Time Course of Stent Endothelialization After Intravascular Radiation Therapy in Rabbit Iliac Arteries

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Background—Late total occlusion after vascular brachytherapy (VBT) continues to be a serious complication. Delayed reendothelialization was suggested as a pivotal cause, but the time course for complete healing is unknown.

Methods and Results—Seventy-two rabbit iliac arteries underwent stent implantation and were treated with γ-radiation using 125I. The prescribed doses were 0 Gy (controls, n = 24 arteries), 15 Gy (n = 24), or 30 Gy (n = 24) at 2 mm. Animals were killed at 1 month (n = 24), 3 months (n = 24), or 6 months (n = 24) and were analyzed for histomorphometry or scanning electron microscopy. Intimal area was reduced after VBT at 3 months with 15 and 30 Gy (0.66 ± 0.07 and 0.66 ± 0.04 mm², respectively) compared with controls (1.01 ± 0.11 mm², P < 0.05) and at 6 months with 30 Gy (0.75 ± 0.09 versus 1.28 ± 0.26 mm² in controls, P < 0.01). Intimal area was similar at 6 months between 15 Gy and controls. At 1 month, 92 ± 4% of the control stented segment was covered with endothelial cells, whereas only 37 ± 4% and 37 ± 8% was covered in the 15- and 30-Gy arteries, respectively. Similarly, at 3 and 6 months, there was a difference in the extent of reendothelialized areas (at 3 months, 95 ± 2%, 32 ± 12%, and 29 ± 13%; and at 6 months, 98 ± 2%, 40 ± 8%, and 35 ± 12% in control, 15-Gy, and 30-Gy arteries, respectively). Excess platelets and leukocytes were seen in irradiated arteries without complete coverage of endothelium.

Conclusions—Reendothelialization after VBT is not completed at 6 months after VBT. Special care with prolonged antiplatelet therapy should be considered beyond that time point. (Circulation. 2003;107:2153-2158.)

Key Words: brachytherapy • stent • endothelium • thrombosis

Vascular brachytherapy (VBT) has demonstrated efficacy for the treatment of in-stent restenosis. Randomized clinical trials have demonstrated reduction of the restenosis rate with VBT compared with conventional therapy, primarily by inhibition of smooth muscle cell proliferation.1-6 Despite effective inhibition of neointima formation with VBT, long-term results were associated with high rates of late total occlusions and late thrombosis.7,8 The late thrombosis phenomenon was attributed to delayed vessel healing of the injured area after percutaneous coronary intervention with fibrin deposition, incomplete reendothelialization, and loss of endothelial function.9 This effect was more pronounced in stented vessels.10 Lack of endothelial healing has been reported in vessels treated with radioactive stents.11 This could have been attributed to the sustained radioactivity for >60 days with high-activity radioactive stents. Currently, VBT is delivered only by catheter-based systems, and the rate of healing and reendothelialization is unknown for a single dose administration and a higher dose rate. In the absence of an intact endothelium, the vessel is vulnerable to platelet adhesion and inflammatory cell infiltration, events that contribute to thrombotic closure.12 To eliminate late thrombosis, prolonged antiplatelet therapy regimens were initiated for patients treated with VBT, but the optimal duration of antiplatelet therapy is unknown because of the lack of information on the time course of stent reendothelialization after VBT.13

The purpose of this study was to determine the influence of radiation and its dose on the pace of reendothelialization after VBT in rabbit iliac arteries.

