Continuous periaortic infusion improves doxycycline efficacy in experimental aortic aneurysms

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Objective: **We created a novel continuous infusion system to evaluate the efficacy of juxta-aortic doxycycline delivery as a transitional step toward developing hybrid drug/device treatment strategies for abdominal aortic aneurysm (AAA) disease.**

Methods: **Controlled comparison of treatment outcomes was studied in animal models with molecular and morphologic tissue analysis in a collaboration between university and corporate research laboratories. Rat AAAs were created via porcine pancreatic elastase (PPE) infusion and grouped and analyzed by subsequent treatment status (either doxycycline in vehicle or vehicle alone) and drug delivery method (continuous infusion via periaortic delivery system [PDS] or twice-daily subcutaneous injection). The main outcome measures were AAA diameter via direct measurement, medial elastin lamellar preservation via light microscopy, mural smooth muscle cell (SMC) proliferation and SMC and macrophage density via immunostaining and counting, expression of matrix metalloproteinases 2, 9, and 14 and tissue inhibitors of metalloproteinases 1 and 2 via real-time reverse transcriptase–polymerase chain reaction, and enzymatic activity via substrate zymography. Serum drug levels were analyzed via liquid chromatography/mass spectroscopy.**

Results: **PDS (1.5 mg/kg/day) and subcutaneous (60 mg/kg/day) delivery methods caused comparable reductions in AAA diameter during the period of 14 days after PPE infusion. PDS rats gained more weight during the postoperative period ($P < .001$), possibly as a result of reduced serum drug levels and systemic toxicity. Doxycycline treatment reduced AAA macrophage infiltration and SMC proliferation significantly. Despite reduced diameter, circumferential elastic lamellar preservation was not apparent in doxycycline-treated AAAs.**

Conclusions: **Continuous periaortic infusion lowers the effective doxycycline dose for experimental AAA limitation. Alternative biologic inhibition strategies might also be amenable to direct intra-aortic or juxta-aortic delivery. Periaortic infusion might improve the clinical outcome of minimally invasive AAA treatment strategies. (J Vasc Surg 2004;39: 1312-21.)**

Clinical Relevance: **Aneurysm remodeling may continue after successful endovascular AAA exclusion. Continued proteolytic activity within the aneurysm wall potentiates late graft migration and failure. The doxycycline infusion system developed in these experiments may serve as a prototype for adjuvant treatment modalities that complement endovascular AAA exclusion. Local delivery of doxycycline or other agents active in AAA disease, either continuously or at selected intervals after graft implantation, may stabilize the wall and aid in maintaining aneurysm exclusion. Alternative delivery methods could include passive diffusion from either the graft material itself or treatment reservoirs incorporated into endografts. Given the recognized limitations of current technologies, adjuvant biologic therapies have the potential to improve long-term patient outcome significantly after endovascular exclusion.**

Abdominal aortic aneurysm (AAA) disease is a chronic condition characterized by progressive aortic medial vascular smooth muscle cell (SMC) loss, elastin and collagen degradation, impaired aortic integrity, and ultimate rupture. Although the initiating influences of AAA disease remain poorly characterized, aneurysmal dilatation is primarily attributed to progressive proteolytic depletion of medial and adventitial elastin.[1-3] Elastolytic proteinases are up-regulated in AAA tissue, including matrix metalloproteinases (MMPs)–2 and 9, and expressed principally by infiltrating macrophages, vascular smooth muscle cells (SMCs), and endothelial cells.[4-6] The co-occurrence of elastolytic activity, elastin degradation, and aneurysm enlargement has led to the observation that pharmacologic inhibition of MMP activity might retard aortic wall matrix degradation and aneurysm growth in patients with small AAAs or those not amenable to surgical repair.

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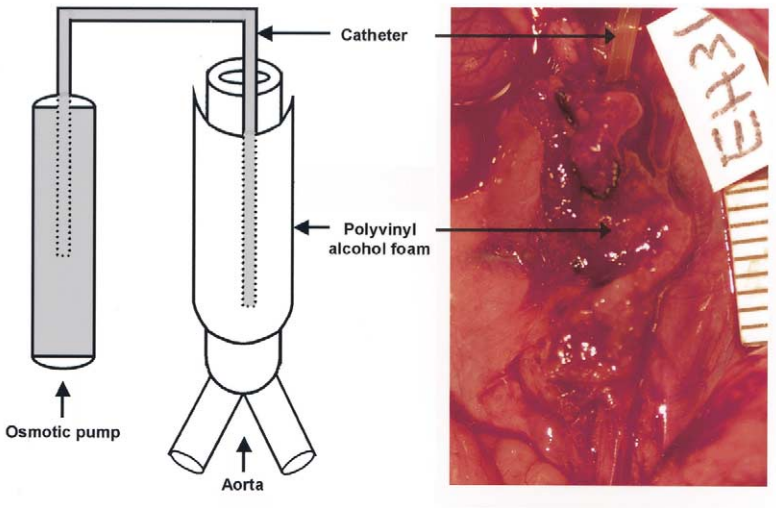

Fig 1. PDS. The PE-10 tubing is placed adjacent to the aorta immediately after intraluminal elastase infusion, wrapped with polyvinyl alcohol foam, and tunneled across the abdominal wall to connect with a reservoir containing either doxycycline solution or vehicle alone (representative photograph).

