Intracoronary $\beta$-Radiation Exacerbates Long-Term Neointima Formation in Balloon-Injured Pig Coronary Arteries

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Background—Long-term biological effects of ionizing radiation on coronary arteries remain poorly defined. We examined late arterial responses 6 months after balloon angioplasty and $\beta$-radiation in normal pig coronary arteries.

Methods and Results—Coronary arteries of 25 adult pigs were randomized to receive 20 Gy (n = 8) or 30 Gy (n = 9) of $^{186}$Re $\beta$-radiation or sham radiation (n = 8) immediately after balloon angioplasty. Aspirin was given daily during follow-up. The study vessels were analyzed histopathologically at 6 months. $\beta$-Radiation decreased lumen area (20 Gy, $1.55\pm0.99$ mm$^2$; 30 Gy, $1.03\pm0.82$ mm$^2$; and 0 Gy, $2.05\pm0.80$ mm$^2$; $P<0.05$) but not overall vessel area. The neointimal area was significantly larger within the injured segment with $\beta$-radiation (20 Gy, $1.92\pm1.23$ mm$^2$; 30 Gy, $1.51\pm0.97$ mm$^2$; and 0 Gy, $0.89\pm0.31$ mm$^2$; 0 Gy versus 20 Gy, $P<0.05$), and a significant increase of edge stenosis was observed with $\beta$-radiation. Irradiated vessels also had larger thrombus areas within the neointima (30 Gy, $0.24\pm0.61$ mm$^2$; 20 Gy, $0.98\pm1.57$ mm$^2$; and 0 Gy, $0.00\pm0.01$ mm$^2$; $P<0.05$) and larger adventitial areas (20 Gy, $2.25\pm0.75$ mm$^2$; 30 Gy, $2.38\pm0.98$ mm$^2$; and 0 Gy, $1.23\pm0.29$ mm$^2$; 0 Gy versus 20 or 30 Gy, $P<0.05$) that showed substantial collagen accumulation.

Conclusions—Intracoronary $\beta$-radiation did not inhibit neointima formation in balloon-injured normal pig coronary arteries 6 months after the interventional procedure. Unresorbed thrombus contributed to, but was not the sole component of, augmented neointima formation. Irradiated vessels demonstrated more adventitial thickening and fibrosis. These observations may have relevance for long-term clinical outcomes after intracoronary $\beta$-radiation.

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Key Words: angioplasty ■ restenosis ■ radioistopes ■ pathology

Restenosis has risen as the major limitation of percutaneous coronary interventions and can occur in up to 45% of patients. Intracoronary brachytherapy performed by catheter-based technology has proven to be efficacious in inhibiting neointima formation at 1 month in animal models. However, the long-term effects of ionizing irradiation on the vessel wall remain poorly defined.

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Long-term follow-up in animals with radioactive stents has been reported. Carter et al described increased neointima formation and adventitial fibrosis at 6 months using high-activity $^{32}$P radioactive stents in atherosclerotic pig coronary arteries. A larger neointima and delayed neointimal healing 15 weeks after radioactive stent implantation in dog coronary arteries were also reported by Taylor et al. However, a sustained reduction of neointimal proliferation but incomplete vessel wall healing 1 year after the implantation of high-activity $^{32}$P radioactive stents in rabbit iliac arteries was communicated by Farb et al.

Compared with radioactive stents, long-term animal data on catheter-based intracoronary radiation are incomplete. Initial studies with $^{192}$Ir in balloon-injured pig arteries showed sustained inhibition of neointima formation at 6 months. Recently, however, Kaluza et al failed to demonstrate such effect 6 months after balloon angioplasty or stenting and catheter-based $^{32}$P $\beta$-radiation in adult pig coronary arteries. Half of the animals with irradiated vessels died during follow-up due to late thrombotic coronary events, despite chronic aspirin therapy.

