Low-Molecular-Weight Heparin Reduces Neointimal Proliferation After Coronary Stent Implantation in Hypercholesterolemic Minipigs

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Background. Intracoronary stents have been suggested as a method of reducing the restenosis rate after balloon angioplasty. Proliferation of vascular smooth muscle cells is a major contributing factor to the restenosis process. Heparin and some of its derivatives have been shown to inhibit smooth muscle cell proliferation. We investigated the effect of low-molecular-weight heparin on the proliferative response after implantation of a balloon-expandable tantalum stent in previously deendothelialized coronary artery segments of hypercholesterolemic minipigs.

Methods and Results. Minipigs were fed a diet containing 2% cholesterol, starting 1 month before balloon denudation of the endothelium in a coronary artery. One month later, a stent was implanted at this site. Animals were killed after 4 weeks (group 1, n=6) or 3 months (group 2, n=6). Animals in group 3 (n=6), also followed for 4 weeks after stenting, received subcutaneous low-molecular-weight heparin at a dose of 200–300 units/kg anti-factor Xa activity per day in addition to the chronic acetylsalicylic acid (100 mg/day) also administered to groups 1 and 2. Eighteen of 22 animals survived to the end of the study. Angiography revealed patent stents in all surviving animals. In group 1, histological analysis showed extensive neointimal proliferation around stent struts. Maximal neointimal thickness seen in group 1 averaged 0.93±0.11 mm, was lower after 3 months (0.8±0.14 mm) in group 2, but was significantly reduced (0.44±0.18 mm, p<0.01) in group 3.

Conclusions. These data show a significant reduction of the neointimal proliferative response to coronary stent implantation by low-molecular-weight heparin. (Circulation 1992;86:531–537)

Key Words • stents • coronary artery • restenosis • smooth muscle cells

Restenosis after coronary angioplasty remains the Achilles heel of this technique; frequency of restenosis ranges =20% to =40% after coronary balloon angioplasty.1,2

Clinical trials of restenosis rate reduction by pharmacological means have been inconclusive or unsuccessful.3–8 Use of nonballoon devices for angioplasty, including mechanical, thermal, and laser devices, have not resulted in a reduction of the restenosis rate.9,10

Restenosis is a complex phenomenon involving elastic recoil of the vessel wall, possible local thrombosis with subsequent organization of the clot, and, probably as the major component, local proliferative reaction of the vessel wall.11–13

Autopsy studies and analysis of atherectomy specimens from restenotic lesions have demonstrated proliferating smooth muscle cells (SMCs) as the major cellular constituent of the restenotic lesion.13–15

Intravascular stents have been suggested as a method of dealing with occlusive dissection after angioplasty and preventing restenosis.16–18 Intravascular stents act as an internal scaffold of the vessel wall to cope with elastic recoil. The proliferative reaction of the vessel wall, however, is not significantly blunted after stent implantation and, again, is basically caused by proliferating SMCs migrating through the meshes of the stent.19,20 Accordingly, given the results of uncontrolled clinical trials with different types of coronary stents, there remains a significant percentage of restenosis. Thus, even in stented vessels, there is a need to pharmacologically modulate the proliferative vessel wall response.

Heparin, by a mechanism not completely elucidated,21 has been shown to inhibit SMC proliferation both in vitro and in animal experiments without adversely affecting endothelial cell proliferation.22,23

In this study, we assessed the effect of low-molecular-weight heparin on the restenotic process in coronary arteries of hypercholesterolemic minipigs after implantation of the Wiktor stent.

Methods

Animal Model

Minipigs of the Göttingen strain of either sex with a mean±SD weight of 37±6 kg (range, 28–49 kg) were
used. They were fed a standard diet (Altromin, FRG) supplemented with 2% cholesterol and 10 g Na-cholate per day beginning 4 weeks before the first intervention. Blood samples for measurement of serum cholesterol were drawn before starting the animals on the cholesterol diet, when the coronary arteries ballooned, at stent implantation, and at the end of the experiments.

