Therapeutic and mechanistic explorations of in-stent restenosis in the rat aortic stenting model
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CHAPTER 6
Pattern of neointimal healing in drug-eluting versus bare metal stents in the rat aortic stenting model.


submitted
ABSTRACT

Objectives: Histological changes in the neointima might be associated with late stent thrombosis. We wanted to determine if the neointima of drug-eluting stents is different from their bare metal controls.

Methods: Male Wistar rats were randomized to one of four groups: Express® Stent, Taxus® Express² Stent, Bx Velocity® Stent and Cypher® Stent. Stents were implanted in the abdominal aorta. Histological analyses were performed after 1 and 4 weeks. After 1 week the inflammation score, neointimal cell density and signs of incomplete healing were measured. After 4 weeks the same measurements were performed plus the neointimal area, neointimal thickness, media area and lumen area. Results: no differences were observed after 1 week between bare metal stents and drug-eluting stents. At 4 weeks neointimal cell density was lower, inflammation-score was higher in the drug-eluting stents. The healing at 4 weeks was complete in the bare metal stent groups and incomplete in more than 90% of the drug-eluting stents. Neointima area was reduced in the drug-eluting stents. No differences in the neointima were found between the paclitaxel and the sirolimus eluting stent.

Conclusions: These results show that differences in inflammation, cell density and signs of incomplete healing exist between drug-eluting stents and bare metal stents, especially adjacent to the stent struts. Over time incomplete healing persists in drug-eluting stents but resolves in bare metal stents.
INTRODUCTION

Drug-eluting stents are successful in reducing in-stent restenosis(1;2). However there have been some concerns over the safety of drug-eluting stents especially since there have been reports of increased late stent thrombosis(3). Joner et al found persistent fibrin deposition and delayed re-endothelialization in patients who received a drug-eluting stent and had late stent thrombosis(4). This suggests that late stent thrombosis might arise from incomplete endothelial healing. Alternatively, one could speculate that not only incomplete healing of the endothelium but also incomplete healing and structural changes in the neointima could lead to increased risk of late stent thrombosis. Although fibrin content of the neointima was studied in drug-eluting stents, changes in the structure of the neointima (e.g. cell density) have not been well characterized.(5) The goal of this study was to determine if the neointima of drug-eluting stents is structurally different from the neointima of bare metal stents. Therefore we measured neointimal cell density, inflammation-score and looked for signs of incomplete healing (hemorrhage and acellularity) in the Taxus® Express™ and the Cypher® Stent and their bare metal controls (Express® and Bx Velocity® Stent). Furthermore to assess extracellular matrix formation and tissue strength we measured collagen content in both drug-eluting and bare metal stents. To assess incomplete healing of the neointima these measurements were performed after 1 week (inflammation phase) and 4 weeks (maximum neointimal area). To this end, we employed the rat abdominal stent model, previously shown to display reduced neointimal formation in the Cypher® Stent(6).

METHODS

Animals
All procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Specific pathogen-free, Male Wistar WU rats (Charles Rivers) weighing 450-520 g were fed standard rat chow and water ad libitum. Stent implantation was performed on fifty-one rats. Overall mortality was approximately 8%. Most animals died of perforation of the aorta, vena cava puncture or aortic thrombosis during or shortly after stent implantation.

Stent Implantation
Animals were anesthetized with O2, N2O, and isoflurane 2% (Abbott International Ltd). Premounted stents were implanted in the abdominal aorta as described previously all 2.5 x 8 mm (6). There were 4 groups: one group received the Express® Stent manufactured by Boston Scientific (the same stent as the Taxus® Express™ Stent only without the paclitaxel-eluting polymer), one group received the Taxus® Express™ Stent, one group received the Bx Velocity® Stent manufactured by Cordis (the same stent as the Cypher® Stent only without the sirolimus-eluting polymer) and the last group received the Cypher® Stent. We gave clopidogrel to all rats to prevent early thrombus and inflammation around stent-struts and to mimick the situation in humans. All animals received rat chow mixed with clopidogrel (Plavix, Sanophi) 0,33 mg· gram rat chow starting 5 days before stent-implantation until termination of the animals. After 7 and 28 days, animals were anesthetized and heparinized with 500 IU intravenously (Leo Pharma B.V.). Abdominal aortas were harvested, fixed, embedded in methylmetacrylate, sectioned, and stained for histological analysis.

