Sulodexide reduces senescence-related changes in human endothelial cells

Katarzyna Suminska-Jasinska[ABCDEFG], **Alicja Polubinska**[ABCD], **Marta Ciszewicz**[BC], **Adam Mikstacki**[DEF], **Artur Antoniewicz**[EF], **Andrzej Breborowicz**[ABCDEFG]

Department of Pathophysiology, Poznan University of Medical Sciences, Poznan, Poland

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Summary

Background: Senescent endothelial cells acquire functional properties that make the vascular wall more prone to atherosclerotic changes. We tested whether senescence of the endothelial cells maintained in *in vitro* culture can be moderated by their simultaneous exposure to sulodexide.

Material/Methods: Replicative aging of the endothelial cells was studied during their 15 passages performed every 4 days in cells cultured in standard medium or in medium supplemented with sulodexide 0.5 LRU/mL. Changes in population doubling time and β-galactosidase activity were used as indexes of aging and compared with other cellular parameters.

Results: Repeated passages of endothelial cells induce their senescence, as reflected by prolongation of the population doubling time, increased β-galactosidase activity, oxidative stress and release of cytokines. Healing of the injured endothelial monolayer is impaired in senescent cells. Sulodexide partially prevents oxidative stress and totally eliminates other senescence-related changes such as increased release of MCP-1, lengthening of the population doubling time, and impaired healing of the cellular monolayer after its mechanical injury.

Conclusions: Sulodexide prevented cellular senescence in cultured endothelial cells, moderating features of the cellular senescence in endothelial cells in *in vitro* conditions, which potentially may have practical application. The administration of sulodexide could potentially be used in prevention of atherosclerotic changes.

key words: endothelial cells • senescence • sulodexide

Author's address: Andrzej Breborowicz, Department of Pathophysiology, Poznan University of Medical Sciences, Swiecickiego 6 Str., 60-781 Poznan, Poland, e-mail: abreb@ump.edu.pl

BACKGROUND

Sulodexide is a mixture of the natural glycosaminoglycans – heparin and dermatan sulfate. It has application in treatment of various diseases, such as prevention of thrombosis, [1] and reduction of albuminuria in diabetic nephropathy [2]. Sulodexide has an antilipemic effect, which suggests it may be useful in treatment of patients with atherosclerosis [3]. Experimental results showing the protective effect of sulodexide against reperfusion injury suggest that the effectiveness of that drug was related to its anti-inflammatory, and not anticoagulant, activity [4]. However, there is a lack of direct evidence that sulodexide exerts its pharmacological effect in the blood vessels due to its action on the endothelial cells. In previous experimental studies in animals, the protective effect of sulodexide against factors injuring the endothelium was demonstrated in rabbit arteries perfused with the stimulated polymorphonuclear cells, as well as in diabetic rats [5,6].

Adequate function of the endothelial cells lining the blood vessels determines intravascular homeostasis and blood pressure, and prevents progression of atherosclerosis. Sulodexide has high affinity to the endothelial cells, causing release from their surface and from the subendothelial matrix of active substances such as tissue factor pathway inhibitor [7] and hepatocyte growth factor [8]. We previously found that sulodexide reduces the toxic effect of hyperglycemia in these cells [2]. However, in the same study we found that sulodexide reduced the oxidative stress and release of cytokines in the cells cultured in control medium without high glucose content. Anti-inflammatory action of sulodexide was also demonstrated in other experimental models, such as peritonitis [10] and myocardial infarction [4].

Abnormalities in function of the vascular endothelial cells are caused not only by their exposure to noxious substances (i.e., glucose in high concentration and homocysteine), but also appear in the process of aging. Senescence of the endothelial cells is linked with enhanced inflammatory reaction and oxidative stress [11]. Cellular senescence was first described by Hayflick and Moorhead, who found that primary cultures of human fibroblasts exhibit a limited capacity for proliferation when they are serially subcultivated [12]. Cellular senescence also occurs *in vivo* and contributes to progression of cardiovascular diseases [13].

We hypothesized that chronic exposure of endothelial cells to sulodexide may partially prevent their aging, due to the anti-inflammatory action of sulodexide. We evaluated these changes in *in vitro* cultured endothelial cells during their senescence as a consequence of replication in the presence of sulodexide.