The adult animals (older than 1 year) reached a stable body weight before starting the diet, and no significant weight changes were observed during the study period (as long as 5 months).

In preliminary experiments, two animals were fed this diet for 4 weeks after ballooning of two coronary arteries and then were killed. On visual inspection of the intimal surface, areas of lipid deposition in the ballooned segments were evident. Angiography before death showed no luminal narrowing.

**Wiktor Stent**

The Wiktor stent is a 15-mm-long balloon-expandable device comprising a single tantalum wire with a diameter of 127 μm. It is crimped onto standard percutaneous transluminal coronary angioplasty (PTCA) balloons 2.0–4.0 mm in diameter in 0.5-mm increments. Stents were implanted via the surgically exposed right carotid artery using a 9F giant-lumen guiding catheter (El Gamal) under sterile conditions. The balloon containing the stent was advanced over a coronary guide wire placed distally into the selected coronary artery. Stent placement at the target site could be achieved easily because of the excellent radiopacity of the tantalum wire.

**Protocol**

After 4 weeks on the cholesterol diet, animals were intubated under general anesthesia (10 mg/kg azaperone s.c. and 8 mg/kg metomidate, followed by 8–10 mg/kg metamizole, 0.5 mg atropine, and 15 mg priritramide i.v.). They were ventilated with a 2:1 mixture of nitrous oxide and oxygen (Sulla 19 respirator, Dräger, FRG). Blood gas analysis was then performed, and ventilation was adjusted as necessary to maintain physiological conditions for pH, Pco2, and Po2. The left carotid artery was exposed, and endothelial injury of the left circumflex artery (LCx) or left anterior descending coronary artery (LAD) was performed through three consecutive inflations of a PTCA balloon 0.3–0.5 mm larger than the vessel diameter at 6 atm inflation pressure. Vessel diameter before balloon placement was determined from two orthogonal views using an electronic caliper. No view was accepted where a side branch projected over the segment of interest. Results given in Table 1 represent the worst of two views, i.e., that showing the smaller vessel diameter.

Thereafter, the artery was repaired or ligated, and the animals were returned to their cages after recovery from anesthesia. After 4 weeks, angiography was repeated in the same exact views for assessment of luminal narrowing after balloon endothelial injury. A stent was then implanted at the site of the previous balloon dilatation via the right carotid artery (see above).

Either 4 weeks or 3 months after stent implantation, control angiography was performed to confirm patency of the stented artery and determine luminal narrowing within the stent using the same two orthogonal views. Coronary artery diameters proximal, distal to, and within the stent were determined. Again, results given in Table 1 represent the worst of two views.

Thereafter, the heart was excised, immediately perfused with phosphate-buffered saline at a pressure of 100 mm Hg, and then perfusion-fixed with buffered glutaraldehyde.

**Antiplatelet Therapy and Anticoagulation in Experimental Groups**

All animals received 100 mg/day acetylsalicylic acid added as a tablet to the diet each morning starting on the day before balloon dilatation and continuing throughout the study. Before insertion of the arterial catheter for balloon dilatation, stent implantation, and control angiography, animals were administered a single bolus of 100 units/kg heparin i.v. In two experimental groups (n=6 each), experiments were terminated either 4 weeks (group 1) or 3 months (group 2) after stent implantation.

Group 3 (n=6) received a daily subcutaneous injection of low-molecular-weight heparin (Fragmin, Kabi, FRG) at a dose of 200–300 units anti-factor Xa activity in addition to the heparin bolus at implant and the chronic administration of acetylsalicylic acid. Plasma anti-factor Xa activity was measured 24 hours after administration, and subsequent doses were adjusted to achieve levels between 0.5 and 1.5 units/ml. Treatment was started on the day after stent implant and continued throughout the 4 weeks until death. Initially, two of six animals had suboptimal levels. In these two, the fragmin dose was increased to achieve levels in the desired range. Anti–Xa factor activity was subsequently measured at least once per week, and all values were in the desired range at weeks 1–4.

