Radiosurgery inhibition of the Notch signaling pathway in a rat model of arteriovenous malformations

Laboratory investigation

JIAN TU, M.D., Ph.D.,1 YANG LI, Ph.D.,1 ZHIQIANG HU, M.D., Ph.D.,2 AND ZHONGBIN CHEN, Ph.D.3

1Australian School of Advanced Medicine, Macquarie University, Sydney, New South Wales, Australia; 2Department of Neurosurgery, the 9th Medical Clinical College of Beijing University; and 3Department of Electromagnetic and Laser Biology, Beijing Institute of Radiation Medicine, Beijing, China

Object. Notch signaling has been suggested to promote the development and maintenance of arteriovenous malformations (AVMs), but whether radiosurgery inhibits Notch signaling pathways in AVMs is unknown. The aim of this study was to examine molecular changes of Notch signaling pathways following radiosurgery and to explore mechanisms of radiosurgical obliteration of “nidus” vessels in a rat model of AVMs.

Methods. One hundred eleven rats received common carotid artery–to–external jugular vein anastomosis to form an arteriovenous fistula (AVF) model. Six weeks postoperatively, dilated small vessels and capillaries formed a nidus. The rats with AVFs received 25-Gy radiosurgery. The expression of Notch1 and Notch4 receptors and their ligands, Delta-like1 and Delta-like4, Jagged1, Notch downstream gene target HES1, and an apoptotic marker caspase-3 in nidus vessels in the AVF rats was examined immunohistochemically and was quantified using LAS-AF software at 7 time points over a period of 42 days postradiosurgery. The interaction events between Notch1 receptor and Jagged1, as well as Notch4 receptor and Jagged1, were quantified in nidus vessels in the AVF rats using proximity ligation assay at different time points over 42 days postradiosurgery.

Results. The expression of Notch1 and Notch4 receptors, Delta-like1, Delta-like4, Jagged1, and HES1 was observed in nidus vessels in the AVF rats pre- and postradiosurgery. Radiosurgery enhanced apoptotic activity (p < 0.05) and inhibited the expression of Notch1 and Notch4 receptors and Jagged1 in the endothelial cells of nidus vessels in the AVF rats at 1, 2, 3, 7, 21, 28, and 42 days postradiosurgery (p < 0.05). Radiosurgery suppressed the interaction events between Notch1 receptor and Jagged1 (p < 0.001) as well as Notch4 receptor and Jagged1 (p < 0.001) in the endothelial cells of nidus vessels in the AVF rats over a period of 42 days postradiosurgery. Radiosurgery induced thrombotic occlusion of nidus vessels in the AVF rats. There was a positive correlation between the percentage of fully obliterated nidus vessels and time after radiosurgery (r = 0.9324, p < 0.001).

Conclusions. Radiosurgery inhibits endothelial Notch1 and Notch4 signaling pathways in nidus vessels while inducing thrombotic occlusion of nidus vessels in a rat model of AVMs. The underlying mechanisms of radiosurgery-induced AVM shrinkage could be a combination of suppressing Notch receptor signaling in blood vessel endothelial cells, leading to a reduction in nidus vessel size and thrombotic occlusion of nidus vessels.

(http://thejns.org/doi/abs/10.3171/2013.12.JNS131595)

Key Words • arteriovenous fistula • arteriovenous malformation • endothelial cell • Notch signaling pathway • stereotactic radiosurgery • vascular disorders

RADIOSURGERY has been used to manage patients with cerebral arteriovenous malformations (AVMs) for more than 4 decades. It offers an important option for patients with AVMs that are unsuitable for resection or embolization. Studies reveal that radiosurgery induces thrombosis in AVM vessels, and by 2–3 years approximately 75% of AVM vessels are completely obliterated. Although overexpression of endothelial adhesion and inflammatory and thrombotic molecules has been reported in irradiated endothelial cells in an AVM model in rats, the underlying molecular signaling network remains largely unknown. A growing body of

Abbreviations used in this paper: AVF = arteriovenous fistula; AVM = arteriovenous malformation; BSA = bovine serum albumin; CCA = common carotid artery; EJV = external jugular vein; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NICD = Notch intracellular domain; PLA = proximity ligation assay; RBP-J kappa = J-kappa recombination signal-binding protein; VEGFR = vascular endothelial growth factor receptor.

