Different responses by cultured aortic and venous smooth muscle cells to gamma radiation

SEUNG-JUNG KIM, TAKAHISA MASAKI, ROY ROWLEY, JOHN K. LEYPOLDT, SYED F. MOHAMMAD, and ALFRED K. CHEUNG

Division of Nephrology, Ewha Women’s University, Seoul, Korea; Department of Medicine, University of Utah School of Medicine, Salt Lake City, Utah; Department of Radiology, University of Utah School of Medicine, Salt Lake City, Utah; Veterans Affairs Salt Lake City Health Care System and Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah; Veterans Affairs Salt Lake City Healthcare System and Department of Medicine, University of Utah School of Medicine, Salt Lake City, Utah

Different responses by cultured aortic and venous smooth muscle cells to gamma radiation.

Background. Stenosis of hemodialysis arteriovenous grafts is usually focal and caused by the proliferation of vascular smooth muscle cells (SMCs). External radiation of the graft is a potential strategy to prevent stenosis; however, the relative responsiveness of arterial and venous SMCs to radiation is unknown.

Methods. Human aortic and saphenous vein SMCs were cultured in a medium containing growth factors and serum and treated with 0 to 50 Gy in a γ irradiator. At 2 to 20 days post-irradiation, cell counting, methylthiazoletetrazolium dye reduction, [3H]-thymidine uptake, and bromodeoxyuridine (BrdU) incorporation assays were performed.

Results. All assays showed that 1 to 50 Gy inhibited the proliferation of both aortic and venous SMCs in a dose-dependent manner. Importantly, venous cells were less susceptible to radiation in all assays, compared to aortic cells. At day 10, 1 to 50 Gy of radiation inhibited the increase in the number of aortic cells by 24% to 66% and venous cells by 8% to 25% (P < 0.01) (aortic vs. venous). The differences between aortic and venous cells varied among different assays and were most pronounced in the BrdU assay.

Conclusion. Inasmuch as myointimal hyperplasia occurs at both arterial and venous anastomoses, future strategies using radiation to prevent hemodialysis vascular access stenosis should take these differences into consideration.

Vascular access failure is a common problem in chronic hemodialysis patients, leading to substantial morbidity and medical expenses [1]. Failure of the synthetic arteriovenous graft is often due to stenosis at the venous, and to a lesser extent the arterial, anastomosis as a result of myointimal hyperplasia, which is largely proliferation of vascular smooth muscle cells (SMCs) with matrix deposition [2]. Although balloon angioplasty has become an accepted treatment for graft stenosis, recurrence rates following this procedure are high, reported to be 37% to 62% at 6 months [3–5]. This is partly because balloon angioplasty induces injury to the vessel wall, which leads to cell proliferation and a vicious cycle. Therefore, inhibiting vascular SMC proliferation is a better approach to prevent myointimal hyperplasia and graft stenosis. One potential approach is ionizing radiation.

Ionizing radiation induces DNA damage, which leads to cell death. At sublethal doses, it appears to induce the expression of both early- and late-responding genes associated with growth control [6–8]. Further, radiation may induce apoptosis and inhibition of normal cell cycle progression in various cell types [6, 7, 9]. Proliferating SMCs may be particularly susceptible to the effects of ionizing radiation, such as those found in intimal hyperplastic lesions in hemodialysis vascular access. Several reports have shown that radiation inhibits arterial SMC proliferation in culture and in whole animals [8–12]. Little is known, however, about the effect of radiation on cultured venous SMCs, even though most stenotic lesions are found at the venous anastomosis of synthetic vascular grafts and the literature accumulated in the last few years have demonstrated that arterial SMCs and venous SMCs are phenotypically different from each other [13–16]. Our recent study has further shown that human aortic SMCs are more resistant to antiproliferative drugs than venous SMCs [17]. In the present study, we examined the differences in susceptibility to the inhibitory effects of γ radiation between human aortic and venous SMCs in culture.

