Storage of Tissue-Type Plasminogen Activator in Weibel-Palade Bodies of Human Endothelial Cells

Corinne Rosnoblet, Ulrich M. Vischer, Robert D. Gerard, Jean-Claude Irminger, Philippe A. Halban, Egbert K. O. Kruithof

Abstract—Tissue-type plasminogen activator (t-PA) is acutely released by endothelial cells. Although its endothelial storage compartment is still not well defined, t-PA release is often accompanied by release of von Willebrand factor (vWF), a protein stored in Weibel-Palade bodies. We investigated, therefore, whether t-PA is stored in these secretory organelles. Under basal culture conditions, a minority of human umbilical vein endothelial cells (HUVEC) exhibited immunofluorescent staining for t-PA, which was observed only in Weibel-Palade bodies. To increase t-PA expression, HUVEC were infected with a t-PA recombinant adenovirus (AdCMVt-PA). Overexpressed t-PA was detected in Weibel-Palade bodies and acutely released together with endogenous vWF by thrombin or calcium ionophore stimulation. In contrast, plasminogen activator inhibitor type 1 and urokinase were not detected in Weibel-Palade bodies after adenovirus-mediated overexpression. Infection of HUVEC with proinsulin recombinant adenovirus resulted in the storage of insulin in Weibel-Palade bodies, indicating that these organelles can also store nonendothelial proteins that show regulated secretion. Infection of AtT-20 pituitary cells, a cell type with regulated secretion, with AdCMVt-PA resulted in the localization of t-PA in adrenocorticotropic hormone–containing granules, indicating that t-PA can be diverted to secretory granules independently of vWF. Coinfection of AtT-20 cells with AdCMVt-PA and proinsulin recombinant adenovirus resulted in the colocalization of t-PA and insulin in the same granules. Taken together, these results suggest that HUVEC have protein sorting mechanisms similar to those of other regulated secretory cells. Although the results did not exclude an alternative storage site for t-PA in HUVEC, they established that t-PA can be stored in Weibel-Palade bodies. This finding may explain the acute coordinate secretion of t-PA and vWF. (Arterioscler Thromb Vasc Biol. 1999;19:1796-1803.)

Key Words: tissue-type plasminogen activator ■ von Willebrand factor ■ Weibel-Palade bodies ■ endothelial cells ■ immunofluorescence

Tissue-type plasminogen activator (t-PA) is a key enzyme for the removal of incipient thrombi in the vascular system, and recombinant t-PA is now widely used for the thrombolytic therapy of myocardial infarction. Endothelial cells (EC) are the main source for plasma t-PA. Although a variety of stimuli such as venous occlusion, exercise, or injection of vasoactive substances are known to acutely increase plasma levels of t-PA, the precise mechanisms responsible for this increase are poorly defined. They may involve an acute release of t-PA from EC, variations in the rate of production of t-PA by EC, or changes in the clearance rate. Several in vivo and ex vivo studies give clear evidence for the occurrence of acute release of t-PA. Experimental induction of disseminated intravascular coagulation in chimpanzees or baboons results in a 50-fold increase in t-PA plasma levels within a few minutes. The rapidity and the magnitude of the increase in the t-PA concentration, as well as the rapid return to normal levels, are consistent only with a massive release of t-PA from storage pools. The findings that injection of vasoactive agents such as thrombin or calcium ionophore leads to an acute increase of t-PA in isolated vascular systems are also consistent with an endothelial t-PA storage pool.

Acute release of t-PA in vivo or in vitro is often accompanied by the release of von Willebrand factor (vWF), an adhesive glycoprotein involved in primary hemostasis. Plasma vWF originates mainly from EC, which store the protein in specific rod-like secretory granules known as Weibel-Palade bodies. Concomitant release of t-PA and vWF has been observed in vivo after injection of 1-desamin-8-arginine vasopressin (DDAVP) or after experimental disseminated intravascular coagulation, ex vivo in isolated rat hindlegs and in vitro in human umbilical vein endothelial cells (HUVEC). Furthermore, patients with severe von Willebrand’s disease are deficient in acute t-PA release. Taken together, these data suggest that t-PA...
and vWF are released either from the same storage granules or from distinct granules responsive to common stimuli. Support for the existence of distinct granules is provided by studies that have observed a discrepancy in the release of t-PA and vWF. In the rat hind leg model, adenosine diphosphate stimulated the release of t-PA but not of vWF. In cultured HUVEC, the thrombin-induced release of t-PA and vWF revealed subtle differences in calcium requirement and per-tussis toxin sensitivity. Furthermore, cell fractionation and immunofluorescence suggest that t-PA and vWF are stored in distinct granules. However, the heterogeneity in t-PA expression by EC does not allow firm conclusions to be drawn as to whether t-PA and vWF have distinct cellular localization(s).

