Original Paper

Sulodexide Slows Down the Senescence of Aortic Endothelial Cells Exposed to Serum from Patients with Peripheral Artery Diseases

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Key Words
Human arterial endothelial cells • Senescence • Atherosclerosis • Sulodexide

Abstract
Background/Aims: Aging of the arterial endothelial cells results in the appearance of their inflammatory phenotype, which may predispose patients to the acceleration of arteriosclerosis. We studied the effect of serum from patients with peripheral artery disease (PAD) on the senescence of human aortic endothelial cells (HAEC) and how that process is modulated by sulodexide. *Methods:* HAEC replicative aging *in vitro* was studied in the presence of 10% PAD-serum (PAD Group) or10%PAD serum and Sulodexide 0.5 LRU/mL (PAD-SUL group). In control group cells were cultured in medium supplemented with 10% fetal bovine serum. All studied parameters were evaluated at the beginning and at the end of the study, in all experimental groups. Population doubling time (PDT) was studied from the cells growth rate after repeated passages, and senescence-associated beta- galactosidase activity (SA-β gal activity) was measured with the fluorescence flow cytometry. Expression of IL6, vWF, p21 and p53 genes was measured with the real-time polymerase chain reaction (Real-Time PCR). Concentrations of IL6 and vWF were measured with the standard ELISA kits. *Results:* PAD serum accelerated the senescence of HAEC as reflected by increased, compared to control, expression of the IL6 gene (+43%, $p < 0.05$) vWF gene (+443%, $p < 0.01$), p21 gene (+ 124%, $p < 0.01$) and p53 gene (+ 85%, $p < 0.01$). Secretion of IL6 and vWF was higher in that group: + 101%, $p < 0.01$ and + 78%, $p < 0.01$, respectively, as compared to control. Also, SA-β gal activity was higher in the PAD group (+33%, $p < 0.05$) than in the control group. In the PAD group PDT was longer (+108%, $p < 0.01$) as compared to control. Simultaneous use of Sulodexide with PAD serum significantly reduced all the above described senescent changes in HAEC. *Conclusions*: PAD serum accelerates the aging of HAEC which may result in the faster progression of arteriosclerosis. Sulodexide reduces PAD induced senescence of HAEC, which results in lower inflammatory and thrombogenic activity of these cells.

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Introduction

Dysfunction of the arterial endothelial cells results in altered intravascular homeostasis which predisposes patients to the progression of atherosclerotic changes. Inflammation and oxidative stress are the main disorders which contribute to impaired function of the vascular endothelium [1]. Cellular senescence, which is a natural process, being parallel t body aging, also happens in the endothelial cells and accelerates the formation of atherosclerotic changes [2]. Hayflick state that cells in *in vitro* culture have limited ability to replicate [3], and later it was shown that progressive loss of telomeres during repeated replication is responsible for that effect [4]. Cells which become senescent not only have reduced ability to proliferate, but also develop the senescence –associated secretory phenotype, which results in the increased expression and secretion of the inflammatory mediators, growth factors and proteases [5].

Senescence of the endothelial cells is not only due to shortening of their telomeres but may also be induced by such factors as high glucose, Angiotensin II or oxidative stress [6, 7, 8]. These factors play an important role in various types of pathologies of the cardiovascular system in humans. Warboys described the presence in aortae from healthy pigs of enhanced senescence-associated β-galactosidase staining at sites of the disturbed blood flow [9]. The presence of senescence-associated β-galactosidase (SA-βGal) was also detected in the endothelium lining atherosclerotic human arteries [10]. Slowing down endothelial senescence may be a potential approach in the prevention of arterial atherosclerosis. The protective action towards the endothelial cells of some drugs such as ACE inhibitors and antioxidant agents may be linked with their antisenescent activity [11].

Glycosaminoglycans are the component of the endothelial glycocalyx regulating interactions between the cells and the intravascular space [12]. Damage to the endothelial glycocalyx is an important factor initiating the process of atherosclerosis [13]. It was shown that the age-related reduced ability of the endothelial progenitor cells to migrate is linked with the alteration in the structure of heparan sulphate proteoglycans located in the glycocalyx [14]. Previously, we found that sulodexide, which is a mixture of low molecular weight heparin and dermatan sulphate, reduces the senescence of human venous endothelial cells in *in vitro* conditions [15]. Recently, we described the protective effect of sulodexide against the proinflammatory action of serum from patients with peripheral artery disease (PAD) towards human aortic endothelial cells [16]. In the present paper we present results from the experiments in which the effect of sulodexide on the aging of human aortic endothelial cells exposed chronically to serum from patients with PAD was evaluated.