MATERIAL AND METHODS

Experiments were performed on human umbilical vein endothelial cells in *in vitro* culture. Primary cell lines were obtained from Cascade Biologics (Paisley, UK). Cells were grown in medium 200PRF supplemented with fetal 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 from Cascade Biologics, Paisley, UK). Culture of the cells was initially established in

75 cm² culture flasks; afterwards, cells were seeded in quadruplicates into 25 cm² culture flasks at density 1.25×10^5 cells/flask, in which they were propagated during the entire study. Cells were divided into 2 experimental groups that were exposed during the experiment to different media:
1. Control group – exposed to standard culture medium (as described above).
2. Sulodexide group – exposed to standard medium supplemented with + Sulodexide 0.5 LRU/mL.

The concentrations of sulodexide used in this study were selected based on the results from our previous experiments [9]. The replicative model of cellular aging applied in these experiments was similar to our previous study [14]. Every 96 hours, growing cells were harvested from the culture flasks; they were counted in a hemocytometer and afterwards were reseeded at density 1.25×10^5 cells/flask into new culture flasks. Population doubling time (PDT) of the cultured cells was calculated according to the following formula:

$$PDT = \ln2/[\ln (N/N_o)/t]$$

Where:
N – number of cells harvested from the flask;
N_o – number of cells seeded into the flask;
T – time of culture.

During the study, 15 passages of the endothelial cells were performed. Functional properties of the cells were evaluated before the start of their exposure to the studied solutions and after their last passage. Cells were seeded into 24-well and 6-well culture plates and into Labtec® wells for further immunohistochemical study.

The following parameters of cellular function were evaluated:
1. Intracellular generation of free radicals was measured with a 2'7'-dichlorodihydrofluorescein diacetate probe, which in the presence of free radicals is converted to the fluorescent 2'7'-dichlorodihydro-fluoroscein. Fluorescence was measured using a microplate fluorescent reader (Victor-2, Perkin Elmer Life Sciences, Finland), with excitation at 485 nm and emission at 530 nm. Generation of free radicals was expressed per number of cells in each well, counted in a hemocytometer.
2. Synthesis of interleukin-6 (IL-6) and monocyte chemoattractant peptide-1 (MCP-1), reflected by concentration of these cytokines in the medium supernatant, were measured with commercially available kits from R&D (UK). Results were recalculated per number of cells in the well.
3. Ratio of total cell protein in the monolayer (measured in cell lysates using the Lowry method) to number of cells in the monolayer (calculated in the corresponding well) was used as an index of the cellular hypertrophy.
4. Number of cells cultured in Labtec® wells that were positively stained for β-galactosidase was used for evaluation of cellular senescence. Staining was performed with a kit available from Mirus (USA).
5. Kinetics of healing of the injured endothelium (cells were removed from a part of the monolayer with a cell scraper) was evaluated in an inverted microscope (Axio Observer D1, Zeiss, Germany). Cells were cultured in chamber for *in vitro* culture attached to the microscope and digital camera. Every 15 minutes for 2 hours, pictures of the healing endothelium were taken. Speed of
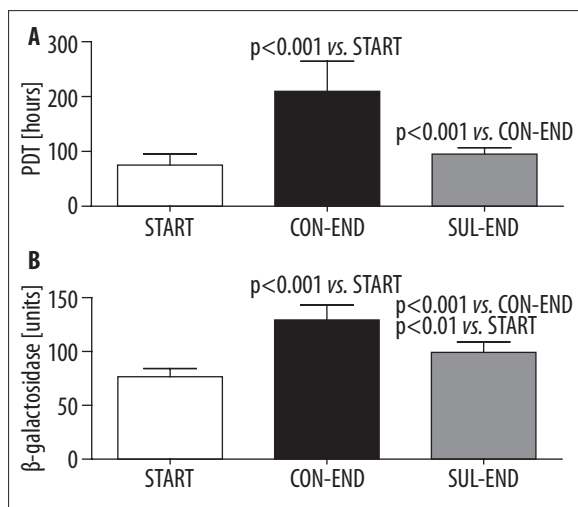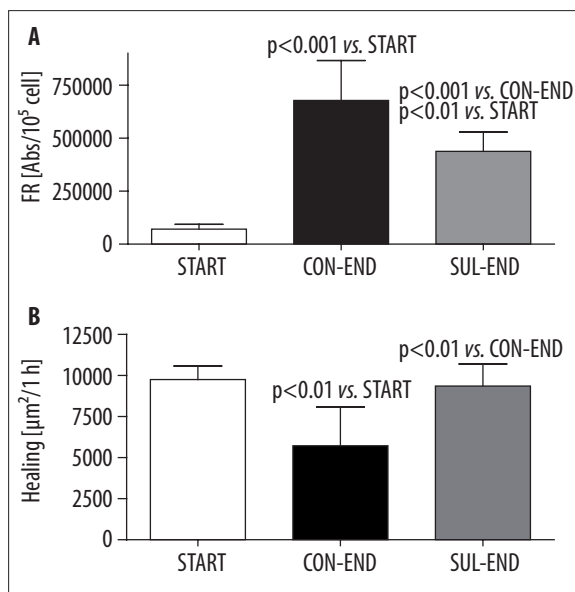
Figure 1. Population doubling time (PDT) – (**A**) and positive staining for β-galactosidase – (**B**) in endothelial cells at the beginning of the experiment (START) and after 15 passages in control medium (CON-END) or in medium supplemented with sulodexide 0.5 LRU/mL (SUL-END).