Key words: hemodialysis, vascular access, smooth muscle cell, radiation.
METHODS

Materials

Human aortic and saphenous vein SMCs and SMC Growth Medium-2 Bullet Kit were purchased from Clonetics (Walkersville, MD, USA). The medium contained 5 µg/mL insulin, 0.5 ng/mL human recombinant epidermal growth factor (EGF), 2 ng/mL human recombinant fibroblast growth factor (FGF), 50 µg/mL gentamicin, 50 ng/mL amphotericin-B, and 10% fetal bovine serum (FBS). Fluorescein isothiocyanate (FITC)-conjugated anti-human smooth muscle actin (SMA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-human von Willebrand factor was purchased from Serotec (Raleigh, NC, USA). FITC-conjugated antirabbit IgG used as the second antibody for von Willebrand factor detection was purchased from Sigma-Aldrich. Methylthiazol tetrazolium (MTT) was purchased from Calbiochem (La Jolla, CA, USA). The bromodeoxyuridine (BrdU)-labeled DNA colorimetric enzyme-linked immunosorbent assay (ELISA) kit and [3H]-thymidine were purchased from Amersham (Piscataway, NJ, USA).

Cell culture

Cells were cultured at 37°C in a humidified 5% CO2 incubator. SMCs were identified by their typical morphology under light microscopy, positive staining with FITC-conjugated anti-SMA and negative staining with anti-von Willebrand factor. Cells from passages 7 to 10 were used in the experiments, similar to those used in our recent studies on antiproliferative drugs [17].

γ Irradiation

Cells were harvested from a T-75 culture flask using digestion with trypsin. The cells were transferred to a 50 mL culture tube, centrifuged and resuspended in the SMC Growth Medium-2. Suspensions of various concentrations of cells were then irradiated with a single dose of 1, 5, 10, 20, or 50 Gy in a Mark 1-30137 Cs irradiator (JL Shepherd and Associates, San Fernando, CA, USA) delivering 7.3 Gy/min at room temperature. Irradiated cells were seeded onto 96-well culture plates at 1 × 10^5 cells per well and incubated at 37°C in 5% CO2. After the cells became adherent, the medium was changed every 2 to 3 days.

Cell activity assay

Four different assays were used to assess the effect of radiation on the vascular SMCs. At 2, 5, 10, 15, and 20 days post-irradiation, the cells were harvested from the culture plates by digestion with trypsin and the number of cells was determined using a Coulter counter (Beckman Coulter, Inc., Allendale, NJ, USA). In addition, the mitochondrial dehydrogenase activity of the cells was assessed using the MTT dye reduction assay [18, 19]. In brief, the cells were incubated with 25 µL of MTT solution (0.5 mg/mL) for 4 hours at 37°C. The resultant formazan product was solubilized in 100 µL of 0.1 N HCl and isopropyl alcohol. The absorbance was measured using a microtiter plate reader at 540 nm test wave length and at 690 nm wave length as reference. The results are presented as absorbance.

DNA synthesis was also determined as a marker of cell proliferation, using the BrdU incorporation and the [3H]-thymidine uptake assays. The BrdU incorporation assay was performed using the commercial ELISA kit, following the manufacturer's instructions after incubation with BrdU for 4 hours. To measure the uptake of [3H]-thymidine by cells, 1 µCi of [3H]-thymidine was added to each well and the cells were incubated for an additional 24 hours. After washing with Hepes-buffered saline, the cells were harvested by digestion with trypsin and transferred to 5 mL of scintillation fluid. The radioactivity was determined in a liquid scintillation counter. The results are presented as counts per minute (CPM).

Statistical analysis

For each assay, the replicate values of three to nine samples in the experiment on a given day were first averaged. The percent inhibition was then calculated as the percent decrease from the corresponding value with no radiation at the same time point in the same experiment. The mean ± SD of the values from experiments performed on different days was then calculated. Differences among the effect of various doses of radiation and differences between aortic and venous cells were evaluated by analysis of variance (ANOVA) with Bonferroni correction and t test.