The drug most widely investigated for AAA inhibition is doxycycline, a member of tetracycline antibiotic family.[7] Petrinec et al[8] first demonstrated that doxycycline therapy inhibited MMP-9 activity, preserved elastic lamellar structure, and reduced enlargement of experimental AAAs. More recently, the efficacy of oral doxycycline therapy in preventing small AAA expansion and modifying serum MMP levels has been tested in small-scale clinical trials.[9-11] AAA limitation in small mammalian models has generally required doxycycline dosages nearly 10-fold higher (30-60 mg/kg/day) than the typical human antimicrobial dose range (2-4 mg/kg/day). Clinical efficacy in limiting AAA progression with doxycycline in the antimicrobial dose range remains unconfirmed. To date, no attempt has been made to optimize aortic MMP inhibition and to limit systemic drug levels by delivering doxycycline directly onto the aneurysm.

If continuous juxta-aortic or intra-aortic doxycycline therapy does limit MMP activity and AAA progression with lower systemic doxycycline levels, this technique might prove to be an effective adjunctive therapy for endovascular AAA repair (EVR). Limiting mural proteolytic activity in or around the aneurysm sac after endovascular exclusion might significantly reduce late remodeling, loss of graft fixation, and distal migration. In addition to doxycycline-mediated MMP inhibition, a wide range of pharmacologic strategies has been proposed to modify or to stabilize AAA progression.[12-14] Adjunctive EVR drug delivery systems could potentially deliver multiple agents concurrently or in sequence in the months or years after device placement, guided by ongoing image- or sensor-guided analysis of aneurysm morphology, diameter, or sac endotension.

We developed a periaortic drug delivery system (PDS) for the rat porcine pancreatic elastase infusion (PPE) AAA model to compare the efficacy of continuous juxta-aortic infusion versus traditional twice-daily subcutaneous doxycycline injections in limiting aneurysm progression as the first step in the potential future development of hybrid drug/device AAA treatment strategies.

MATERIAL AND METHODS

AAA model. Male Sprague-Dawley rats (250-350 g) underwent aortic PPE infusion as previously described.[15,16] Briefly, the infrarenal aorta was isolated, mobilized, and controlled via laparotomy. A polyethylene catheter (PE-10) was advanced into the 15-mm long distal aortic segment from the right femoral artery. One milliliter of normal saline containing 7 μm type I PPE (E-1250; Sigma Chemical, St Louis, Mo) was infused via syringe pump into the controlled segment during a 1-hour period. After infusion the catheter and any residual elastase were withdrawn, and inline aortic flow was restored. All experimental surgical procedures were approved by the Institutional Animal Care and Use Committee of the Veterans Affairs Palo Alto Health Care System and were conducted in compliance with Stanford University Administrative Panel on Laboratory Animal Care Guidelines, including but not limited to those related to Rodent Survival Surgery (http://labanimals.stanford.edu/guidelines/rodent_surg.html) and Endpoint Monitoring and Humane Termination ((http://labanimals.stanford.edu/guidelines/endpoint.html) (both updated October 1998).

PDS. The PDS was constructed in the following manner. After the PPE infusion was complete and the aortic catheter was withdrawn, a second PE-10 tube was secured to the anterior aortic surface with a 1-mm thick sheet of circumferential periaortic porous polyvinyl alcohol foam (2.5 cm^2 total area). The foam was positioned to capture doxycycline infused through the PE-10 tubing and to maintain relatively high periaortic concentration gradients

Table I. Oligonucleotides used for real time reverse transcriptase-polymerase chain reaction

	GeneBank No.	*Forward primer*	*Reverse primer*	*product (base pair)*
MMP-2	NM_031054	5′-TGCTGGAGAACCTGAAGTGT-3′	5′-AGATTGATGCCGTGTACGAG-3′	101
MMP-9	NM_031055	5′-TGGCTCTAGGCTACAGCTTTG-3′	5′-CGACACCAAACTGGATGACAA-3′	101
MMP-14	NM_031056	5′-GCCATGCAAAGGTTCTATGGTT-3′	5′-CGCCTCATAGCCTTCATCGT-3′	71
TIMP-1	U06179	5′-GGCCTCTGGCATCCTCTTG-3′	5′-CCAGGTCCGAGTTGCAGAAA-3′	101
TIMP-2	NM_021989	5′-GCTGGACGTTGGAGGAAAGA-3′	5′-TGTCCCAGGGCACAATAAAGT-3′	101
β-actin	NM_031144	5′-GGGAAATCGTGCGTGACAT-3′	5′-CAGGAGGAGCAATGATCTT-3′	101

without restricting aneurysm progression (Fig 1). The other end of the PE-10 tubing was tunneled into a lateral subcutaneous pocket through the abdominal wall. A 2-mL osmotic pump (Model 2ML2; Azlet Osmotic Pumps, Cupertino, Calif) was secured in the pocket and connected to the tubing. The osmotic pumps were preloaded with either doxycycline in saline or saline alone as described below.

