Cellular Effects of β-Particle Delivery on Vascular Smooth Muscle Cells and Endothelial Cells
A Dose-Response Study
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Background—Although endovascular radiotherapy inhibits neointimal hyperplasia, the exact cellular alterations induced by β irradiation remain to be elucidated.

Methods and Results—We investigated in vitro the ability of 32P-labeled oligonucleotides to alter (1) proliferation of human and porcine vascular smooth muscle cells (VSMCs) and human coronary artery endothelial cells (ECs), (2) cell cycle progression, (3) cell viability and apoptosis, (4) cell migration, and (5) cell phenotype and morphological features. β radiation significantly reduced proliferation of VSMCs (ED 50 1.10 Gy) and ECs (ED 50 2.15 Gy) in a dose-dependent manner. Exposure to β emission interfered with cell cycle progression, with induction of G 0/G 1 arrest in VSMCs, without evidence of cell viability alteration, apoptosis, or ultrastructural changes. This strategy also proved to efficiently inhibit VSMC migration by 80% and induce contractile phenotype appearance, as shown by the predominance of α-actin immunostaining in β-irradiated cells compared with control cells.

Conclusions—32P-labeled oligonucleotide was highly effective in inhibiting proliferation of both VSMCs and ECs in a dose-dependent fashion, with ECs showing a higher resistance to these effects. β irradiation–induced G 0 arrest was not associated with cytotoxicity and apoptosis, thus demonstrating a potent cytostatic effect of β-based therapy. This effect, coupled to that on VSMC migration inhibition and the appearance of a contractile phenotype, reinforced the potential of ionizing radiation to prevent neointima formation after angioplasty. (Circulation. 1999;99:1477-1484.)

Key Words: restenosis ■ radiation ■ angioplasty ■ cells ■ endothelium ■ apoptosis

It is well established that restenosis after angioplasty is characterized by significant neointimal formation and geometric remodeling of the artery. Since the stent-based strategy is effective in preventing vessel shrinkage, excessive neointimal proliferation through the metallic prosthesis continues to reduce lumen size. To prevent neointimal hyperplasia, various therapeutic strategies have been developed on the basis of pharmacological approaches, gene transfer, and more recently on ionizing radiation (β- and γ-emitting sources). Several animal studies with either β particle–emitting stents and wire-mediated endovascular 9 10 and γ emissions 11 13 have shown great promise in the field of restenosis prevention. β-Radioactive stents allow a long-term delivery of β particles within the arterial wall with a low dose rate, whereas endovascular brachytherapy is characterized by high dose rates over a short-term period. Although there is a great interest in developing an effective clinical endovascular radiation strategy to prevent restenosis, several questions pertinent to the use of such strategies remain unanswered. Indeed, the cellular consequences of ionizing radiation leading to the prevention of neointima formation after balloon angioplasty remain to be elucidated.

Accordingly, we developed a strategy combining radiotherapy and gene transfer approaches to effect an in situ delivery of pure β particles in vascular cells in a homogeneous manner. We propose in the present study to investigate in vitro the effect of β emission on (1) proliferation of vascular smooth muscle cells (VSMCs) and endothelial cells (ECs), (2) cell cycle progression, (3) cytotoxicity and apoptosis induction, (4) cell migration, (5) vascular cell phenotype, and morphology features. We demonstrate in vitro the impact of local β irradiation on both proliferation and migration of vascular cells in a dose-dependent manner and describe for the first time the interference of β radiation with VSMC cell cycle progression, phenotype modulation, and absence of cytotoxic effects.

Methods

Cell Culture
All experimental protocols were approved by the Institutional Committee for Animal Protection of the Centre Hospitalier de l’Université de Montréal. Porcine VSMCs were isolated from aortic explants of Yorkshire Swine (Charles Rivers, St Constant, Québec, Canada) as previously described. Human VSMCs were harvested from unused specimens of saphenous veins obtained after bypass surgery, with informed consent of patients. Cell purity was estimated to be >95%, with the use of VSMC α-actin immunostaining (Sigma). Human coronary artery ECs (Clonetics Corp, San Diego, CA).
Calif) were grown in MCDB 131 medium (Sigma) and U937 human cells (monocyte-like leukemic tumor, ATCC, Rockville, Md) in RPMI medium.

**Dosimetry for 32P-Labeled Oligonucleotides**

In vitro–predicted dose was calculated considering both the specific activity of 32P-labeled oligonucleotides and the exposure time according to the dose-point-kernel (DPK) prediction. The DPK estimation, derived from the Monte Carlo method, was based on the homogeneous distribution of radioactive particles in the medium. On the basis of autoradiograms, β-particle delivery was shown to be uniform in the 2-cm2 well, and no radiation was detected in adjacent wells (data not shown).

