Effects of Recombinant Human Type I Pancreatic Elastase on Human Atherosclerotic Arteries

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Rationale: At physiologic pressures, elastic fibers constrain artery diameter. Local treatment of atherosclerotic arteries with PRT-201, a recombinant type I elastase, could result in fragmentation and removal of elastin fibers and increased vessel diameter.

Objective: To investigate the use of PRT-201 as a treatment for human atherosclerotic arteries.

Methods and Results: Arteries were harvested from donor legs amputated due to severe peripheral artery disease or from recently deceased persons who donated their bodies to science. Three- to four-centimeter artery segments were studied on a perfusion myograph to obtain baseline diameter data. After treatment with PRT-201 3.6 mg/mL or saline for 30 minutes myography was repeated. PRT-201 treatment resulted in an increase in vessel diameter across a range of transmural pressures. Average anterior tibial artery diameter increased by 0.78 ± 0.21 mm ($27\% \pm 12\%$), whereas average posterior tibial artery diameter increased by 0.58 ± 0.30 mm ($21\% \pm 11\%$), both $P < 0.001$. Elastin content as measured by desmosine radioimmunoassay was reduced by approximately 50%, $P < 0.001$.

Conclusions: The results suggest that PRT-201 treatment of atherosclerotic peripheral arteries in patients could increase artery diameter, and thus luminal area, possibly alleviating some of the symptoms of peripheral artery disease.

Key Words: elastase, elastin, elastic fiber, atherosclerosis, peripheral artery disease, myography

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INTRODUCTION

Peripheral artery disease (PAD) affects approximately 8 million Americans and is associated with significant morbidity and mortality.[1] Guidelines recommend that patients with intermittent claudication due to PAD should be treated with risk-factor modification, supervised exercise, and if necessary pharmacological therapy with the vasodilator cilostazol. Patients with proximal, flow-limiting lesions routinely receive revascularization. The preferred choice for revascularization is percutaneous transluminal angioplasty of hemodynamically significant stenosis, with stent placement for acute failure of percutaneous transluminal angioplasty.[2] Patency loss after this procedure is high and principally occurs because of restenosis from acute elastic recoil, constrictive arterial remodeling, and neointima formation.[3–5]

Elastin is a hydrophobic monomeric protein that cross-links with other elastin molecules and organizes with a group of accessory proteins to create a meshwork of elastic fibers and sheets in the extracellular matrix of tissues, including arteries and veins. At physiologic pressures and in most clinical situations, the elastic fiber network imparts elasticity and constrains vessel diameter.[6] PRT-201, recombinant human type I pancreatic elastase, is a novel drug candidate that can fragment and remove elastin from selected segments of arteries, resulting in local and permanent dilation of the treated segment. Proteon Therapeutics is currently developing PRT-201 as a possible treatment for PAD in a phase 1–2 clinical trial (www.clinicaltrials.gov, NCT01616290).

The purpose of this research was to determine whether PRT-201 could fragment and remove elastin from atherosclerotic arteries and result in larger arterial diameters. Atherosclerotic human peripheral arteries were studied in an ex vivo model system as a means to predict in vivo responses. PRT-201 was manufactured to pharmaceutical grade quality using recombinant DNA technology. PRT-201 is a highly purified human protein and has not been shown to be immunogenic when administered to humans in hemodialysis access clinical trials.[7–9]

METHODS

Human Tissue Harvest

Before conducting the main study, a pilot study examining the dose–response curve for PRT-201 fragmentation and removal of elastin was conducted to guide dose selection for the main study. In the pilot and main study, all harvested blood

vessels used for controls and drug treatment experiments were placed in ice-cold physiological saline solution immediately after harvest and transported by a courier. The tissues were processed and used in the experiments on arrival. In the pilot study, a popliteal artery was harvested immediately after surgery from a leg amputated from a patient with severe PAD. The artery was divided into several arterial ring segments that were subsequently treated in microfuge tubes with varying concentrations of PRT-201 for 30 minutes. Elastin content before and after treatment was determined by histology and desmosine quantification.

