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TOLERANCE OF ARTERIES TO MICROPLANAR X-RAY BEAMS

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Running title: Microplanar X-ray beam irradiation of arteries

Conflicts of interest

The authors declare no conflict of interest

Abstract

The **purpose** is to evaluate effects of a new radiotherapy protocol: Microbeam radiation therapy, on the artery wall. In previous studies on animal models, it was shown that capillaries recover well from hectogray doses of X rays delivered in arrays of narrow (≤ 50 µm) beams with a minimum spacing of 200 µm. Here, short and long term effects of comparable microplanar beam configurations on the saphenous artery of the mouse hind leg were analyzed *in situ* using nonlinear optics and compared with histopathologic findings.

Methods and Materials. The left hind leg of normal mice including the saphenous artery was irradiated by an array of 26 microbeams of synchrotron X rays (50 µm-wide, spaced 400 µm on center) with peak entrance doses of 312 Gy and 2000 Gy.

Results. The artery remained patent, but narrow arterial smooth muscle cell layer segments that were in the microplanar beams paths became atrophic and fibrotic in a dose-dependent pattern. The wide tunica media segments between those paths hypertrophied, as observed in situ by 2-photon microscopy and histopathologically.

Conclusions. Clinical risks of long-delayed disruption or occlusion of non-targeted arteries from microbeam radiation therapy (MRT) will prove less than corresponding risks from broad-beam radiosurgery, especially if peak doses are kept below 3 hectogray.

Keywords: Artery smooth muscle cells, Microbeam radiation, Two-photon microscopy

Introduction

The effects of ionizing radiation on blood vessels after broad-beam radiotherapy are dose-and time dependent[1] and may result, in human arteries, in delayed arteriosclerotic plaque formation, stenosis, thrombosis, delayed necrosis and death[2]. Therefore, radiotherapy protocols that decrease the risk of arterial damage would be useful.

Typically, MRT uses arrays of narrow (~25-100 µm -wide) microplanar beams (MPBs) separated by wider (100-400 µm on center) microplanar spaces. The height of these microbeams typically varies from 1 mm–100 mm, depending on the target size. Peak entrance doses of several hectogray are surprisingly well tolerated by normal tissues, particularly capillaries[3-6], up to ~2 yr after irradiation, while a preferential damage of malignant tumor tissues occurs. These events have been extensively studied over nearly two decades in preclinical trials based on different animal models, including mice, rats, piglets and rabbits. More recently, sporadically, some biological *in vivo* effects of synchrotron X-ray beams in the millimeter range (0.68 mm-0.95 mm, spaced 1.2 mm-4 mm on center) have been followed up to ~7 months after irradiation. Comparisons between broad beam irradiation and MRT indicate lesser normal tissue damage[3-8] and a higher tumor control for the same sparing of normal tissue in the latter, even if a substantial fraction of tumor cells are not receiving radiotoxic levels of radiation.

This high tolerance has been tentatively explained by rapid cytoplasmic stretching of adjacent endothelial cells (ECs) in the nominally unirradiated valley regions between the MPBs in order to replace ECs in the MPB paths[9,10]. Therefore, the distances between the MPBs should be large enough (≥200 µm center-to-center (c-t-c)) to avoid high doses of scattered radiation to valley regions, which may disable repair. *In vivo*, c-t-c spaces of at least 200 µm between 25 µm-wide MPBs for peak doses up to 1000 Gy allow maintenance of structural and functional integrity of cerebral capillaries of normal mice for at least 3 months[10]. Serduc et al[11] have shown that 50 µm-wide microbeams (in comparison to widths of 25 or 75 µm) deliver the best compromise between brain tumor control and normal brain toxicity in rats.

The comparatively deleterious effects of radiation doses of up to few hectogray delivered to arteries in the broad beam mode have been described[12-15].

