Characterization of Recombinant Plasminogen Activator Production by Primate Endothelial Cells Transduced With Retroviral Vectors

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Retroviral vector-mediated expression of plasminogen activators (PAs) from endothelial cells (ECs) has been proposed as a potential therapeutic approach for intravascular thrombosis. To define the potential for gene transfer to increase fibrinolytic activity in a primate system, baboon ECs were transduced with retroviral vectors expressing wild-type and glycosylphosphatidylinositol-anchored urokinase, as well as wild-type and serpin-resistant tissue PA (t-PA). Expression of either t-PA or urokinase was increased by one log over baseline levels. There was no specific effect of either t-PA or urokinase overexpression on endogenous t-PA, urokinase, or PA inhibitor 1 (PAI-1) expression. Recombinant urokinase could be anchored to the cell surface at a level eight-fold above that of receptor-bound urokinase. The majority of secreted urokinase accumulated in conditioned medium as a free proenzyme, whereas both wild-type and serpin-resistant t-PA accumulated almost exclusively in complexes with PAI-1. In most but not all of the assays, the urokinase vectors conferred PA activity above that of the t-PA vectors. These data show that PA synthesis and activity are specifically increased subsequent to retroviral vector-mediated gene transfer in primate ECs. However, definition of an optimal PA vector will require in vivo experimentation. This is a US government work. There are no restrictions on its use.

The vascular endothelium is thought to play a major role in the regulation of intravascular coagulation and fibrinolysis.1,2 Endothelial cells (ECs) synthesize a variety of procoagulant and anticoagulant molecules, including tissue plasminogen activator (t-PA), protein S, plasminogen activator inhibitor 1 (PAI-1), thrombomodulin, and heparan sulfates. ECs also possess surface binding sites for several molecules important in coagulation or fibrinolysis, including factors IX and X, plasminogen, urokinase, and t-PA. Accordingly, the balance between local intravascular clotting and fibrinolysis may be at least partially dependent on endothelial function, i.e., whether ECs in a specific vascular bed express a relative excess of either prothrombotic or antithrombotic molecules.

We have previously suggested that gene transfer and overexpression of fibrinolytic molecules might be used to enhance the antithrombotic activity of ECs.3-5 We and others have shown that the PA activity of sheep, bovine, and canine6 ECs may be increased by retroviral-mediated gene transfer of cDNA sequences expressing either t-PA or urokinase. These fibrinolytically enhanced cells might be implanted along denuded vessel walls or seeded onto the surfaces of prosthetic devices to decrease local thrombosis in vivo. Alternatively, using recently described techniques,7 genetic material might be delivered to ECs in vivo, decreasing the thrombogenicity of a vascular segment without the need for endothelial denudation and subsequent seeding.

Several PA molecules, either wild type or genetically engineered, have been proposed as candidates for expression in ECs to enhance cellular fibrinolytic activity.8-11 Before the initiation of in vivo studies, we characterized and compared the retroviral vector-mediated expression of several of these recombinant PA in cultured baboon ECs. We chose baboon ECs because preclinical studies of human antithrombotic and fibrinolytic agents are most appropriately performed in a primate model.9,10 In addition, studies with primate cells (in which both nucleic acid sequences and protein products are efficiently detected with assays developed for the human homologs) permit a convenient and accurate assessment of the effect of overexpression of PA on the other components of the EC fibrinolytic system. In the present study, we compare wild-type t-PA, serpin-resistant t-PA, wild-type urokinase, and glycosylphosphatidylinositol-anchored urokinase for (1) their ability to increase primate EC PA activity either in conditioned medium (CM) or at the cell surface and (2) the effect of overexpression of these exogenous PAs on the individual components of the endogenous EC fibrinolytic system.

MATERIALS AND METHODS

Cell harvest and culture. Baboon ECs were a kind gift from Dr Laurence Harker and Johanna Anderson of Emory University, Atlanta, GA. Cells were harvested by collagenase digestion of an external jugular vein of a 12-kg adolescent male baboon, according to protocols approved by the animal care committees of Emory University and the Yerkes Regional Primate Center. EC identity was established by positive staining for von Willebrand's factor antigen. Cells were grown in RPMI-1640, 5 mM HEPES with 20% fetal calf serum (HyClone, Logan, UT), 100 IU/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B (Biofluids, Rockville, MD), 90 U/mL porcine heparin (Sigma Chemical Co, St Louis, MO), and 100 μg/mL EC growth factor (Biomedical Technologies, Stoughton, MA).

Retroviral vector construction. The construction of the retroviral vectors SAX (expressing adenosine deaminase),12 LBGSN (expressing β-galactosidase),13,14 B2Nst (expressing wild-type t-PA),15 LUKSN (expressing wild-type urokinase), and LUK+ASN (expressing a mutant cell surface-anchored urokinase)6 has been described previously. To construct retroviral vectors expressing serpin-resistant t-PA mutants, plasmids containing cDNA clones of two PA mutants ("Δ296-302" and "K296, R298, R299 → E, E, E") were obtained from Drs. J. Sambrook and E. Madison (University of Texas Southwestern, Dallas, TX). The first of these mutants16 contains a seven-aminoc acid deletion (Lys296→Gly296) and the second16 contains three amino acid substitutions (Lys296→Arg, Arg298→Arg and Arg299→Glu) which render the mutants resistant to binding by PAI-1, while preserving plasminogen activation activity. Both cDNAs were removed at 2.0-kb XbaI fragments, blunt ended and ligated into the HpaI I site of...
the plasmid pLXSN.  The vectors thus constructed were named Ld3SN and Ld3+SN and contain the deletion and triple-point mutants, respectively. For each of these two vectors, the presence of the appropriate DNA sequences conferring PAI-1 resistance was confirmed by DNA sequencing of the plasmid vectors using the dideoxy-chain termination method. The LSN vector, expressing wild-type t-PA from the viral long terminal repeat (LTR) promoter was constructed by excision from plasmid pGEM4Xt1 of a 2.2-kb I-Sal fragment containing a human t-PA cDNA, followed by blunt-ending and ligation into the site of pLXSN. All vectors are illustrated in Fig 1.

