Report on the project funded by the Austrian Heart Funds:
The role of the multifunctional protein pigment-epithelium derived factor and its receptors in smooth muscle cell biology and in the development of restenosis.
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Introduction

As outlined in our original proposal a possible role for PEDF in restenosis has been suggested from animal experiments. The underlying molecular mechanisms and signalling pathways, however, have not been characterized in much detail yet. Our own preliminary results have shown that PEDF is present in human smooth muscle cells (SMC) isolated from aorta and its expression is downregulated in a time dependent manner after stimulation with the potent smooth muscle cell mitogen platelet derived growth factor (PDGF). Thus the aim of our project was to more closely define and evaluate the role of PEDF in the development of restenosis in an experimental as well as in a clinical setting. Therefore we proposed to investigate the regulation of PEDF and its receptors adipose triglyceride lipase/ patatin-like phospholipase containing 2 (ATGL/PLPLA2) and ribosome protein SA/laminin receptor (LR/RPSA) in human SMC.

Methods

Cell culture:

We have isolated human coronary artery SMC (HCASMC) from coronary arteries, obtained from patients undergoing heart transplantation (Figure 1). HCASMCs were isolated by explant technique as described below. Human coronary artery was cut in small pieces and placed on a petri dish coated with 1% gelatin. Tissue pieces were covered with minimal essential medium (M199) containing 20% fetal calf serum (FCS), 100 U/ml penicillin, 100U/ml streptomycin, 0.25 µg/ml fungizone and 2 mM L-glutamin and incubated at 37°C in a humidified atmosphere of 5% CO_2: 95% air for one to four weeks. After cell attachment the pieces were removed. Cells were cultured in cell culture flasks coated with 1% gelatine in M199 containing 20% FCS 100 U/ml penicillin, 100 U/ml streptomycin, 0.25 µg/ml fungizone and 2 mM L-glutamin at 37°C in humidified atmosphere of 5% CO_2: 95% air. Cells were for our experiments were used at low passages.

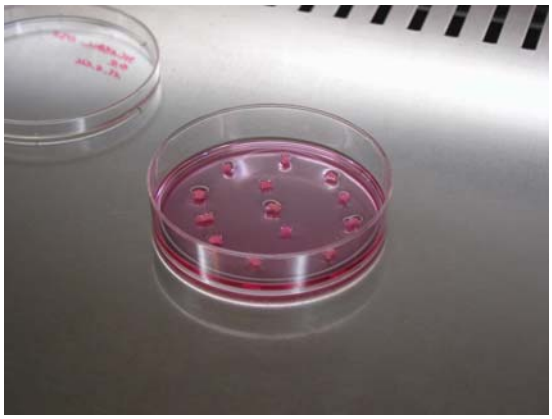

Figure 1: Incubation of the smooth muscle cells

Treatment of cells with PDGF and RT-PCR:

24 hours before treatment with platelet derived growth factor (PDGF) HCASMC were incubated in M199 containing 0.1% bovine serum albumin (BSA). Thereafter medium was replaced with fresh M199 containing 0.1% BSA and PDGF at the indicated concentrations for time periods between 4h and 48h.

Cells were stimulated as described above, supernatant was removed, and mRNA was isolated with the High Pure RNA Isolation Kit according to the manufacturer's instructions. Specific mRNA levels for PEDF, PNPLA2 and RPSA were determined by real time PCR. Samples were analyzed in triplicates on a 96-well reaction plate (Roche, Basel, Switzerland) applying the real-time LightCycler Probe Master, LightCycler TaqMan Master UniversalProbeLibrary Kit. Real-time polymerase chain reaction (PCR) was performed with the LightCycler 480 Real-Time PCR. Primers were designed using the Roche Universal ProbeLibrary Assay Design Centre (http://www.universalprobelibrary.com).

Statistical analysis:

Data was compared statistically by t-test. Values of $P<0.05$ were considered significant.

Results:

Figure 2 shows that PDGF-treatment at the concentration of 200ng/µl of HCASMC reduced mRNA levels for PEDF (upper panel) and for the PEDF-receptors PNPLA2 (middle panel) and RPSA (lower panel) significantly.

