Effects of cytochalasin D-eluting stents on intimal hyperplasia in a porcine coronary artery model

Koen J. Salu, Johan M. Bosmans, Yanming Huang, Marc Hendriks, Michel Verhoeven, Anita Levels, Susan Cooper, Ivan K. De Scheerder, Chris J. Vrints, Hidde Bult*

Division of Cardiology, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium
Division of Pharmacology, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium
Interventional Cardiology, University Hospital Gasthuisberg, Leuven, Belgium
Medtronic Bakken Research Center, Maastricht, The Netherlands
Department of Anatomical Pathology, University of the Orange Free State, Bloemfontein, South Africa

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Abstract

Objective: To investigate whether cytochalasin D-eluting stents (CDES) suppress intimal hyperplasia in porcine coronary arteries and to compare the efficacy of paclitaxel and cytochalasin D as inhibitors of vascular smooth muscle cell (SMC) proliferation and platelet aggregation in vitro.

Methods: Rabbit platelet-rich plasma and SMC cultures derived from rabbit aortas were exposed to $10^{-8} - 10^{-5}$ M cytochalasin D or paclitaxel. Stents directly coated with $2 \mu$g cytochalasin D (low-dose CDES, $n=12$) and bare stents ($n=12$) were randomly deployed in the right and left coronary artery of 12 pigs. Six weeks later, neointima was studied using quantitative coronary angiography (QCA) and morphometry. To examine a ten-fold higher dose, polybutyl methacrylate/polyvinyl acetate-coated stents were loaded with $20 \mu$g cytochalasin D. High-dose CDES ($n=10$) and polymer-only stents ($n=11$) were deployed in 11 pigs.

Results: After 7 days, cytochalasin D (IC$_{50}$ 9.9 ± 0.4 $10^{-8}$ M) and paclitaxel (IC$_{50}$ 1.1 ± 0.4 $10^{-8}$ M) inhibited SMC proliferation in vitro ($n=4$). In contrast, cytochalasin D ($10^{-6} - 10^{-5}$ M, $n=5$), but not paclitaxel, attenuated platelet shape change and aggregation induced by ADP. In vivo QCA showed less late lumen loss in low-dose CDES (0.08 ± 0.07 vs. 0.32 ± 0.08 mm, $P=0.05$), but morphometry demonstrated only a tendency toward a decreased intimal area. High-dose CDES inhibited both late lumen loss (0.31 ± 0.08 vs. 0.91 ± 0.06 mm, $P<0.01$) and intimal area (1.57 ± 0.20 vs. 2.46 ± 0.22 mm$^2$, $P<0.01$). Immunohistochemistry revealed that CDES suppressed peri-strut macrophage recruitment (CD68, $P=0.04$) and cell proliferation (Ki67, $P=0.03$) as compared to polymer-only stents without interfering with endothelial cell recovery or the density of $\alpha$-SMC actin staining. Thromboses or edge effects were not observed in either study.

Conclusions: CDES inhibited in-stent hyperplasia. The reduction (39%) with $20 \mu$g CDES was equivalent to that reported for paclitaxel-eluting stents in pigs. Interference with platelet aggregation, SMC migration, SMC proliferation, and leukocyte recruitment could contribute to the benefit. The data indicate that targeting of actin microfilaments has a potential to suppress in-stent restenosis.

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Keywords: Cytochalasin; Restenosis; Drug-eluting stents; Intimal hyperplasia

1. Introduction

Although coronary stents reduce the incidence of restenosis by opposing elastic recoil and late constrictive remodelling, the occurrence of “in-stent” neointima formation is a drawback. It results from excessive smooth muscle cell (SMC) migration and proliferation, extracellular matrix deposition and a chronic inflammatory response to the stent struts [1,2]. The cytoskeleton is an interesting target to suppress restenosis and stents eluting the microtubule stabilizer paclitaxel have indeed shown to prevent in-stent...
restenosis in animal models [3–5] and in humans [6,7]. However, cytotoxic effects were seen with high doses and in long-term animal experiments [3–5].