Long-term follow-up in early clinical trials with $^{192}$Ir intracoronary radiation in patients with in-stent restenosis suggested a sustained reduction in restenosis that lasted up to 3 years, although a late “catch-up” phenomenon has also
been reported in irradiated patients. Moreover, increased late (>30 days) thrombotic coronary occlusions have also been reported in patients, possibly as a result of radiation-induced delayed re-endothelialization, leading to recommendations for extended antiplatelet therapy.13,14,15

It has become clear that ionizing irradiation delays or even abolishes the normal healing of the vessel wall after injury. Whether this is a beneficial or deleterious effect over the long term is unclear. The present study reports 6-month histopathological findings after balloon-overstretch injury and intracoronary β-radiation using a 186Re liquid-filled balloon system in the normal coronary arteries of adult pigs.

Methods

Study Design
Animal care and handling conformed to National Institute of Health and American Heart Association guidelines and were approved by the Institutional Animal Care and Use Committee of the Atlanta Veterans Administration Medical Center. Twenty-five female and castrated male adult (aged 6 to 9 months; body weight, 33.5±4.8 kg) miniature pigs (Yucatan strain, Lone Star Swine, Seguin, Tex) were assigned by constrained randomization to receive 20 Gy (n=8), 30 Gy (n=9), or sham treatment (0 Gy; n=8) immediately after balloon angioplasty of the left anterior descending and left circumflex coronary arteries. Animals were killed at 6 months, hearts were harvested, and coronary arteries were processed for histopathological evaluation.

Irradiation Procedure
Cardiac catheterization was performed using a femoral artery approach according to standard procedures. All animals received periprocedural aspirin (2 mg/kg) and heparin (200 U/kg). Irradiation was performed immediately after coronary artery balloon overdistention angioplasty. The 186Re-liquid-filled balloon device (Mail winskrodt Inc, St Louis, Mo) consisted of (1) a 25-mm-long radiation treatment balloon (RTB) catheter, (2) a shielded internal manifold subassembly device for loading the balloon catheter, and (3) a clear, colorless solution of sodium [186 Re]-perrhenate, a predominantly high-energy β-emitter.16,17

On the basis of a prior dose-response study, doses selected for testing were 20 and 30 Gy at 0.5 mm radial distance from the RTB catheter surface.16 Treatment times were provided as individual vessel target times on the basis of balloon size used for injury, the assigned dose, and the isotope radioactive decay. Details of the irradiation procedure were described previously.17 The average dwell time was 571±155 s in the 20 Gy group and 647±55 s in the 30 Gy group. Control animals received a sham radiation procedure with a fixed dwell time of 570 s. Fractionation was necessary in every animal. The average number of RTB inflation/deflation cycles was 6 in the control group, 6 in the 20 Gy group, and 7 in the 30 Gy group. Long-term antiplatelet therapy with aspirin (81 mg PO) was given once daily until termination.

Histomorphometric, Histopathological, and Immunohistochemical Analysis
Histomorphometry of vessels with medial fracture on the injured segments and of adjacent, noninjured but irradiated segments proximal and distal to the ruptured zone (edge regions) was performed. Elastin-trichrome–stained sections were measured using computer-assisted morphometry, as previously described.4 The diameters of the arterial lumen, external elastic lamina, outer adventitia, and intima (bordered by the lumen, dissected medial edges, and the exposed external lamina) were traced and, from these, cross-sectional area measurements were obtained. Furthermore, if present, regions of fibrinoid deposits within the neointima were traced independently for determination of neointimal thrombus area and, by subtraction from the neointima area, the fibrocellular neointima area.

Maximal intimal thickness and arc length of the medial fracture were also traced. The adventitial area was calculated by subtracting the external elastic lamina area from the outer adventitia area. The intima area, normalized to the extent of injury, as assessed by the length of the medial fracture (intimal area/fracture area), was calculated; the internal elastic lamina area was calculated from the sum of the lumen area and intimal area. To evaluate the incidence of edge stenosis, the percent area stenosis [1−(lumen area/initial elastic lamina area)×100] was calculated in sections exposed to β-radiation proximal and distal to the medial fracture zone.