For the present study, we chose the MPB widths and spacings specified for future preclinical trials on animal patients at the ESRF, and two entrance doses, one conservative, the other excessive, to generate extreme damage. *In situ* effects of MPBs on arteries of mice were measured using nonlinear optical imaging tools, i.e., two-photon microscopy (2PM) and Second Harmonic Generation Imaging (SHGI)[10,16,17]. We used 2PM to observe changes in the elastic fiber

configuration and nuclei of vascular smooth muscle cells (VSMCs)[17]. SHGI permits direct analysis of the collagen type I and III fibers during the appearance of wall fibrosis16. All *in situ* data were compared with the results of histological analysis.

Materials and Methods

Animals – Saphenous artery

Experiments were performed in accordance with the French Government guidelines (license A3851610008) on 31 normal, 6 week old Balb/c white mice (Charles River, France) with a mean body weight of 20 ± 3 g. The saphenous artery in their left hindlimb was used as a model. The arterial intima is composed by endothelial cells with no or minimal subendothelial connective tissue. The tunica media (TM) consists of VSMCs[18]. Elastic fibers are present in the internal and external elastic lamina and also between the VSMCs[17].

Microplanar beam irradiation

The central cone of the synchrotron beam (0.5 mm-high, 2 cm-wide) at ID 17 of the European Synchrotron Radiation Facility (ESRF, Grenoble, France) was spatially fractionated into parallel microbeams by using a multislit collimator[19] positioned 80 cm upstream from the mice. The maximum dose rate after the filters was 17000 $Gy.s^{-1}$, the median beam energy 107 KeV. A computerized z-goniometer stage automatically adjusted the translation speed to correct for the decaying storage ring current.

The mice were anaesthetized with an intraperitoneal injection of xylazine / ketamine (0.1% /1 % in saline buffer, 10 µl per g of body weight) and placed in a vertical position on a computer-controlled goniometer with the left hindlimb in the beam (figure 1). The left hind limb, underneath the knee, was irradiated ventro-dorsally by an array with a cross section of 10 mm x 10 mm, composed of 26 vertically oriented, 50 µm-wide MPBs spaced 400 µm on center in the collimator. The in-beam skin-entrance peak doses were 312 Gy and 2000 Gy.

Dose-distribution calculations

The MC code PENELOPE[20] (version 2006) simulates coupled photon and electron/positron transport through arbitrary amorphous media and was used to simulate the radiation dose deposited by a single x-ray MPB in a 12mm-high, 30mm-wide and 15mm-deep leg phantom. The composition of the leg (skeletal muscle) was taken from ICRP report 89[21]. Doses were scored in the leg phantom at all depths between 0.5 and 1.5 mm.

2PM

Animal preparation

One, 3, 6 or 12 month(s) after irradiation, mice were anesthetized with a gas mixture of isoflurane (2%) in 30% O^2 and 70% N_2O; their body core temperature was maintained at 37° C. The skin above the irradiated site of the left hind limb, and of the right hind limb for control measurements, was removed. The exposed saphenous artery was covered with 100 µl solution of sulforhodamine-B (SRB) in 0.9 % saline (10 mg/ml, Lambda Physics). In most experiments, 100 µl of Hoechst 33342 in 0.9 % saline (1 mg/ml, SIGMA) was added sequentially. After 5 minutes, the solutions were removed by rinses of 0.9 % saline (5 ml). The mice were killed by neck dislocation and positioned within 5 minutes in a 37° C saline (0.9 %) bath on the motorized stage of the twophoton microscope[17]. Images were processed and displayed using ImageJ software (v.1.41o, Public Domain Software) (see appendix el).

Data analysis

Most data were acquired as z-stacks in dorso-ventral direction covering half the saphenous artery (depth = 200 µm). The thickness of the TM and TA was measured on cross sections in MPB paths and in the center of adjacent valleys. In MPB paths, the thinnest TM was measured from the center of the internal to the center of the external elastic lamina (two values per 2 or 4 selected sections/mouse).