Stent thrombosis (ST) is another, often catastrophic complication of stent implantation. (Sub)-acute ST is largely, but not entirely, prevented by dual anti-platelet therapy (aspirin plus a thienopyridine). Moreover, drug-eluting stents (DES) may even increase the risk of late ST by delaying stent endothelialisation [2,6,8]. Hence, a DES with anti-thrombotic properties could be an attractive option.

Actin filaments are another component of the cytoskeleton. They are required for intracellular signalling, cell migration, replication and protein synthesis [9]. Their formation is reversibly blocked by cytochalasins, lipophilic fungal metabolites that prevent actin monomer addition at the rapidly growing end of actin filaments, thereby interfering with cell functions, including platelet aggregation [10–14]. Previously we showed that cytochalasin D, the most potent derivative, inhibited vascular SMC migration in vitro and in vivo during collar-induced intimal thickening in the rabbit carotid artery [15]. Furthermore, addition of 90 μg cytochalasin D to a biocompatible stent coating caused 39% inhibition of the neointima in porcine coronary arteries [16]. However, in vivo lumen diameters and late lumen loss could not be determined in that study. Those parameters are clinically more relevant and also display a greater sensitivity to detect anti-restenotic effects of DES than post mortem morphometry [4,17]. Hence the current studies investigated whether lower doses of cytochalasin D could inhibit in-stent neointima formation using both angiography and morphometry. Furthermore, in order to reveal the cellular mechanisms, we used immunohistochemistry and investigated potency and efficacy of cytochalasin D as inhibitor of platelet aggregation and SMC proliferation in vitro in comparison to the reference drug paclitaxel.

2. Materials and methods

The ethical committees of the Universities of Antwerp and Leuven approved the studies that were done in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Concentrations are given as final concentrations.

2.1. SMC proliferation and viability

SMCs were isolated from New Zealand White rabbit aorta and cultured as described [15]. SMCs (passage 3–5, 50,000/well) were seeded in six-well plates in Ham F10 medium supplemented with 10% foetal bovine serum. On day 0, 3 and 6 saline, 1% ethanol or 10^{-8}–10^{-5} M cytochalasin D (n=4, Sigma) or paclitaxel (n=4, Bristol-Meyers-Squibb) was added in combination with changes of medium and foetal bovine serum. On day 7 SMCs were harvested by trypsinisation and counted (Coulter Counter) and in parallel monolayers neutral red deposition was measured for 2 h [18] and expressed as nmol neutral red/μg protein (BCA assay, Pierce). Concentrations inhibiting cell proliferation by 50% (IC_{50}) were calculated using non-linear regression.

2.2. Platelet aggregation

Platelet-rich plasma was obtained by centrifugation (900 g, 10 min) of citrate blood collected from pentobarbital (30 mg/kg) anaesthetized rabbits [19] and incubated with cytochalasin D, paclitaxel (10^{-8}–10^{-5} M) or ethanol (1%) for 3 min at 37 °C in an aggregometer (Chronolog Corp.). Light transmission was quantified 10 s after injection of 1, 3, 10 or 30 μM ADP [19]. In parallel experiments, one volume 0.15 M sodium cacodylate-buffered 1% glutaraldehyde was added 10 s after injection of 30 μM ADP or 0.9% NaCl for transmission electron microscopy.

2.3. Stent loading

2.3.1. Low dose (2 μg)

Balloon-mounted V-Flex Coronary Stents (3.0–16 mm; Cook Inc.) were dipped in ethanol (bare) or in 1 mg cytochalasin D/ml ethanol for 60 s and afterwards air-dried using warm laminar flow for 30 s [4]. To determine drug content, balloon-mounted stents (n=3) were placed in 1 ml ethanol to extract cytochalasin D. Afterwards, the stents were coated again, deployed to remove the stent and stent and balloon were placed separately in 1 ml ethanol. The cytochalasin D content of the extracts was quantified on the basis of UV absorbance (λ 207 nm) using a standard curve (0–25 μg/ml).