**Local β-Particle Delivery**

We used 32P-labeled DNA oligonucleotides as a molecular delivery mode of β particles to vascular cells. In the present in vitro report, the sense sequence of c-myc was used to ensure that the observed effects were not sequence specific but mainly the result of β-particle emission. Previous studies conducted to compare sense and antisense sequences reported that the efficacy of both 32P-labeled sequences was statistically similar in inhibiting VSMC proliferation (unpublished data). Sense c-myc phosphorothioate oligonucleotides (5'-ATGCCCTCAACGTG-3') were synthesized on a DNA/RNA automated synthesizer (Applied Biosystems) and purified according to the supplier’s protocol (Glen Research). Oligonucleotides were internally labeled with [32P]ATP (3000 Ci/mmol, Du Pont Canada Inc) by T4 polynucleotide kinase (Gibco-BRL). The stability of the labeled oligomer was assessed by incubating VSMCs with the probe (0.4 nmol/L) for 1 to 7 days. Extracts were loaded on 20% urea-polyacrylamide gel and revealed by autoradiography. To determine the cellular localization of the radioactive source, VSMCs were transfected with oligomer 5'-labeled with fluorescein-isothiocyanate molecules (FITC, 2 μmol/L).

**External γ- and Electron-Particle Deliveries**

To compare the low dose-rate strategy obtained with the use of labeled oligonucleotides to a high dose-rate approach, we exposed VSMCs, ECs, and U937 cells to either 60Co (Cobalt-60) or electron beam sources, leading to γ- (1.6 Gy/min) or electron-particle (4 Gy/min) deliveries, respectively (3, 10, 20, and 30 Gy).

**VSMC Viability**

Cell integrity was evaluated by the trypan blue exclusion test (Gibco-BRL). Increasing β doses were tested (1, 3, 5, 10, 20, and 30 Gy) by incubating VSMCs with labeled oligonucleotides for 72 hours. Cell viability assay was performed in triplicate on 4 cell preparations and expressed as a percentage of total cell counts.

**Cell Proliferation Assay**

VSMCs and ECs were synchronized with serum-deprived medium for 48 hours and then stimulated with FBS in the presence of increasing levels of 32P-labeled oligonucleotides (0.4 to 10 Gy). Nonirradiated cells received equal concentrations of unlabeled oligonucleotides (<20 nmol/L). After [methyl-3H]-thymidine incorporation (6.7 Ci/mmol, NEN Life Science Products), cells were treated as previously described. To evaluate the cell regrowth after removal of the β source from the medium, VSMCs were first incubated with labeled oligonucleotides (5.5 Gy), leading to severe inhibition of proliferation (80%), then the β source was removed from the cell layer for 3 and 5 days. The effects of local irradiation on proliferation was calculated as a percentage of that obtained with nonirradiated cells, on the basis of quadruplicates with 5 to 7 cell preparations. Because similar effects of labeled oligonucleotides on porcine and human VSMCs were seen (Figure 3), we then decided to perform all the following experiments on porcine VSMCs because porcine explants are more readily available.

**Cell Cycle Progression**

VSMCs were synchronized in serum-deprived medium for 48 hours. Labeled oligonucleotides were simultaneously added with 10% FBS. Cells were incubated with β sources (2, 10, and 30 Gy) and harvested 6, 10, 16, 20, 24, 48, 72, 96, and 120 hours after serum activation. Cells were fixed in 70% ethanol and treated with 0.1% sodium citrate, 0.3% NP-40, 0.02 mg/mL RNase, and 0.05 mg/mL propidium iodide. Stained cells were analyzed by flow cytometry with a FACScan model (Becton Dickinson Immunocytometry Systems).

**Internucleosomal DNA Fragmentation**

VSMCs, ECs, and U937 cells were incubated with labeled oligonucleotides for 72 hours (1, 3, 5, 10, 20, and 30 Gy). The 3 cell types were also exposed to external 60Co and electron beam sources (3, 10, 20, and 30 Gy) and processed 24 and 72 hours after irradiation. As a positive control of DNA fragmentation, we treated U937 cells with 1 μmol/L camptothecin, a DNA topoisomerase I inhibitor (Sigma) for 6 hours. Genomic DNA was extracted from treated cells as described.