Materials and Methods

Experiments were performed on human aortic endothelial cells (HAEC) obtained from Life Technologies Corporation (Carlsbad, USA). Cells were maintained in standard conditions of the *in vitro* culture in air atmosphere with 5% CO_2 at 37°C. Culture was established in medium M200 supplemented with 2% foetal bovine serum, hydrocortisone 1μg/ml, heparin 10μg/ml, human epidermal growth factor 10ng/ml and basic fibroblast growth factor 3 ng/ml.

During the experiments we evaluated the effect of serum from patients with PAD (III or IV Fontaine stage) on the replicative aging of HAEC. The study was approved by the University Bioethical Committee. Sera obtained from 10 patients were pooled (PAD serum) and used during the study. Replicative senescence of the endothelial cells was performed according to the standard method used in our lab [17]. In the experimental groups, during the whole study, the following media were used:

A. Control group – standard culture medium supplemented with 10%FBS

B. PAD Serum group – standard culture medium supplemented with 10% PAD serum

C. PAD Serum – Sulodexide group – standard culture medium supplemented with 10% PAD serum and Sulodexide 0.5 LRU/mL

In all groups experiment was started with the cells from the second passage.

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Induction of cellular senescence in vitro

The senescence of HAEC was induced by serial passaging at 7-day intervals for 10 weeks. Cells were seeded into 25 cm^2 flasks at density 3 x 10^3/cm^2. At each subcultivation, the cells were counted and the population doubling time (PDT) was estimated according to the following formula: PDT = ln2/[ln(N/N$_0$)/t] where N indicates the number of cells harvested at the end of the incubation, N$_0$ – number of cells seeded and t – time of culture.

At the beginning of the study and after 10 weeks cells were also seeded into 6 wells plates for analysis of genes expression, and into 24 wells plates for evaluation of their secretory activity. Also SA-βGal activity in the cells was measured with fluorescence analysis. Additionally, at the end of the study the ability of the cells from all experimental groups to proliferate in standard culture was evaluated.

Analysis of gene expression

Experiments were performed on HAECs monolayers in 6-well plates, at the beginning of the study and after 10 weeks of cellular replicative aging. Cell monolayers were incubated for 24 hours in the following media:
- Standard culture medium supplemented with 10% FBS
- Standard culture medium + 10% PAD serum
- Standard culture medium + 10% PAD serum + Sulodexide 0.5 LRU/mL

After exposition, total RNA from the cells was isolated using the ReliaPrep™ RNA Cell Miniprep System (Promega, USA) method according to the manufacturer's instruction. RNA samples were treated with DNase I using DNA-free DNase Treatment and Removal Reagent (Ambion). RNA quality and concentration were assessed by spectrophotometry using a NanoDrop (NanoDrop, Thermo Scientific, DE, USA).

One microgram of total RNA was reverse-transcribed to cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland). Relative levels of the mRNA of 4 genes: Interleukin -6 [IL6], Von Willebrand factor [VWF], Tumour protein p53 [p53] and Cyclin-dependent Kinase Inhibitor 1 [p21] were analyzed using Sybr green real-time quantitative PCR (Applied Biosystems) in triplicates and normalized to levels of internal house-keeping genes: Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH) and Hypoxanthine Phosphoribosyltransferase 1 (HPRT)

Specific primers for the amplification of each gene were designed using Primer-BLAST [18]. Primer characteristics are presented in Table 1. The PCR parameters were as follows: initial denaturing for 10 min at 95°C, followed by 35 cycles of denaturation (95°C for 10 s), annealing (56°C for 45 s) and extension (60°C for 20 s). After completed real-time PCR reactions, a melting curve analysis was performed for each sample to confirm that a single, specific product was generated. Relative gene expression was calculated using the 2-ΔΔCt method [19].

Evaluation of the HAEC secretory activity.

At the beginning and at the end of the study cells were seeded into 24-wells plates and grown in media adequate for each group until they reached monolayers. Then 24 hour incubation in the studied media was performed. Afterwards in all groups cells were exposed in standard culture medium, with 2%FBS for 24 hours to evaluate their secretory activity. At the end of the incubation supernatant was collected for further measurements of IL6 (ELISA kit, R&D, Minneapolis, USA) and vWF (ELISA kit, Sigma-Aldrich, St. Louis, USA) concentration. Cells were detached with trypsin 0.05%- EDTA 0.02% solution, and the number of cells in each well was counted in a hemocytometer.