This article contains some figures that are displayed in color online but in black-and-white in the print edition.
Evidence has supported that activated Notch signaling plays a pivotal role in the development and maintenance of AVMs in humans.\textsuperscript{43,70} Activation of Notch1 or Notch4 in mice causes AVM-like abnormalities.\textsuperscript{17,21,42} Recently, normalization of Notch4 has been suggested as a strategy to reduce blood vessel size in a mouse model of AVMs.\textsuperscript{41}

The Notch1 receptor is expressed in both vascular endothelial cells and smooth muscle cells, while the Notch4 receptor is expressed primarily in endothelial cells.\textsuperscript{46} Notch ligands Delta-like1, Delta-like4, and Jagged1 are also expressed in both vascular endothelial cells and smooth muscle cells.\textsuperscript{34,66} Both Notch receptors and their ligands are transmembrane proteins; therefore, signaling is restricted to neighboring cells. Notch signaling between endothelial cells and smooth muscle cells occurs via cell-to-cell communication and has critical influences on vascular development, including proliferation, migration, angiogenic processes, and arterial-venous differentiation.\textsuperscript{2,17,23,38} Although the intracellular transduction of the Notch signal is remarkably simple, with no secondary messengers, this pathway functions in an enormous array of developmental processes. Defects in Notch signaling cause abnormal vascular development.

The specific roles of individual Notch receptors and their ligands in human vascular homeostasis are not well known. The majority of knowledge implicating Notch signaling in vessel homeostasis and development has arisen through gain and loss of function studies in mice.\textsuperscript{14,36} Observations in mice suggest that Notch1 plays a role in angiogenesis\textsuperscript{40} and AVM pathogenesis.\textsuperscript{43} Notch4 is involved in the initiation and maintenance of arteriovenous communications,\textsuperscript{32} although Notch4 is not critical to vascular development, but shares functional redundancy with Notch1 in vascular development.\textsuperscript{34} Delta-like1 is suggested to be critical to vascular maturation and vessel integrity.\textsuperscript{18} Delta-like4 plays a critical role in early vascular remodeling, arterial and venous specialization, and Notch1-mediated signaling in early vascular development.\textsuperscript{11,12} Jagged1 contributes to vascular maturation and plays a distinct role in Notch1 signaling.\textsuperscript{15,68} The direct targets of Notch signaling remain vague. Notch expression activates transcription of HES family genes and subsequently results in repression of HES target genes,\textsuperscript{24} many of which are tissue specific transcriptional activators.\textsuperscript{33} Thus, Notch activation of HES can modulate cellular differentiation. It has been reported that the Notch signaling pathway responds to the Notch1 activator by increased angiogenesis and Jagged1 inhibitor by reduced angiogenesis in adult rats.\textsuperscript{26} However, the knowledge of how the Notch signaling pathway responds to radiosurgery in AVM vessels remains absent in the literature. This study was undertaken to examine whether radiosurgery inhibits endothelial Notch signaling and molecular mechanisms of radiosurgical obliteration of “nidus” vessels in a rat model of AVMs.

**Methods**

**Cell Viability Assessment**

To evaluate the effect of radiation on cell viability, bEnd.3 cells were examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Roche Applied Science) assay following irradiation as previously reported.\textsuperscript{19,33} Briefly, bEnd.3 cells (ATCC) were seeded in 96-well plates at a density of 8 × 10\textsuperscript{3} cells/well for 24 hours and were then exposed to radiation delivered by an orthovoltage x-ray generator (Philips RT100, Royal Philips Electronics) with a dose of 25 Gy. Sham controls were treated identically but were not exposed to radiation. The MTT was prepared at 5 mg/ml in RPMI1640 phenol red free medium. The assay activity was determined at 6, 12, 24, 72, 120, and 168 hours after irradiation and was performed in triplicate and repeated 6 times. The viability of cells in irradiation sham controls was considered as 100%.

**Rat AVF Model**

Animal experimentation was approved and performed in accordance with the guidelines of the institutional experimental animal care and ethics committee, *Guide for the Care and Use of Laboratory Animals* (Institute for Laboratory Animal Research, National Research Council. Washington, DC: National Academy Press, 1996), and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. An AVF was created in 111 Sprague-Dawley male rats (7 weeks old, weight 230 ± 9 g) by an anastomosis of the left common carotid artery (CCA) to the left external jugular vein (EJV) as shown in Fig. 1 and as previously reported.\textsuperscript{69} The rats were allowed to acclimatize to their new surroundings before the experiment began. The surgical procedures were not performed in the presence of other rats. General anesthesia was induced using a mixture of 4% isoflurane and oxygen (2 L/min) via a nose cone. The depth of anesthesia was assessed using the respiratory rate and by checking hindlimb withdrawal to pain. No procedure was commenced until there was a consistent absence of response to pain. A heating blanket was used for the duration of the procedure.

