Krueger-Genge, A.; Hiebl, B.; Franke, R.P.; Lendlein, A.; Jung, F.:

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In: Clinical Hemorheology and Microcirculation (2017) IOS Press

DOI: 10.3233/CH-179211

Effects of Tacrolimus or Sirolimus on the adhesion of vascular wall cells: controlled *in-vitro* comparison study

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Key words: Endothelial cells, Sirolimus, Tacrolimus, DMSO

Abstract

In drug eluting stents the cytostatic drugs Sirolimus or Tacrolimus are used to inhibit blood vessel restenosis by limiting the proliferation of smooth muscle cells. However, the cytostatic activity of both drugs was shown to be not cell specific and could also affect the stent endothelialisation, respectively. Currently, only limited *in vitro* data are available about the impact of Sirolimus and Tacrolimus on endothelial cell proliferation over a broad concentration range. To answer this question the following study was performed.

Commercially obtained HUVEC were expanded with DMEM cell culture medium (GIBCO, Germany) supplemented with 5 vol% fetal calf serum on non-coated regular polystyrene-based 24-multiwell plates. For drug testings 2×10^4 cells/cm^2 were seeded and grown for 24 h until 30-40% of the multiwell surfaces were covered and then exposed to Sirolimus (1.0×10^{-11} - 1.0×10^{-5} mol/l) or Tacrolimus (2.0×10^{-8} - 6.2×10^{-5} mol/l), both dissolved in DMSO. 12, 24 and 48 h after adding the drugs, cell numbers per area were quantified by counting the cells in six wells with four fields of view per well, representing 0.6 mm^2, using a confocal laser microscope.

After 48 h of cell growth in the drug-free cell culture medium, the HUVEC number increased from 2.0×10^4 to 3.55×10^4 cells/cm^2 (mean cell doubling time: 53.6 h, n=6). At lower concentrations ($\leq 2.0\times10^{-6}$ mol/l) Tacrolimus reduced the number of adherent HUVEC significantly less than Sirolimus ($p<0.05$). However, at higher concentrations ($\geq 2.07\times10^{-5}$ mol/l) the effect of Tacrolimus on the number of adherent endothelial cells was significantly greater than that of Sirolimus ($p<0.05$). At the highest concentration applied (6.22×10^{-5} mol/l), Tacrolimus induced detachment of all HUVECs within 12 h after drug application. The number of adherent HUVEC decreased only slightly (about 9%) after Sirolimus application at the highest concentration (1.09×10^{-5} mol/l).

These data show that in a non-flow model the cytostatic drug Tacrolimus reduced the number of adherent endothelial cells less than Sirolimus, as long as the drug concentration did not surpass 10^{-6} mol/l. At the limits of solubility, Sirolimus (1×10^{-5} mol/l) reduced the number of adherent endothelial cells less than Tacrolimus (6×10^{-5} mol/l), which induced detachment of endothelial cells.

1. Introduction

Local strategies directed against vascular smooth muscle cell proliferation such as drug-eluting stents demonstrated that Sirolimus-eluting stents (SES) and Paclitaxel-eluting stents (PES) in combination with antiplatelet therapies significantly reduced the restenosis rates compared to bare metal stents (BMS) [1, 2, 3]. Drug-eluting stents (DES) quickly became the standard of care for the percutaneous treatment of symptomatic coronary artery disease. The enthusiasm for DES, however, has been dampened by safety concerns regarding their association with significantly higher rates of late stent thromboses appearing after 30 days of implantation compared to the rates with BMS [4, 5].

Histopathologic studies on cardiovascular tissues of patients dying from late stent thrombosis revealed that the primary substrate in all cases was delayed arterial healing characterized by persistent fibrin deposition and poor endothelialisation [6]. A comparison of histological findings from patients receiving either DES or BMS showed that DES implantation resulted in substantial impairment of arterial healing [7].

