Diffusion of Alexa Fluor 488-Conjugated Dendrimers in Rat Aortic Tissue

BRENDA S. CHO,[a,b] KAREN J. ROELOFS,[b] ISTVAN J. MAJOROS,[c] JAMES R. BAKER, JR.,[c] JAMES C. STANLEY,[b] PETER K. HENKE,[b] AND GILBERT R. UPCHURCH, JR.[b]

[a]*Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI, USA*

[b]*Department of Surgery, Jobst Vascular Research Laboratories, University of Michigan, Ann Arbor, MI, USA*

[c]*Department of Internal Medicine, University of Michigan, Ann Arbor, MI, USA*

ABSTRACT: In this study, the distribution of labeled dendrimers in native and aneurysmal rat aortic tissue was examined. Adult male rats underwent infrarenal aorta perfusion with generation 5 (G5) acetylated Alexa Fluor 488-conjugated dendrimers for varying lengths of time. In a second set of experiments, rats underwent aortic elastase perfusion followed by aortic dendrimer perfusion 7 days later. Aortic diameters were measured prior to and postelastase perfusion, and again on the day of harvest. Aortas were harvested 0, 12, or 24 h postperfusion, fixed, and mounted. Native aortas were harvested and viewed as negative controls. Aortic cross-sections were viewed and imaged using confocal microscopy. Dendrimers were quantified (counts/high-powered field). Results were evaluated by repeated measures ANOVA and Student's *t*-test. We found that in native aortas, dendrimers penetrated the aortic wall in all groups. For all perfusion times, fewer dendrimers were present as time between dendrimer perfusion and aortic harvest increased. Longer perfusion times resulted in increased diffusion of dendrimers throughout the aortic wall. By 24 h, the majority of the dendrimers were through the wall. Dendrimers in aneurysmal aortas, on day 0 postdendrimer perfusion, diffused farther into the aortic wall than controls. In conclusion, this study documents labeled dendrimers delivered intra-arterially to native rat aortas *in vivo*, and the temporal diffusion of these molecules within the aortic wall. Increasing perfusion time and length of time prior to harvest resulted in continued dendrimer diffusion into the aortic wall. These preliminary data provide a novel mechanism whereby local inhibitory therapy may be delivered locally to aortic tissue.

Address for correspondence: Gilbert R. Upchurch, Jr., M.D., University of Michigan Hospital, 2210 THCC, 1500 East Medical Center Drive, Ann Arbor, MI 48109-0329. Voice: 734-936-5790; fax: 734-647-9867.
e-mail: riversu@umich.edu

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BACKGROUND

Local drug delivery has been used to treat many diseases. Local intra-arterial drug delivery is difficult because it may be diluted away from the target site rapidly due to high pressure and flow within the arteries.[1] In one study, extracellular matrix (ECM)-directed local drug delivery was achieved via a polylysine vehicle for treating restenosis in the vessel wall. This was done by maintaining a high concentration of the drug within the target tissue.[2] An alternative drug carrier that has not previously been used to deliver agents to aortic tissue is a poly(amidoamine) (PAMAM) dendrimer. PAMAM dendrimers have been used as carriers for delivering drugs in cancer therapies.[3] PAMAM dendrimers can also be used as imaging agents in humans.[4] Their multifunctional structure makes them appealing for drug delivery applications.[5] In addition, to be useful in biological systems, materials need to be biocompatible and water soluble.[6] Dendrimers are synthetic and very biocompatible with a highly branched structure (FIG. 1).[5] This structure allows for the attachment of multiple agents by covalent linkage or by encapsulation.[4] In this study, dendrimers were acetylated to neutralize some of the primary amine groups and to reduce nonspecific binding. Their shapes and sizes are well defined and uniform, and their surfaces are readily chemically functionalized and modifiable.[5,7]

One potential clinical application for these dendrimers is targeted drug delivery for treating abdominal aortic aneurysms (AAAs). AAAs are the 10th leading cause of death of men and the 14th leading cause of overall death in the United States.[8] Although there are currently no proven effective nonsurgical medical therapies to treat patients with AAAs, doxycycline has been shown to limit the enlargement of experimental AAAs[9–12] and decrease levels of plasma matrix metalloproteinase 9 (MMP-9), an enzyme known to degrade the aortic wall in patients with AAAs.[13] By delivering locally rather than systemically, required doses of compounds such as doxycycline could be reduced, thereby eliminating the risk of unwanted side effects.

