



Human pulmonary artery endothelial cells in the model of mucopolysaccharidosis VI present a prohypertensive phenotype



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ABSTRACT

Background: Mucopolysaccharidosis type VI (MPS VI) is an autosomal recessive lysosomal disorder caused by a deficient activity of N-acetylgalactosamine-4-sulfatase (ARSB). Pulmonary hypertension (PH) occurs in MPS VI patients and is a marker of bad prognosis. Malfunction of endothelium, which regulates vascular tonus and stimulates angiogenesis, can contribute to the occurrence of PH in MPS VI.

Aim: The aim of the study was to establish a human MPS VI cellular model of pulmonary artery endothelial cells (HPAECs) and evaluate how it affects factors that may trigger PH such as proliferation, apoptosis, expression of endothelial nitric oxide synthase (eNOS), natriuretic peptide type C (NPPC), and vascular endothelial growth factor A (VEGFA).

Results: Increasing concentrations of dermatan sulfate (DS) reduce the viability of the cells in both ARSB deficiency and controls, but hardly influence apoptosis. The expression of eNOS in HPAECs is reduced up to two thirds in the presence of DS. NPPC shows a biphasic expression reaction with an increase at 50 µg/mL DS and reduction at 0 and 100 µg/mL DS. The expression of VEGFA decreases with increasing DS concentrations and absence of elastin, and increases with increasing DS in the presence of elastin.

Conclusion: Our data suggest that MPS VI endothelium presents a prohypertensive phenotype due to the reduction of endothelium's proliferation ability and expression of vasorelaxing factors.

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1. Introduction

Mucopolysaccharidosis type VI (MPS VI, Maroteaux–Lamy syndrome, OMIM# 253200) is an autosomal recessive lysosomal storage disorder affecting many organs of the human body. It is determined by mutations in the arylsulfatase B gene (*ARSB*) gene located on chromosome 5 (5q13–5q14) [1]. Pathogenic mutations of this gene result in a reduced or absent activity of arylsulfatase B (4-sulfatase, arylsulfatase B, ARSB, EC 3.1.6.12), and lead to incomplete degradation and accumulation of glycosaminoglycans (GAGs): dermatan sulfate (DS) and chondroitin 4-sulfate (CS). The negative role of DS on the cardiovascular system has been widely documented; the excess of DS in the heart causes valvular heart disease with all consequences [2,3]. The occurrence of pulmonary hypertension (PH) in MPS patients is a

marker of increased mortality [4] whereas the etiology and mechanisms causing PH in MPS VI patients still remain unknown.

Pulmonary hypertension is caused by the reduction of pulmonary vessels. Reduced total cross-section of pulmonary vessels causes the given blood volume from the right ventricle to be ejected with higher pressure to retain the constant flow. Moreover, increased wall stiffness of pulmonary vessels contributes to increased pulmonary pressure as the vascular bed cannot receive blood volume quickly enough. There is a large heterogeneity among patients with PH in terms of disease development, including genetic and clinical predispositions and clinical responses to available therapeutics [5]. PH is characterized by vasoconstriction and vascular remodeling including medial thickness and muscularization of non-muscularized vessels [6–8]. Most patients with severe pulmonary arterial hypertension also have structural changes in pulmonary arterioles caused by the angiogenic proliferation of endothelial cells forming plexiform lesions [9–11]. Endothelium plays a key regulatory function in the vascular system by controlling vascular tone, platelet activity, leukocyte adhesion and angiogenesis [12]. For occurrence and progression of severe pulmonary hypertension the dysfunction of endothelium is crucial; it affects the reduction of vasodilator production, whereas vasoconstrictors are overexpressed [13,14]. Moreover, pulmonary artery endothelial cell apoptosis, induced by a

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variety of factors (i.e. hypoxia), is the initial step triggering PH [15–17]. Most endothelial functions depend, to various extent, on changes in intracellular calcium [18]. The endothelial nitric oxide synthase (eNOS) producing nitric oxide (NO) remains the main regulator causing vascular smooth muscle relaxation and blood pressure reduction. It is well documented that the increase of intracellular calcium stimulates (via calmodulin) the eNOS to produce NO [19]; on the other hand a calcium independent eNOS pathway has also been reported [20]. Another factor that proved to cause vasodilatation is the natriuretic peptide type C (NPPC) [21]. NPPC involves the calcium/nitric oxide synthase/nitric oxide pathway for vascular relaxation [21]. Apart from vasodilatory properties, NPPC has anti-inflammatory [22–24] and anti-mitogenic properties [22,23,25] and promotes regeneration of endothelial cells in injured vessels [25–27]. Vascular endothelial growth factor (VEGF) is the main proangiogenic factor. VEGF's under-expression is linked to PH occurrence [28,29]. It could be observed that in the monocrotaline PH model a drop of pulmonary expression of VEGF causes the decrease of pulmonary vessel number and increase in vessel wall thickness [29]. VEGF gene transfer in this model was an effective method of preventing the PH development and progression [30].