Large randomized clinical trials powered to prove late thrombotic end points are slowly emerging so that comparative animal studies or *in vitro* studies are effective methods for assessing the influence of cytostatic agents on the degradation of fibrin deposition and on endothelial development and maturation. In this study, the effects of Tacrolimus and Sirolimus in comparison to a vehicle solution alone (culture medium supplemented with DMSO) on the endothelial cell performance were analyzed.

2. Material and Methods

2.1. Cell Culture

Human umbilical venous cells (HUVEC) were commercially obtained (Lonza, Cologne, Germany) and expanded with DMEM cell culture medium (life technologies, Carlsbad, CA, USA) supplemented with 5 vol% fetal calf serum (FCS, Sigma-Aldrich, Saint Louis, MO, USA). HUVECs of the third passage were used [8].

2.2 Study design

2×10^4 cells/cm^2 were seeded on regular non-coated polystyrene-based 24-multiwell plates under static conditions for 24 hours until a confluence of 30 – 40% was reached. Then the culture medium was supplemented with Tacrolimus or Sirolimus and the drug testings started for 48 h.

The drugs were solved in DMSO and aliquots were produced between 2.0×10^{-8} and 6.22×10^{-5} mol/l for Tacrolimus and 1.0×10^{-11} and 1.0×10^{-5} mol/l for Sirolimus. The maximum concentrations of Tacrolimus or Sirolimus were chosen so that the solubility products of both substances in cell culture medium supplemented with DMSO were not surpassed. The influence of the solvent DMSO without drug addition was used as reference system/control.

After 12, 24, and 48 h cell numbers per area were quantified by counting the cells in six wells with four fields of view per well, representing 0.6 mm^2, using a confocal laser microscope (cLSM, LSM 510 META, Zeiss, Oberkochen, Germany). The absolute cell numbers per area were then related to control cultures at the beginning of the experiments (24 h after seeding) supplemented only with DMSO and given as relative density in percent.

Additionally, the influence of the tested macrolides on HUVEC membrane integrity was assessed after 48 h of cell exposition on the multiwell plates (LDH-assay, Roche, Penzberg, Germany) at a drug concentrations of 10^{-6} mol/l, measuring the extracellular concentration of the enzyme lactate dehydrogenase (LDH) – normally only found in the cytoplasm - using a TECAN SpectraFluor Plus spectrophotometer at 492 nm [9].

The study was performed in accordance with the ethical guidelines of *Clinical Hemorheology and Microcirculation* [10].

2.3 Statistics

For all samples, mean value and standard deviation are given (mean value ± standard deviation). Gaussian distributions were tested for all samples using Kolmogorov and Smirnov test. For three sample problems a two-way variance analysis was performed. For two sample problems unpaired two-sided t-tests were used. P values less than 0.05 were considered significant. Because of the explorative character of the study no Bonferoni-adjustment was performed.

3. Results

Figure 1 shows adherent HUVEC numbers per cm^2 of culture area in percent (related to control cultures supplemented without DMSO), between the start of the experiment and 48 h later. 48 hours after the start of the experiments, the relative number of adherent HUVEC increased from 99.12±9.3% to 129.3±3.6% without addition of DMSO, increased from 99.9%±4.1% to 141.3%±3.8% after addition of 10^{-6} vol% DMSO, increased from 98.3%±5.9% to 139.3%±7.6% after addition of 10^{-2} vol% DMSO, increased from

93.6%±3.8% to 141.4%±4.8% after addition of 0.5 vol% DMSO, and increased from 68.7%±5.9% to 82.5%±9.6% after addition of 1 vol% DMSO.

The addition of DMSO influenced the relative density of adherent HUVEC over the time ($p=0.001$) and with increasing amounts of DMSO ($p=0.001$). Up to a DMSO concentration of 0.5 vol% the numbers of HUVEC increased over time but without a difference of the different DMSO concentrations. They decreased however, at a DMSO concentration of 1 vol%.