Histopathological evaluation of balloon-induced vessel wall injury and the effects of β-radiation on the cellular and extracellular matrix components within the neointima, media, and adventitia were made using sections stained with elastin-trichrome, hematoxylin-eosin, and modified Russell-Movat pentachrome. The Movat pentachrome staining was previously validated for the detection of platelet/fibrin rich thrombi within the neointima.8 To further aid in thrombus identification, immunohistochemical staining using a polyclonal rabbit anti-human von Willebrand factor (vWf) antibody (1:100, DAKO) was performed.18 To evaluate inflammation at 6 months, inflammatory cells were identified with a mouse anti-porcine CD45 antibody (1:20, Serotec). Porcine spleen was used as a positive control. Briefly, paraffin-embedded, formalin-fixed tissue sections were deparaffinized and predigested with protease K (1 mg/mL; Sigma) for 5 minutes. Sections to be stained for CD45 were pretreated with antigen retrieval buffer (DAKO) for 20 minutes at 90°C. Primary antibody was applied at the indicated dilution in 1.0% BSA in PBS and incubated in a humidified chamber for 60 minutes at room temperature. The sections were washed in PBS and then incubated with a biotinylated secondary antibody (Link biotinylated anti-rabbit IgG; DAKO) in PBS for 15 minutes at room temperature. This was followed by washing with PBS and incubation with the avidin-biotin enzyme complex and chromogenic substrate (Vector red), as described by the manufacturer (Vector Laboratories). Serial sections treated with secondary antibodies only or with nonimmune immunoglobulins showed no staining.

Statistical Analysis
Measurements were averaged per vessel, data were grouped by radiation dose, and group means and standard deviations were calculated. To test for the effects of β-radiation, one-way ANOVA was used. When the F-statistic indicated a significant treatment effect, post hoc, pair-wise, between-groups comparisons were made using Tukey’s test. When assumptions of normal distribution or equal variance were violated, ANOVA on ranks was performed with follow-up comparisons using Dunn’s method. A critical value of P<0.05 was considered indicative of a treatment effect or between-groups difference. All statistical analyses were performed with SigmaStat for Windows 2000 software (SPSS Inc). Continuous variables are presented as means±SD.

Results
Twenty-five animals received balloon-overstretch injury of the left anterior descending and left circumflex coronary arteries. Subsequently, 17 were subjected to intracoronary β-radiation (20 Gy, n=8; 30 Gy, n=9) and 8 to the placement and inflation of the delivery balloon with sterile water instead of the radioactive solution. Two animals died during follow-up, one in the 20 Gy group and another one in the 30 Gy group at 2 and 5 months after treatment, respectively. Histopathological examination of the coronary arteries revealed total occlusion by partially organized multilayered thrombus with little fibrocellular neointimal growth. Histomorphometric data were obtained from vessels that exhibited complete medial fracture (40 of 46 vessels; 0 Gy, n=13; 20 Gy, n=13; 30 Gy, n=14). Micrographs of elastin-trichrome–stained sections from the 3 groups are shown in Figure 1.
Vessels from animals that received balloon injury and sham radiation demonstrated eccentric fibrocellular neointima formation associated with the medial defects. Neointimal cells were stellate or spindle shaped, often showing elongation and circumferential long axis orientation in the more luminal aspect, where cell density was also higher. Smooth muscle cells and extracellular matrix of the tunica media, where it was intact, had normal morphology. Adventitial changes were minimal; samples showed moderate fibrosis and adventitial thickening that was mostly localized to the site of medial fracture. The luminal surface displayed a confluent endothelial-like cell layer.

In marked contrast, coronary arteries from animals that received balloon injury and \( \beta \)-radiation consistently showed the following 3 morphological features: (1) thrombus, mostly appearing as partially organized fibrinoid deposits within the neointima but occasionally presenting as small submedial hemorrhages, and often displaying stratification suggestive of repeated thrombotic episodes; (2) paucity or lack of cytoplasmic staining of medial smooth muscle cells, with occasional focal medial thinning and atrophy; and (3) marked enlargement and circumferential fibrosis of the adventitia. These features were virtually absent from balloon-injured, sham-irradiated arteries. Some vessels showed luminally adherent platelets and leukocytes, suggesting an absence of functional endothelium.

Movat pentachrome staining revealed substantial matrix within the neointima, \( \text{vWF} \) was identified by immunohistochemistry. Positive \( \text{vWF} \) immunostaining was observed in the neointima of irradiated vessels (Figures 2e and 2f). Control, nonirradiated arteries showed no staining in the neointima (Figure 2d); only the endothelium of the vasa vasorum and the endothelium and subendothelial space of the arterial lumen were positively stained for \( \text{vWF} \) (Figure 2).

