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Expression of OATP2B1 as determinant of drug effects in the microcompartment of the coronary artery

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ABSTRACT

Clinical success of coronary drug-eluting stents (DES) is hampered by simultaneous reduction of smooth muscle cell (HCASMC) and endothelial cell proliferation due to unspecific cytotoxicity of currently used compounds. Previous in vitro data showing SMC-specific inhibition of proliferation suggested that statins may be suitable candidates for DES. It was aim of this study to further investigate statins as DES drug candidates to identify mechanisms contributing to their cell-selectivity. In vitro proliferation assays comparing the influence of various statins on HCASMC and endothelial cells confirmed that atorvastatin exhibits HCASMC-specificity. Due to similar expression levels of the drug target HMG-CoA reductase in both cell types, cellular accumulation of atorvastatin was assessed, revealing enhanced uptake in HCASMC most likely driven by significant expression of OATP2B1, a known uptake transporter for atorvastatin. In accordance with the finding that endogenous OATP2B1 influenced cellular accumulation in HCASMC we used this transporter as a tool to identify teniposide as new DES candidate drug with HCASMC-specific effects. We describe OATP2B1 as a determinant of pharmacokinetics in the coronary artery. Indeed, endogenously expressed OATP2B1 significantly influences the uptake of substrate drugs, thereby governing cell specificity. Screening of candidate drugs for interaction with OATP2B1 may be used to promote SMC-specificity.

1. Introduction

Atherosclerosis is a major risk factor of coronary artery disease which finally may lead to myocardial infarction [1]. In order to restore the blood flow in obstructed arteries the percutaneous coronary transluminal angioplasty (PTCA) is commonly used in clinics. However, after dilatation a variety of cellular mechanisms take place including aggregation of platelets, release of growth factors, infiltration of inflammatory cells, and proliferation and migration of smooth muscle cells [2,3] finally resulting in restenosis of the vessel often with fatal impact on the outcome. In order to prevent restenosis after transluminal artery dilatation the implantation of bare metal stents (BMS) was introduced in this clinical procedure. Although prospective multicenter studies demonstrated an improved clinical outcome with reduced rates of restenosis after stenting [4,5], success of BMS was significantly hampered by the manifestation of subacute thrombosis and in-stent restenosis [6]. Indeed, it is assumed that the injury of the vessel wall during stent deployment triggers an inflammatory process resulting in production and secretion of various growth factors that stimulate smooth muscle cell proliferation resulting in the pathological phenomenon of neointima formation [2,7–9]. The first-generation drug-eluting stents (DES), loaded with sirolimus (Cypher, Cordis, Warren, New Jersey, USA) or paclitaxel (Taxus, Boston Scientific, Natick, USA) were introduced with the idea that cytotoxic compounds inhibit the formation of a neointima due to their antiproliferative activity, thereby reducing the adverse event of in-stent restenosis [10,11]. In accordance with this notion are findings of controlled clinical trials reporting reduced in-stent late lumen loss and in-stent angiographic restenosis comparing DES and BMS [12]. However, the adverse event of stent thrombosis remains a major safety problem, where late stent thrombosis (>1 year after stenting) is more likely after implantation of DES [13,14]. Numerous factors have been associated with the risk of thrombotic events in DES-treated arteries, including reduced endothelialization of the stent strut due to inhibition of migration and proliferation of endothelial cells. Subsequent developments of DES focused on agents demonstrating not only antiproliferative, but also enhanced anti-inflammatory activity as observed with everolimus or zotarolimus, which are both currently used on second-generation DES. However, even if there is a lower risk of late stent thrombosis in patients treated with second-generation DES the incidence of this adverse event has not been abolished [15]. Despite the fact that limus derivatives reduce inflammation after stenting, from a pharmacological perspective there is still the need for

Abbreviations: HCASMC, human coronary artery smooth muscle cells; HCAEC, human coronary artery endothelial cells; HMGCR, HMG-CoA reductase.

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compounds which specifically inhibit the migration and proliferation of smooth muscle cells without affecting endothelial cells in order to enhance healing of vascular implants.

One approach to improve the outcome of DES may be the use of drugs showing benefits in therapy of atherosclerosis. Several studies have previously reported an improved clinical outcome of patients orally treated with statins during the time of DES deployment. Especially the treatment with atorvastatin or fluvastatin has been associated with a reduced risk of adverse cardiac events and other ischemia-related outcomes in patients undergoing PTCA [16,17], thereby being the basis for previous reports suggesting that statin-coated stents may be an option for improved clinical outcome [18,19]. In this context, it seems noteworthy that several in vitro and in vivo studies have reported a positive impact of delivery devices coated with statins. Particularly, for cerivastatin a pronounced effect on smooth muscle cell proliferation and migration [20] without impeding endothelial cell proliferation has been shown [21], supported by in vivo findings in a porcine coronary model [22]. Even if statins are all primarily targeting HMG-CoA reductase thereby modulating cholesterol levels, there are significant differences in their physicochemical and pharmacokinetic properties, which include passive permeability and active transport [23]. Assuming that distribution in the area of the coronary artery, defined as microcompartment, surrounding the drug-delivery devices also depends on passive diffusion or active transport those mechanisms may significantly influence cell-type specificity of drugs used on DES. In order to test this hypothesis we compared the impact of different statins on cellular proliferation, showing pronounced differences comparing smooth muscle and endothelial cells.

