Stable thrombus formation on irradiated microvascular endothelial cells under pulsatile flow: pre-testing an annexin V-thrombin conjugate for treatment of brain arteriovenous malformations

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Abstract

Background: Our goal is to develop a vascular targeting treatment for brain arteriovenous malformations (AVMs). Externalized phosphatidylserine has been established as a potential biomarker on the endothelium of irradiated AVM blood vessels. We hypothesize that phosphatidylserine could be selectively targeted after AVM radiosurgery with a ligand-directed vascular targeting agent to achieve localized thrombosis and rapid occlusion of pathological AVM vessels.

Objective: The study aim was to establish an *in vitro* parallel-plate flow chamber to test the efficacy of a pro-thrombotic conjugate targeting phosphatidylserine.

Methods: Conjugate was prepared by Lys-Lys cross-linking of thrombin with the phosphatidylserine-targeting ligand, annexin V. Cerebral microvascular endothelial cells were irradiated (5, 15, and 25 Gy) and after 1 or 3 days assembled in a parallel-plate flow chamber containing whole human blood and conjugate (1.25 or 2.5 µg/mL). Confocal microscopy was used to assess thrombus formation after flow via binding and aggregation of fluorescently-labelled platelets and fibrinogen.

Results and conclusions: The annexin V-thrombin conjugate induced rapid thrombosis (fibrin deposition) on irradiated endothelial cells under shear stress in the parallel-plate flow device. Unconjugated, non-targeting thrombin did not induce fibrin deposition. A synergistic interaction between radiation and conjugate dose was observed. Thrombosis was greatest at the highest combined doses of radiation (25 Gy) and conjugate (2.5 µg/mL). The parallel-plate flow system provides a rapid method to pre-test pro-thrombotic vascular targeting agents. These findings validate the translation of the annexin V-thrombin conjugate to pre-clinical studies.

Introduction

Brain arteriovenous malformations (AVMs) result from abnormal vascular development resulting in direct arterial-to-venous connections without intervening capillaries, forming tangled collections of vessels called the 'nidus'. This causes high pressure arterial blood to flow directly to veins increasing the risk of vessel rupture [1]. Hemorrhage can lead to significant neurological morbidity or mortality [2, 3]. Current treatment approaches include surgical resection [4], endovascular embolization [5] and radiosurgery [6] with the goal to achieve complete AVM vessel removal or occlusion, and prevent hemorrhage. Depending on size and location of the lesion, over 90% of large AVMs are not treated safely using current treatments [7]. Hence there is a need to develop a new treatment approach to achieve complete obliteration. This could be made possible using a vascular-targeting approach by inducing rapid thrombosis precisely on targeted AVM vessels without affecting surrounding normal vessels.

In vascular targeting, a target molecule is recognized on the surface of the endothelium and a ligand or antibody to the target is bound to a drug or effector of interest [8]. The concept was initially developed for the treatment of cancer, where the inherent difference between normal and tumor vessels is utilized to direct therapeutic agents selectively to the abnormal tumor endothelium [9]. To date, no valid targets have been found on the AVM endothelial surface that make it significantly different from normal vessels. Hence, we aim to use radiosurgery to prime the AVM endothelium to induce potential targets. Our previous studies demonstrated that radiation can induce up-regulation of surface biomarkers on the AVM endothelium thus discriminating it from normal vessels [10-14]. These proteins may serve as potential targets for ligand-directed pro-thrombotic conjugates, enhancing the thrombotic response when induced with radiation. Our overall aim is to develop pro-thrombotic conjugates that target these radiation-stimulated proteins. However, it is

not economical to test multiple combinations of target molecules with thrombotic agents using an animal model. Hence we are developing an *in vitro* parallel-plate flow chamber model to simulate *in vivo* fluid shear stresses which rapidly tests the potency of conjugate-driven thrombus formation on irradiated endothelial cells when exposed to dynamic fluid flow [15]. Parallel-plate flow perfusion devices are widely used to evaluate platelet function, endothelial cell function, and thrombus formation [16-19].