Study groups. AAA groups were defined on the basis of treatment modality, either continuous retroperitoneal infusion via the PDS or subcutaneous injection (SC). PDS rats were treated with either doxycycline in saline (PDS-DOX subgroup, n = 9; 5 mg/mL infused at 5 μL/h delivering 1.5 mg/kg/day) or control saline solution (PDS-CON subgroup, n = 9; 5 μL/h). SC rats were treated with twice-daily injections of either doxycycline in saline (SC-DOX subgroup, n = 9; 15 mg/kg in 0.5 mL) for a total doxycycline dose of 30 mg/kg/day or saline alone (SC-CON subgroup, n = 9; 0.5 mL). Treatment was continued for 14 days after PPE infusion. Three additional AAA rats were created for molecular analysis and sacrificed at 14 days without any postoperative infusions or injections. Rats were sacrificed by intentional anesthetic overdose. Aortic external diameter was the primary index of aneurysm progression, measured in situ before and after PPE infusion and immediately before sacrifice by using electronic microcalipers. Measurements were consistently obtained at the point of maximum transverse diameter. Observers were blinded to treatment status at the time of measurement.

Serum drug concentration. One milliliter of rat tail-vein blood was drawn on days 2, 4, 7, and 14 after PPE infusion/treatment initiation and saved in 10% ethylenediaminetetraacetic acid. Serum doxycycline concentration was determined in all groups at all time points via liquid chromatography/mass spectroscopy (PPD Discovery, Morrisville, NC).

Metalloproteinase and inhibitor expression. Before sacrifice the aneurysmal aortic segment was dissected free of surrounding retroperitoneal tissue via repeat laparotomy. After diameter measurement and sacrifice, aneurysms were harvested, snap frozen in liquid nitrogen, and stored at −80°C. Real-time reverse transcriptase–polymerase chain reaction (PCR) was performed with the GeneAmp 7700 sequence detection system (Applied Biosystems, Foster City, Calif).[15] High quality total RNA was extracted by using TRIzol reagent (Gibco BRL, Rockville, Md) and used to generate cDNA for oligo-deoxythymidine oligodeoxynucleotide primer (T12-18) by using the Superscript II reverse transcriptase system (Invitrogen, Carlsbad, Calif). The following primers were designed by using Primer Express software (Applied Biosystems) and synthesized: MMP-2, MMP-9, MMP-14, tissue inhibitor of metalloproteinases (TIMP)-1, and TIMP-2 (Table I). Equal amounts of cDNA were used in duplicate and amplified with the SYBR Green I Master Mix System (Applied Biosystems). PCR was performed after thermal activation for 10 minutes at 95°C followed by 40 cycles of warming for 15 seconds at 95°C and annealing/extension for 1 minute at 60°C. Amplification efficiencies were validated and normalized against β-actin. Correct PCR product size was confirmed by electrophoresis through 2% agarose gel with ethidium bromide.

Gelatinase activity. Aortic proteins were extracted as previously described.[16] For detection of MMP-2 and MMP-9 activity, equal amounts of samples standardized for protein concentration (20 μg) were loaded on each lane and run in parallel in 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis gels containing 1% gelatin (Invitrogen). The molecular sizes of gelatinolytic activities were determined with prestained protein standards (Invitrogen). Gels were washed with Triton X-100 (2.5%) and then incubated overnight (37°C) in developing buffer (50 mmol/L Tris Base and 10 mmol/L $CaCl_2$). Zones of lysis were visualized after staining the gels with 0.5% Coomassie blue R-250. Densitometric analysis of lytic bands for MMP-2 and MMP-9 was performed by public domain software NIH Image version 1.61 (http://rsb.info.nih.gov/nih-image/index.html).

Cellular proliferation and inflammatory cell infiltration. To investigate intrinsic aortic cell proliferation, selected rats in all groups received an intraperitoneal administration of bromodeoxyuridine (BrdU) at 50 mg/kg (Sigma Chemical, St. Louis, Mo) in physiologic saline solution (5 mg/mL) 1 hour before sacrifice to "pulse label" cells in DNA synthesis (S) phase. After sacrifice these rats were pressure perfusion fixed with 4% paraformaldehyde solution in 0.1 mol/L phosphate buffer (pH, 7.4; 20°C) via the left ventricle for 30 minutes at an intra-arterial pressure of 100 mm Hg. Following the immunohistochemical staining protocol, the tissue sections were prepared and incubated with anti-BrdU mouse monoclonal antibody (Becton Dickinson) at 1:50 dilution in 0.1%

Table II. Weight gain following PPE infusion as a function of DOX treatment status

	BW (% increase)		*SP (% delivered)*	
	Day 0	*Day 14*	*Day 0*	*Day 14*
PDS-DOX	343.4 ± 17.1	416.3 ± 14.1 (20.0 ± 3.2)	2.0	0.3 ± 0.03 (84.9 ± 1.5)
PDS-CON	338.5 ± 9.4	395.3 ± 8.7 (17.3 ± 3.6)	2.0	0.3 ± 0.05 (85.2 ± 2.4)
SC-DOX	326.1 ± 9.0	365.1 ± 18.3 (11.9 ± 3.8)*	N/A	N/A
SC-CON	342.1 ± 9.4	412.3 ± 12.8 (20.6 ± 4.7)	N/A	N/A

BW, Body weight (gm); *SP,* total solution in pump (mL); *PDS-DOX,* periaortic doxycycline infusion; *PDS-CON,* periaortic saline infusion; *SC-DOX,* subcutaneous doxycycline injections; *SC-CON,* subcutaneous saline injections.
*P < .001 vs other groups.

