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# Gene therapy to promote thromboresistance: Local overexpression of tissue plasminogen activator to prevent arterial thrombosis in an *in vivo* rabbit model

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**ABSTRACT** **Tissue-type plasminogen activator (tPA) catalyzes the rate-limiting initial step in the fibrinolytic cascade. Systemic infusion of tPA has become the standard of care for acute myocardial infarction. However, even the relatively short-duration protocols currently employed have encountered significant hemorrhagic complications, as well as complications from rebound thrombosis. Gene therapy offers a method of local high-level tPA expression over a prolonged time period to avoid both systemic hemorrhage and local rebound thrombosis. To examine the impact of local tPA overexpression, an adenoviral vector expressing tPA was created. The construct was characterized functionally *in vitro*, and the function of the vector was confirmed *in vivo* by delivery to the rabbit common femoral artery. Systemic coagulation parameters were not perturbed at any of the doses examined. The impact of local overexpression of tPA on *in vivo* thrombus formation was examined subsequently in a stasis/injury model of arterial thrombosis. The construct effectively prevented arterial thrombosis in treated animals, whereas viral and nonviral controls typically developed occluding thrombi. This construct thus offers a viable technique for promoting a locally thromboresistant small-caliber artery.**

Cardiovascular disease remains the leading cause of death in the U.S., in spite of preventative efforts (1). Thus, a great deal of research has been focused on studying the pathogenesis, prevention, and treatment of atherosclerosis as the underlying cause for the majority of cardiovascular diseases. Current management relies on combinations of dietary modification, precise control of blood glucose, lipid-lowering drugs, behavioral modification including smoking cessation, and balloon dilation or surgical manipulation of advanced disease. Although each of these interventions has benefited survival or quality of life, astounding morbidity and mortality remain.

Because most acute myocardial infarctions and cerebrovascular accidents are precipitated by acute thrombotic events superimposed on underlying lesions (2), a great deal of attention has been focused recently on the process of arterial thrombosis. Clinical research has shown that thrombolytic therapy improves survival in acute myocardial-infarction and cerebrovascular-accident patients (3–6), that heparin improves survival in certain high-risk subpopulations (7), and that chronic low-dose aspirin therapy may decrease the incidence of all such events (8, 9). In addition, a number of new antithrombotic modalities, including enhancement of nitric oxide production (10), tissue-factor pathway inhibitors (11), hirudin (7), antibodies to glycoprotein IIb/IIIa (2, 12), and TNK-tissue-type plasminogen activator (tPA; Genentech; ref. 13), have shown great promise in initial studies. Recently, primary coronary angioplasty has been examined in several trials with mixed results (14, 15).

tPA, a 527-aa residue serine protease, performs the primary, though not exclusive, role in fibrinolysis by preferentially catalyzing the conversion of the proenzyme plasminogen to plasmin in the presence of fibrin (16, 17). tPA function remains relatively clot-specific because of its high affinity for fibrin–plasminogen complexes via a fibronectin-like finger domain of lysine binding sites near the amino terminus and because of its relatively poor activity in the absence of fibrin (16, 18). Hepatic metabolism represents the primary clearance mechanism for tPA, and the enzyme has a half-life on the order of 5–10 min in the circulation (19).

Thrombolytic therapy using systemically administered recombinant tPA (rtPA) has become the standard clinical treatment for acute myocardial infarction. This treatment approach has resulted in reductions in infarct size, preservation of ventricular function, and overall reductions in mortality (3–5). Treatment with tPA has also proven effective in improving the neurologic outcome in patients with acute ischemic stroke (6).