The procedure was performed in a sterile field using aseptic technique. The left CCA was exposed, and blood flow was measured through the CCA using a 1-mm Doppler ultrasonic probe (Transonic Systems). The left EJV was then exposed and ligated with 10-0 nylon suture at its junction with the subclavian vein. An aneurysm clip was placed across the rostral EJV. Microclips were also applied proximally and distally on the CCA, and a small arteriotomy was made on the lateral aspect. An end-to-side anastomosis of the EJV to the CCA was performed using a continuous 10-0 nylon suture. The clips were sequentially removed from the EJV, distal CCA, and proximal CCA. Blood flow was measured through the proximal CCA and the vein using 1- and 2-mm Doppler ultrasonic probes (Transonic Systems). The wound was closed, and isoflurane was turned off, allowing the animal to breathe oxygen until the time of awakening. Once awake, the animal was placed in an individual cage and housed singly for 1 week postoperatively. Observations were carried out daily for the 1st week and then weekly thereafter. Observations included weight, assessment of motor function, behavior, and wound health. Six weeks after surgery, di-
1). There was no evidence of significant morbidity.

lated small vessels and capillaries formed a “nidus” (Fig. 1). There was no evidence of significant morbidity.

### Radiosurgery Dosimetry

The planning of radiosurgery in the rat AVF model was performed 6 weeks after the surgical creation of the AVF. Anesthesia for radiosurgery was initially induced with a mixture of isoflurane (4%) and oxygen (2 L/min) and maintained with an intramuscular injection of ketamine and midazolam. Twenty minutes prior to the procedure, ketamine (40 mg/kg) and midazolam (1.5 mg/kg) were given intramuscularly. Once the righting response was lost, the rats were given another dose of ketamine (20 mg/kg) and midazolam (0.75 mg/kg) to ensure an adequate duration of anesthesia. While sedated, the rats breathed oxygen (2 L/min) via a nose cone. The depth of anesthesia was assessed using the respiratory rate and by checking the hindlimb withdrawal to pain. The respiratory and heart rates were routinely observed. The rats remained sedated for 40 minutes.

Once the rat was sedated, the location of the AVF was identified using Doppler ultrasound (SonoSite). The surgical wound was reopened to expose the AVF, nidus, and surrounding structures. Microclips were placed across the distal fistula and the proximal and distal CCA, after which 1 ml of Omnipaque contrast agent (GE Healthcare) was injected into the AVF. The rat was placed on a custom-made stage attached to the head ring of the X-Knife linear accelerator (Radionics), and CT angiography was performed. The contrast agent–filled AVF was visualized on CT angiography, and its 3D location in a coordinate system defined by the head ring was determined. According to the alignment of the AVF at the intersection of the 3 laser beams, radiation arcs delivered a dose of 0, 5, 15, 25 or 50 Gy of gamma irradiation to the AVF with the 90% isodose line encompassing the AVF. Fifteen rats with AVFs were randomly divided into 5 groups, and 3 rats with AVFs per group received a dose of 0, 5, 15, 25, or 50 Gy. The delivery of radiation took 30 minutes. The wound was closed, and the rats were kept in a quiet area until recovery. The rats were returned to the holding facility, and there were no reports of death.

Three days postradiosurgery, the rats were killed, and their AVFs, nidi, and surrounding tissue were collected and embedded in tissue freezing medium (ProSciTech) with liquid nitrogen for immunohistochemical analysis of caspase-3 and Notch1 and Notch4 receptors. Compared with the fluorescent intensity of caspase-3 at 0 Gy, caspase-3 intensity increased by 1.4% ± 0.2% (p < 0.01), 17% ± 0.9% (p < 0.01), 28% ± 2% (p < 0.01), and 21% ± 2% (p < 0.01) at 5, 15, 25, and 50 Gy, respectively. The expression of Notch1 was downregulated by 6% ± 1% (p < 0.05), 32% ± 4% (p < 0.01), 61% ± 5% (p < 0.01), and 49% ± 4% (p < 0.01) at 5, 15, 25, and 50 Gy, respectively. The expression of Notch4 was inhibited by 1% ± 0.3%, 27% ± 2% (p < 0.01), 26% ± 1% (p < 0.01), and 9% ± 2% (p < 0.05) at 5, 15, 25, and 50 Gy, respectively. These preliminary data suggested a maximal dose of 25 Gy to the AVF suitable for irradiation of the rat AVF model. Twenty-five Gy is the same dose that a patient would receive to his/her AVM.

### Irradiation of the Rat AVF

Six weeks after surgery, radiosurgery was performed under anesthesia as previously reported. The AVF rats were randomly divided into radiation and sham-radiation control groups. Once the AVF rat was sedated using intraperitoneal ketamine and midazolam, the animal was placed on the stage attached to the head ring of the X-Knife linear accelerator (Radionics). The fistula was located using Doppler ultrasound (SonoSite; Fig. 1D) and positioned at the intersection of the 3 targeting laser beams that designate the center of the radiation delivery arcs. The AVF rats received a dose of 25 Gy to the AVF, while contralateral CCAs and EJVs received a dose of 2–4 Gy. The delivery of radiation took 30 minutes. The
sham-radiation controls were treated identically but did not receive radiation.

