Validation of a Vasculogenesis Microfluidic Model for Radiobiological Studies of the Human Microvasculature

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The therapeutic ratio of radiotherapy is limited by acute or chronic side effects with often severe consequences to patients. The microvasculature is a central player involved in both tumor responses and healthy tissue/organ radiological injuries. However, current preclinical vascular models based on 2D culture offer only limited radiobiological insight due to their failure in recapitulating the 3D nature experienced by endothelial cells within the human microvasculature. To address this issue, the use of a 3D microvasculature-on-a-chip microfluidic technology is demonstrated in radiobiological studies. Within this vasculogenesis model a perfusable network that structurally mimics the human microvasculature is formed and the biological response to ionizing radiation including cellular apoptosis, vessel tight adherens junction breakage, DNA double strand break, and repair is systematically investigated. In comparison to cells grown in a 2D environment, human umbilical vein endothelial cells in the 3D microvasculature-on-a-chip displays significant differences in biological responses, especially at high X-ray dose. This data confirms the feasibility of using microvascular-on-a-chip models for radiobiological studies. Such vasculogenesis models have strong potential to yield more accurate prediction of healthy tissue responses to ionizing radiation as well as to guide the development of risk-reducing strategies to prevent radiation-induced acute and long-term side-effects.

1. Introduction

More than half of all cancer patients should receive radiotherapy at least once during their treatment.[1,2] Despite significant advances in cancer radiation modalities, which allow for more accurate dose delivery, the therapeutic ratio of cancer radiotherapy remains limited by off-target damages to normal tissues. In this regard, the microvasculature plays a critical role, not only because in addition to inherently influencing the overall tumor response, radiation-induced damages to vascular cells directly or indirectly result in both acute and long-term side effects with often severe consequences for patients.[3] The organs and tissues most relevant to cancer radiotherapy, including the liver, kidney, gastrointestinal tract, skin, brain, and lungs, are indeed characterized by a high concentration of radiosensitive microvascular structures.[3] The nature of radiation induced injuries to vascular cells as well as their tissue-specific microenvironment dictate the clinical manifestation.
and extent of radiological side-effects. The acute response to irradiation occurs within hours and is characterized by structural and functional endothelium dysfunction, including apoptosis, endothelial swelling, increased vascular permeability, and edema, as well as lymphocyte adhesion and infiltration.[4] Endothelial cell senescence is also implicated in long-term vascular dysfunctional states, including tissue fibrosis.[5,6] In addition, epidemiological data suggests that even low ionizing radiation doses are associated with increased risks of cardiovascular disease. For example, radiation was shown to induce proatherosclerotic processes in endothelial cells in a dose-dependent manner,[7] even at doses well below those commonly used in fractionated treatment protocols.

Despite the well-established importance of microvasculature damage on the overall response to ionizing radiation, the understanding of the microvasculature radiobiology and its clinical impact remains limited. This is especially the case for advanced radiotherapy modalities involving very high X-ray doses or high linear energy transfer (LET) radiation. This is a significant and pressing issue as stereotactic body radiation therapy (SBRT) and stereotactic radiosurgery (SRS) modalities have become mainstream in radiotherapy. Through computer-aided field shaping and careful treatment planning, dosimetry and improved radiation delivery systems, X-ray doses as high as 20 Gy in a single fraction and 60 Gy in 3–5 fractions are delivered in contemporary SBRT and SRS, and the therapeutic mechanisms associated to such high doses as well as their effects on the microvasculature remain controversial.[8,9] Conversely, studies have highlighted profound radiation quality-dependent radiobiological differences between X-ray and high LET particle therapies. For example, the endothelial cell response to radiation was found to be more pronounced and longer lasting for Fe ions than for X-rays.[8] C-ions were also reported to induce more acute damages to endothelial cells than X-rays.[9]