Table III. Influence of delivery method on serum doxycycline concentrations (ng/mL)

	Day 2	*Day 4*	*Day 7*	*Day 14*
PDS-DOX	17.5 ± 3.8	17.4 ± 4.3	16.4 ± 4.6	21.1 ± 8.5
SC-DOX	725.6 ± 242*	776.0 ± 192.1*	1359.7 ± 137.1*†	1243.7 ± 187.9*†

PDS-DOX, Retroperitoneal infusion system; *SC-DOX,* subcutaneous injections.
*P < .00001 vs PDS-DOX group.
†P < .001 vs day 2 and day 7 within SC-DOX group.

bovine serum albumin in phosphate-buffered saline (PBS) for 1 hour. After washing, sections were incubated 1 hour with a biotinylated anti-mouse immunoglobulin G (KPL Lab Inc, Gaithersburg, Md) at room temperature following the ABC method according to the manufacturer's protocol and counterstained with hematoxylin. Medial SMC proliferation was defined as all BrdU-positive SMCs/section (2 sections/case). All sections were counted twice and averaged for a final value.

Mouse anti-rat ED-1 monoclonal antibody was obtained from Serotec, Inc (Raleigh, NC). Biotinylated anti-mouse secondary antibody and ABC kits were both obtained from Vector Laboratories, Inc (Burlingame, Calif). Tissue sections were prepared for immunohistochemical staining in the following manner; after blocking nonspecific binding with horse serum (1:200 diluted in PBS) for 30 minutes at room temperature and rinsing with PBS, sections were incubated with an antigen-specific primary antibody followed by biotinylated second antibody according to the manufacturer's protocol. The sections were counterstained with hematoxylin and examined via light microscopy. Medial macrophage infiltration was determined via cell counting throughout the entire media (2 sections/rat) as ED-1 positive cells/cross section. Adventitial macrophage infiltration was determined on the entire cross section (ED-1 positive cells/sections). All sections were counted twice and averaged for a final value.

Statistical analysis. All data were expressed as mean ± standard deviation. Statistical analysis was performed by using the one-way analysis of variance for non-normally distributed populations with the Bonferroni-Dunn correction for multiple comparisons. Differences were considered statistically significant at $P < .05$.

RESULTS

The PPE infusion dose and dwell time were reduced in these experiments to minimize artifactual influences related to infusion pressure and aortic ischemia and to emphasize inflammation as the primary etiologic agent for aneurysmal degeneration. Despite these protocol modifications, AAAs were produced in adequate numbers in all groups; 89% of the rats (32/36) developed at least 60% diameter enlargement between the end of the infusion and sacrifice. One rat in each of the four groups failed to reach this threshold, leaving eight rats per group for further analysis.

In both the PDS-DOX and PDS-CON groups greater than 85% of the pump reservoir was delivered to the periaortic foam collar during the 14-day postoperative period (Table II). Despite 60-fold lower serum doxycycline levels (Table III; day 14, $P < .001$) in PDS versus SC-DOX rats, the degree of AAA diameter reduction achieved versus suitable controls was comparable between the two groups (P = not significant; Table IV). SC-DOX rats also gained significantly less weight during the 14-day treatment period than PDS-DOX rats ($P < .01$; Table II). Serum doxycycline levels also varied more widely as a function of time in SC-DOX versus PDS-DOX rats (Table III).

Histologically, aortic endothelial cell and medial SMC populations were reduced immediately after PPE infusion (90% and 43%, respectively). The modified infusion protocol did limit initial postinfusion internal elastic lamellar degeneration compared to previous experiments[15] (Fig 2), but at 14 days the characteristic transmural monomorphonuclear and polymorphonuclear inflammatory cell infiltrate, near-complete elastin dissolution, and progressive diameter enlargement associated with PPE infusion were uniformly present. Regenerating and possibly proliferating endothelial cells and SMCs were present along the inner

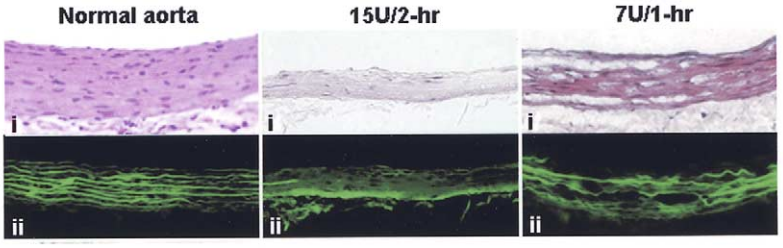

Fig 2. Infusion-related endothelial, smooth muscle, and lamellar injury as a function of PPE dose and infusion time. (**i**) Hematoxylin-eosin stain; (**ii**) aortic elastin autofluorescence, both original magnification ×400.