**Histology**

Vessel segments 10 mm proximal to 10 mm distal of the stent were excised, dehydrated in increasing alcohol concentrations, and embedded in methacrylate. This procedure allowed the preparation of slices through the stented segments without prior removal of the stent. Thus, the vessel structures around the stent remained intact. Slices were stained with toluidine blue.

In five slices per stent and in a proximal and distal segment adjacent to the stent, morphometric analysis of the free luminal area and vessel area (lumen plus intima plus media) was performed. The smallest free luminal area of the five sections per stent was used for between-group comparison. The difference between vessel and luminal area, representing vessel wall area, was calculated. The mean values of five sections per stent were compared between groups. The maximal thickness of the neointima was determined in the five sections of individual stents. Here, the maximal values of each stent were used for comparison between experimental groups. Histological analysis was performed by an investigator blinded to the treatment group.

**Statistical Analysis**

Results are expressed as mean±SD. Data of group 1 were compared with those of group 2 or 3 using the Mann-Whitney U test. Vessel diameters before stent implantation and stent sizes were compared with
TABLE 1. Angiographic Vessel Diameter and Stent Size

<table>
<thead>
<tr>
<th>Group</th>
<th>Vessel diameter (mm)</th>
<th>4*/12 Weeks</th>
<th>Stent size (mm)</th>
<th>%DS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before balloon</td>
<td>Before stent</td>
<td>After stent</td>
<td></td>
</tr>
<tr>
<td>1 (4 weeks)</td>
<td>3.1 ± 0.4</td>
<td>2.6±0.3</td>
<td>2.9±0.3</td>
<td>2.1±0.6</td>
</tr>
<tr>
<td>2 (3 months)</td>
<td>2.6±0.5</td>
<td>2.7±0.3</td>
<td>2.8±0.3</td>
<td>2.3±0.3</td>
</tr>
<tr>
<td>3 (fragmin; 4 weeks)</td>
<td>2.6±0.3</td>
<td>2.5±0.3</td>
<td>2.7±0.3</td>
<td>2.6±0.3</td>
</tr>
</tbody>
</table>

Vessel diameters and the nominal sizes of the inflated stent-carrying balloons are given in millimeters. Vessel diameters given are the smallest within the segment assigned to be stented or carrying the stent, respectively. %DS, percent diameter stenosis after 4 weeks (group 1 and 3) or 3 months (group 2) relative to the vessel diameter proximal to the stent. By ANOVA, vessel diameters before stenting and stent sizes are not different between the groups.

ANOVA. A value of $p<0.05$ was considered statistically significant.

Results

Serum Cholesterol

Baseline serum cholesterol levels ranged from 72±14.3 to 93.6±16.8 mg/100 ml. After 4 weeks on the cholesterol diet, levels increased about twofold. They reached a plateau value three to four times higher than baseline after 8 weeks (Table 2).

Angiography

All 18 animals in which the study protocol was completed had patent stented vessels at control angiography. Two animals were found dead in their cage within 24 hours of balloon angioplasty, and two were dead after stent implantation (thrombotic stent occlusion at autopsy). Of the latter, one animal was intended to be killed after 3 months, and one animal had been assigned to the fragmin group.

Vessel diameters before balloon dilatation in the target segments were 2.0–3.1 mm. Accordingly, balloon and stent sizes were either 2.5, 3.0, or 3.5 mm. Mean vessel diameters are given in Table 1. Luminal narrowing of less than 50% of the diameter proximal to the stent was observed in two animals in group 1 (68% and 54% diameter stenoses).