To date, most in vitro radiobiological studies have been performed using 2D culture of vascular cells. However, the importance of mimicking the cells–cells and cells-extracellular matrix 3D environments of tissues in radiobiological investigation is now well established.[10–13] Cells cultured within a 3D environment are more radioresistant than when cultured in 2D and likely provide more relevant radiobiological insights. This cell-adhesion-mediated-radioresistance is linked for instance to the differences in chromatin structure between cells 2D surfaces and cell grown within a 3D environment.[14] However, despite the strong data supporting the use of more physiologically relevant tissue models in radiobiological studies, to date their application to investigate in vitro the effect of ionizing radiation on the microvasculature is scarce. The secretion of inflammatory markers was found to differ significantly for endothelial cells cultured in standard 2D plastic well-plates and on nonadherent surfaces promoting 3D clustering.[15] On the other hand, the kinetics of p53 binding protein 1 linked to DNA double-strand breaks were similar for human umbilical vein endothelial cells (HUVECs) cultured either in 2D or within tube-like vascular structures.[16]

Recent advances in the organs-on-a-chip field enable the fabrication of advanced in vitro tissue models[17] that recapitulate the key structural and phenotypical complexity of the in vivo environments. Perfusion histovascular networks can be prepared through the self-organization of endothelial cells when provided the correct biochemical cues within a microfluidic environment.[18] either through coculture with human lung fibroblasts or by exposure to interstitial flow. Microvasculature-on-chip models have been successfully applied to investigate several important physiological processes, providing for example new insights about endothelial barrier dysfunction associated with sickle cell disease, malaria, and other inflammatory and haematological diseases,[19] as well as better knowledge of tumor cell extravasation dynamics[20] and the mechanisms through which inflamed neutrophils exert proextravasation effects.[21] Microfluidic vasculogenesis models closely mimic the structure of small blood vessels and capillaries densely dispersed throughout all human tissues and known to be more radiosensitive than large vascular structures.[22] In addition, microvasculature-on-chip models can be perfused continuously, which expose the vascular cells to fluid shear stress that is essential to the obtainment of fully functional endothelial phenotypes.

Toward validating vasculogenesis microfluidic models in the radiobiological studies of the human microvasculature, we endeavored to systematically compare the response of HUVECs to ionizing radiation when cultured in a standard 2D environment or in a microvasculature-on-chip model. We investigated cell apoptosis (caspase 3/7), vessel tight adherens junction breakage (VE-cadherin) and DNA double-strand break and repair (γ-H2AX) (Figure 1). Significant differences were measured between the 2D and 3D environments, especially at high X-ray doses in the range typically used in clinical SBRT and SRS. In addition, we also demonstrate the feasibility of measuring inflammatory markers resulting from irradiation in the spent medium. This data confirms the feasibility of using microvasculature-on-a-chip model in radiobiological studies. Microfluidic vasculogenesis models have strong potential to assist in rigorously comparing the effects on microvascular structures of radiation quality, doses, and administration regimen. They could also facilitate the development of risk-reducing strategies to prevent ionizing radiation-induced acute and long-term side-effects, for example through the use of cell cycle modulators and anti-inflammatory agents as radioprotectors and/or radiation mitigators.

2. Results

2.1. Formation of the Microvasculature-On-A-Chip

The microvasculature-on-a-chip model used in this study is derived from the model developed by Kamn and co-workers.[23] The poly(dimethylsiloxane) (PDMS) microfluidic device is made up of three cell channels (1.3 mm) with adjoining media channels (1 mm), which are connected to the media reservoirs to allow for the perfusion of growth media and compounds (Figure 1). The perfusable microvasculature is formed through the self-organization of HUVECs with the support of normal human lung fibroblasts (NHLFs) within 5 days. In this design, the HUVECs are encapsulated within the fibrin gel in the
central channel, while the NHLFs are encapsulated in fibrin gel in the two side channels.

The formation of the microvascular network was monitored over 5 days and characterized by confocal imaging of the microvasculature at day 5 (Figure 2). In agreement with previous reports, fully developed tube-like-structures were obtained after 5 days of culture within the microfluidic device. It also worth noting that an optimal amount of NHLFs is required to allow for the reliable formation of the perfusable vasculature. The endothelial marker VE-cadherin was strongly expressed at day 5, demonstrating the formation of adherens junction proteins within the tube-like perfusable network. Staining of actin filaments with FITC-phalloidin showed the presence of stress fibers parallel to the main axis of the tube-like-structures. Some NHLFs could be observed within the HUVECs central channel.