The aim of the study was to investigate whether cellular uptake may be a determinant of pharmacodynamics of statins in the micro compartment of the vessel wall, and whether transport by a membrane transporter might be predictive for cell specificity of a compound used on coronary drug delivery devices.

2. Methods

2.1. Materials

[^{3}H]-atorvastatin (specific activity 10 Ci/mmol), [^{3}H]-atorvastatin lactone (specific activity 10 Ci/mmol) and [^{3}H]-estrone 3-sulfate (specific activity 50 Ci/mmol) were obtained from Hartmann Analytic (Braunschweig, Germany). Mevastatin, pravastatin, and simvastatin were purchased from Sigma-Aldrich (Deisenhofen, Germany) and cerivastatin from Bayer (Leverkusen, Germany). Farnesyl pyrophosphate and geranylgeranyl pyrophosphate were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA) and atorvastatin from Pfizer Pharma (Karlsruhe, Germany). All other chemicals were obtained from Sigma-Aldrich (St. Louis, USA) unless otherwise stated.

2.2. Cell culture

Human coronary artery smooth muscle cells (HCASMC) and endothelial cells (HCAEC), isolated from different human individuals and genders, and their optimized media i.e., Smooth Muscle Cell Growth Medium 2 and Endothelial Cell Growth Medium MV were obtained from PromoCell GmbH (Heidelberg, Germany). Cells were cultivated in a humidified atmosphere at 37 °C and 5% CO_2 from passage four to ten. For proliferation assays, cells were seeded in 96-well plates at a density of 5×10^3 cells/well. After 24 h in growth medium, HCASMC or HCAEC were maintained for further 24 h in basal medium (PromoCell GmbH) supplemented with only 0.05% or 0.5% FCS, respectively. To simulate cellular stimulation after stent implantation, cells were then treated with growth medium in presence or absence of the respective drug for 48 h. For transport studies and expression analysis cells were seeded in respective well plates at a density of 1.2×10^4 cells/cm^2. Madin–Darby canine kidney II (MDCKII) cells overexpressing OATP2B1 as previously reported [24] were cultivated in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 1% GlutaMax and 250 μg/ml Hygromycin B, both obtained from Life Technologies (Carlsbad, USA), at 37 °C and 5% CO_2.

2.3. Bromodesoxyuridine (BrdU) cell proliferation assay

Cell proliferation of HCASMC and HCAEC in the presence of statins was determined using the commercially available BrdU Cell Proliferation Kit (Roche, Basel, Switzerland). In brief, BrdU labeling solution was added to the medium after 24 h treatment followed by incubation for 24 h. Subsequently, BrdU ELISA was performed according to the manufacturer's instructions and luminescence was measured with the microplate reader (Infinite® 200 Pro-Tecan, Männedorf, Switzerland). Cells treated with the solvent served as control. Data are presented as percent of control.

2.4. Adenoviral infection

For adenoviral infection the previously reported Ad-OATP2B1 [25] was used. To study the influence on cell proliferation, HCASMC were seeded in 96-well plates 24 h prior to infection with 50 pfu/cell Ad-OATP2B1 followed by incubation with atorvastatin for 48 h. For short-time incubation with teniposide, cells were treated for 10 min, washed with PBS and cultivated in growth medium for 48 h. Transport activity in HCASMC and HCAEC was assessed 48 h after adenoviral infection. Cells infected with Ad-lacZ served as reference for all experiments if not otherwise stated.

2.5. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from six different donors of both naïve HCASMC and HCAEC was isolated using NucleoSpin® RNA (Machery-Nagel, Düren, Germany) and reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions. For qRT-PCR, we used TaqMan® gene expression assays from Life Technologies detecting HMGCR (Hs00168352_m1), 18S rRNA (Hs99999901_s1), or GAPDH (4326317E). PCR reactions were carried out using the Viia 7™ (Life Technologies). Expression relative to human liver was calculated using the ΔΔCt-method.

2.6. Immunoblot analysis

For Western blot analysis cells were harvested in 100 μl of the commercially available RIPA buffer supplemented with 10 μg/ml aprotinin and 10 μg/ml leupeptin. Protein content in cell lysate was quantified by a bicinchoninic acid assay (Pierce, Thermo Fisher Scientific Inc., Rockford, USA). Protein samples were separated by SDS-PAGE and electrotransferred to nitrocellulose membranes using a Tank blotting system (Bio-Rad Laboratories, Berkeley, USA). After protein transfer, membranes were placed in 5%-FCS in TBS-T (0.25 M Tris–base, 0.026 M KCl, 0.04% Tween 200) for 1 h prior to incubation with the primary antibody. The antibodies used were anti-HMGCR (sc-27578, 1:2000 Santa Cruz Biotechnology, Inc.), anti-β-actin (sc-1616, 1:2000 Santa Cruz Biotechnology, Inc.), anti-van Willebrand factor (vWF, ab6994, 1:5000 Abcam® Cambridge, UK) and anti-α-smooth muscle actin (α-SM-actin, ab7817, 1:2000 Abcam®) diluted in TBS-T supplemented with 0.5% BSA. The previously reported anti-OATP2B1 serum [24] was used in a dilution of 1:5000. Incubation was performed at 4 °C overnight followed by several washing steps with TBS-T and incubation with the respective HRP-labeled secondary antibody for 1 h at room temperature. Luminescence was visualized using the ECL Western blotting substrate (Pierce, Thermo Fisher Scientific Inc.) and digitalized with the ChemiDoc XRS system (Bio-Rad Laboratories). Densitometry was conducted using Image Lab 4.1 software from Bio-Rad Laboratories.