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EXPERIMENTAL STUDIES

External Beam Radiation After Stent Implantation Increases Neointimal Hyperplasia by Augmenting Smooth Muscle Cell Proliferation and Extracellular Matrix Accumulation

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OBJECTIVES We sought to examine the effects of high volume external beam radiation (EBR) after stent implantation on neointimal hyperplasia, smooth muscle cell (SMC) proliferation, presence of inflammatory cells and expression of extracellular matrix (ECM).

BACKGROUND Endovascular irradiation has been shown to reduce restenosis rates after angioplasty in preliminary trials, but conflicting results have been reported for the effects of external beam irradiation.

METHODS Forty-three Palmaz-Schatz stents were implanted into iliac arteries of New Zealand White rabbits. The arteries were externally irradiated after stent implantation with a single dose of 8 Gy (at day 3) or 16 Gy in two fractions (8 Gy at days 3 and 4) by means of a linear accelerator. In the control rabbits, no radiation was applied after stent implantation. Smooth muscle cells, macrophages and ECM were studied by immunohistochemistry at one and 12 weeks after stent implantation. Collagen type I and biglycan messenger ribonucleic acid (mRNA) levels were assessed by Northern blot analysis at one week. Neointimal cell densities and arterial lumen stenosis were measured by histomorphometry at 12 weeks.

RESULTS At 1 week, SMC proliferation at the site of stent implantation was increased after EBR with 8 and 16 Gy (26 ± 5%, 32 ± 3% vs. 17 ± 8%; $p < 0.01$, 16 Gy vs. control). External beam radiation with 8 and 16 Gy augmented SMC proliferation proximal and distal to the angioplasty site (11 ± 3%, 14 ± 3 vs. 6 ± 1%; $p < 0.01$, 16 Gy vs. control). Collagen type I and biglycan mRNA levels were elevated in stented arteries after EBR with 16 Gy. At 12 weeks, a marked decrease in neointimal cell density (248 ± 97 vs. 498 ± 117 SMCs/0.1 mm^2 neointima; $p < 0.005$ vs. control) was noted after EBR with 16 Gy. Irradiation with 8 and 16 Gy increased arterial lumen stenosis compared with nonirradiated control rabbits (45 ± 7%, 55 ± 9% vs. 33 ± 7%; $p < 0.05$, 8 Gy and $p < 0.001$, 16 Gy vs. control).

CONCLUSIONS High volume external beam radiation at doses of 8 or 16 Gy causes restenosis by augmenting proliferative activity at and adjacent to the site of stent implantation, and by dose-dependent up-regulation of extracellular matrix expression. The study suggests that excessive matrix accumulation is an important determinant of failure of radiation therapy to prevent restenosis. (J Am Coll Cardiol 1999;34:561–6) © 1999 by the American College of Cardiology

Restenosis after angioplasty continues to be a clinical problem despite the use of endovascular stents and increasing insight in the pathophysiology of the restenotic process (1–3). Neointimal hyperplasia is regarded as the main factor responsible for lumen renarrowing after stent implantation (4). Radiation therapy has been shown to inhibit neointimal hyperplasia and plaque formation in experimental studies and in preliminary clinical trials (5–14). We and others found that radioactive stents markedly reduce neointimal hyperplasia in rabbit and pig restenosis models (15–17).

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One of the mechanisms by which radioactive stents may inhibit neointimal thickening is the suppression of local smooth muscle cell (SMC) proliferation (15).

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Abbreviations and Acronyms

CIA = common iliac artery
EBR = external beam radiation
ECM = extracellular matrix
EIA = external iliac artery
MAC = macrophage
mRNA = messenger ribonucleic acid
SMC = smooth muscle cell

However, conflicting results were reported on the effects of external beam radiation (EBR) for restenosis prevention. In some studies, balloon angioplasty was performed in rats and rabbits, and EBR was found to reduce neointimal hyperplasia (18–21). Other investigators, however, observed an increase in neointima formation after angioplasty and radiation therapy (22,23). One study documented an increase of neointimal hyperplasia after stent implantation in pig coronary arteries when subsequent EBR was performed (23). Arteries undergoing angioplasty and irradiation may develop alterations in their biologic functions. In fact, only few data exist on radiation effects after angioplasty with respect to changes in extracellular matrix (ECM) formation. The purpose of this study was: 1) to evaluate whether EBR at a single dose of 8 Gy or a dose of 16 Gy delivered in two fractions reduces or increases neointimal thickening after stent implantation in rabbits; and 2) to investigate the mechanisms that may be involved in neointima formation after radiation therapy such as smooth muscle cell proliferation, adhesion of macrophages, and ECM accumulation.