2.2. Radiation Induced Structural Changes in HUVEC Monolayers and Microvasculature-On-Chip

HUVECs cultured either in 2D or within the 3D environment of the microvasculature-on-chip were first qualitatively observed under bright field microscopy at 24 h after exposure to different radiation doses (0 Gy control and 2 to 25 Gy). A dose-dependent increase in nonviable cells presenting a rounded morphology could be observed for HUVECs grown in a 2D environment (Figure 3). Irradiation can induce morphological changes,
including cell detachment, fragmentation, and generation of spaces in endothelial cell monolayers, observable as early 24 h post irradiation. In contrast, no obvious morphological changes could be observed in the HUVEC 3D culture at 24 h even at the highest tested dose (25 Gy).

Next, to investigate the morphological changes induced by radiation on the microvascular structures, the expression of VE-cadherin was monitored for both HUVEC monolayers (2D) and HUVECs in the 3D environment (Figure 4). VE-cadherin is a key protein involved in the maintenance of the endothelial barrier and its expression was imaged by confocal microscopy following radiation treatment. As shown in Figure 4A following radiation doses of 15 and 25 Gy, adherens junctions were found to be more fragmented than in the case of the sham control (0 Gy) for HUVECs in 2D monolayers. In addition, no obvious differences were observed for the HUVECs within the 3D microvasculature. To obtain more quantitative insight of the effect of radiation in both 2D and 3D HUVECs, the AngioTool algorithm (National Cancer Institute) was employed to automatically segment the VE-cadherin from the background. In this process, the average length of VE-cadherin adherens junction is calculated by algorithm segmentation, as shown in Figure 4B. AngioTool analysis confirmed that in the case of 2D HUVEC culture, the average length of adherens junctions is significantly reduced after treatment with high radiation dose ($p < 0.05$ for 15 Gy and 25 Gy vs 0 Gy). The result is consistent with the previous finding reporting loss of expression of the cell adhesion molecule (PECAM-1) after high dose irradiation. However, in agreement with the qualitative observation, no significant differences could be measured between the sham control and high radiation dose groups in the case of the HUVEC microvasculature.

2.3. Radiation Induced Apoptosis

Endothelial cell apoptosis plays a critical role in radiation induced microvascular damage and death. We therefore investigated the effects of radiation on the apoptotic response for endothelial cells cultured in both the 2D and 3D microvasculature-on-a-chip environments. The CellEvent Caspase-3/7 staining kit, which measures the well-established early apoptosis indicators caspase-3 and caspase-7 activity and shown to be effective in 3D microfluidic device, was used to investigate the apoptosis of HUVECs following radiation. Upon activation of the caspase-3 and caspase-7 apoptotic cascade, cells fluoresce green and can be imaged using confocal microscopy. To account for the more densely populated cells in the 3D versus the 2D environment, the number of caspase-3/7-positive cells was enumerated from confocal images and then divided by the total cell number (determined from Hoechst staining) in each field of view to obtain the ratios of apoptotic cells. Both in 2D and 3D, the numbers of caspase-3/7-positive cells increased in a dose dependent manner (Figure 5A), although to a much lesser extent in the case of the 3D microvasculature. The percentage of apoptotic cells was statistically higher in 2D as compared with that in 3D for all doses above 2 Gy (Figure 5B). At 25 Gy, more than 40% of HUVECs cultured in 2D were positive for caspase-3/7, while the apoptotic ratio was approximately only 10% in the 3D environment.
These structural and apoptosis results demonstrate that HUVECs are more radioresistant when in a 3D microenvironment mimicking that of the real microvascular structure compared cell cultured within 2D monolayers. This suggests that conventional radiobiological studies based on endothelial cells cultured in 2D model overestimate the damage effects of radiation to healthy microvasculature.

