Rat model of pulmonary arteriovenous malformations after right superior cavopulmonary anastomosis

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PULMONARY ARTERIOVENOUS MALFORMATIONS (PAVMs) are a cause of progressive cyanosis in children after the anastomosis of the superior vena cava to the pulmonary artery (bidirectional cavopulmonary anastomosis) often causing significant morbidity in these children. The bidirectional cavopulmonary anastomosis provides excellent hemodynamic palliation for single ventricle physiology; however, the durability of this palliation is limited due to the likelihood of developing PAVMs and progressive cyanosis. There is evidence that the development of PAVMs represents a form of abnormal angiogenesis that occurs due to the absence of hepatic-derived factors directly perfusing the lungs when the superior vena cava provides the sole source of pulmonary blood flow (2, 5, 10, 11); however, the exact etiology of these lesions remains unknown. Research into this phenomenon would be facilitated by the development of a suitable animal model. We have developed a small animal model of unilateral PAVMs following a right-sided superior cavopulmonary anastomosis in rats, and we have studied these animals with angiography and microvessel density determination to confirm the validity of this model. We found this model to be valid by successfully reproducing the human condition angiographically and histologically.

MATERIALS AND METHODS

Operative technique. Eight male Sprague-Dawley rats (200–300 g) underwent a right superior cavopulmonary anastomosis (classic Glenn shunt) allowing the left (unshunted) lung of each animal to serve as a control for all subsequent studies. Animals were anesthetized with 50

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mg/kg of pentobarbital sodium intraperitoneally, and a left internal jugular catheter was placed for intravenous fluid administration. After endotracheal intubation, animals were ventilated with a high-frequency oscillating ventilator (Sensormedics model 3100A, Sensormedics; Yorba Linda, CA). A right anterolateral thoracotomy was performed, and the right lung was retracted inferiorly (Fig. 1A). With the use of an operating microscope (Nikon model SMZ-10, Nikon; Tokyo, Japan), the right superior vena cava was mobilized, and the azygous vein was divided. The superior vena cava was then clamped and divided after ligation of the atrial end with 8-0 polypropylene suture. The right pulmonary artery was mobilized from the main pulmonary artery to the hilum, clamped, and divided after ligation of the proximal end with 8-0 polypropylene suture (Fig. 1B). An end-to-end anastomosis was then performed between the superior vena cava and distal right pulmonary artery by using interrupted 10-0 nylon suture (Fig. 1C). After the clamps were removed, the chest was closed, and a single chest tube was placed in the right hemithorax until the animal was extubated. Postoperatively, the animals were given rat chow and water ad libitum. During the first postoperative week, animals were given lactated Ringer solution in place of water, and acetaminophen was administered for pain.

Animals were euthanized at time points between 2 and 13 mo postoperatively (2 mo, 1 animal; 4 mo, 1 animal; 8 mo, 2 animals; 11 mo, 1 animal; 12 mo, 2 animals; and 13 mo, 1 animal). Animals were first anesthetized with 50 mg/kg pentobarbital sodium intraperitoneally, and bilateral pulmonary angiography was performed through catheters in the left and right superior venae cavae. The animals were then euthanized, and the lungs were removed and immediately processed for histologic examination. This project received institutional review and approval (Animal Care and Use Committee, Children's Hospital and Regional Medical Center, Seattle, WA), and all animals received humane care in compliance with The Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Publication No. 86-23).

**Immunohistochemistry.** Lung sections were immediately fixed in 4% neutral buffered formalin and embedded in paraffin. Five-micrometer sections were cut in a coronal plane from matching levels along the longitudinal axis of the lower lobe from each shunted and control lung. Sections were rehydrated through graded alcohols, and endogenous peroxidase was quenched with 3% hydrogen peroxide for 15 min. To improve antigen retrieval, sections were microwaved for 10 min in citrate buffer, pH 6.0. After being allowed to cool for 30 min, sections were washed in PBS (pH 7.4). A rabbit polyclonal antibody against an endothelial specific antibody [von Willebrand factor (vWF, Dako; Carpinteria, CA)] diluted 1:250 in 1% bovine serum albumin/PBS was applied to sections. After incubation for 60 min in a humidity chamber, a goat anti-rabbit secondary antibody (1:500) was applied for 30 min, followed by avidin biotin enzyme complex (both from Vector Laboratories; Burlingame, CA). Slides were developed with diaminobenzidine tetrahydrochloride (Sigma; St. Louis, MO) and counterstained with methyl green.