2.3.2. High dose (20 μg)

Stainless steel AVE S670 coronary stents (Medtronic Inc., 18 mm) were coated with a 7–8 μm layer of nonerodable polybutyl methacrylate (PBMA)/polyvinyl acetate co-polymer (90/10) and a 1–1.5 μm PBMA top layer to slow down drug release. This platform was loaded with 20 μg cytochalasin D. All stents were sterilised with gamma irradiation.

2.3.3. In vitro drug release

CDES were placed in 2 ml TBS (0.01 M Tris pH = 7.4, 0.154 M NaCl) at 37 °C and transferred to 2 ml fresh TBS at regular intervals (1 h to 7 d). The cytochalasin D content (μg ml^{-1}) of the solutions was measured by HPLC and multiplied by two to calculate μg released in the preceding interval. The cumulative elution profiles were fitted to a one-phase exponential delivery model to assess half-life and maximum release.
2.4. In vivo studies

Male domestic pigs (Sus scrofa, age 10 weeks) were anaesthetized (ketamine IM 5 mg/kg, followed by IV ketamine 0.1 mg/kg/hr and IV pancuronium 0.4 mg/kg/hr), ventilated, instrumented and treated with heparin (5000 IU bolus, 400 IU/h infusion) and aspirin (250 mg). In the low dose study a bare (n = 12) and a CDES (2 µg, n = 12) was randomly placed in the right (RCA) or left ascending coronary artery (LAD) of 12 pigs as described [20]. In the high dose study 10 pigs received a polymer-only (n = 10) and a CDES (20 µg, n = 10) in the RCA or LAD. Stents were deployed (3.5 mm RCA, 3.0 mm LAD) at nominal pressure (8 atmospheres, 30 s) to obtain a 1.0–1.1 balloon was randomly placed in the right (RCA) or left ascending arterial lesion (n = 10) [19]. Therefore, the balloon was deployed over the stent [20]. Additionally, the balloon used in the high dose study [20]. In the RCA or LAD. Stents were deployed (3.5 mm RCA, 3.0 mm LAD) at nominal pressure (8 atmospheres, 30 s) to obtain a 1.0–1.1 balloon-tovessel ratio [21]. In vivo studies

Quantitative coronary angiography (QCA) was performed before stenting, immediately thereafter and at follow-up using the CAAS-II-automated-QCA system (Pie Medical). An experienced investigator, who was blinded to the treatment, measured the reference diameter and the minimal luminal diameter (MLD) of the stent segment. Late lumen loss (MLDpost-implantation − MLDfollow-up) and percentage stenosis (100 × [1 − (MLDfollow-up /MLDreference, follow-up)]) were calculated to evaluate in-stent restenosis [20]. Presence of edge effects, defined as >50% stenosis within 5 mm of proximal or distal stent edges, aneurysms and luminal thrombus were also evaluated.

2.5. Histology

The heart was excised, coronary arteries were pressure fixed (10% formaldehyde, 80 mmHg, 20 min). Stent segments were carefully dissected together with 1 cm vessel both proximal and distal to the stent, fixed in 10% formaldehyde for 7 days and then divided into 3 blocks containing the proximal, middle and distal parts of the stent [20]. The three regions were embedded in (2-hydroxyethyl)-methacrylate (low dose study, Technovit 7100) or methylmethacrylate (high dose study, Technovit 9100, Heraeus Kulzer GmbH, Germany). Sections (5 µm, tungsten wolfram carbide knife, Rotary heavy-duty microscope HM 360, Microm, Germany) were stained with haematoxylin–eosin stained sections. The average scores of each section were calculated as the sum of filament scores/total number of filaments. Focal fibrin deposition, intra-intimal haemorrhages and media necrosis were examined at every struts on Movat’s pentachrome stained sections [5] and quantified as percentage positive struts. Finally, luminal coverage of endothelial-like cells was evaluated using light microscopy without (low dose study, magnification 600x) or with lectin staining (high dose study).