In 16 of 18 animals, side branches were located within the stented segment. Twelve of these were patent at control angiography. One diagonal branch of the LAD

Table 2. Serum Cholesterol

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>4 Weeks on diet</th>
<th>8 Weeks on diet</th>
<th>12 Weeks on diet</th>
<th>20 Weeks on diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (4 weeks)</td>
<td>93.5±16.8</td>
<td>207.5±61.2</td>
<td>309.8±92.0</td>
<td>282.0±46.0</td>
<td></td>
</tr>
<tr>
<td>2 (3 months)</td>
<td>79.7±22.4</td>
<td>134.0±36.1</td>
<td>299.7±75.5</td>
<td>281.8±69.0</td>
<td></td>
</tr>
<tr>
<td>3 (fragmin)</td>
<td>72.0±14.3</td>
<td>145.5±32.9</td>
<td>330.0±63.2</td>
<td>319.0±56.7</td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean±SD measurements in six animals per time point. None of the differences reached the level of statistical significance.
in group 1 and one in group 3 (fragmin) and one posterolateral branch of the LCx in group 1 and one in group 2 were occluded; in each case, occlusions were associated with a small myocardial infarct in the respective region.

In fragmin-treated animals, bleeding complications at injection sites or at the surgical wounds were not observed.

**Histological Analysis**

A typical section through a stented LAD in group 1 is shown in Figure 1. In all animals, the media was severely thinned by the stent struts. Occasionally, disruption of the lamina elastica interna and fragmentation of the media was observed. A fibrocellular proliferation was present, which was maximal around the stent struts and less pronounced between struts.

In the two animals that died acutely after stent implantation, the relation between stent and vessel diameter was similar to that in the other animals. At autopsy, the stented arteries were occluded by thrombus. The stent struts indented the media, which was found partially disrupted.

**Morphometry**

In segments proximal and distal to the stent, the vessel wall area accounted for \(18\pm3\%\) of the total vessel area. This relation of vessel wall to total vessel area increased dramatically 4 weeks after stent implantation (group 1; Table 3). However, this increase in vessel wall area was not associated with a corresponding decrease of the free luminal area because significant luminal narrowing was observed in only two stents in group 1 (Table 4). Maximal intimal thickness averaged \(0.93\pm0.11\) mm (Table 5). After 3 months, both vessel wall area and intimal thickness showed a tendency toward lower values compared with group 1, but these differences were not statistically significant.

Although the proliferative response in group 3 (fragmin) was morphologically similar to that in group 1, the extent of this response was markedly lower. Maximal intimal thickness was less than 50\% of that in group 1 and vessel wall area was \(\approx50\%\) less. Data are given in Tables 3, 4, and 5.

**Discussion**

Balloon dilatation of coronary arteries is followed by proliferation of SMCs, which migrate from the media to the intima.\(^{21-22}\) Studies on specimens of human athero-
TABLE 3. Vessel Wall Area

<table>
<thead>
<tr>
<th>Vessel wall area (of total vessel area)</th>
<th>Group 1 (4 weeks)</th>
<th>Group 2 (12 weeks)</th>
<th>Group 3 (fragmin; 4 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal segment</td>
<td>18.3±2.9</td>
<td>17.5±2.1</td>
<td>18.9±3.2</td>
</tr>
<tr>
<td>Stents</td>
<td>60.9</td>
<td>57.7</td>
<td>25.8</td>
</tr>
<tr>
<td></td>
<td>58.4</td>
<td>60.2</td>
<td>42.1</td>
</tr>
<tr>
<td></td>
<td>51.2</td>
<td>46.3</td>
<td>44.7</td>
</tr>
<tr>
<td></td>
<td>68.9</td>
<td>40.4</td>
<td>23.5</td>
</tr>
<tr>
<td></td>
<td>71.4</td>
<td>58.1</td>
<td>38.8</td>
</tr>
<tr>
<td></td>
<td>49.3</td>
<td>61.9</td>
<td>42.3</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>60.0±9.0</td>
<td>54.1±8.7</td>
<td>36.2±9.2</td>
</tr>
</tbody>
</table>

Vessel wall area (difference between vessel area and free luminal area) is expressed in percentage of total vessel area. Five sections per stent were analyzed. Mean value of the five sections is given. *p<0.01 for the difference from group 1.

