Durable thrombosis in a rat model of arteriovenous malformation treated with radiosurgery and vascular targeting

Laboratory investigation

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Object. Radiosurgical treatment of brain arteriovenous malformations (AVMs) has the significant shortcomings of being limited to lesions smaller than 3 cm in diameter and of a latency-to-cure time of up to 3 years. A possible method of overcoming these limitations is stimulation of thrombosis by using vascular targeting. Using an animal model of AVM, the authors examined the durability of the thrombosis induced by the vascular-targeting agents lipopolysaccharide and soluble tissue factor conjugate (LPS/sTF).

Methods. Stereotactic radiosurgery or sham radiation was administered to 32 male Sprague-Dawley rats serving as an animal model of AVM: 24 hours after this intervention, the rats received an intravenous injection of LPS/sTF or normal saline. The animals were killed at 1, 7, 30, or 90 days after treatment. Immediately beforehand, angiography was performed, and model AVM tissue was harvested for histological analysis to assess rates of vessel thrombosis.

Results. Among rats that received radiosurgery and LPS/sTF, induced thrombosis occurred in 58% of small AVM vessels; among those that received radiosurgery and saline, thrombosis occurred in 12% of small AVM vessels (diameter < 200 μm); and among those that received LPS/sTF but no radiosurgery, thrombosis occurred at an intermediate rate of 43%. No systemic toxicity or intravascular thrombosis remote from the target region was detected in any of the animals.

Conclusions. Vascular targeting can increase intravascular thrombosis after radiosurgery, and the vessel occlusion is durable. Further work is needed to refine this approach to AVM treatment, which shows promise as a way to overcome the limitations of radiosurgery.

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Key Words • arteriovenous malformations • radiosurgery • vascular targeting • vascular disorders

Arteriovenous malformations (AVMs) of the brain are a leading cause of nontraumatic intracranial hemorrhage among children and young adults. The prevalence of AVMs is 10–500 AVMs per 100,000 persons (0.01%–0.5% of the population), yet these lesions are the most common cause of neurological impairment or death among patients younger than 20 years.

The goal of AVM treatment is to obliterate flow through the abnormal vessels and, therefore, eliminate the threat of intracranial hemorrhage, while maintaining normal circulation and preserving neurological function.

Each of the current treatment modalities (microsurgical resection, endovascular therapy, and radiosurgery) has its own benefits and risks. Microsurgical resection is curative for small, superficial lesions in noneloquent parts of the brain with low complication rates. However, after decades of advances and refinement, the techniques of microsurgical resection will probably not improve substantially, and resection will always be invasive. Although endovascular therapy can be considered minimally invasive and in theory can obliterate flow, it is technically possible in only a few cases; its main use is as an adjunct to resection or radiosurgery.
Radiosurgery has the benefits of low initial risk and can be administered on an outpatient basis. For treatment of AVMs of 3 cm in diameter or smaller, obliteration rates of 70%–80% have been obtained. The limitations of radiosurgery include a delay to obliteration and decreased effectiveness for larger AVMs. For AVMs larger than 3 cm, all current treatment modalities have drawbacks, and very large AVMs are often untreatable. Potential advances in radiosurgery could offer a treatment option for these cases. Radiosurgical planning and delivery methods are improving along with advances in computing and technology.

The effectiveness of radiosurgery could also be improved with the addition of biological methods. One such approach is enhancement of thrombosis. In the field of cancer research, various techniques are used to stimulate thrombosis within tumor vessels, resulting in tumor necrosis. These vascular-targeting techniques rely on constitutive molecular or physical differences between tumor vessels and normal vessels. The concept of using similar techniques to induce thrombosis within AVM vessels is attractive, but this approach is limited by the fact that AVM vessels do not differ enough from normal vessels to enable selective thrombosis. This limitation could be overcome if the radiosurgery-induced molecular changes within the targeted vessels were sufficient to differentiate them from normal vessels. We have been investigating this approach in an animal AVM model and have recently demonstrated proof of principle of a radiosurgery and vascular-targeting agent strategy to enhance early thrombosis.

The aim of this study was to further evaluate the durability and long-term effectiveness of thrombosis obtained by using radiosurgery and vascular targeting.

Methods

Approval for animal experimentation was obtained from the Animal Care and Ethics Committee of the University of New South Wales. Animal experimentation was performed in accordance with Animal Care and Ethics Committee guidelines and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Thirty-two male Sprague-Dawley rats were used as the animal model of AVM. This model has been shown to share hemodynamic, angiographic, morphological, and molecular characteristics with human AVMs. The AVM model creation has been described in detail. Briefly, rats were anesthetized, and an anterior cervical approach was used to expose the left common carotid artery and external jugular vein. Blood flow through the common carotid artery was measured using a 1-mm Doppler ultrasonic probe (Transonic Systems Inc.). The caudal external jugular vein (at its junction with the subclavian vein) was ligated, and it was anastomosed (end-to-side) to the common carotid artery with continuous 10-0 nylon sutures. Blood flow was then measured through the proximal common carotid artery and the arterialized vein by using 1-mm and 2-mm Doppler ultrasonic probes, respectively (Transonic Systems Inc.).