In this study, generation 5 (G5) acetylated Alexa Fluor 488-conjugated dendrimers were delivered intra-arterially to native and aneurysmal aortas to examine the diffusion of these labeled dendrimers through the aortic wall.

METHODS

Adult male Sprague–Dawley rats weighing 190–210 g were obtained from Charles River Laboratories (Wilmington, MA, USA) and were used for all experiments in this study. Surgical procedures and experiments were approved

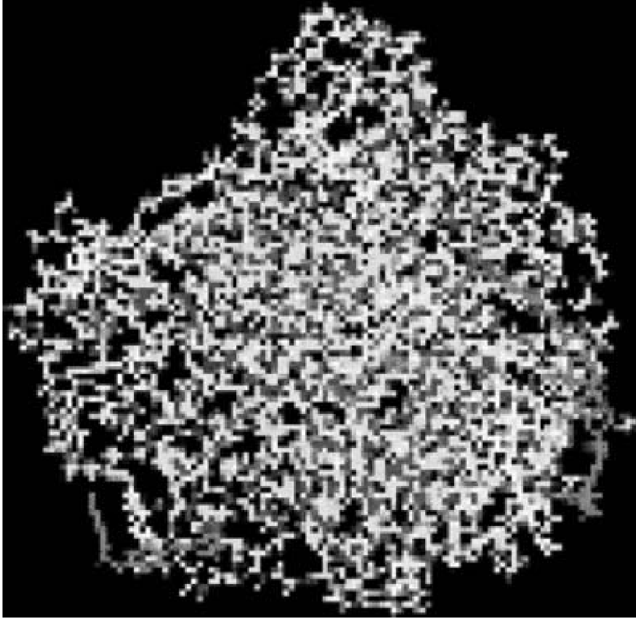

FIGURE 1. Structure of dendrimer. Dendrimer synthesis is attained via stepwise additions and reactions; each layer is referred to as a "generation." In this study, generation 5 dendrimers were partially acetylated and conjugated to Alexa Fluor 488 molecules.[3]

by the University of Michigan Universal Committee on the Use and Care of Animals, per Protocol #8833.

Rodent Elastase Perfusion AAA Model

Rats were anesthetized with 2–3% isoflurane with 100% oxygen. Using sterile techniques, a ventral midline incision was made. The infrarenal abdominal aorta was isolated and perfused with pancreatic porcine elastase as previously described (FIG. 2 A, B).[14] Briefly, the aorta was exposed from the iliac bifurcation (distal) to the level of the left renal vein (proximal), and all side branches within the exposed length of the aorta were tied off with 7–0 silk suture (Ethicon Inc., Somerville, NJ, USA) to ensure pressurization of the aorta. Using 4–0 cotton suture (Genzyme Corp., Fall River, MA, USA) and a vascular clip, temporary distal and proximal aortic control was obtained. A 30-gauge needle was used to make an aortotomy just proximal to the iliac bifurcation. A polyethylene-10 (PE-10) tube was inserted through the aortotomy, and pancreatic porcine elastase (6 units/mL; Lot Number 083K7655; Sigma, St. Louis, MO, USA) was infused into the isolated infrarenal aorta for 30 min. Following perfusion, a 10–0 nylon monofilament suture (Surgical Specialties Corp., Reading, PA, USA) was used to repair the aortotomy in the aorta.