The cross-sectional areas of lumen, intima, media and stent were determined in each proximal, middle and distal stent region by means of stereological point counting (square grid, 0.09 mm² per point, 4× objective) [23]. Subsequently, the areas within the IEL (IEL area=lumen area+intima area), within the EEL (EEL area=IEL area+media area) and percentage stenosis (100 × [IEL area−lumen area]/IEL area) were calculated. In addition, segments within 5 mm proximal and distal from each stent were fixed in 4% formaldehyde and paraffin embedded. In sections (5 µm) stained with haematoxylin–eosin lumen, intima and media area were measured for evaluation of edge-or remodelling-effects as recommended [21].

For immunohistochemistry, central regions embedded in Technovit 9100 (high dose study) were stained with monoclonal antibodies against α-SMC-actin (1A4, 1/2700, Sigma), macrophages (CD68, 1/300, Serotec) or Ki67 (1/100, Serotec) a nuclear antigen expressed during late G1, S, G2 and M phases [24]. α-SMC-actin content of intima and media was determined using stereological point counting and expressed as percentage. Ki67 and CD68 positive cells were counted at four stent struts (3, 6, 9 and 12 o’clock) as recommended [1] and the average density (positive cells/mm²) was calculated. Lectin staining was graded (I ≤ 10%, II = 11−50%, III = 51−90% and IV > 90% of the lumen circumference) to determine endothelial cell coverage.

Finally, in the high dose study a second mid region of two stents (1 RCA, 1 LAD) from each group was separately fixed in phosphate buffered (pH 7.2) 2.5% glutaraldehyde and post-fixed in osmium tetroxide. After dehydration through a graded series of acetone concentrations, critical point drying with carbon dioxide and gold sputtering, the lumen was examined in a Phillips XL40 scanning electron microscope (SEM) for endothelial coverage and maturity, adhering platelets and fibrin deposits.

2.6. Statistics

Data are presented as mean±S.E.M; n represents the number of stents or animals. In vitro data were evaluated using an analysis of variance (ANOVA, SPSS 10.0) and Dunnett’s post hoc test. Morphometrical data, injury and inflammatory scores were analysed using an ANOVA with repeated measurements: proximal, middle and distal stent region. A two-tailed P < 0.05 was considered statistically significant.
3. Results

3.1. In vitro SMC proliferation and platelet aggregation

Exposure to cytochalasin D (IC_{50} 9.9±0.4 \times 10^{-8} \text{ M}, n=4) or paclitaxel (IC_{50} 1.1±0.4 \times 10^{-8} \text{ M}, n=4) for 7d inhibited SMC proliferation (Fig. 1). Cell loss was observed at 10^{-5} \text{ M} paclitaxel and the remaining, adherent SMCs displayed 50% reduction of the uptake of neutral red. Cytochalasin D did not attenuate this viability index.

ADP-induced platelet shape change was inhibited by cytochalasin D (10^{-6} \text{ M} - 16±4%, 10^{-5} \text{ M} - 41±4%, \text{ F}=14.94, P<0.001, all four ADP concentrations combined; 1 \mu\text{M ADP} shown in Fig. 2). Transmission electron microscopy confirmed that more cytochalasin D-treated platelets maintained a discoid shape with less pseudopodia and less inter-platelet contacts compared to control platelets (Fig. 3). Cytochalasin D also attenuated aggregation (−17±3% at 10^{-5} \text{ M}, all ADP concentrations combined). The inhibition became more prominent at threshold ADP concentrations (1 \mu\text{M shown in Fig. 2}), when 10^{-6} \text{ M} cytochalasin D also displayed anti-aggregating activity. Paclitaxel was without effect on either shape change (\text{ F}=1.37, P=0.26, all four ADP concentrations) or aggregation (Fig. 2).

3.2. Low dose (2\mu g) study

Balloon-mounted stents contained 2.74±0.04 \mu\text{g cytochalasin D} (n=3), from which 1.9±0.1 \mu\text{g} was bound to the stent and 1.1±0.2 \mu\text{g to the balloon-surface. Maximum in vitro release amounted to 1.9 (95% confidence interval (CI) 1.7–2.0) \mu\text{g with a half-life of 0.4 (CI 0.3–1.1) h} (Fig. 4).