Methods

Animals

Thirty-six female or male New Zealand White rabbits (3 to 3.5 kg; Thomas D. Morris, Inc, Reisterstown, Md) were used in this study. The protocol was approved by the Institutional Animal Care and Use Committee of the Medstar Research Institute. All animals received aspirin 81 mg/d for 7 days before the initial intervention and until they were killed. Rabbits were anesthetized with an intramuscular injection of ketamine (25 mg/kg) and xylazine (2.5 mg/kg). During the experimental procedure, anesthesia was maintained by intermittent injection of ketamine (20 mg/kg IM).
Catheterization Procedure
The left carotid artery was exposed, and a 5F pediatric arterial sheath was inserted via an arteriotomy. An intra-arterial injection of 500 IU heparin was given. A baseline aortic and iliac angiogram was recorded, and stents (3.0×9.0 mm onto a 3.0×15-mm balloon, Tristar Stent, Guidant) were deployed in iliac arteries by high-pressure balloon inflation to achieve a 1.1:1.0 to 1.2:1.0 stent-to-artery ratio.

Radiation
After stent deployment, radiation treatment was performed by positioning a delivery catheter (0.79 mm in diameter over a flexible 0.014-in wire). Stented segments of arteries were irradiated with 6 seeds of 192 Ir source (length, 23 mm; range of activity, 200 to 300 mCi) inserted into the lumen of the delivery catheter. The radiation source was left for a period sufficient to deliver 0 Gy as controls (dummy seeds; number of arteries, 24), 15 Gy (n=24), or 30 Gy (n=24) at 2 mm from the center of the source axis. After the irradiation or control treatment, rabbits were allowed to recover.

Follow-Up
Animals were killed at 1 month (control, n=8 arteries; 15 Gy, n=8; 30 Gy, n=8), 3 months (control, n=8; 15 Gy, n=8; 30 Gy, n=8), and 6 months (control, n=8; 15 Gy, n=8; 30 Gy, n=8). Before they were killed, all rabbits received 2000 U heparin via the ear vein, and an angiogram was recorded. A cannula was inserted into the abdominal aorta to perfuse 100 mL of 5% dextrose solution containing 100 U/mL heparin, followed by 0.25% silver nitrate for 20 seconds, followed then by 5% dextrose. The iliac arteries were then perfusion-fixed at 100 mm Hg for 2 hours with 10% buffered formalin. Half of the arteries were used for morphometric studies, and the rest were submitted for electron microscopy.

Morphometric and Histological Analysis
The stented portion of the vessel was placed intact into processing vials and dehydrated with a graded series of alcohols. Arteries containing stents were then infiltrated and embedded in methylmethacrylate plastic. Vials were sealed airtight and placed in a 38°C waterbath for polymerization. After polymerization, 1- to 2-mm segments from the proximal, middle, and distal portions of the methylacrylate blocks were cut with a diamond-edge rotating precision saw. Plastic sections (4 to 5 μm thick) were then cut, adhered to glass slides, and allowed to dry. The plastic was then removed, and the sections were rehydrated and stained with hematoxylin-eosin and Movat pentachrome stains.

All histological sections were magnified and digitized with the observer blinded to the treatment group. The neointimal thickness at and between each stent wire site was measured by computerized morphometry, and the mean neointimal thickness for each arterial segment was calculated. Computerized planimetry was performed to