The means (± standard deviations) of pooled values were calculated. For both tunicae, the ratios of “thickness in the MPB path” over “thickness in midvalley (V)” were calculated (RTM = $tTM_{(MPB)}$ / $tTM_{(V)}$, and RTA = $tTA_{(MPB)}$ / $tTA_{(V)}$) to correct for small variations of the tunica’s thickness along the artery; the ratio is 1 in unirradiated arteries. Non linear fitting and unpaired student t-tests were performed using GraphPad PRISM (GraphPad Software, Inc, version 3). Significance was established at the 95 % confidence interval with a P-value < 0.05.

Histology

The *in situ* data were compared with histopathological findings. The saphenous artery, saphenous vein, nerve and surrounding soft tissues of 16 irradiated and 16 sham-irradiated mice, from knee to ankle, were fixed in neutral buffered 10% formalin and embedded in paraffin. The intervals between irradiation and autopsy were one month (3 mice: 312 Gy; 3 control mice), 3 months (2 mice: 2000 Gy; 3 mice: 312 Gy; 5 control mice) and 6 months (3 mice: 2000 Gy; 5 mice: 312 Gy; 8 control mice). Sections were stained by hematoxylin and eosin (HE) for the detection of nuclei (dark blue) and cytoplasm (pink); Elastica van Gieson for the detection of elastic fibers (dark violet) and collagen fibers (red); Masson Trichrome for the simultaneous detection of collagen (blue), cytoplasm, mainly of VSMC (light red or pink), and cell nuclei (dark brown to black). The pathologist (JL) was blinded to the irradiation data.

Results

Mice in the 312 Gy group appeared normal. No skin reactions, behavioral changes or weight loss were detected. In the 2000 Gy group, a transient edema was observed in the left hind limb between 12 hours and 1 month after irradiation, and deficient use of their left hind paw and toes for ≥ 6 months after irradiation. Muscle atrophy appeared one year after irradiation.

Dose-distribution

Simulations (Table 1, figure 2) indicate that a doubling of the c-t-c spaces from 200 to 400 µm results in 3-fold decrease of the minimum dose in the center of valley, whereas doubling the MPB width nearly doubles that midvalley dose. The configuration with the lowest valley doses (< 10 Gy) for both peak doses is: 25 µm peak width and 400 µm c-t-c spacing. The highest estimated valley dose in our experiment was 17.6 Gy (50 µm peak width, 400 µm c-t-c spacing, 2000 Gy peak dose).

2PM

Elastic fibers and smooth muscle cells in the tunica media

In both groups, the SRB staining of the elastic fibers in the MPB paths was more diffuse than in the valley regions,

in which discrete, thin elastic fibers alternated with dark rings of VSMCs (figure 3B). In the 312 Gy group, the mean c-t-c spacing of MPB paths was 430 ± 21 µm (figures 3A/C), versus 450 ± 15 µm in the 2000 Gy group (figures 4C and 5D). Histopathological results were comparable: 312 Gy; 380 ± 34 µm, 2000 Gy; 310 ± 11 µm. Neither internal nor external elastic lamina displayed disruptions after exposure to both radiation doses (fig. 4 D). In both groups, fewer VSMC nuclei (Hoechst) per surface area were seen in the MPB paths than in the valleys 1 month after irradiation (figures 3B, 4B). Thinning of the TM was significant 1 month after irradiation (table 2). Changes in the mean RTM ($tTM_{(MPB)}$ / $tTM_{(V)}$) are shown in figure 6. The data fitted a one-phase exponential decay (see legend of figure 6). For the decrease in thickness, the half life in the 312 Gy group was slightly longer than that in the 2000 Gy group: 1.6 and 1.2 months, respectively. In the 2000 Gy group, VSMCs in the irradiated microslices of the TM had disappeared 6 months after irradiation (figure 4D). This loss was directly correlated to a significant and delayed increase of the media thickness in the valley regions after 6 months (table 2 and figure 6). At 6 and 12 months after irradiation, VSMCs in the MPB paths had been replaced by connective tissue containing collagen type I and type III fibers (arterial wall fibrosis, see figures 4C and 5D). Similar changes were not observed in mice exposed to 312 Gy, where the decrease of the TM thickness in the MPB path remained stable 6 months after MPB irradiation (table 2).