**Histology and Immunocytochemistry**

Rats were anesthetized and perfused with 4% paraformaldehyde. Specimens, including carotid-jugular anastomosis, arterialized feeding vein, nidus, and draining vein, were embedded in paraffin for histology or in tissue freezing medium (ProSciTech) with liquid nitrogen for immunohistochemistry and proximity ligation assay. Hematoxylin and eosin staining was performed as previously described. Immunohistochemistry was performed as previously described. Briefly, sections were washed, and nonspecific binding was blocked by 10% horse serum. Anti–rat primary antibody, Notch1 (dilution 1:50, R&D Systems), Notch4 (dilution 1:50, Cell Signaling), Delta-like1 (dilution 1:500, Rockland), Delta-like4 (dilution 1:500, Rockland), HES1 (dilution 1:200, Abcam), caspase-3 (dilution 1:500 Abcam), or CD31 (dilution 1:1000, Abcam) was applied and incubated at 4°C overnight. Slides were washed and incubated with Alexa Fluor 488–conjugated goat anti–rabbit immunoglobulin G (dilution 1:800, Molecular Probes) or Alexa Fluor 594 goat anti–mouse immunoglobulin G (dilution 1:800, Molecular Probes) secondary antibody for 2 hours in the dark, and examined using a confocal microscope (Leica SP5). Images were obtained using a confocal microscope (TCS SP5 X, Leica). Z-Stacks were composed of 6 consecutive images with a total Z volume of 12 μm.

**Proximity Ligation Assay**

Proximity ligation assay (PLA) was applied to examine the interaction between the Notch receptor and its ligand. All reagents used for the PLA were purchased from Olink Bioscience. The in situ PLA was performed according to the manufacturer’s instructions. Briefly, tissue sections were permeabilized in 0.2% TX-100, 0.5% bovine serum albumin (BSA) in phosphate-buffered saline, and then blocked in 10% BSA in phosphate-buffered saline and incubated with anti–rat primary antibodies, Notch1 and Delta-like1, Notch1 and Delta-like4, Notch1 and Jagged1, Notch1 and HES1, Notch4 and Delta-like1, Notch4 and Delta-like4, Notch4 and Jagged1, or Notch4 and HES1. Duolink MINUS and PLUS conjugated secondary antibody incubation, ligation, and amplification steps for PLA were performed as suggested by Olink using 40–μl volumes. Following amplification, slides were washed for 10 minutes in Olink Buffer B, pH 7.5, followed by a 10-minute wash in 0.5% BSA. Fluorescent images were obtained using a confocal microscope (TCS SP5 X, Leica). Z-Stacks were composed of 6 consecutive images with a total Z volume of 12 μm.

Images captured for PLA events were analyzed using Leica LAS AF software (version 2.4.1, Leica Microsystems GmbH). First, Z-stack images were converted into maximum representations. Three polygon regions of interest were drawn evenly along the vessel lumen 5 μm into the tunica intima as to envelop the vessel’s endothelium. Three more circular regions of interest were evenly placed within the tunica media. The regions of interest were between 0.5 and 1.0 mm² in size. The positive PLA events were observed as fluorescent particles (2–50 pixels in diameter). When PLA events merged to create particles larger than 50 pixels, the area was measured, and the number of events was assumed to be the particle area divided by 10 since 10 pixels were the median size of most PLA events. The number of fluorescent spots obtained from PLA in regions of interest were automatically quantified and recorded. A threshold of 100 (gray values) was set for a positive signal prior to signal counting. To account for nonspecific signals, “background” signal per square millimeter values for each specimen’s endothelial and medial regions of interest were generated from each specimen’s negative control and then subtracted from each respective regions of interest signal per square millimeter value. The number of PLA events was assessed by 2 observers blinded to the sample nature.

**Statistical Analysis**

Data are expressed as the mean ± SEM (number of experiments). The statistical difference between groups was determined using the unpaired 2-tailed t-test. When there were more than 2 groups, differences were analyzed using ANOVA if the variances were equal and the Mann-Whitney nonparametric test if variances were unequal. Linear regressions were calculated using a statistical computer package, (Number Cruncher Statistical Systems). A value of p < 0.05 was considered statistically significant.

**Results**

**Cell Viability Postradiosurgery**

Radiation effect on bEnd.3 cell viability was assessed as shown in Fig. 2. The cell viability of sham irradiation controls was considered as 100%. Following a single dose of 25-Gy irradiation, the cell viability was significantly reduced compared with sham irradiation controls at 6-, 12-, 24-, 48-, 72-, and 120-hour time points. The cell viability was recovering over a period of 168 hours postradiosurgery. There was a positive correlation between the cell viability and time required for recovery (r = 0.85, p < 0.04).