**Transmission Electron Microscopy**

VSMCs, ECs, and U937 cells were incubated with labeled oligonucleotides for 72 hours (3 and 30 Gy). Cells were fixed in Millonig’s sodium phosphate solution containing 2.5% glutaraldehyde and stored in 0.1 mol/L Millonig’s solution until processing (50 to 70 nm thick). Transmission electron microscopy was performed with a Zeiss Em10 CA microscope (JFE Enterprises).

**Cell Migration and Immunofluorescence**

VSMC and EC migration assays were performed with modified Boyden chambers (Neuroprobe Inc) in which cells were allowed to migrate through a polycarbonate membrane (8-μm pore size, Costar Corp). Immunostaining was performed to investigate effects of β irradiation on cell phenotype by incubating cells with either anti–α-actin or anti–von Willebrand factor antibodies (Sigma) for VSMCs and ECs, respectively. After nuclei staining with 0.01% Hoechst solution (Sigma), slides were observed with an epifluorescence microscope. Low β-irradiation doses (0.4 and 1 Gy) were used to evaluate the setting of migration and phenotype modulation.

**Statistical Analysis**

All values are expressed as mean±SEM. Differences between control and irradiated cells were assessed by ANOVA, with subsequent Student-Newman-Keuls test for multiple comparisons. Statistical significance was established at a value of P<0.05.

**Results**

**Local β-Particle Delivery**

We used a phosphorothioate-modified oligonucleotide because its high stability has been previously demonstrated. The labeled oligomer showed no evidence of radiolysis or degradation within the media even after 7 days (Figure 1), demonstrating the high stability of 32P-labeled oligonucleotides in vitro. As illustrated in Figure 2A, >95% of cells were positive for fluorescent label, showing that the transfection was highly effective and uniform in vitro. FITC oligomers were localized mainly in the nucleus in accordance with previous studies. In agreement with the DPK prediction, no radiation was reported in adjacent wells (Figure 2B); the level and the distribution of β-particle emission were reinforced by autoradiography analysis (data not shown). Together, these results demonstrated that stable labeled oligonucleotides homogeneously emit β particles.
Cell Viability

After β irradiation, viable cell proportion was maintained to an average of 95%±1% (1, 3, and 10 Gy), compared with 96%±0.6% in control VSMCs. Interestingly, molecular β irradiation, even at high doses (20 and 30 Gy), did not alter significantly VSMC viability (94%±0.9% for 30 Gy, P=NS, vs 1 to 10 Gy), suggesting that 32P-labeled oligonucleotides were not lethal for VSMCs.

Vascular Cell Proliferation

The effect of unlabeled oligonucleotides (<20 nmol/L) on cell proliferation was evaluated to be a reduction of <10%, in accordance with previous studies. For the first time, we report a significant inhibition of VSMC proliferation with β irradiation in a dose-dependent manner (0.4 to 10 Gy, Figure 3A), with an ED50 at 1.10±0.07 Gy and 1.08±0.12 Gy for porcine and human, respectively, an ED50 statistically similar in both species. Increasing β dose induced comparable patterns of growth inhibition in ECs, with an ED50 at 2.15±0.10 Gy (Figure 3B). However, ED50 comparison of VSMC and EC demonstrated that the ability of β irradiation to inhibit proliferation was significantly higher in VSMCs compared with that with ECs (P<0.01), except at the highest dose (10 Gy). Removal of 32P-labeled oligonucleotides did not completely abolish the potential inhibitory effect of β irradiation on VSMC proliferation over 3 to 5 days (P<0.05 vs nonirradiated cells, Figure 4).

Cell Cycle Progression in VSMCs

As shown in Figure 5, serum-deprived cells were mainly distributed in the G0/G1 phase of the cell cycle (57%±2%,
panel A) with reduced DNA synthesis activity (32% ± 2%) and low level of cell division (8% ± 1% in the G2/M phase). Between 3 to 20 hours after serum activation, quiescent cells did not progress into the S phase (data not shown). As previously described,27 24 hours after serum addition, we observed that most of VSMCs progressed into the S phase (85% ± 3%, Figure 5B), and to a lesser extent, cells remained in the G0/G1 state. Interestingly, VSMCs remained in the G0/G1 phase after β exposure (2, 10, and 30 Gy), with a concomitant reduction in cell cycle progression into the S phase (Figures 5 and 6). β irradiation did not modify significantly the G2/M phase of the cell cycle (Figure 6). As illustrated in Figure 5, no apoptotic peak corresponding to DNA degradation was noted at low or high β doses. Investigation of long-term effects of β exposure on cell cycle progression showed that cells remained mainly in the G0/G1 state (57% ± 3%) for up to 120 hours.