The key role of t-PA in protecting the vascular system from thrombotic occlusions makes it important to better understand the mechanisms of t-PA storage and release. The present immunofluorescence study was undertaken to investigate the intracellular sorting of t-PA in HUVEC. Because endogenous t-PA expression is very low in HUVEC, we enhanced t-PA expression by adeno-virus-mediated t-PA gene transfer. This approach was chosen because primary human EC are known to be resistant to other transfection methods. We also investigated the localization of t-PA in AtT-20 cells, a model cell system that is used to study the sorting of proteins into the constitutive or the regulated secretory pathway. A further aim was to determine whether Weibel-Palade bodies can store non-EC proteins known to follow the regulated pathway of secretion. Our observations indicate that both t-PA and other regulated proteins can be sorted to Weibel-Palade bodies.

Methods

Materials

Unless stated otherwise, all biochemical reagents and chemicals used in this study were from Sigma or Fluka and of the highest grade available. The following antibodies were used: monoclonal anti-human t-PA antibody PAM-3 (directed against the protease domain) was from Biopool; monoclonal anti-human t-PA antibody ESP-4 (kringle 2 domain) was from American Diagnostica; monoclonal anti-human u-PA antibodies 7C7, 2L3, and 4D1E823 were a gift from Dr Paul de Clerck (Institute for Pharmacological Sciences, University of Leuven, Leuven, Belgium); rabbit anti-human vWF antibodies were from Dako and immunopurified on immobilized vWF before use; rabbit anti-human adrenocorticotropic hormone (ACTH) antibodies and monoclonal anti-human insulin (used for insulin and vWF double label experiments) and anti-rat Golgi 58K protein antibodies were from Sigma; monoclonal anti-human Lamp-2 H4B4 used as a lysosomal marker was from the Developmental Studies Hybridoma Bank; monoclonal 6C4 antibody used as a marker for late endosomes24 was a gift from Dr Toshhide Kobayashi and Jean Gruenberg, (University of Geneva, Geneva, Switzerland); monoclonal anti-human PAI-1 antibody was from Monozyme; guinea pig anti-human insulin antibodies (used for insulin and t-PA double label experiments) were a gift from Dr Paolo Alcorn of UT Southwestern Medical Center (Dallas, Texas). The resulting plasmid, pACskCMV-PAI, was cotransfected with ClaI digested Ad5dl309 into 293 cells using standard methods29 to yield AdCMV-PAI. The recombinant adenoviruses were propagated on a monolayer of 293 cells (CR1 1573, ATCC) and titrated by plaque assay.

HUVEC or AtT-20 cells were grown to 50% confluency in 24-well plates or on 12-mm diameter glass coverslips. They were then infected for 1 hour at 37°C in 300 µL RPMI 1640/10% FBS or DMEM/10% FBS with 100 pfu/mL recombinant adenovirus (AdCMVPAI-1),27 and proinsulin recombinant adenovirus.28 Recombinant u-PA adenovirus was prepared as follows: a plasmid containing the human pro-u-PA cDNA, pu-PA, was made available by William Bennett of Genentech Inc, San Francisco, CA. The uPA cDNA fragment from HindIII to SspI, containing 75 bp of 5′ untranslated sequence and 600 bp of 3′ untranslated sequence was inserted into the adenovirus construction plasmid, pACskCMV2. This plasmid contains the left end of adenovirus type 5 including the origin of replication and packaging sequences (nt no. 1-454), an SV40 ori/hGH terminator, a polylinker to facilitate cDNA insertion, the CMV promoter, and flanking adenovirus sequences (nt no. 3334-5779) serving as the target for homologous recombination. It was kindly provided by Joseph Alcorn of UT Southwestern Medical Center (Dallas, Texas). The resulting plasmid, pACskCMV-PAIu, was cotransfected with ClaI digested Ad5dl309 into 293 cells using standard methods29 to yield AdCMV-PAIu. The recombinant adenoviruses were propagated on a monolayer of 293 cells (CR1 1573, ATCC) and titrated by plaque assay.