Following the pilot study, additional superficial femoral and anterior and posterior tibial arteries were harvested immediately after surgery from legs amputated from patients with severe PAD or within 24 hours of death from donors who gave their bodies to science. The arteries were sectioned to create 2–4 mm length ring segments that were used for pretreatment histology and desmosine analysis. The remaining longer arterial segments were used for perfusion myography experiments.

The study was approved by an institutional review committee. Patients gave informed consent for the amputated tissue to be used for research.

Perfusion Myography

The PM-1 perfusion myograph is designed to measure artery diameter at various transmural pressures.[10] Segments of artery 3–4 cm long were mounted onto the cannulae of the perfusion myograph and secured with silk or nylon ligatures. Mounted artery segments were bathed in a physiological saline solution (Krebs solution) at 37°C and gassed with a mixture of 95% O_2 or 5% CO_2 in a stainless steel chamber.

Pressures were continuously monitored both upstream (proximal pressure) and downstream (distal pressure) of the artery. To measure vessel diameters, the edges of the rounded vessels were continuously recorded at predefined points using an optical imaging system. Luminal diameters were not measured. Transmural pressures were increased in 10–20 mm Hg increments from 10 to 120 mm Hg (donor 2) or from 10 to 80 mm Hg (donors 6–10). The diameter was allowed to stabilize for at least 1 minute at each pressure before making measurements, and an initial pressure–diameter curve was created. In donors 6–10, after recording artery diameter at 80 mm Hg, the luminal pressure was reduced to 10 mm Hg, and the artery diameter was recorded to measure recoil.

Drug Treatment

For the pilot study, popliteal artery rings from a single patient were transferred to microfuge tubes. The artery rings were then treated in duplicate with vehicle or PRT-201 0.1, 0.5, 1.0, 5.0, and 10.0 mg/mL in a volume of 0.5 mL for 30 minutes. At the end of the incubation period, the artery rings were placed into fresh tubes containing phosphate buffered saline with 0.01% polysorbate 80 (PBSP) and rinsed by gently inverting the tubes for 30 seconds. This wash process was then repeated in fresh PBSP, and the artery rings were transferred to vials containing 10% formalin for storage at ambient temperature before shipping. One replicate at each condition was sent for measurement of protein content and elastin content by desmosine quantification. The other replicate was sent for histological analysis.

In the main study, the perfusion myograph bath chamber was drained of fluid after obtaining the initial diameter data. At a transmural pressure of 40 mm Hg, PRT-201 was applied dropwise at a concentration of 3.6 mg/mL every 30 seconds over 30 minutes. The total volume administered was 2.5 mL. For donor 2, an additional arterial segment was treated with PBSP as a control, following the same protocol. After treatment, arterial segments were washed 3 times with Krebs solution to remove any remaining treatment solution, the bath was refilled, and the second set of diameter data was obtained following the same protocol.

Statistical Analysis

Pressure–diameter curves were generated from the data and compared by testing the mean artery diameters at each pressure increment using 2-way analysis of variance. Overall average change and percentage change in diameter were also compared by testing the mean diameters measured across all pressures using 2-sided, paired t tests. Mean artery desmosine content before and after PRT-201 treatment were compared using a paired t test.

Desmosine Quantification

After completion of the experiment, the ends of the vessel that were mounted on the cannula of the pressure myograph were trimmed off and discarded, and the remaining vessel was cut into 3 rings for measurement of protein content and elastin content by desmosine quantification.

Desmosine is a protein cross-link unique to elastin. Desmosine levels in the artery rings from the experiments were determined by radioimmunoassay and reported as picomoles of desmosine per milligram protein.[11] Protein content of the sample was measured using a ninhydrin-based protein assay.[12]

Histology

Formalin-fixed artery rings were embedded in plastic, sectioned, and stained with Verhoeff–Van Gieson stain at Charles River Pathology Associates (Frederick, MD). The resulting glass slides were examined by a pathologist for evidence of elastic fiber fragmentation and removal. Elastic fibers stain dark blue or black with the Verhoeff–Van Gieson stain.