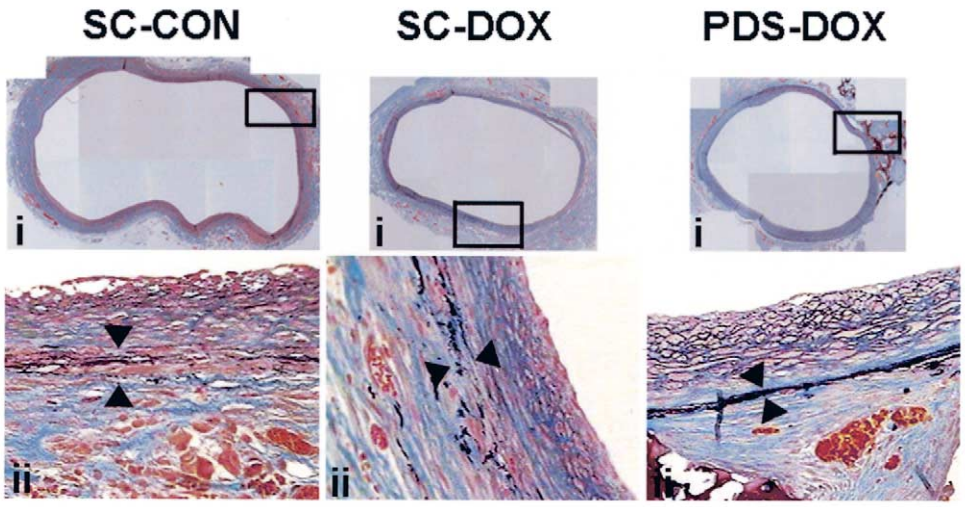

Fig 3. Effect of doxycycline treatment status on elastin preservation. AAA diameter (**i,** original magnification 4×) was reduced with either twice-daily subcutaneous injections (SC-DOX, 60 mg/kg/day) or continuous periaortic infusion (PDS-DOX, 1.5 mg/kg/day) doxycycline therapy. SC-CON, AAA treated with vehicle alone. **ii,** Medial elastic lamellar attenuation as a function of doxycycline treatment status *(arrowheads)*. Proliferating SMCs are present on the luminal side of the degenerating media surrounded by newly synthesized elastin fibers (original magnification ×400; elastin Masson trichrome stain).

Table IV. Influence of doxycycline treatment status/method on AAA diameter

	Pre-PPE	*Post-PPE (increase %)*	*Death (increase %)**	*AD reduction* †
PDS-CON	1.73 ± 0.04	2.09 ± 0.04 (21.1 ± 3.0)	4.52 ± 0.52 (116.8 ± 25.5)	
PDS-DOX	1.71 ± 0.03	2.11 ± 0.03 (24.3 ± 1.3)	3.84 ± 0.51 (80.8 ± 24.1)‡	35.2%
SC-CON	1.74 ± 0.02	2.12 ± 0.03 (21.8 ± 2.4)	5.25 ± 1.14 (148.0 ± 53.9)§	
SC-DOX	1.67 ± 0.08	2.07 ± 0.06 (23.6 ± 6.2)	3.86 ± 0.72 (86.2 ± 34.2)‖	45.9%

Pre-PPE, Aortic diameter before PPE infusion (mm); *Post-PPE,* diameter immediately after PPE infusion (day 0, mm); Death, diameter 14 days after PPE infusion (mm).
*Percent increase vs Post-PPE diameter.
†Diameter reduction vs saline treatment alone.
‡$P = .019$ vs PDS-CON group.
§$P = .19$ (not significant) vs PDS-CON group.
‖$P = .025$ vs SC-CON group.

margins of the inflamed and degenerating media, although to a lesser degree than that previously reported to occur under high aortic flow conditions.[16] Well-differentiated SMCs in this region produced and were surrounded by newly synthesized elastic fibers as confirmed by Masson trichrome staining (Fig 3).

In addition to limiting AAA enlargement, doxycycline treatment status influenced aortic cellularity. Macrophage transmural infiltration was reduced in the medial layer in both the PDS-DOX and SC-DOX groups as compared to PDS-CON and SC-CON groups ($P < .01$, Fig 4). No difference in macrophage density was noted as a function of

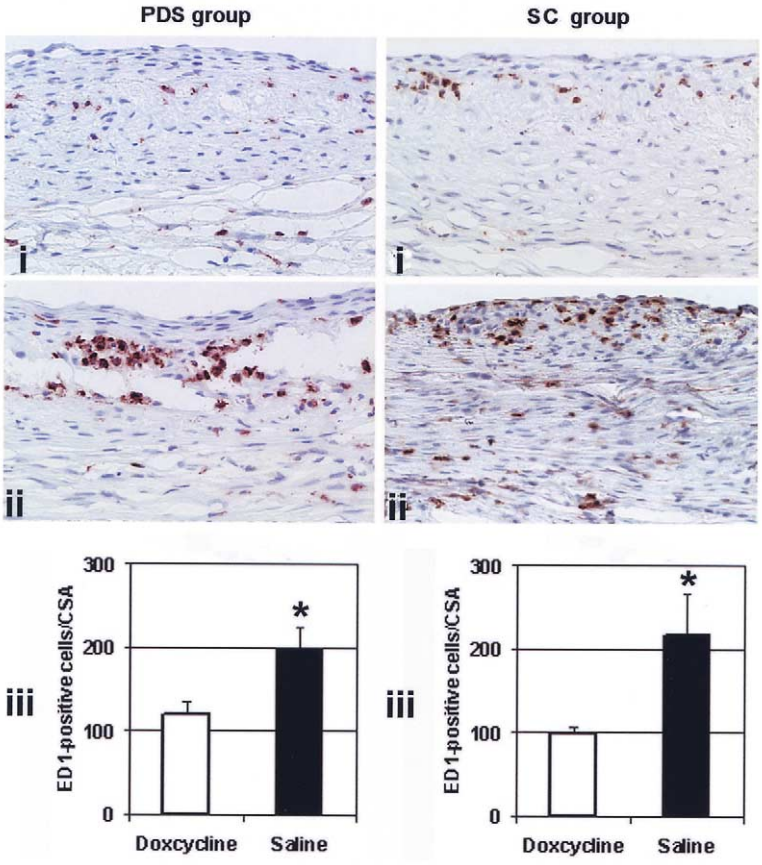

Fig 4. Effect of doxycycline treatment status on transmural macrophage density. (**i**) Doxycycline treatment; (**ii**) saline treatment; (**iii**) index of macrophage medial infiltration. *Brown nuclei,* ED-1 positive cells; *CSA,* cross-sectional area of media. *$P < .01$ vs doxycycline treatment group. Original magnification ×400.