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase digestion25 and grown at 37°C in a humidified atmosphere containing 5% CO2. Briefly, the umbilical vein was washed with Krebs-Ringer–bicarbonate buffer (KRBH, 120 mmol/L NaCl, 4.75 mmol/L KCl, 1.2 mmol/L KH2PO4, 0.6 mmol/L MgSO4, 1.2 mmol/L CaCl2, 25 mmol/L NaHCO3, and 25 mmol/L HEPES, pH 7.4) and incubated for 10 minutes with 1 mg/mL collagenase (CLS, type 1, Worthington Biochemical). Cells were collected by flushing the vein with 50 mL RPMI 1640 supplemented with 10% FBS (Life Technologies) and then grown in RPMI 1640 containing 90 µg/mL heparin (Boehringer Ingelheim), 15 µg/mL endothelial cell growth supplement (Upstate Biotechnology), 10 mmol/L HEPES, 100 µmol/L penicillin, and 100 µg/mL streptomycin and supplemented with 10% FBS. Cells were passaged by trypsin/EDTA (Biochrom KG, Berlin, Germany) treatment at a split ratio of 1/3 and used at passage 1 or 2. Tissue culture dishes (Falcon Becton Dickinson Labware, Lincoln Park, NJ), 24-well plates (Costar, Cambridge, MA) and glass coverslips were coated with 0.1% gelatin. Cultured cells were identified as endothelial by the presence of vWF antigen and labeling of Weibel-Palade bodies using indirect immunofluorescence.

AtT-20 mouse pituitary tumor-derived cells were grown in Dulbecco’s modified Eagle’s medium containing 4.5 g/L glucose (DMEM, Life Technologies) and supplemented with 10% FBS. AtT-20 cells were passaged by trypsin/EDTA at a split ratio of 1:10.

Cell Infection With Recombinant Adenoviruses

The following recombinant adenoviruses have been previously described: t-PA recombinant adenovirus (AdCMVt-PA),29 PAI-1 recombinant adenovirus (AdCMVPAI-1),27 and proinsulin recombinant adenovirus.28 Recombinant u-PA adenovirus was prepared as follows: a plasmid containing the human pro-u-PA cDNA, pu-PA, was made available by William Bennett of Genentech Inc, San Francisco, CA. The uPA cDNA fragment from HindIII to SspI, containing 75 bp of 5′ untranslated sequence and 600 bp of 3′ untranslated sequence was inserted into the adenovirus construction plasmid, pACskCMV2. This plasmid contains the left end of adenovirus type 5 including the origin of replication and packaging sequences (nt no. 1-454), an SV40 ori/hGH terminator, a polylinker to facilitate cDNA insertion, the CMV promoter, and flanking adenovirus sequences (nt no. 3334-5779) serving as the target for homologous recombination. It was kindly provided by Joseph Alcorn of UT Southwestern Medical Center (Dallas, Texas). The resulting plasmid, pACskCMV-PAIu, was cotransfected with ClaI digested Ad5dl309 into 293 cells using standard methods29 to yield AdCMV-PAIu. The recombinant adenoviruses were propagated on a monolayer of 293 cells (CR1 1573, ATCC) and titrated by plaque assay.

HUVEC or AtT-20 cells were grown to 50% confluency in 24-well plates or on 12-mm diameter glass coverslips. They were then infected for 1 hour at 37°C in 300 µL RPMI 1640/10% FBS or DMEM/10% FBS with 100 pfu/mL recombinant adenovirus (AdCMVPAI-1), or with proinsulin recombinant adenovirus at a titer of 108 pfu/mL. These virus titers were determined by indirect immunofluorescence study was undertaken to investigate the sorting of proteins into the constitutive or the regulated secretory pathway. A further aim was to determine whether Weibel-Palade bodies can store non-EC proteins known to follow the regulated pathway of secretion. Our observations indicate that both t-PA and other regulated proteins can be sorted to Weibel-Palade bodies.