Histopathology

Longitudinal sections: At 6 months after irradiation, the TM in the MPB paths was thinner in the high-dose group (mean thickness 5 ± 1 µm) than in the 312 Gy group (20 ± 3 µm). These thin segments of the TM were wider in the 2000 Gy group (34 ± 9 µm) than in the 312 Gy group (19 ± 9 µm, $p = 0.02$), and tended further to widen slightly with time. With increasing distance from the beam path, the TM progressively thickened to a maximum value at middistance (midvalley) from the next MPB, slightly exceeding the value in unirradiated arteries. Thus, the TM between two MPB paths bulged into the arterial lumen and towards the adventitial layer. The arteries acquired a “segmented” or “beaded” appearance (figure 5A). In MPB paths, especially in the 2000 Gy group, the muscular TM was replaced by connective tissue, the latter forming circular indentations into the TM. The arterial “segmentation” was arbitrarily graded from 0 (no segmentation) to 4 (obvious segmentation). The segmentation grade for all arteries exposed to 2000 Gy was 4, regardless of the time after irradiation, versus 1.4 ± 0.9 for all those exposed to 312 Gy ($p < 0.05$). Proximally and distally of the irradiated site the arteries were indistinguishable from normal, unirradiated arteries. The internal elastic lamina and the endothelial layer appeared intact in the MPB path. Subendothelial fibrosis, plaques or thrombi were

absent in ~200 µm-wide arteries. Rarely, nuclear debris or pyknotic nuclei were noted in MPB paths. Infiltrates of lymphoid cells were rarely seen around irradiated arteries, and mainly in the 2000 Gy group. Veins and nerves appeared normal.

Discussion

The salient findings of this study are: i) The high tolerance of small arteries to doses up to 2000 Gy, without any consecutive occlusion within 1 year, in contrast to the deleterious consequences of comparable or lower doses delivered by seamless X rays[12-15]. ii) Signs of dose-dependent damage to the wall of small arteries in the microslices of irradiated tissue, particularly to the VSMCs of the normal media. Main events that herald the arterial response to radiation injury can be evaluated rapidly by 2PM and SHGI in situ or in vivo[17], without specific fluorescent dyes. In the 312 Gy group, the TM thinned by about 25 % in the path of the MPBs, but disappeared in the 2000 Gy group, most likely after radiation-induced cell death. The extent of tissue damage was obviously dose dependent. In comparison, Dilmanian et al[22], did not observe, 25 days after irradiation, any damage to carotid arteries of rats exposed to an array of 27 µm-wide MPBs spaced at 200 µm with peak doses of 150 Gy, delivered in a single fraction one hour after angioplasty. We did not detect signs of proliferation and/or migration of VSMCs from the valley into the MPB regions. This might be related to the absence of noticeable radiation damage to elastic fibers and VSMC in the valleys for the 312 and 2000 Gy group, despite delivery of midvalley doses up to 17.6 Gy. Disruption of elastic fibers would trigger the release, migration and proliferation of VSMCs near the arterial lumen. Elastin-VSMC interactions are deemed to be a critical factor for maintenance of vascular homeostasis, avoidance of arterial wall thickening, plaque formation, and thrombosis of the artery at long term after radiation[23, 24]. Histologic signs of ongoing VSMC death such as presence of nuclear debris and pyknotic nuclei in the beam path were rarely seen; this observation may point to necrobiosis of VSMCs of the media in MPB paths. The loss of VSMCs in the media and their replacement by fibrous tissue, particularly after exposure to 2000 Gy, resulted in segmentation of the arteries, which displayed a thicker TM than in the lower dose group. These changes have an impact on the resilience of the artery needed to absorb the hemodynamic stress of the cardiac systole, to propagate the pulse wave and to sustain blood pressure during diastole. The presence of the internal and external elastic lamina may help in maintaining elasticity. The local lack of VSMCs and their replacement by collagen fibers