METHODS

Animal model. Animal studies were carried out according to the guidelines of the American Heart Association and were approved by the local ethics committee. Forty-three Palmaz-Schatz stents at a length of 7 mm were implanted into both iliac arteries of 23 New Zealand White rabbits (2.5 kg to 3.2 kg) as described previously (15). Palmaz-Schatz stents of 15-mm length were cut in two pieces at the bridging strut, which was then completely removed. After anesthesia with ketamine (35 mg/kg) and xylazine (5 mg/kg), the femoral arteries were exposed and ligated distally. The protocol included placement of one half-stent into each iliac artery. A 4-F pediatric sheath was inserted into one femoral artery and the rabbits received heparin 500 IU and aspirin 60 mg IV. Angiograms of the iliac arteries were obtained by retrograde injection of contrast agent to determine the vessel diameter. A stent was mounted onto a standard angioplasty catheter (balloon length 20 mm) and implanted into the iliac artery with a balloon inflation for 2 min. The stent was placed either into the common or the external iliac artery, positioned under fluoroscopy control and inflated to a mean balloon expanded stent to artery ratio of 1.2:1. Thereafter, the contralateral iliac artery was stented. The stents were placed into contralateral sites of the common and external iliac arteries to obtain nonstented proximal and distal iliac segments for evaluation of radiation injury. In addition, these two different sites of stent implantation (common vs. external iliac artery) were chosen to evaluate the effects of the radiation in vessels of different size and diameter. In three rabbits, stents could not be placed contralaterally. After the wounds were closed, the rabbits were given aspirin 60 mg IM every third day for four weeks. The rabbits were killed after one and 12 weeks for histologic examination and messenger ribonucleic acid (mRNA) extraction from the iliac arteries.

Radiation protocol. The rabbits underwent anesthesia and were placed in supine position before irradiation. Four square segments were marked on the depilated skin surface of the abdomen after simulator-fixed 4×4 cm^2 irradiation fields including the stents and the adjacent uninjured iliac artery were determined. External beam radiation was performed with a Siemens linear accelerator using 6-MV photons. The dose was specified to a depth of 15 mm, which corresponds to the average distance from the iliac arteries to the skin. A radiation dose of 8 Gy at day 3 or 8 Gy at days 3 and 4 after stent implantation was delivered at a dose rate of 2.5 Gy/min.

Histomorphometry. After the animals were sacrificed with a lethal dose of sodium pentobarbital, the iliac arteries with the stents were harvested, and two thirds of the stented region was cut off of each artery. The segments were immersed in 4% paraformaldehyde. After stepwise dehydration with graded alcohols, specimens were embedded in epoxy-araldit resin. The stented arteries were serially sectioned into six to eight slices (70 μm) with a rotating diamond-coated saw (Leica) with the stent struts remaining in situ. The sections were stained with toluidine blue. The length of the external elastic lamina, the area confined by the internal elastic lamina and the cross-sectional neointimal area were measured by morphometry using a light microscope connected to digital image analyzer (Pavlov), as described previously (15). The vessel injury score was determined according to the method devised by Schwartz et al. (24). The percent area stenosis was calculated by the formula: (Neointimal Area/Stent) ÷ (Area Confined by Internal Elastic Lamina) $\times$ 100. Two independent observers measured the total cell number in the neointima of the arterial cross sections and determined the cell density by computer-assisted cell counting at $\times$400 light magnification in 10 to 15 randomly chosen 0.1-mm^2 neointimal or medial areas. Frequencies of proliferating smooth muscle cells are expressed as percentage of total number of cells.

Immunohistochemistry. After the wires had been removed from the remaining one third of the stented segment, the specimens were cryofixed with liquid nitrogen. Irradiated arterial segments at a longitudinal distance of 1.5 cm from the proximal and distal ends of the stents were

Table 1. Iliac Artery Morphometry 12 Weeks After Stent Implantation and Subsequent External Beam Irradiation