RESULTS

Tissue Harvest

Artery donors were from the United Kingdom. Table 1 lists the individual donors and the actual use of the arteries. Tibial arteries from donors 3–5 were harvested after limb amputation for PAD. These arteries failed to hold pressure on the perfusion myograph because of leaking. As a result of this, the protocol was amended to source tibial arteries from recently deceased donors, and subsequent to this amendment, arteries from donors 6 through 10 were successfully studied.

**TABLE 1.** Experimental Data From Each Individual Donor

Donor	Artery Source	Artery Type and Comments
1	Amputation	Popliteal artery
2	Amputation	Posterior tibial artery
3	Amputation	Anterior and posterior tibial artery, too atherosclerotic to test
4	Amputation	Anterior and posterior tibial artery, too atherosclerotic to test
5	Amputation	Anterior and posterior tibial artery, too atherosclerotic to test
6	Postmortem	Anterior and posterior tibial artery
7	Postmortem	Anterior and posterior tibial artery
8	Postmortem	Anterior and posterior tibial artery
9	Postmortem	Anterior tibial artery
10	Postmortem	Anterior and posterior tibial artery

All tibial artery donors were white, 6 were men, and their ages ranged from 56 to 88 years. All arteries were atherosclerotic by visual inspection. Figure 1 is a representative image of an anterior tibial artery from a postmortem donor. The artery wall was thickened with yellow atherosclerotic plaque containing areas of white calcification. The texture was mainly firm with interspersed softer areas.

Pilot Study

Table 2 summarizes the desmosine content of popliteal artery rings from donor 1. Artery rings were untreated or treated with vehicle or PRT-201 at varying concentrations for 30 minutes.

Histology demonstrated a PRT-201 dose-related reduction in elastic fiber staining. Figure 2 displays representative histological images of a vehicle-treated artery ring and an artery ring treated with PRT-201 5 mg/mL for 30 minutes. In the vehicle-treated artery ring, there is an abundance of blue-black elastic fibers evident in the internal and external elastic lamina, and adventitia. In contrast, there are fewer elastic fibers and almost complete removal of the internal and external elastic laminae in the PRT-201–treated artery ring.
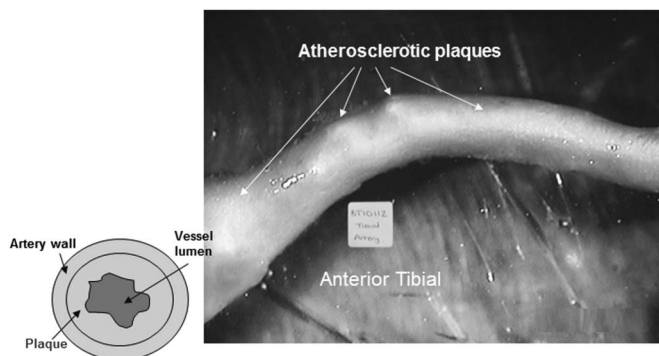
FIGURE 1. Representative image of an anterior tibial artery from a postmortem donor showing the presence of atherosclerotic plaques within the vessel.

TABLE 2. Desmosine Content of Artery Rings That Were Untreated or Treated With Vehicle or PRT-201 for 30 Minutes

| Test Condition	Desmosine pmol/mg Protein
Untreated	2072
Vehicle	2088
PRT-201 0.1 mg/mL	2383
PRT-201 0.5 mg/mL	1678
PRT-201 1.0 mg/mL	1304
PRT-201 5 mg/mL	959
PRT-201 10 mg/mL	1100

Main Study

For donor 2, the transmural pressure in the posterior tibial artery was increased from 10 to 120 mm Hg in 10–20 mm Hg increments during the first and second pressure–volume measurements. After treatment with either PRT-201 or vehicle, the diameters of the posterior tibial artery segments were greater at all transmural pressures. The greater diameter after treatment with vehicle indicated that the pressure applied during the first measurement resulted in persistent pressure-induced alteration in artery diameter. To avoid such effects, a lower maximum pressure of 80 mm Hg was used in subsequent tests.