In this study, we aimed to investigate the pro-thrombotic efficacy of a phosphatidylserine (PS)-targeting annexin V-thrombin conjugate in irradiated human microvascular endothelial cells using the parallel-plate flow system. PS is a negatively charged phospholipid present in the membrane of all cells. It normally localizes to the inner membrane leaflet that faces the cytosol, but under certain conditions will translocate to the outer leaflet of the plasma membrane where it plays a role in various cellular processes [20, 21]. PS plays a vital role during apoptosis where it is exposed on the surface of apoptotic cells to trigger phagocytosis [22]. PS externalization has been shown to occur on tumor endothelium, allowing it to be exploited for imaging and vascular targeting using PS-directed antibodies and immunoconjugates [23-25]. In addition, radiation therapy has been reported to enhance PS exposure on tumor endothelium, serving as a potential radiation-stimulated target in future clinical applications [25, 26]. Our recent studies also demonstrated significant externalization of endothelial PS upon radiation both *in vitro,* in brain microvascular endothelial cells, and *in vivo,* using a rat model of AVM [11, 12]. This suggests PS could be used as a vascular target in irradiated AVMs.

In the current study, we investigated the efficacy of a PS-targeting annexin V-thrombin conjugate at inducing thrombus formation under parallel-plate flow on irradiated human brain microvascular

endothelial cells. Using whole human blood perfusion, we investigated the effect of conjugate dose, radiation dose and methods of thrombus analysis, in this system.

Materials and methods

Annexin V-thrombin conjugate preparation

Thrombin (Jomar Life Research) was conjugated with annexin V (Jomar Life Research) using a Lys-Lys protein-protein conjugation kit (Click Chemistry Tools Bioconjugate Technology Company) according to the manufacturer's instructions. Briefly, 300 µL thrombin (2 mg/mL) in BupH buffer (pH 7.5) was labelled using 12-fold molar excess tetrazine (Tz) reagent, and 300 µL annexin (1 mg/mL) in BupH buffer was labelled using 12-fold molar excess trans-cyclooctene (TCO) reagent. Annexin-TCO and thrombin-Tz were mixed together (ratio 1:2) and the conjugation reaction analyzed by SDS-PAGE (Supplementary Fig S1). Image J (Version 1.5, Rasband, W.S., ImageJ, National Institute of health, USA) was used to determine efficiency of conjugation as 40%. Thrombin activity was also measured before and after conjugation using a commercially available thrombin activity assay kit (Ana Spec, CA, USA).

Blood collection

All experimental procedures using human blood were approved by the Macquarie University Human Ethics Committee (approval number HREC: 5201300883) and were carried out following the Australian Code of Practice for the Care and Use of Human tissues for Scientific Purposes (version 4) with informed consent. Fresh human whole blood was collected by venepuncture in a sodium citrate tube (BD Bioscience) from healthy volunteers. Blood was kept at room temperature while 3 µg/mL rhodamine 6G (Sigma-Aldrich) was added to label the platelets and 1.3 mg/mL Fibrinogen-FITC was added to monitor thrombus formation. The fibrinogen from human plasma (Sigma-Aldrich) was commercially purchased and conjugated to FITC using the FluoroTag™

FITC Conjugation Kit (Sigma-Aldrich) according to the manufacturer's instructions. The blood was then re-calcified with 10 mM calcium chloride and 5 mM magnesium chloride immediately prior to use in the parallel-plate flow system.

Cell culture and irradiation

HCMEC/D3 (CELLutions Biosystems Inc), an immortalized cell line from cerebral microvascular endothelial cells, were routinely cultured in EBM-2 endothelial basal medium (Lonza) supplemented with 5% fetal bovine serum, 1% Penicillin/Streptomycin, 10 mM HEPES (Life Technologies), and 1 ng/mL human basic fibroblast growth factor (Sigma-Aldrich) at 37˚C in 5% carbon dioxide and passaged at 90% confluence with trypsin-EDTA. All culture vessels were pre-coated with 100 µg/mL rat tail collagen (In Vitro Technologies).

For irradiation, cells were seeded at $1x10^4$ cells/mL onto collagen-coated 35 mm glass bottom petri dishes (MatTek Corporation) containing 1.5 mL EBM-2 medium and allowed to grow for 2 days to achieve 100% confluency. Cells were treated with a single dose of 5, 15 or 25 Gy of ionizing radiation by linear accelerator (LINAC; Elekta Synergy, Crawley, UK) at Macquarie University Hospital (Sydney, Australia), as previously described [11, 13]. Non-irradiated cells treated identically but without radiation (sham) were used as controls. At 1, 3 or 5 days after radiation or sham treatment, the cells were transferred to the parallel-plate flow chamber.