All current clinical protocols employ systemic routes of tPA administration. However, despite the many advantages of systemically administered tPA relative to other systemic therapies, significant complications remain. Central among these problems are hemorrhages, particularly pulmonary and intracerebral hemorrhages (3–6), each of which can be life-threatening. The extended 12- to 72-h infusion regimens of tPA previously examined for venous and thromboembolic disease have proven to be virtually useless because of these complications (20). Moreover, significant hemorrhage has been observed consistently in the 1.5- to 3-h regimens commonly employed in acute myocardial infarction (3–6). Although systemic tPA therapy remains a viable treatment, restricted overexpression of tPA in the local environment of the clot might prevent or lyse a thrombus effectively without systemic side effects. In addition, the longer duration of therapy in this environment could improve results and prevent rebound thrombosis after cessation of therapy. Several laboratories have already reported retroviral (21, 22), adenoviral (23), and liposome-mediated (24) gene transfer to the vessel wall with varying efficiencies. Our laboratory and others have

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Abbreviations: Adv/RSV-βgal, recombinant adenovirus containing the *Escherichia coli* β-galactosidase gene driven by the RSV promoter; Adv/RSV-tPA, adenovirus expressing human tPA under the control of a RSV promoter; cfa, common femoral artery; moi, multiplicity of infection; pfu, plaque-forming unit; RSV, Rous sarcoma virus; rtPA, recombinant tissue-type plasminogen activator; tPA, tissue-type plasminogen activator.

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found adenovirus-based models to be high-efficiency *in vivo* expression systems.

Our laboratory thus has chosen to pursue local vascular adenoviral delivery of tPA to control thrombosis. In this investigation, we assess the *in vitro* and *in vivo* function of a replication-deficient adenovirus expressing human tPA under the control of a Rous sarcoma virus (RSV) promoter (Adv/RSV-tPA). In endothelial cell cultures, the Adv/RSV-tPA construct consistently produces very high levels of functional tPA expression relative to controls. Comparable function of the construct in a rabbit model is confirmed by local *in vivo* delivery to the common femoral artery (cfa), followed by local assay of tPA activity; systemic coagulation parameters were not elevated at any viral dose. Further, efficacy of the construct in preventing *in vivo* arterial thrombus formation was confirmed and compared with results from viral and nonviral controls.

## MATERIALS AND METHODS

Chromogenic assay kits for tPA activity were obtained from American Diagnostica (Greenwich, CT). Reference tPA of known activity was obtained from Calbiochem for generation of standard curves. Additional tPA for systemic infusion was obtained from Genentech. *Not*I linkers were obtained from New England Biolabs, and restriction endonucleases were from Boehringer Mannheim. Cell culture medium (DMEM), fetal bovine serum, and M199 were obtained from GIBCO/BRL. Recombinant adenovirus containing the *Escherichia coli* β-galactosidase gene driven by the RSV promoter (Adv/RSV-βgal) was kindly provided by Michel Perricaudet (Institut Gustave Roussy, Ville-Juif, France).

**Construction of Adv/RSV-tPA.** The complete cDNA of human tPA, a kind gift of Sandra Degen (University of Cincinnati, Cincinnati), was cloned into the *Not*I site of the E1A$^-$ adenoviral transfer vector pAdLI, under the control of the RSV promoter (25). This construct was cotransfected with the plasmid pJMI7 into the adenovirus packaging cell line 293. Recombinant adenovirus clones were assayed initially for tPA function, as described below, after plaque purification and amplification as described (26, 27).

***In Vitro* tPA Expression Assay.** Human umbilical-vein endothelial cells were harvested by the method of Jaffe *et al.* (28) and grown on gelatin-coated plates in M199, supplemented with 20% fetal bovine serum, 0.1 mg/ml porcine heparin, 5 μg/ml extracellular matrix, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37°C. All studies were performed on cells at passage 2. Human umbilical-vein endothelial cells were infected (at $n = 3$ each) with Adv/RSV-tPA, Adv/RSV-βgal, or PBS for 4.5 h at 300 multiplicities of infection (mois), then incubated in dye-free DMEM for 3 days. Medium was then changed, and the cells were incubated for 30 min. Aliquots were then withdrawn and employed as serial dilutions in a commercially available chromogenic assay of tPA activity (American Diagnostica), and results were standardized to curves obtained with commercially obtained tPA of known activity (Calbiochem).