Cells interact with the extracellular matrix adhesively, however various signals can be transduced through the plasmalemma using cell surface receptors [31,32]. A non-integrin receptor, which is present on the cell surface for interactions with elastin, consists of two cell-membrane-associated proteins immobilizing the third protein – elastin binding protein (EBP) [33–35]. This endothelial elastin–laminin receptor [36] is linked to cytoskeletal actin microfilaments and contains a lectin domain that, when occupied by galactosugar or N-acetylgalactosamine-containing glycosaminoglycans (i.e. dermatan sulfate), inhibits elastin binding [34,37,38]. The binding of elastin to the receptor causes a rapid transient intracellular calcium (Ca^{2+}) increase and thus the intracellular calcium signaling contributes to the regulation of various endothelial functions [39]. Such a mechanism can at least partially explain the pathology of MPS VI on a cellular basis. Using this information we constructed MPS VI cellular model of human pulmonary artery endothelial cells where the arylsulfatase B was silenced with corresponding small interfering ribonucleic acid (siRNA) and the cells were cultured in the presence of dermatan sulfate with or without elastin [40].

The aim of the study was to establish a human pulmonary artery endothelial model of MPS VI and evaluate how it alternates proliferation, apoptosis, expression levels of eNOS, NPPC and vascular endothelial growth factor A (VEGFA) – factors that can contribute to an elevated incidence of PH in MPS VI patients. New data obtained on endothelial cell dysfunction in MPS VI could contribute to the development of novel strategies for the treatment of this disorder.

2. Material and methods

Dermatan sulfate (chondroitin sulfate B sodium salt) from porcine intestinal mucosa and soluble elastin from bovine neck ligament obtained were from Sigma-Aldrich St. Louis, MO, USA.

Human pulmonary artery endothelial cells (HPAECs) were obtained from Lonza, Switzerland. EBM-2 Basal Medium supplied with EGM-2 BulletKit from Lonza, Switzerland was the growth medium. 13% Bovine Fetal Serum Gold from GE Healthcare Life Sciences (former PAA Laboratories) was added to the growth medium to maintain the proliferation of the cells. The cells were cultured at 37 °C with 5% CO₂ in humidified conditions.

The silencing of arylsulfatase B (ARSB) was conducted using commercially available siRNA sequence. Arylsulfatase B short interference RNA (siARSB) was obtained from GE Healthcare Life Sciences (former Dharmacon Inc.), Pittsburg, USA: 1xSMARTpool:siGENOME ARBS siRNA (human) for ARSB RNA silencing; the negative control was 1xsiGENOME Non-Targeting siRNA Pool 1 (human). Oligofectamine transfection reagent and OptiMEM transfection medium were obtained from Life Technologies Corporation, Carlsbad, CA, USA.

The siRNA transfection was conducted according to the Oligofectamine protocol. Shortly, cells were plated and incubated overnight in complete growth medium. The mixture containing Oligofectamine and siRNA in serum free OptiMEM after incubation was added to the cells in serum-free OptiMEM and was incubated for the next 24 h. All measurements were done in triplicates with control group transfected with non-targeting siRNA. The gene expression analyses were done 36 h after transfection.

To analyze the effect of DS and elastin on HPAECs the transfection medium was changed to growth medium after 24 hour transfection, than DS was added. After following 12 hour incubation elastin was added. The measurements were completed after the next 24 hour incubation.

RealTime PCR was done using RealTime PCR Mix EvaGreen Kit (A&A Biotechnology, Gdynia, Poland) and CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The expression levels of individual mRNA were analyzed using the cycle threshold method. Relative quantification of mRNA expression levels was calculated using the $2^{-\Delta\Delta Ct}$ Ct method [41]. For RealTime PCR primers with the following sequences were used:

ARSB – Forward (F): ctgcctttccaccgtcctcc, Reverse (R): cgcgtctcctgtaaagcctg,

VEGFA – F: ctgctctgagttgccagg, R: caggggatggaggaaggtc,

eNOS – F: gtccaacatgctgctgaaa, R: ggtatccaggtccatgcaga.