Parallel-plate flow chamber assembly and operation

The flow apparatus used in this study is the parallel-plate flow chamber (Glycotech Co., Gaithersburg, ML) encompassing an enclosed circulation system consisting of: 1) flow deck with inlet and outlet port connected to tubing; 2) silicon rubber gasket with a flow width of 1 cm and a thickness of 0.010 cm fitted beneath the deck; 3) 35 mm glass-bottom petri dish which contained the cultured cells. A vacuum port was used to seal the dish with gasket to the petri dish containing

100% confluent HCMEC/D3 cells which was then connected to the pulsatile syringe pump using silicone tubing filled with whole blood. To optimize flow conditions, the pump was allowed to run with the confluent cells in EBM-2 medium rather than blood with variations in flow rate (20 strokes/min, 38 strokes/min, 52 strokes/min, or 66 strokes/min), volume (1.4 mL/min, 2.4 mL/min, 3.4 mL/min or 4.4 mL/min) and flow time (5 min, 10 min, 15 min, or 20 min) where stability, behavior, and confluency of the cells were observed after each experiment using brightfield microscopy.

Average wall shear stress determination

To determine the average wall shear stress within the flow chamber, 6 µm fluorescent beads (FluoroSpheres sulphate microspheres, Molecular Probes, Ore, USA) were introduced in a non-cellularized circulatory system at a volume of 20 µL (500x dilution) and images were captured at 100 ms frame intervals with 10 ms exposure time where the peak speed of the beads in the center line of the flow chamber was determined over the duration of the pump cycle. The wall shear stress (WSS, in dynes/cm^2) can be related to the peak (center line) velocity (V_{Peak} in m/s) by: $WSS = 10 \times (4\mu V_{Peak}/t)$, where μ is viscosity of water (8.90×10^{-4}) in Pascal seconds (Pa.s) which is 10 times less compared to blood viscosity, and t is the chamber thickness in meters.

Thrombosis under flow

The pump was filled with 12 mL of the labelled whole human blood and assembled with the petri dish containing sham or irradiated cells. At time zero, different concentrations of conjugate were injected into the system to achieve final concentrations of 1.25 µg/mL, 2.5 µg/mL or, 5 µg/mL. The pump was operated under optimized flow conditions (described in results). After 15 min, the pump was stopped, the unit was disassembled, and the cells washed three times with phosphate-buffered saline followed by fixation with 50% ethanol for 15 min at room temperature. The cells

were stained with 100 µg/mL Hoechst 33342 solution (Thermo-Fisher Scientific), washed, and mounted for confocal microscopic analysis. Experiments were also carried out with free unconjugated 1.5 µg/mL thrombin and free unconjugated 0.9 µg/mL annexin V as control, where percent loss of thrombin activity (5%) was taken into consideration. An equivalent volume of saline injected into the system was used as a non-conjugate control.

Microscopy and image analysis

Cells were viewed under bright-field and using ex358nm/em461nm channels for Hoechst. Platelets and fibrin were viewed at ex550nm/em570nm and ex495/em519nm respectively. Z stacks were taken and 3D image reconstruction was done using Bitplane (IMARIS software version 8) for measuring thrombus volume. First, the image was selected by adding its surface into the analysis toolbar and absolute intensity was applied after selecting the source channel. Threshold was applied and adjusted manually until the image distribution over the surface was observed with all deposited fibrin. The volume was obtained by unifying whole fibrin volume in a field of view. 2D analysis of platelet area was obtained with the same software tool settings. Five fields of view from each experiment were selected and the average measurements were considered for analysis. Data analysis was performed using Prism 6.01 (Graphpad software Inc., La Jolla CA). Values are given as mean ± standard deviation (SD) of 3 independent experiments. Multiple comparisons were performed using two-way ANOVA with Tukey's post-hoc analysis and the level of significance was set at $p<0.05$.

Analysis of fibrin degradation product (FDP)

As a further measure of thrombotic activity in the flow system, whole blood was analyzed after flow for the presence of fibrin degradation product (FDP). Whole blood was collected after each flow experiment, centrifuged at 1000 x g for 10 min to separate plasma which was stored at -80˚C

until analysis using a fibrin degradation product (FDP) enzyme-linked immunosorbent assay (ELISA) kit (Jomar Life Research) according to the manufacturer's instructions.

