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Pulmonary Angiography for the Diagnosis of Thromboembolic Events in the Non-Human Primate

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Abstract

Background—Evaluating possible thromboembolic events in the non-human primate has traditionally required euthanasia, significantly limiting the ability to conduct longitudinal studies. We hypothesized that pulmonary angiography could offer a safe, reproducible, and non-lethal means to assess for pulmonary embolus in the non-human primate.

Methods—Eleven rhesus primates were studied using standard pulmonary angiography techniques. Five animals studied had previously received humanized anti-CD154 antibodies (associated with thromboembolism risk) in the context of skin transplantation 2 years before the angiography study. Four primates were studied after receiving mouse anti-human CD154 antibody following allogeneic islet or skin transplantation.

Results—Angiography was successful in all primates. We observed no complications, and all animals promptly recovered from the procedure. Angiographic findings consistent with thromboembolism were demonstrated in the three primates actively receiving anti-CD154 antibody and in one primate that last received anti-CD154 nearly 2 years before the study. The study was normal in both the streptozotocin-induced diabetic control animals. Histopathology of the lungs confirmed thrombus in two of the four primates, but no thromboembolus was identified in the other two. The first had limited pathologic evaluation without fine slices, and in the second (treated 2 years before with a humanized anti-CD154), ascariasis was found in the area identified as abnormal by the angiogram.

Conclusions—Minimally invasive pulmonary angiography is a safe, reproducible, and inexpensive method to assess possible thromboembolic events in the non-human primate. This method may allow for the longitudinal assessment of non-human primates given novel agents that may promote thromboembolism.

When evaluating a new biologic agent, especially ones thought to predispose to thromboembolic events, a reliable means for identifying those events in vivo is much needed. Assessing thromboembolic events in primates has traditionally required that the animal be euthanized for detailed postmortem analysis. This approach means that early thromboembolic events may be missed. For instance, an animal euthanized months after the experimental agent was administered may have allowed for a vessel occluded from a prior clot to have recanalized. In clinical practice, pulmonary angiography is the safe, “gold standard” for the diagnosis of

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pulmonary emboli (1). The aim of this study was to assess feasibility of this procedure in non-human primates. We successfully performed a percutaneous pulmonary angiography upon 11 rhesus macaques and now describe both the pulmonary angiography procedure and our findings in the 11 animals.

Our laboratory is interested in studying ways to promote immunologic tolerance. Monoclonal antibodies (e.g., anti-CD154 and anti-CD40) and receptor-based fusion proteins (e.g., CTLA4-Ig) represent promising agents in the quest for immunologic tolerance toward a health restoring allograft or against a tissue or organ undergoing autoimmune attack (2). Although these agents have appeared safe and effective in animal models, some (e.g., anti-CD154) have been associated with a high frequency of thromboembolic events in early clinical trials (3,4). The thromboembolic complications reported after the initial use of many antibodies largely disappeared when more highly purified antibody preparations were administered (3). Non-human primates are highly relevant for the study of such novel therapeutic agents because of their close phylogenetic relationship to humans (5,6) and because many of the newer immunomodulatory agents (antibodies and receptor fusion proteins) are specific for human epitopes that will cross-react with the corresponding primate epitopes (7,8). We have therefore adapted pulmonary angiography for use in the non-human primate.

MATERIALS AND METHODS

The procedures described in this study were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals*, Institute of Laboratory Animals Resources, National Research Council, DHHS, Pub. No. (NIH) 86–23 (1985), and were approved by the Animal Care and Use Committee of the National Institute of Health (NIH).

Animals

Rhesus macaques (Table 1) were obtained through the NIH primate facility in Poolesville, MD. The animals (n=11) were from 2 to 8 years old and ranged in weight from 3.7 to 7.7 kg. Five of the animals had previously been given streptozotocin (a pancreatic beta cell toxin) to induce diabetes, and three of these five were given a subsequent islet transplant followed by a murine anti-CD154 monoclonal antibody to prevent islet allograft rejection. The other six primates had received skin allografts 5 to 24 months before, followed by treatment with various anti-CD154 antibodies. The two diabetic primates that were not given an islet transplant served as controls for this study because these primates received no immunomodulatory agents.