The 32 rats were divided into 3 treatment groups: vascular-targeting agent plus radiotherapy (LPs/sTF+RTX), vascular-targeting agent plus sham radiotherapy (LPs/sTF−RTX), and saline plus radiotherapy (saline+RTX). For each of the 4 time points (1, 7, 30, and 90 days after treatment), the LPs/sTF−RTX group contained 3 rats (total 12), the LPs/sTF+RTX group contained 3 rats (total 12), and the saline+RTX group contained 2 rats (total 8).

At 6 weeks after creation of the AVM model, radiosurgery was administered. A custom-made stage was attached to the X-Knife LINAC device (Radionics). Radiation (25 Gy) was delivered to the model nidus with the 90% isodose line encompassing the fistula. The contralateral carotid arteries and jugular vein received a radiation dose less than 4 Gy.

At 24 hours after administration of radiosurgery, either LPs/sTF or saline was injected into the femoral vein of each rat that had received radiosurgery. At 24 hours after sham radiosurgery, LPs/sTF was injected; LPs was given at 0.1 mg/kg body weight, and sTF was given at 0.4 mg/kg body weight. The LPs and sTF were suspended in 1 ml 0.9% sterile saline; injection of the suspension was followed by 1.5 ml 0.9% sterile saline to flush the injection line. Rats in the saline group received only 1.5 ml of 0.9% sterile saline.

At 1, 7, 30, or 90 days after injection, angiograms (via a mini-laparotomy and iliac artery cut down) were obtained to assess the nidus and draining vein; immediately afterward, rats were killed by paraformaldehyde perfusion fixation. After perfusion, vascular tissue from the neck region bilaterally was harvested for analysis. Tissue samples were embedded in paraffin. Sections of 10-μm thickness were taken from the proximal carotid artery, carotid–jugular anastomosis, distal carotid artery, arterialized feeding vein, infracranial AVM nidus, contralateral infracranial tissue, contralateral carotid artery, and draining vein. For confirmation of the presence of antemortem thrombi in the vessel lumen, sections were stained with H & E or with Martius scarlet blue.

The end points examined were fistula blood flow (measured by Doppler flowmetry), draining vein diameter (measured angiographically), and intravascular thrombosis rates (assessed histologically). Thrombosed and patent vessels were counted on 5 representative high-power fields of sections from each vascular region and from each of the harvested organs by 2 observers blinded to the animal treatment group.

Using H & E–stained sections and Martius scarlet blue–stained sections, the same 2 observers counted thrombosed and patent vessels on 5 representative high-power fields of sections from each AVM nidus. The results of each observer were compared; if results differed, those sections were reexamined by both observers together, and the number of thrombi was decided. We performed ANOVA using SPSS version 13 software (SPSS Inc.). A value of $p < 0.05$ was considered statistically significant.

Results

Blood Flow and Angiography

The Doppler measurements of flow through the fis-
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tula obtained before treatment and at 1, 7, 30, and 90 days after treatment are shown in Table 1. Doppler assessment of the blood flow was performed at 2 points during the experiment. Before fistula formation, blood was measured through the common carotid artery, and after treatment (before the animal was killed), blood flow was measured through the arterialized vein by using a 1- to 4-mm ultrasonic probe (Transonic Systems). Results of Doppler flow measurement analyses were not statistically significant.

**Thrombosis Rates**

Intravascular thrombosis was detected in rats in all 3 groups (Fig. 1). Average thrombosis rates were highest (58%) among animals in the LPS/sTF+RTX group, second highest (43%) among those in the LPS/sTF–RTX group, and lowest (13%) among those in the saline+RTX group. Among animals in the control saline+RTX group, an initial absence of thrombosis was followed by a steady increase in thrombosis over time.

Statistical analysis of pooled data from the experimental groups revealed an association between LPS/sTF (with or without radiation) and development of thrombi (p = 0.03). At 90 days, thrombosis rates among rats in the LPS/sTF+RTX group were significantly higher than rates among rats in the other 2 groups (p < 0.01).