Histopathological evaluation and CD45 staining were performed to evaluate chronic inflammatory processes in the vessel wall 6 months after injury. Although cells with morphological features typical of neutrophils (ie, lobate nuclei and granular cytoplasm), monocyte-macrophages (ie, large, indented nuclei and agranular cytoplasm), and lymphocytes (ie, small, round nuclei and scant cytoplasm) were often observed in the thrombus area within the neointima of irradiated vessels, no CD45-positive cells were found in any area of irradiated or nonirradiated arteries (data not shown).

Histomorphometric findings are presented in the Table. \( \text{\textsuperscript{186}Re} \) \( \beta \)-radiation was associated with a significant decrease in lumen cross-sectional area, such that the lumen size of 30 Gy-treated vessels was significantly smaller than that of control arteries. No effect of \( \beta \)-radiation was found on vessel cross-sectional area. Both 20 Gy- and 30 Gy-treated vessels had a larger adventitial area compared with sham-irradiated arteries. In addition, a significant treatment effect of \( \beta \)-radiation on neointima formation was observed (\( F=4.062, P=0.025 \)); the neointimal area in 20 Gy-treated vessels was significantly larger than that in control vessels. Neointima area normalized to the extent of vessel injury (intima area/...
fracture length) was also increased in irradiated vessels (0 Gy, 0.54±0.25 mm; 20 Gy, 0.84±0.43 mm; and 30 Gy, 0.79±0.19 mm; \( P<0.05 \) for 20 Gy versus 0 Gy). Despite chronic antiplatelet therapy, the thrombus area within the neointima was increased by \( \beta \)-radiation and was significantly greater in 20 Gy-treated arteries than in control vessels.

Histological examination revealed that 10 (77%), 8 (62%), and 10 (71%) vessels from the sham, 20 Gy-, and 30 Gy-treated groups, respectively, showed neointimal growth outside the fracture region but within the expected radiation fall-off zone or similar area of sham treatment. The mean percent area stenosis in these edge regions was significantly greater in 20 Gy- and 30 Gy-treated arteries compared with sham-irradiated control vessels (0 Gy, 12±8.0%; 20 Gy, 30±14%; 30 Gy, 22±21%; \( P<0.05 \) versus 0 Gy; Figure 3).

**Discussion**

Incidental radiation of the heart has been shown to accelerate coronary artery disease years after the initial exposure. Catheter-based intracoronary radiation is being considered as a potential therapy to reduce neointimal hyperplasia after balloon angioplasty. However, the effects of radiation on arterial remodeling and the potential for further stenosis in adjacent areas need to be carefully evaluated. The data presented here, which demonstrate increased neointimal growth and edge stenosis, suggest that careful consideration of radiation dosage and timing is necessary to minimize the risk of adverse outcomes after catheter-based radiation therapy.
the treatment of choice for in-stent restenosis. Substantial concerns, therefore, have arisen regarding the long-term effects and safety of this procedure. Potential late adverse effects include arterial thrombosis with associated myocardial infarction and/or sudden death. In the study of Kaluza et al., "rebound" restenosis with a return of angina pectoris, radiation-induced arteriosclerosis, and coronary aneurysm formation.

In the present study, we examined the late arterial responses after balloon angioplasty and catheter-based beta-radiation in the normal coronary arteries of adult miniature pigs. A high-energy beta-emitter, $^{186}$Re, was used at 2 different doses (20 Gy and 30 Gy at 0.5 mm from the RTB catheter surface). Both 20 Gy and 30 Gy of $^{186}$Re beta-radiation resulted in marked adverse histopathological effects at 6 months, including decreased lumen cross-sectional area, exacerbated neointima area, thrombus accumulation in the neointima, focal medial atrophy, and adventitial/periarterial fibrosis. Irradiated arteries showed a marked positive immunohistochemical vWF staining within the neointima, whereas control vessels showed staining restricted to the endothelium and subendothelial space of the coronary artery luminal surface and to the endothelial cells of the vasa vasorum. These results suggest that thrombus accumulation and persistence were consequences of irradiation. Moreover, the morphological appearance of the neointima in many irradiated vessels, with stratification of fibrinoid deposits, suggested episodic mural thrombus deposition, as described in the study by Kaluza and coworkers.