2.4. DNA Damage and Repair Kinetics

To further obtain mechanistic insights and better understand the causes of the increased radio-resistance of 3D microvasculature, we studied the DNA double-strand break (DSB) and repair kinetics of HUVECs both in 2D and 3D after radiation. DNA damage is the main cellular effector of ionizing radiation and the major pathway inducing cell death or cell cycle arrest.[31,32] Immunofluorescent staining of the phosphorylated form of H2AX (γ-H2AX) distributed in cell nucleus is a well-established marker of double stranded DNA damages. The number of γ-H2AX foci is well correlated with the presence of DSB, and the kinetics of foci disappearance matches the kinetics of DSB repair.[33,34] Immunofluorescent staining and confocal microscopy imaging of γ-H2AX were used to quantify the foci formation in HUVECs 2D monolayers and within the microvasculature-on-a-chip after being exposed to different radiation doses (Figure 6A). The number of foci at different time intervals (1, 4, and 24 h) were also quantified to establish the repair kinetics of DSBs in both 2D and 3D environments. To quantitatively compare the degree of DNA damage between the 2D and 3D environments, we used the TrackMate plugin from ImageJ, which is capable of recognizing particles from background, to segment particle like foci in each cell nucleus. As shown in Figure 6B, no significant differences were measured 1 h after irradiation, demonstrating that the levels of DNA damages are similar between 2D and 3D immediately after radiation. These results also confirmed that the PDMS microfluidic device does not scatter the radiation dose received
by cells in any significant way. After 4 h irradiation, a reduction in foci numbers was measured in both 2D and 3D as expected. However, the number of DSBs in HUVECs within the 3D microvasculature was significantly lower than those in 2D monolayers. A similar result was obtained at 24 h post irradiation. We can conclude that faster DNA repair occurs postirradiation for HUVECs within the 3D microvasculature-on-chip environment in comparison to HUVECs in a 2D monolayer. This is consistent with the observed cellular morphological changes and cellular apoptotic rates and confirms the higher radio-resistance of the endothelial cells in this 3D environment.

2.5. Measurements of Inflammatory Cytokines from Spent-Medium

The proinflammatory cytokines, IL-6 and IL-8 are key cytokines involved in the inflammatory response of endothelial cells in the vasculature to radiation damage.\[35\] To determine whether the 3D microvasculature-on-chip model can be utilized to monitor the inflammatory response of endothelial cells to radiation, we assessed the levels of IL-6 and IL-8 in the spent medium eluted from the device prior to and following irradiation (Table 1). The concentrations of both IL-6 and IL-8 increased in a dose dependent manner following irradiation, indicating that the 3D microvasculature-on-chip model can provide greater insight into the radiobiological inflammatory response of endothelial cells.

3. Discussion

Damages caused by ionizing radiation to vascular cells and structures within tissues and organs are directly linked to both acute and long-term side effects of radiotherapy with often severe and debilitating consequences for patients. Microvasculature-on-a-chip models have been recently successfully used to shed light in important biological processes.\[18-22\] The vascular tube-like-structures formed within microvasculature-on-chip devices closely mimic the structures of small capillaries present throughout all tissues and organs. This led us to postulate that vasculogenesis microfluidic models have significant potential to assist in obtaining better radiobiological insights and consequently in designing better and safer radiation algorithms. Better radiobiological models of the human
microvasculature are urgently needed to foster the implementation of contemporary radiotherapy protocols, including those based on high doses or high LET particles. In addition, there are significant knowledge gaps in the understanding of radiobiological incidents and resulting disease processes and progression, and an equally important need to develop medical countermeasures that mitigate radiation vascular injury.\(^{[6,36]}\)