Comparative Effects of Local and External Irradiation on DNA Fragmentation

Labeled oligonucleotides at low (3 Gy) and elevated dose ranges (10, 20, and 30 Gy) did not induce DNA fragmentation in VSMCs, ECs, and U937 cells (Figure 7A). Exposure to 60Co or electron beam sources did not alter DNA integrity in VSMCs and ECs, whereas this type of delivery induced DNA laddering in U937 cells (Figures 7B and 7C). These data suggest that U937 cells may be more sensible than vascular cells after high dose-rate radiation. Camptothecin also led to severe DNA fragmentation in leukemic cells after 6 hours (Figure 7A, lane 4), as previously reported.21

Morphological Changes and Apoptosis

Electron micrographs showed that after labeled-oligonucleotide irradiation (3 and 30 Gy), VSMCs, ECs, and U937 cells conserved normal ultrastructural features (Figure 8). Presence of apoptotic cells was identified by the following features: chromatin compaction, cell surface blebbing, cytoplasmic shrinkage, and condensation. Morphological studies reported that the frequency of apoptotic cells in control and β-irradiated VSMCs, ECs, and U937 cells was <3%, whereas after camptothecin treatment, 50% to 60% of leukemic cells met the apoptosis criteria.21

Cell Migration and Phenotype

Our results reported similar inhibitory effect in migration of VSMCs (43% ± 0.4% and 20% ± 3.5% of control cell level) and ECs (47.3% ± 2.5% and 13% ± 2.3% of control cell level) for doses of 0.4 and 1 Gy, respectively (P < 0.001 vs control cells). Proliferating VSMCs showed a weak immunostaining of α-actin proteins, corresponding to the synthetic phenotype (Figure 9A). Local β irradiation induced an increase in α-actin fibers that appeared to be dose dependent (Figures 9B and 9C) and occurred in nearly all cells (>95%, panel D), suggesting the appearance of a contractile phenotype in irradiated VSMCs. With ECs, β irradiation (0.4 and 1 Gy) did not modify von Willebrand factor immunostaining (data not shown).

Discussion

The development of endovascular radiotherapy initiated a series of seminal experiments demonstrating the effectiveness of a γ source to prevent restenosis over short-term9,10,12 and long-term observation periods.9,11,28 Verin et al29 and Waksman et al10,30 subsequently demonstrated the effectiveness of a β source in reducing intimal hyperplasia in rabbit and pig models, respectively. Stent-based approaches were also proposed as platforms from which β emission could be delivered to the vessel wall.6–8 In a landmark trial, Teirstein et al14 reported the successful reduction of restenosis rates in injured arteries with iridium-192. Although the concept of radiotherapy to prevent restenosis recently reached the clinical arena,
several questions regarding its mechanism of action on vascular cells remain unanswered. Therefore, we proposed in this study to examine the consequences of \( \beta \) emission radiation on various fundamental functions of vascular cells such as cell viability, proliferation, and migration.

We also dissected the effects of \( \beta \) radiation on cell cycle progression, apoptosis induction, phenotype modulation, and morphological features in VSMCs and ECs.

To deliver \( \beta \) particles in a homogenous fashion, we developed a novel approach based on the local delivery of radiolabeled short DNA single strands. We demonstrate that oligomers internally labeled with phosphorus-32 \((^{32}\text{P})\) provided a stable source of radiation and an uniform dose distribution to target cells. Comparison of the effects of sense and antisense sequences demonstrated that both labeled sequences had similar inhibitory effects, supporting the concept that the oligonucleotides only served as a molecular vector to locally deliver \( \beta \) particles. The \(^{32}\text{P}\)-isotope (half-life 14.3 days) has already been adopted by 2 different platforms in coronary radiation clinical trials (wire based and stent based).

To date, few data are available on the biological effects of radiation on vascular cells. Fischell et al\(^{13}\) observed that \(^{32}\text{P}\)-radioactive wires inhibited the growth and migratory functions of VSMCs by use of a nonuniform dose distribution. A dose-response effect of a cesium-137 source on vascular cells.

**Figure 5.** Effects of \( \beta \) irradiation on cell cycle progression of VSMCs. Representative cell cycle progression (measured on 6500 cells) of quiescent (A), proliferating (B), 2 Gy–irradiated (C), and 30 Gy–irradiated (D) cells are illustrated. Treatment of 10 Gy gave similar results.