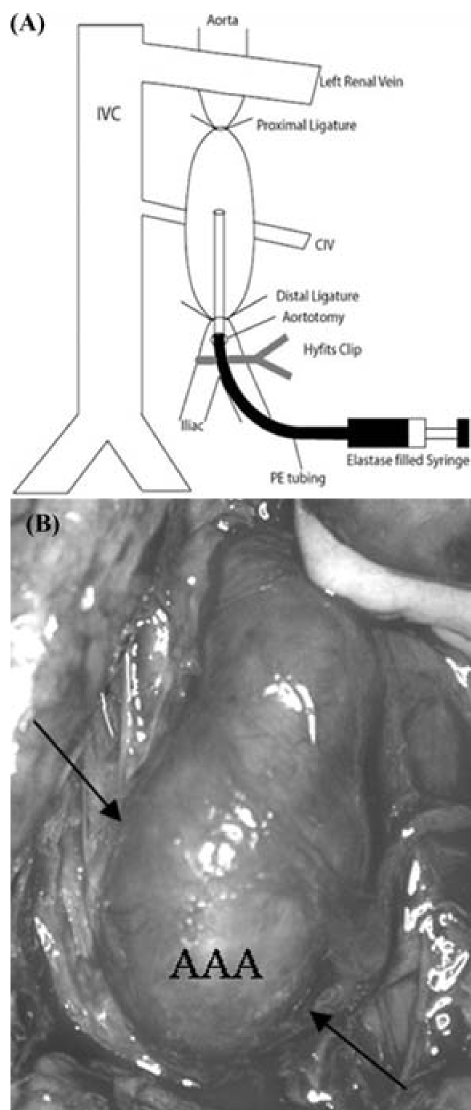

FIGURE 2. (**A**) Schematic of dendrimer and elastase perfusion setup. (**B**) Image of elastase-perfused aortic aneurysm.

Images were taken using a Spot Insight Color Optical Camera (Diagnostic Instruments, Sterling Heights, MI, USA) that was attached to the operating microscope (Nikon, Melville, NY, USA), and aortic diameters were measured using Image Pro Express Software (Media Cybernetics Inc., Silver Spring, MD, USA). To close the abdomen, a 3–0 vicryl suture (Ethicon Inc., Somerville, NJ, USA) was used and the rats were allowed to recover.

Dendrimer Perfusion

The design and synthesis of the G5 acetylated Alexa Fluor 488-conjugated dendrimers (molecular weight of 27,000 g/mol) are described according to Majoros *et al*.[3] To improve water solubility, and decrease aggregation and nonspecific delivery of these dendrimers, the terminal primary amino functionality was acetylated.[15,16] Nonacetylated primary amino groups were used for conjugation of Alexa Fluor 488, an imaging agent.[3,17]

Experiment 1

Rats were anesthetized as described above for elastase perfusion, and underwent infrarenal aortic perfusion with G5 acetylated Alexa Fluor 488-conjugated dendrimers (200 nM in 1X PBS) at a rate of 2 mL/h for varying lengths of time (10, 30, 60 min; $n = 4$ per perfusion time). Upon dendrimer perfusion, blood was allowed to flush out excess dendrimer solution. Aortas were harvested 0, 12, or 24 h postperfusion ($n = 4$ per time point).

Experiment 2

Rats were anesthetized and underwent infrarenal aortic elastase perfusion. Seven days following elastase perfusion, rats underwent aortic dendrimer perfusion as described above. Seven and 8 days postelastase perfusion, rats were anesthetized, infrarenal aortas were exposed, and aortic diameters were measured prior to aortic explantation.

For all experiments, upon harvesting, aortic tissue was rinsed with 1X PBS, pH 7.2–7.4, fixed in formalin (10%) for 1 h at room temperature, and mounted in ProLong Gold Antifade Reagent (Molecular Probes, Eugene, OR, USA). Aortic cross-sections were viewed and imaged using confocal microscopy. Dendrimers were quantified within the aortic wall by manually counting them in captured images, and are presented as dendrimers per high-powered field. Native aortas (i.e., not perfused with dendrimers) were harvested and viewed as negative controls.