Results

Average flow rate and shear stress

Flow conditions were optimized to ensure that cells remained adherent to the surface until the end of the experiment. The stroke rate was optimized at 38 strokes/min. The flow volume and flow time were found to be optimal at 2.4 mL/min and 15 min respectively (Supplementary Fig S2A, B, C). The average wall shear stress using these parameters was calculated based on bead velocity over time and measured as 3.1 dynes/cm^2. Increasing any of these parameters led to decreased cell confluency. The shear stress profile confirmed the pulsatile flow of the system simulating the *in vivo* environment (Supplementary Fig S2D).

Effect of radiation on HCMEC/D3 cell confluency

Cell confluency after 15 min of flow at optimized conditions was monitored over a period of 5 days post radiation (Supplementary Fig S3). No decrease in cell confluency was observed at day 1 and day 3 compared to the control at all radiation doses after flow. At day 5, cell loss and cellular hypertrophy were observed. The enlargement and flattening of cells is consistent with radiation-induced acquisition of a senescent phenotype [27]. While the cells spread to provide greater coverage of the collagen-coated dish, confluency was still reduced, hence all subsequent experiments were performed at day 1 and 3 post-irradiation to reduce non-specific induction of thrombosis via collagen exposure.

Annexin V-thrombin conjugate enhances platelet binding and aggregation on irradiated endothelial cells under flow

Evidence for thrombus formation was determined by measuring platelet and fibrinogen aggregation and accumulation by confocal microscopy (Fig 1). We first determined platelet adhesion and aggregation on the endothelial surface, one of the first steps in thrombus formation. The average size of the platelet aggregates as well as the total area of platelet deposition per field of view was measured. Overall, increased platelet binding and aggregation was observed with increasing doses of both conjugate and radiation (Fig 2A – D). The average size of platelet aggregates (Fig 2A, B) increased significantly (2.1-fold, $p<0.0001$) at the higher doses of 15 and 25 Gy using 2.5 µg/mL conjugate dose at both day 1 and day 3. No major differences were observed between day 1 and day 3 values. Total platelet area (per field of view) increased modestly with each radiation dose in the absence of conjugate (up to 1.5 – 2-fold), though this was not statistically significant (Fig 2C, D). However, in the presence of conjugate, significant increases in total platelet area were observed relative to the non-irradiated, saline control at all radiation doses (3 – 4-fold; Fig 2C, D).

Figure 1: Platelet aggregation and fibrin formation on irradiated endothelial cells. Representative confocal images of platelet aggregation and fibrin formation on irradiated endothelial cells in the parallel-plate flow system at day 1 or day 3 post-irradiation or sham. Platelets were pre-stained in whole blood with R6G (red), and FITC-labelled fibrinogen (green) was added prior to circulation. Cell nuclei were stained post-flow with Hoechst 33342 (blue). Bar = 50 µm; magnification = 200x.

Figure 2: Platelet aggregation on irradiated endothelial cells. Average size of platelet aggregates (μm^2) at day 1 (A) and day 3 (B) post-irradiation. Total platelet area per field of view at day 1 (C) and day 3 (D). Data are shown as mean ± SD (3 independent experiments) and were analyzed

using two-way ANOVA with Tukey's post-hoc analysis. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.5 comparisons relative to saline, non-irradiated control. ####p<0.0001, #p<0.5, comparisons within radiation dose group.

Annexin-V thrombin conjugate enhances fibrin deposition on irradiated endothelial cells under flow.

Fibrin deposition is representative of a more mature and stable thrombus hence we used incorporation of fluorescently labelled fibrinogen as a measure of stable thrombus formation (Fig 1 and Fig 3A – G). We used 3D reconstruction using z stacks of confocal microscopy (Fig 3A, B, C) to determine the average volume of individual fibrin thrombi (Fig 3D, E) as well as the total fibrin volume per field of view (Fig 3F, G) at day 1 and 3. At both post-irradiated time points, no fibrin deposition was observed on non-irradiated controls with or without conjugate addition (Fig 1 and Fig 3D – G). No fibrin deposition was observed at any radiation dose in the absence of conjugate. A dose of 1.25 µg/mL conjugate showed significant fibrin development at 15 Gy and 25 Gy with minimal to no fibrin deposition at 5 Gy on both day 1 and day 3 (Fig 3D – G). A dose of 2.5 µg/mL initiated fibrin formation at 5 Gy, followed by formation of a more interlinked fibrin network at 15 and 25 Gy. At both 15 Gy and 25 Gy using 2.5 µg/mL conjugate, average fibrin volume increased 32 – 64-fold (P<0.0001) compared to non-irradiated saline control and respective irradiated saline control (at same radiation dose) (Fig 3D, E). No major differences were noted between day 1 and day 3 except significant differences between the two conjugate doses were more apparent at day 1 (P<0.0001). Values of total fibrin volume (Fig 3F, G) showed a similar trend to average volume (Fig 3D, E).