**Local *in Vivo* Delivery of Recombinant Adenovirus for *in Vivo* tPA Expression.** *In vivo* adenoviral delivery to an isolated vessel segment was performed as described below. Briefly, the left cfa of each New Zealand White rabbit was exposed. The left inferior epigastric artery (IEA) was ligated, cannulated, and divided, and a 3.0-cm segment of the cfa beginning just proximal to the left IEA stump was then isolated via atraumatic microvascular clamps. The contents of the lumen were aspirated via the IEA stump, and the lumen was washed gently with PBS before incubation of 200 μl of PBS alone, 200 μl of $5 \times 10^9$ plaque-forming units per ml (pfu/ml) Adv/RSV-βgal, 200 μl of $5 \times 10^8$ pfu/ml Adv/RSV-tPA, 200 μl of $1 \times 10^9$ pfu/ml Adv/RSV-tPA, or 200 μl of $5 \times 10^9$ pfu/ml Adv/RSV-tPA ($n = 3$ each). These titers were selected based on earlier β-galactosidase marker experiments, which identified minimal expression below $1 \times 10^8$ pfu/ml and evidence of significant toxicity above $7.5 \times 10^9$ pfu/ml. The segment was allowed to stand for 15 min before incubation solutions were aspirated, and the lumen was washed before the clamps were removed.

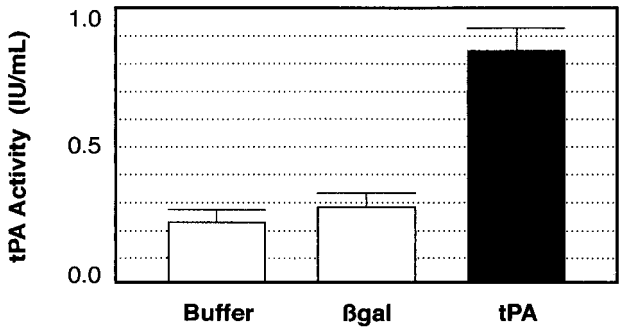


FIG. 1. *In vitro* tPA activity in human umbilical-vein endothelial cells. Cells were incubated with buffer alone, 300 moi Adv/RSV-βgal, or 300 moi Adv/RSV-tPA, then employed 3 days later in a chromogenic assay for tPA activity. Values represent mean plus standard error.

On day 5 after viral delivery, the cfa was reexposed, and the previously treated vessel segment was reisolated with microvascular clamps and cannulated via the superficial femoral artery just distal to the treated segment, with care to place the needle distal to the segment itself. A solution of PBS was then infused and incubated *in vivo* for 30 min. This solution was then withdrawn and employed as 20-μl aliquots and serial dilutions to 20-μl aliquots in a chromogenic assay as above.

**Perturbation of Systemic Coagulation Parameters After Local *in Vivo* Viral Delivery.** Blood samples were withdrawn from the animals via ear vein both before viral delivery and after 5 days (before reexposure of the cfa). Samples were used to determine fibrin split product levels. Additional controls of normal, unmanipulated, age-matched controls and age-matched controls were treated with systemic tPA infusion according to the widely clinically employed accelerated-infusion protocol (a 0.15-mg/kg bolus of tPA was given i.v. via ear vein, followed by i.v. infusion of 0.75 mg/kg over 30 min, then by 0.5 mg/kg over 60 min).

***In Vivo* Thrombus Initiation After Adenoviral Delivery.** Local *in vivo* delivery of recombinant adenovirus was accomplished as described above for the following groups ($n = 4$ each): (*i*) 200 μl of PBS, (*ii*) 200 μl of Adv/RSV-βgal at a titer of $5 \times 10^9$ pfu/ml, and (*iii*) 200 μl of Adv/RSV-tPA at a titer of $5 \times 10^9$ pfu/ml. On day 3 after viral delivery, each animal