RESULTS

Dendrimer Diffusion in Native Aortas

In native aortas, dendrimers penetrated the aortic wall at all time points. For all perfusion times, fewer dendrimers were present as time between dendrimer perfusion and aortic harvest increased (TABLE 1, FIG. 3). Longer perfusion times resulted in increased diffusion of dendrimers throughout the aortic wall. By 24 h, the majority of the dendrimers were through the wall.

TABLE 1. Dendrimer quantification and location in normal aortic tissue

Perfusion time (min)	Time before harvest (h)	Average ± SEM number of dendrimers*	Location of dendrimers
10	0	99 ± 21	Media
10	24	61 ± 4	Mostly media, some adventitia
30	0	71 ± 7	Inner media
30	12	60 ± 4	Adventitia
30	24	35 ± 1	Adventitia
60	0	46 ± 2	Outer media, adventitia
60	12	34 ± 1	Adventitia

*Mean counts from four samples per group.

When native aortas were perfused for 10 min, dendrimers were concentrated in the media (toward lumen) on day 0, and in the media and adventitia 24 h postperfusion. More dendrimers were present on day 0 than 24 h postperfusion. Upon perfusing normal aortas for 30 min with dendrimer, the majority of the dendrimers were in the media on day 0, and in the adventitia 12 h and 24 h postperfusion. There were fewer dendrimers present 12 h postperfusion compared with day 0, and even fewer on 24 h postperfusion. After a 60-min perfusion of normal aortas, the dendrimers were primarily in the media (toward adventitia) and in the adventitia at day 0, and only in the adventitia 12 h postperfusion. Representative images of dendrimer-perfused normal aortas on day 0 are shown in FIGURE 4. Native aortas that were not perfused with dendrimers were harvested and demonstrated no fluorescence.

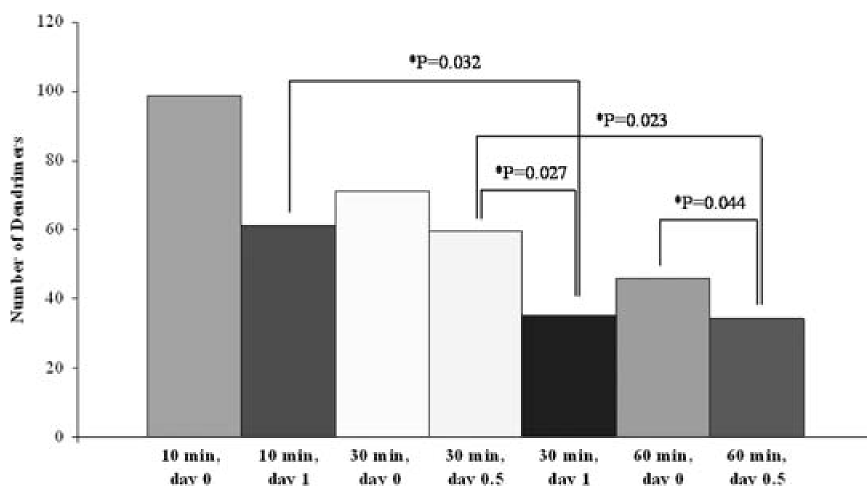

FIGURE 3. Dendrimer quantification in normal aortic tissue.