Figure 3: Fibrin thrombus formation on irradiated endothelial cells. (A) Representative confocal image of interlinked fibrin fibers in 2D (green); (B) 3D reconstruction of Z stacks showing

deposited fibrin on the surface; (C) individual 3D fibrin thrombi (yellow) could be measured independently to give average fibrin volume (D, E) or summed to give total fibrin volume per field of view (F, G) at day 1 (D, F) or day 3 (E, G). Data are shown as mean ± SD (3 independent experiments) and were analyzed using two-way ANOVA with Tukey's post-hoc analysis. ***p<0.001, **p<0.01, comparisons relative to non-irradiated control. ####p<0.0001, ##p<0.01, #p<0.5, comparisons with saline control within radiation dose group. Scale bar = 30 µm. Magnification = 200x.

Free thrombin or annexin V induces low level activation of blood coagulation

We also examined thrombosis in the presence of free, non-targeted thrombin and free annexin V at activity levels/doses equivalent to that of the conjugate. Base levels of platelet binding and aggregation were observed at all radiation doses using saline, free thrombin and free annexin under flow at both time points (Fig 4). Average platelet area was not generally affected by free thrombin or annexin V at any radiation dose at either time point (Fig 4A, B). Total platelet area increased 2 – 3-fold in response to free thrombin in non-irradiated controls and at all radiation doses in the range relative to the non-irradiated saline control (Fig 4C, D). Similarly, annexin V increased total platelet area at the higher radiation doses relative to the non-irradiated saline control, however was not significantly different to the respective saline control at the equivalent radiation dose.

Figure 4: Platelet and fibrin deposition in response to free annexin and free thrombin. Average platelet area (A, B), total platelet area (C, D), average fibrin volume (E, F), and total fibrin volume (G, H) in response to saline control, free thrombin and free annexin at each radiation dose on both day 1 (A, C, E, G) day 3 (B, D, F, H). Data are shown as mean ± SD (3 independent experiments) and were analyzed using two-way ANOVA with Tukey's post-hoc analysis. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.5, comparisons relative to sham-irradiated, saline control.

####p<0.0001, ###p<0.001, ##p<0.01, #p<0.5, comparisons relative to saline control within radiation dose group.

Average fibrin volume and total fibrin volume increased between 4 – 5-fold in response to thrombin alone relative to the non-irradiated saline control at both days, however, minimal significant difference was seen relative to the respective irradiated saline controls (at same dose) (Fig 4E – H). However, when compared to levels of fibrin deposition in response to conjugate, maximal levels of total fibrin deposited in response to free thrombin and free annexin V were in the range $0.05 – 0.1 \times 10^5$ μm^3, while the conjugate resulted in volumes in the range of $1 – 2 \times 10^5$ μm^3, a 10 – 20-fold increase.

Plasma FDP level

The FDP concentration was measured in plasma from whole blood after flow in blood treated with 2.5 µg/mL conjugate at the day 1-time point (Fig 5). Significant increases were observed at both 5 Gy (28-fold, p<0.01) and 25 Gy (68-fold, p<0.0001). FDP levels increased 9-fold in response to 15 Gy however this did not reach statistical significance with the 3 replicate samples.

Figure 5: Plasma FDP concentration after flow. Presence of FDP analyzed in plasma treated with 2.5 µg/mL conjugate at day 1 post-irradiation or sham under flow. Data are shown as mean ± SD (3 independent experiments). Statistical differences were analyzed using one-way ANOVA with Tukey's post-hoc analysis. ****p<0.0001, **p<0.01 comparisons relative to sham-irradiated control.