doxycycline delivery method (PDS-DOX vs SC-DOX, $P =$ not significant). BrdU pulse-labeling showed reduced SMC proliferation and regeneration in doxycycline-treated rats ($P < .05$) (Fig 5), suggesting that doxycycline limited medial SMC regeneration after aneurysm formation. No differences in aortic elastin preservation were noted between either DOX group or control AAAs. Increased expression of mRNA for MMP-2, -9, and -14 and TIMP-1 and -2 were noted in all AAA groups compared to control aorta. Doxycycline treatment status affected only MMP-9 expression; message for MMP-2 and -14 and TIMP-1 and -2 was similar to that present in AAAs treated with saline alone (Fig 6). MMP-9 expression was lower in SC-DOX than PDS-DOX rats ($P = .015$), perhaps because of the higher serum levels associated with subcutaneous injection. Gelatin zymography of aortic extracts showed lytic bands at 72 and 62 kd in all AAA groups, consistent with proteolytic activity of the pro- and active forms, respectively, of MMP-2. Significant gelatinolysis was also noted at 92 and 86 kd, indicating increased pro- and active MMP-9 activity. Activity of both MMP-9 isoforms was reduced in doxycycline-treated rats. MMP-2 activity, however, was apparently not influenced by doxycycline treatment (Fig 6). PDS-DOX treatment resulted in reduced pro–MMP-9 activity compared with SC-DOX–treated rats ($P < .002$).

DISCUSSION

This study demonstrated the feasibility, initial effectiveness, and potential advantages of a novel drug delivery system for experimental aortic aneurysm therapy. Low dose doxycycline delivered continuously to the periaortic retroperitoneal space attenuated aortic expansion, reduced macrophage infiltration, and limited SMC proliferation. These effects were achieved with dramatic reductions in serum drug levels compared to standard systemic doxycycline dosing protocols.

The PDS method evolved from previously tested prototypes that used drug-eluting fabric patches or viscous gels to deliver doxycycline to the periaortic space. PDS proved least likely among these methods to precipitate premature rupture or artifactually to limit diameter enlargement. Any local infusion method might influence AAA progression, if only as a consequence of the extra dissection required for placement. Although polyvinyl alcohol itself is not biologically inert, in this experiment the presence of the foam

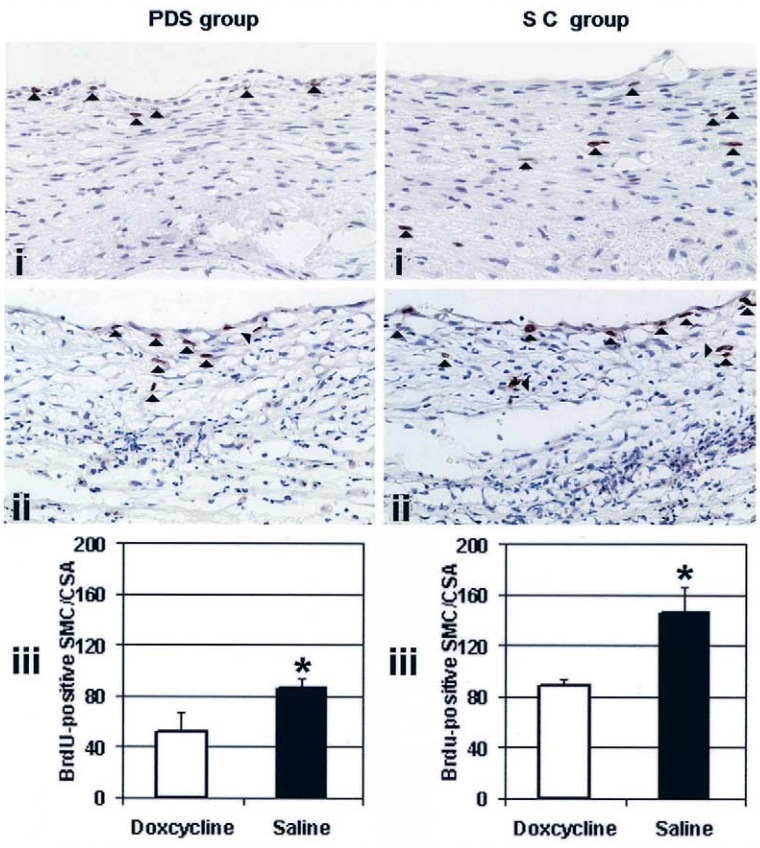

Fig 5. Effect of doxycycline treatment status on aortic SMC proliferation. *Arrows,* BrdU-labeled SMC nuclei in the actively remodeling aortic wall after PPE infusion. **i,** AAA after doxycycline treatment; **ii,** AAA after saline treatment (original magnification ×400); **iii,** index of BrdU-labeled SMCs in AAA wall. *CSA,* Cross-sectional area of media; * $P < .01$ vs doxycycline treatment group.

alone did not significantly influence enlargement (Table IV).