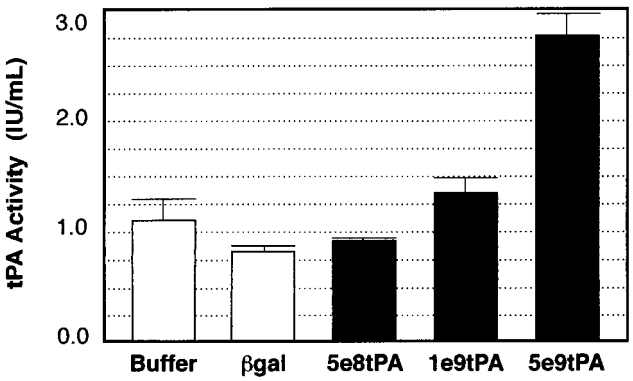


FIG. 2. tPA activity in treated cfas 5 days after delivery buffer alone, 200 μl of $5 \times 10^9$ pfu/ml Adv/RSV-βgal, 200 μl of $5 \times 10^8$ pfu/ml Adv/RSV-tPA, 200 μl of $1 \times 10^9$ pfu/ml Adv/RSV-tPA, or 200 μl of $5 \times 10^9$ pfu/ml Adv/RSV-tPA. Standard error as depicted.

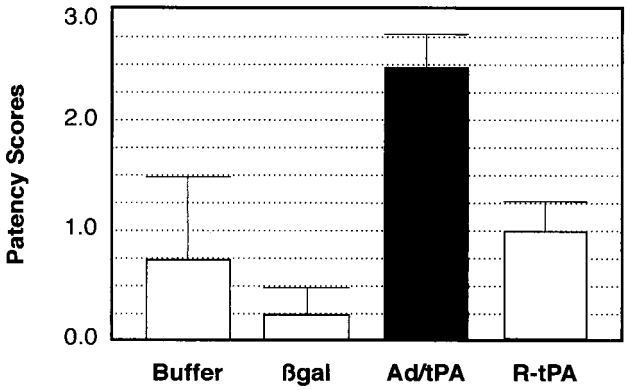


FIG. 3. Gross-patency scores after delivery of buffer alone, 200 μl of $5 \times 10^9$ pfu/ml Adv/RSV-βgal, 200 μl of $5 \times 10^9$ pfu/ml Adv/RSV-tPA (Ad/tPA), or accelerated systemic infusion of rtPA (R-tPA) followed by thrombus initiation as described in *Materials and Methods*. Values represent mean plus standard error.

was reexplored and a thrombus initiated as described below. Briefly, at a point 1.0 cm distal to the IEA in the cfa segment previously isolated, the cfa was divided and reanastomosed with a 10-0 nylon suture by using standard microsurgical techniques. After the anastomosis was completed, two loose perpendicular loops of 8-0 silk suture were placed 0.5 cm distal to the anastomosis to introduce an element of stasis and elements that were directly thrombogenic. An additional control group included age-matched animals treated with systemic tPA infusion according to the widely clinically employed accelerated-infusion protocol (described above) at the time of thrombus initiation. On day 3 after thrombus initiation (day 6 after viral delivery), all animals were assessed for the degree of thrombus formation in the treated vessel segments as described below.

On day 6 after viral delivery, the animals were killed, and patency was assessed by direct inspection as well as by histologic examination. The following scoring system was employed to assess gross patency and thus grade gross efficacy: 0 = total thrombosis of vessel (>2.5 cm); 1 = incomplete thrombosis of vessel with no distal refill on "milking" (<2.5 cm); 2 = incomplete thrombosis of vessel with slow refill; and 3 = completely patent vessel without evidence of thrombosis. At the end of the experiment, the average patency scores were determined for each treatment group.

To examine these effects quantitatively and to define better the spatial distribution of any alterations in thrombus formation within the vessel segments, segments from 5 mm proximal to the anastomosis to 10 mm distal to the stasis element were divided into three roughly equal zones. For all animals, zone A contained the anastomotic line (the most proximal injury); zone B contained the perpendicular silk loops (stasis element); and zone C was defined as the area downstream of B. Serial sections were performed in each zone of each artery. A Minolta RD-175 SLR Digital camera was used to record images of each slide at high resolution. The resulting images were analyzed with the IMAGEPRO PLUS analysis system, version 3.0.1 (Media Cybernetics, Silver Spring, MD) to determine the average percent of luminal area that represented intravascular thrombus (cross-sectional thrombus area normalized to available lumen for each). Briefly, thrombus margins were determined and defined as primary area of interest. The number of pixels contained in this region was determined. The length of the internal elastic lamina (visualized by Voerhoff–von Gieson staining for elastin) was calculated as a secondary area of interest. The internal elastic lamina was assumed to be nondistensible, and the circular "available lumen" was calculated from lamina length. This method was used to assess accurately available areas in segments with small thrombus percentages, even for those areas with vessel collapse (fixation and sectioning artifacts). The method used here