**Microvessel density.** Ten high-power fields (×400) from each microscopic section were imaged with a digital camera and digitizing software (Leica D-200 camera and Leica DC digitizing software, version 3.0, Leica Mikroskopie und Systeme; Wetzlar, Germany) from the shunted and non-shunted lungs from each animal. The examined fields were from matching areas obtained from the periphery of the midportion of shunted and control lungs. The microvessel density of each specimen was then determined by identifying vessels staining positively for vWF by using an automated image analyzer (Image-Pro Plus, Media Cybernetics; Silver Spring, MD).

**Statistical analysis.** Student's t-test was used to compare microvessel densities between the right lung (ipsilateral to the cavopulmonary anastomosis) and left lung (control) for animals at each time point. Simple linear and quadratic regression models were applied to evaluate the association between time following the Glenn shunt and the ratio of microvessel density between the right lung and control lungs. All statistics were carried out with JMP statistical software, version 4.0 (SAS Institute; Cary, NC).

**RESULTS**

Before the 11-mo time point, pulmonary angiography revealed no appreciable differences between the animals' shunted and control lungs. Pulmonary angiography at 11 mo after surgery and at all later time points demonstrated findings typical of PAVMs in the lung ipsilateral to the cavopulmonary anastomosis (abnormal capillary phase with a coarse and dilated appearance of the microvasculature), whereas the contralateral lung was normal (Fig. 2). Microvessel den-
sity was greatly increased in the shunted lung compared with the control lung in all animals beyond the 4-mo time point (Fig. 3). Figure 4 presents representative sections stained for vWF from a shunted lung and a control lung from the same animal, demonstrating greatly increased small blood vessels extending far into the periphery of the shunted pulmonary parenchyma. A strong linear relationship was found between the time following surgery and the magnitude of increase in microvessel density between the shunted and control lungs for each animal (Fig. 5). Inclusion of a quadratic term worsened the overall fit of the model; therefore, the simple linear model is reported. For each month following the Glenn shunt, there was a 29% increase (95% confidence interval, 17–41%) over baseline in the ratio of shunted to control pulmonary microvessels \( (P = 0.003) \) with an \( R^2 \) value of 0.79.

**DISCUSSION**

The development of PAVMs after cavopulmonary anastomosis is a well-recognized clinical phenomenon that has not been attributable to a precise etiology (6–9). These lesions may arise due to an angiogenic stimulus resulting from the absence of hepatic venous effluent directly perfusing the pulmonary arterial circulation (3, 5). However, palliated single-ventricle physiology is complex, and multiple other factors that are present in these children may contribute to PAVM development. Specifically, hypoxia and nonpulsatile pulmonary blood flow have been implicated as contributing to PAVM formation (4). The successful development of an animal model would allow the etiology and pathophysiology of this phenomenon to be systematically approached. This report details our attempt to develop a small animal model of this phenomenon.
Animal model considerations. There are several attributes that a successful animal model for any human condition should possess. The ideal animal model should be inexpensive, easy to maintain, hardy enough to withstand experimental manipulation, and successful in its reproduction of the human pathology under study. The rat model that we have created fulfills these criteria. Rats are inexpensive and easy to maintain, which is especially important to minimize per diem animal care costs for a process that requires prolonged survival as in the present case. Sprague-Dawley rats are particularly hardy and are able to tolerate the extensive surgical procedure required for a cavopulmonary anastomosis. These animals recover quickly and almost immediately demonstrate minimal lasting effects from the surgical procedure. In addition, vast numbers of reagents are available for molecular analysis for use in the rat to enable the further study of PAVM development. Performing a unilateral cavopulmonary anastomosis facilitates this process, allowing each animal to serve as its own control by comparison with the nonshunted lung.

Angiography. The ultimate goal for any experimental model, however, is its faithful reproduction of the human condition under study. This rat model demonstrates the same angiographic and histologic features demonstrated by children with PAVMs following cavopulmonary anastomosis. As in humans, there is a time-dependent angiographic evolution of these lesions. Before 11 mo, angiographic evidence of PAVMs was consistently absent, whereas angiographically apparent PAVMs were present in all of the animals at this time point or beyond. In addition, these animals demonstrated the same angiographic features that typify PAVMs in humans, namely, an abnormal capillary phase with coarsening and dilatation of the peripheral pulmonary microvasculature.