impedes an active local dilation and constriction. A thickened TM in the valley regions might compensate for blood pressure losses along the artery. The functional deficits and the atrophy of the hind leg in mice exposed to 2000 Gy was probably related to changes in blood pressure and perfusion in feeding arteries. There were no signs of endothelial damage such as apoptosis, presence of recent or old hemorrhage, focal hemosiderosis, recent thrombosis and/or intimal plaques. Further, a transient hind limb edema occurred within one month after exposure to a peak dose of 2000 Gy. These observations are in accordance with the impressive repair capacity of brain capillary endothelia and the occurrence of minimal and transient brain edema after microplanar irradiation with entrance doses up to 1000 Gy[5, 25]. Peak doses of several hundred Gy, multidirectional intersecting microplanar beams of about 50-µm width, and beam spacing distances of ≥200 µm have been used to treat radioresistant brain tumors in rats[3,5,11]; arterial damage was not described. Here, we have shown that arteries can tolerate a 2000 Gy entrance dose if the c-t-c spacing is ≥ 400 µm. Arrays of microbeams applied from different ports on a target form a radiotoxic field in depth and cause minimal normal tissue damage within single arrays before and after the tumor[5,28]. For brain tumors, in the first phase of clinical trials at the ESRF with dog and cat patients, only unidirectional or simple cross-fired MRT will be applied to a depth of ≤7cm[4] , using arrays of 50 µm-wide microplanar beams, c-t-c spacing of 400 µm.

The limited tissue penetration of low energy x-ray photons produced by the synchrotron may be unfavorable when compared with megavoltage photons. However, peak doses in the order of hectogray will produce a sufficiently high differential effect in depth, as valley doses have a considerably lower fall-off in dose with increasing tissue depth than peak doses[25,27]. Evolution of the MRT technique by interlacing microplanar arrays combined with image-guided, confocal MRT permits creation of small lesions, in the millimeter range, that may radiosurgically remedy Parkinson disease or epileptogenic foci[28].

MPB irradiation effects described in our study are minor compared to the typical lesions of small and medium-sized arteries after exposure to seamless ionizing radiation; transmural arterionecrosis may take place within 2 to 3 weeks after stereotactic gamma irradiation (1 to 3 hectogray)[14]. If the irradiated arterial segment is large enough, thrombosis, obliteration, aneurysmatic dilatation and possibly wall rupture may also occur. Ensuing tissue injuries such as fibrosis, atrophy and/or necrosis may result in death, particularly if larger vessels[12-14] are involved and may manifest themselves very late[13]. In humans, a major artery might develop stenosis years after low-dose irradiation (10 Gy) delivered with radiosurgery[15].

Conclusions

Mice hind leg arteries tolerate doses of up to 2000 Gy delivered by spatially fractioned MPBs in a single session without developing occlusions within 1 year, in contrast to the deleterious consequences of comparable doses delivered by seamless X rays. The permanent sequelae of microplanar irradiation appear to be confined to microscopic segments of the TM and TA. However, the 1-year time for observation should be extended, possibly to the lifetime of longer-lived mammals to model arterial radiation damage in humans.

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Titles and legends to figures

Figure 1

Mice were positioned vertically, their abdomen facing the beam; the left hind limb was slightly stretched in a plastic cylinder. The radiochromic film displays the 10mm x 10mm array of 26 microbeams (50 µm-wide, c-t-c spacing 400 µm), skin entrance dose 2000 Gy.

Figure 2

Simulated dose distribution in the mouse leg at a depth of 1 mm from the skin surface for peak doses of 312 Gy (--) and 2000 Gy (-).