NPPC – F: gaagaaggcgacaaggctc, R: gattcgtccagcttgaggc.

Relative quantification of cell viability was done using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, Fitchburg, USA) referred to as MTT Test). Shortly, the assay is based on the reduction of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. Only living and metabolic active cells can produce the formazan dye. The intensity of the product color measured at 490 nm is directly proportional to the number of living cells in the culture. The formazan dye product was quantified by a microplate spectrophotometer (EPOCH, BioTek Instruments, Winooski, VT, USA).

For quantification of apoptosis FITC Annexin V Apoptosis Detection Kit from BioLegend, San Diego, CA, USA was used. Shortly, the assay is based on the translocation of phosphatidylserine (PS) from the inner (cytoplasmic) leaflet of the plasma membrane to the outer (cell surface) leaflet in early stages of apoptosis. FITC-Annexin V binds PS and stained (apoptotic) cells are counted by flow cytometric analysis. To distinguish apoptotic (FITC-Annexin V staining) from necrotic cells FITC-Annexin V was used in conjunction with propidium iodide. Analysis of stained cells was performed using cytofluorometric FACS Aria III (Becton Dickinson, Franklin Lakes, NJ, USA).

The statistical analyses were conducted using Microsoft Excel software (Microsoft Corporation, Redmond WA, USA). The value was expressed as mean \pm standard deviation of three independent experiments. Two-tailed Student t-test was used for analyzing unpaired data, and p value below 0.05 was considered statistically significant.

3. Results

Using the commercially designed siRNA sequence against the Arylsulfatase B we were able to achieve 41.1% silencing of ARSB mRNA in HPAECs.

The results of the viability assays are presented in Figs. 1 and 2. Both tests showed reduced viability of HPAECs in high DS concentrations. However, the cells incubated without elastin in growth medium showed a biphasic viability response. First the viability of both siARSB transfected and control cells decreased in low concentrations of DS. After reaching the lowest viability at DS 12.5 μ L/mL the viability of the cells increased reaching its maximum at 50 μ g/mL. Above this concentration the viability of the cells consecutively decreased again. There was also a difference in viability between siARSB and control – at the