TABLE 4. Maximal Reduction of the Free Luminal Area Within Stents

<table>
<thead>
<tr>
<th>Reduction (%)</th>
<th>Group 1 (4 weeks)</th>
<th>Group 2 (12 weeks)</th>
<th>Group 3 (fragmin; 4 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>68</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>38</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>54</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>27.3±26.5</td>
<td>16.3±11.7</td>
<td>5.7±6.3*</td>
</tr>
</tbody>
</table>

Maximal reduction of the free luminal area is expressed as the difference to the luminal area in the segment proximal to the stent, which was taken as 100%. Five sections per stent were analyzed, and the smallest area of five was taken. *p<0.05 for the difference from group 1.

sclerotic, restenosed plaques obtained during atherectomy have shown this to occur in a similar fashion in humans. SMCs were identified by their characteristic positive immunohistochemical staining as the predominant proliferating cell type in restenotic lesions. The process of intimal SMC proliferation starts about 3 days after balloon injury, peaks at 1 week, and continues until reendothelialization is complete. This process results in intimal thickening, most likely due to extracellular matrix synthesized by proliferating cells. Implantation of stents has been proposed as a possible remedy for the restenosis problem. However, it has become evident that restenosis still occurs in 15–30% of cases using different stent devices.

Our study shows a proliferative neointimal response induced by implantation of a coronary stent in hypercholesterolemic minipigs. Extensive neointimal proliferation is present after 4 weeks, as reflected by a severalfold increase of wall thickness. Three months after stent implantation, wall thickness is reduced, which is compatible with the reported inhibitory effect of reendothelialization on SMC proliferation.

Furthermore, the present data demonstrate a significant reduction of this proliferative vessel wall response after coronary stent implantation by subcutaneous administration of low-molecular-weight heparin.

TABLE 5. Maximal Intimal Thickness

<table>
<thead>
<tr>
<th>Thickness (mm)</th>
<th>Group 1 (4 weeks)</th>
<th>Group 2 (3 months)</th>
<th>Group 3 (fragmin; 4 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.10</td>
<td>0.59</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>0.98</td>
<td>0.88</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>0.93</td>
<td>0.98</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>0.94</td>
<td>0.81</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>0.96</td>
<td>0.72</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>0.79</td>
<td>0.79</td>
<td>0.22</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>0.93±0.11</td>
<td>0.80±0.14*</td>
<td>0.44±0.18†</td>
</tr>
</tbody>
</table>

For each animal, five sections through the stent were analyzed, and the maximal intimal thickness is shown in millimeters. *0.1>p>0.05 for the difference from group 1. †p<0.01 for the difference from group 1.

Stent Placement and Thrombosis

Positioning of the radiopaque tantalum stent used in this study was easily controlled. Stent embolization, reported for stainless-steel stents that are less easily seen under fluoroscopy, did not occur. There were two cases of stent thrombosis in this study, emphasizing the thrombogenic potential of stents. Although stent thrombosis is observed in the clinical setting, incidence has been low in animal models, even without the aggressive anticoagulation used in humans. Two recent animal studies also used this tantalum coil balloon-expandable stent. Although Schwartz et al reported four stent-related deaths, van der Giessen et al saw no such adverse events. Because all deaths occurred within the first hours after stent implantation, severe vessel damage and subsequent thrombus formation by severely oversized stents may be the underlying cause, as pointed out by Schwartz et al.