Doxycycline inhibits proteolytic activity via direct enzyme inhibition, as well as down-regulation of MMP-9 gene transcription.[17] In addition to these direct effects, doxycycline also reduces interleukin-1 levels,[18] inhibits murine macrophage nitric oxide synthase activity,[19] induces macrophage apoptosis,[20] and reduces reactive oxygen species in vitro,[21] activities that might also influence aortic inflammation, diameter enlargement, and wall fragmentation in AAA disease. In contrast with reports from earlier studies,[8,22,23] the medial elastic lamellae were not well preserved in either doxycycline treatment group in this experiment. This apparent inconsistency might reflect limitations inherent to the model. These aneurysms demonstrate significant circumferential and longitudinal heterogeneity in the degree of aortic inflammation, elastolysis, and mural thrombus formation present after PPE infusion. As a result, well-formed lamellae are clearly seen in isolated sections in some aneurysms (as demonstrated in the low power sections in Fig 3, as an example), irrespective of doxycycline treatment status. We also used lower PPE doses and shorter infusion times than those reported in previous experiments, changes that might limit artifactual lamellar dissolution during the aneurysm initiation phase. Taken as a whole, however, no consistent circumferential evidence of lamellar preservation attributable to treatment status is present in doxycycline-treated AAAs.

If the elastic lamellae are not preserved, what alternative mechanisms account for doxycycline-mediated AAA suppression? We noted a consistent reduction in transmural macrophage density in doxycycline-treated AAAs compared to control, findings at odds with those reported by Petrinec et al[8] with a similar model but consistent with in vitro observations regarding the ability of tetracycline-class drugs to induce macrophage apoptosis. Reduced macrophage infiltration has also been associated with reduced experimental AAA progression under high flow aortic conditions,[24] underscoring the important role that inflammation plays in aneurysmal degeneration. Relative adventitial collagen retention might also account for some degree of diameter reduction present in doxycycline-treated AAAs. In addition to elastolysis, MMP-2 and -9 also have enzymatic activity against arterial collagens types I and III, and

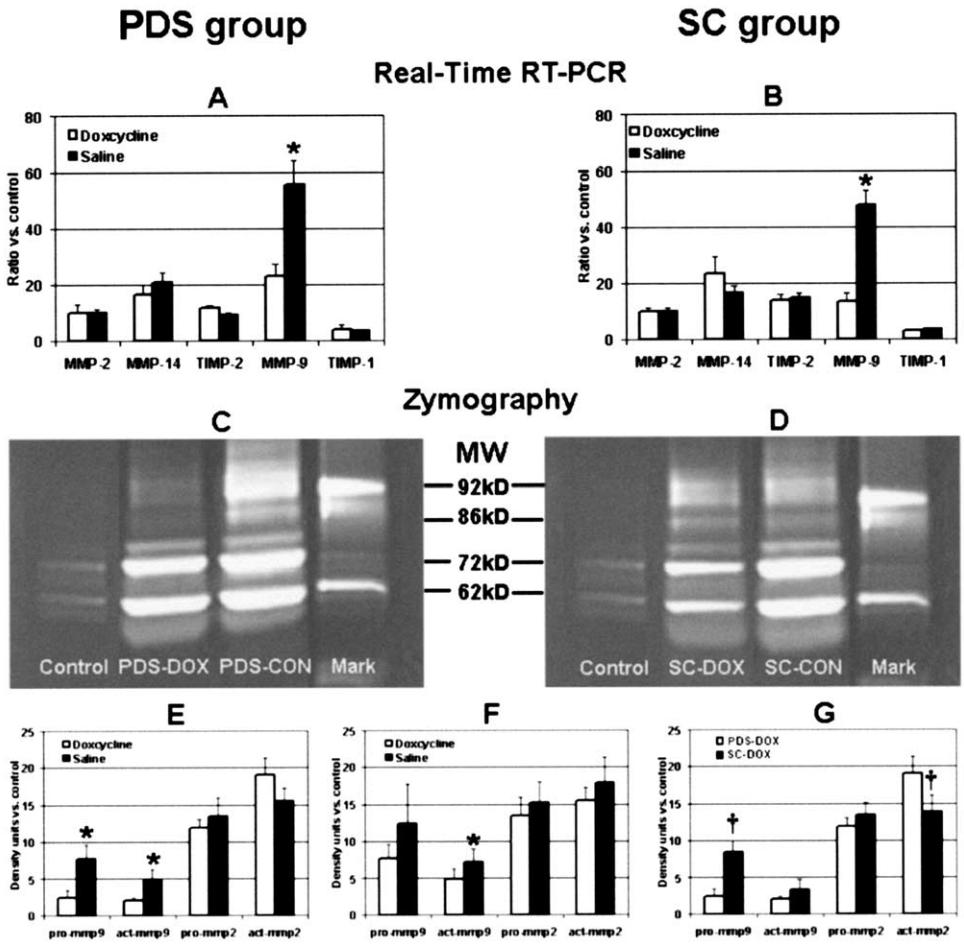

Fig 6. MMP gene expression and activity in AAA tissue with or without doxycycline treatment. Doxycycline therapy reduced AAA MMP-9 gene expression in PDS-DOX group (**A**) and SC-DOX group (**B**), but it did not affect MMP-2 and -14 or TIMP-1 and -2. Gelatin gel zymography demonstrated reduced activity of pro- and active forms of MMP-9 after doxycycline treatment (**C**-**F**). The PDS method was more effective in this regard than subcutaneous injection (**G**). MMP-2 activity was not affected by doxycycline treatment. Lytic bands corresponding to apparent molecular weight *(MW)* of MMP-2 and MMP-9 were quantified by densitometry. $*P < .01$ vs doxycycline treatment; $\dagger P < .05$ vs PDS-DOX group.

although AAA enlargement has generally been mechanistically linked to elastin rather than collagen depletion,[25] relative adventitial collagen retention might also account for some degree of the diameter reduction evident in the doxycycline-treated rats. Confirmation of this hypothesis will require quantification of AAA collagen and elastin content in subsequent experiments.