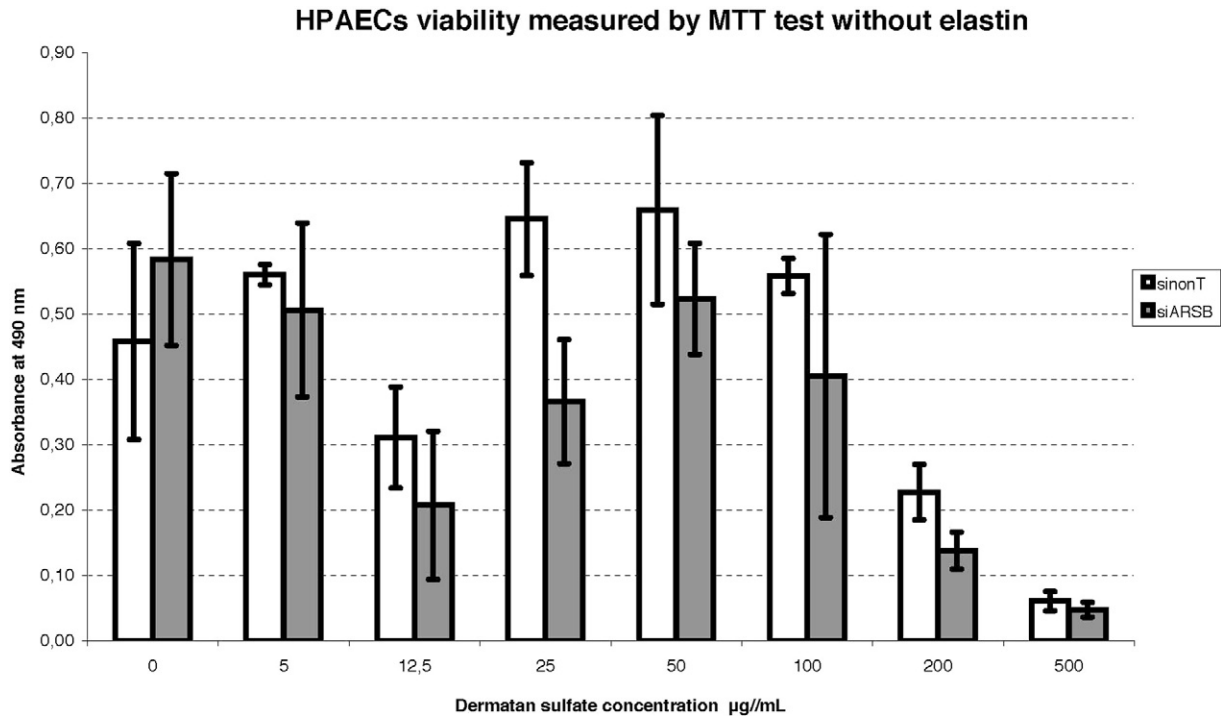


Fig. 1. HPAEC viability measured by MTT test without elastin. Influence of various concentrations of dermatan sulfate on the viability of HPAECs transfected with ARSB siRNA without elastin. The corresponding controls are HPAECs transfected with non-targeting siRNA. Data were presented as means and standard errors. Horizontal bars show statistical significant differences with p-values between values at the bar ends.

25 µg/mL DS concentration the viability of the control group was significantly higher. Other DS concentrations showed also this trend. 50 µg/mL elastin concentration caused a significant reduction viability of HPAECs already at 25 µg/mL with no transient viability gain. The trend of higher HPAECs viability in the control group in comparison to siARSB transfected cells, however statistically non-significant, could also be seen in the experiment with elastin.

The results of the apoptosis measurements as a ratio of corresponding measurements of siARSB transfected cells and their controls are presented in Fig. 3. The cells were analyzed in three subgroups: late apoptotic – labeled with propidium iodide (IP) and FITC Annexin V, early apoptotic – labeled with FITC Annexin V and non-apoptotic – not labeled. The most signal counted in fluorocytometry came from the not labeled cells – non-apoptotic cells (70–75% of whole cell pool –

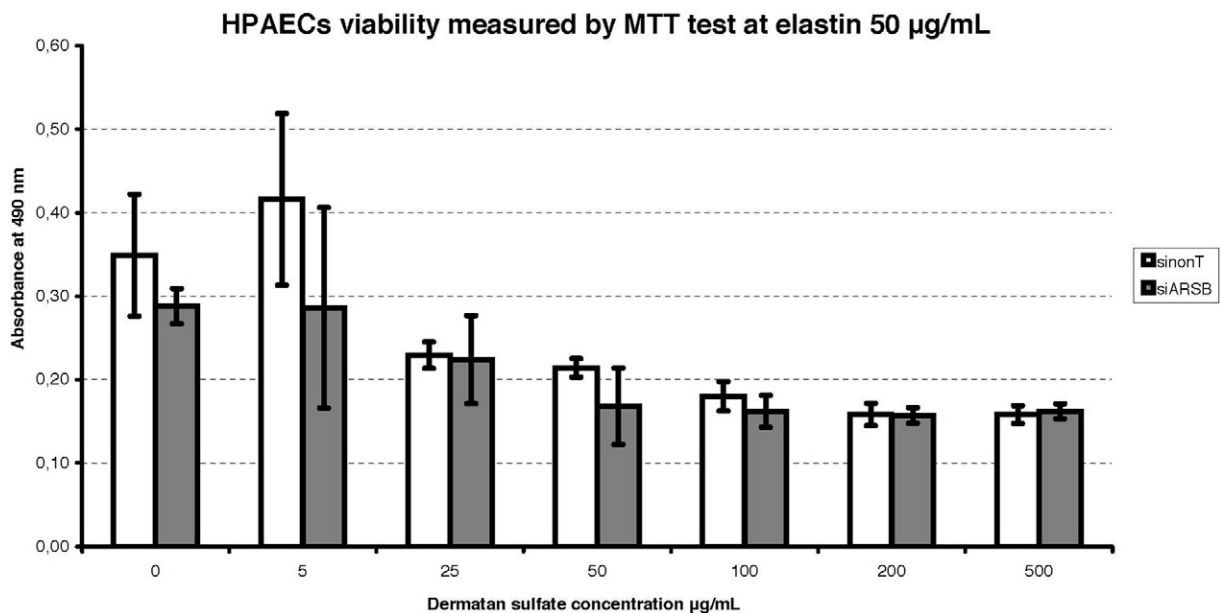


Fig. 2. HPAEC viability measured by MTT test at elastin 50 µg/mL. Influence of various concentrations of dermatan sulfate on proliferation and viability of HPAECs transfected with ARSB siRNA in the presence of elastin (50 µg/mL). The corresponding controls are HPAECs transfected with non-targeting siRNA. Data were presented as means and standard errors. Horizontal bars show statistical significant differences with p-values between values at the bar ends.