Radiation Dose	n	Injury Score	Neointimal Area (mm^2)	% Lumen Stenosis	# Neointimal Cells	% Proliferating Cells (Neointima)	Neointimal Cell Density (n/0.1 mm^2)
Control	7	1.2 ± 0.2	1.2 ± 0.2	33 ± 7	2,403 ± 390	3.6 ± 0.7	488 ± 117
8 Gy	5	1.4 ± 0.1	1.7 ± 0.2†	45 ± 7*	2,367 ± 360	3.1 ± 1.3	396 ± 77
16 Gy	5	1.3 ± 0.2	1.5 ± 0.1†	55 ± 9§	1,971 ± 696	3.1 ± 0.7	248 ± 97‡

*$p < 0.01$, †$p < 0.05$, ‡$p < 0.001$, §$p < 0.005$ vs. control. Values are mean ± SD.

removed and cryofixed. These specimens were processed to assess radiation effects in direct vicinity to the stenting procedure. Species-appropriate biotinylated secondary antibodies were applied followed by a streptavidin horseradish peroxidase complex (Amersham). Antibody binding was visualized with 3,3-diaminobenzidine yielding a brown color. Counterstaining was performed with Gill's hematoxylin. To detect SMCs, mouse immunoglobulin G2a monoclonal antibody to rabbit SMC-actin (Boehringer Mannheim) was applied at a 1:800 dilution. Proliferating SMCs were detected by double-staining of the sections showing alpha-actin–positive cells with a monoclonal mouse antibody (clon PC10, Dako) against proliferating cell nuclear antigen (1:100 dilution). Macrophages were visualized using the RAM-11 antibodies at a 1:100 dilution (mouse monoclonal, Dako), and collagen type I was detected with monoclonal mouse antibodies (Calbiochem, 1:100 dilution). Uninjured arteries, lung with alveolar macrophages and ileum were used as positive controls. Omission of the first antibody abolished the immunoreaction completely and was used as a negative control.

Northern blot analysis. One week after stent implantation, the arterial tissue of nonirradiated stented segments (n = 4), and stented segments irradiated with 8 Gy (n = 4) and 16 Gy (n = 4) were cryofixed with liquid nitrogen and then homogenized. Total RNA was isolated using spin columns (RNeasy, Qiagen), and RNA concentration was measured spectrophotometrically. Agarose/formaldehyde gels were loaded with 5 μg RNA per lane, and RNA was resolved by electrophoresis. Ribonucleic acid was transfered to a nylon membrane (Hybond N+, Amersham) and cross-linked by ultraviolet radiation. Membranes were prehybridized in a hybridization oven (Amersham) for 1 h at 65°C in prehybridization solution (0.25 mol/liter Na2HPO4, pH 7.2, 1 mmol/liter ethylenediaminetetraacetic acid, 20% sodium dodecyl sulfate, 0.5% blocking reagent [Boehringer Mannheim]). Membranes were hybridized with digoxigenin-labeled complementary deoxyribonucleic acid probes for human procollagen type 1 (American Type Culture Collection) and biglycan (25,26) (generous gift of Dr. Larry Fisher, Bethesda, Maryland). Hybridization was performed overnight at 65°C and was followed by 3 × 15 min washings in 20 mmol/liter Na_2HPO_4, in 1 mmol/liter ethylenediaminetetraacetic acid and in 1% sodium dodecyl sulfate at 65°C. Membranes were then incubated at room temperature in buffer 1 (0.1 mol/liter maleic acid, 0.15 mol/liter NaCl, pH 7.5) for 5 min, followed by 2 h in buffer 2 (1% blocking reagent in buffer 1). For detection of hybridized complementary deoxyribonucleic acid, an alkaline phosphatase-conjugated antidigoxigenin Fab fragment (Boehringer Mannheim) was applied for 30 min (dilution 1:15,000 in buffer 2), after which membranes were washed 4 × 10 min in buffer 1. This was followed by a 5-min washing in buffer 3 (0.1 mol/liter Tris-HCl, 0.1 mol/liter NaCl, pH 9.5). Finally, membranes were incubated for 5 min with the luminescence reagent CSPD (Boehringer Mannheim), diluted 1:100 in buffer 3. Membranes were then exposed to X-ray film. Following detection, the 28-S and 18-S rRNA bands on the membranes (controls) were visualized by staining with 0.03% methylene blue (in 0.3 mol/liter Na-acetate, pH 5.2).

Statistical analysis. Data are given as mean ± SD. Data were analyzed using the Student *t* test. To compare multiple group means, analysis of variance followed by Fisher's or Scheffé's F test was applied. A p value of <0.05 was considered significant.