For the angiography, the non-human primates were anesthetized with a mixture of ketamine hydrochloride (7 mg/kg i.m.), xylazine (6 mg/kg i.m.) (Moby Corporation, Animal Health Division, Shawnee, KS), and atropine (0.04 mg/kg i.m.) (Elkins-Sinn, Inc, Cherry Hill, NJ.). A 22-gauge, 1-inch angiocath (Becton Dickinson, Infusion Therapy Systems Inc., Sandy, UT) was inserted into the saphenous vein for hydration with normal saline or 5% dextrose. In addition, the animals were given intravenous cefazolin sodium 400 mg (Ancef, SmithKline Beecham Pharmaceuticals, Philadelphia, PA) and 44,000 IU/kg i.m. of a long-acting penicillin (AMBI-PEN, G. C. Henford Mfg. Co., The Butler Co., Columbus, OH). Propofol injectable emulsion 1% (Gensia Sicor Pharmaceuticals, Inc., Irvine, CA) was administered intravenously if needed to allow for intubation with a 4- or 5-mm endotracheal tube (Mallinckodt Critical Care, Glens Falls, NY). Tears Renewed (Akkorn, Buffalo Grove, IL), an ophthalmic lubricant, was placed in the conjunctival sac to prevent corneal drying. Anesthesia was maintained with Isoflurane (0.5–1.5%) (Aerrane, Anaquest, Madison, WI) and oxygen at a total flow rate of 2 L/min.

The inguinal areas and upper rear legs were clipped, and monkeys were positioned in a supine position. The aseptic field was scrubbed with Alcare (Steris Corporation, St. Louis, MO) and

then sterilely draped. Using real time ultra-sound guidance (Sonosite Inc., Bothell, WA), the common femoral vein (CFV) was identified, evaluated for patency (compression and color doppler), and then accessed percutaneously using the sterile modified Seldinger technique (9). Materials included the following: a 4Fr micropuncture introducer set (Cook Incorporated, Bloomington, IN) containing a 21G, 7-cm long micropuncture introducer needle, a stainless steel/nitinol 0.018-inch, 40-cm wire with a platinum tip, and a 4Fr coaxial catheter 10-cm long; a 0.035-inch, 140-cm long Bentson wire (Cook Incorporated, Bloomington, IN); a 0.035-inch 150-cm long angled glide wire (Boston Scientific, Watertown, MA); and a 4Fr 70-cm long pediatric pigtail catheter (Cook Incorporated, Bloomington, IN). The pigtail catheter was advanced under fluoroscopy over the Bentson wire using stiff guide wire technique, directing the catheter and positioning it in the main pulmonary artery. This technique is well known to most interventional radiologists (10) and involves a 180° bend at the stiff end of a heavy duty guide wire that is inserted into a pigtail catheter while in the right atrium of the heart, thereby providing a 60° to 70° deflection of the catheter and easier placement of the latter into the right ventricle of the heart. Once the pigtail catheter is in the right ventricle, the stiff end of the wire is removed, and the soft end is inserted up to the pigtail. While holding on the wire in place, the pigtail catheter is rotated counter-clockwise and advanced into the main pulmonary artery. Further selective catheterization of the upper and lower branches of the left and right pulmonary arteries with the 4Fr pigtail catheter was achieved using a Benson or a glide wire. The monkeys were momentarily disconnected from the ventilator during the angiographic run.

Approximately 4 cc of nonionic, iso-osmolar full strength, non-dilute contrast material (Isovue 300, Bracco diagnostics Incorporated, Princeton, NJ) was used per single digital hand injected run. Digital subtracted images (4/sec) of the upper lobes arterial vasculature were obtained using one view (P-A), and of the mid to lower lobes arterial vasculature using two or three views (P-A, right and/or left obliques). The pigtail catheter was then withdrawn over a Benson or a glide wire and moderate external pressure over the CFV was applied for 20 minutes. We then applied a pressure dressing over the access site for an additional 30 minutes. All radiographs were reviewed in digital format as a cine loop and individual frames by at least two radiologists.