To demonstrate the radiobiological relevance of microfluidic vasculogenesis models, we systematically compared the effects of ionizing radiation on HUVECs, both for X-ray doses relevant to fractionated irradiation protocols and SRBT/SRS. More specifically, we investigated the structural changes, apoptosis, and DNA damage and repair kinetics resulting from the irradiation of HUVECs cultured within either a 2D environment or the 3D environment of the microvasculature-on-chip. HUVEC monolayers were used for the 2D studies to ensure that cells were in a nonproliferating phase comparable to that of the 3D vascular tube-like-structure of the microfluidic model and eliminating the effect of cell cycle on DNA double break and repair. Significant differences were observed between the 2D and 3D environments, especially at high X-ray doses. Significant and dose-dependent changes to the integrity of the VE-cadherin cell–cell tight junctions were measured in 2D monolayers. On the other hand, no changes were detected for HUVECs in the 3D environments, even at doses as high as 25 Gy. VE-cadherin is an endothelial-specific adherens junctions protein required for the formation of endothelial barriers and in turn plays a central role in vascular functions. Dose-dependent cleavages to VE-cadherin was previously measured in monolayers formed from endothelial cells from human coronary artery and linked to increased endothelium permeability.\(^{[17]}\) Irradiation induced shortening of the width of VE-cadherin-positive areas at the cell–cell contacts was also observed for human dermal lymphatic endothelial cell monolayers at 5 Gy.\(^{[38]}\) Further studies are warranted to establish the relevance of the increased resistance to irradiation induced adherens junction damages in 3D microvasculature models in comparison to 2D monolayers to in vivo endothelial microvessels. Such studies are important as radiation induced vasculature damages increase significantly its permeability to macromolecules which is linked in turn to a broad range of biological responses, especially at high doses relevant to SRBT and SRS.\(^{[19,40]}\) For example, irradiation with a single 20 Gy dose increased the permeability in a mice model of the blood-brain barrier to FITC-dextran with molecular weights below 150 kDa.\(^{[40]}\) The perfusability of the microfluidic-on-chip is

Figure 5. Cell apoptosis at 24 h after irradiation. A) Representative confocal images of Caspase-3/7 staining for HUVECs in 2D and 3D; Scale bar: 50 µm B) Ratios of apoptotic cells in 2D and 3D. (*\(p<0.05\); **\(p<0.01\)). Error bar: SEM.
ideally suited to investigate the complex interplay between ionizing radiation and vascular integrity and resulting permeability increases. The feasibility of using microfluidic vasculogenesis models to quantitatively measure in vitro the vascular permeabilities of not only macromolecules but also live cells has been already demonstrated.

We also assessed apoptosis levels in both 2D and 3D environments. While significant apoptosis was observed in HUVEC monolayers in the high dose range (41.6% at 25 Gy vs 7.7% in unirradiated control group), much milder effects were observed for cells within the microvasculature-on-chip with an apoptosis rates of 12.2% 24 h postirradiation with 25 Gy.

Figure 6. DNA DSB induced at different time points after irradiation. A) Representative confocal images showing γ-H2AX foci in cell nucleus at 1, 4, and 24 h after irradiation (0, 2, and 6 Gy). B) Average foci numbers per cell nucleus versus time in 2D and 3D. (*** p < 0.001; **** p < 0.0001). Error bar: S.D.
shown to significantly influence the response of vascular cells and flow, shear stress and mechanotransduction signaling in the microvasculature-on-chip. In addition, hemodynamic pressure and flow, shear stress and mechanotransduction signaling play a key role in endothelial pathophysiology and have been observed to modulate these effects, but further studies are needed to validate new therapeutic approaches. For example, blocking of ICAM-1 (intracellular adhesion molecule-1) with monoclonal antibodies in a mouse brain blood barrier model reduced the radiation induced microvascular permeability to normal level.

Moreover, the apoptosis rate was strongly dose-dependent for the HUVEC monolayers. The observed high rate of apoptosis in the 2D model is qualitatively in agreement with previous similar studies with HUVEC monolayers as well as other vascular cells. For example, 44% of capillary endothelial cells within a 2D monolayer were apoptotic 6 h after 10 Gy X-ray irradiation. The striking difference measured between the monolayer and microvasculature-on-chip demonstrates the importance of the microenvironment in the resistance of endothelial cells to ionizing resistance. This is consistent with previous studies that have shown that treatment of endothelial cell monolayers with proangiogenic growth factors basic fibroblast growth factor (bFGF) and vascular endothelial growth factor significantly reduces apoptosis rates.

The presence of bFGF more than halved the apoptosis rate at 10 Gy in capillary endothelial cell monolayer (44% vs 20%).