One stent was lost in the RCA of one pig and an additional CDES was placed in the circumflex artery. QCA indicated that lumen diameters before stenting and lumen expansion after stent deployment were similar in both groups (Table 1). At follow-up all stents were patent and without signs of luminal thrombus formation, “edge-effects” or aneurysm formation. Late lumen loss was statistically not different from zero (CI –0.08–0.24 mm) in CDES and 75% (CI 19–131%) less compared to bare stents. However, morphometry failed to show a statistically significant reduction of intima formation in CDES. The other arterial dimensions, the injury and inflammatory scores were not different (Table 2). Luminal coverage by endothelial-like cells was virtually complete in both groups and the percentage struts showing fibrin deposits (4.3±1.3% versus 6.3±2.8%, P=0.64) was low and not different either.
Luminal thrombus, late malapposition, intra-intimal hemorrhage or focal medial necrosis was not observed in either group. In view of favourable tendencies for intimal area (−28%) and area stenosis (−31%), the experiment was repeated with a ten-fold higher dose.

3.3. High dose (20 μg) study

Polymer-coated stents contained 21.9 ± 1.8 μg cytochalasin D (n = 3). Maximum in vitro release was 18 (95% limits 16.6–19.4) μg with a half-life of 43 (95% limits 33–60) h (n = 5, Fig. 4).

One CDES was lost in a RCA and spare CDES (RCA) and polymer-only (LAD) stents were implanted in an additional pig. QCA pointed to similar lumen diameters before stenting and an equal lumen increase after stent deployment in both groups (Table 1). At follow-up, all stents were patent and without signs of luminal thrombus, “edge-effects” or aneurysm formation. Percent stenosis (−62%, P < 0.001) and late lumen loss (−65%, P = 0.003)

Table 1

QCA of stented coronary arteries at 6 weeks follow-up

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Low dose study</th>
<th>High dose study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bare 2 μg cytochalasin D</td>
<td>Polymer 20 μg cytochalasin D</td>
</tr>
<tr>
<td>Reference</td>
<td>n = 12</td>
<td>n = 11</td>
</tr>
<tr>
<td>MLDpre</td>
<td>2.80 ± 0.14</td>
<td>2.58 ± 0.17</td>
</tr>
<tr>
<td>Reference</td>
<td>3.01 ± 0.13</td>
<td>3.01 ± 0.13</td>
</tr>
<tr>
<td>MLDpost</td>
<td>2.80 ± 0.15</td>
<td>2.80 ± 0.15</td>
</tr>
<tr>
<td>Reference</td>
<td>2.93 ± 0.16</td>
<td>2.93 ± 0.16</td>
</tr>
<tr>
<td>Late lumen loss</td>
<td>0.91 ± 0.06</td>
<td>0.91 ± 0.06</td>
</tr>
<tr>
<td>Stenosis 6 wks, %</td>
<td>35.7 ± 2.4</td>
<td>35.7 ± 2.4</td>
</tr>
</tbody>
</table>

MLD = minimal lumen diameter; data represent mm (except for stenosis, %) and are given as mean ± S.E.M.
Data represent mm² (except for stenosis and scores) and are given as