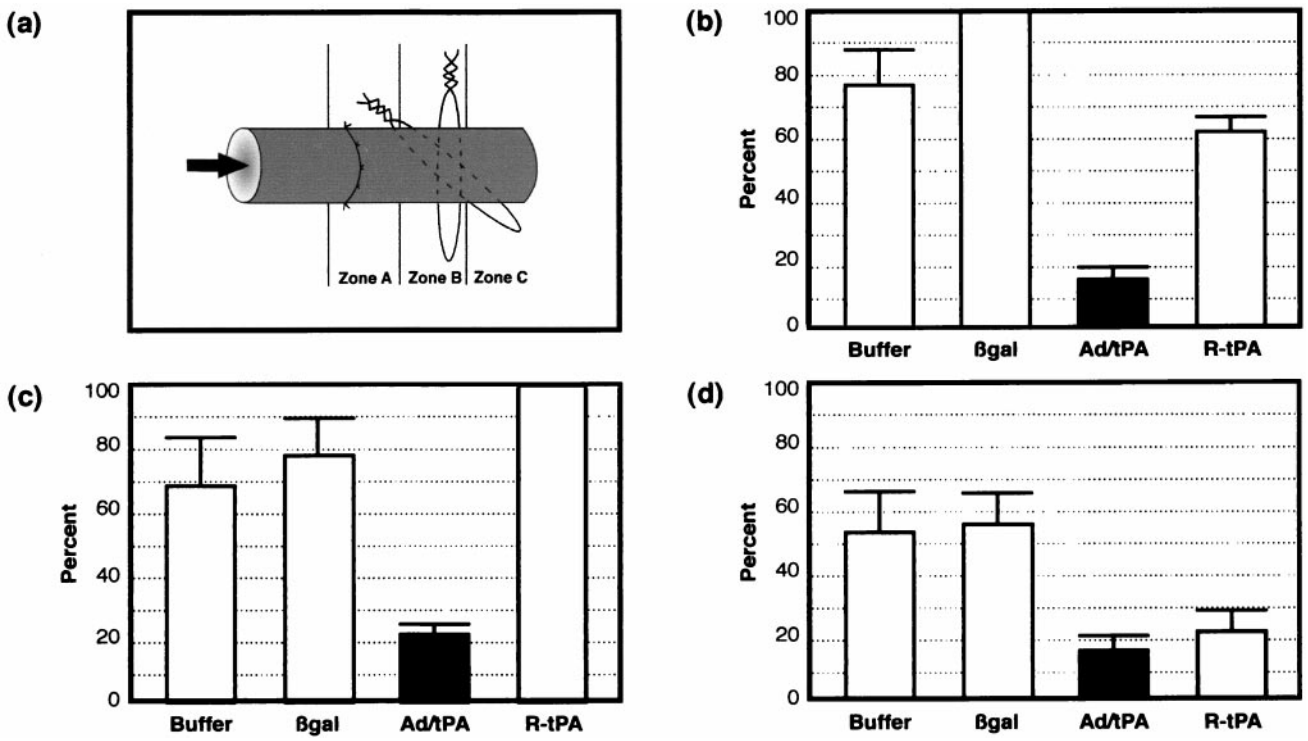


FIG. 4. Percentages of cross-sectional thrombus by zone after delivery of buffer alone, 200 μl of $5 \times 10^9$ pfu/ml Adv/RSV-βgal (βgal), 200 μl of $5 \times 10^9$ pfu/ml Adv/RSV-tPA (Ad/tPA), or accelerated systemic infusion of rtPA (R-tPA) with thrombus initiation as described in *Results*. (*a*) Illustration of anatomic segments corresponding to zones A–C. Cross-sectional thrombi in zones A (*b*), B (*c*), and C (*d*). Standard error as depicted.