Clinical trials with oral doxycycline therapy to retard small AAA growth have been reported, are planned, or are already underway.[8,26,27] Adjunctive doxycycline might also prove effective in limiting aortic remodeling and subsequent device migration after endovascular AAA exclusion.[28] To date, oral doxycycline doses used in human trials have been far lower than those shown to be effective in experimental models (Table V). Even at these lower dosages, however, doxycycline use might precipitate serious side effects,[9] potentially limiting long-term therapeutic utility. Continuous periaortic retroperitoneal infusion substantially reduces overall dose, reduces potentially dose-related systemic side effects (such as reduced weight gain in rodent models), and minimizes serum level variability while maintaining therapeutic efficacy in experimental AAA. Continuous delivery might also improve therapeutic compliance via utilization of periodic dosing and reservoir capabilities similar to those pioneered by continuous insulin infusion systems in diabetic patients. If proven significant and reproducible in the clinical setting, these advantages might dramatically improve the therapeutic index and ultimate clinical efficacy of doxycycline or other tetracycline derivatives as adjunctive or stand-alone therapy for AAA suppression.

Table V. Summary of doxycycline dosages reported effective in limiting experimental AAA

Investigator	*Animal model*	*Local delivery*	*Systemic delivery*	*Diameter reduction**
Thompson RW (1996)[8]	Rat-PPE	N/A	63 mg/kg/day SC	56%
(1998)[23]	Rat-PPE	N/A	7.5–60 mg/kg/day SC	38%–60%
(2000)[31]	Rat-PPE	N/A	500 mg/L/day PO	51%
(2000)[32]	Mouse-PPE	N/A	30 mg/kg/day PO	34%
Baxter BT (2002)[9]	Mouse-$CaCl_2$	N/A	50–100 mg/kg/day PO	44%–66%
Daugherty A (2003)[13]	Mouse-Ang II	N/A	30 mg/kg/day PO	No diameter data
Present study (2003)	Rat-PPE	1.5 mg/kg/day	60 mg/kg/day SC	27%–38%

For the present series the diameter differences were calculated from preoperative aortic diameter rather than post infusion diameter, as is the case in Table IV to conform with methods used in other. All measurements at 14 days except reference 23 (7 days).
SC, Doxycycline subcutaneous injection; *PO,* doxycycline dissolved in drinking water for oral delivery.
*Reduction in doxycycline-treated group vs control group; the average degree of percent reduction in treated AAAs was calculated from the difference between the preinfusion and final diameter.

Although effective in short-term rodent models, the long-term clinical utility of doxycycline in human AAA disease treatment remains unknown. Although MMP inhibition might limit destructive matrix remodeling, MMP activity is also crucial for SMC proliferation and migration, potentially critical compensatory responses in AAA disease. SMC depletion is a prominent feature of human AAA disease, and MMP-9–deficient mice[29] and doxycycline-treated rats (30 gm/kg/day)[30] demonstrate reduced aortic SMC proliferation and migration after aortic injury. In our experiments doxycycline-treated rats also demonstrated reduced SMC proliferation, although the balance of doxycycline therapy remained antianeurysmal as indicated by reduced aneurysm diameter. In human disease, AAA recognition and treatment begin years or decades after disease onset after extensive cellular depletion and matrix degeneration. To replicate the human condition more accurately, experimental models should initiate therapy at late intervals after PPE infusion, a staggered approach yet to be tested in any model and a significant goal for further investigations. Regardless of the results reported in small mammalian models, the ultimate consequences of prolonged doxycycline administration for human AAA inhibition will need to be determined in valid clinical trials.

Although doxycycline was chosen for these experiments, continuous periaortic infusion is likely to offer distinct advantages over oral or parenteral administration of any suppressive agent in regard to local tissue concentrations, systemic effects, and toxicity indexes. A number of alternative drugs, such as angiotensin-converting enzyme and 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins), limit experimental AAA progression and have great potential utility for human AAA therapy.[12-14] Synchronous or staged infusions of multiple agents at a variety of time points might ultimately prove most effective for aneurysm suppression, either for preclinical lesions identified by screening or as adjunctive therapy after EVR exclusion.

In summary, this study demonstrates the utility of continuous periaortic infusion as an alternative method of achieving doxycycline-induced MMP-9 inhibition and aneurysm progression in experimental AAAs. Reduced serum doxycycline levels associated with this delivery method might limit systemic side effects while maintaining therapeutic efficacy. The efficacy of doxycycline as a clinical AAA suppression agent awaits confirmation by ongoing and future clinical trials. Studies to further define the role of medial SMC apoptosis, proliferation, and differentiation in aortic aneurysms might lead to additional potentially complementary adjunctive therapies for this serious and life-threatening disease.

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