Reoxygenation of Hypoxic Coronary Smooth Muscle Cells Amplifies Growth-Retarding Effects of Ionizing Irradiation

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Background—Hypoxic human coronary smooth muscle cells (HCSMCs) are possible targets for brachytherapy to prevent restenosis after percutaneous transluminal coronary angiography. It is unclear whether growth kinetics and gene expression of these cells undergoing γ-irradiation are changed by reoxygenation.

Methods and Results—Hypoxic (H) and hypoxia-reoxygenated (H-R) HCSMCs were irradiated with γ-radiation at single doses of 4, 8, and 16 Gy using a 60Co-source. Vascular endothelial growth factor gene expression of HCSMCs was dramatically suppressed in H-R versus H cells independent of the radiation dose (15±7% versus 2183±2023%, P<0.01, H-R versus H cells). An oxygen enhancement ratio of 1.8 was calculated after irradiation from the retarded growth of H-R versus hypoxic HCSMCs. Production of reactive oxygen species by HCSMCs after irradiation increased by 15±2% in H-R cells versus 7±1% in H cells (P<0.05).

Conclusions—Reoxygenation of hypoxic HCSMCs markedly amplifies growth-retarding effects of ionizing irradiation. On the basis of these findings, oxygenating radiosensitizers should be analyzed with regard to suitability for coronary brachytherapy to prevent restenosis. (Circulation. 2004;109:1036-1040.)

Key Words: hypoxia ■ muscle, smooth ■ restenosis

Radiotherapy after angioplasty has been shown to be an effective treatment to lower restenosis rates in patients with excessive neointimal hyperplasia after stent placement.1-4 In the Scripps Coronary Radiation to Inhibit Proliferation Post Stenting (SCRIPPS) trials, a wide range of doses between 8 and 30 Gy was prescribed to the media of the coronary arteries.5 However, in-stent neointimal hyperplasia does not seem to be eliminated with the present dosing strategies, and in most previous radiation trials to prevent restenosis, negative results of coronary brachytherapy were attributable to underdosing.6 Conversely, high radiation doses may cause excessive thrombosis and aneurysm formation.7 Thus, the dilemma of applying an optimal radiation dose for coronary brachytherapy presently is not solved.

Medial smooth muscle cells are considered target cells for vascular brachytherapy. However, it has been suggested that most of these targets cells are hypoxic. Several studies indicated that medial cells of coronary arteries exist at least in part in a state of chronic hypoxia. Physiological studies in the late 1970s demonstrated that the avascular media of a human thoracic aorta is chronically hypoxic and that smoking can additionally reduce oxygen tension in the media.8 If a stent is deployed to the arterial wall, hypoxia of medial smooth muscle cells increases and returns to normal after a period of 28 days in the rabbit.9 Hypoxia stimulates proliferation of human vascular smooth muscle cells (VSMCs).10 Moreover, supplemental oxygen may reduce exaggerated proliferative responses in the vasculature.11 We hypothesized that human coronary smooth muscle cells (HCSMCs) in a state of hypoxia irradiated during coronary brachytherapy would react differently than reoxygenated cells. To prove this hypothesis, isolated HCSMCs were subjected to hypoxia and reoxygenation in culture and were then γ-irradiated at different doses.

Methods

Cell Culture and Irradiation Protocol

HCSMCs and low serum growth medium were purchased (PromoCell) and incubated at 37°C in room air and 5% CO2. Cells were counted with a Neubauer counter (Brand) seeded into 12 culture dishes (2×104 cells/cm2, Sarsted) and were subjected to hypoxia in an air-tight chamber continually gassed with 95% N2 and 5% CO2 for 6 hours (0.1% <Po2 <0.2%). Megacolonies were created by depositing 10 μL of cell suspension into the center of each dish. The oxygen content was continuously monitored with an O2 Meter (Oxi320, WTW). Cells were then reoxygenated with room air for 30 minutes. After reoxygenation, smooth muscle cells were irradiated with 4, 8, and 16 Gy γ-radiation using a 90Co-source. Hypoxic (H) and hypoxia-reoxygenated (H-R) HCSMCs were cultured for an additional 48 hours for RNA analysis.

RNA Isolation and cDNA Synthesis

HCSMCs were lysed in Trizol reagent (Invitrogen), and RNA was extracted according to the manufacturer’s protocol. Residual DNA...
was removed by treatment with DNaseI (Clontech). RNA 1 μg was reverse transcribed with cDNA Synthesis Kit for reverse transcription–polymerase chain reaction (RT-PCR) (Roche Diagnostics). cDNA 1 μL was used for PCR amplification.