Although quantitative in vivo data is scarce, the apoptosis dose-dependency observed in the microvasculature-on-chip model appears to be more consistent with that observed in real tissues. For example, relatively high doses (>22 Gy, 100 kV X-ray) were required to obtain detectable loss of endothelial in a rat spinal cords, suggesting that 2D culture studies overestimate ionizing radiation damages to endothelial cells.

Next, we compared the effects of X-ray irradiation on DNA double-strand breaks and repair kinetics. Unrepaired DNA double-strand breaks directly lead to loss of vascular cells through either apoptosis, senescence, mutation or genomic instability. DSBs were assessed using standard γ-H2AX immunostaining combined with confocal microscopy. The number of foci 1 h postirradiation was found to be dose-dependent for both the 2D and 3D cultures. No differences were measured at this time-point between HUVECs in the 2D and 3D environments for the tested doses of 2 and 6 Gy. However, the number of foci decreased more rapidly in HUVECs within the microvasculature-on-chip model, suggesting a faster DSB repair kinetic in comparison to 2D monolayers. A study by Grabham et al. showed no observed differences in DSB repair kinetic between HUVEC monolayers and tube-like-structured obtained for HUVECs grown within collagen/Matrigel gel in presence of vessel-promoting growth factors, although 2D cultures resulted in a small increase in DSBs at the earlier time point after irradiation with Gamma irradiation.

The cause for this discrepancy is unclear but could be linked to the significant differences between the static monoculture model used in this work and the dynamic coculture characteristic of the microvasculature-on-chip. In addition, hemodynamic pressure and flow, shear stress and mechanotransduction signaling play a key role in endothelial pathophysiology and have been shown to significantly influence the response of vascular cells to ionizing radiation.

Finally, we demonstrated the feasibility of measuring secreted inflammatory cytokines within the spent media eluted from the microvasculature-on-chip. As expected, sharp and dose-dependent increases in IL-6 and IL-8 were measured at 24 h postirradiation. No attempt was made to compare cytokine expression between the 2D and 3D environments owing to the difficulty in normalizing the data between the two models. The perfusable nature of the microfluidic microvasculature-on-chip facilitates such measurements which can be performed without disturbing the culture conditions. This is an important feature as the immunomodulatory effects of ionizing radiation on the human vasculature remains to be fully elucidated.

In addition to radiation-induced secretion of soluble factors, increases in the expression of adhesion molecules post irradiation play a key role in the recruitment of inflammatory cells from the bloodstream. Blocking specific pathways can be used to modulate these effects, but further studies are needed to validate new therapeutic approaches. For example, blocking of ICAM-1 (intracellular adhesion molecule-1) with monoclonal antibodies in a mouse brain blood barrier model reduced the radiation induced microvascular permeability to normal level.

While beyond the scope of the present study, this is another important element of the vascular radiobiological response that could be investigated using microvasculature-on-chip models. Finally, while the objective of the present work is to investigate the effect of ionizing radiation on the established microvasculature, the dynamic vasculogenesis progression within the microfluidic devices (Figure 2A) also provides a good mimicry of the overall angiogenesis process. The effects of radiotherapy on tumor angiogenesis largely influence the prognosis, however these effects are complex and highly dependent on dosage and modality of radiation.

In this regard, although it is beyond the scope of this work, vasculogenesis models have strong potential to provide new and relevant insights about the effects of ionizing radiation quality and dose on angiogenic processes.

4. Conclusion

In conclusion, we demonstrated the feasibility of using a microvasculature-on-a-chip model to investigate the radiobiological effects of ionizing radiation on the human microvasculature. The microvasculature formed within this microfluidic vasculogenesis model mimics closely the structure of small vascular structures including capillaries that are known to be more radio-sensitive than larger vascular vessels. The systematic comparison between HUVECs cultured within conventional 2D model and the microvasculature-on-a-chip revealed significant differences upon irradiation with X-ray, especially at high doses relevant to SRBT and SRS. While objective comparison with human microvasculature is difficult owing to the lack of quantitative data, the structural similarity and qualitative data suggest that such microvasculature-on-chip models are more physiologically relevant than standard 2D cultures. Better preclinical models of the microvasculature models are

Table 1. Concentrations of IL-6 and IL-8 inflammatory markers in spent medium eluted from microvasculature-on-a-chip.