RESULTS

Vascular Injury Score

In general, the injury scores were different for stents placed in the common iliac artery compared with stents placed into the external iliac artery (0.9 ± 0.4 vs. 1.2 ± 0.3, $p < 0.005$, respectively). The injury scores related to the treatment groups are summarized as follows. One week after implantation of nonirradiated control stents (n = 5), the injury score was 1.2 ± 0.1. The score was 0.6 ± 0.2 in stented arteries irradiated with 8 Gy (n = 4), and 0.8 ± 0.3 for arteries irradiated with 16 Gy (n = 5, $p < 0.05$ control stents vs. stents irradiated with 8 Gy). Twelve weeks after implantation of nonirradiated control stents (n = 7), the injury score was 1.2 ± 0.2. The score was 1.4 ± 0.1 in stented arteries irradiated with 8 Gy (n = 5), and 1.3 ± 0.2 in arteries irradiated with 16 Gy (n = 5, p = NS vs. control, Table 1), indicating that the radiation dose did not affect the injury score after stenting.

Histologic Analysis of the Arteries at One Week

Arteries with nonirradiated and irradiated stents were endothelialized. In general, a similar rate of macrophages

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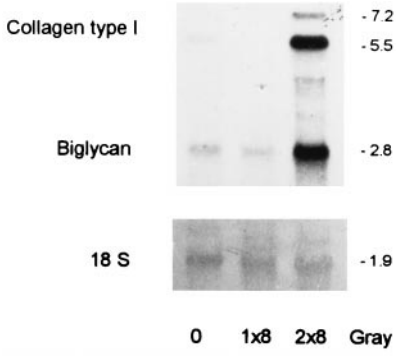

Figure 1. Northern blot demonstrating increased expression of collagen type I (7.2- and 5.5-kDa band) and biglycan (2.8-kDa band) in the stented arteries exposed to external beam radiation at a dose of 2 × 8 Gy (16 Gy) compared with 8 Gy and nonirradiated arteries. The 18-S rRNA band documents equal loading and RNA transfer (control).

adhered to the stented region of irradiated arteries (8 Gy and 16 Gy) and the nonirradiated arteries (42 ± 15, 51 ± 17 vs. 37 ± 5 macrophages/cross section, p = NS vs. control). Mural thrombus formation in the stented region was minimal. Thrombotic stent occlusions were not found. The neointima within the stents consisted predominantly of fibrin. Each of the irradiated but nonstented arterial segments adjacent to the angioplasty site were endothelialized, and no neointimal hyperplasia was found. Detached endothelial cells or mural thrombi as a sign for major radiation damage at the irradiated sites were not detected.

Proliferating SMCs in neointima of stented arteries. The frequency of proliferating SMCs in nonirradiated stented arteries (n = 5, control) was 17 ± 8%, 26 ± 5% in stented arteries irradiated with 8 Gy (n = 4, $p < 0.05$ vs. control) and 32 ± 3% in stented arteries irradiated with 16 Gy (n = 5, $p < 0.01$ vs. control).

Proliferating SMCs in arterial segments adjacent to stent implantation. Smooth muscle cell proliferation markedly increased after EBR proximal and distal to the site of stent implantation. The frequency of proliferating cells in the media of nonirradiated arteries proximal or distal to the site of stent implantation (n = 5) was 6 ± 1%, in arterial segments irradiated with 8 Gy (n = 4) 11 ± 3% ($p < 0.05$ vs. control) and in segments irradiated with 16 Gy (n = 5) 14 ± 3% ($p < 0.01$ vs. control).

Collagen type I and biglycan mRNA levels. Both collagen type I and biglycan mRNA levels markedly increased in arteries with a dose of 2 × 8 Gy EBR at days 3 and 4 (16 Gy) compared with stented arteries irradiated with 8 Gy and the control arteries. This finding shows an augmented expression of extracellular matrix after a dose of 16 Gy (Fig. 1).

Histologic Analysis of the Arteries at 12 Weeks

Neointimal thickening consisted predominantly of alpha-actin–positive SMCs, some macrophages and ECM. The neointimal volume in the stented region after EBR with 8 or 16 Gy was greater than without EBR (Table 1). Diffuse extracellular collagen type I immunostaining was found in the neointima covering nonirradiated stents and those irradiated with 8 Gy. More intense immunostaining was present in the neointima of stented arteries irradiated with 16 Gy.