<table>
<thead>
<tr>
<th>Follow-Up/Dose</th>
<th>Stent Area, mm²</th>
<th>Intimal Thickness Between Struts, μm</th>
<th>Intimal Thickness at Struts, μm</th>
<th>Fibrin Score</th>
<th>Inflammation Score</th>
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<tbody>
<tr>
<td>1 Month</td>
<td></td>
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<tr>
<td>0 Gy</td>
<td>7.72±0.65</td>
<td>0.86±0.21</td>
<td>6.81±0.45</td>
<td>11.2±1.9</td>
<td>33±10</td>
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<tr>
<td>15 Gy</td>
<td>7.65±0.58</td>
<td>0.80±0.42</td>
<td>6.76±0.44</td>
<td>10.3±4.6</td>
<td>26±9</td>
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<tr>
<td>30 Gy</td>
<td>7.33±0.74</td>
<td>0.57±0.27</td>
<td>6.76±0.38</td>
<td>7.6±3.1</td>
<td>19±2</td>
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<tr>
<td>3 Months</td>
<td></td>
<td></td>
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<tr>
<td>0 Gy</td>
<td>6.84±0.34</td>
<td>1.01±0.11</td>
<td>5.83±0.36</td>
<td>14.8±1.3</td>
<td>38±4</td>
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<tr>
<td>15 Gy</td>
<td>6.83±0.56</td>
<td>0.66±0.07†</td>
<td>6.17±0.48</td>
<td>9.6±1.6†</td>
<td>23±9†</td>
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<tr>
<td>30 Gy</td>
<td>7.32±0.38</td>
<td>0.66±0.04†</td>
<td>6.66±0.44*</td>
<td>9.1±1.4†</td>
<td>17±6†</td>
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<tr>
<td>6 Months</td>
<td></td>
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<tr>
<td>0 Gy</td>
<td>7.71±0.66</td>
<td>1.28±0.26</td>
<td>6.32±0.29</td>
<td>16.9±3.5</td>
<td>37±23</td>
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<tr>
<td>15 Gy</td>
<td>7.79±0.36</td>
<td>1.35±0.37</td>
<td>6.37±0.28</td>
<td>17.6±4.8</td>
<td>63±31</td>
</tr>
<tr>
<td>30 Gy</td>
<td>8.12±0.22</td>
<td>0.75±0.09†</td>
<td>7.33±0.14*</td>
<td>9.3±1.4†</td>
<td>9±1†</td>
</tr>
</tbody>
</table>

*P<0.05 and †P<0.01 vs 0 Gy at the same time point.
determine the area of the internal elastic lamina (IEL), external elastic lamina, and lumen (IPLab Spectrum Software). Percent luminal stenosis was calculated as \[\frac{1 - (\text{lumen}/\text{IEL})}{100}\]. Inflammation around each strut was assessed, and the following scale for inflammatory cells adjacent to struts was used: 0, no inflammatory cells per strut; 1+, 1 to 10 inflammatory cells per strut; 2+, 11 to 20 inflammatory cells per strut; and 3+, >20 inflammatory cells per strut. The intimal fibrin content was graded as 0, no fibrin deposition; 1+, focal residual fibrin involving <25% of the circumference of the artery; 2+, moderate fibrin deposition involving ~25% of the circumference of the artery; or 3+, heavy deposition of fibrin involving >25% of the circumference of the artery.

**Ultrastructural Analysis**

To confirm the extent of endothelialization and thrombosis, the remaining half of the specimen was processed for scanning electron microscopy. The cut specimens longitudinally were rinsed in 3 changes of sodium phosphate, fixed in 1% osmium tetroxide, and rinsed in distilled water. The specimens were dehydrated in a graded series of alcohol, critical-point dried, placed in a vacuum coater, and coated with 30 to 40 nm gold. Both halves of the entire luminal surface were photographed at high and low power. Photographs were digitized, and the percentage of the luminal surface that was endothelialized was measured. Endothelialized surface was calculated as (endothelialized surface within stented segment/total stented segment surface)\(\times 100\). The relative growth rate between intima and endothelium was assessed by intimal area/endothelialized surface ratio (mm\(^2\)).

**Statistical Analysis**

Results are expressed as mean\(\pm\)SEM. Comparisons of continuous variables between groups were performed by ANOVA with Bonferroni’s multiple comparison test for post hoc analysis. Significance was established by a value of \(P<0.05\).

**Results**

All animals were alive at scheduled follow-up, and 72 arteries were available for analysis. All arteries were patent angiographically.

**Histomorphometric Analysis**

Thirty-six stented sites were examined by histomorphometry. None of the arteries analyzed in this study had incomplete stent apposition, excess of intimal thickening at the stent edges, or intraluminal thrombus. Intimal area was reduced at 3 months with 15 and 30 Gy (0.66\(\pm\)0.07 and 0.66\(\pm\)0.04 mm\(^2\), respectively, versus 1.01\(\pm\)0.11 mm\(^2\) in controls, \(P<0.05\)).
However, at 6 months, only a dose of 30 Gy demonstrated reduction of intimal area ($0.75 \pm 0.09$ versus $1.35 \pm 0.37$ mm$^2$ with 15 Gy and $1.28 \pm 0.26$ mm$^2$ in controls, $P<0.01$) (Table).