RESULTS

Cell counting assay

Cell counts in the control (no radiation) and most of the radiation doses increased with time after radiation and appeared to reach a plateau at 15 days (Fig. 1A and B). The increase in the cell number for both aortic and venous cells, however, was inhibited by radiation in a dose-dependent manner. At 10 days post-irradiation, the number of cells was significantly (P < 0.01) lower at doses ≥5 Gy for aortic cells and ≥10 Gy for venous cells, compared to control. At all doses, the aortic cells were more sensitive to radiation than the venous cells; the percent inhibition was significantly greater (P < 0.01) at 20 Gy and 50 Gy (Fig. 1C).

MTT reduction assay

In the MTT assay, the absorbance (mitochondrial dehydrogenase activity) for both aortic and venous cells
increased with time and reached a plateau or began to decrease at 10 days after irradiation (Fig. 2A and B). The increase in the mitochondrial activity of both cell types was inhibited by radiation in a dose-dependent manner. At 10 days post-irradiation, the activity was significantly ($P < 0.01$) lower at doses $\geq 5$ Gy in aortic cells and $\geq 10$ Gy in venous cells. At 10 Gy and 50 Gy, the percent inhibition in aortic cells was greater than that in venous cells (Fig. 2C), but the differences were only marginally significant ($P = 0.042$ and 0.059, respectively).

**BrdU incorporation assay**

The BrdU assay also showed that the DNA synthesis in both aortic and venous cells was inhibited by radiation in a dose-dependent manner, although the time-dependent pattern of response varied substantially depending on the dose (Fig. 3A and B). With no radiation or low-dose radiation (1 Gy or 5 Gy), the BrdU incorporation curves for aortic cells were multiphasic, but the direction of change always paralleled each other among the three dose groups (no radiation, 1 Gy, and 5 Gy). At higher radiation doses ($\geq 10$ Gy), the level of BrdU incorporation was rather constant over time (Fig. 3A).

In the venous cells, the BrdU incorporation curves were also multiphasic with no or 1 Gy radiation, but there was a general downward trend with time. Again, the direction of change paralleled each other for no radiation and 1 Gy (Fig. 3B). At higher doses of radiation ($\geq 5$ Gy), the time-dependent curves of BrdU incorporation were consistently biphasic.

At 10 days post-irradiation, proliferation was significantly ($P < 0.01$) inhibited at all doses $\geq 1$ Gy for aortic cells and at all doses $\geq 10$ Gy for venous cells. At all doses, the aortic cells were more sensitive to radiation than the venous cells; the percent inhibition was consistently and significantly greater in the aortic cells ($P < 0.01$) (Fig. 3C).

**[$^3$H]-thymidine uptake assay**

The thymidine uptake assay in the aortic cells (Fig. 4A) showed time-dependent patterns of response similar to those in the BrdU incorporation assay. For the venous cells, however, the time-dependent patterns in the thymidine uptake assay were somewhat different than those in the BrdU assay. Despite this apparent difference, the thymidine assay also showed that DNA synthesis in both aortic and venous cells was consistently inhibited by radiation in a dose-dependent manner. Further, at 10 days post-irradiation, the percent inhibition in the aortic cells was significantly greater than that in the venous cells at 10 Gy and 20 Gy ($P < 0.01$) (Fig. 4C).
Kim et al: γ radiation and smooth muscle cells

DISCUSSION

Since balloon angioplasty accelerates the formation of myointimal hyperplasia, the development of strategies to prevent hyperplasia in synthetic arteriovenous grafts is urgently needed. Animal models suggest that both local drug delivery [20] and external radiation [21] are promising strategies for this purpose.