Lumen stenosis, neointimal cell number and cell density. Seven stents placed in the common iliac artery (CIA) and 10 stents placed in the external iliac artery (EIA) were compared by histomorphometry after 12 weeks. The neointimal area (mm^2) was 1.2 ± 0.2 in the stented CIA and 1.6 ± 0.2 in the stented EIA ($p < 0.0001$ CIA vs. EIA). Thus, neointimal hyperplasia was greater in the stented EIA compared with the stented CIA, independent of the applied radiation doses.

In nonirradiated stents (n = 7), the lumen stenosis in common and external iliac arteries due to neointimal hyperplasia was 33 ± 7%. In the stented arteries irradiated with 8 Gy (n = 5) and 16 Gy (n = 5), the lumen stenosis was significantly greater than in nonirradiated arteries (45 ± 7%, 55 ± 9% vs. 33 ± 7%; $p < 0.001$ after 16 Gy vs. nonirradiated stents, Table 1). The total number of neointimal cells/cross section was not different between the study groups (2,403 ± 390 in the stented region of nonirradiated arteries, 2,367 ± 360 after 8 Gy and 1,971 ± 696 after 16 Gy; p = NS, Table 1). However, the density of the cells in the neointima covering the stented region of nonirradiated arteries was markedly smaller after irradiation with 16 Gy (248 ± 143 SMCs/0.1 mm^2 neointima vs. 488 ± 117 SMCs/0.1 mm^2 neointima; $p < 0.005$ vs. control) (Fig. 2). Irradiation with 8 Gy did not cause a significant reduction in neointimal cell density (396 ± 77 vs. 488 ± 117 SMCs/0.1 mm^2 neointima; p = NS vs. control).

DISCUSSION

External beam radiation markedly increased lumen stenosis by neointima formation compared with nonirradiated arteries at a dose of 8 Gy or 16 Gy delivered at days 3 and 4 after stent implantation. This result contrasts with those of previous reports of beneficial effects of EBR with similar dose ranges in rat and rabbit models of restenosis after angioplasty (6,18,19,21). In addition, our experience with endovascular radiation in the rabbit model clearly shows that radiation therapy can prevent lumen narrowing by neointima formation (15,16). In this study, it was attempted to delineate some of the mechanisms that may be responsible for the observed adverse effect of EBR on neointimal hyperplasia in stented arteries of rabbits.

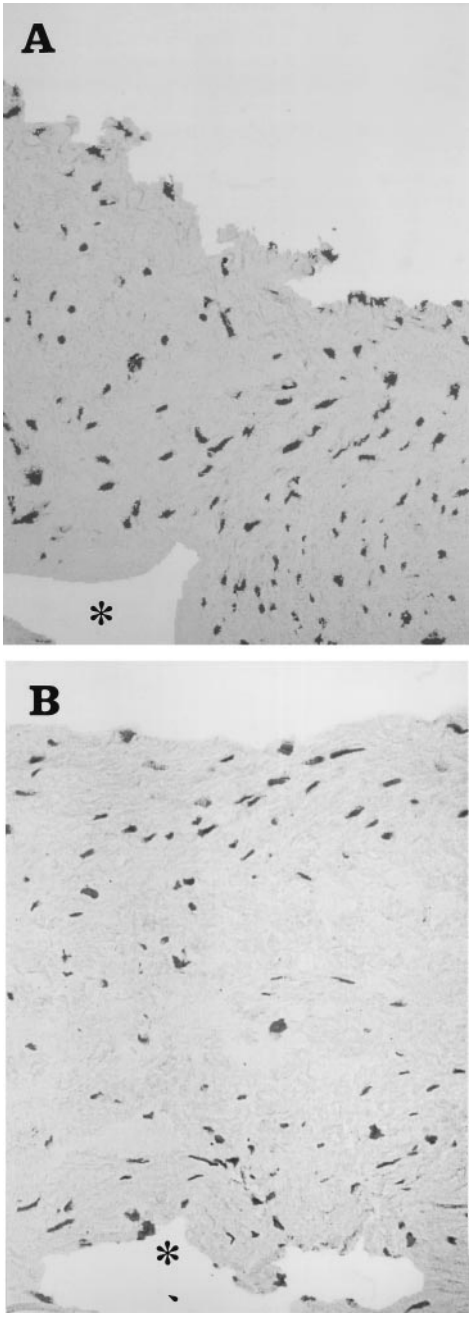

Figure 2. (A) Histologic image showing neointima covering a nonirradiated stent 12 weeks after implantation in the rabbit iliac artery. *Denotes area of removed stent wire. **(B)** Histologic image illustrating extracellular matrix accumulation in the neointima at 12 weeks after stenting and external beam radiation with 16 Gy, indicated by the low density of neointimal cells. *Denotes region of removed stent wire. Magnification ×400.