Fibrin deposition and inflammation around stent struts were observed more frequently in irradiated arteries than in controls at all time points. Control arteries presented mild degrees of fibrin deposition at 1 month, although irradiated arteries showed persistent inflammation and fibrin deposition in both doses up to 6 months after VBT.

**Ultrastructural Analysis of Endothelialization**

Thirty-six stents were analyzed for scanning electron microscopy. One month after stent implantation, control arteries were fully endothelialized; the luminal surface of the vessel wall and the stent struts were covered by confluent endothelial cells (Figure 1). In control arteries, endothelial cells covered $92 \pm 4\%$ of the surface at 1 month. Similar results were observed at 3 months and 6 months ($95 \pm 2\%$ and $98 \pm 2\%$, respectively, $P=NS$).

In contrast, 1 month after VBT, arteries irradiated with 15 or 30 Gy were incompletely endothelialized. Arteries demonstrated large nonendothelialized or partially endothelialized areas with exposed stent wires and nonconfluent endothelial cells (Figure 2, A–C). Irradiated arteries presented less endothelialized surface than control arteries: endothelialized surface at 1 month was $37 \pm 4\%$ with 15 Gy and $37 \pm 8\%$ with 30 Gy ($P<0.001$ versus control).

Similarly, irradiated arteries were not fully endothelialized at 3 and 6 months. Large areas not covered by endothelial cells were observed in arteries irradiated with 15 and 30 Gy. Endothelialization occurred predominantly at the edges of the stents, where endothelial cells lined the stent struts, whereas the middle of the stents showed extensive denuded struts (Figure 3). At 3 months, endothelialized surface was $32 \pm 12\%$ and $29 \pm 13\%$ in arteries irradiated with 15 and 30 Gy, respectively, and $40 \pm 8\%$ and $35 \pm 12\%$ at 6 months ($P<0.001$ versus control at the same time points) (Figure 4). Endothelialized surface was similar between 15 and 30 Gy at all time points and did not show any increase from 1 month to 6 months.

At 1, 3, and 6 months, the endoluminal surface of nonirradiated arteries had few or no cells of hematopoietic origin attached to it. In irradiated arteries, isolated platelets lined the luminal surface not covered by endothelial cells (Figure 5A). Maximal cell adhesion was localized on bared metallic struts and included red blood cells, platelets, and macrophages (Figure 5B).

Intimal proliferation and endothelial healing paces differed between irradiated and control arteries. The intimal proliferation was proportionally more pronounced than the endothelial heal-
ing in irradiated arteries than in controls at 1, 3, and 6 months (Figure 4). At 6 months, intimal area/endothelialized surface was 2.13 mm² in 30 Gy, 3.32 mm² in 15 Gy, and 1.02 mm² in controls (15 Gy versus control, \(P = 0.02\); 30 Gy versus control, \(P = 0.09\)).

Discussion

This study demonstrates incomplete reendothelialization and vessel healing 6 months after stent implantation with adjunct VBT. Lack of endothelial healing was seen with both doses of 15 and 30 Gy despite regrowth of neointima and loss of effectiveness of radiation with the 15-Gy dose at 6 months.

Animal and clinical studies have demonstrated the efficacy of intravascular radiation to inhibit neointimal hyperplasia after stent placement. However, late total occlusion has been observed as a major complication of VBT. Thus, along with delaying neointimal hyperplasia, VBT also delays endothelial regrowth and function. These factors may contribute to the cause of late thrombosis. Prolonged antiplatelet therapy up to 6 or 12 months had shown benefit in reducing late total occlusions, but the optimal duration of this treatment is not yet well defined.