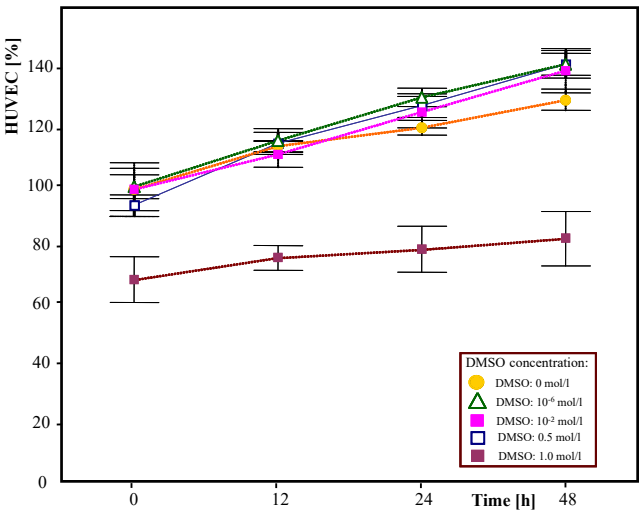

Figure 1: Relative density of HUVEC per cm^2 in percent related to the initial number of adherent endothelial cells without addition of DMSO immediately after the start of the experiment and after 12, 24 and 48 h; DMSO was added in concentrations between 10^{-6} vol% and 1 vol% to the culture medium

Figure 2 shows HUVEC seeded for 24 h, directly after incubation (Fig. 2a, left) in fresh culture medium supplemented with 0.5 vol% DMSO (without addition of cytostatic drugs) and 48 h later (Fig. 2b, right).

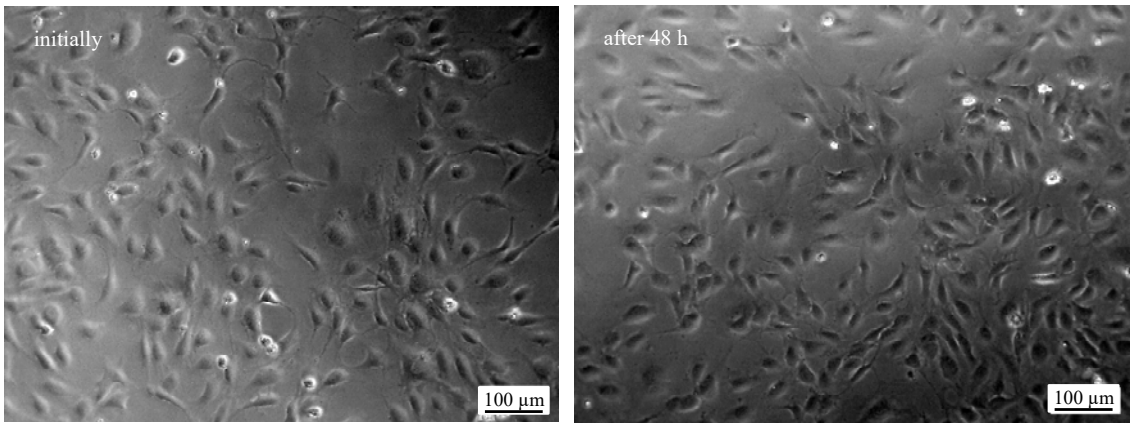

Figure 2: HUVEC 24 h after cell seeding and directly after incubation (Fig. 2a, left) in fresh culture medium supplemented with 0.5 vol% DMSO and 48 h later (Fig. 2b, right)

HUVEC seeded on polystyrol (24-well plates) displayed typical aspects of cells early in culture and among them a few larger cells, typically 1.5 to 4%, of vascular wall descendant cells like myofibroblastic precursor cells or smooth muscle cells (SMC). 48 hours later there were increased numbers of more or less healthy looking spindle-shaped migrating HUVEC and the total cell number was clearly greater than 48 h before addition of new culture medium supplemented with DMSO.