For donors 6–10, the luminal pressure was increased from 10 to 80 mm Hg in increments of 10–20 mm Hg during the first diameter measurement. Before application of test article and before the second pressure–volume measurement, artery diameter at 10 mm Hg was measured the second time to confirm that arterial diameter had returned to approximately the same diameter observed in the first pressure–diameter measurement at that same pressure, indicating that arterial elasticity was not persistently altered during the first pressure–diameter measurement. Comparison of artery diameters at 10 mm Hg before and after increasing the pressure to 80 mm Hg showed no significant difference; the average diameter of anterior tibial artery segments increased by only 6% ± 12% (paired t test, $P = 0.29$), and the average diameter of posterior tibial artery segments increased by only 3% ± 5% ($P = 0.32$). Pressure–diameter curves for anterior and posterior tibial arteries before and after PRT-201 treatment are shown in Figures 3 and 4.

After PRT-201 treatment, the diameters of anterior and posterior tibial arteries were significantly increased at all pressures tested. The anterior tibial artery diameter was greater on average by 0.78 ± 0.21 mm, and the percentage increase was on average 27% ± 12%. The posterior tibial artery diameter was greater on average by 0.58 ± 0.30 mm, and the percentage increase was on average 21% ± 11%. The changes and percentage changes were statistically significant ($P < 0.0001$). No arteries ruptured at the pressures tested in these experiments.

Table 3 shows the desmosine (elastin) content of arteries treated with PRT-201 during the experiments and compared with baseline values. Treatment with PRT-201 resulted in a mean reduction in desmosine content of approximately 50% ($P < 0.001$).
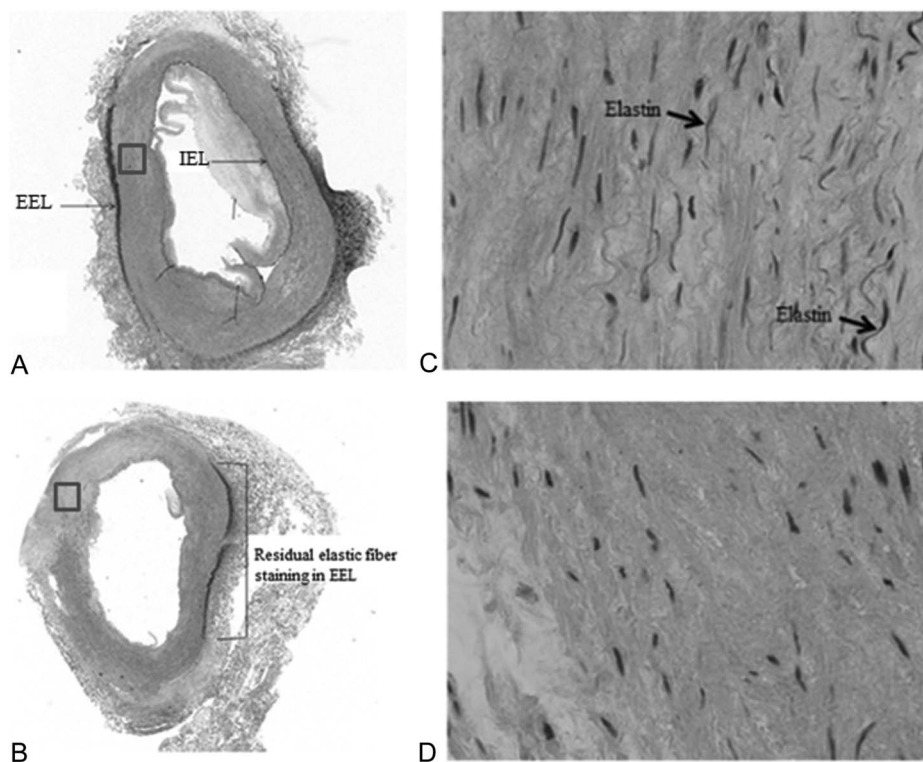
FIGURE 2. Photomicrograph of transverse sections of human popliteal artery treated with vehicle (A) (×2) and (C) (×40) or PRT-201 5 mg/mL for 30 minutes (B) (×2) and (D) (×40). EEL, external elastic lamina; IEL, internal elastic lamina.