Proliferation of HAEC

After 10 weeks of replicative aging, cells from each group were seeded in 24-well plate in a standard culture medium (8 wells per group). After 24 hours when the cells were attached to the bottom of the well, the medium was

Table 1. Characteristic of the primers used in the study

Gene	Name	Gene ID	Amplicon length (bp)	Primer sequence (5'→3')
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase	2597	231	F:TTCGTCATGGGTGTGAACC R:GATGATGTTCTGGAGAGCCC
HPRT	Hypoxanthine Phosphoribo-syl-transferase 1	3251	192	F: TGCTCGAGATGTGATGAAGG R: TCCCCTGTTGACTGGTCATT
IL6	Interleukin 6	3569	264	F:ATGAACTCCTTCTCCACAAGC R:GTTTTCTGCCAGTGCCTCTTTG
VWF	Von Willebrand factor	7450	232	F:CCAGATTTGCCACTGTGATG R:AAAGGCCTTCAGCACTTCAA
p53	Tumor protein p53	7157	99	F:TTGCAATAGGTGTGCGTCAG R:TCCCCACAACAAAACACCAG
p21^{Cip1}	Cyclin-dependent Kinase Inhibitor 1	1026	113	F:TGATTAGCAGCGGAACAAGG R:TGCCAGGAAAGACAACTACTCC

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replaced with the standard culture medium + 2% FBS supplemented with ^{3}H- methyl thymidine (Sigma-Aldrich, St. Louis, USA) in an amount sufficient to obtain the final concentration of the isotope of 2μCi/mL. HAEC were cultured in the presence of ^{3}H- methyl thymidine for 24 hours, and then the medium was removed, and cells were harvested from the wells with trypsin 0.05%- EDTA 0.02% solution and precipitated with 10% trichloroacetic acid (TCA). The cell precipitate was washed twice with TCA and lysed with 0.1 N NaOH. Radioactivity of the lysed cell precipitate was measured in a β-counter, and its value reflected the amount of the radiolabelled thymidine incorporated into the DNA of the proliferating cells.

Fluorescence detection of SA-β gal activity

The quantitative assessment of the senescence-associated beta-galactosidase activity was performed using flow-cytometry analysis according to the Debacq-Chainiaux et al. protocol [20]. Briefly, cells were seeded in 6-well culture plates and incubated with 100 nM bafilomycin A1 for 1 h in fresh cell culture medium at 37°C and 5% CO_2. After lysosomal alkalinization, the fluorogenic substrate for β-galactosidase (C_{12}FDG, dodecanoylaminofluorescein di-β-D-galactopyranoside) was added to the culture medium to the final concentration of 33 μM and incubated for an additional 2 h. After the incubation, the solution was removed and the cell monolayers were washed twice for 40 s with 2 ml of PBS at room temperature. Afterwards the cells were harvested with trypsin 0.05%-EDTA 0.02% solution, counted and subjected to flow-cytometry analysis using FACS ARIA III (Becton-Dickinson, USA). An appropriate negative control was used to determine the level of autofluorescence. The determination of positive cells was carried out on a two-parameter display of FSC versus C_{12}-FL (FITC/488nm).

Statistical analysis

Results are presented as mean ± SD. Analysis of the data was performed with one way analysis of variance, with the post hoc Newman Keuls test. A p value less than 0.05 was considered significant.

Results

Repeated 10 passages of HAEC from the control group resulted in their senescence as reflected by hypertrophy of the cells, increased accumulation of SA-β-Gal (+643%, p<0.0001), and prolongation of their PDT (+46%). Also we observed increased expression of genes for IL6 (+280%, p<0.001) and vWF (+200%) as well as increased secretion of these compounds from the cells, by 31%, p<0.01 and by 46 %, p<0.01, respectively. Expression of genes responsible for the process of cellular senescence was also increased: p53 (+30%) and p21 (+110%), as compared to the beginning of the study.

Senescence of the cells replicated in the presence of PAD serum was stronger than in control. The addition of Sulodexide (0.5 LRU/mL) resulted in a weaker prosenescent effect of PAD serum. Mean values of PDT for cells exposed to PAD serum was higher by 108%, p<0.0001 vs. control and by 72%, p<0.05 vs. cells treated simultaneously with Sulodexide (Fig. 1). HAEC exposed for 10 weeks to PAD serum had weaker ability to proliferate in standard culture conditions: - 21% vs. control, p<0.01 and -26% vs. cells treated with PAD serum and Sulodexide p<0.05. (Fig. 2). HAEC chronically exposed to PAD serum had a higher increase of SA-β-Gal activity than cells from the control group (+33%, p<0.05) or cells treated with PAD serum and Sulodexide (+44%, p<0.01) (Fig. 3).