Amphotropic vector packaging lines were obtained from Dr Evelyn Karson (National Heart, Lung, and Blood Institute [NHLBI], Bethesda, MD) (SAX), Genetic Therapy Inc (Gaithersburg, MD) (LBgSN), and, for the remainder of the vectors, generated in our laboratory by transfection of PA-317 cells, as described previously. Retroviral vector stocks used to transduce baboon EOCs had titers of approximately 10^8 G418-resistant colony-forming units/mL, and were free of helper virus as determined by the S"/L" assay. All transduced cells were selected in 0.2 mg/mL G418 for a period of at least 14 days before the performance of any assays, and were processed as described previously. The levels of t-PA, u-PA, and PAI-1 antigen were determined using enzyme-linked immunosorbent assays (ELISAs) (Immundi-5 t-PA, u-PA, and PAI-1 (American Diagnostica, Greenwich, CT). Of note, whereas these assays were developed for the detection of human proteins, they all detected the analogous baboon protein in CM from untransduced cells. The precise extent of cross-reactivity of the baboon proteins in these assays was not determined directly; however, the validity of our assumption that cross-reactivity is high seems justified based on the concordance of the results of the ELISA, immunoprecipitation, and RNA analyses (see below). In addition, when human immunosassays have been used to quantitate baboon plasma t-PA and PAI-1, values within the known human ranges have been obtained, suggesting cross-reactivity is high. Total cellular protein in 0.1% NP-40 lysates was measured with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, VA), using bovine y-globulin as a standard.

**Quantification of anchored and receptor-bound urokinase.** The amount of anchored urokinase was measured in a manner similar to that described previously. Briefly, cells were grown to confluence in RPMI-1640 containing 3 mg/mL bovine serum albumin (BSA; Sigma). Medium samples were collected and processed as described previously. The levels of t-PA, u-PA, and PAI-1 mRNA, blots were probed with a 1.5-kb BamHI fragment HindIII fragment of plasmid pULCUPA.22 Beta-actin mRNA was detected by hybridization with BamHI human beta-actin cDNA fragment (a gift of Dr Roger Cohen, NHLBI).

**Zymography.** Zymographic analysis of medium conditioned by cultured cells was performed after incubating the cells overnight in RPMI-1640. Two different electrophoretic/zymographic techniques were used. We first used the Laemmli buffer system, with casein as the plasmin substrate, as described previously.4 Because of the consistent presence of a broad band of PA activity in the 60- to 80-kD range thought to be at least partially caused by dissociation of covalent PAI complexes at relatively high pH, we adopted the buffer system of Moos et al.26 Separation of proteins in this system occurs under these conditions would be both more highly resolved and more representative of the PA species present in the CM samples. Attempts to incorporate casein into gels run in this buffer system resulted in technically unsatisfactory gels. Substitution of bovine fibrinogen (Calbiochem, La Jolla, CA) for casein as a plasmin substrate at a final concentration of 0.2 mg/mL resulted in technically satisfactory, high-resolution zymograms without the broad band suggestive of enzyme-inhibitor complex dissociation. Final polyacrylamide concentrations in the stacking and running gels of the pH 7.28 buffer system were 4% and 12%, respectively. Methodology for the zymography was otherwise identical with each of the two gel-buffer systems.5 Prestained molecular-weight standards (Diversified...
Biotech, Newton Centre, MA) were used including β-galactosidase (116 kD), phosphorylase B (96 kD), and human serum albumin (68 kD), as well as a mixture of human t-PA (68 kD), high molecular-weight urokinase (54 kD), and low molecular-weight urokinase (35 kD) (American Diagnostica). This latter mixture served both as additional molecular-weight standards and as positive activity controls for the zymography.

**Immunoprecipitation.** Cells were plated onto 100-mm tissue culture dishes at 1.5 × 10⁶ cells per dish. Two days later, the cells were incubated for 1 hour in 5.0 mL methionine-free RPMI (Selectamine; Gibco, Grand Island, NY). 5S-methionine (500 μCi; Amersham, Arlington Heights, IL) per dish was then added for a 3-hour incubation. Labeled medium was centrifuged to remove cellular debris, then precleared by addition of 230 μL of protein G-Sepharose 4B conjugate (Zymed, San Francisco, CA) along with 100 μg of either nonimmunone goat IgG (Sigma) or goat antibodies to human t-PA or human urokinase (American Diagnostica). In later experiments, species containing PAI-1 were precleared with 250 μg of goat antibody to human PAI-1 (American Diagnostica), whereas control aliquots were precleared with 250 μg of either the anti-uPA (for the t-PA immunoprecipitations) or anti-t-PA IgG (for the urokinase immunoprecipitations). Preclearing was at 4°C for 4 hours, followed by centrifugation at 12,000g for 10 minutes. Remaining supernatants were then immunoprecipitated overnight with 100 μL of protein G-Sepharose 4B conjugate and 50 μg of goat antibodies to either human t-PA or urokinase (American Diagnostica). In this manner, parallel preclearing of aliquots of a sample with either anti-uPA or anti-PAI-1, followed by immunoprecipitation of each of the aliquots with anti-t-PA, resulted in precipitation of either all t-PA containing species or immunoprecipitation of only those t-PA species not complexed with PAI-1. Free u-PA species were also differentiated from those containing PAI-1 using an analogous technique.

After immunoprecipitation, complexes were washed and then resuspended in loading buffer without dithiothreitol or β-mercaptoethanol. The samples were then analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using the pH 7.28 buffer system described above. 14C-labeled molecular-size markers were phosphorylase b (97 kD), BSA (69 kD), ovalbumin (46 kD), and carbonic anhydrase (30 kD), and were obtained from Amersham. After staining with Coomassie Blue, gels were dried and autoradiographed.

**PA activity assays.** PA activity was measured using two methods: (1) by collection of CM that was subsequently assayed for PA activity; and (2) by addition of a plasminogen substrate reagent (PSR) mixture directly to cultured cells, followed by continuous measurement of plasmin activity in the presence of the cultured cells.