RESULTS

Pulmonary angiography was performed successfully in all 11 non-human primates with no complications and produced images satisfactory to exclude large or segmental pulmonary emboli. In one monkey, we found angiographic evidence for bilateral segmental acute pulmonary emboli to the lower lobes (Fig. 1A and B), and in two other monkeys, we found evidence for unilateral segmental lower lobe acute pulmonary emboli. Three of the four animals with positive angiographic findings were given an anti-CD154 antibody within the preceding 2 months. In one of the four animals, no additional immunomodulatory agent was given and we repeated the pulmonary angiogram 6 weeks later, demonstrating evidence for recanalization (Fig. 1C). Five non-human primates were given a skin allograft 5 to 24 months before the pulmonary angiography, and these animals were treated with either humanized or mouse anti-human anti-CD154 antibody after their skin graft (11). All had received anti-CD154 antibody for 1 month after skin graft transplantation, and these five animals' pulmonary angiograms were all read as negative.

Three non-human primates with pulmonary angiograms read as positive were killed for histologic examination. In two of the three, a pulmonary embolus was confirmed (Fig. 2). In one, the pathologist did not recognize a pulmonary embolus, possibly because of undirected lung sampling.

One primate (primate number 580) (Table 1) underwent repeat angiography 21 months after the initial negative one, revealing an abrupt change in caliber in the artery supplying the right

lower lobe medial basal segment. The cut-off, and the few attenuated vessels distal to that region, suggested the possibility of old pulmonary embolus with partial recanalization. This primate was killed 1 week later, and careful necropsy failed to demonstrate a thrombus, but rather the pathologist identified findings consistent with chronic lung mite lesions (ascariasis) and perivascularitis in the lung region identified angiographically.

DISCUSSION

Pulmonary angiography is the gold standard for the diagnosis of pulmonary emboli in humans (1). We are now the first to report the technique in the non-human primate model. Our data suggests that angiography may be used accurately and safely in primates and, as such, allows for an easy, reproducible, and inexpensive method to evaluate and study thromboembolic side effects of certain monoclonal antibodies. Although primate experiments are appropriately tightly regulated, experiments in primates are critical as agents designed to induce immunologic tolerance are tested.

Several factors necessitate the use of primates in studies designed to test for immunologic tolerance (7,12-15):

1. Rodent models are too permissive. That is, many tolerance techniques that prove robust in rodent models fail when tested in higher species like non-human primates and humans.
2. Many antibodies being developed for human clinical testing cross-react with the analogous epitopes in non-human primates but not with corresponding epitopes from other less closely related species.
3. Primate models allow observations regarding adverse effects like delayed wound healing and susceptibility to infection (16,17).
4. The non-human primate genes encoding MHC proteins targeted during rejection are well conserved between non-human primates and humans (18-20).

Before our report, only euthanizing the primate and examining the pulmonary vessels and lung using both gross and histologic means could diagnose thromboembolic events. Apart from being a terminal and costly procedure, we suggest the current approach may be inaccurate. Even in humans, pulmonary emboli are difficult to diagnose, and considerable data suggest that the entity remains quite under-diagnosed (1). One reason is that autopsy based diagnosis may be difficult to differentiate from artifactual *in situ* thrombosis. In addition, pulmonary emboli may remain sub-clinical. Further, many consider histologic diagnosis of pulmonary emboli more sensitive early after the event since recanalization may occur with time. Finally, scrupulous inspection by the pathologist is required because an otherwise nondirected lung survey may overlook a small embolus.

Our study has several limitations. The five primates given a skin allograft and the humanized anti-CD154 months earlier had negative angiographic studies. Anti-CD154 antibodies have been reported, however, to be associated with acute thromboembolic events in primates (4). As we studied the primates 5 to 24 months after the last anti-CD154 dose, we cannot rule out a previous thrombosis that may have recanalized by the time the study was conducted. In fact, this may explain why thrombosis was not seen in previous reports describing the long-term success of this agent in primate allograft models (11,12). On the other hand, the pathologist failed to find an embolus in one out of three primates with a positive angiography study. This disparity may be caused by either an overly sensitive angiography or an incompletely sensitive system for identifying emboli at autopsy. Nevertheless, the pulmonary angiography approach we now describe should allow a prospective means for diagnosing pulmonary emboli in non-human primates that does not require euthanasia. That is, no primate found to have a pulmonary

embolus at autopsy had a normal angiogram, so the technique appears sensitive. Our data does raise some question about the test's specificity, however, because one animal with an abnormal pulmonary angiogram had a different diagnosis at autopsy (ascariasis), and the pathologist reported no pulmonary vascular abnormality in another primate with an abnormal angiogram.