Histology
Histomorphometrical analysis was performed on Lawson (elastin staining), hematoxyline-eosine and Sirius red stained sections by measurements of the proximal, middle, and distal parts of each stent. Lawson-stained sections were used to measure neointimal formation and injury score. Hematoxyline-eosine stained sections were used to measure neointimal cell density, inflammation score, and to look
for signs of incomplete healing. The neointimal area, neointimal thickness, media area and lumen area were measured or calculated as described previously (6). In short, the areas within the external elastic lamina (EEL), IEL and lumen were measured by using digital morphometry by means of an Olympus BX-50F4 microscope, an Olympus c-3030 zoom digital camera and Olympus DP-Soft version 3.0 software (Olympus, Tokyo, Japan). The lumen area was subtracted from the IEL area to give the neointimal area. The IEL area was subtracted from the EEL area to give the media area. The neointimal thickness (length perpendicular from the bottom of the stent strut to the roof of the stent strut) was measured at each stent strut and averaged for all struts. For each stent part the mean of six sections was calculated. The injury and inflammation scores were assessed as described by Schwartz et al and Kornowski et al (7;8). Neointimal cell density was determined in hematoxylin-eosin–stained sections at x400 magnification and expressed as x100/mm2 as described previously (9). Signs of incomplete (neointimal) healing were defined as acellularity of neointima or the presence of a hemorrhage in the neointima. Hemorrhage was defined as the presence of more than one hundred grouped biconcave discs in the neointima. Acellularity of neointima was defined as absence of cells in an area with an estimated width of half the nearest stent strut and one-third the neointimal thickness. Signs of incomplete healing were scored in the proximal, middle, and distal parts of each stent. Each part was scored four times at 90 degree rotation apart. If none of the parts of the stent showed either a hemorrhage or an acellular neointimal area a score 0 was assigned. If only one of the parts showed either a hemorrhage or an acellular neointimal a score of 1 was assigned. The total score of incomplete healing in each group was averaged, multiplied by 100 and expressed as a percentage. After 1 week the inflammation score, signs of incomplete healing and neointimal cell density were measured. After 4 weeks the same measurements were performed plus the neointimal area, neointimal thickness, media area, lumen area and injury score.

Collagen staining and analysis
0.1% Sirius Red F3B stained sections were used to measure collagen content using computerized image analysis. The expression of collagen was measured using computer-assisted morphometry. A total of 5 fields per slide were evaluated at a magnification of 200 x. Image analysis was performed by a technician blinded to the source of the sample. Image analysis was performed using an automated macro written with the software package Leica Qwin. A background image of a blank area of the slide was obtained and background correction was performed to adjust for subtle irregularities in the illumination of the microscope field. The software was set to subtract background staining from the stained sections, subsequently staining intensity was measured and used as an indirect measure for collagen content.

Statistical methods
Data are expressed as mean ±SEM. Differences between groups were determined by Student’s t-test for unpaired samples with the bonferroni correction for multiple comparisons (dichotomous variables were tested with Chi-square). All P-values were two-tailed, and a P-value of <0.05 was considered statistically significant. Analyses were performed using SPSS software (SPSS version 12.0, Chicago, IL, USA).

RESULTS

Weight
Baseline weight (mean±SD) was 508±44, weight at 4 weeks was 519±64.
**Histological analysis**

*Incomplete healing*

After 1 week no differences exist between bare metal stents and drug-eluting stents in measurements representing incomplete healing of the neointima as shown in Table 1 and Figures 2 and 3. However after 4 weeks drug-eluting stents had a higher inflammation score, lower cell density and more signs of incomplete healing (hemorrhage or acellularity of neointima) compared to bare metal stents as shown in Table 2 and Figures 1, 2 and 3. These differences were mainly observed in the neointima adjacent to the stent struts, but they were also found further from the stent struts. In Figure 4 the irregularity of the pattern of incomplete healing in drug-eluting stents is shown. Collagen content was decreased in drug-eluting stents compared to bare metal stents as shown in Table 4. In bare metal stents collagen was evenly distributed over the neointima. On the contrary in the drug-eluting stents collagen was not evenly distributed: almost absent in acellular areas but present in almost normal quantities (compared to bare metal stents) in more cellular areas as shown in Figure 5.