Two CM PA activity assays were used. The first was based on the protocol described by Chandler et al., with minor adaptations. Confluent 35-mm wells of cells were incubated overnight with 1.5 mL of RPMI with 3 mg/mL BSA. CM was either assayed immediately without acidification or acidified by addition of an equal volume of 0.5 mol/L sodium acetate pH 4.2 (to prevent further association of t-PA with PAI-1). Using this methodology, we have found that activity of acidified t-PA is stable over at least four freeze-thaw cycles. CM (5 μL; either acidified or nonacidified) was added to 250 μL of PSR, present in a 96-well plate, prewarmed to 37°C. The PSR consists of 0.075 mol/L TRIS pH 8.15, 0.01% Triton X-100, 0.5 μmol/L human Glu-plasminogen, 0.1 mg/mL CNBr-cleaved fibrinogen fragments, and 0.65 mmol/L S-2251. A baseline reading of OD₅₀₄₄ was made, and the plate maintained in the tissue culture incubator. OD₅₀₄₄ was measured approximately every 10 to 20 minutes, and the assay was terminated at 3 hours or sooner if all of the samples had attained an OD₅₀₄₄ of at least 0.8. When this first PSR was used, the cells remained in monolayers as observed by phase contrast microscopy. After the completion of the absorbance readings, the cells were rinsed and then lysed for determination of protein. Because of the heterogeneous nature of the PA activity expressed by the multiple types of transduced cells, rates of plasminogen activation were not further converted to IU/mL of either urokinase or t-PA. These rates were simply calculated for individual wells as ΔOD₅₀₄₄/hr/mg cellular protein.

Addition of the second PSR mixture to the cultured cells (formulated as described above to optimize the activity of the serpin-resistant t-PA mutants) resulted in concentrations of 0.05 mol/L Tris pH 7.4, 0.005% Triton X-100, 0.5 μmol human Glu-plasminogen, 0.1 mg/mL CNBr-cleaved fibrinogen fragments, and 0.65 mmol/L S-2251. A baseline reading of OD₅₀₄₄ was made, and the plate maintained in the tissue culture incubator. OD₅₀₄₄ was measured approximately every 10 to 20 minutes, and the assay was terminated at 3 hours or sooner if all of the samples had attained an OD₅₀₄₄ of at least 0.8. When this first PSR was used, the cells remained in monolayers as observed by phase contrast microscopy. After the completion of the absorbance readings, the cells were rinsed and then lysed for determination of protein. Because of the heterogeneous nature of the PA activity expressed by the multiple types of transduced cells, rates of plasminogen activation were not further converted to IU/mL of either urokinase or t-PA. These rates were simply calculated for individual wells as ΔOD₅₀₄₄/hr/mg cellular protein.

**RESULTS**

Secretion of recombinant human t-PA and urokinase by cultured baboon ECs. Immunoreactive t-PA was detect-
able in the CM of all baboon ECs, including untransduced cells as well as cells transduced with either the t-PA or urokinase-expressing vectors. In initial experiments designed to determine the optimal structure of retroviral vectors used to express PA from baboon ECs, cells transduced with a vector expressing t-PA from the viral LTR secreted at least 3 to 4 times more t-PA than did cells transduced in parallel with the B2NST vector, in which t-PA is expressed from an internal simian virus 40 (SV-40) promoter (Fig 1, and data not shown). Based on these data and on Northern analysis in which this increased t-PA protein expression correlated with an increase in specific transcripts encoding t-PA (see below), we constructed the remainder of the vectors using the LTR to drive expression of the various PA cDNAs.

Cells transduced with the LtSN, Ld3+SN, and LdtsN vectors (expressing wild-type and serpin-resistant human t-PA) secreted t-PA at a rate approximately 5-fold to 15-fold above that of both the untransduced and urokinase-transduced cells under either serum-free or serum-containing conditions (Fig 2A, and data not shown). Transduction of baboon ECs with either urokinase-expressing or control vectors resulted in either unchanged or slightly increased t-PA secretion, less than twofold above that of the untransduced cells (Fig 2A, and data not shown).

Under either serum-free or serum-containing conditions, cells transduced with the LUKSN vector secreted urokinase at rates ranging from 6 to 15 times that of untransduced and control-transduced cells, whereas cells transduced with the LUK+ASN vector secreted urokinase at rates 10 to 25 times higher than the controls (Fig 2B, and data not shown). Thus, similar to our findings with cultured bovine ECs, baboon ECs transduced with the LUK + ASN vector secreted urokinase at a rate typically twofold to threefold higher than that of LUKSN-transduced cells (Fig 2B, and data not shown). Transduction of baboon ECs with either t-PA–expressing or control vectors did not affect urokinase secretion (Fig 2B and data not shown).

Detection and quantification of GPI-anchored and receptor-bound urokinase. We measured both GPI-anchored and receptor-bound urokinase on ECs transduced with the LUKSN and LUK+ASN vectors (Table 1). Treatment of LUKSN-transduced cells with glycine buffer (pH 3) resulted in the accumulation of 19.8 ± 1.7 ng urokinase/mg cellular protein in the supernatant, whereas treatment with DMEM (pH 7) resulted in the accumulation of only 3.8 ± 1.0 ng urokinase/mg cellular protein. In contrast, treatment of LUK + ASN-transduced cells with these same reagents resulted in the accumulation of 10 ± 2.8 and 9.0 ± 2.2 ng urokinase/mg cellular protein, respectively. These data suggest the presence of acid-releasable, receptor-bound urokinase on LUKSN, but not LUK+ASN-transduced cells. The higher level of urokinase that accumulated with DMEM treatment of LUK+ASN versus LUKSN-transduced cells most likely reflects the increased rate of urokinase secretion found with the LUK+ASN vector (see Fig 2B).