Although technical artifacts or iatrogenic catheter thrombus could conceivably simulate a pulmonary embolus, these are rare, and the cases we report do not fit previous descriptions of those confounding diagnoses. One non-human primate with an abnormal angiogram but no evidence of pulmonary artery clot at autopsy warrants further comment. This animal had a normal pulmonary angiogram 4 months after its last dose of humanized anti-CD154, followed by a positive study 2 years later. At autopsy, the pathologist diagnosed pulmonary ascariasis involving the area that appeared abnormal during angiography. Ascariasis of the non-human primate lung may be associated with vessel hyperplasia and thrombus formation (21). Therefore, when interpreting the positive angiographic findings in the non-human primate, one should include ascariasis in the differential diagnosis. When studying new drugs, primates displaying positive findings on pulmonary arteriograms may require a lung necropsy, with the pathologist instructed to carefully look for a thrombus or for local evidence of ascariasis at the area with the positive findings.

Besides pulmonary angiography, transthoracic and transesophageal echocardiography, magnetic resonance angiography, spiral computerized tomography, and ventilation-perfusion lung scanning (22) have all been used to diagnose pulmonary thromboembolic events. Non-human primate pulmonary perfusion scans (23,24) and intraoperative pulmonary arterial angiograms (25) have been reported as techniques useful for the hemodynamic evaluation of xenogeneic heart or lung and allogeneic lung immediately following transplantation. One study of long-term survivors of cardiopulmonary allograft and autograft procedures described a common femoral vein (CFV) or an internal jugular vein (IJV) cut-down technique for the placement of a 5F Swan-Ganz pediatric balloon-tipped catheter, which was advanced into the main pulmonary artery after balloon inflation, under fluoroscopic control. Pulmonary arteriography was performed following measurement of pulmonary pressures (26). To our knowledge, there are no reports of ultrasound and fluoroscopic guided percutaneous selective catheterization of the upper and lower, right and left pulmonary arteries using the sterile modified Seldinger technique in non-human primates. The Seldinger technique enables vascular access without a vascular cut-down and therefore with minimal trauma to the surrounding tissues (9).

CONCLUSION

Minimally invasive pulmonary angiography is a feasible, safe, reproducible, and inexpensive method (approximately \$150.00 of disposable angiographic equipment per procedure) to assess possible thromboembolic events in the non-human primate. We suggest that thin section pathology studies should be performed only in primates with positive findings based upon the screening angiography.

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FIGURE 1.

(A) Primate 3405. Digital subtracted angiographic image of a right side lower lobe arterial embolus (*black arrow*), as demonstrated on an oblique view. (B) Primate 3405. Non-digital subtracted angiographic image of a left side lower lobe arterial embolus (*black arrow*), as demonstrated on a P-A view. (C) Primate 3405. Digital subtracted angiographic image in the P-A view, demonstrating minimal, subtle recanalization of the left side lower lobe arterial embolus.

FIGURE 2.
Lungs of primate 3405, positive for thrombus in the left caudal lung lobe (H&E, 400× magnification).

TABLE 1

Primates Studied: Procedures and Findings

Primate ID	Diabetes induction	Transplant date	Transplant type	Anti-CD154 ab source	Angiography dates	Results	Euthanasia
3405	6/26/02	9/25/02	Islet	Murine	10/24/02 12/18/02	Positive/positive	1/17/03
3051	3/14/02	7/17/02	Islet	Murine	8/13/02	Positive	8/18/02
3288	8/13/02	1/22/03	Islet	Murine	1/17/03 2/12/03	Negative/ positive	2/14/03
2912	1/15/02	None	None	None	2/21/03	Negative	N/A
3378	4/23/02	None	None	None	2/21/03	Negative	N/A
AK6A	N/A	4/5/1999	Skin	Lyophilized 5U8	11/7/01	Negative	N/A
AK4C	N/A	11/8/1999	Skin	Lyophilized 5U8	11/14/01	Negative	N/A
866	N/A	7/24/2001	Skin	Murine	12/10/01	Negative	N/A
AK5X	N/A	11/15/1999	Skin	Lyophilized 5U8	12/19/01	Negative	N/A
580	N/A	8/14/2001	Skin	IDEC 131	12/19/01 7/23/03	Negative/ positive	7/29/03
AK77	N/A	9/1/1999	Skin	Lyophilized 5U8	7/23/03	Negative	N/A