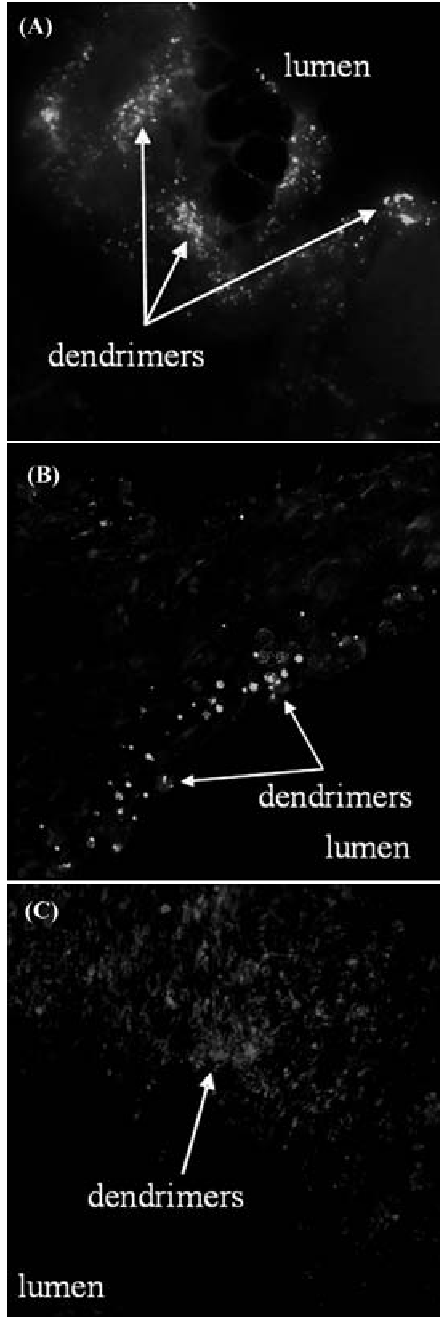

FIGURE 4. Dendrimer-perfused normal aorta on day 0, 10 min perfusion (**A**) day 0, 30 min perfusion (**B**) day 0, 60 min perfusion (**C**) 40×.

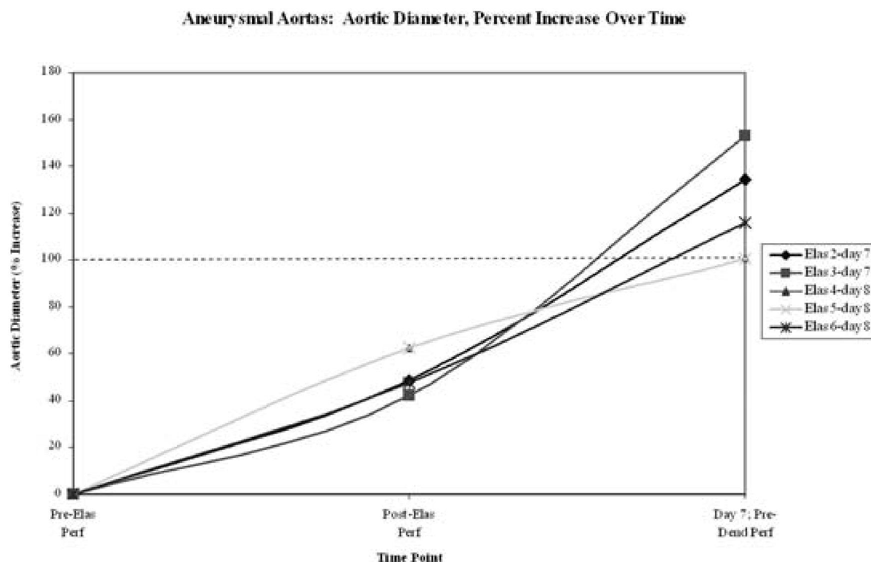

FIGURE 5. Percent increase in aortic diameter in elastase-perfused aortas postperfusion and 7 days following perfusion. Each curve represents one animal. All animals formed AAAs (defined as 100% increase in diameter) on postoperative day 7.

Experimental AAA Formation

The mean percentage increase (±SEM) in aortic diameter in the aneurysmal aortas 7 days following elastase perfusion was 121 ± 10% (FIG. 5). Each animal formed an aneurysm (defined as 100% increase in aortic diameter) by day 7 postelastase perfusion.