Changes in Arterial Morphology

Smooth muscle cell proliferation and neointima formation. High volume EBR (4×4 cm^2 fields) including those segments of the iliac artery not subjected to the angioplasty procedure was performed to evaluate the effects of radiation injury. The early wave of SMC proliferation in the "neointima" covering the stented region was enhanced by EBR at one week, and proliferation in the media of the iliac artery was augmented distal to the site of stent implantation. Therefore, high volume external irradiation stimulated SMC proliferation not only at the site of the stent implantation but also in adjacent nonstented segments. Neointima formation was potentiated 12 weeks after EBR in the EIA compared with the CIA, indicating a more deleterious effect of EBR in smaller vessels.

Extracellular matrix accumulation. Two major matrix components of arteries have been evaluated in this study. Collagen type I and biglycan mRNA levels were elevated at one week after EBR with 16 Gy. At 12 weeks, the increase in neointimal volume was associated with a reduction in cell density of arteries irradiated with 16 Gy. The absolute cell numbers in the neointima were not significantly different between the study groups after 12 weeks. Therefore, the increase in neointimal volume in arteries treated with 16 Gy was predominantly due to enhanced ECM accumulation. A marked increase in neointimal ECM was noted only at doses of 16 Gy but not at 8 Gy. Previous studies indicated that extracellular matrix remodeling is essential in neointimal lesion formation after angioplasty (27). An upregulation of collagen type I and III after radiation therapy has been previously found in other tissues (28). This study suggests that accumulation of extracellular matrix after stent deployment is augmented by EBR, and that excessive matrix formation is a determinant of failure of radiation therapy to prevent restenosis.

Related Previous Studies

One might presume that the doses of externally applied radiation should be higher after stent implantation than after balloon angioplasty to effectively reduce neointimal hyperplasia because stents induce more neointimal hyperplasia than balloon angioplasty alone (24). In addition, macrophage infiltration is increased after stent implantation compared with balloon angioplasty alone (29). Therefore, doses of 7 and 14 Gy, which were effective after balloon angioplasty alone in rabbits as reported by Abbas et al. (21), may not be sufficient to prevent restenosis after stent implantation. Wiedermann and coworkers noted that endovascular gamma radiation markedly reduced neointimal hyperplasia only with doses as high as 20 Gy, and reported stimulatory effects of radiation therapy using low doses (8). Schwartz et al. had already found that EBR at doses of 4 and 8 Gy increase neointimal hyperplasia after stent implantation in pig coronary arteries (23). This study was criticized for evaluating arteries subjected to excessive vessel injury. However, the adverse effect of external beam irradiation after stent implantation in the previous study is corroborated by the results at lower degrees of vascular injury from this rabbit study. The radiation dose delivered to the adventitia may be critical with respect to success or failure of radiation therapy to reduce restenosis (11).

High Volume External Beam Radiation—An Option for Restenosis Prevention?

Endovascular irradiation before and after angioplasty or stent implantation potently inhibits SMC proliferation and neointimal hyperplasia in rabbit and pig restenosis models (7,9,10,15). Recent clinical trials with endovascular gamma radiation or beta radiation clearly show that a brief irradiation period before or shortly after angioplasty can substantially reduce restenosis rates in human coronary arteries (13,14,30). The ongoing clinical trials with radioactive stents will show whether an endovascular continuous low level irradiation is also effective in reducing restenosis rates (17). One preliminary clinical trial with a small number of patients reported that external beam radiation at 12.5 Gy, fractionated into 5 × 2.5 Gy, resulted in a reduction of restenosis rates after peripheral angioplasty (31).

This experimental study suggests that doses of 8 and 16 Gy of EBR delivered after stent implantation are too low to reduce restenosis. A stimulatory effect on SMC proliferation after EBR may not be observed with doses greater than 16 Gy. The critical issue with respect to the appropriate dose ranges for restenosis prevention may be delivering the dose that sufficiently induces cell killing. This study indicates that EBR of large "nontarget" tissue volumes actually increases the proliferative activity of cells located adjacent to the site of stent implantation. Different applications of EBR, that is, as used in brachytherapy before or shortly after stent implantation, or given as multiple fractions, should also be studied as a therapeutic option. The hazards of irradiating healthy arteries or neighboring organs clearly should stimulate further research in precise target lesion treatment for restenosis prevention.

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