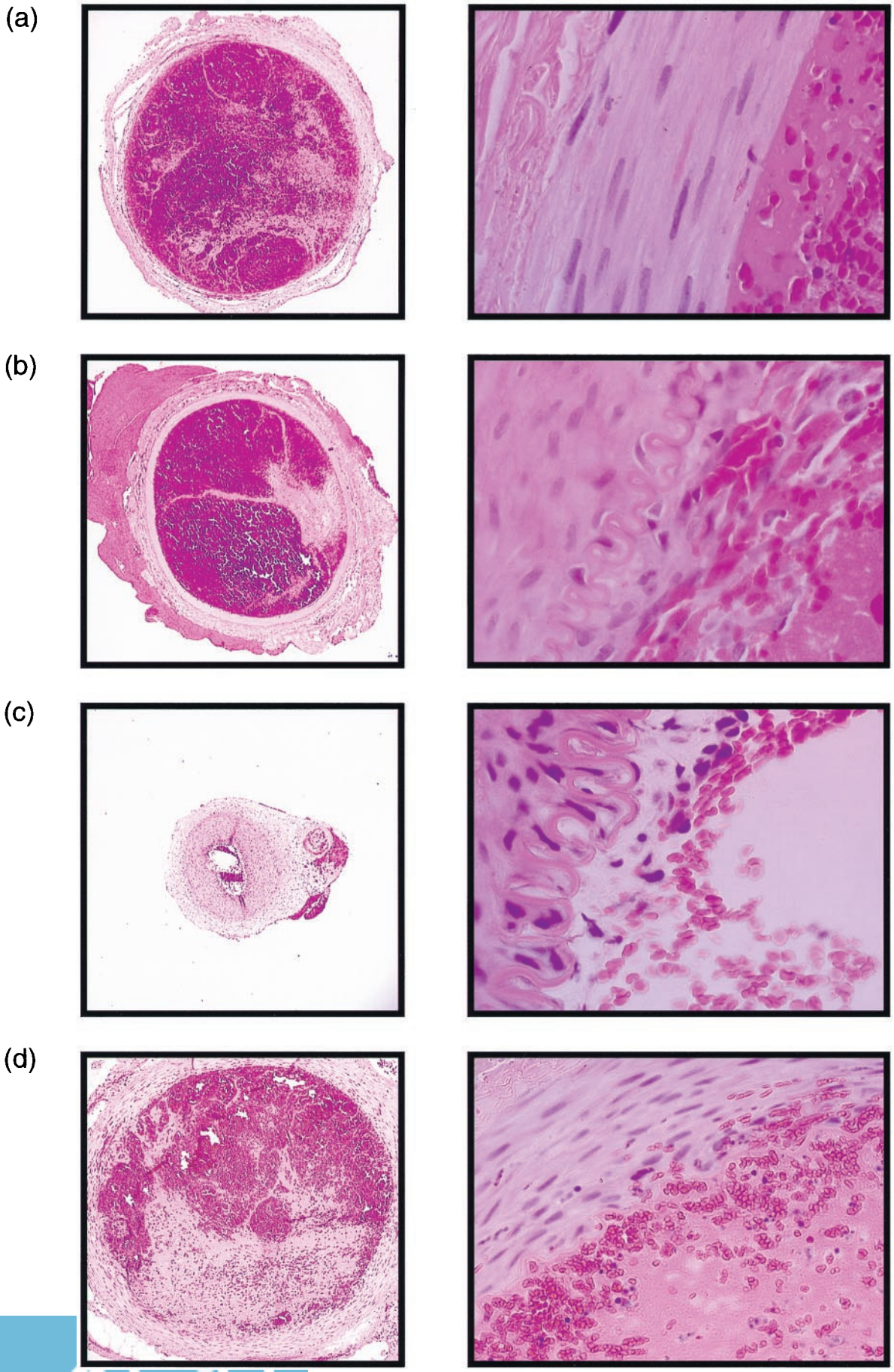


FIG. 5 (*Legend appears at the bottom of the opposite page.*)

is inaccurate only for segments that do not have a fully distending thrombus. In such segments, this method will overestimate the degree of thrombus. Hence, the simplified method tends to minimize indications of therapeutic effect by overestimating the degree of thrombus in low-thrombus cross sections only but accurately assessing full-thrombus controls. Each image was analyzed manually to confirm automatic processing accuracy, and the results were tabulated as average percentage of cross-sectional thrombus for each segment of each vessel. In this manner, quantitation of thrombus formation was completed for each of the various zones for each animal group. Statistical significance was analyzed with ANOVA, and the difference in the mean value between the various groups was calculated with $P$ values, as reported in *Results*.