Dendrimer Diffusion in Aneurysmal Aortas

In aneurysmal aortas, dendrimers diffused farther into the aortic wall than in control aortas (30 min perfusion time, day 0 harvest). Fewer dendrimers were present in aortas that were harvested 24 h postdendrimer perfusion than when harvested immediately (FIG. 6). By 24 h, the majority of the dendrimers were through the wall.

Upon perfusing aneurysmal aortas (day 7 postelastase perfusion) for 30 min with dendrimer, the majority of the dendrimers were in the adventitia on both days 7 and 8 (postelastase perfusion). At both time points, dendrimers in aneurysmal tissue diffused farther out than in native tissue (TABLE 2). Representative images of dendrimer-perfused aneurysmal aortas on day 0 are shown in FIGURE 7.

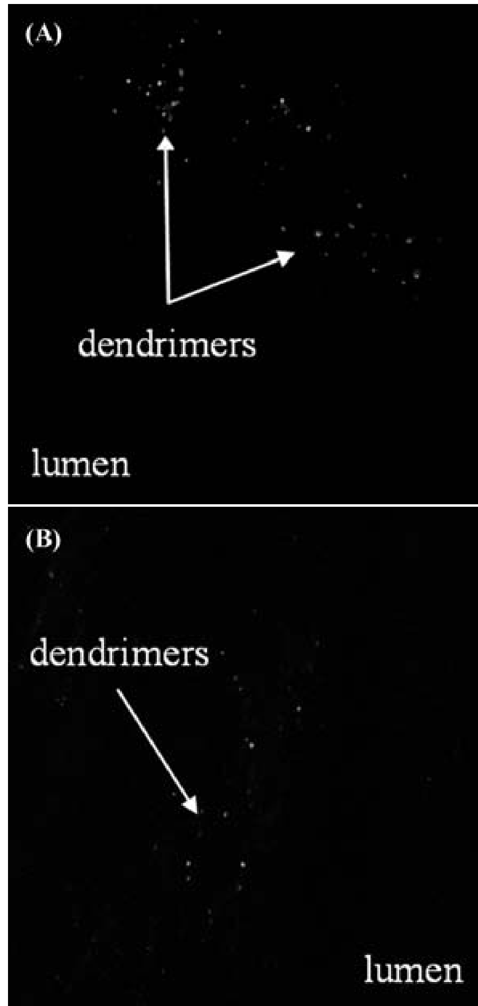

FIGURE 6. Dendrimer-perfused aneurysmal aorta on day 7 postelastase perfusion and harvested on day 7, 30 min perfusion (**A**) and day 8, 30 min perfusion (**B**), 40×.

DISCUSSION

The objective of this study was to determine if dendrimers could be delivered intra-arterially to normal and aneurysmal rat aortas *in vivo*, and to examine the distribution of the labeled dendrimers within the aortic wall. This preliminary study indicates that labeled dendrimers diffuse into the aortic wall from the lumen. These dendrimers are not specific to the aorta, but at early times since they are delivered intra-arterially remain in the aortic wall and do not diffuse

TABLE 2. Dendrimer quantification and location in aneurysmal aortic tissue

Perfusion time (min)	Time before harvest (h)	Average ± SEM number of dendrimers*	Location of dendrimers
30	0	50 ± 13	Adventitia
30	24	34 ± 9	Adventitia

*Mean counts from four samples per group.

out into other tissues. Both normal and aneurysmal aortic tissues are permeable enough to allow for the passage of nanoparticles through the wall. Additionally, varying dendrimer perfusion times, and lengths of time between dendrimer perfusion and explantation affected the concentration gradient-dependent diffusion patterns.