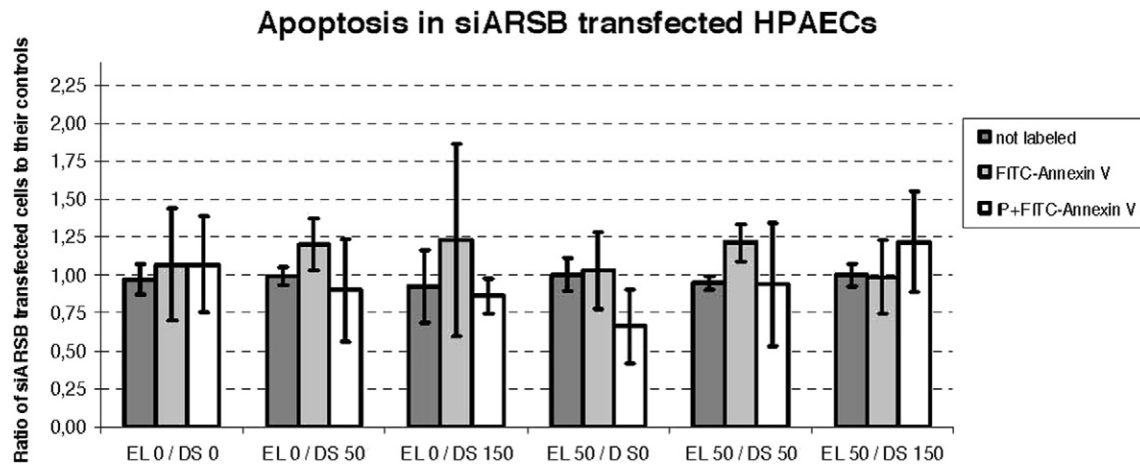


Fig. 3. Apoptosis in siARSB transfected HPAECs. Analysis of apoptosis in HPAECs in vitro using FITC-Annexin V and propidium iodide (IP). For the quantification of apoptosis cytofluorometry was used and the data was presented as a ratio of siARSB transfected cells to their controls. The particular fractions of cells (late apoptotic, not labeled and early apoptotic) correspond to labeling (IP + FITC-Annexin V, not labeled and FITC-Annexin V). The necrotic cells labeled with IP only were omitted due to only a trace amount of IP labeled cells. The analysis was conducted by various concentrations of dermatan sulfate and elastin. EL0/DS 0 – without elastin and dermatan sulfate, EL 0/DS 50 – without elastin and with 50 $\mu\text{g}/\text{mL}$ dermatan sulfate, EL 0/DS 150 – without elastin and with 150 $\mu\text{g}/\text{mL}$ dermatan sulfate, EL 50/DS 0 – with elastin 50 $\mu\text{g}/\text{mL}$ and without dermatan sulfate, EL 50/DS 50 – with 50 $\mu\text{g}/\text{mL}$ elastin and 50 $\mu\text{g}/\text{mL}$ dermatan sulfate, EL 50/DS 150 – with 50 $\mu\text{g}/\text{mL}$ elastin and 150 $\mu\text{g}/\text{mL}$ dermatan sulfate. Data were presented as means and standard errors.

the differences between all groups were not significant), and to the less extent from the early apoptotic cells labeled with Annexin V solely (about one fourth of the cell pool; the differences between all groups were also not significant). Late apoptotic cells labeled with both Annexin V and IP consisted not more than 2.75% of the counts. There has been no influence found for ARSB silencing on the apoptosis of HPAECs in the majority of elastin/dermatan sulfate constellations. Solely the incubation with elastin of siARSB transfected HPAECs showed the trend to reduce the apoptosis in the absence of dermatan sulfate (the number of late apoptotic cells dropped by 33%); however the consecutive increase of apoptosis (measured by Annexin V and IP) with rising DS concentrations could further be observed – the number of the late apoptotic cells increased by 55% in probes with 150 $\mu\text{g}/\text{mL}$ DS in comparison to probes with no DS. Furthermore the observed trend to increase the number of non-apoptotic cells with increasing concentrations of DS without elastin was not significant. The necrotic cells labeled with IP solely counted only for a trace signal in the fluorocytometry (less than 0.5% of the counts) and were omitted in Fig. 3.