Exposure of LUKSN-transduced ECs to PIPLC (see Materials and Methods) resulted in the release of 10.5 ± 0.98 ng urokinase/mg cellular protein. Exposure to enzyme buffer alone released 5.0 ± 0.75 ng urokinase/mg cellular protein (Table 1). In contrast, exposure to LUK+ASN-transduced cells to PIPLC for 1 hour resulted in the release of 160 ± 32.0 ng urokinase/mg cellular protein, with enzyme buffer alone resulting in the accumulation of 27 ± 5.3 ng urokinase/mg cellular protein. These data suggest the presence of GPI-
anchored urokinase on the LUK+ASN-transduced cells, in an amount approximately eight times greater than the number of receptor-bound urokinase molecules on LUKSN cells. The low apparent level of PIPLC-releasable urokinase in the LUKSN cells (5 ng/mg cellular protein) most likely results from the detection by the ELISA of additional urokinase molecules that are secreted during the PIPLC incubation and have bound to surface urokinase receptors. These molecules are released by the PIPLC treatment (the urokinase receptor is also attached via a GPI anchor\(^1\)), collected with the supernatant, and could possibly be detected in the urokinase ELISA. In the absence of PIPLC (glycine, pH3, + buffer alone), secreted urokinase that has bound to cell-associated receptors is left behind when the supernatant is collected.

Effect of transduction on secretion of PAI-1 antigen. PAI-1 antigen was detectable in CM from both untransduced and transduced cells. In all samples, PAI-1 was present both in mass and molar excess over both t-PA and urokinase (Fig 2, and data not shown). PAI-1 secretion by cells transduced with the control vectors, the urokinase vectors, and two of the three t-PA vectors (Ld3+SN and Ld3+SN) was mildly increased (20% to 50%) over that of untransduced cells. PAI-1 secretion by the LdtSN vector was increased by an additional 50% above this level (Fig 2C, and data not shown). The pattern of results obtained under serum-containing conditions was similar to that shown in Fig 2C in that there was no increase in PAI-1 antigen secretion consistently associated with expression of t-PA or urokinase, when compared to ECs transduced with the control vectors (data not shown).

Effect of transduction on PA and inhibitor mRNA. All human cDNA probes detected both the introduced human transcripts as well as the corresponding endogenous baboon transcripts. The endogenous baboon t-PA and urokinase mRNA species are distinguished from the human mRNAs by a difference in size. Therefore, cross-hybridization of the probes to the baboon mRNAs allowed us to determine the effects (if any) of increases in PA expression on the transcript levels of endogenous PA and inhibitor genes. The following representative cells lines were examined by Northern analysis: untransduced cells, cells transduced with the control vectors LBgSN and SAX, cells transduced with two human t-PA vectors (B2NST and Ld3+SN, which express wild-type and serpin-resistant t-PA, respectively), and one human urokinase vector (LUKSN).

Transcription of a t-PA cDNA from the viral LTR resulted in higher mRNA levels than those obtained when the t-PA cDNA was driven from an internal SV-40 promoter (Fig 3A; Ld3+SN versus B2NST). The relative abundance of the LTR-driven transcript in the B2NST-transduced cells versus the SV-40 driven transcript (Fig 3A) also suggests that the LTR may be more active than the SV-40 promoter in the context of a double-promoter retroviral vector integrated into baboon

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Table 1. Detection of Receptor-Bound and GPI-Anchored Urokinase

<table>
<thead>
<tr>
<th>Treatment of Cells</th>
<th>Vector</th>
<th>Glycine pH 3</th>
<th>DMEM pH 7</th>
<th>Glycine pH 3 + PIPLC</th>
<th>Glycine pH 3 + Buffer Alone</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>LUKSN</td>
<td>19.8 ± 1.7</td>
<td>3.8 ± 1.0</td>
<td>10.5 ± 0.98</td>
<td>5.0 ± 0.75</td>
</tr>
<tr>
<td></td>
<td>LUK+ASN</td>
<td>10.0 ± 2.9</td>
<td>9.0 ± 2.2</td>
<td>160 ± 32.0</td>
<td>27 ± 5.3</td>
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Data are pooled from two separate experiments with n = 6 or 7 for each determination. Data are presented as mean ± SEM.
ECs. This result is similar to our findings in sheep large-vessel ECs, but different from results we reported previously in human umbilical-vein ECs, wherein both transcripts were equally abundant. Expression of endogenous baboon t-PA mRNA was similar in all transduced cell lines examined, at a level modestly above that present in untransduced cells (Fig 3A). This increase parallels the small increases in t-PA antigen secretion found consequent to transduction with the control and urokinase vectors (Fig 2A). For both the B2NSt and Ld3+SN-transduced cells, the increase in amount of total mRNA species encoding t-PA (human plus baboon) appeared similar to the increase in t-PA antigen secretion (approximately twofold for B2NSt and 10-fold for Ld3+SN, respectively; Figs 2A, 3A, and data not shown).

Analysis of urokinase mRNA showed the LTR-driven human urokinase mRNA to be present in significant excess over the endogenous baboon urokinase mRNA (Fig 3B). The increase in urokinase mRNA in the LUKSN-transduced cells appeared quantitatively similar to the increase in secreted urokinase protein (=10-fold; see above). Expression of endogenous baboon urokinase mRNA was unaffected by any of the vectors examined.

Analysis of endogenous baboon PAI-1 expression showed two mRNA species, with a very small increase in PAI-1 mRNA in all transduced cell lines examined, compared with levels in untransduced cells (Fig 3C). Therefore, PAI-1 mRNA levels were not specifically affected by the expression of human t-PA or urokinase. This finding is again quantitatively similar to the results of the CM assays for PAI-1 antigen (see above).

Zymographic analysis of CM. The molecular masses of species with PA activity present in the CM of transduced cells was determined by zymography. We first examined the expression of the serpin-resistant t-PA expressed by the vector Ld3+SN. As one of the primary goals of these studies was to determine the potential contribution of serpin resistance to the enhancement of EC fibrinolytic activity, we characterized the expression of this t-PA mutant by casein-plasminogen zymography in the murine PA-317 producer cell line, before transduction of ECs. CM from these PA-317 cells contained a doublet with PA activity of ~70 kD. This doublet was not found in the CM of control PA-317 cells expressing the SAX vector (Fig 4A). Similarly, human t-PA antigen was present in the medium of the Ld3+SN producer cells, but was absent from the medium of the SAX producer line (data not shown). Therefore, cells transduced with the Ld3+SN vector secrete active human t-PA of the appropriate molecular mass, an important consideration in interpreting the data that follow.