**Histological Analyses**

Histological examination demonstrated the presence of antemortem thrombi and postmortem clots within the vessel lumens. In addition to staining the sections with hemotoxylin and eosin (Fig. 2), to confirm the presence of fresh, mature, and old fibrin antemortem thrombi, we also used Martius scarlet blue stain (Fig. 3). Occlusion was occasionally seen in larger vessels; however, most thrombi were seen in small vessels (less than 200 μm). The antemortem thrombi were a composition of fibrin–platelet bands and erythrocyte-rich accumulations. In addition, lines of Zahn were occasionally seen; the paucity of this finding could be explained by thrombi occurring in the smaller vessels, with less area for lamination to occur. Among rats in the saline+RTX control group, very few thrombosed vessels were found; no thrombosis was seen at day 7 (Fig. 2 A2), but thrombosis gradually and steadily increased over time, as did evidence of radiation effect, such as intimal hypertrophy and subendothelial proliferation (Fig. 2 B2 and C2). In contrast, among rats in the LPS/sTF–RTX sham radiation group, rates of thrombosis were higher (Fig. 2 A1, B1, and C1). Among rats in this group, rates of thrombosis varied according to time, although not with a progressive increase. Among rats in the LPS/sTF+RTX full-treatment group, rates of formation of fibrin and erythrocyte thrombi were the highest; some variation occurred over time (Fig. 2 A3, B3, C3; Fig. 3A and B). The highest rate of thrombosis was observed at the final time point (Day 90).

**Discussion**

As the leading cause of nontraumatic intracranial hemorrhage among children and young adults, intracranial AVMs are clinically significant. Hemorrhage accounts for a considerable short-term mortality rate of 10%–29% and a risk for permanent disability of 14%–85%. The classification of AVMs according to size, pattern of venous drainage, and neurological eloquence of adjacent brain enables stratification of treatment risk. Low-grade AVMs (Grade I–II) account for approximately 40% of AVMs and can be treated with low risk. One-third of AVMs are high grade (Grade IV–V), and over 90% of these are untreatable. Of the intermediate-grade AVMs (Grade III), many are not treatable without high risk. New treatments are needed for these patients.

A promising new therapeutic technique is the pro-

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**TABLE 1: Doppler flow through arterialized jugular vein of rats, before and after treatment**

<table>
<thead>
<tr>
<th>Time (days)†</th>
<th>Pre-Tx‡</th>
<th>Post-Tx‡</th>
<th>Change</th>
<th>Pre-Tx‡</th>
<th>Post-Tx‡</th>
<th>Change</th>
<th>Pre-Tx‡</th>
<th>Post-Tx‡</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>43 ± 6.66</td>
<td>99 ± 25.53</td>
<td>56</td>
<td>35 ± 5.51</td>
<td>128 ± 37.9</td>
<td>93</td>
<td>100 ± 19</td>
<td>72 ± 21.21</td>
<td>−28</td>
</tr>
<tr>
<td>7</td>
<td>53 ± 12.67</td>
<td>176 ± 39.26</td>
<td>123</td>
<td>72 ± 20.07</td>
<td>296 ± 46</td>
<td>224</td>
<td>67 ± 13</td>
<td>113 ± 7.7</td>
<td>46</td>
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<tr>
<td>30</td>
<td>21 ± 6.5</td>
<td>17 ± 9</td>
<td>−4</td>
<td>NM</td>
<td>NM</td>
<td>0</td>
<td>NM</td>
<td>NM</td>
<td>0</td>
</tr>
<tr>
<td>90</td>
<td>29 ± 4.36</td>
<td>249 ± 25.4</td>
<td>220</td>
<td>105 ± 9.71</td>
<td>291 ± 41.63</td>
<td>186</td>
<td>44 ± 12</td>
<td>16 ± 11.31</td>
<td>−28</td>
</tr>
</tbody>
</table>

* NM = flow not measured because of equipment failure; Tx = treatment.
† Indicates days posttreatment.
‡ Values are presented as the mean ± SEM.
motion of intravascular thrombosis by use of molecular therapies. Similar strategies have been used for cancer therapy, in which deliberate stimulation of intravascular thrombosis has been investigated as a way to achieve tumor necrosis.\(^{11,14,17,30,51}\) This vascular targeting can use a ligand-directed strategy, combining a targeting moiety and an effector moiety. The targeting moiety is an antibody or peptide that binds to a marker that is selectively expressed on tumor vessel endothelium, and the effector moiety induces thrombosis.\(^{51}\) Vascular targeting can also use a nonligand strategy, which involves administration of agents that directly interact with the target cells because of their molecular and ultrastructural differences from normal cells. Vascular targeting is conceptually attractive for the treatment of AVMs because vascular thrombosis is the primary aim and is potentially curative. For selective thrombosis of AVM vessels with either a ligand-directed or a nonligand strategy, endothelial surface molecules that highly discriminate between AVM vessels and normal vessels are required. The differences in molecular characteristics and ultrastructure between AVM vessels and normal vessels have been investigated. Expression of interstitial cell adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin is higher in human AVM endothelium than in normal cerebral vessel endothelium.\(^{55}\) In addition, AVM endothelial cells demonstrate loss of tight junctions, abnormal cytoplasmic processes, and production of Weibel-Palade bodies.\(^{57}\) However, unlike vascular targeting in cancer research, in which inherent differences between tumor and normal endothelial cells are sufficient to enable selective targeting, no marker has been detected in AVMs that would be sufficiently discriminating to allow production of thrombosis within AVM vessels only.\(^{28,48,49,55–58}\)