<table>
<thead>
<tr>
<th>IL-6 [pg mL⁻¹]</th>
<th>0 Gy</th>
<th>2 Gy</th>
<th>6 Gy</th>
<th>15 Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before IR</td>
<td>0</td>
<td>27</td>
<td>29</td>
<td>N.A.</td>
</tr>
<tr>
<td>24 h after IR</td>
<td>18</td>
<td>107</td>
<td>204</td>
<td>860</td>
</tr>
<tr>
<td>IL-8 [pg mL⁻¹]</td>
<td>0</td>
<td>1.8</td>
<td>2</td>
<td>N.A.</td>
</tr>
<tr>
<td>Before IR</td>
<td>3</td>
<td>16</td>
<td>58</td>
<td>81</td>
</tr>
<tr>
<td>24 h after IR</td>
<td>15</td>
<td>16</td>
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urgently needed to both advances our understanding of the effects of contemporary ionizing radiation modalities as well as to assist in developing new approaches that mitigate their acute and long-term effects on healthy tissues and organs. With this in mind, the perfusable nature of these models will enable the straightforward testing of radio-protectant and radiosensitizer as well as allow for continuous monitoring of secretion profile, yielding more comprehensive radiobiological insights.

5. Experimental Section

Materials and Reagents: PDMS and curing agent (Sylgard 184) were acquired from Dow Corning. Poly-fluorosilane was obtained from Sigma Aldrich. Glass coverslip (60 mm × 24 mm × 0.17 mm) were obtained from ProSciTech. Cell adhesion polymer slides were purchased from ibidi. Trypsin-EDTA, Dulbecco’s PBS (D-PBS), PE-antihuman VE-cadherin, and CellEvent caspase-3/7 green detection reagent were obtained from Thermo Fisher Scientific. AlexaFluor 488-goat antirabbit IgG, FITC-Phalloidin, Hoechst, 4% Paraformaldehyde, Triton X-100, Bovine Serum Albumin (BSA), fibrinogen, thrombin, and Poly-fluorosilane were obtained from Sigma Aldrich. Fibrinogen and thrombin were dissolved in D-PBS to a concentration of 6 mg mL⁻¹ and 100 U mL⁻¹ and stored at −20 °C. Anti-γ-H2AX (phospho S139) was obtained from Abcam.

Cells Culture and Reagents: HUVECs and NHLFs were purchased from Lonza and maintained in EGM-2 and FGM-2 (Lonza) supplemented with EGM-MV BulletKit and FGM-2 Bullet Kit, respectively. Cells were maintained in 75 cm² flasks in a 37 °C incubator for at least 1 h before performing downstream processing. For apoptosis detection, 20 µg mL⁻¹ of FITC-Phalloidin for 1 h and then incubated with Hoechst for 10 min.

Preparation of Microvasculature-On-A-Chip: The chromium mask was designed according to the previous literature with slight modification and fabricated by Australian National Fabrication Facility (ANFF). The microfluidic device master was fabricated by photolithography using the chromium mask with SU-8 as photoresist. The channel height is approximately 130 µm.

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Cell Culture: Polymer coverslips with cell adhesion coating mounted on 8-well silicone removable chamber (ibidi) were used to culture cells on 2D to allow confocal microscopy observation. After reaching 70–80% confluence HUVECs were detached and 20 000 cells were seeded in each well and then kept in chamber for another 3–5 days until HUVECs were confluent for radiation. 400 µL medium was added in each well and replenished every 2 days.