Indirect Immunofluorescence Staining

HUVEC or AtT-20 cells grown on glass coverslips were fixed for 20 minutes in 4% freshly depolymerized paraformaldehyde in phosphate buffered saline (PBS: 10 mmol/L NaH2PO4/Na2HPO4, 136 mmol/L NaCl, and 4 mmol/L CaCl2, pH 7.4), washed with PBS, quenched for 20 minutes with 0.27% NH4Cl/0.38% glycine in PBS, pH 7.4 and permeabilized for 30 minutes with 0.1% saponin/0.1% BSA in PBS, pH 7.4. For insulin staining, HUVEC were fixed for 30 minutes in Bouin solution (75% saturated picric acid solution, 7.4% formaldehyde, and 5% acetic acid) and permeabilized by progressive 3 minutes ethanol dehydration (30%, 50%, 70%, and 90% ethanol in water) and rehydration (70%, 50%, 30%, and 0% ethanol in water).

For single label analyses, cells were incubated sequentially with the primary antibody and the fluorescent second antibody. For double label analyses, cells were incubated sequentially with a mixture of primary antibodies and a mixture of fluorescent second
antibodies. Antibodies were diluted in 0.1% BSA in PBS, pH 7.4. Monoclonal antibodies for primary staining were used at 10 μg/mL for detection of t-PA, u-PA, and PAI-1, 5 μg/mL for late endosomal membranes and at dilutions of 1/5000 for lysosomal membranes, 1/50 for insulin, and 1/100 for Golgi; polyclonal antibodies for primary staining were used at 2 μg/mL for detection of vWF, 1/100 for ACTH, and 1/200 for insulin. Rhodamine-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit antibodies were used at a dilution 1/100. FITC-conjugated sheep anti-guinea pig antibodies was used at a dilution of 1/400. All incubations were performed for 1 hour at room temperature. After washing, coverslips were mounted in polyvinyl alcohol. Pictures were taken with a Zeiss Axioshot (Carl Zeiss) photomicroscope equipped with epilumination and specific filters for fluorescein and rhodamine using a plan apochromate×63/1.40 objective and Tmax black and white film (Eastman Kodak).

Negative control experiments were performed by omitting the primary antibodies or by using an irrelevant primary antibody of the same species or IgG subclass (for monoclonal antibodies). For double label analyses, we verified that the FITC fluorescence gave no signal in the rhodamine channel and conversely. We also confirmed the absence of cross-reactivity between mouse antibodies and FITC-conjugated goat anti-rabbit antibodies as well as between rabbit antibodies and rhodamine-conjugated goat anti-mouse antibodies.

**Secretion Studies**

Forty-eight hours after infection with recombinant AdCMVt-PA, HUVEC grown on 24-well plates were washed 3× with KRBH/BSA 0.1%, pH 7.4, preincubated for 30 minutes in 1 mL KRBH/BSA, and then incubated again for 30 minutes in 0.5 mL fresh KRBH/BSA alone, KRBH/BSA containing 1 NIH U/mL of human thrombin (Sigma), or KRBH/BSA containing 2 μmol/L calcium ionophore A23187 (Sigma). The cell supernatants were centrifuged and kept at −20°C until determination of t-PA and vWF antigen concentrations.

**Antigen Determinations**

Human t-PA antigen was determined by ELISA (Imulyse t-PA, Biopool). vWF was measured by ELISA as described elsewhere.14 Data are presented as means±SEM. The significance of differences was calculated using the Student t test.

**Results**

**Localization of t-PA in Endothelial Cells**

To study the intracellular localization of t-PA in HUVEC grown under basal culture conditions, we performed double label indirect immunofluorescence analysis for t-PA and vWF. All data shown in this article concerning the immunolocalization of t-PA were obtained using monoclonal antibody ESP-4 (similar data were obtained using monoclonal antibody PAM-3). A minority of the cells (<2%) were weakly but distinctly stained by anti–t-PA antibodies. These antibodies stained elongated organelles spread throughout the cytoplasm and positive for vWF, which identifies them as Weibel-Palade bodies, or 2) there is a heterogeneity in t-PA expression levels, with only a minority of cells expressing staining was observed in all cells (Figure 2, left panel). It was mainly restricted to a distinct perinuclear region that was similar to that labeled with an antibody against protein 58 K, a membrane protein of the Golgi apparatus (data not shown). No staining of elongated organelles was observed using anti–PAI-1 antibodies.

Many cell types are able to internalize and degrade t-PA. To test whether endocytosis of t-PA by some of the cells could have contributed to the immunofluorescence signal for t-PA, we compared the morphology of late endosomes (labeled with antibody 6C4, Figure 2, middle panel) and lysosomes (labeled with anti–Lamp-2 antibody, Figure 2, right panel) with that of the t-PA–labeled organelles. The morphology of the numerous endocytic/lysosomal structures stained in all cells by these antibodies was clearly different from that of the t-PA–positive organelles.