We have proposed that stereotactic radiosurgery could be used as a priming technique, selectively altering the surface molecule expression of the endothelial cells in AVMs without affecting normal brain vessels.\(^{27,48,55}\) In this paradigm, radiosurgery is not being used as the main treatment modality; it is simply being used to create discriminating endothelial changes within AVM endothelial cells.

In a previous study, we showed that radiosurgery and vascular targeting can induce selective intravascular thrombosis in an animal model of AVM.\(^{47}\) In that study, we used a nonligand approach and administered LPS/sTF systemically 1 day after radiosurgery. Among its many effects, LPS directly stimulates endothelial expression of tissue factor, interstitial and vascular cell adhesion molecules, and E-selectin.\(^{6,7,24,37}\)

Tissue factor expression induced by LPS is insufficient to initiate coagulation; however, the coadministration of sTF, which binds to the endothelial surface after association with coagulation factor VIIa, allows the surface density of
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tissue factor to reach sufficient levels to initiate thrombus formation. This effect is also dependent on the endothelial cell membrane altering its structure so that phosphatidylserine is on the external surface.8 Externalization of phosphatidylserine occurs in tumor vessels, which allows the LPS/sTF treatment to produce thrombosis selectively in those vessels. It has been shown that radiation causes phosphatidylserine externalization in lung endothelium,26 and we have shown that radiosurgery causes externalization of phosphatidylserine in the endothelial cells in the AVM vessels (J. M. Fairhall et al., personal communication, 2010).

The previous study showed that radiosurgery with vascular-targeting therapy is feasible, but that study did not address the durability of the induced thrombosis. The results of the study reported here demonstrate that the induced thrombosis is durable, and even increases, up to at least 3 months after treatment. The highest thrombosis rates were observed for rats in the LPS/sTF+RTX group. Thrombosis was localized to vessels within the tissue volume treated with radiosurgery.

Thrombosis did occur in rats in the LPS/sTF–RTX group. It is possible that sufficient endothelial changes are caused by the shear stress from the high blood flow in these vessels23 to enable the LPS/sTF treatment to induce thrombosis. Among rats in this group, thrombosis durability appeared less robust; rates of vessel occlusion at the later time points were lower.

As with the earlier study, the radiosurgery and vascular-targeting treatment successfully created small vessel thrombosis but did not result in occlusion of larger vessels. This finding accounts for the lack of blood flow changes detected by using Doppler flow measurements and angiography. Possible explanations for the lack of large vessel thrombosis include a difference in the endothelial response to radiation and, more likely, sufficiently high flow through these vessels to prevent thrombus formation.

This work supports the concept of radiosurgery and vascular targeting as a possible new treatment method for AVMs. We are currently working to identify other discriminating endothelial surface molecular changes that could be targeted by using a ligand-based technique. Although the nonligand approach reported here produced significant thrombosis, using LPS in humans raises safety concerns. A ligand approach has many theoretical advantages, including the ability to use different effector moieties and the ability to attach multiple effector molecules to a single antibody. Using nanoparticles containing large concentrations of an effector moiety attached to a targeting antibody is an attractive approach that is worth exploring.

In this study, we did not achieve complete occlusion of the model AVM, and large vessels were not thrombosed. In addition to using a ligand-based treatment, other possible methods to overcome these limitations include increasing the dose of the targeting agent, using multiple treatments, increasing the effector concentration by using nanoparticle technology, and combining the vascular-targeting treatment with endovascular techniques.

Conclusions

The results of this study support the concept of using radiosurgery as a primer to enable vascular-targeting treatment for AVMs. Durable microvessel thrombosis was achieved after radiosurgery and administration of LPS/sTF. The thrombosis was localized, and no systemic effects were seen. These findings confirm that the effect of radiosurgery used to induce thrombosis can be enhanced and localized by using vascular-targeting agents. Although more work is needed before human clinical trials can be conducted, confirmation of this proof of principle warrants investigation of more vascular-targeting agents.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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Author contributions to the study and manuscript preparation include the following. Conception and design: Smee, Stoodley. Acquisition of data: Reddy. Analysis and interpretation of data: Reddy. Drafting the article: Duong, Reddy. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Duong. Statistical analysis: Reddy, Fairhall. Administrative/technical/material support: Duong. Study supervision: Stoodley.

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