**Radiosurgery Induces Apoptosis**

Caspase-3 is selected as a marker for apoptosis. The levels of caspase-3 expression in nidus vessels in the rats with AVFs over a period of 42 days postradiosurgery at 25 Gy are shown in Figs. 3 and 4. There was significant upregulation of caspase-3 expression in nidus vessels after irradiation. The levels of caspase-3 overexpression were 72% at 1 day postirradiation and peaked at 154% at 21 days after radiation (p < 0.01). The level of caspase-3 expression was negatively correlated to the cell viability at 1 and 3 days postradiosurgery as shown in Figs. 2 and 4.
Radiosurgery inhibition of Notch signaling pathway

Expression of Notch Receptors and Their Ligands in the Rat AVF Model

CD31 was selected as a marker for the endothelium of nidus vessels, and its expression is shown in Fig. 3. The expression of Notch1 receptor, Notch4 receptor, Jagged1, Delta-like1, Delta-like4, and HES1 were predominantly expressed in the endothelium of nidus vessels (Fig. 3).

Radiosurgery Inhibits Notch Receptors

CD31 was expressed postradiosurgery (Fig. 3). The expression of Notch1 receptor was significantly downregulated postradiosurgery (Fig. 3). The levels of Notch1 expression decreased over a period of 42 days postradiosurgery and by 68% on Day 42 (p < 0.01). There was a negative correlation between the levels of Notch1 expression and time over the first 3 days postradiosurgery (r = -0.99, p < 0.009; Fig. 4). The expression time course of Notch1 was different from that of cell viability as shown in Fig. 2. The expression time course of Notch1 was also different from those of caspase-3 and CD31 as shown in Fig. 4.

Prior to radiosurgery, the level of Notch4 receptor expression was greater than that of Notch1 expression (p < 0.01; Fig. 4). The expression of Notch4 was significantly downregulated postradiosurgery (Fig. 4). The levels of Notch4 expression were declined over a period of 42 days postradiosurgery. The lowest level of Notch4 expression was observed on Day 28 postradiosurgery and was reduced by 60% compared with the level prior to irradiation (p < 0.01). The expression time course of Notch4 was different from that of Notch1 (Fig. 4).

Radiosurgery Inhibits Notch Receptor Ligands

The expression of Jagged1 was significantly decreased at every time point over a period of 42 days postradiosurgery (p < 0.002; Fig. 4). The lowest level of Jagged1 expression was observed on Day 1 after irradiation, which reduced by 85% compared with that of the sham radiation controls (p < 0.001). The expression of Delta-like1 was significantly decreased on Days 1 and 28 postradiosurgery (p < 0.05; Fig. 4). The expression of Delta-like4 was significantly decreased on Days 1 and 28 postradiosurgery (p < 0.05; Fig. 4). The expression time-course of Jagged1, Delta-like1, and Delta-like4 were similar to that of Notch4.

Radiosurgery Inhibits HES1

HES1 represents the overall activity of Notch signaling. The levels of HES1 expression were varied over a period of 42 days postradiosurgery. On Days 1 and 28 after irradiation, the levels of HES1 expression were significantly decreased compared with the sham radiation controls (p < 0.001). This phenomenon was also observed in the expression time courses of Notch4 and Jagged1 and Delta-like1 and Delta-like4 (Fig. 4), suggesting that the activated Notch4 signaling pathway responded to radiosurgery in a greater degree than that of Notch1 signaling pathway.

Radiosurgery Inhibits Interaction Between Notch Receptors and Their Ligands

Based on the results obtained from Radiosurgery Inhibits Notch Receptor Ligands and Radiosurgery Inhibits Notch Receptors (Fig. 4), confirmation of interaction events between Notch receptor1 or 4 and Jagged1 was performed using in situ proximity ligation assay. Proximity ligation assay revealed that the number of interaction events between Notch1 receptor and Jagged1 or Notch4 receptor and Jagged1 in the irradiated AVM vessels was significantly lower than the number of interaction events between Notch1 receptor and Jagged1 or Notch4 receptor and Jagged1 in the sham-radiation controls over a period of 42 days postradiosurgery (Figs. 5 and 6; p < 0.001).

Radiosurgery Induces Thrombosis in Nidus Vessels in the Rat AVF Model

Compared with the sham radiation controls, radiosurgery-induced thrombus formation was observed in small vessels (< 50 µm in diameter) and medium vessels (50–200 µm) of nidus vessels in the AVF rats (Fig. 7). The thrombi formed were sustainable, and the percentage of fully occluded AVM vessels increased with time postradiosurgery (r = 0.93, p < 0.001).