We next performed casein-plasminogen zymography on CM from baboon ECs both untransduced and transduced with representative vectors: SAX and LbGSN (controls), B2NSt (wild-type t-PA), Ld3+SN (serpin-resistant t-PA), and LUKSN (wild-type urokinase) (Fig 4B). CM of untransduced ECs contained sharp bands of PA activity at ~33, 55, 90, and 110 kD as well as a broad band between 65 and 80 kD. Zymograms of CM from ECs transduced with the SAX, LbGSN, and Ld3+SN vectors were essentially identical to the zymograms of untransduced cells. CM from the B2NSt-transduced cells contained increased activity in both the sharp 110-kD band and the broad 65- to 80-kD band, but was otherwise identical to those described previously. Finally, CM from the LUKSN-transduced cells contained increased activity in the sharp 33-, 55-, and 90-kD bands as well as in the broad 65- to 80-kD band. In none of the CM samples was a distinct 65- to 70-kD doublet suggestive of free t-PA present.

Based on the molecular masses of these PA species, and by analogy with previous immunoprecipitation analyses we interpret these results as follows: the 33- and 55-kD bands represent free low molecular-weight and free high molecular-weight baboon urokinase. The 110-kD band is a complex of t-PA (either baboon or human) and baboon PAI-1, the 90-kD band is a complex of high molecular-weight baboon urokinase and baboon PAI-1. Finally, the broad 65- to 80-kD band consists primarily of either t-PA and/or u-PA released from covalent complexes with PAI-1 during the electrophoresis run (see Materials and Methods). Therefore, in the CM of ECs transduced with the B2NSt vector, there is an increase in the t-PA/PAI-1 complex consequent to the increase in t-PA secretion, but there is little or no detectable free t-PA. In the CM of ECs transduced with the Ld3+SN vector, there is neither evidence of additional free t-PA nor is there evidence of additional activity present in association with a t-PA/PAI-1 complex. In the CM of ECs transduced with the LUKSN vector, there is evidence both of an increase in free urokinase and in the activity associated with the urokinase–PAI-1 complex.

We repeated certain of the zymograms using the pH 7.2F buffer system and a fibrinogen substrate, as described in Materials and Methods (Fig 4C). Adoption of this technique resulted in superior resolution of the lysis zones and in elimination of the broad 65- to 80-kD band. A new band appeared at approximately 80 kD, most prominently in the LUKSN-transduced cells. The loss of the 65- to 80-kD band was presumably a result of increased stability of PAI complexes at this lower pH. The 80-kD doublet is most likely a low molecular-weight urokinase–PAI-1 complex that is unstable at high pH. Therefore, the results of the fibrinogen/plasminogen zymograms extended those obtained from the casein-plasminogen zymograms: (1) CM from untransduced cells contains very faint bands at 90 and 80 kD (u-PA/PAI-1 complexes) and a faint doublet at 65 to 70 kD (presumably free t-PA); (2) CM from Ld3+SN-transduced cells is essentially identical to that from untransduced cells, whereas in CM from B2NSt-transduced cells the 105-kD band appears as a doublet, the upper band of which may be a specific human t-PA/baboon PAI-1 complex; (3) CM from ECs transduced with the LUKSN vector contains increased amounts of activity at 33, 55, 80, and 90 kD (all thought to be urokinase-containing species); (4) there are very low levels of a 65- to 70-kD doublet present in all CM samples (most likely a small amount of free baboon and/or human t-PA). Therefore, the fibrinogen-plasminogen zymograms confirm that expression of neither the wild-type nor the serpin-resistant t-PA vectors results in the accumulation of free t-PA in CM.

Immunoprecipitation of t-PA and urokinase-containing molecular species. To determine more conclusively the
The position of migration of molecular size markers (in kilodaltons) is indicated. Light bands represent lysis zones caused by activation of cells transduced with the LdJ+SN vector (which expressed serpin-resistant human t-PA) and the SAX vector (which expresses human adenosine deaminase as a control). (B) Casein-plasminogen zymography of CM from baboon ECs either untransduced or transduced, using the Laemmli buffer system. Vector used is indicated across top: “None” for untransduced cells, SAX, LGbSN, LD3+SN, B2NSf, and LUKSN express adenosine deaminase, β-galactosidase, serpin-resistant t-PA, wild-type t-PA, and single-chain urokinase, respectively. (C) Fibrinogen-plasminogen zymography of CM from baboon ECs, using pH 7.28 buffer system (see Materials and Methods). Vectors are as described in A and B. For all zymograms, the position of migration of molecular size markers (in kilodaltons) is indicated. Light bands represent lysis zones caused by activation of plasminogen; control gels with water substituted for plasminogen had no lysis zones (not shown).

Identification of the t-PA–containing species that were detected at significant levels by the ELISA, but were virtually absent on zymography, we performed immunoprecipitation of metabolically labeled CM. Certain samples were precleared with antibodies to PAI-1 to help identify t-PA–PAI-I complexes.

T-PA immunoprecipitation of CM from a clone of PA-317 cells transduced with the B2NSf vector (and shown by specific assays to contain very high levels of both human t-PA activity and antigen*) yielded three major bands, of ≈100, 80, and 65 kD (Fig 5). When the CM was precleared with antibodies to PAI-1, the 100- and 80-kD bands were specifically depleted, identifying these two bands as t-PA–PAI-I complexes. The exact nature of the 80-kD species is uncertain, and may represent either a degradation product of the 100-kD species, or as suggested by others, a complex of the B chain of t-PA and PAI-I. The 65-kD band was identified as free human t-PA both by its immunoreactivity and the fact that it comigrated with purified human t-PA loaded in an adjacent lane and stained with Coomassie blue (data not shown). Therefore, this protocol could detect both free and complexed t-PA.

Immunoprecipitation of t-PA species from CM of untransduced baboon ECs or ECs transduced with the control SAX vector yielded a few faint bands, consistent with the relatively low level of t-PA secretion from these cells (Fig 5, and data not shown). Two of these bands, at 100 and 80 kD, were specifically removed by preclearing with anti–PAI-1. Immunoprecipitation was also performed on CM from baboon ECs transduced with either LtsN (wild-type t-PA), LD3+SN, and LdltSN (both serpin-resistant t-PAs). At the time of the medium collections, cells transduced with these vectors were secreting t-PA at levels ≈5 to 10 times higher than the untransduced cells. CM of cells transduced with all three vectors again contained immunoreactive t-PA only in association with PAI-1 (Fig 5, and data not shown). In contrast to the CM from the PA-317 cells, no appreciable t-PA antigen precipitated as a band migrating near 65 to 70 kD, suggesting the absence of free t-PA.