## RESULTS

***In Vitro* tPA Expression Assay.** To demonstrate production of functional tPA, human umbilical-vein endothelial cell cultures were infected with buffer alone, 300 moi Adv/RSV-βgal, or 300 moi Adv/RSV-tPA. Incubations were performed for 30 min at 72 h post infection, and aliquots were employed in a chromogenic assay of tPA activity as detailed in *Materials and Methods*. The results are depicted in Fig. 1. Functional tPA expression reached 356.2% of buffer controls and 290.7% of viral controls ($P < 0.05$ and $P < 0.05$, respectively).

***In Vivo* tPA Expression Assays.** To confirm these results in an *in vivo* system quantitatively, 3.0-cm segments of New Zealand White rabbit cfas were infected *in vivo* with 200 μl of $5 \times 10^9$ pfu/ml Adv/RSV-tPA, $1 \times 10^9$ pfu/ml Adv/RSV-tPA, $5 \times 10^8$ pfu/ml Adv/RSV-tPA, $5 \times 10^9$ pfu/ml Adv/RSV-βgal, or PBS alone. At 5 days after infection, *in vivo* functional tPA assays were performed as above. The results are presented in Fig. 2. The $5 \times 10^9$ pfu/ml Adv/RSV-tPA titer produced the highest levels of tPA activity (2.778 ± 0.209) and was significant relative to all other groups ($P < 0.05$). This titer was thus selected for all subsequent experiments. In these assays, buffer-treated arteries were able to produce 1.129 ± 0.209 units/ml tPA over 30 min.

**Perturbation of Systemic Coagulation Parameters After Local *in Vivo* Viral Delivery.** To examine the systemic impact of local overexpression of this secreted product, the animals for each of the above doses had blood samples withdrawn on day 5, before the *in vivo* assays described above were performed. Controls of normal age-matched unmanipulated animals were added to the controls above. Further, additional age-matched controls underwent systemic infusion of tPA on day 5, according to the accelerated-infusion protocol. All unmanipulated, PBS-, β-galactosidase-, and tPA-treated animals had undetectable fibrin split products levels, whereas systemic tPA infusion resulted in elevation to 18 ± 12.3 μg/ml. Based on these results, a titer of $5 \times 10^9$ pfu/ml was selected to achieve high-level overexpression without systemic perturbation for subsequent experiments.

**Prevention of *in Vivo* Thrombus Formation.** To examine the impact of local *in vivo* overexpression of tPA on thrombus formation, rabbit cfas were treated *in vivo* with 200 μl of $5 \times 10^9$ pfu/ml Adv/RSV-tPA, $5 \times 10^9$ pfu/ml Adv/RSV-βgal, or PBS alone at the time of thrombus initiation; another group of animals received an administration of systemic tPA at the time of thrombus initiation. On day 3 after infection, a thrombus was initiated by using a combination of a nondenuding injury and a stasis element as described. This model of thrombus induction was selected, because the endothelium remains relatively preserved despite both injury and stasis elements. On day 6 after infection, the vessels were reexposed, and gross patency was assessed. The gross-patency scores are presented in Fig. 3. The PBS controls exhibited a patency score of 0.75. The Adv/RSV-βgal group had a score of 0.25, and systemic rtPA-treated animals exhibited a score of 1.00. The Adv/RSV-tPA group, which had a score of 2.5, was thus significantly different from all other groups ($P < 0.05$). Although the Adv/RSV-βgal-treated group appeared to have longer and more significant thrombus formation than nonviral controls, these observations were not statistically significant (95% confidence level).