Figure 3 shows the alterations of adherent HUVEC numbers per cm^2 in percent (related to control cultures supplemented only with DMSO in amounts necessary to solve the aliquots of the macrolides) over time after addition of DMSO-solved Sirolimus to the culture medium in concentrations between 1.0×10^{-11} mol/l and 1.0×10^{-5} mol/l. The latter concentration was chosen so that the solubility product of the drug in the solvent was not surpassed. The addition of Sirolimus influenced the numbers of adherent HUVEC significantly over the time of the experiment (p=0.01) and depending on the amount of Sirolimus added (p=0.01).

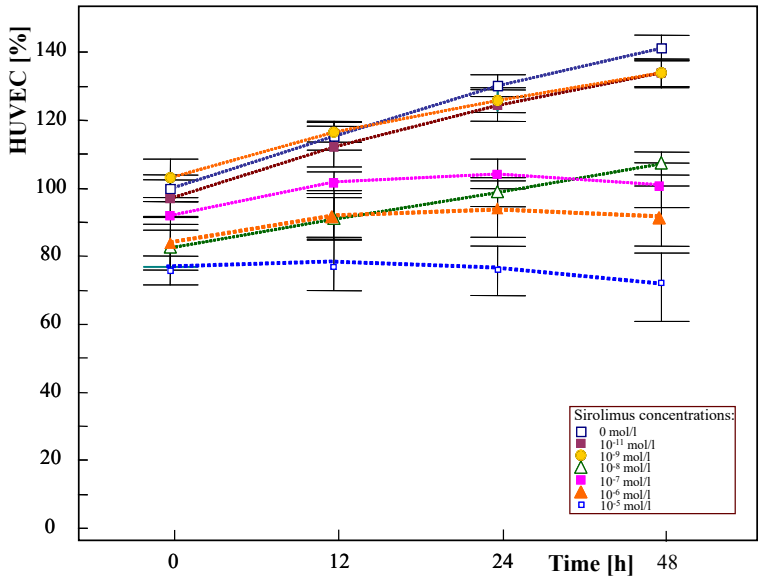

Figure 3: Relative density of adherent HUVEC per cm^2 in percent (related to control cultures supplemented only with DMSO in amounts necessary to solve the aliquots of Sirolimus), immediately after the addition of Sirolimus in concentrations between 1.0×10^{-11} and 1.0×10^{-5} mol/l and then 12, 24, and 48 h later.

The relative number of adherent HUVEC increased from 97.1%±5.6% to 133.5%±4.3%, 48 h after the addition of 1.0×10^{-11} mol/l Sirolimus solved in DMSO to the culture medium. The increase in numbers of adherent HUVEC continuously decreased with increasing Sirolimus concentration added to the culture medium. At a Sirolimus concentration of 1.0×10^{-6} mol/l the relative increase in adherent HUVEC amounted to 7.6%±13.7%, at a concentration not

surpassing the solubility product (1.09×10^{-5} mol/l) the relative density of adherent HUVEC decreased by 4.9%±7.4%. At the same time the number of initially adherent HUVEC decreased from about 100% (at concentrations of 10^{-11} and 10^{-9} mol/l) to 75.9% (Sirolimus concentration of 1.09×10^{-5} mol/l).

Figure 4 shows HUVEC 24 h after cell seeding and directly after incubation (Fig. 4a, left) in fresh culture medium supplemented with 1.09×10^{-5} mol/l Sirolimus solved in DMSO and 48 h later (Fig. 4b, right).

After the addition of Sirolimus, the HUVEC on polystyrene (24-well plate) initially displayed typical aspects of not so dense HUVEC early in culture and among them a few larger cells, typically 1.5 to 4%, of vascular wall descendant cells like myofibroblastic precursor cells or smooth muscle cells. 48 h later, there were increased numbers of spindle-shaped migrating HUVEC where several of these cells exhibited more or less damages. Interestingly, the larger cells seemed to be unaffected. Cell morphology indicated elevated numbers of proliferating cells, but also increased numbers of damaged cells and an increase in cell debris.