**Table 2**
Morphometry of stented arteries at 6 weeks follow-up

<table>
<thead>
<tr>
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<th></th>
<th>High dose study</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bare</td>
<td>2 µg cytochalasin D</td>
<td>ANOVA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=12</td>
<td>n=12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lumen area</td>
<td>5.40±0.44</td>
<td>5.93±0.37</td>
<td>0.373</td>
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<tr>
<td>Intima area</td>
<td>1.29±0.18</td>
<td>0.93±0.13</td>
<td>0.114</td>
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<tr>
<td>Media area</td>
<td>1.82±0.11</td>
<td>1.88±0.16</td>
<td>0.778</td>
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<tr>
<td>IEL area</td>
<td>6.69±0.34</td>
<td>8.66±0.32</td>
<td>0.726</td>
<td></td>
</tr>
<tr>
<td>EEL area</td>
<td>8.51±0.42</td>
<td>8.73±0.42</td>
<td>0.714</td>
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<tr>
<td>Stent area</td>
<td>6.86±0.29</td>
<td>6.98±0.28</td>
<td>0.786</td>
<td></td>
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<tr>
<td>Stenosis, %</td>
<td>21±4</td>
<td>14±2</td>
<td>0.130</td>
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<tr>
<td>Injury score</td>
<td>0.53±0.10</td>
<td>0.48±0.04</td>
<td>0.690</td>
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<tr>
<td>Inflammatory score</td>
<td>1.00±0.08</td>
<td>0.87±0.04</td>
<td>0.146</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Polymer</td>
<td>20 µg cytochalasin D</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=11</td>
<td>n=10</td>
<td></td>
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<tr>
<td>Lumen area</td>
<td>2.77±0.43</td>
<td>3.16±0.35</td>
<td>0.489</td>
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<tr>
<td>Intima area</td>
<td>2.46±0.22</td>
<td>1.57±0.20</td>
<td>0.007**</td>
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</tr>
<tr>
<td>Media area</td>
<td>1.71±0.11</td>
<td>1.43±0.09</td>
<td>0.067</td>
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<tr>
<td>IEL area</td>
<td>5.23±0.31</td>
<td>4.73±0.22</td>
<td>0.207</td>
<td></td>
</tr>
<tr>
<td>EEL area</td>
<td>6.94±0.34</td>
<td>6.17±0.22</td>
<td>0.073</td>
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<tr>
<td>Stent area</td>
<td>5.62±0.30</td>
<td>5.35±0.18</td>
<td>0.446</td>
<td></td>
</tr>
<tr>
<td>Stenosis, %</td>
<td>50±5</td>
<td>35±5</td>
<td>0.063</td>
<td></td>
</tr>
<tr>
<td>Injury score</td>
<td>1.69±0.14</td>
<td>1.67±0.19</td>
<td>0.945</td>
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<tr>
<td>Inflammatory score</td>
<td>2.22±0.21</td>
<td>1.80±0.20</td>
<td>0.160</td>
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</tbody>
</table>

Data represent mm² (except for stenosis and scores) and are given as mean ± S.E.M of the average values of proximal, mid and distal stent region.

were significantly reduced in CDES as compared to controls.

Morphometry documented a significant decrease (−36%) of the intimal area in CDES (Table 2; Fig. 5A and B), whereas IEL, EEL, media, lumen and stent areas were not different (Table 2). Inflammatory score and area stenosis tended to be lower in CDES (Table 2, Fig. 5G and H). The injury score was almost identical for CDES and polymer-only stents, but in agreement with the smaller artery size it was greater than in the first study. When the individual injury score of each region was included as covariate in an analysis of covariance, the inhibitory effects of CDES on inflammatory score (\(P = 0.007\)) and percentage stenosis (−30%, \(P = 0.003\)) became highly significant. The frequencies of struts showing fibrin deposits (6.5±1.6% and 4.9±2%) or intra-intimal haemorrhages (23±7% and 12±7%) were statistically not different (Fig. 5C and D).

“Edge effects”, luminal thrombus, late stent malapposition or focal medial necrosis was not observed in any group.

Immunohistochemistry showed no difference among CDES and polymer-only stents for intimal (87±7% vs. 88±3%) or medial (81±6% vs. 80±6%) \(\alpha\)-SMC-actin content (Fig. 5I and J). However, the proliferation marker Ki67 (5±1 vs. 13±3 cells/mm², \(P = 0.032\), Fig. 5K and L) and CD68-stained macrophages (20±5 vs. 88±29 cells/mm², \(P = 0.041\), Fig. 5M and N) were reduced in CDES compared to polymer-only stents. Lectin staining was virtually circumferential in both groups (median values 3.7–4.0, i.e., ≥90% coverage; Fig. 5E and F). SEM confirmed a smooth and completely recovered endothelium in mid-stent regions of both groups (Fig. 6). Immature endothelial cells with bulging nuclei were observed in all specimens without indication of differences of endothelium coverage and maturity, fibrin deposition or platelet adhesion between groups.