decrease in wound size was calculated with a computer program (Axio Vision Release 4.6, Zeiss, Germany) and the final result was expressed in μm^2/min.

Results were expressed as mean ±SD. Statistical analysis was performed with one-way analysis of variance with *post hoc* analysis done with the Tukey test. A p value less than 0.05 was considered statistically significant.

RESULTS

Cells from the control group demonstrated signs of hypertrophy during replicative aging: 237 ± 61 µg protein/10^5 cells at the beginning of experiment and 487 ± 119 µg protein/10^5 cells at the end (p<0.01). However, in the presence of sulodexide, hypertrophy of the cells at the end of the experiment, despite some increase, was not significantly different from at the beginning of the study: 351 ± 86 µg protein/10^5 cells (n.s.). Population doubling time was increased at the end of the experiment in the control group, but in cells treated with sulodexide its value was comparable to that at the beginning of the study. Positive staining for β-galactosidase increased during replicative aging in both studied groups, but that increase was significantly weaker in the sulodexide-treated cells (Figure 1). Replicative aging resulted in increased oxidative stress in both studied groups, but in cells exposed to sulodexide oxidative stress was weaker (–35%, p<0.01) than in the control group (Figure 2). Healing of the mechanically injured endothelial layer was slower in control cells exposed to replicative aging, but healing of the endothelial cells monolayer was not affected by aging when these cells were exposed to sulodexide (Figure 2). Replicative aging of the control cells resulted in increased spontaneous release of IL-6 and MCP-1 (Figure 3). In the presence of sulodexide, only spontaneous release of IL-6 was enhanced at the end of the experiment, but it was still lower than in the control group (–47%, p<0.01). Cells treated with sulodexide did not show increased release of MCP-1 during replicative aging (Figure 3).

Figure 2. Intracellular generation of free radicals (FR) – (**A**) and healing of the injured endothelial monolayer – (**B**) in endothelial cells at the beginning of the experiment (START) and after 15 passages in control medium (CON-END) or in medium supplemented with sulodexide 0.5 LRU/mL (SUL-END).
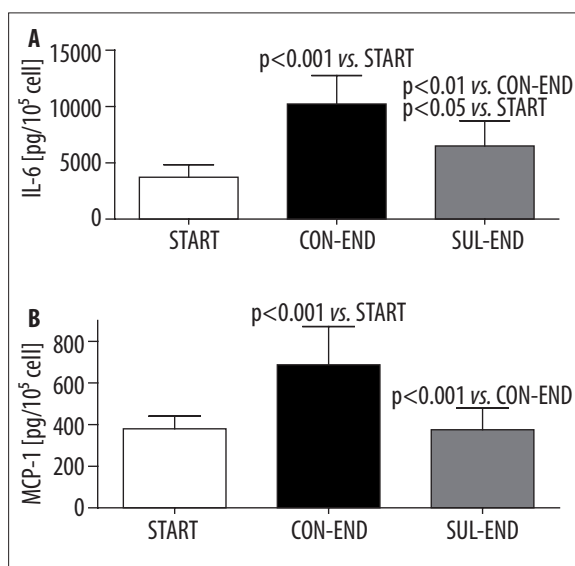
Figure 3. Unstimulated synthesis of interleukin-6 (IL-6) – (**A**) and monocyte chemoattractant protein -1 (MCP-1) – (**B**) in endothelial cells at the beginning of the experiment (START) and after 15 passages in control medium (CON-END) or in medium supplemented with sulodexide 0.5 LRU/mL (SUL-END).