**Localization of Overexpressed t-PA in Endothelial Cells**

The results presented above for t-PA can be interpreted in 2 ways: 1) there is heterogeneity in the intracellular sorting of t-PA with only a minority of EC able to target t-PA to Weibel-Palade bodies, or 2) there is a heterogeneity in t-PA expression levels, with only a minority of cells expressing

![Image](http://atvb.ahajournals.org/DownloadedFrom/a2h-ahajournals.org.by-guest-on-october-30-2017/article-figures/fig1.jpg)
sufficient t-PA to be detected by our technique. To increase t-PA expression in HUVEC we used a recombinant adenovirus vector expressing human t-PA (AdCMVt-PA). A multiplicity of infection was chosen such that approximately 30% to 50% of the cells over-expressed t-PA to preserve some noninfected cells as negative controls in the immediate vicinity of cells that overexpressed t-PA. Double label immunofluorescence experiments for t-PA and vWF were then performed. In infected cells, the staining pattern for overexpressed t-PA, namely elongated organelles and a bright perinuclear pattern, was identical to that for vWF (Figure 3). No staining for t-PA was detected in areas devoid of vWF. The perinuclear staining for vWF was identical to that previously described and similar to the pattern seen in studies using antibodies specific for the rough endoplasmic reticulum. There were no endosome or lysosome-type structures labeled with anti–t-PA antibody in AdCMVt-PA–infected HUVEC. This suggested that endocytosis of overexpressed t-PA did not contribute to the immunofluorescence signal. The presence of cells positive for vWF but negative for t-PA again provided the intra-image negative control. At higher AdCMVt-PA titers, almost all HUVEC expressed high amounts of t-PA in Weibel-Palade bodies (data not shown).

To verify whether overexpressed t-PA follows the regulated pathway of secretion, we measured t-PA release in AdCMVt-PA infected HUVEC after stimulation with known secretagogues. Treatment with thrombin or the calcium ionophore A23187 led to an acute increase in release of t-PA (3- to 4-fold) and of vWF (6-fold) as compared with untreated HUVEC (Table 1).

Localization of Overexpressed PAI-1 and u-PA in Endothelial Cells

To analyze whether adenovirus-mediated overexpression of a protein would artifactually result in a detectable signal in Weibel-Palade bodies, we infected HUVEC with recombinant adenoviruses that encode PAI-1 or u-PA under control of the same promoter (cytomegalovirus [CMV]) as that used for the t-PA recombinant adenovirus. We then performed double immunofluorescence analysis for vWF and either PAI-1 or u-PA.

The staining pattern for overexpressed PAI-1 was similar to that for endogenous PAI-1 and markedly different from that for vWF (Figure 4). We observed strong staining for PAI-1 in the perinuclear region corresponding the Golgi apparatus and diffuse staining throughout the cell. As ob-

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<tr>
<th>T-PA Release (Fold Increase)</th>
<th>vWF Release (Fold Increase)</th>
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<tbody>
<tr>
<td><strong>Thrombin</strong></td>
<td>4.0 ± 0.9†</td>
</tr>
<tr>
<td><strong>A23187</strong></td>
<td>2.8 ± 0.7*</td>
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Cells cultured in 24-well plates were infected with t-PA recombinant adenovirus (AdCMVt-PA), further cultured for 48 hours, preincubated 30 minutes with Krebs-Ringer–bicarbonate buffer (KRBH) for equilibration and then incubated for 30 minutes again with KRBH alone (basal secretion), human thrombin (1 NIH U/mL), or A23187 (2 μmol/L). Values are presented as fold increases over basal secretion (mean of 6 independent triplicate experiments ± SEM).

*P<0.05; †P<0.01.
served in uninfected HUVEC, the anti–PAI-1 antibodies did not label Weibel-Palade bodies.

HUVEC cultured under basal conditions exhibited a weak immunofluorescence signal for u-PA (data not shown). Infection of these cells with AdCMV-u-PA led to a marked increase in u-PA expression. The staining pattern for overexpressed u-PA, corresponding to the Golgi apparatus and to focal contact points was distinct from that for vWF (Figure 5). The anti–u-PA antibodies did not label Weibel-Palade bodies.