### 4. Discussion

#### 4.1. Rationale for cytochalasin D as anti-restenotic agent

In-stent intimal hyperplasia results from a complex cascade, wherein platelet activation and aggregation, recruitment of inflammatory cells and proliferation and migration of \(\alpha\)-actin positive cells play pivotal roles [1,2]. Molecules that suppress those cell types could therefore be of interest to prevent in-stent restenosis. Cytochalasins inhibit polymerisation of actin-filaments, thereby interfering with essential cell functions [10]. Previously, we demon-
strated that cytochalasin D inhibits rabbit SMC migration [15]. The present study shows that cytochalasin D inhibits SMC proliferation as well, which is in accordance with the observation that the drug blocks extra-cellular signal-regulated kinases [25]. Moreover, the SMCs remained viable at all concentrations. Paclitaxel was more potent as proliferation inhibitor, but caused ≥50% loss of SMC number and viability at 10⁻⁵ M.

Unlike paclitaxel, cytochalasin D attenuated in vitro platelet shape change, pseudopodium formation and aggregation, particularly at low ADP concentrations. The current study employed rabbit platelets, but others have demonstrated suppression of aggregation or accelerated disaggregation in humans [11–14] and other species. Cytochalasins inhibit pivotal platelet activation steps, such as store-mediated Ca²⁺ entry [26], recruitment of αIIbβ₃ integrin and fibrinogen binding [27]. Therefore, the inhibition occurs also in aspirin-treated platelets [12] and is not restricted to ADP but is seen with almost every agonist [11]. These properties constitute a major difference with paclitaxel and are potentially interesting since mural thrombus is an early response to stenting [2]. Moreover, (sub)acute ST is not entirely prevented by dual therapy with aspirin plus a thienopyridine to block P2Y₁₂ ADP receptors and, more importantly, cases of late ST have been reported for paclitaxel-and sirolimus-eluting stents [8].

4.2. In vivo effects of cytochalasin D-eluting stents

To study the effects of cytochalasin D on in-stent intimal hyperplasia, we first used direct stent-coating. This technique is easy to apply, but is limited to lipophilic drugs and small quantities and results in a fast release. Indeed, in vitro release of 2 μg cytochalasin D was virtually complete after 2 h, thus resembling the “bolus-like” pattern seen with delivery balloons [28]. In spite of the rapid release, direct stent application of 2 μg cytochalasin D resulted in less late lumen loss 6 weeks after stenting. For directly applied paclitaxel it has been proposed that the fast release results in a rapid vascular uptake that is essential to inhibit the earliest events in the restenotic cellular cascade and that retention of the drug by membrane lipids may possibly act as a depot for prolonged release [4]. Since intimal area (−28%) and area stenosis (−31%) showed favourable tendencies in 2 μg CDES, the effects of a ten-fold higher dose were investigated. Direct stent coating was inappropriate for this purpose because of its restricted loading capacity. A polybutyl methacrylate/polyvinyl acetate co-polymer was selected as matrix carrier for cytochalasin D, since beneficial outcomes for thrombogenicity and biocompatibility have been shown for methacrylate polymer-coatings in porcine coronary arteries [17,29,30]. The polymer was indeed capable to retain 20 μg cytochalasin D and displayed a slow, controlled release in vitro (days vs. hours) in comparison to the direct coating. The higher cytochalasin D dose also inhibited late lumen loss at 6 weeks follow-up, as compared to polymer-only stents. Moreover, morphometry showed significantly less intimal area — with or without adjustment for the injury score. In accordance with reports on other DES in pig porcine coronary arteries [4,17], angiographic analysis of late lumen loss showed a greater effectiveness of cytochalasin D than morphometry of the intima in both studies. The 36% reduction in intimal area by 20 μg cytochalasin D is comparable to the inhibition (39%) seen with 90 μg [16] and equivalent to that obtained with paclitaxel [4] or the pleiotropic drug sirolimus [30] in pig coronary arteries.