DISCUSSION

The current treatment of PAD involves risk-factor modification by smoking cessation, diet, exercise, and the prescription of HMG-CoA reductase inhibitors and antithrombotic agents.[2] These measures are often unsuccessful in part because of poor adherence. Therefore, angioplasty is commonly performed to improve walking performance.[2] However, long-term patency after angioplasty is generally poor.[3–5] Thus, there is a need for new and more durable therapies.

Cilostazol, a vasodilator, is one of the few rigorously studied treatments for intermittent claudication in PAD. Cilostazol increases blood vessel diameter, which decreases total peripheral resistance leading to increased blood flow in the affected limb. Cilostazol has been shown to increase walking distance in patients with intermittent claudication.[13] Since the blood flow is related to the fourth power of the lumen radius, even small changes in the diameter of a blood vessel will significantly increase flow. A 5% increase in radius theoretically would increase blood flow by 22%. A 20% increase in radius would theoretically increase blood flow by approximately 100%.

PRT-201 acts as a vasodilator and could improve blood flow and walking distance by a similar mechanism as cilostazol. Cilostazol requires chronic oral dosing, which is moderately effective, and associated with side effects.[13] In contrast, PRT-201 requires a single application to cause local and possibly permanent vasodilation. In clinical trials, PRT-201 was found to be well tolerated and nonimmunogenic in patients undergoing surgery to create a hemodialysis access.[7–9]

Previous publications suggested that application of type I pancreatic elastase to normal blood vessels could lead to increased vessel diameter.[14,15] However, this has not been demonstrated in atherosclerotic arteries, and it was feared that the treatment could result in vessel rupture or aneurysm formation. Before proceeding into clinical trials, it was necessary to confirm safety in toxicology studies and also confirm that dilation was possible in atherosclerotic arteries. The current
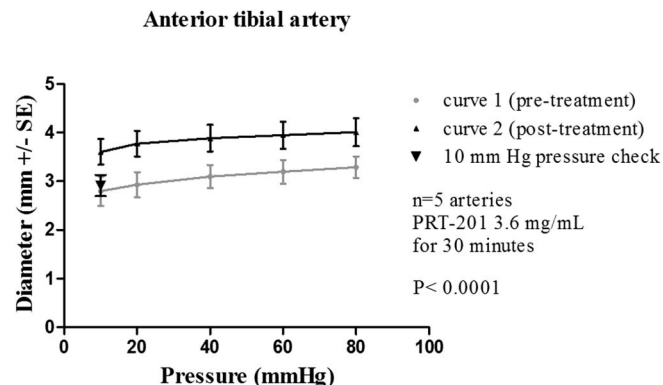
FIGURE 3. Effect of PRT-201 at a concentration of 3.6 mg/mL for 30 minutes on the external diameter of human anterior tibial arteries.
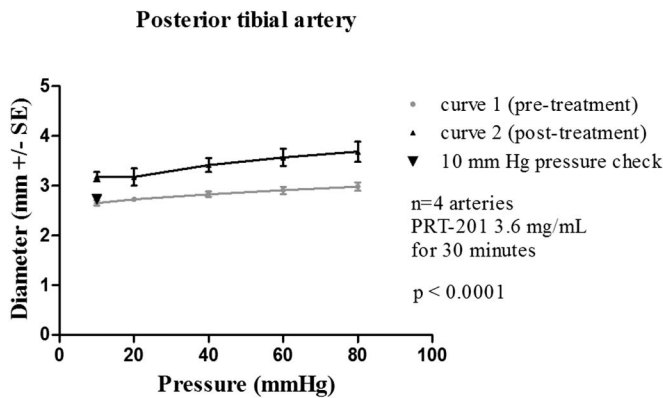
FIGURE 4. Effect of PRT-201 at a concentration of 3.6 mg/mL for 30 minutes on the external diameter of human posterior tibial arteries.

investigation reported here provides strong evidence that the latter is possible.