After 10 weeks of the replicative aging cells exposed to PAD serum showed higher expression of genes for IL6 (+43%, p<0.05), vWF (+443%, p<0.01), p21 (+124%, p<0.01) and p53 (+85%, p<0.01) than cells cultured in control medium. The addition of Sulodexide prevented PAD-serum induced changes of the above listed genes expression in HAEC undergoing replicative aging (Fig. 4). In the case of p21 and p53 genes, their expression after exposure to PAD serum but supplemented with Sulodexide was lower than in the control group (respectively – 53%, p<0.05 and -38%, p<0.05).

The secretion of IL6 studied at the end of the experiment was highest in the PAD group, as compared to control (+101%, p<0.01) and the Sulodexide group (+104%, p<0.01). The same effect was observed for vWF, where the synthesis of that molecule in cells from the PAD

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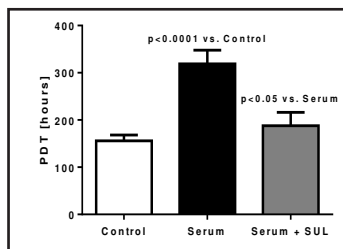Sosińska-Zawierucha et al.: Endothelial Senescence and Sulodexide

Fig. 1. Population doubling time, after 10 weeks of the experiment, in control group (Control), cells exposed to PAD serum (Serum) and cells exposed to PAD serum + Sulodexide 0.5 LRU/mL (Serum-SUL).
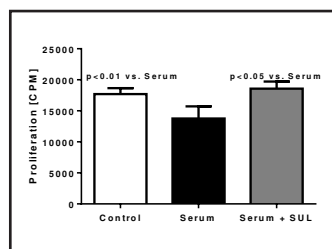
Fig. 2. Proliferation of HAEC, in standard culture medium + 2%FBS, after 10 weeks of replicative aging in standard culture medium (Control) to medium supplemented with PAD serum (Serum) and to medium supplemented with PAD serum and Sulodexide 0.5 LRU/mL.
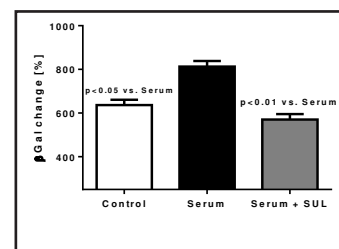
Fig. 3. Relative change, vs. start of the experiment, of SA-β-Gal activity in control group (Control) and in cells exposed to PAD serum (Serum) or PAD serum and Sulodexide 0.5 LRU/mL (Serum+SUL).
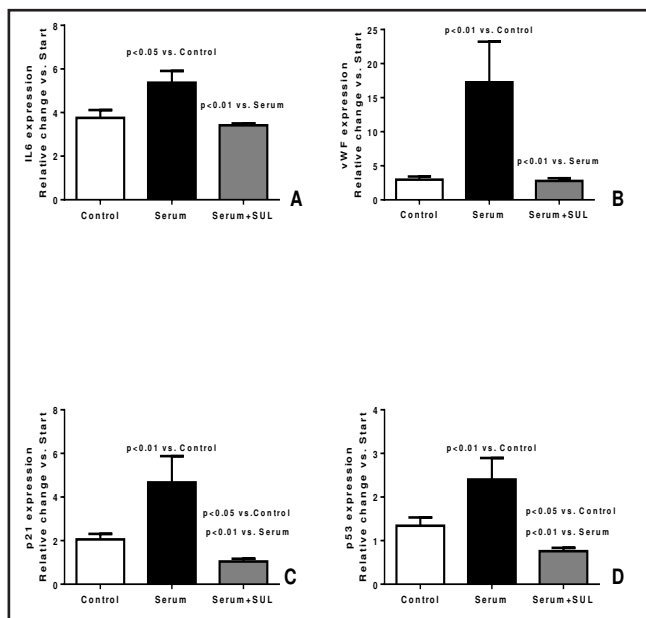

Fig. 4. Relative vs. start of the experiment, expression of IL6 (A) and vWF (B) p21 (C) and p53 (D) in cells undergoing replicative aging during 10 passages in control medium (Control), medium supplemented with PAD serum (Serum) or PAD serum and Sulodexide (Serum + Sul).
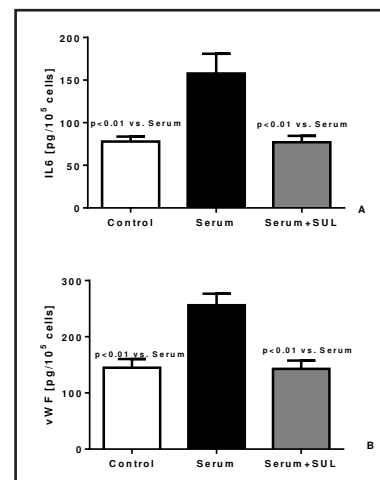
Fig. 5. Secretion of IL6 (A) and vWF (B) after 10 weeks of replicative aging of HAEC in control medium (Control), in medium with PAD serum (Serum) or in medium with PAD serum and Sulodexide (Serum + SUL).