CM from baboon ECs (both untransduced and transduced with urokinase vectors) was immunoprecipitated with antibodies to human urokinase. Preclearing was again performed with both anti–PAI-1 and control antibodies (Fig 6). CM of ECs that were either untransduced or transduced with the LUKSN or LUK+ASN vectors showed four dominant bands at ≈90, 80, 50, and 35 kD (Fig 6). Preclearing with antibodies to PAI-1 resulted in specific depletion of the 90- and 80-kD bands, identifying these bands as urokinase–PAI-I complexes and the 50- and 35-kD bands as free urokinase (high and low molecular-weight urokinase, as judged by their appropriate molecular masses). Therefore, secreted urokinase accumulates in CM to a significant degree as a free enzyme, unbound by PAI-1.

**Measurement of PA activity in CM.** Overnight (16 hours) collections of CM from both untransduced and transduced cells were initially assayed for PA activity with Glu-plasminogen as a substrate, in the presence of CNBr-cleaved fibrinogen. CM from untransduced cells contained a low but measurable level of PA activity (Table 2). PA activity in the CM of ECs transduced with the t-PA–expressing vectors LtsN, LD3+SN, and LdltSN was similar to that present in the untransduced cells. Despite this absence of increased activity, antigen assays performed on the same CM samples...
Fig 5. Detection of t-PA containing species by metabolic labeling and immunoprecipitation. Medium conditioned by either PA317 or baboon ECs grown in the presence of ^35S-methionine was subjected to immunoprecipitation with antibodies to human t-PA. PA317 cells were transduced with the B2NSt vector, which expresses wild-type human t-PA. Baboon cells were either untransduced ("None"), or transduced with the LtSN or Ld3+SN vectors (which express wild-type and serpin-resistant human t-PA, respectively). Paired aliquots of CM were precleared either with antibodies to human PAI-1 ("+" lanes) or antibodies to human urokinase, as a control ("−" lanes). Species present in the "−" lanes and absent in the "+" lanes are identified as containing PAI-1.

showed that during the CM collections, the LtSN, Ld3+SN, and Ld+tSN-transduced cells secreted t-PA at rates 15 to 18 times higher than did the untransduced cells (Table 2). In contrast, CM from ECs transduced with the urokinase-expressing vectors LUKSN and LUK+ASN contained PA activity ≈10-fold and 15-fold higher than that present in CM from untransduced cells. This increase in activity could be completely eliminated by performing the assay in the presence of neutralizing antibodies to urokinase, but not with an equal concentration of antibodies to t-PA (data not shown).

The activity assay was also performed with CM samples that had been acidified immediately on collection. Whereas immediate acidification prevents further association of t-PA with PAI-I, thereby preserving the activity of any free t-

Fig 6. Detection of urokinase-containing species by metabolic labeling and immunoprecipitation. Medium conditioned by baboon ECs grown in the presence of ^35S-methionine was subjected to immunoprecipitation with antibodies to human urokinase. Baboon cells were either untransduced ("None"), or were transduced with the LUKSN or LUK+ASN vectors (which express wild-type and glycosylphosphatidylinositol-anchored urokinase, respectively). Paired aliquots of CM were precleared either with antibodies to human PAI-1 ("+" lanes) or antibodies to human t-PA (as a control; "−" lanes). Species present in the "−" lanes and either absent or significantly depleted in the "+" lanes are identified as containing PAI-1.
cells transduced with the wild-type and deletion mutant t-PA vectors contained plasminogen and Desafib. Under these assay conditions, CM kinase vectors allow the accumulation of free PA in the CM. Bradykinin activation was nearly undetectable in samples taken from the untransduced as well as all of the transduced cell lines (data not shown). Therefore, the CM activity assays were in agreement with the zymograms and immunoprecipitation; only the urokinase vectors allow the accumulation of free PA in the CM of the baboon ECs.

Because of a concern that we were failing to detect activity of the serpin-resistant t-PA mutants because of our assay conditions, we repeated the CM activity assay using Lys-plasminogen and Desafib. Under these assay conditions, CM from cells transduced with the Ld3+SN vector contained significant PA activity, whereas both untransduced cells and cells transduced with the wild-type and deletion mutant t-PA vectors contained virtually no measurable activity (Table 2). The specific activity of the CM of the Ld3+SN transduced cells was 180,000 IU/mg t-PA. This result is in striking contrast to those obtained with the aforementioned zymography, immunoprecipitation, and activity assays, which all suggested there was virtually no t-PA activity in these CM samples (see above). When the assay was repeated with CNBr-cleaved fibrinogen (80 or 400 μg/mL) substituted for Desafib and Lys-plasminogen maintained, virtually no PA activity was detected in any of the CM samples assayed, reflecting a decrease in PA activity of over two orders of magnitude in the CM from the Ld3+SN-transduced cells. These CM activity assays were repeated with purified single-chain t-PA. Substitution of CNBr-cleaved fibrinogen for Desafib resulted in a decrease in the rate of plasminogen activation (ΔOD405/hr) by one log over a wide range of concentrations of wild-type t-PA (Table 3, and data not shown). Thus the triple-point t-PA mutant expressed by the Ld3+SN vector appears to have detectable activity only if Desafib is used as a stimulus. The rate of plasminogen activation by wild-type t-PA is also higher in the presence of Desafib than CNBr-fibrinogen; however, this increase is far less than that found for the Ld3+SN CM.

Measurement of PA activity in the presence of cultured cells. In vivo intravascular fibrinolysis occurs in the presence of endothelium and is thought to include participation of the EC surface in the localization and enhancement of PA-mediated fibrinolysis.33,34 Therefore, a PA activity assay was performed using an assay in which the reactions could take place in the presence of the cultured ECs.