This study demonstrated that a single treatment of PRT-201 ex vivo increased the diameter of atherosclerotic human arteries across a range of pressures raising the possibility that the local administration of PRT-201 could be used as an adjunct to angioplasty, or as an alternative to angioplasty in selected patients.

One limitation of this research is that the arteries were not severely atherosclerotic, and the presence of atherosclerosis was determined visually without quantification of the extent of plaque. Attempts were made to study arteries from limbs amputated from patients with severe atherosclerosis, but arterial segments from these patients leaked perfusate from the cannulated ends of the artery and would not adequately hold pressure. Wire myography is an alternative method to measure PRT-201–dependent dilation of arteries. In separate studies, the authors used wire myography to measure diameter changes in severely atherosclerotic arteries after ex vivo angioplasty and treatment with PRT-201, demonstrating increases in vessel diameter with PRT-201 treatment (data not shown).

The local administration of PRT-201 after balloon angioplasty of severely atherosclerotic and narrowed segments of the femoral and popliteal arteries is currently being explored in a human phase-1 clinical study (www.clinicaltrials.gov, NCT01616290), where PRT-201 is administered to the adventitia by a Mercator Bullfrog (San Leandro, CA) catheter after successful balloon angioplasty.

In less severe cases, patients with PAD and claudication might benefit from PRT-201 treatment alone. Even a modest increase in arterial diameter with PRT-201 could significantly increase lumen area and blood flow in a stenotic artery segment.

Before PRT-201 could be embraced, it would need to be shown that the treatment is technically feasible and safe. In toxicology studies, PRT-201 administered to the carotid artery of rabbits removed elastin without clinical toxicity. The longest follow-up in the studies was 6 months. PRT-201 administered to the superficial femoral artery of swine also removed elastic fibers and caused arterial dilation without evidence of chronic inflammation, collagen degradation, or aneurysm formation. The longest follow-up in these studies was 90 days. Although elastase has been used in the literature to create aneurysms, in retrospect, it is not the elastolytic activity which creates aneurysm but the contaminants and impurities in the elastase which caused inflammation, and collagen degradation which led to aneurysms.[16] PRT-201 is a novel and highly purified formulation and has not been shown to cause aneurysms in hemodialysis access clinical trials.[7–9] PRT-201 monotherapy treatment would also need to show a measurable improvement in function (eg, walking distance) and demonstrate safety in the longer term. These questions will need to be addressed in clinical trials.

CONCLUSIONS

In conclusion, PRT-201 is a novel drug candidate, which can significantly dilate atherosclerotic human blood vessels by over 20%. PRT-201 was not immunogenic and did not cause vessel rupture or aneurysms in hemodialysis access clinical trials. PRT-201 treatment could potentially increase the walking performance of PAD patients by increasing artery lumen diameter and blood flow. This will need to be confirmed in clinical trials.

TABLE 3. Desmosine Content (pmol desmosine/mg protein) of Arteries Treated With PRT-201

Donor Number	Artery Type	Desmosine at Baseline	Desmosine After PRT-201 Treatment	Change in Desmosine	Percentage Change in Desmosine
6	Anterior tibial	2645	723	−1922	−73
	Posterior tibial	2875	1832	−1043	−36
7	Anterior tibial	3284	1130	−2154	−65
	Posterior tibial	2876	1956	−920	−32
8	Anterior tibial	3952	1984	−1968	−50
	Posterior tibial	3028	1524	−1504	−50
9	Anterior tibial	5662	1888	−3774	−67
10	Anterior tibial	3038	1870	−1168	−38
	Posterior tibial	4260	2006	−2254	−53
Mean ± SD		3420 ± 965	1613 ± 449	−1856 ± 872	−52 ± 14

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