory reaction, free radical production and metalloproteinase expression is sulodexide. Laboratory studies have documented the use of sulodexide to suppress IL-6 and monocyte chemoattractant protein-1 (MCP-1) in cell cultures, as measured via glucose cytotoxicity tests. Other studies have determined that sulodexide decreases senescence-related changes in endothelial cell cultures and decreases both MMP-9 secretion from the white blood cells and its activity levels. In the literature, clinical confirmation of the beneficial effect of the sulodexide on the chronic venous insufficiency complications can also be found. In the randomized SUAVIS study, an improvement of venous ulcer healing in patients treated with sulodexide was observed.

In our in vitro experiments, the serum from patients with CVD was investigated. We demonstrated that chronic exposure to CVD serum has a deleterious effect on the human endothelial umbilical cells in these cultures. The phenotype of the endothelial monolayers with low replicative activity related to chronic serum exposure became more inflammatory than the control cells, exhibiting the highest unstimulated release of inflammatory mediators such as IL-6, MCP-1 and s-ICAM-1 at the end of the experiment (Figure 1). These cells also possessed the strongest reactivity to inflammatory stimulation with interleukin-1 (Figure 2). The increased release of inflammatory mediators may influence intravascular inflammation, leukocyte activation and their increased adherence to the endothelial cells.

Both oxidative stress and inflammation are considered to be important factors responsible for the ageing of endothelial cells, which results in the loss of functional properties required for intravascular homeostasis. In our study, the ageing of endothelial cells using a model of replicative senescence was evaluated. Under proper in vivo conditions, vascular endothelial cells are quiescent. However, after exposure to noxious effects such as hypoxia, hypertension, endotoxins or mechanical injury, these cells become activated and start to proliferate, replacing the damaged cells. In this study, we found that upon exposing endothelial cells to the serum of CVD patients, appearance of the senescent phenotype of the endothelial cells undergoing replicative ageing was observed. When compared with the control group, hypertrophy of these cells was stronger, and their PDT was longer. Additionally, these endothelial cells exhibited a strong inflammatory phenotype due to increased intracellular generation of free radicals and increased release of inflammatory mediators, which occurs even when these senescent cells are incubated in the control medium (Figure 3). Our results clearly show that even without the altered shear stress effect or the increased hydrostatic pressure (which are normally present in vivo in the veins of the CVD patients), CVD patient serum alone causes progressive damage to the endothelial cells, accelerating their senescence.

Sulodexide, which was studied in our experiments, contains a mixture of natural glycosaminoglycans with potential pleiotropic action in the vascular pathologies. Due to its composition, sulodexide displays anticoagulant activity, lowers plasma lipids levels, reduces blood viscosity, suppresses inflammatory reactions in leukocytes and endothelial cells and inhibits metalloproteinase activity. All of these effects are potentially beneficial to vascular system homeostasis in pathological conditions including CVD and in particular, diabetes. Senescence of the endothelial cells is described not only in in vitro conditions but also in
in vivo settings; therefore, it must be considered as an inevitable process. Interestingly, several pathological factors can accelerate the process of senescence, which was also confirmed in our present study. Several approaches aimed at reducing endothelial senescence were proposed. Previously, we found that sulodexide reduces senescence in the endothelial cells when they are exposed to repeated replications during in vitro culture. In this study, we demonstrated that modification of the CVD serum with orally applied sulodexide treatment for 8 weeks results in a weaker pro-ageing effect of the serum on the endothelial cells. Reduced intracellular oxidative stress and reduced inflammatory activity of the endothelium can potentially result in lower leucocyte recruitment, which may result in reduced intravascular inflammation. The results from our study agree with this finding and explain the success of clinical trials in which patients with CVD showed improved clinical parameters when treated with sulodexide.

Conclusions

In conclusion, the endothelium is a promising target for therapy in patients with CVD. The chronic in vitro exposure of HUVEC to medium supplemented with CVD patient serum induces an inflammatory phenotype. Sulodexide treatment significantly reduces that effect and slows HUVEC senescence in the milieu of CVD serum.

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