DISCUSSION

Endothelial cells age when maintained in *in vitro* culture and exposed to repeated passages [15]. Under normal conditions, endothelial cells rarely divide, but their replication is initiated after injury. Balloon denudation of the endothelial cells in rabbits resulted in increased numbers of these

cells that were positively stained for β-galactosidase [16]. Accelerated senescence of the endothelial cells is detected in humans in areas of the blood vessels exposed to hemodynamic forces of shear and stress, called "atherosclerotic-prone areas" [11]. During the lifespan, endothelial cells are exposed to various types of the oxidative stress, which cause their injury, followed by healing of the endothelium [17]. Prevention, or at least slowing, of endothelial senescence is highly recommended and its final result may be reduction of the cardiovascular risk and disease in humans [18]. Several approaches were proposed to achieve that goal, such as mild physical exercise [19], supplementation of magnesium [20], estrogens [21] or use of antioxidants [22]. We propose that sulodexide can be added to that list.

In our experiments, control cells exhibited morphological and functional changes typical of the process of aging – hypertrophy and increased positive staining for β-galactosidase, together with increased oxidative stress and release of the inflammatory cytokines (Figures 1–3). Exposure of the cells to an identical experimental procedure, but in medium supplemented with sulodexide 0.5 LRU/mL, partially prevented changes in structure and function of the cells related to their aging. When the sulodexide end data were compared to the measured cellular parameters at the beginning of the experiment, only staining for β-galactosidase, oxidative stress and release of Il-6 were significantly increased, but less than in the control group. However, in the sulodexide group we did not observe cellular hypertrophy, and population doubling time was comparable to the values at the beginning of the experiment. The beneficial effect of sulodexide, preventing senescence of the endothelial cells, could be due to its antioxidant and anti-inflammatory action [9,23,24]. Bilinska et al observed an antioxidant effect of sulodexide in patients with stable coronary artery disease, but no change in the systemic inflammation [24]. However, they use markers of inflammation different from those our study used, which may explain the differences between our results. Additionally, patients in the clinical study reported by Bilinska were already being treated with other drugs that could suppress inflammation [24]. In our experiments, we found that aging of the endothelial cells results in reduced ability of these cells to heal the injured monolayer. In clinical conditions, dysfunction, injury and desquamation of the endothelial cells lining the blood vessels occurs due to turbulent shear stress at areas of abrupt curvatures or narrowings in the vasculature, as well as after intravascular therapeutic procedures such as angioplasty in treatment of vascular stenosis. Endothelial cells undergoing replicative aging, when chronically exposed to sulodexide, preserve their ability to heal the injured cells' monolayer, which may be important in situations of increased damage to these cells in *in vivo* conditions. On the other hand, it is well documented that healing of the injured endothelial monolayer depends not only on proliferation and migration of the *in situ* localized cells, but also depends strongly on availability of the circulating progenitor cells [25]. Therefore, our results can be extrapolated only to a single mechanism of the *in vivo* endothelial healing.

The anti-inflammatory action of sulodexide documented in this study confirms data from our previous studies [4,9,10]. Low release of MCP-1 from cells treated with sulodexide may result in decreased expression of adhesion molecules and decreased leukocyte-mediated injury of the endothelial cells [26]. MCP-1 is one of the main humoral factors determining progression of atherosclerotic changes in the vasculature [27]. Therefore, inhibition by sulodexide of MCP-1 release from endothelial cells and simultaneous anticoagulant activity may result in significant inhibition of atherosclerosis.

CONCLUSIONS

Cellular senescence in the vasculature is one of the processes which contribute to initiation and/or progression of atherosclerosis. There is correlation between shortening of telomers and progression of atherosclerosis [28]. In fact, senescent endothelial cells can be found in almost any atherosclerotic plaque [29]. Elimination of senescence in the endothelial cells is not possible, but slowing of that process may contribute to reduction of progression of atherosclerotic changes in blood vessels. We found that sulodexide slows senescence of the endothelial cells, which results in reduced proinflammatory action of these cells, and their improved healing after mechanical injury. These observations may have important clinical implications, but require further studies, first in animals experimental models, and then in clinical studies.

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