Primary data from an individual cell-associated PA activity experiment are illustrated in Fig 7, showing the more rapid time course of plasminogen activation by the LtSN and LUK+ASN-transduced cells in this assay. The pooled results of several assays of ECs transduced with the various vectors are also shown (Fig 8). Untransduced EC activated plasminogen in the cell-associated assay at a low but measurable level. EC transduced with the control vectors SAX and LBGSN activated plasminogen at the same rate as untransduced cells. Cells transduced with either the wild-type t-PA vector LtSN or the anchored urokinase vector LUK+ASN each had a 4.4-fold increase in PA activity over the untransduced controls (P < .0001 for each compared to untransduced cells). In contrast, cells expressing the serpin-resistant mutant vectors Ld3+SN and LdtSN activated plasminogen at rates 1.7 and 0.7 times that of the untransduced controls, respectively. The rate for wild-type urokinase was slightly increased at 1.3 times that of the untransduced ECs.

To exclude the possibility that our failure to detect increased activity of certain of the transduced cells might result from a loss of transgene expression, on two occasions CM collections were performed just before the initiation of the

| Table 2. PA Activity of CM, Assayed With Glu-plasminogen and CNBr-Cleaved Fibrinogen Fragments |
|-----------------|-----------------|-----------------|-----------------|
| Vector          | Activity (IU/mg protein) | t-PA Secretion (ng/mg protein/d) |
| None            | 9.0 ± 1.6        | 40 ± 4.0        |
| LtSN            | 6.5 ± 2.0        | 596 ± 36        |
| LUK+ASN         | 84 ± 34          | ND              |
| LUK+ASN         | 133 ± 61         | ND              |
| Ld3+SN          | 4.9 ± 1.1        | 710 ± 15        |
| LtSN            | 3.6 ± 2.1        | 614 ± 54        |

Assays were performed on two separate medium collections with n = 3 to 6 for each vector. Pooled data from the two collections are expressed as mean ± SD.

Abbreviation: ND, not done.

Table 3. PA Activity of CM, Assayed With Lys-plasminogen and Either Desafib or CNBr-Cleaved Fibrinogen Fragments

<table>
<thead>
<tr>
<th>Vector</th>
<th>(ΔOD405/hr)*</th>
<th>t-PA Antigen (ng/mL)</th>
<th>Specific Activityt (IU/mg t-PA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>.010 ± .006</td>
<td>.003 ± .001</td>
<td>5.2 ± 1.5</td>
</tr>
<tr>
<td>LtSN</td>
<td>.009 ± .006</td>
<td>.003 ± .001</td>
<td>54 ± 1.2</td>
</tr>
<tr>
<td>Ld3+SN</td>
<td>.74 ± .027</td>
<td>.003 ± .001</td>
<td>33 ± 5.8</td>
</tr>
<tr>
<td>LtSN</td>
<td>.020 ± .002</td>
<td>ND</td>
<td>18 ± 7.6</td>
</tr>
<tr>
<td>sc-tPA (4.2 IU/ml)</td>
<td>.70 ± .036</td>
<td>.004 ± .009</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>510,000</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.

* Activity and antigen assays were performed on two separate medium collections with n = 5 samples for all except LdtSN, which was collected from triplicate wells on one occasion (n = 3). Both activity and antigen data are mean ± SD. Desafib was present as 25 μg/mL and CNBr-fibrinogen at 80 μg/mL. Essentially identical results were obtained with CNBr-fibrinogen present at 400 μg/mL (not shown).

† Specific activity of CM, with reference to a standard curve performed with single-chain t-PA. Specific activity designated "—" was too low to convert accurately to IU/mg.

‡ Single-chain t-PA activity standard (American Diagnostica). Specific activity given by manufacturer; not determined directly.
assay. On both occasions significant vector-associated increases in either t-PA or urokinase were present, in the ranges described above (data not shown). The cell-associated PA activity assay was repeated using Desafib and Lys-plasminogen in place of CNBr-cleaved fibrinogen fragments and Glu-plasminogen. Untransduced cells as well as cells transduced with the LUK+ASN, LtSN, Ld3+SN, and LdtSN vectors were assayed. The relative amounts of PA activity of the cells were in the same order as was found with the CNBr-fibrinogen/Glu-plasminogen reagent (data not shown).

**DISCUSSION**

The major findings of this work are: (1) primate ECs can be genetically modified to increase PA expression by 10- to 20-fold; (2) the effects of overexpression of both t-PA and urokinase are specific. There is no effect of t-PA or urokinase overexpression on endogenous t-PA or urokinase expression and no specific effect of expression of either of these PA on PAI-1 expression; (3) enzymatically active urokinase can be attached to the surface of primate EC via a GPI anchor, and the level of GPI-anchored urokinase is significantly greater than the level of receptor-bound urokinase; (4) both native and serpin-resistant t-PA molecules expressed from ECs accumulate in CM almost exclusively as inactive complexes with PAI-1, whereas expressed urokinase accumulates to a significant extent as a free enzyme; and (5) substantial activity of a serpin-resistant t-PA mutant is detectable only if Desafib rather than CNBr-cleaved fibrinogen is used as a stimulator.

In previous work, we showed that expression of human PA cDNAs in sheep and bovine ECs resulted in enhanced PA activity. Podrazik et al. extended these results in a system of gene transfer into cultured canine ECs. The use of primate ECs in the present study permitted us to answer specific additional questions concerning the absolute increase over baseline levels of PA synthesis and activity and the effect of vector-mediated PA expression on endogenous EC PA and inhibitor synthesis. The premise of vector-mediated transfer of genes such as t-PA and urokinase that are already present in ECs at two copies per diploid genome is that expression from the transgenes will be greater than that from the endogenous genes. Were this not the case, increases in protein production consequent to retroviral vector-mediated gene transfer of an additional cDNA sequence would be fairly modest. Our analysis of t-PA and urokinase mRNA and protein confirmed that the constructs confer levels of expression approximately one log above endogenous expression levels. This level of increase in PA production, if achievable in vivo, may be sufficient to be clinically useful in preventing intravascular thrombosis.
Relatively small increases in endogenous PA and inhibitor expression were found in cells transduced with control as well as t-PA and urokinase vectors, an effect most likely caused by the increased age in culture (caused by the G418 selection step) of transduced versus untransduced cells. However, we found no specific effect of vector-mediated PA expression on endogenous EC PA and inhibitor synthesis. This finding is also encouraging as concerns potential in vivo applications of PA gene transfer, for if PAI-1 production increased as a result of exposure of ECs to increased levels of PA (as has been found by some authors, but not by others), there might be no net effect of PA expression on cellular fibrinolytic activity. Previous data showing consistent increases in fibrinolytic activity in EC transduced with PA-expressing vectors have argued that PAI-1 expression does not increase proportionately; however, this has never been shown directly.