*Neointimal formation*

Neointimal area and control parameters are presented in Table 3. Neointimal area and thickness were decreased in drug-eluting stents.

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**Figure 1:** Hematoxyline-eosine stained photomicrographs (x400) of stented abdominal aortas after 4 weeks showing the lumen, the neointima and the media. A striking difference in neointimal cell density is present between the bare metal stents (A= Express 2 and C= Bx Velocity) and the drug-eluting stents (B=Taxus Express 2 and D=Cypher) Furthermore hemorrhage and acellular areas are seen in the neointima of the drug-eluting stents (#=hemorrhage, *=acellular areas).
Table 1. Incomplete healing after 1 week:

<table>
<thead>
<tr>
<th>Variable</th>
<th>(A=Express 2) (N=5)</th>
<th>(B= Taxus) (N=6)</th>
<th>(C= Bx Velocity) (N=6)</th>
<th>(D=Cypher) (N=6)</th>
<th>P-value A:B/C:D/A:C/B:D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neointimal cell density (× 100/mm²)</td>
<td>24.0±1.5</td>
<td>28.9±4.9</td>
<td>26.1±1.60</td>
<td>27.0±3.7</td>
<td>NS/NS/NS/NS</td>
</tr>
<tr>
<td>Inflammation score</td>
<td>0.69±0.05</td>
<td>0.80±0.15</td>
<td>0.56±0.02</td>
<td>0.56±0.08</td>
<td>NS/NS/NS/NS</td>
</tr>
<tr>
<td>Signs of incomplete healing (% of rats)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>NS/NS/NS/NS</td>
</tr>
</tbody>
</table>

DES versus DES (B:D): There was no significant difference in inflammation score, cell density and signs of incomplete healing between the Taxus® Express2™ and the Cypher® Stent.

BMS versus BMS (A:C): There was no significant difference in inflammation score, cell density and signs of incomplete healing between Express® and the Bx Velocity® Stent.

BMS’s versus DES’s (A:B and C:D): the Express® did not differ from the Taxus® Express2™ Stent in inflammation score, cell density and signs of incomplete healing. The Bx Velocity® did not differ from the Cypher® Stent in inflammation score, cell density and signs of incomplete healing.

Table 2. Incomplete healing after 4 weeks:

<table>
<thead>
<tr>
<th>Variable</th>
<th>(A=Express 2) (N=6)</th>
<th>(B= Taxus) (N=7)</th>
<th>(C= Bx Velocity) (N=7)</th>
<th>(D=Cypher) (N=8)</th>
<th>P-value A:B/C:D/A:C/B:D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neointimal cell density (× 100/mm²)</td>
<td>37.2±1.1</td>
<td>22.2±2.5</td>
<td>42.4±2.5</td>
<td>28.7±4.9</td>
<td>P&lt;0.05/P&lt;0.05/NS/NS</td>
</tr>
<tr>
<td>Inflammation score</td>
<td>0.06±0.02</td>
<td>0.26±0.04</td>
<td>0.11±0.03</td>
<td>0.30±0.06</td>
<td>P&lt;0.05/P&lt;0.05/NS/NS</td>
</tr>
<tr>
<td>Signs of incomplete healing (% of rats)</td>
<td>0±0</td>
<td>82±11</td>
<td>4±4</td>
<td>96±4</td>
<td>P&lt;0.05/P&lt;0.05/NS/NS</td>
</tr>
</tbody>
</table>

DES versus DES (B:D): There was no significant difference in inflammation score, cell density and signs of incomplete healing between the Taxus® Express2™ and the Cypher® Stent.

BMS versus BMS (A:C): There was no significant difference in inflammation score, cell density and signs of incomplete healing between Express® and the Bx Velocity® Stent.

BMS’s versus DES’s (A:B and C:D): the Taxus® Express2™ had a higher inflammation score, lower cell density and more signs of incomplete healing compared to the Express® Stent. The Cypher® Stent had a higher inflammation score, lower cell density and more signs of incomplete healing compared to the Bx Velocity® Stent.

Table 3 Neointimal area and control parameters.