Several factors likely influenced the distribution of the dendrimers, including the pressure within the isolated portion of the aorta during dendrimer perfusion, the resistance of the aortic tissue, and any convective forces present in the aorta. The physical and chemical properties of the dendrimers contribute to their diffusion patterns in the aortic wall. These dendrimers diffuse into the aortic wall because of their small size, and are attractive for local delivery of agents since they are biocompatible and have a highly branched structure, allowing for the attachment of multiple agents.[5] For the present study, dendrimers were acetylated to neutralize some of the primary amine groups. Their shapes and sizes are well defined and uniform, and their surfaces are readily chemically functionalized and modifiable.[5,7] The pressure within the isolated portion of

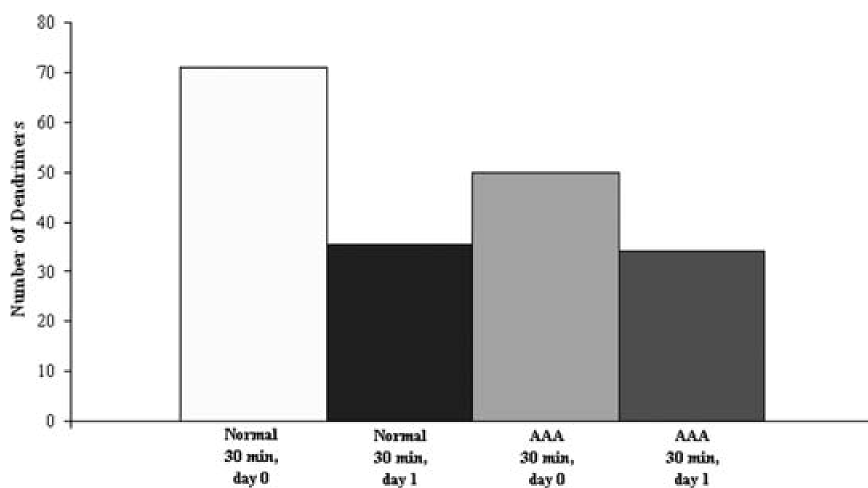

FIGURE 7. Dendrimer quantification in aneurysmal aortic tissue (normal data shown for comparison).

the aorta during dendrimer perfusion and any convective forces present in the aorta were similar in the native and aneurysmal aortas.[18] However, due to the remodeling of the aortic ECM during AAA formation, the change in the resistance of the aortic tissue most likely contributed to the different distribution patterns seen in the normal and aneurysmal tissue samples.

These preliminary results warrant further investigation of the use of dendrimers for targeted drug delivery, especially in the area of AAA, where there are no proven effective medical therapies. Because of their biocompatibility and ability to attach these particles to a wide variety of agents, their potential as drug-carrying vehicles is excellent. The size of these dendrimers also makes them good candidates for delivering these agents. In previous studies, liposomes with a mean diameter of approximately 500 nm represented the maximum size that could be used for drug delivery.[19]

Future experiments may include binding agents to dendrimers, and delivering them intra-arterially to experimental AAAs during development and following formation of small AAAs. Candidate agents include: (1) doxycycline, which has been shown to inhibit formation of experimental AAAs[9–12] and slow the growth of small AAAs in humans[13] and (2) 17-beta estradiol, which has been shown to inhibit the development of experimental AAAs.[20]

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REFERENCES

1. BRIEGER, D. & E. TOPOL. 1997. Local delivery systems and prevention of restenosis. Cardiovasc. Res. **35:** 405–413.
2. SAKHAROV, D.V., A.F. JIE, M.E. BEKKERS, *et al*. 2001. Polylysine as a vehicle for extracellular matrix-targeted local drug delivery, providing high accumulation and long-term retention within the vascular wall. Arterioscler. Thromb. Vasc. Biol. **21:** 943–948.
3. MAJOROS, I.J., T.P. THOMAS, C.B. MEHTA & J.R. BAKER. 2005. Poly(amidoamine) dendrimer-based multifunctional engineered nanodevice for cancer therapy. J. Med. Chem. **48:** 5892–5899.
4. MALIK, N., R. WIWATTANAPATAPEE, R. KLOPSCH, *et al*. 2000. Dendrimers: relationship between structure and biocompatibility *in vitro*, and preliminary studies on the biodistribution of 125I-labelled polyamidoamine dendrimers *in vivo*. J. Control Release. **65:** 133–148. Erratum in: J. Control Release 2000. **68:** 299–302.
5. PATRI, A.K., I.J. MAJOROS & J.R. BAKER. 2002. Dendritic polymer macromolecular carriers for drug delivery. Curr. Opin. Chem. Biol. **6:** 466–471.