Using a stent implantation injury model, Rogers et al demonstrated that despite a 60% loss in the endothelial cell monolayer within 1 hour after direct stent implantation, there is complete regeneration of the damaged endothelial cell layer within 14 days. In the present study, the nonirradiated arteries were almost completely covered by endothelial cells (with few or no platelets adhered to the luminal surface) by 1 month after stent injury. On the contrary, in irradiated arteries, only 35% to 40% of the luminal surface was covered by endothelial cells at 1 month, indicating that reendothelialization is delayed by VBT. The presence of endothelial cells on the luminal surface of stent struts of irradiated arteries indicates that the recovery process of the endothelium starts as early as 1 month after VBT. However, at 6 months after VBT, large areas of stent struts still remained uncovered, and endothelial cells lined only 40% of the luminal surface. Early reports showed that VBT inhibited cell proliferation in the vessel wall and reduced intimal proliferation but did not assess its effect on endothelium. Later, lack of endothelium was observed in radioactive 32P stents at 6 and 12 months after implantation. However, because radioactivity of 32P stents is sustained weeks after stent implantation with a very low dose rate, one cannot extrapolate about the course of reendotheliali-
eralization for catheter-based irradiation with a single administration of radiation with a much higher dose rate and lower cumulative dose to the target site. Salamé et al. showed that platelet adhesion is sustained up to 30 days after VBT. Our results demonstrate that the thrombogenic risk after VBT is maintained beyond 6 months and may continue as long as endothelial cells do not cover the luminal vessel surface. Even though we observed the signs of endothelial healing as soon as 1 month after VBT, the endothelialized surface of the lumen did not increase significantly from 1 to 6 months, indicating that a significantly long time would be required for endothelial cells to completely cover the vessel lumen. However, this experiment was not able to determine the duration for complete reendothelialization or even whether complete reendothelialization occurs.

For the most part, bare stent struts exposed to the vessel lumen appeared to be the most frequently observed site of large platelet adhesion. This phenomenon was less frequently observed on the nonendothelialized vessel wall or on the struts covered by smooth muscle cells and may explain the increased risk for late thrombosis associated primarily with new stent implantation at the time of VBT in clinical practice.10

In our study, we observed that radiation inhibited neointima at 3 months with both 15 and 30 Gy, but at 6 months, only arteries irradiated with 30 Gy continued to show reduction of neointima compared with controls. These findings corroborate previous animal studies in which the antiproliferative effect of irradiation was no longer observed at 6 months with 15 Gy,19,20 Thus, higher doses may be necessary to prolong the effectiveness of irradiation over time. Interestingly, the present study did not find differences in the absolute and the rate of reendothelialization of the examined doses to support a longer duration of antiplatelet therapy for 30 Gy over 15 Gy. Because endothelial cells are more sensitive than vascular smooth muscle cells,21 they may require a longer time to recover from radiation injury. This difference in radiosensitivity may explain the lack of reendothelialization in the presence of exuberant neointima formation, as seen with 15 Gy at 6 months.

Limitations
This study was performed in the animal model of juvenile rabbits and might not fully represent the mechanism of reendothelialization in human atherosclerotic arteries treated with radiation. Because of possible different rates of healing among species, a longer reendothelialization process might be observed in humans compared with animal models.22 This experiment was performed in stented arteries, and this model might be relevant only for patients receiving a stent at the time of the irradiation procedure.

The study was limited to 6 months of follow-up. Thus, it is limited in identifying a time point for complete reendothelialization and for determination of the optimal antiplatelet therapy after VBT. However, the endothelial regrowth between 3 and 6 months suggests that this process may take years and be beyond the scope of a feasible animal study.

Conclusions
Complete reendothelialization is not achieved at 6 months after VBT. Therefore, antiplatelet therapy should be prolonged beyond this time point. Special care and consideration on the duration of antiplatelet therapy should be given to patients who are undergoing stenting and adjunct vascular brachytherapy.

References
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_Circulation_. 2003;107:2153-2158; originally published online April 14, 2003;
doi: 10.1161/01.CIR.0000062648.39025.09
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/107/16/2153