Discussion

In this study, we investigated the efficacy of an annexin V-thrombin conjugate to bind selectively to irradiated endothelial cells and induce thrombosis under flow using an *in vitro* parallel-plate flow chamber. This demonstrated the successful development and use of an *in vitro* flow device to test the effect of a pro-thrombotic conjugate in stimulating platelet aggregation and fibrin formation. Here, we successfully used fresh human whole blood with irradiated endothelial cells to demonstrate *in vitro* shear-dependent thrombosis in the presence of a PS-targeting pro-thrombotic conjugate. The current study demonstrates that durable platelet adherence to HCMEC and stable fibrin thrombi formation is possible by priming the endothelial cells with radiosurgery and using our pro-thrombotic vascular targeting agent under flow conditions.

Cell irradiation causes cellular changes that tend to up-regulate various cell adhesion, pro-inflammatory, and pro-thrombotic molecules on the endothelial surface [10, 14, 28, 29], and this includes the externalization of PS [24]. We reported on post-irradiation PS externalization on microvascular endothelial cells *in vitro*, using live cell imaging, and *in vivo*, using near-infrared fluorescent optical imaging in a rat AVM model [11, 12]. Thus, PS appears a valid target for a vascular targeting approach to AVM treatment and we developed a PS-targeting, annexin V-thrombin conjugate for testing. In agreement with earlier studies, we have now demonstrated that this conjugate can bind effectively on irradiated endothelial cells, and further, that this occurs under high flow and initiates platelet aggregation and fibrin clots in the presence of whole blood. Not unexpectedly, higher radiation doses (15 and 25 Gy) caused significantly greater thrombus formation when compared to the lowest dose (5 Gy) and when in combination with a conjugate dose of 2.5 µg/mL. However, our data suggest that there was a significant synergistic interaction between the effects of radiation dose and conjugate dose, and that low radiation dose could still

induce endothelial PS exposure and thrombosis when used in combination with the higher conjugate concentration. This is considered noteworthy for future applications to AVM treatment in the clinic. Treatment of large AVMs by stereotactic radiosurgery alone is often limited by the high dose requirement to achieve complete obliteration (>15 Gy) [30]. This is often not achievable in large lesions without significant off-target effects to the surrounding brain. The potential ability to use a lower dose of radiation when combined with a vascular targeting agent would potentially increase the number of patients considered treatable. It was important in this study to examine not just thrombus formation but also thrombus stability in the presence of the developed conjugate. Binding and aggregation of platelets which occurs early in the clotting cascade does not necessarily indicate that a stable thrombus will be formed [33]. Formation of fibrin polymers occurs later in the coagulation cascade to attain a mature, stable clot. Thrombin causes activation of platelets allowing binding of soluble fibrinogen in the blood, which is then cleaved to insoluble fibrin by active thrombin and polymerized and cross-linked to achieve greater stability. Fluorescently labelling fibrinogen with FITC gave evidence of these later thrombotic events and clear fluorescent imagery of fibrinogen deposition and aggregation and what appeared to be the development of fibrin branching representative of growing thrombi. This technique of labelling thrombus through fibrinogen has been used previously as an advanced method of studying platelet activation in whole blood [31, 32]. Fibrinogen labelled with near-infrared fluorophores has also been used *in vivo* as a marker of clot formation in animal models (Ohnishi et al 2006) ~~[33].~~ It must be noted however that in the current study fibrinogen deposition was used as a surrogate marker of thrombus formation. We did not specifically assess fibrinogen cleavage to fibrin by thrombin *in situ* and in future inclusion of post-flow immunostaining with a fibrin-specific antibody would further clarify fibrinogen to fibrin cleavage rates by the targeted thrombin. However we did observe the

progressive accumulation of what appeared a branched fibrin network characteristic of insoluble polymerized fibrils.