Similar results for the vessel wall response to implantation of a metal coil stent in coronary arteries have been reported for both this and other stent types. In a study in normolipidemic pigs, Schwartz et al recently reported a more intense proliferative response for the same stent type in those segments where subintimal damage around the stent was evident. This observation is confirmed by our data in hypercholesterolemic animals, where wall thickness was lowest in those segments with a circumferentially intact media. Van der Giessen et al reported an increase in intimal thickness in pigs 1–4 weeks after implantation of this stent. Our study shows that proliferation ceases thereafter. No further increase but rather a tendency to lower wall thickness was evident after 3 months. The absolute wall thickness in group 1 4 weeks after stent implantation in our study was higher compared with the results of van der Giessen et al. This difference may be due to the hypercholesterolemia induced in our protocol because the animals were normolipidemic in the former study. In the report by Rodgers et al, who implanted a differently designed tantalum stent in hypercholesterolemic pigs, luminal narrowing to =40% was observed, but absolute wall or neointimal thickness was not detailed.
Influence of Low-Molecular-Weight Heparin on the Proliferative Response

An ideal pharmacological approach to reduce the proliferative response should act selectively on SMC proliferation while leaving the reendothelialization unimpaired or enhanced. In addition to its anticoagulatory effects, heparin has an inhibitory effect on SMC proliferation in vitro.22 Clowes and Karnovsky21 first showed an inhibition of vascular SMC proliferation after endothelial injury by heparin in vivo. In a rat carotid angioplasty model, localized adventitial delivery of heparin equally diminished SMC proliferation without systemic anticoagulation.23,35

Experimental evidence suggests the inhibitory effect of endothelium on SMC proliferation to be mediated by heparin-like substances.36,37 Although the exact mechanism of action is not clear, heparin and its derivatives appear to block the cell cycle at the G1 stage.21 This antiproliferative action is independent of the anticoagulatory sites on heparin and is conserved in low-molecular-weight fragments.22 Compared with standard heparin, the bleeding risk associated with anticoagulative therapy is reduced for doses of low-molecular-weight heparin that are effective in the treatment of venous thromboembolism.38 In the present study, we did not observe bleeding either locally or at the site of surgery after starting the low-molecular-weight heparin injections. In addition, the pharmacokinetic profile of low-molecular-weight heparin is more favorable due to a half-life of anti–Xa factor activity, which is twice that of unfractionated heparin.39 Thus, the need for continued administration through indwelling catheters is avoided.

In our model, a reduction of the proliferative vessel wall response after coronary stenting by low-molecular-weight heparin is obvious. Intimal thickness and total vessel wall area were significantly reduced in the low-molecular-weight heparin–treated group. Recently, in a rabbit iliac artery model, low-molecular-weight heparin administered subcutaneously over 4 weeks has been shown to significantly reduce restenosis after balloon angioplasty.40 In the clinical setting, heparin is given routinely in large doses during all angioplasty procedures; nevertheless, restenosis still occurs. Administration of heparin usually is not extended beyond 24 hours after the procedure. Because of the short biological half-life of heparin, an antiproliferative effect lasting beyond 2 days cannot be expected. Because reendothelialization is not complete before at least 14 days in humans,25 and thus the endogenous heparinoid inhibition of SMC proliferation is lacking, this could be the appropriate time window for prolonged heparin administration. Accordingly, in a study of the effect of heparin infused throughout the first 48 hours after angioplasty, no effect on restenosis rate was observed.41

Study Limitations

As with any animal study in the field of restenosis, it must be kept in mind that no model exists that resembles exactly human coronary atherosclerosis. In the present study, high cholesterol diet and balloon denudation resulted in some degree of vessel wall reaction within 4 weeks. These observations closely resemble observations in the same animal model42 in which cholesterol feeding was continued for 3 months after balloon denudation. The effect of prolonged low-molecular-weight heparin administration on restenosis after vessel wall injury is under clinical investigation. Our study shows a reduction by low-molecular-weight heparin of intimal proliferation induced by coronary artery stenting in minipigs when given subcutaneously over 4 weeks after stent implantation.

References

Low-molecular-weight heparin reduces neointimal proliferation after coronary stent implantation in hypercholesterolemic minipigs.
A B Buchwald, C Unterberg, K Nebendahl, H J Gröne and V Wiegand

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