<table>
<thead>
<tr>
<th>Variable</th>
<th>(A=Express 2) (N=6)</th>
<th>(B= Taxus) (N=7)</th>
<th>(C= Bx Velocity) (N=7)</th>
<th>(D=Cypher) (N=8)</th>
<th>P-value A:B/C:D/A:C/B:D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injury score</td>
<td>0</td>
<td>0.05±0.05</td>
<td>0.05±0.05</td>
<td>0</td>
<td>NS/NS/NS/NS</td>
</tr>
<tr>
<td>Internal elastic lamina area (mm²)</td>
<td>3.87±0.06</td>
<td>3.77±0.25</td>
<td>3.56±0.09</td>
<td>3.68±0.11</td>
<td>NS/NS/NS/NS</td>
</tr>
<tr>
<td>Neointima area (mm²)</td>
<td>0.77±0.04</td>
<td>0.56±0.02</td>
<td>0.73±0.04</td>
<td>0.52±0.03</td>
<td>P&lt;0.05/P&lt;0.05/NS/NS</td>
</tr>
<tr>
<td>Neointimal thickness (µm)</td>
<td>165±6.3</td>
<td>122±7.8</td>
<td>169±9.4</td>
<td>144±5.5</td>
<td>P&lt;0.05/P&lt;0.05/NS/NS</td>
</tr>
<tr>
<td>Media area(mm²)</td>
<td>0.22±0.02</td>
<td>0.20±0.02</td>
<td>0.23±0.01</td>
<td>0.21±0.02</td>
<td>NS/NS/NS/NS</td>
</tr>
</tbody>
</table>

In the drug-eluting stents the neointimal area and neointimal thickness were significantly decreased compared to the bare metal stents. There are no differences in the control parameters (injury score, internal elastic lamina area, media area) of all groups.
Table 4. Collagen content and distribution

<table>
<thead>
<tr>
<th>Variable</th>
<th>(A=Express)</th>
<th>(B=Taxus)</th>
<th>(C=BxVelocity)</th>
<th>(D=Cypher)</th>
<th>(E=A+C)</th>
<th>(F=B+D)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N=6)</td>
<td>(N=6)</td>
<td>(N=7)</td>
<td>(N=8)</td>
<td>(N=13)</td>
<td>(N=14)</td>
<td>A:B/C:D/A:C/B:D/E:F</td>
</tr>
<tr>
<td>Collagen</td>
<td>8.6±2.3</td>
<td>3.3±1.1</td>
<td>6.5±1.5</td>
<td>3.3±2.1</td>
<td>7.5±1.3</td>
<td>3.3±1.3</td>
<td>NS/NS/NS/NS/P&lt;0.05</td>
</tr>
</tbody>
</table>

No significant differences in total collagen content were found when all 4 groups were separately compared however there was a trend towards less collagen content in the drug-eluting stents. Furthermore a significantly lower collagen content was found in the drug-eluting stents after we combined both drug-eluting and bare metal stent groups and compared the two groups.

Figure 2: Neointimal cell density after 1 week and 4 weeks. After 1 week no difference is seen between the bare metal stent groups and the drug-eluting stent groups. However after 4 weeks cell density is lower in the drug-eluting healing stent groups.

Figure 3: Signs of incomplete healing after 1 week and 4 weeks. After 1 week no difference is seen between the bare metal stent groups and the drug-eluting stent groups. However after 4 weeks still signs of incomplete are seen in the drug-eluting stent groups.
Figure 4 Hematoxyline-eosine stained photomicrographs (x400) of stented abdominal aortas after 4 weeks showing the lumen, the neointima and the media. We observed an irregular pattern of incomplete healing in drug-eluting stents: in the same cross section of neointima normal cell density areas existed next to completely acellular areas (A=Taxus, B=Cypher, #=normal cell density, *=acellular areas).