With this assumption in mind, confocal microscopy and digital image reconstruction allowed us to reconstruct a 3D image of this networked fibrinogen deposition to give us more detailed information on whole thrombus volume using fibrin structure. Similar analysis approaches were suggested by other authors previously [19]. It was possible to observe the initiation of fibrin and branched fibrin network on activated platelets in response to increases in radiation and conjugate dose. Measuring thrombosis using the deposited fibrin was far superior to assessing platelet binding and aggregation. However, by examining both platelet and fibrin deposition in this study, we could see that though free thrombin caused platelet activation with more platelet aggregates under flow, the level of activation was still well below the baseline activation of combined conjugate and radiation dose groups. In addition, there was no significant fibrin formation observed in response to thrombin alone, in comparison to that induced by conjugate. Radiation alone (in the absence of conjugate), caused a dose-dependent increase in platelet adhesion and aggregation, however did not significantly increase fibrin deposition. Similarly, conjugate had a dose-dependent effect on platelet aggregation but not fibrin deposition in the absence of radiation. Thus, in alignment with the literature [14, 34], radiation appears to induce a pro-thrombotic, platelet-binding surface on the endothelium but does not necessarily induce rapid formation of more stable thrombi. All thrombus formation is a balance between the activities of pro- and anti-coagulant factors present on the endothelial surface and in the plasma. The normal, healthy endothelium provides an anti-coagulant milieu which our approach utilizes to prevent off-target activation of thrombin on normal vessels. However on the irradiated endothelium, proclivity toward a pro-coagulant state tips the balance toward coagulation through down-regulation of

molecules such as thrombomodulin and subsequent limitation of protein C activation which normally acts to restrain the coagulation cascade (Wang 2007; Wang 2002). Our results show that this alone may not be enough to reproducibly overcome the inherent thromboresistance of the endothelium, however, together with delivery of excess, targeted thrombin saturates the irradiated region, tipping the balance toward a pro-coagulative state. Here we show that delivery of PS-targeted thrombin to the irradiated cells is far superior to adding free thrombin in the blood. This further suggests that systemic thrombosis would be unlikely to occur in the presence of conjugate on non-irradiated vessels without the initial pro-thrombotic radiation stimulus. Further *in vivo* studies will be required to assess long term thrombus stability and specificity.

In addition, we used FDP analysis of the unconditioned (post-flow) plasma to assess thrombus stability since a fine balance between clot formation and fibrinolysis determines the thrombus maturity [35]. The determination of FDP in plasma or serum has been used previously and considered as an effective predictor showing patient's coagulative-fibrinolytic state in thrombosis, acute myocardial infarction, or sepsis [36] . Our findings clearly indicate the presence of increasing levels of FDP in response to radiation at the 2.5 µg/mL conjugate dose which confirmed the fibrin deposition data and formation of highly stable thrombus under flow conditions. This suggests the possibility of achieving fully developed thrombosis even at low radiation doses.

The development of this parallel-flow system has significant advantages over prior studies. The inclusion of 3 different methods for analysis of thrombus formation was important to fully determine the effects of combined conjugate and radiation doses and for their comparison to the effects of thrombin alone. The pulsatile nature of the pump further added advantage to this study by simulating the *in vivo* hemodynamic condition using its pulsatile flow pattern. However, the system is limited with respect to achieving a completely physiological flow rate and shear stress

level, which was limited by the ability of the endothelial cells to adhere to the artificial collagen substratum. Average WSS measurements lie in the range of 2 – 67.4 dynes/cm^2 for the large cerebral feeder vessels of AVMs [37], and in the range of 7 - 23 dynes/cm^2 for normal intracranial vessels [38]. The ability to culture human AVM endothelial cells isolated from surgically excised human AVMs would also be more physiologically relevant but we consider not necessary for early development and pre-testing of conjugate once a valid target is identified.

These preliminary investigations in the flow system naturally require further validation *in vivo*. Future testing of the PS-targeting conjugate at similar dose combinations in our AVM animal model will allow assessment of thrombus formation in appropriately sized vessels under deranged hemodynamic conditions that reflect those in human AVMs (Raoufi Rad 2017, Tu et al 2010). This will not only advance the approach toward translation once target expression is validated using imaging in AVM patients after irradiation, but will validate the utility of the established parallel-plate flow system for pre-assessment of other ligand-guided pro-thrombotic agents targeting other putative radiation-stimulated targets.

Conclusions

Our findings demonstrate the first evidence of stable thrombus formation on irradiated endothelial cells in a parallel-flow device by designing and testing a pro-thrombotic conjugate targeting PS. Using this system, we established dose combinations of conjugate and radiation that could effectively induce highly stable thrombus formation in the presence of whole blood that can now be applied in a pre-clinical animal study. Further, development of this system also enables us to rapidly screen other potential target molecules and pro-thrombotic conjugates in future, prior to testing in pre-clinical models. Improving the treatment potential of stereotactic radiosurgery in

AVM therapy by combining with ligand-directed vascular targeting remains a viable objective with PS a potential target.

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Conflicts of Interest

The authors declare no conflicts of interest.

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