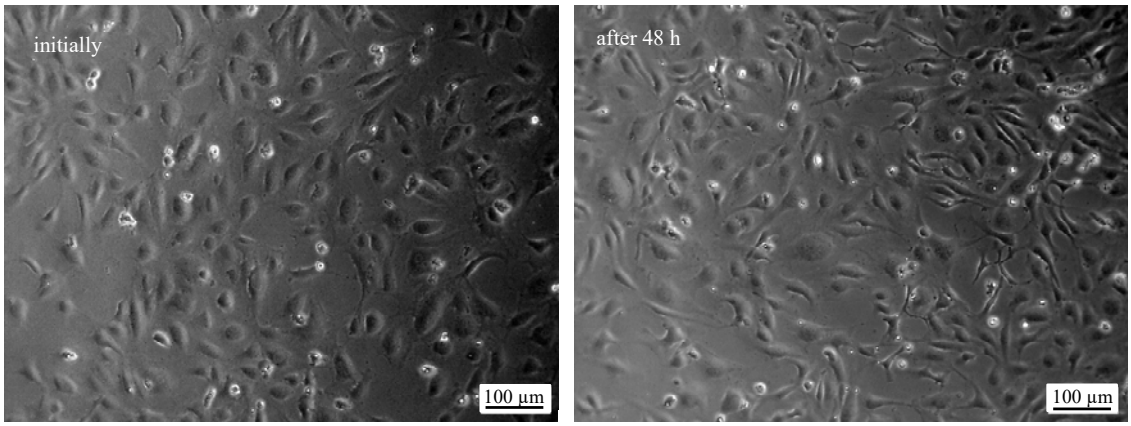

Figure 4: HUVEC 24 h after cell seeding and directly after incubation (Fig. 4a, left) in fresh culture medium supplemented with 1.09×10^{-5} mol/l Sirolimus solved in DMSO and 48 h later (Fig. 4b, right).

Figure 5 shows the alterations of relative numbers of adherent HUVEC per cm^2 in percent (related to control cultures supplemented only with DMSO in amounts necessary to solve the aliquots of Tacrolimus) over time after addition of DMSO-solved Tacrolimus to the culture medium in concentrations between 2.0×10^{-8} mol/l and 6.22×10^{-5} mol/l. The latter concentration was chosen so that the solubility product of Tacrolimus in the solvent was not exceeded. The addition of Tacrolimus influenced the relative numbers of adherent HUVEC

over the time (p=0.001) and depended also on the amount of Tacrolimus added (p=0.001) significantly.