**Primers and Real-Time PCR**
Transcripts for vascular endothelial growth factor (VEGF) and 28S rRNA were amplified by PCR using specific primers that have been described previously.12 PCR was performed using a LightCycler (Roche Diagnostics) according to the manufacturer’s instructions. All reactions were carried out in a total volume of 20 μL per capillary with 0.5-μmol/L primers and 4 mmol/L MgCl₂. Nucleotides, Taq DNA polymerase, and buffer were included in the Fast Start DNA Master SYBR Green I mix (Roche Diagnostics). The standard amplification consisted of an initial denaturation step (95°C for 10 minutes) followed by 55 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 10 seconds, and extension processes at 72°C for 13 seconds. Detection of the fluorescent product was carried out after an additional 2-second step at 81°C. The raw data were analyzed with the LightCycler software version 5, as described previously.13 Briefly, relative copy numbers for transcripts of all unknown samples were determined according to a calibration curve. To correct differences between samples, data were normalized by dividing the copy numbers of the VEGF transcripts by the copy numbers of the 28S rRNA transcripts.

**Measurement of Intracellular Reactive Oxygen Species Production**
To measure the intracellular production of reactive oxygen species (ROS) after irradiation under H and H-R conditions, HCSMCs were cultured on glass cover slips. A method based on the oxidation of DCFH-DA resulting in the formation of the fluorescent compound DCF described by Zulueta et al14 was used. Before HCSMCs were exposed to H and H-R, cells were preincubated in cell medium supplemented with 10 μmol DCFH-DA (Sigma) for 45 minutes. After exposure, cells were irradiated and fixed in 1% paraformaldehyde and double-stained for ROS and nuclear DNA and were analyzed with a Zeiss Axioscope fluorescent microscope (Zeiss) using a FITC and DAPI fluorescence filter cube, respectively. Digital images were quantified for ROS activity with the KS300 software (Zeiss).

**Smooth Muscle Cell Growth Assessment Protocol**
Growth area of HCSMC megacolonies (1.5×10⁶ cells) was determined during 17±4 consecutive days and was planimetrically traced via a light microscope (EL Mikroskop Leitz Fluovert FS) twice per week. Growth areas were compared between the groups with a microscale image processor (Scion Image Version Beta 4.0.2, Scion Corporation, and Zeiss). The end point of the study was the specific growth delay at 6-fold growth, accounting for different growth rates of individual cell lines, as previously described.15,16 The isoeffect level for comparison was defined as time to end point. Specific growth delay of H versus H-R (normoxic) cells was calculated by the equation (TTEhypoxic − TTEnormoxic)/TTEhypoxic.

The results were plotted as dose-response curves, ie, specific growth delay versus dose shown in Figure 2, A and B. The oxygen enhancement ratio, which displays the dose-modifying effect of oxygen, was obtained by dividing the dose given under hypoxic conditions by the isoeffective dose under reoxygenated conditions.

**Statistical Analysis**
The data are expressed as mean±SEM. An unpaired t test was used to compare independent variables of hypoxia and reoxygenation. ANOVA with Scheffe’s correction was applied to assess dose dependence among the study groups. The statistical analysis was performed with the SAS statistics software package (SAS System for Windows, version 6.12, SAS Institute).

**Results**

**VEGF Gene Expression After Ionizing Irradiation of HCSMCs under H and H-R Conditions**
The PCR products of the VEGF gene were markedly elevated in H cells versus H-R cells. When HCSMCs were irradiated with 4, 8, and 16 Gy, for example, VEGF gene expression rose significantly in a dose-dependent fashion 30 minutes after irradiation. The increased gene expression was sustained at 3 days after irradiation. VEGF gene expression was severely depressed in reoxygenated HCSMCs (2183±2023% versus 15±7%, P<0.01; H-R versus H cells), independent of the radiation dose (Figures 1 through 3).