Discussion

The postradiosurgical responses of human AVM endothelial cells appear to be highly heterogeneous, which results in variable outcomes. Radiosurgically induced AVM vaso-occlusion could occur shortly after irradiation or as late as 5 years, or it may never occur by the end of the follow-up period. Studies suggest that radiosurgery-related vasculopathy in AVMs includes stenosis and thrombosis, altered gene expression of endoglin and endothelial nitric oxide synthase, and inflammation. However, the mechanism remains a mystery. In this study, we compared the changes of Notch signaling before and after radiosurgery in an AVF model in rats. We found that radiosurgery inhibits the interaction events between Notch receptors and their ligands, resulting in a significant decline of Notch signaling in nidus vessels in the rat AVF model. The mechanisms of radiosurgery-
induced AVM shrinkage could be a combination of suppressing Notch signaling in blood vessel endothelial cells and thrombotic occlusion of AVM vessels.

Notch Signaling in the Rat AVF Model

The pathogenesis of AVMs was related to the alteration of molecular signaling pathways that regulate vascular homeostasis. Notch signaling pathway is hypothesized to contribute to AVM pathogenesis via abnormal regulation of vascular development and maintenance. The activated Notch signaling pathway has been found to induce the formation of arteriovenous lesions in mice. Notch signaling activation was ubiquitous in that activation was observed in both Notch1 and Notch4 via interaction with their ligands Delta-like1, Delta-like4, and Jagged1. The expression of Notch1, Notch4, Delta-

**Fig. 3.** Molecular changes in the nidus vessel wall pre-and post-radiosurgery at 25 Gy. The intensity of immunofluorescence of caspase-3 staining was increased in vessels post-radiosurgery, suggesting elevated apoptotic activity. CD31 stained positively, indicating intact endothelium. The intensity of immunofluorescence of Notch1 and -4 receptors, their ligands Jagged1 and Delta-like1 and -4, and Notch downstream target HES1 declined in vessels 7 days post-radiosurgery. L = lumen. Bar = 50 μm.
Radiosurgery inhibition of Notch signaling pathway

like1, Delta-like4, and Jagged1 and Notch downstream target HES1 was observed in the endothelial cells of nidus vessels in the rat AVF model. The expression of multiple receptors and ligands indicates that activated Notch1 and Notch4 signaling pathways interact with one another. HES1 is a downstream target of both activated Notch1 and Notch4 signaling pathways, and its expression in the endothelial cells of nidus vessels in the AVF rats indicates overall activation of Notch signaling in both pathways.

Notch1 signaling was activated through interaction with Delta-like1, Delta-like4, and Jagged1 in the endothelial cells of AVMs. Evidence obtained from loss of function studies supports a critical function of Notch1- and Notch4-mediated signaling in vascular maintenance, and

Fig. 4. The intensity of immunofluorescence of caspase-3, CD31, and Notch1 and -4 receptors, their ligands Jagged1 and Delta-like1 and -4, and Notch downstream target HES1 in nidus vessels was quantified using a confocal microscope over a period of 42 days postradiosurgery. Data are expressed as the mean ± SEM of 4 rats at each time point. *p < 0.05 paired comparison between pre- (-1 day) and postradiosurgery at different time points.
disruption of Notch1 results in vascular immaturity and hyperplasia, and even death, due to vascular complications. Activation of Notch1 signaling has been reported to result in vessel enlargement and arteriovenous communication. Notch4 signaling was also activated through interaction with Delta-like1, Delta-like4, and Jagged1 in the endothelial cells of an AVM model. Previous studies have demonstrated that increasing Notch4 activation in the mouse vasculature produces dilated vessels and reduces smooth muscle cell populations and arteriovenous communications within the cerebral circulation. Studies have also demonstrated that the cessation of Notch4 activation stops the progression of vascular abnormalities and promotes reversion to the normal vasculature. The observed expression of Notch1 and Notch4 activation in the endothelial cells of nidus vessels in the rat AVM model suggests the observed expression’s similarity to the mouse model of AVMs.