In the present study, we have demonstrated that, while external beam γ radiation was effective in inhibiting the proliferation of both human aortic and venous SMCs in culture, the venous cells were consistently less susceptible to radiation than the aortic cells. Four different assays were performed to assess the effects of radiation: cell count, MTT dye reduction, BrdU-labeled DNA uptake, and [3H]-thymidine incorporation assays. The MTT dye reduction assay measures the cleavage of the tetrazolium salt MTT into formazan by the mitochondrial enzyme succinate dehydrogenase in living cells [18, 19]. Traditionally, cell proliferation has been assessed using the incorporation of thymidine as an indicator of DNA synthesis. The BrdU-labeled DNA colorimetric ELISA [22] is based on the incorporation of the pyrimidine analogue BrdU instead of thymidine into the DNA of proliferating cells, and is simple, fast, and sensitive. In the current study, the thymidine and BrdU assays showed rather similar results. The similar chronologic pattern of response in the aortic cells between the two assays (Figs. 3A and 4A) and the similar chronologic pattern in the venous cells between the two assays (Figs. 3B and 4B) add credence to the results.

The results from the thymidine and BrdU assays were nonetheless different from those in the cell count and MTT reduction assays, suggesting these assays complement each other in the assessment of cellular activities and their responses to radiation. For example, although the venous cells were still incorporating thymidine after an extensive period of time, the rate of incorporation began to decline after day 2 (Fig. 4B). It should be emphasized that this observation does not imply that these cells were dying at this stage; in fact, they continued to synthesize DNA, albeit at a slower rate. This decline in DNA synthesis was nonetheless eventually manifested by no further increase in the number of cells at a later time point (day 15) (Fig. 1B). In contrast, the MTT reduction assay assesses the mitochondrial activity of the cells, regardless of the cell number and whether the cells are actively proliferating or not. The trend of the changes in the MTT assay (Fig. 2A and B) in general paralleled that for the cell counts (Fig. 1A and B), although the MTT reduction began to decline at day 10 (Fig. 2B) while the cell counts were still increasing (Fig. 1B). This latter observation suggests that the mitochondrial activity was less active in the cells at days 10 to 20 compared to the earlier time points. In both cell counting and MTT assays,
Fig. 3. Bromodeoxyuridine (BrdU) assay. (A) Effect of various doses of radiation on the proliferation of aortic smooth muscle cells (SMCs). Presented are mean ± SD for each data point (N = 5 separate experiments). Although the time-dependent profile of BrdU incorporation varied among the groups exposed to different doses of radiation, there was a delay for the cells to reach confluence that is dependent on the dose of radiation. A similar inability of human aortic SMCs to reach confluence after a single dose of 6 Gy radiation has been reported [23].

The control group (no radiation) displayed multiphasic patterns in both thymidine and BrdU assays, indicating that, while the cells continued to grow at all time, the rate of growth changed during the course of the experiments (Figs. 3 and 4). The discrepancies in results between assays that assess thymidine incorporation and mitochondrial activities have been previously reported by other investigators. For example, Buttke, McCubrey, and Owen [19] compared thymidine incorporation assay to a tetrazolium dye reduction assay using an interleukin (IL)-3–dependent FDC-P1 cell line. In that study, thymidine uptake decreased in day 2, while the mitochondrial activity was still increasing.

The most important finding of the present study was the lower susceptibility of venous SMCs to radiation in all assays examined, compared to aortic SMCs. This observation represents a distinct contrast to our earlier studies which showed that cultured human venous SMCs were more, rather than less, susceptible to pharmacologic inhibition than aortic SMCs [17]. The SMCs used in the present study were derived from multiple passages. Therefore, the differences observed between the aortic and venous SMCs could potentially be due to differences in the changes in characteristics that occurred during in vitro culture. An alternative explanation of the differences between aortic and saphenous venous cells may be related to the size and location of the blood vessels from which the SMCs were obtained, instead of differences between the arterial and venous trees. It should be emphasized that we used the same batches of SMCs and similar passages in both the drug studies [17] and the present radiation studies. Yet, the susceptibility of aortic SMCs to radiation relative to venous SMCs was in the opposite direction as their susceptibility to drugs relative to venous SMCs. These observations strongly suggest that human aortic SMCs and venous cells are inherently different from each other. It should be emphasized that our results were obtained in vitro and may be different from the behavior of SMCs in vivo.