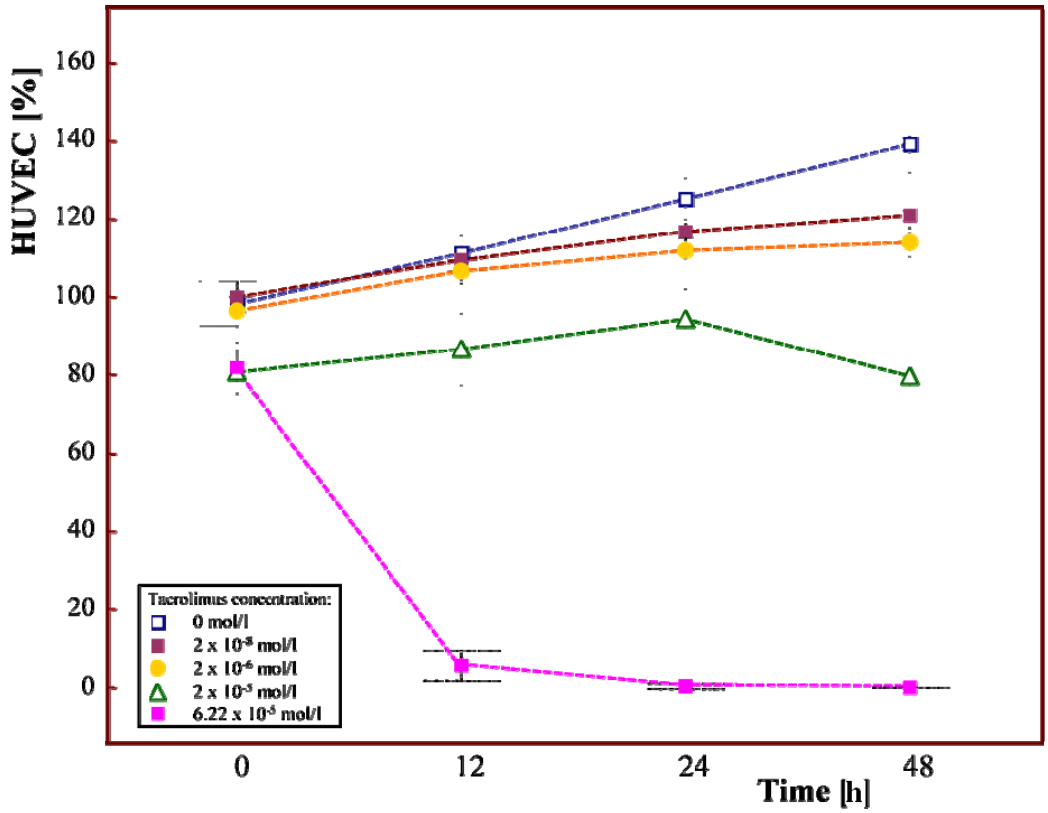

Figure 5: Relative numbers of adherent HUVEC per cm^2 in percent (related to control cultures supplemented only with DMSO in amounts necessary to solve the aliquots of Tacrolimus) after the addition of Tacrolimus in concentrations between 10^{-11} and 6.22×10^{-5} mol/l, immediately after Tacrolimus addition and then 12, 24, and 48 h later

With increasing Tacrolimus concentration the relative numbers of initially adherent HUVEC as well as the increase in relative numbers of adherent HUVEC decreased over time. After addition of the maximum concentration (solubility product not surpassed) of 6.22×10^{-5} mol/l Tacrolimus, the number of HUVEC decreased down to zero.

Figure 6 shows HUVEC 24 hours after cell seeding and directly after incubation (Fig. 6a, left) in fresh culture medium supplemented with 2×10^{-7} mol/l Tacrolimus solved in DMSO and 48 h later (Fig. 6b, right).

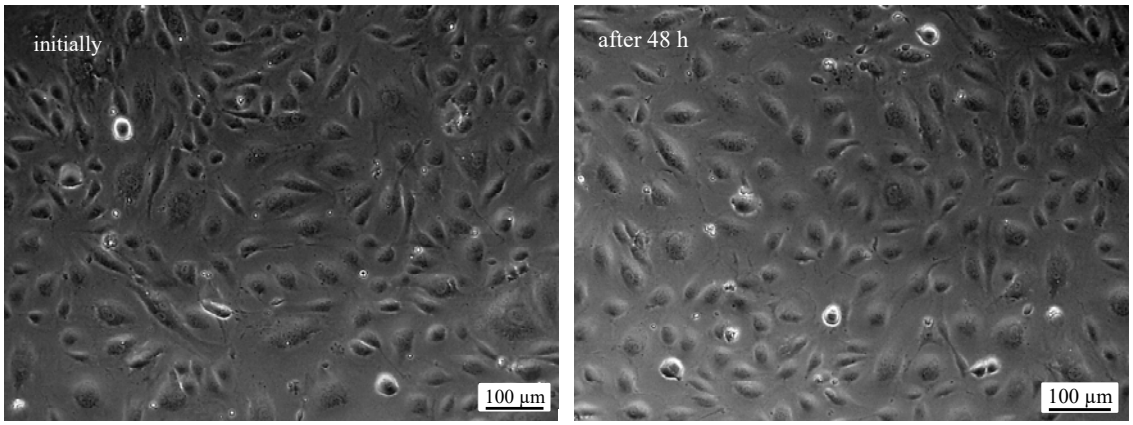

Figure 6: HUVEC 24 h after seeding and directly after incubation (Fig. 6a, left) in fresh culture medium supplemented with 2×10^{-7} mol/l Tacrolimus solved in DMSO and 48 hours later (Fig. 6b, right).