Figure 5 Corresponding sirius red stained photomicrographs (B,D,F and H) and hematoxyline-eosine stained photomicrographs (A,C,E and Gx400) of stented abdominal aortas after 4 weeks showing the lumen, the neointima, media and adventitia. Absent collagen (less red) staining (B,F) in acellular neointima (A,E) and more collagen staining (D,H) in neointima areas with higher cell density (C,G). (A,B,C,D=Cypher, E,F,G,H=taxus).
DISCUSSION

In this study we explored the differences in neointimal healing between bare metal and drug-eluting stents, 1 and 4 weeks after stenting. After 1 week neointimal cell density, inflammation score and signs of incomplete healing were not different between bare metal and drug-eluting stents. However after 4 weeks neointimal cell density was lower, signs of incomplete healing were higher and the inflammation score was higher in the drug-eluting groups, especially in the neointimal area adjacent to the stent struts. To interpret these results understanding of the development of in-stent restenosis in our model is needed. At 1 week smooth muscle proliferation and neointimal formation is incomplete and inflammation is at its peak(6). At 4 weeks neointimal formation has reached its peak and inflammation is low. The normal complete healing pattern of bare metal stents in our model is a high neointimal cell density, low inflammation and absence of acellular areas and hemorrhages after 4 weeks(6). This pattern was found only in the bare metal stents and not in the drug-eluting stents. These results suggests that not only endothelial healing (as observed in other studies) but also neointimal healing is delayed in drug-eluting stents. In this study incomplete healing was characterized by low cellular density, acellular areas, hemorrhage and higher inflammation in the neointima of drug-eluting stents. Low neointimal cell density was also found by Finn et al in drug-eluting stents, but this was found in overlapping stent segments (drug and/or polymer concentrations are likely to be significantly higher at sites of stent overlap). Also they did not compare their measurements of neointimal cell density in drug-eluting stents with control bare metal stents.(10) Farb et al showed that systemic everolimus treatment in rabbits reduced neointimal formation but they also noticed hypocellularity. This suggests that the inhibiting effect of anti-restenotic drug (everolimus, sirolimus, paclitaxel) on proliferating cells is responsible for the low neointimal cell density rather than the polymer or the stent itself (11). However the exact mechanisms underlying incomplete neointimal healing in drug-eluting stents are still unknown.

A link between late stent thrombosis and incomplete endothelial healing was suggested by Joner et al: incomplete reendothelization and high fibrin content in drug-eluting stents could be a potent thrombogenic stimulus. Our results showing low collagen content in neointimal areas with incomplete healing suggest a second hypothesis: the neointimal tissue of drug-eluting stents is weaker and could rupture more easily thereby exposing tissue factor and inducing thrombus. The presence of hemorrhages (Figure 1) within the neointima of drug-eluting stents seems to support this hypothesis. The low neointimal cell density could be correlated to tissue strength: there are not enough cells to produce extracellular matrix and ensure a strong neointima. Both sirolimus and paclitaxel have a direct effect on the extracellular matrix production in cells. Sirolimus inhibits collagen synthesis in rat vascular smooth muscle cells and paclitaxel also reduces tenascin (a extracellular matrix glycoprotein) in human arterial smooth muscle cells (12). Collagen and tenascin are both positively correlated with tissue strength(13;14). Interestingly we found a lower collagen content in drug-eluting stents especially in acellular areas.

We did not find significant differences in the neointima between the paclitaxel and sirolimus-eluting stents at both 1 and 4 weeks. According to our findings both sirolimus and paclitaxel stents have incomplete healing and prolonged inflammation after 4 weeks. After 4 weeks both the Taxus® Express™ and the Cypher® Stent still elute some of their drug(4;15). So it could be that differences in the neointima between the Taxus® Express™ and the Cypher® Stent would occur later after stenting due to different release profiles of both stents.

There are some limitations to this study. Firstly, this study was conducted with juvenile rats without preformed atherosclerotic plaque in arteries. The hydrophobic nature of sirolimus and paclitaxel could
increase drug concentrations in atherosclerotic plaques with high lipid content. So it is possible that we underestimated the effects of drug-eluting stents on the neointima due to lower tissue concentrations in our non-atherosclerotic model. Secondly, due to the low incidence of late stent thrombosis it is difficult to measure differences in the incidence of late stent thrombosis between drug-eluting stents and bare metal stents in a small animal study (16). This study therefore can not directly link the differences in the neointima with late stent thrombosis. It would be interesting to study to what extent the differences in the neointima between drug-eluting stents and bare metal stents found in this study are related to the incidence of late stent thrombosis.

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Reference List