The mechanisms by which sublethal doses of radiation inhibit the proliferation of SMCs are not fully understood. Some studies have shown that radiation arrested varied among the groups exposed to different doses of radiation, there was a dose-dependent inhibition of cell proliferation by radiation. (C) Comparison between aortic and venous cells in response to various doses of radiation. Presented are % inhibition compared to control (no radiation) at 10 days post-irradiation. *P < 0.01 vs. the corresponding control; **P < 0.01 vs. venous cells with the same dose of radiation. Note the difference in the scale of the vertical axis between (A) and (B).
cell cycles at the G2/M phase [9, 24], while others showed that the arrest occurred at the transition between the G1/S and G2/M phases [25] or at both the G0/G1 and G2 phases [6]. Recently, Scott et al [12] reported a G0/G1 block in rat aortic and human coronary artery SMCs at 24 hours after radiation, but an S phase arrest by 48 hours and a G2/M phase arrest by 96 hours after radiation. Thus, the point of arrest within the cell cycle appears to depend on the time of examination after irradiation.

In various cell types, radiation can also induce apoptosis and subsequent cell death [6, 26]. It is, however, unlikely that apoptosis plays a significant role in the inhibition of SMC proliferation by radiation [10]. In prior studies of rat aortic [8] and human coronary arterial [12] SMCs, there was no increase in DNA laddering and proteolytic adenosine diphosphate (ADP)-ribose polymerase (PARP) cleavage was not detected in irradiated rat aortic SMCs [10]. Further, terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) biotin nick end labeling (TUNEL) was not increased in the arterial wall of porcine coronary artery after irradiation [27]. Additional studies will be needed to explore the mechanisms by which radiation inhibits the proliferation of vascular SMCs and the mechanisms underlying the different responses between aortic and venous SMCs. Perhaps even more intriguing are the cellular mechanisms that allow one type of SMC to be more responsive to radiation and another type of SMC to be more responsive to pharmacologic agents.

A recent in vivo study is of particular relevance to the present findings. Kelly et al [21] employed a porcine model of synthetic arteriovenous dialysis grafts and showed that external beam radiation applied immediately after placement of the graft reduced the percentage of luminal stenosis by 47% (P = 0.051) in the treated arteries and by 23% (P = 0.039) in the treated veins. These data were interpreted by the authors to represent a greater effectiveness of external radiation in preventing stenosis at the arterial anastomosis than at the venous anastomosis. They also appear to be consistent with the present study showing a greater responsiveness of cultured aortic SMCs to γ radiation compared to venous cells.

CONCLUSION

The present results showed that external beam γ radiation was effective in inhibiting the proliferation and mitochondrial enzymatic activity of both human aortic and venous SMCs in culture; however, the venous cells were less susceptible to radiation than the aortic cells. Therefore, the effective radiation dosages used in vivo likely vary depending on the target location. Future strategies using radiation to prevent stenosis in hemodialysis vascular access and other vessels should take these differences...
into consideration. Further investigations are needed to determine the mechanisms by which various vascular SMCs respond differently to antiproliferative drugs and radiation.

ACKNOWLEDGMENTS

This work was supported by the Medical and Research Services of the Department of Veterans Affairs, National Heart, Lung and Blood Institute (RO1HL67646), Dialysis Research Foundation, and the National Kidney Foundation of Utah. Dr. Donald Blumenthal provided helpful comments in the interpretation of data and preparation of this manuscript.

Reprint requests to Alfred K. Cheung, Dialysis Program, 85 North Medical Drive East, University of Utah, Salt Lake City, UT 84112. E-mail: alfred.cheung@hsc.utah.edu

REFERENCES