The interaction between the Notch receptor and its ligand in vascular development and homeostasis has yet to be fully characterized. Notch ligands are known to display different binding affinities and suborgan patterns of expression. It would appear that ligand-specific function is dictated by the geographic location of the receptor. Delta-like1 is expressed in the venous and arterial vasculature during angiogenesis. Inactivation of Delta-like1 has been observed to impact the overall strength and integrity of the vascular wall. It has been hypothesized that activation of Notch signaling through Delta-like1 might be associated with the abnormal vascular maturation and arteriovenous specification. Delta-like4 is expressed throughout the development of both venous and arteries. Delta-like4 mimics the expression of Notch1 and has been suggested to be the primary activator of Notch1 during angiogenesis. Inactivation of Delta-like4 has been shown to disrupt remodeling of the vascular plexus with complications in the organization of the vascular bed, vessel diameter, arterial branching, and arteriovenous communication, and inhibition of vessel sprouting during angiogenesis. Jagged1 is expressed throughout the development of vasculature. Inactivation of Jagged1 results in insufficient remodeling of the vascular plexus with loss of vascular integrity and a depleted smooth muscle cell population. Jagged1 is involved in insufficient homeostatic maintenance of the tunica media in AVM pathogenesis. Notch activation controls endothelial cell behavior via which receptor-ligand interaction is modified independent of transcriptional regulation, posttranslational modification, and cellular trafficking. Briefly, Notch ligands on the signal-sending cell trigger the Notch1 or Notch4 receptor on the adjacent
Radiosurgery inhibition of Notch signaling pathway

signal-receiving cell, leading to sequential receptor cleavages within the transmembrane domain, resulting in the release of the Notch intracellular domain (NICD). The NICD moves into the signal-receiving cell nucleus and binds to transcriptional factor J-kappa recombination signal-binding protein (RBP-J kappa). Association of NICD and RBP-J kappa replaces the corepressor with a coactivating complex containing Mastermind-like protein and activates the transcription of target genes such as HES.

The activated Notch signaling downregulates vascular endothelial growth factor receptor (VEGFR)–2 and upregulates VEGFR1, leading to cell differentiation during angiogenesis.10,14,44 In this study, the expression of Notch1 and Notch4 receptors, Jagged1, Delta-like1, Delta-like4, and HES1 suggests that upregulation of Notch signaling is occurring via a “universal” modulator that does not discriminate between ligand or receptor type.

A question that pertains to the implication of Notch signaling in the rat AVF model is whether Notch activation is due to angiogenesis or is a secondary effect of endothelium response to an altered state of hemodynamic stress following AVF formation. Vascular endothelial growth receptor was upregulated in the nidus vessels of the rat AVF model over a period of 84 days after the creation of an AVF,27 suggesting that angiogenesis occurred. In the same AVF model, the blood flow through the carotid-jugular fistula increased from 12 ml/min to 161 ml/min over a period of 84 days post-AVF formation.62 In the current study, examination of nidus vessels in the same AVF model revealed that Notch signaling was activated in the endothelial cells of AVM-like vessels. It is possible that an increased hemodynamic stress after AVF formation induces the activation of Notch signaling, contributing to the angiogenesis of an AVM nidus. Human cerebral AVMs exhibit an altered state of hemodynamic stress,40 which induces changes in endothelial cell and smooth muscle cell signaling profiles. Therefore, the AVF rat model has a “nidus” with endothelial molecular changes similar to those observed in human AVMs, supporting its use as a model for studying Notch signal responsiveness of radiosurgery.

**Radiotherapy Inhibits Notch Signaling in the Rat AVF Model**

We found that the expression of Notch molecules and the interaction between the Notch receptor and its ligand were decreased in irradiated AVFs. An obvious question was whether reduced expression of Notch molecules and their function are due to decreased cell viability and/or elevated apoptosis. The reduction in cell viability was observed in the first 5 days after irradiation; however, it was reversible. Cell damage recovered with time, and a full recovery was evidenced 7 days after radiosurgery. Low cell viability may partially explain radiation-induced Notch inhibition in the first 5 days but is unlikely to explain Notch suppression over a period from Day 7 to Day 42 postirradiation. Increased apoptosis was reflected by elevated caspase-3 activity. Caspase-3 is responsible for partial or total proteolytic cleavage of many key proteins needed for cell survival. Increased caspase-3 activity was observed in the endothelium of AVF vessels exposed to radiation. However, it is not clear whether the inhibition of Notch signaling was the primary cause or a consequence of activated apoptosis. Quillard et al.52 reported that inflammatory cytokines elicit a switch in Notch expression characterized by Notch2 predominance over Notch4, leading to reduced Notch activity and promoting apoptosis. Overexpression of Notch2 and silence of Notch4 induces the cleavage activity of caspase-3, indicating that both events are proapoptotic in vascular endothelial cells. Ding et al.9 also reported that puromycin aminonucleoside activates Notch signaling and induces apoptosis in renal proximal tubular cells. It has been confirmed that inactivation of Notch signaling protects neuronal cells from apoptotic death and further brain injury following ischemic stroke.3 There is a positive correlation between Notch signaling and apoptosis. Thus, the inhibition of Notch signaling is the biological response to radiosurgery, not the secondary negative effect of apoptosis.
This study shows that both Notch1 and Notch4 signaling pathways were downregulated by radiosurgery. The Notch signaling pathway is heavily regulated by factors that modulate Notch pathway transcription, post-translational modification, and signal transmission.22 The Notch signaling pathway is modulated by ubiquitin ligases,20,45,56,57 glycosyltransferases,1,6,39 and γ-secretase coactivators.5,55 Since the Notch pathway is under the regulation of these contributory factors, the presence or absence of a pathway modulator could be the limiting factor of pathway activation and affects signaling interaction events between the Notch receptor and its ligand. Radiosurgery could have a direct or indirect effect on Notch molecules and/or their modulator(s), resulting in inhibition of Notch signaling pathways.