Microvessel density. Sensitive diagnostic tools demonstrate evidence of right to left shunting in the lungs of children after cavopulmonary anastomosis much earlier than the development of angiographic changes. For example, Bernstein and co-workers (1) detected significant right to left shunting by contrast echocardiography, possibly through early PAVMs, as soon as 6 wk after cavopulmonary anastomosis. We demonstrated the histologic correlate to this clinical finding in an analysis of peripheral lung biopsies in children after cavopulmonary anastomosis by determination of the microvessel density in these specimens (11). Microvessel density is a known marker of angiogenesis that has been used extensively in the oncologic literature to document the metastatic potential of tumors (12, 13). Early after bidirectional cavopulmonary anastomosis, children demonstrate increased microvessel density compared with controls even in the absence of
angiographically evident PAVMs. The results of that clinical study confirmed the angiogenically active nature of PAVMs and confirmed that proliferative vascular changes can be detected microscopically long before changes in angiography.

In the present study, we used microvessel density to determine whether this animal model demonstrated a similar early increase in microvessel density preceding angiographically apparent PAVMs. In this small animal model, increased microvessel density appeared in the shunted lung for the first time between 4 and 8 mo after surgery. As in the human condition, this occurred before angiographic evidence of PAVMs, which occurred for the first time 11 mo after surgery. A strong positive correlation was evident between the length of time after cavopulmonary anastomosis and the microvessel density, which demonstrated progressive increases in magnitude at each further time point after the performance of the shunt. In addition, the microscopic appearance of these lesions was similar to that of humans with greatly increased numbers of small blood vessels that extended far into the periphery of the pulmonary parenchyma (2).

As with any biological system in whole animals, there was significant variability in microvessel density between each animal. In fact, the largest microvessel density in either shunted or control lungs was observed in the animal euthanized 2 mo after cavopulmonary anastomosis. This large variability between animals is another feature that makes the use of the contralateral lung as a control an attractive approach. Despite the large variation between animals, when the ratio of the microvessel density of the shunted lung to the control lung was plotted versus time, a nearly linear relationship was found. The use of the nonshunted lung as an internal control for each animal allowed the time-dependent increase in microvessel density to be demonstrated for this model.

Study limitations. This rat model differs from the human condition in a number of ways. First, these are young adult rats that have not possessed single ventricle physiology before the creation of the cavopulmonary anastomosis. Second, the pulmonary vasculature of infant humans who have single ventricle physiology and chronic cyanosis might be expected to respond differently after cavopulmonary anastomosis than the pulmonary vasculature in these rats. Finally, large animal models might allow the performance of a cavopulmonary anastomosis in immature animals; however, large animal models such as sheep or calves have a limitation in the number of reagents that are available for molecular analysis of this lesion. From a practical standpoint, large animal models are substantially more expensive to maintain. The creation of a surviving animal model with single ventricle physiology, followed by the performance of a cavopulmonary anastomosis in the same animal, will probably be prohibitively difficult in an animal of any size. The fact that these animals develop PAVMs that appear to be angiographically and histologically the same as those seen in the human condition suggests that the absence of hepatic venous effluent directly perfusing the pulmonary arteries is of central importance regardless of the underlying cardiac physiology.

In addition to differences in physiology, important differences exist in the anatomy of this small animal model compared with humans. Specifically, rats possess bilateral superior vena cavae; however, the presence of a left superior vena cava probably allows these animals to survive after cavopulmonary anastomosis. Pressure in the superior vena cava is always higher after a bidirectional cavopulmonary anastomosis, sometimes prohibitively so, which may result in the development of a “superior vena cava syndrome.” This can be a significant problem that requires expert intensive care unit management postoperatively. If the rats possessed a single superior vena cava, the acute rise in superior vena cava pressure postoperatively might cause fatal cerebral edema; a problem for which there are relatively few therapies in this simple model. The fortuitous presence of the left superior vena cava in rats allows the cerebral veins to decompress and minimizes the occurrence of cerebral edema. Another problem from bilateral superior vena cavae could potentially arise if the left-sided superior vena cava provided a collateral source of hepatic venous effluent to reach the right-sided superior vena cava and thereby the right lung. This is unlikely to occur in this model; however, in that after a superior cavopulmonary anastomosis, superior vena cava pressure is higher than pressure in the inferior vena cava and hepatic veins. In this model, lower pressure hepatic venous effluent in collateral venous channels should not flow toward the higher pressure in the right superior vena cava and right lung.

In summary, we have successfully created a small animal model of PAVMs developing after cavopulmonary anastomosis that faithfully reproduces the human condition. The importance of this study is the demonstration of the validity of this animal model that possesses the same time-dependent angiographic and histologic features as the human condition. As such, this may represent a new tool that provides an opportunity to study molecular mechanisms involved in the development of this condition. Most importantly, this model may be used to test the effects of inhibitors of angiogenesis that may eventually lead to the development of medical therapy for this difficult problem.

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