Immunohistochemical staining with CD45, a pan-leukocyte marker, revealed no positive cells in any arterial layer, despite the fact that cells showing the typical morphology of inflammatory cells (neutrophils, monocyte-macrophages, and lymphocytes) were observed in irradiated vessels. These results suggest that the infiltrating leukocytes may have lost the surface epitope CD45 after infiltrating the tissue space. Further time-course studies with a range of leukocyte markers are needed to address this conundrum and to characterize and assess the extent and role of chronic inflammation in irradiated coronary arteries.

Our 6-month findings are quite distinct from short-term, 1-month animal studies with intracoronary radiation. Short-term observations in juvenile pigs have consistently demonstrated an inhibition of neointima formation with appropriate doses of gamma- and beta-radiation. We previously documented a significant increase in platelet recruitment and a larger thrombus area at 1 month in injured irradiated vessels compared with nonirradiated injured arteries in adult miniature pigs. The current experiments showed that, unlike nonirradiated arteries in which a complete resolution of thrombus and thin neointima was seen by 6 months, thrombus persisted in irradiated arteries and neointima was augmented.

Differential cell radiosensitivity and cell doubling times between the young pigs (6 to 8 weeks) used in short-term studies and in long-term studies of catheter-based gamma-radiation and the adult Yucatan pigs (6 to 9 months) used in the current protocol and the study of Kaluza et al. may account at least partially for these discrepancies. Brenner and Miller recently demonstrated that a clinically effective gamma-radiation dose prevented adult human aortic smooth muscle cells from dividing, without causing immediate cell death. Such "clonogenic inactivation," they postulated, is the dominant mechanism for the delay of the restenosis process. However, in the long-term, radiation therapy might not prevent restenosis because surviving cell fractions may eventually repopulate the neointima.

Our results are comparable to the long-term data reported on intracoronary beta-radiation by Kaluza et al., who used a centered $^{32}$P source. We have also found similar results with another beta-emitter, $^{90}$Sr/Y, in the same animal preparation at 6 months and 1 year of follow-up. Whether gamma-emitters used for intracoronary radiation could evoke similar long-term arterial responses remains unknown. It is important to note that the long-term studies with $^{192}$Ir were performed only on a small scale and in juvenile pigs. If young animals have an enhanced capacity to re-endothelialize the balloon-damaged arterial surface and resorb mural thrombus with cellular invasion and fibrinolytic activity in the irradiated vessel, our results and those of Kaluza et al. might not be specific to beta-radiation. However, the higher luminal surface-to-deep-tissue dose inherent with the less-penetrating forms of beta-radiation, especially in the present system with the emitter in virtual contact with the luminal surface, might slow re-endothelialization by delivering a higher dose to remnant endothelium at the angioplasty site. This could impair the capacity for endothelial cell mitosis necessary for surface recovery and protection against ongoing mural thrombus formation. Episodic mural thrombus accumulation without substantial fibrinolysis and resorption would be expected to substantially contribute to neointima formation. Further studies are required to determine whether previously reported beneficial results with $^{192}$Ir or with other gamma-emitters can be reproduced in adult pig coronary arteries.

**Study Limitations**

Direct extrapolation of the long-term outcome of endovascular irradiation in the pig coronary artery preparation of restenosis to the human setting is not appropriate. The porcine coronary arteries used in this study were initially normal, whereas human coronary arteries subjected to angioplasty and irradiation have atherosclerotic disease. However, because the vascular effects of ionizing irradiation may occur many years after the initial exposure and healing is still not achieved 6 months after irradiation in pigs, additional and longer-term animal studies with intracoronary radiation are warranted.

**Conclusions**

Intracoronary beta-radiation in balloon-injured adult pig coronary arteries has deleterious long-term effects. Neither an inhibitory effect on neointima formation nor a positive effect on vascular remodeling was found. On the contrary, in this animal preparation, these components of the restenosis process were augmented by beta-radiation. Persistent neointimal thrombus and adventitial fibrosis were present in irradiated arteries. These observations may be relevant to long-term outcomes in human patients.
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