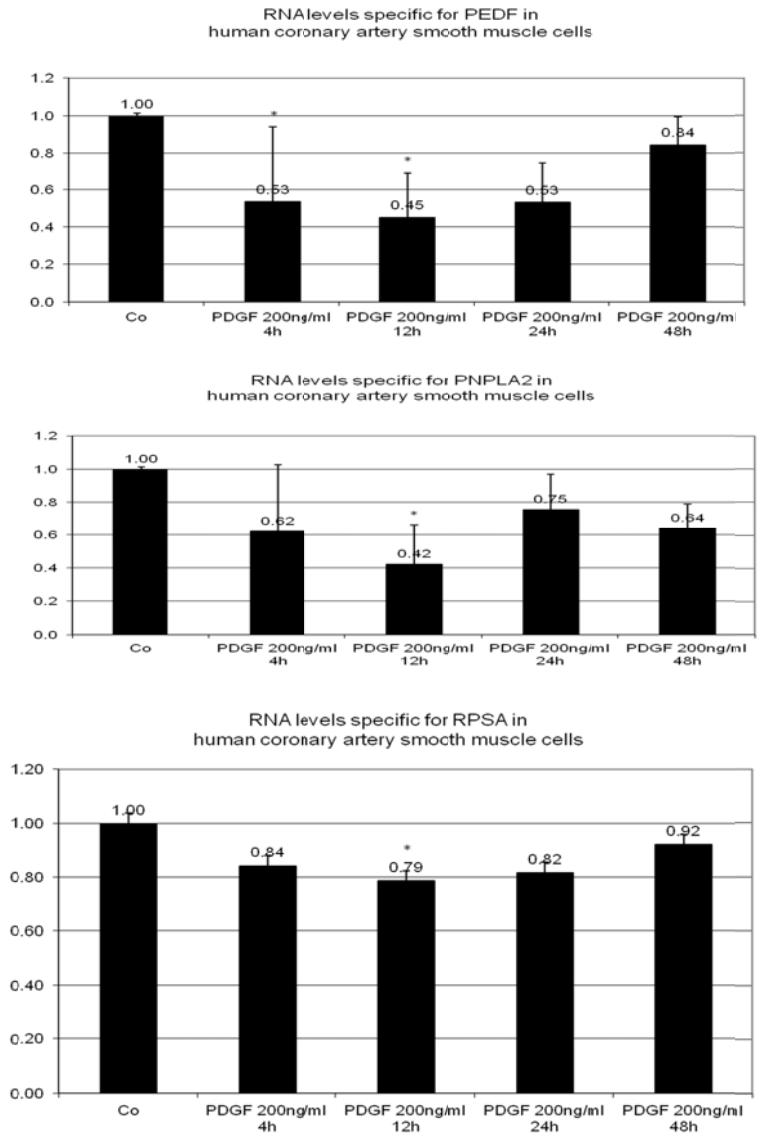

*Figure 2: Effects of PDGF stimulation on PEDF, PNPLA2 and RPSA expression in HCASMC. Confluent monolayers of HCASMC were incubated for 4, 12, 24 or 48 hours in absence or in presence of PDGF at the concentration of 200ng/µl. Experiments were performed 2-times with cells obtained from 2 different donors and gave similar results. A representative experiment is shown. PEDF mRNA level was normalized according to the respective GAPDH. mRNA levels are given as fold of control which was set as 1-fold. Values represent the mean +/- SD of three determinations. (*p<0.05)*

Conclusion:

So far we could show that a strong mitogen for smooth muscle cells, namely PDGF decreases the expression of the antiangiogenic modulator PEDF and its receptors in vascular smooth muscle cells. If operative *in vivo* this effect could impact on smooth muscle cell proliferation and plaque vascularization with PDGF not only regulating the former directly but also supporting both processes indirectly by inhibiting an antiangiogenic modulator and its receptors thereby creating a proangiogenic microenvironment in the wall of the affected vessel.

Outlook:

As outlined in our original proposal, we will in the remaining part of this project aim to elucidate the signal transduction pathways responsible for the downregulation of PEDF and its receptors by PDGF. Given the experimental evidence that PEDF and its receptors are downregulated through a growth factor for smooth muscle cells such as PDGF the planned clinical part of our study seems highly warranted. Thus we will determine plasma levels of PEDF in patients undergoing stent implantation and thus study a possible association of PEDF with restenosis in these patients.