To exclude cytotoxic drug effects, a lactate dehydrogenase (LDH) assay was performed in HUVEC at drug concentrations of 10^{-6} mol/l. This concentration was chosen as a clear reduction of the HUVEC density was found. Neither Sirolimus nor Tacrolimus led to pathologic changes of the integrity of the HUVEC membrane.

4. Discussion

Drug eluting stents (DES) have markedly decreased the rate of in-stent restenosis [11, 12, 13]. First examples of this therapeutical strategy are immunosuppressant substances like Sirolimus or Tacrolimus released from stents, which were reported to inhibit smooth muscle cell proliferation, and are assumed to be a key process for neointima formation in the context of in-stent restenosis [14, 15]. Recent reports have associated drug-eluting stents with subacute or late thrombosis [4, 6]. As primary substrate of late stent thrombosis, delayed and poor endothelialisation were reported [6].

Sirolimus and Tacrolimus are macrolides, antibiotics that share potent immunosuppressive properties. Both were shown to bind to the immunophylin FK binding protein (FKBP12) but trigger different downstream mediators [16]. While the binding affinity of Sirolimus or Tacrolimus to the prototypical FKBP, hFKBP12, is almost the same [17], the protein hFKBP25 showed a 200-fold higher binding affinity to Sirolimus in comparison to Tacrolimus [18]. These differences in the binding affinity have long remained elusive on a molecular basis but could recently be elucidated by Prakash et al. [19].

Sirolimus inhibits the mTOR (mammalian target of rapamycin), which plays an important role in connecting extracellular signals with intracellular pathways critical for arterial repair.

mTOR may also have less well characterized effects on the regulation of gene transcription [20]. The mechanism of action of paclitaxel involves binding to the β-subunit of the tubulin heterodimer, promoting tubulin polymerization, cell cycle arrest, and, eventually, inhibition of cell migration and proliferation [21]. Although tubulin cycling is critical for cell division, it is independent of transmission of extra-/intracellular signalling. Thus, in contrast to the effects of Sirolimus on the diverse actions of the mTOR pathway, the action of Paclitaxel may make it independent of these signalling mechanisms. However, both drugs inhibit the proliferation of smooth muscle cells [22] and of endothelial cells [15, 20]. Matter and coworkers showed that Tacrolimus exerted markedly less antiproliferative effects on endothelial cells than Sirolimus [23].

It is not known so far, which concentration of these drugs are present at the luminal stent surface. Implantation of Sirolimus eluting stents in animals resulted in maximum plasma Sirolimus concentrations of about 2.63 ng/ml, which was clearly below the minimum concentration (9.14 ng/ml) applied in the present study [24]. To overcome the complex problems in measuring macrolide concentrations at surfaces of implanted DES, the present study applied macrolide concentrations between 10^{-11} mol/l and up to the limits of macrolide solubility in order to gain information on possible effects of macrolides on vascular wall cells over the whole range of possible drug concentrations.

Commercially available HUVEC of the 3rd passage were seeded on 24-well plates (without any coating or additional growth factors) and incubated in the cell culture medium DMEM containing 5 vol% fetal calf serum [25]. The highly lipophilic drugs [26] were solved in dimethyl sulfoxide **(**DMSO) and then added to the cell culture medium up to a maximum concentration of 1 vol% of the medium.