Figure 3 A-D

2PM image of the saphenous artery (longitudinal sections) in the left hind limb one month (**Figures 3A/B**) and 3 months (**Figures 3C/D**) after microplanar irradiation (312 Gy). Results at 6 months, similar to those at 3 months, are not shown here.

Figure 3A: Raw image of elastic fibers staining whitish with SRB, 12 µm below the external surface of the artery. The MPB paths are indicated with white arrows (c-t-c spacing 450 µm). The elastic fiber staining is diffuse in these paths compared to the pattern of discrete fibers in the valley regions. **Figure 3B**: Merged color image of elongated VSMC green-stained nuclei (Hoechst 33342) and red-stained elastic fibers (SRB). In the beam path, note the relative paucity of elongated nuclei. **Figure 3C**: Merged color image of cell nuclei and elastic fibers of the artery wall at a depth of 32 µm. The distance between the beam paths (white arrows) is 430 µm. **Figure 3D**: Corresponding histological image (Elastica van Gieson): In the beam path (black arrows), the VSMC layer (pale, yellowish) is slightly narrower and fibrotic, indented by adventitial connective tissue (red).

Figure 4 A-F

Three months (A-B) and 6 months (C-F) after microplanar irradiation (2000 Gy). All images are longitudinal sections with exception of inserts in **A** (see arrows) and in **D** and **E** which are cross sections. **Figure 4A**: Elastic fibers (red) and collagen fibers type I and III (SHG, green) 26 µm below the external surface of the artery. Upper insert: Cross section at the level of the MPB path; lower insert: cross section of the valley; the (dark) TM in the

beam path is thinner than in the valley region. **Figure 4B**: Image 16 µm below the external surface of the artery. Elastic fibers (red) are diffusely stained in the beam path. The density of the green, elongated-shaped VSMC nuclei is higher in the valleys than in the darker MPB path. **Figure 4C**: Elastic (red) and collagen type I / III (green) fibers 26 µm beneath the external surface of the artery. The distance between the irradiated sections is 450 µm. **Figure 4D and 4E**: Cross sections of **4C** in the MPB path (**4D**) and in the central valley region (**4E**) (white arrows). The TM has completely disappeared in **4D** and the TA is thicker than in figure **4E**. The internal and the external elastic lamina are not disrupted and enclose the well-preserved dark muscular TM. **Figure 4F**: corresponding histological sections showing a segmented saphenous artery (Elastica-van Gieson). The black arrows indicate the MPB paths.

Figure 5A-D

Macroscopic image (**5A**) of the left hind limb and 2PM images (**5B-D**) of its saphenous artery 12 months after exposure to MPBs (entrance dose 2000 Gy).

Figure 5A: The left hind limb shows slight muscle atrophy. The left saphenous artery (insert) is segmented.

Figure 5B-D: merged color images 14 µm (**5B**), 26 µm (**5C**) and 28 µm (**5D**) below the external surface of the artery. The white arrows indicate the MPB paths with the presence of collagen fibers (fibrosis) and cell nuclei. The dashed arrow in figure **5B** indicates presence of extra collagen fibers (green) in the central region of the valley (see text). The distance between the paths is 415 µm.

Figure 6

Mean TM thickness ratio (microbeam path / valley, ± one standard deviation) versus time after irradiation, 312 Gy (■) and 2000 Gy (▲) dose groups. Asterisks: significant difference ($p < 0.05$) between the means at 1, 6 and 12 month(s) (Table 2). Data fitted to a one phase exponential decay (solid lines, Y = Span (exp (-K.t)) + Plateau);

312 Gy: Span = 0.31 ± 0.04; K = 0.45 ± 0.22; Plateau = 0.65 ± 0.04; Half-life (0.69/K) = 1.55 months, R2 = 0.9998.

2000 Gy: Span = 0.91 ± 0.06; K = 0.57 ± 0.08; Plateau = 0.14 ± 0.02; Half-life = 1.22 months, R2 = 0.9989.