Radiation: Both 2D and 3D microvasculature-on-a-chip were irradiated at the Radiation Oncology Department of the Lyell McEwin Hospital using a 6MV X-ray beam from a Varian iX linear accelerator (Varian Medical System, Palo Alto, CA). The linear accelerator was calibrated using the clinical IAEA TRS398 protocol and the radiation dose output checked on the day of irradiation with Daily QA 3 device (Sun Nuclear, USA). To achieve an electronic equilibrium at the microvasculature, samples were placed on top of a 13 mm slab of solid water (RW3; ρ = 1.0459 g cm⁻³ PTW, Freiburg, Germany) with the table top at the level of the isocenter and irradiated from below with the gantry at 180°. A radiation field size to cover the samples was used. 50 mm of solid water was placed on top of the sample to provide full scatter conditions. Under these conditions, the radiation doses discrepancy between this in vitro configuration and the in vivo one was significantly minimized and the delivered doses were expected to be accurate. During transportation to hospital samples were kept in styrofoam box and wrapped by 37 °C prewarmed gel pack. The irradiation and transportation process was completed within 2 h. The 0 Gy control sample (sham) was also transported in the same manner. Thereafter samples were kept in incubator for at least 1 h before performing downstream processing.

Fluorescence and Immunofluorescence Staining: For both VE-cadherin and γ-H2AX immunofluorescence staining, cells in both 2D and 3D were fixed with 4% (v/v) paraformaldehyde for 20 min and permeabilized with 0.2% (w/v) Triton X-100 for 15 min, and blocked with 5% (v/v) BSA at room temperature for 1 h. For immunofluorescent staining of VE-cadherin, cells in both 2D and 3D were incubated with PE-antihuman VE-cadherin antibody diluted in 3% BSA (1:100 dilution) for 1 h, then incubated with Hoechst for 10 min for nuclei staining, both were under light protect condition. For γ-H2AX immunofluorescent staining, cells were incubated with anti-γ-H2AX primary antibody diluted in 5% BSA (1:500 dilution) for 1 h, AlexaFluor 488-goat antirabbit IgG secondary antibody diluted in 1% BSA (1:500 dilution) for another h then stained with Hoechst for 10 min.

For F-actin staining, cells were fixed and permeabilized and blocked as described above, and incubated with 20 µg mL⁻¹ of FITC-Phalloidin for 1 h and then incubated with Hoechst for 10 min.

For apoptosis detection, 4 × 10⁴ µL (1:500 dilution) CellEvent Caspase-3/7 Green Detection Reagent was added to cells both in 2D and 3D 2 h after radiation. 24 h after radiation, Hoechst dissolved in 4% paraformaldehyde was added to cells and incubated for 20 min and then washed by D-PBS once to avoid extensive washing, which may flush away apoptotic cells that were not well-attached.

Confocal Microscopic Imaging: Confocal microscopic imaging, samples were typically imaged at 20 × for VE-cadherin and caspases-3/7 and 6 × for γ-H2AX. Image depths of 1–1.5 µm (0.8 µm for γ-H2AX) were used to create a 3D stack, then projected to 2D by maximum projection. During imaging, parameters (laser intensity, gain, image depth, etc.) were kept constant when characterizing a specific marker.

For the image analysis, segmentation of VE-cadherin and length measurement was performed by AngioTool Image software plugin. Caspase-3/7 images were analyzed manually, positive cells were identified when green fluorescent spots were visible and colocalized with or neighboring to the cell nucleus. γ-H2AX foci counting was performed automatically by TrackMate plugin from ImageJ. γ-H2AX foci images were converted to 8 bit format, and LOG detector was selected to detect foci from the background. During detection, estimated blob diameter was set at 7 pixels (pixel size: 0.132 µm) and threshold at 8, matching with typical foci size and filtering nonspecific background spots, respectively.

Detection of Inflammatory Markers in Culture Medium: Supernatants were collected from the medium reservoirs prior to and after radiation exposure and stored at −80 °C for future use. The inflammatory IL-8, IL-6 cytokine secretion was measured by Peprotech Human IL-8 and IL-6 TMB ELISA development kit, according to the manufacturer’s instructions. Sample concentrations were determined using the equation generated for each individual cytokine and reported in pg mL⁻¹.

Statistics: Each 2D experiment was performed with at least 3 replicate wells and in each well at least 3 images were taken. In 3D microvasculature, at least 2 devices were used in each experiment and at least 5 images...
were taken for each device. For a comparison of 2 independent variables, unpaired two tailed students’ t-tests were used and \( p < 0.05 \) is considered to be statistically significant. GraphPad Prism 7.04 was used to analyze statistical data.

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### Conflict of Interest

The authors declare no conflict of interest.

### Keywords

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