Immunohistochemistry revealed less peri-strut staining of the cell cycle marker Ki67 in CDES. This confirms the in vitro results and indicates that even six weeks after stenting cytochalasin D inhibited cell proliferation in the lesion in vivo. In addition, the peri-strut macrophage number and the inflammatory score were significantly attenuated as well. These anti-inflammatory properties of cytochalasin D are in line with the suppression of leukocyte diapedesis during collar-induced intimal thickening in the rabbit [15]. In contrast, the density of α-actin positive SMCs in media and intima was not different between both groups. Since the intimal cross-sectional area was reduced, this implies that the neointima in CDES contained fewer SMCs due to inhibition of either stent-induced SMC migration, as seen in the rabbit collar model [15] or SMC proliferation (cf. in vitro results).

The luminal coverage by endothelial cells was complete at 6 weeks in both groups as assessed by lectin staining and SEM. Finally, the reduced in-stent neointimal formation in CDES was not accompanied by induction of proximal or
distal edge-effects, luminal thrombus formation, late stent malapposition or focal medial necrosis around stent struts, indicating no thrombotic or local toxic effects of cytochalasin D itself. Focal fibrin deposition and the presence of intra-intimal haemorrhages were not different between both groups. Therefore, the anti-restenotic action of cytochalasin D seems due to inhibition of cell proliferation and recruitment of macrophages and SMCs, without signs of retarded vascular healing or local toxicity, at least at 6 weeks.

4.3. Limitations of the study and conclusions

First, normal porcine coronary arteries may not necessarily predict significant drug effects in atherosclerotic human vessels [21]. However, similar reductions of restenosis in native pig coronary arteries by paclitaxel-[4] and sirolimus-eluting stents [30], have been transferred successfully into clinical trials [6,7,31]. Second, neointimas in the high dose study were more pronounced than in the low dose study. There are several putative explanations for the difference. It is well known that in-stent neointima formation is directly proportional to the degree of arterial injury [2,22]. Both quantitative angiography and morphometry (EEL area) demonstrated that slightly smaller arteries were stented in the high dose study, resulting in a greater insult. Another explanation could be that the polybutyl methacrylate/polyvinyl acetate co-polymer was less biocompatible than other methacrylate polymers used previously [17,29,30]; indeed polymers may promote in-stent proliferation and restenosis [32,33]. Third, the different stent platforms (V-flex versus AVE S670) used in the two studies may elicit distinct vascular responses [2]. Hence it is not clear whether greater benefit would be obtained using other doses or release platforms, though the inhibition of neointima formation was equivalent to that seen with 90 μg cytochalasin D [16]. Determination of the intra-arterial delivery efficiency could help to fine tune the efficacy of CDES. However, even for DES that are currently used in the clinic, data on the arterial delivery efficacy are virtually absent. Finally, it remains to be determined whether the effects are sustained at later time points (3 to 6 months). Indeed, beneficial effects seen 6 weeks after implantation of sirolimus-eluting stents in porcine coronary arteries were not maintained at 6 months follow-up, suggesting a possible “catch-up” phenomenon with DES as reported previously for brachytherapy [34].

Nevertheless, the present study shows that the microfilament inhibitor cytochalasin D inhibits both platelet aggregation and SMC proliferation in vitro, whereas paclitaxel did not affect the platelets. Using direct and polymer coating techniques, cytochalasin D reduced stent-induced intimal hyperplasia in porcine coronary arteries at 6 weeks follow-up without inducing edge effects. Based on intimal area, the high dose (36%, low dose 28% inhibition) seemed favourable, but for late lumen loss the directly coated low dose showed the best outcome. Thus the release platform and pharmacokinetics could be equally or even more important contributors to the efficacy. Immunohistochemistry revealed suppression of cell proliferation and attenuated recruitment of macrophages and SMCs as possible mechanisms responsible for the benefit. Finally, endothelial regeneration was complete at 6 weeks follow-up and local toxicity or ST could not be documented in CDES. In view of its anti-platelet, anti-migratory and anti-proliferative properties, cytochalasin D seems potentially an attractive therapeutic option to suppress both in-stent restenosis and ST.

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