**Figure 6.** Statistical effect of \( \beta \) irradiation on cell cycle distribution of VSMCs. Data are represented as mean±SEM. \( ^*P<0.01 \) when proliferating VSMCs were compared with quiescent cells; \( ^\#P<0.01 \) when treated VSMCs were compared with proliferating cells.
VSMC proliferation was reported and showed "reproductive cell death" without, however, any sign of apoptosis. The current study reports for the first time that \(\beta\)-irradiation efficiently inhibits human and porcine VSMCs and human ECs in a dose-dependent manner. At a moderate range of \(\beta\)-particle delivery (0.4 to 6 Gy), ECs appeared to be more radioresistant than VSMCs. No difference, however, could be seen at doses that induced optimal proliferation inhibition. Protocols used in current clinical trials of brachytherapy thus would not be expected to have a different effect on VSMCs and ECs. VSMC growth inhibition was maintained over at least 5 days after radioactive source removal, suggesting either that the effects of \(\beta\)-irradiation are sustained even after source removal or that remaining incorporated intracellular oligonucleotides continued to inhibit proliferation. To understand the mechanisms of the inhibitory effect of radiation, we investigated cell cycle progression in irradiated VSMCs. For the first time, we report that \(\beta\)-irradiation caused cells to remain in the G0/G1 phase and reduced cell numbers in the S phase, as leukemic cells after radiotherapy. More-\nover, this growth arrest was maintained over 5 days. The mechanisms of \(\beta\)-irradiation–induced G1 arrest in VSMCs remain, however, to be defined. Recent recombinant adenoviral strategies reported that adenovirus containing p21, p53, or retinoblastoma efficiently blocked the cell cycle progression and successfully reduced intimal hyperplasia in animals. It may be hypothesized that p53-induced p21 inhibits the cyclin-dependent kinases, leading to the G1 arrest in irradiated VSMCs.

Previous studies on the effects of external radiation using high dose-rates reported damage leading to "reproductive cell kill" or apoptosis induction. However, "reproductive cell kill" is not synonymous to the loss of physical integrity. The current low dose-rate \(\beta\)-emission approach did not induce signs of loss of organelle integrity or DNA fragmentation, as shown by ultrastructural and FACS analyses. To compare this low dose-rate approach with that used in brachytherapy, we exposed cells to external sources of \(\gamma\) or electron particles. High dose-rate irradiation did not induce DNA laddering in VSMCs and ECs, whereas leukemic cells appeared to die by radiation-induced DNA fragmentation. This is in accordance with the low level of apoptosis found in rabbit \(\beta\)-irradiated arteries and the absence of apoptosis reported in porcine irradiated arteries. The low dose-rate delivery effected by labeled oligonucleotides (2 to 10 cGy/h) is more closely related to that used with radioactive stents than that used in brachytherapy (1 to 5 Gy/min). Consequently, such low dose-rate strategies could be defined as cytostatic, preserving cell integrity and viability and acting as a growth suppressor. Also, no apoptosis could be seen in vascular cells in our high dose-rate experiments. Whether "physical cell kill" is a desirable end point in the objective of preventing clinical restenosis remains unanswered.

In addition to inhibiting cell proliferation, \(\beta\) radiation significantly reduced migration of VSMCs and ECs, at much lower \(\beta\) doses (1 Gy) than those affecting the proliferative properties, suggesting that the migratory function may be
more radiosensible than cell growth. γ irradiation inhibited EC migration but failed to alter von Willebrand factor immunostaining, suggesting that reduced migratory function of irradiated ECs may be responsible for potential endothelium-related dysfunction by altering reendothelialization of injury site after balloon angioplasty. VSMC functions are closely related to their phenotype modulation, and in proliferative disorders such as restenosis, contractile VSMCs become dedifferentiated and develop the synthetic phenotype. This immature stage, similar to the in vitro conditions, is correlated with a high migratory capacity of VSMCs. We demonstrate that low γ irradiation induced the transition from the synthetic to the contractile phenotype as shown by the higher α-actin immunostaining in γ-irradiated cells.

In conclusion, we report that labeled oligonucleotides inhibited proliferation of VSMCs and ECs in a cell-specific and dose-specific manner by acting as a growth suppressor, resulting in G₁ arrest, with no morphological changes and apoptosis induction. γ radiation succeeded in inhibiting cell migration and enhanced the contractile phenotype of VSMCs. By acting on vascular cell function, the γ-radioactive strategy may contribute to prevent intimal hyperplasia after angioplasty.

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References


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