The impact of ARSB silencing on the expression of endothelial nitric oxide synthase, natriuretic peptide type C and vascular endothelial growth factor A was presented in Fig. 4. All expression measurements were done in various concentrations of DS with and without the addition of elastin. The experiments revealed that the expression of the endothelial nitric oxide synthase in the case of Arylsulfatase B silencing is significantly reduced (Fig. 4a). Reduction of expression levels of eNOS varied with concentrations of the elastin and dermatan sulfate. In the absence of elastin and dermatan sulfate the level of eNOS expression was reduced by 35%. The addition of DS to the growth medium caused further reduction of the eNOS expression to about 1/3 of the normal level. The eNOS expression in the presence of elastin showed about 50% reduction in growth medium without dermatan sulfate and in the 150 $\mu\text{g}/\text{mL}$ DS concentration, and in the moderate concentration of DS (50 $\mu\text{g}/\text{mL}$) the reduction was less – about 40%. The expression patterns of NPPC (Fig. 4b) showed a reduced NPPC expression without DS and in high DS concentrations with transient NPPC expression gain up to 20% in middle DS concentrations both with and without elastin. The expression level of VEGFA (Fig. 4c) in siARSB transfected and control cells revealed an opposite effect on increasing concentrations of dermatan sulfate in the presence and absence of elastin. Whereas VEGFA expression level in siARSB transfected HPAECs decreased in raising concentrations of dermatan sulfate, VEGFA expression in the presence of elastin went up with raising concentrations of dermatan sulfate reaching normal level.

4. Discussion

There is little evidence in the literature on the impact of increased amounts of dermatan sulfate and arylsulfatase B shortage on a single cell function. The majority of descriptions of the MPS VI pathology are limited to presenting the pathological processes in the whole systems of the body or organs [42]. However, explanation of the pathology by the DS interference of the elastin receptor has already been proposed – the excess of DS inhibits the calcium signaling dependent on elastin receptor. Elastin is a glycoprotein of cross-linked 72 kD tropoelastin subunits and is an abundant component of extracellular matrix of the arteries, lung and skin [43]. DS enriched extracellular matrix would falsely indicate that the cell resides in an elastin lacking environment or in environment with low concentration of elastin degradation products – elastin derived peptides (EDP) [33–39], that has been shown to promote endothelial cell migration and tubulogenesis [44].

The cardiovascular system is frequently disease-involved in MPS VI; malfunction of endothelium could lead to some of its presentation – valvular disease, coronary artery disease or pulmonary hypertension [45,46]. The pathogenesis of the pulmonary hypertension in MPS VI patients is unknown. Obstructive and restrictive lung disease in concert with upper airway obstruction leading to obstructive sleep apnea [41] may contribute to the hypoxia induced PH in these patients. Moreover, pathology in the left heart i.e. due to mitral valve malfunction and/or dysfunction of the left ventricle might also cause PH [2,3]. Although these factors may be important for the occurrence of PH in MPS VI, the importance of eventual malfunction of the pulmonary artery endothelial cells cannot be omitted during considering PH etiology. This opinion confirms the results of Kelly et al. who documented a significantly impaired endothelial function of MPS children in a non-invasive test measuring digital reactive hyperemia [47].

Endothelium plays a crucial role in many processes within the vascular bed creating an inner layer directly contacting blood. A potential pathology in endothelial cells would have a negative impact on vessels, heart valves, immune and coagulation processes. Thus, the aim of the study was to investigate how arylsulfatase B deficiency changes the endothelial cells of human pulmonary artery. The real conditions on the molecular level in MPS VI patients are unknown and surely vary between separate subjects. To emulate these conditions and having limited reference data from the literature, we decided to create an endothelial cellular model of the MPS VI disease. We tested siARSB transfected HPAECs in various concentrations of storage material