The activity of HES1 represents overall Notch1 and Notch4 signaling,21 which was downregulated by radiosurgery. Loss of function in the Notch pathway usually results in abnormal vascular development.26,30 Embryo homozygous with double mutations of Notch1 and Notch4 showed a more pronounced phenotype than the Notch1 null mutant.28 Although there is no obvious phenotype in Notch1–/– mice, endothelial cell–specific expression of constitutively activated Notch4 causes AVM-like vascular abnormalities in adult mice.7,42 Elevated Notch4 activity in mice did not affect the absolute number of endothelial cells but increased the ratio of arterial to venous endothelial cells.23 Notch4 activation also results in a negative correlation between enlargement of the microvasculature and capillary density.42 Endothelial Notch1 activation reduces capillary density by inhibition of sprouting angiogenesis.13 These observations coincide with the primary characteristic of enlarged and tangled vessels in AVMs. It is generally considered that AVMs are congenital abnormalities that fail to regress;26 however, suppressing the Notch4 transgene could result in reprogramming arterial endothelial cells in the enlarged AVM vessels to a venous endothelial cell specification, leading to a decrease in AVM vessel size.41 In addition to radiosurgery inducing thrombotic occlusion of AVM vessels, this study suggests that radiosurgery could activate reprogramming of vascular endothelial cells of AVMs by a decrease in Notch1 and Notch4 signaling.

Radiosurgery Induces Thrombosis in the Rat AVF Model

A likely mechanism of radiosurgery-induced thrombus formation is that radiation induces apoptosis of AVM endothelial cells to externalize phosphatidylserine from the inner leaflet of the endothelial cell membrane lipid bilayer. Exposed phosphatidylserine binds to soluble tissue factor factor in blood circulation, triggering the coagulation cascade.4,55 Phosphatidylserine–tissue factor initially forms a complex with factor VIIa. The factor VIIa–tissue factor complex catalyzes the activation of factor IX and factor X, leading to the conversion of prothrombin to thrombin, and thence the production of fibrin from fibrinogen. The fibrin thrombi cause vessel occlusion in AVMs. The fibrin thrombi result in sustainable vessel occlusion in the nidus of the AVF rat model for a 90-day follow-up period (J Tu, unpublished data).

Study Limitations

While we reported interesting findings related to the mechanisms of radiosurgery-induced AVM shrinkage, there were a number of limitations in our study. First, the observations were obtained from a rat AVF model rather than from humans as no AVM specimen can be ethically obtained from patients who respond well to radiosurgery. Second, our findings in the rat AVF model have yet to be confirmed in a separate model as it is technically challenging to deliver radiosurgery to a small size transgenic mouse model of AVMs. Third, partial vaso-occlusion does not reduce the risk of hemorrhagic stroke due to AVMs.

Conclusions

Radiosurgery may trigger reprogramming of endothelial cells in AVM vessels by inhibiting Notch1 and Notch4 signaling pathways while inducing the formation of thrombi. The former mechanism induces AVM shrinkage, and the latter mechanism causes thrombotic occlusion of the lumina.

Disclosure

This work was partially supported by a China–New South Wales collaborative research program grant to J.T., Z.C., and Z.C. and a postgraduate scholarship for overseas study from Shanghai Jiao Tong University, Shanghai, China to Y.L. The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Tu. Acquisition of data: all authors. Analysis and interpretation of data: Tu, Li. Drafting the article: Tu. 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: Tu. Statistical analysis: Tu, Li. Administrative/technical/material support: all authors. Study supervision: Tu.

References


Acknowledgments: Tu. Analysis and interpretation of data: Tu, Li. Administrative/technical/material support: all authors. Study supervision: Tu.

Author contributions to the study and manuscript preparation include the following. Conception and design: Tu. Acquisition of data: all authors. Analysis and interpretation of data: Tu, Li. Drafting the article: Tu. 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: Tu. Statistical analysis: Tu, Li. Administrative/technical/material support: all authors. Study supervision: Tu.
Radiosurgery inhibition of Notch signaling pathway