group was highest, as compared to control (+78%, $p < 0.01$) and the Sulodexide group (+80%, $p < 0.01$) (Fig. 5).

Discussion

Aging of the endothelial cells is an important factor contributing to the progression of atherosclerosis due to the development in these cells of the senescence-associated secretory phenotype [21]. Previously, we found that already short, lasting 24 hours exposure of human aortic endothelial cells to PAD serum induces the inflammatory reaction [16].

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The proinflammatory phenotype has been described in the arterial endothelial cells from healthy older individuals, which suggests that cellular senescence of the endothelial cells may promote such a reaction [22]. The results of our present study suggest that chronic exposure of HAEC undergoing replicative aging to PAD serum accelerates the development of their senescence-associated secretory phenotype, reduces their ability to grow, as well as results in increased expression of genes p21 and p53 promoting cellular senescence. The increased expression of p53 genes observed in our study may reflect telomeres shortening in the aging endothelial cells, which may contribute to the progression of atherosclerosis [23, 24]. The expression of p21 genes was also increased, which together with the increased activity of p53 genes, shortens the lifespan of the endothelial cells [25].

Stronger pro-inflammatory and pro-aging genes expression, secretory pattern and inhibition of HAEC growth exposed to the PAD serum may be due to multiple pathomechanisms. Various factors present in the serum of patients with PAD such as dyslipidemia, intravascular inflammatory mediators and oxidative factors, can contribute to aging of the endothelial cells and their dysfunction [26, 27]. There are various pharmacological approaches to prevent or at least reduce endothelial senescence and its contribution to the progression of atherosclerosis, including the use of ACE inhibitors, beta blockers, antioxidant agents, calcium channel blockers and statins [11]. Disorders within the endothelial glycocalyx reflected by a deficient amount of glycosaminoglycans or their altered structure may predispose patients to the progression of atherosclerotic changes [12]. Previously we found that sulodexide slows the senescence of the human venous endothelial cells undergoing replicative aging in standard culture conditions [15], and in the presence of serum from patients with chronic venous insufficiency [28]. The results from our study confirm that sulodexide also decreases senescence of aortic endothelial cells chronically exposed to PAD serum. We suggest that the beneficial effect of sulodexide in patients with peripheral artery diseases [29] may be caused not only by its direct antiinflammatory action in HAEC [16] but also due to the antisenescent effect in these cells.

The results from the present study confirm that sulodexide slows the progression of HAEC senescence, which in consequence results in reduced release of the inflammatory IL6 and prothrombotic factor vWF. All these effects may translate into slower progression of atherosclerosis. An interesting finding was the strong suppression by Sulodexide of p21 and p53 activity in HAEC, which was even more profound than in the control group. It was shown that telomere dysfunction, DNA damage or genomic instability can trigger cell senescence via the p53/p21 pathway [27]. Activation of that pathway, as was shown in our study, may be blocked by Sulodexide. In another study heparin sulfation appeared to be essential for prevention of cellular senescence and that effect was linked with inhibition of p53/p21 pathway [30]. This may suggest that many beneficial effects of Sulodexide in clinical practice may be due to its antisenescent activity.

The results from our studies, together with our previous findings [16], suggest that sulodexide which is a mixture of the natural glycosaminoglycans present in the endothelial glycocalyx, has a pleiotropic effect on arterial endothelial cells. It inhibits the senescence of the arterial endothelial cells, which in consequence decreases intravascular inflammation and the thrombogenic properties of plasma. All these effects may slow the progression of atherosclerotic changes in the arteries. We do not know if the effect of sulodexide on the aortic endothelial cells, as described in the present paper, is the same in other parts of the arterial system i.e.in the coronary vessels. Therefore, further experimental studies should be performed to evaluate the action of that drug on endothelial cells from other arterial vessels. The efficiency of sulodexide as a drug slowing the progression of atherosclerosis should also be further evaluated in clinical trials.

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Disclosure Statement

Dr K. Jasinska - Suminska is an employee of Alfa Wassermann Polska.

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