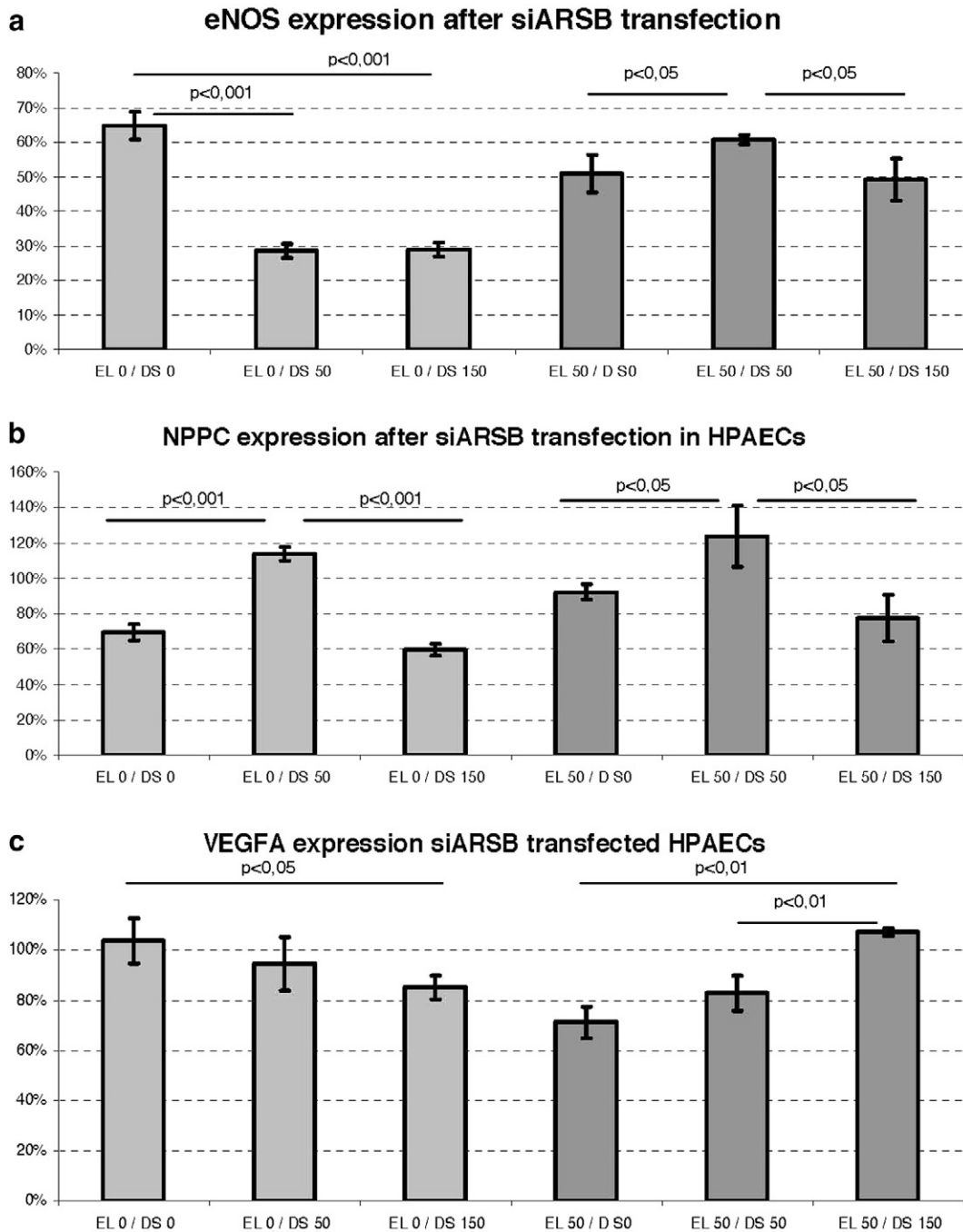


Fig. 4. Analysis of the impact of siARSB transfection in HPAECs on the expression of (a) – endothelial nitric oxide synthase (eNOS), (b) – natriuretic peptide type C (NPPC), and (c) – vascular endothelial growth factor A (VEGFA) in the presence of elastin and dermatan sulfate. The results show, relative to the control (siRNA non-targeting), change of the gene expression in the case of ARSB silencing with corresponding siRNA. EL0/DS 0 – without elastin and dermatan sulfate, EL0/DS 50 – without elastin and with 50 µg/mL dermatan sulfate, EL 0/DS 150 – without elastin and with 150 µg/mL dermatan sulfate, EL 50/DS 0 – with 50 µg/mL elastin and without dermatan sulfate, EL 50/DS 50 – with 50 µg/mL elastin and 50 µg/mL dermatan sulfate, EL 50/DS 150 – with 50 µg/mL elastin and 150 µg/mL dermatan sulfate. Data were presented as means and standard errors. Horizontal bars show statistical significant differences with p-values between values at the bar ends.