**Growth of HCSMCs After Ionizing Irradiation Under H and H-R Conditions**
The growth-retarding effect of oxygen supply with room air to hypoxic HCSMCs in culture was observed independent of the applied radiation dose over a period of 17±4 days (Figure 4). The calculated oxygen enhancement ratio was 1.8 (95% CI, 1.3 to 2.4) for H-R versus H cells.
Production of ROS by HCSMCs After Ionizing Irradiation Under H and H-R Conditions

Dose elevations from 4 to 16 Gy applied to hypoxic HCSMCs increased ROS production by 7 ± 1% versus nonirradiated controls. The production of ROS rose significantly to 15 ± 2% in reoxygenated cells (P < 0.05, H-R versus H cells). An augmented ROS production in H-R cells after irradiation was observed independent of the radiation dose. Thus, both ionizing irradiation and reoxygenation of hypoxic cells increased ROS production (Figures 5 and 6).

Discussion

A potential link between cancer and restenosis radiotherapy in terms of radiosensitization may be derived from the fact that coronary smooth muscle cells as the presumed target cells for vascular brachytherapy are at least in part hypoxic. Several studies in the cancer literature have reported a benefit of radiosensitization with oxygen during ionizing radiation of hypoxic tumor cells.

Tannock et al. 17 were among the first to postulate that oxygen diffusion into tumors is impaired and may contribute to a reduced radiosensitivity. Other authors provided mathematical models for oxygen diffusion distance of tumors, 18 and later several large randomized multicenter trials showed positive effects of carbogen breathing (95% oxygen and 5% carbon dioxide) or hyperbaric oxygen therapy in conjunction with radiotherapy in cancer treatment. 19–21

Several studies in cardiovascular research suggest that coronary smooth muscle cells are hypoxic after percutaneous transluminal coronary angiography and stent implantation. 9,11 It has been postulated that especially medial smooth muscle cells as potential target cells for coronary brachytherapy are hypoxic, 8 requiring larger radiation doses to induce growth arrest or cell death. Denekamp and colleagues 22–24 emphasized that hypoxic conditions of target cells for ionizing radiation may markedly alter the radiation results compared with normoxic or reoxygenated cells. Cancer cells, however, have different proliferation characteristics compared with...
human VSMCs. Thus, therapeutic consequences derived from studies of oxygenated tumors may be applicable only in part to vascular brachytherapy. We therefore studied the influence of oxygen delivery (reoxygenation) on hypoxic HCSMCs before radiotherapy in culture.

The experiments show for the first time that reoxygenation of HCSMCs before \( /H9253\)-irradiation markedly changes growth pattern and the expression of growth factor genes. Reoxygenation of hypoxic HCSMCs clearly elicits growth-retarding effects over a broad dose range relatively independent of the applied radiation doses.

Two apparent mechanisms of action have been found to be involved in this effect, the suppression of VEGF activity and the increased production of ROS. Previous work has shown that VEGF gene expression is enhanced after vascular injury and promotes migration of HCSMCs, neointima formation, and arteriogenesis.\(^{25-27}\) Reoxygenation dramatically suppressed early VEGF expression and substantially reduced the number of HCSMCs capable of migration. This finding was relatively independent of the applied radiation dose and indicates that proliferative stimuli as a response to hypoxia may be altogether reduced by means of supplemental oxygen.\(^{10,11}\)

Secondly, ionizing irradiation of cells increases ROS production per se and induces the expression of tumor suppressor protein p53 expression. This leads to apoptosis and cell death.\(^{28,29}\) As shown is our study, reoxygenation before radiotherapy of VSMCs additionally augments ROS production, and thus cell death signaling pathways may be increasingly stimulated.

One may conclude from these data that ongoing clinical trials increasing the radiosensitivity of tumors in cancer patients using hyperoxic xenon to improve radiation results should be carefully reviewed for potential suitability in the field of coronary brachytherapy for prevention of restenosis. However, at this time it cannot be ruled out that radiation toxicities such as aneurysm formation and increased thrombogenicity will not be observed applying supplemental oxygen together with ionizing radiation to prevent restenosis. Additional animal studies will have to exclude this possibility.

Figure 4. Growth curves as percentage of relative growth areas of megacolonies of HCSMCs over time after irradiation with 4, 8, and 16 Gy. Left, Growth of hypoxic HCSMCs. Right, Delayed growth of the reoxygenated HCSMCs after irradiation.

Figure 5. Photographs taken from fluorescent microscopy of HCSMCs in a state of hypoxia (A) and reoxygenation (B). The more intense cytosolic green stain in B indicates the augmented presence of intracellular ROS in reoxygenated cells after irradiation.

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Clinical studies using supplemental oxygen via a facemask before and during coronary brachytherapy are underway to analyze whether the present observations can be transported from bench to bedside.

References


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