6. Lesniak, W., A.U. Bielinska, K. Sun, *et al*. 2005. Silver/dendrimer nanocomposites as biomarkers: fabrication, characterization, *in vitro* toxicity, and intracellular detection. Nano. Lett. **5:** 2123–2130.
7. Bosman, A.W., H.M. Janssen & E.W. Meijer. 1999. About dendrimers: structure, physical properties, and applications. Chem. Rev. **99:** 1665–1688.
8. Ailawadi, G., J.L. Eliason & G.R. Upchurch Jr. 2003. Current concepts in the pathogenesis of abdominal aortic aneurysm. J. Vasc. Surg. **38:** 584–588.
9. Petrinec, D., S. Liao, D.R. Holmes, *et al*. 1996. Doxycycline inhibition of aneurysmal degeneration in an elastase-induced rat model of abdominal aortic aneurysm: preservation of aortic elastin associated with suppressed production of 92-kD gelatinase. J. Vasc. Surg. **23:** 336–346.
10. Sho, E., J. Chu, M. Sho, *et al*. 2004. Continuous periaortic infusion improves doxycycline efficacy in experimental aortic aneurysms. J. Vasc. Surg. **39:** 1312–1321.
11. Thompson, R.W., S. Liao & J.A. Curci. 1998. Therapeutic potential of tetracycline derivatives to suppress the growth of abdominal aortic aneurysms. Adv. Dent. Res. **12:** 159–165.
12. Curci, J.A., D. Petrinec, S. Liao, *et al*. 1998. Pharmacologic suppression of experimental abdominal aortic aneurysms: a comparison of doxycycline and four chemically modified tetracyclines. J. Vasc. Surg. **28:** 1082–1093.
13. Baxter, B.T., W.H. Pearce, E.A. Waltke, *et al*. 2002. Prolonged administration of doxycycline in patients with small asymptomatic abdominal aortic aneurysms: report of a prospective (phase II) multicenter study. J. Vasc. Surg. **36:** 1–12.
14. Anidjar, S., J.L. Salzmann, D. Gentric, *et al*. 1990. Elastase-induced experimental aneurysms in rats. Circulation **82:** 973–981.
15. Patri, A.K., J.F. Kukowska-Latallo & J.R. Baker. 2005. Targeted drug delivery with dendrimers: comparison of the release kinetics of covalently conjugated drug and non-covalent drug inclusion complex. Adv. Drug Deliv. Rev. **57:** 2203–2214.
16. Quintana, A., E. Raczka, L. Piehler, *et al*. 2002. Design and function of a dendrimer-based therapeutic nanodevice targeted to tumor cells through the folate receptor. Pharm. Res. **19:** 1310–1316.
17. Panchuk-Voloshina, N., R.P. Haugland, J. Bishop-Stewart, *et al*. 1999. Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. J. Histochem. Cytochem. **47:** 1179–1188.
18. Lovich, M.A. & E.R. Edelman. 1995. Mechanisms of transmural heparin transport in the rat abdominal aorta after local vascular delivery. Circ. Res. **77:** 1143–1150.
19. Price, R.J., D.M. Skyba, S. Kaul & T.C. Skalak. 1998. Delivery of colloidal particles and red blood cells to tissue through microvessel ruptures created by targeted microbubble destruction with ultrasound. Circulation **98:** 1264–1267.
20. Ailawadi, G., J.L. Eliason, K.J. Roelofs, *et al*. 2004. Gender differences in experimental aortic aneurysm formation. Arterioscler. Thromb. Vasc. Biol. **24:** 2116–2122.