Dimethyl sulfoxide **(**DMSO) showed numerous biological effects on cell differentiation, and alteration of cell-surface carbohydrate structures [27]. DMSO in higher concentrations (>10 vol% [28]) was described to be cell-toxic [29], in lower concentrations of 1 vol % – 3 vol % it suppressed the capillary tube formation due to suppression of matrix metalloproteinase-2 production [30]. This was shown to coincide with remarkable changes in cell morphology and with the inhibition of cell proliferation revealing an IC50 at 2.9 vol% DMSO [31]. In the study presented here, DMSO was used in far lower concentrations (between 10^{-6} vol% and 1 vol% of the culture medium). Only a slight reduction in the number of adhering HUVEC was shown at a concentration of 1 vol% DMSO, which has to be considered when the effects of Sirolimus and Tacrolimus are discussed.

HUVEC proliferation was not measured directly since Matter et al. showed that the assessment of ^{3}H-thymidine incorporation could well correlate with the numbers of adhering HUVECs [23]. At lower concentrations (up to 10^{-6} mol/l) the relative numbers of adherent HUVEC were higher when cells were exposed to Tacrolimus; they were lower when they were exposed to Sirolimus [23]. Thus, Tacrolimus exerted markedly less antiproliferative effects on HUVEC compared to Sirolimus in this concentration range, while the vehicle solution (DMSO) did not influence the cell numbers up to a concentration of 0.5 vol% of the culture medium. At higher concentrations the situation changed completely. At the highest possible Sirolimus concentration (1.09×10^{-5} M; solubility product not surpassed) the average relative HUVEC numbers fell to only 9% below baseline values. At the application of the highest possible Tacrolimus concentration (6.22×10^{-5} M), however, a drastic reduction of the HUVEC numbers down to zero followed already within 24 h.

For low concentrations the results of the present study are in line with those reported earlier [23]. The low inhibition of proliferation by Tacrolimus in the low concentration range is similar to the results presented here, although there were clear differences in the experimental procedure: no coating versus Fibronectin coating, low FCS concentration versus high FCS concentration, macrolide solubilisation in DMSO versus no use of DMSO, short duration of experiments versus longer duration of experiments, just to name some of the differences. The key difference between the former study [23] and the study presented here is that in the present study both macrolides were tested at maximum concentrations without exceeding the solubility products. At maximum concentration the effects of Sirolimus were markedly different in so far as the inhibition of numbers of adherent HUVEC and possibly of cell proliferation was much lower compared to effects in HUVEC exposed to Tacrolimus. This might be an advantage in stenting of Sirolimus over Tacrolimus. Of course, this does not answer the many questions concerning availability and effects of Sirolimus or Tacrolimus: Is it true that only freely available and solubilized macrolides (only about 0.1%) not bound to erythrocytes (which amounts up to 95%) and plasma proteins exert effects on endothelial cells and smooth muscle cells [32] or can we expect also other effects on cells in direct vicinity to the DES, since it is the in-stent stenosis, which poses the serious problem?

This study is limited to observations *in vitro*, whose relevance to human clinical circumstances is uncertain because the local concentrations of the drugs near the stent surface are not known, platelet function inhibitors or anticoagulants and serious players like erythrocytes and T cells are lacking in our experimental set-up [3, 25]. The use of HUVEC instead of ECs from coronary arteries may pose a potential limitation of the study. We used

HUVEC from a provider, which often are contaminated with a certain amount of smooth muscle cells and myofibroblastic precursor cells [33]. Since it is not completely clear, whether HUVEC or smooth muscle cells and their precursors are at the heart of the in-stent restenosis problem we wanted to test both populations in the same experimental set-up for the effects of the macrolides used in clinical practise.

Despite the limitations, our results document a remarkable difference in the effects of both macrolides on relative numbers of adherent ECs and possibly on cell proliferation.

5. Conclusion

A local over-dosage of Tacrolimus on a stent's surface would bear the risk of suppression of the local endothelialization of the luminal stent surface completely, which does not seem to happen using Sirolimus according to our results.

Optimal drug release or elution from the stent coating is still an important issue and needs to be addressed in the future.

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