(DS) and exposure to stimulation of the elastin receptor by elastin. According to previously published data [32,36,38] DS should interfere the stimulation of elastin receptor and change the intracellular calcium levels causing multiple functional alteration of cells. Such model has its limitations. The main difference between our model of MPS VI and disease itself consists in the way of reduction of ARSB function. In the case of the MPS VI disease the lesion of the function comes from mutation in ARSB gene causing structural and functional alternation of the enzyme. In the proposed MPS VI model the reduction of the enzyme function comes solely from the

reduction of ARSB amount. The kinetics of the gene silencing with siRNA does not allow us to precisely determine the level of enzyme activity in our experiments. However we were able to observe statistically significant changes in most of our experiments. Our MPS VI model consists not only from the silencing of the ARSB gene solely, but we analyzed also the HPAECs' function in the case of DS and elastin excess. In our opinion the functional changes in MPS VI cells come not only from reduced ARSB function exclusively but also from the excess of DS in the cytoplasm and outside cell environment. Moreover the clinically observed abnormalities in MPS VI patients

occurred in a much longer time than the time range of our experiments. Thus minimal abnormalities over a long time would sum up to clinical effect. While analyzing the clinical data we must keep in mind existing possible adaptational mechanisms, which would compensate the negative effect of the DS excess.

Our results show that, although the viability characteristics is alternated in the presence of arylsulfatase B deficiency, it hardly influences the HPAEC apoptosis – an initial step to trigger PH [15–17]. Additionally, viability response of the cells is different between cells under and without exposure to elastin as well as in HPAECs treated with siARSB and their controls. The control cells tended to have higher proliferation than siARSB cells in corresponding DS concentrations. The proliferative reaction to rising DS concentrations in the absence of elastin was biphasic with viability minimum at 12.5 µg/mL, two viability peaks at about 5 and 50 µg/mL and consecutive viability drop starting from 100 µg/mL. HPAECs stimulated by elastin did not show this viability pattern. The experiment with elastin revealed steady drop in cell viability with rising DS concentrations. This general reduction of the HPAEC proliferation may present on the cellular level the pathology observed on the whole organism. If this proliferation reduction would concern all cells in a MPS VI patient, then generally we would have a growth retardation independent from growth hormone deficiency. Such observations are common in MPS VI patients especially with rapidly progressing phenotype. There are various potential mechanisms explaining this observation – i.e. siARSB cells may produce less para- and autocrine growth factors in the presence of intracellular DS excess, and the metabolism of the cells can be reduced or cell cycle may be slowed down. The obtained results in HPAECs viability test let us speculate that not every process in our MPS VI model proceeds straight lineally.

The following step of the study was to analyze the expression of eNOS, NPPC and VEGF. In the case of eNOS we observed a drastic drop of expression, especially in the case of DS excess. Such reduced eNOS would produce less nitric oxide and reduce a vasodilative reaction. Similarly, generally reduced natriuretic peptide level could contribute to the elevated vasospastic reaction. In our experiments high DS concentrations caused up to 40% NPPC expression reduction in siARSB cells. The increase of NPPC levels in both elastin enriched and non-enriched media as well as eNOS expression in moderate DS concentrations in the elastin enriched medium further supported our finding that the correlation between DS concentration and expression of separate proteins is not linear in the case of ARSB deficiency. Our results with VEGF, a prominent angiogenic endothelial cell derived factor, support our results obtained from the viability assay of diminished HPAEC proliferation; obtained data clearly show that the rising concentration of DS in siARSB transfected cells reduces the VEGF expression level. The surprising effect of elastin enrichment – VEGF expression gain with raising DS – remains unexplained. Impaired vasculature growth due to the lack of the proliferative stimuli could further contribute to the reduction of pulmonary vasculature bed size and to the elevation of pulmonary arterial pressure. Thus, a reduced expression of VEGF during the lung development would contribute to the incidence of PH in the adulthood of MPS VI patients.

5. Conclusions

Our results demonstrate that the arylsulfatase B deficient pulmonary artery endothelial cell is alternated and differently reacts to factors stimulating elastin receptor. The results of this research can contribute to the evaluation of further studies on endothelial dysfunction aiming to propose new treatment options and to reduce elevated cardiovascular mortality in MPS VI patients.

Acknowledgments

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