Histologic evaluation was employed quantitatively (as described in *Materials and Methods*) to define any spatial differences in thrombus formation between groups. Microscopic images were obtained and analyzed to determine the average cross-sectional area of thrombus in each zone. These results were then normalized to the available lumen in each corresponding section, with the results reported as average percentage of cross-sectional thrombus for each zone (Fig. 4). Representative histologic findings for each treatment group are presented in Fig. 5. Across all sections, buffer-treated animals averaged 67.57% ± 7.78% cross-sectional thrombus. Viral controls rose to 80.42% ± 5.74%, and animals treated with systemic infusion of rtPA averaged 61.02% ± 6.09%. Vessels treated with the Adv/RSV-tPA construct, in contrast, averaged 19.12% ± 1.90%. Thus, statistically significant overall patency was also achieved with the Adv/RSV-tPA construct relative to all controls ($P < 0.01$). In contrast, current clinical protocols for systemic infusion of rtPA did not afford significant thrombus reduction relative to nonviral controls ($P > 0.05$), corresponding to clinical observations of high-rebound thrombosis at this time point.

## DISCUSSION

In this work, a recombinant adenovirus expressing human tissue plasminogen activator was constructed, shown to be functional both *in vitro* and *in vivo*, and proven effective in prevention of *in vivo* intravascular thrombus formation in a targeted vascular segment.

*In vitro* experiments indicated high-level function of the construct. A limited dose-response experiment confirmed *in vivo* function in the rabbit cfa. Expression of just under 3.0 units/ml was attained at $5 \times 10^9$ pfu/ml, whereas controls remained near 1.0 unit/ml in this system. In the accelerated tPA-infusion protocols employed clinically, peak systemic concentrations of tPA approach ≈6.0 units/ml. However, local concentrations vary dramatically because of quite high liver metabolism and variations in flow patterns, which limit concentrations in a slow-flow thrombosed vessel to levels well below those of the systemic peak concentration in these protocols. Moreover, effective thrombolysis is brief, leading to significant rates of rebound thrombosis (15). Because the Adv/RSV-tPA construct can locally produce 3.0 units/ml, longer-term thrombus prevention becomes feasible. In this experiment, none of the Adv/RSV-tPA groups showed detectable elevations in fibrin split products, despite the local overexpression of tPA. In contrast, the accelerated systemic tPA infusion resulted in elevations in fibrin split product levels, consistent with the systemic hemorrhagic risk associated with clinical use of this protocol. Thus, this construct offers the potential to avoid these limiting complications.

To determine whether these levels of local tPA production would be adequate to limit thrombus formation, the construct

FIG. 5. (*On the opposite page.*) Representative low (×5; *Left*) and high (×50; *Right*) magnification photographs of cfa cross sections 3 days after thrombus initiation for animals treated with buffer alone (*a*), 200 μl of $5 \times 10^9$ pfu/ml Adv/RSV-βgal (*b*), 200 μl of $5 \times 10^9$ pfu/ml Adv/RSV-tPA (*c*), or accelerated systemic infusion of rtPA at the time of thrombus initiation (*d*).

was tested in an *in vivo* model of arterial thrombosis. Although nonviral, mock-virus-treated, and rtPA-treated animals typically developed thrombi with no gross evidence of distal flow, the Adv/RSV-tPA construct grossly prevented the formation of an occluding intraarterial thrombus in this experiment. Histologic analysis confirmed that Adv/RSV-tPA animals consistently maintained significant patency throughout the treated and adjacent vessel segments, whereas occluding thrombi formed in the viral, nonviral, and systemic infusion controls. Thus, local overexpression of tPA through this delivery system results in a vessel segment that is resistant even to a complex, relatively extreme impetus for thrombus formation.

Initial adenoviral delivery systems have been shown to have expression at significant levels for only a 2- to 4-week period, but more recent refinements have extended this time frame. The brief 1.5- to 3-h exogenous thrombolytic regimens have encountered great success, but they are limited in part by rebound thrombosis, which usually occurs within the first few days (15). Because the construct presented here effectively prevents arterial thrombosis even after systemic rtPa controls reocclude, this approach may avert significant rebound thrombosis. Further, this extended time frame of effective thrombolysis may be achievable with simultaneous decreases in the risk of systemic hemorrhagic complication, as observed in the dose-response experiments. After further characterization, this approach might be employed as part of the armamentarium to prevent or treat arterial thrombosis.

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