A major purpose of the present study was to compare the various PA vectors for their ability to increase EC fibrinolytic activity, in order to choose an optimal vector for subsequent in vivo studies. The results obtained with the urokinase vectors were perhaps the most straightforward and can be considered first; results with the t-PA vectors were far more complex. Vector-encoded urokinase accumulated in CM to a significant extent as a free enzyme, in striking contrast to our findings with all forms of t-PA. The presence of free urokinase suggests that under these experimental conditions a significant amount of secreted urokinase accumulates as single-chain urokinase, which does not bind to PAI-1, but can be converted to the two-chain active form by generated plasmin. To define the better urokinase vector, we compared LUKSN- and LUK+ASN-transduced cells in assays measuring secreted protein (Fig 2), CM activity (Table 2), and cell-associated activity (Fig 8). Transduction with LUK + ASN resulted in higher levels of both antigen and activity than did transduction with the wild-type urokinase vector LUKSN. Previous data obtained with bovine cells have suggested that these increased levels do not result from an increase in urokinase mRNA, but rather from posttranscriptional events. A property of the anchored urokinase that likely contributes to an increase in cell-associated activity is its ability to accumulate on the cell surface at a level almost a log above that of receptor-bound wild-type urokinase. Taken together, the present data suggest that GPI-anchored urokinase (rather than wild-type urokinase) is a superior means by which to increase the fibrinolytic activity of primate ECs.

Comparisons made between the t-PA vectors were surprising, yet ultimately equivocal. In a previous study, we found that recombinant human PAI-1 expressed in sheep EC accumulated in CM largely as an inactive complex with sheep PAI-1. Therefore, we anticipated that when expressed in baboon ECs, wild-type human t-PA would also be highly bound by endogenous PAI-1, and that is precisely what was found (Figs 4 and 5, Table 2). Less anticipated was the finding that both of the serpin-resistant t-PA mutants were also present almost exclusively in CM as SDS-stable complexes with baboon PAI-1 (Figs 4 and 5, and data not shown). Apparently even the low reported rates of association of these mutant t-PAs with PAI-1 are adequate to achieve virtually complete binding of secreted t-PA under the conditions of the overnight CM collection. These data should not be interpreted as conflicting with those of Madison et al., in which the incubation time of the mutant t-PAs with PAI-1 was only 20 minutes, rather than overnight, and the assay conditions used to measure residual t-PA activity were different from those with which the data in Table 2 were generated. We attempted to replicate the assay conditions of Madison et al and discovered that despite the existence of SDS-stable complexes between the serpin-resistant t-PAs and PAI-1, significant activity could nevertheless be detected in CM containing the triple-point t-PA mutant if Desafib was substituted for CNBr-cleaved fibrinogen (Table 3). Although it has been shown previously that CNBr-cleaved fibrinogen and Desafib stimulate t-PA activity via different mechanisms, the exact mechanism according to which the activity of the triple-point mutant was detected uniquely in the presence of Desafib is not at all clear, and merits further investigation.

The results of the plasminogen activation assay performed in the presence of cultured ECs (Figs 7 and 8) were unanticipated in two respects. First, cells transduced with wild-type t-PA expressed significant PA activity despite the lack of free t-PA in CM collected from these cells. The presence of the EC surface and the relative absence of PAI-1 in this assay (in which CM is removed before addition of plasminogen and therefore, newly released t-PA encounters a relative abundance of plasminogen over PAI-1) may be responsible for this finding. Second, cells transduced with wild-type t-PA possessed rates of plasminogen activation twofold to sixfold above that of the serpin-resistant mutants. Two possible explanations for this finding are that: (1) there are differences in the interactions of these t-PA molecules with EC surface molecules that bind t-PA and potentially facilitate its interaction with plasminogen; and (2) this assay largely measures initial rates of plasminogen activation rather than enzyme kinetic parameters. In this context, Li et al. have shown that the deletion mutant expressed by the LdtSN vector effects a twofold to threefold lower initial rate of plasminogen activation than does wild-type t-PA. The failure of serpin resistance to result in an increase in activity in this assay system may also be caused by the relative abundance of plasminogen over PAI-1 during the reaction, a setting in which any contribution of PAI-1 resistance is diminished.

Is it possible to extrapolate from these data to choose a “best” vector for in vivo testing? Perhaps, but with great caution. The apparent advantages of the urokinase vectors are most likely caused by the dominant effect of PAI-1 on any assay that involves collection of CM from cultured ECs (secreted single-chain urokinase is highly resistant to PAI-1 binding). PAI-1 comprises up to 12% of secreted protein from cultured ECs, and in the present study, PAI-1 was always present in CM in excess over both t-PA and urokinase (Fig 2). In contrast to this in vitro behavior, PAI-1 expression in ECs in normal vessels appears to be quite low, and may be a minor factor in controlling local fibrinolysis in vivo. Finally, whether the nature of fibrin stimulation of t-PA activity in vivo is best predicted by in vitro assays using CNBr-cleaved fibrinogen or Desafib is not yet clear. Until
GENE TRANSFER INTO BABOON ENDOTHELIAL CELLS

these questions are answered, at least in part by the comparison of future in vivo results to the predictions of these in vitro data, extrapolation from the in vitro assays of plasminogen activation will remain difficult.

In conclusion, although the data herein do not clearly predict a “best” vector for in vivo studies, the characterization of PA expression by transduced primate ECs continues to support the approach of gene transfer as a specific and potentially powerful therapy for in vivo thrombosis in primates.

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Characterization of recombinant plasminogen activator production by primate endothelial cells transduced with retroviral vectors

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