Expression of t-PA in AtT-20 Cells
To test whether the sorting of t-PA into regulated secretory granules requires the presence of vWF, we infected AtT-20 cells with AdCMVt-PA. Double label immunofluorescence analysis showed a strong signal for t-PA in approximately 50% of the cells, whereas no t-PA was detected in uninfected cells. t-PA was localized in round storage granules containing ACTH (Figure 6), an endogenous protein with regulated secretion. Staining was particularly strong at the tips of the extended processes of these cells. The presence of cells positive for ACTH but negative for t-PA provided an intra-image negative control.

To test whether AtT-20 cells can be infected by more than 1 recombinant adenovirus and then express and store different proteins in the same organelle, these cells were concomitantly infected with both AdCMVt-PA and proinsulin recombinant adenoviruses. The AtT-20 cells were then analyzed by double label immunofluorescence for t-PA and insulin (Figure 7). AtT-20 cells simultaneously infected by both t-PA and proinsulin recombinant adenoviruses efficiently expressed these proteins. Furthermore, staining for t-PA and insulin was observed within the same granules.

Expression of Insulin in Endothelial Cells
To determine whether Weibel-Palade bodies are able to store non-EC proteins with known regulated secretion, we infected HUVEC with proinsulin-recombinant adenovirus. Double-label immunofluorescence analysis showed that adenovirus-mediated proinsulin gene transfer in HUVEC resulted in the targeting of insulin in Weibel-Palade bodies (Figure 8).

Discussion
The regulated release of t-PA enables EC to acutely generate a strong, local fibrinolytic response. Indeed, in chimpanzees and baboons a thrombogenic stimulus leads to a massive increase in plasma concentrations of t-PA resulting in the rapid appearance of fibrin degradation products.7,8 From these results it appears that the regulated release of t-PA by EC is a major defense mechanism against thrombosis. However, other proteins stored by EC have a prothrombotic action. Thus, exocytosis from Weibel-Palade bodies leads to exposure of P-selectin at the cell surface, which stimulates
leukocyte adhesion and release of vWF that contributes to platelet aggregation. Many factors—such as thrombin, fibrin, bradykinin, histamine, platelet activating factor, DDAVP, and the calcium ionophore A23187—are known to induce the release of vWF from EC in vitro and in vivo. A majority of these factors also induce the acute release of t-PA. These observations suggest that t-PA and vWF are stored either in the same granules or in distinct granules that respond to similar stimuli.

Our data provide strong evidence that EC are able to sort t-PA into Weibel-Palade bodies. In HUVEC cultured under basal conditions, only a small percentage of the cells had sufficient t-PA to be detected by immunofluorescence analysis. In these t-PA positive cells, t-PA was detected in Weibel-Palade bodies. To increase the expression of t-PA in HUVEC, we used an adenoviral vector containing the t-PA cDNA under control of the strong CMV promoter (AdCMVt-PA). Thrombin and the calcium ionophore A23187 induced the parallel acute release of vWF and of overexpressed t-PA, indicating that both proteins were released from preformed storage compartments. The secretion data obtained in our model of adenovirus-mediated t-PA overexpression are in agreement with previous findings in HUVEC cultivated under basal conditions and using the same secretagogues in which acute t-PA release could be observed using an ultrasensitive t-PA antigen assay. By immunofluorescence analysis, we observed a clear localization of overexpressed t-PA in the Weibel-Palade bodies of AdCMVt-PA infected cells. At high titers of AdCMVt-PA, almost all HUVEC expressed t-PA in Weibel-Palade bodies. This showed that the ability to store t-PA in Weibel-Palade bodies is not restricted to a small subpopulation of the cells. Rather, it suggests that the presence of a t-PA signal in the Weibel-Palade bodies of some HUVEC cultured under basal conditions is due to differences in t-PA expression levels among cells.

The targeting of overexpressed t-PA to Weibel-Palade bodies was unlikely to be an artifact of overexpression because 1) a qualitatively similar staining pattern was observed in HUVEC cultured under basal conditions; 2) we observed no staining of Weibel-Palade bodies using anti–PAI-1 antibody, even though PAI-1 is endogenously expressed by HUVEC at much higher levels than t-PA after AdCMVt-PA treatment; and 3) no PAI-1 or u-PA could be detected in Weibel-Palade bodies after adenovirus-mediated overexpression of these proteins. This implies that protein over-expression by itself is not sufficient to result in protein sorting into Weibel-Palade bodies.

Our finding on the localization of t-PA in Weibel-Palade bodies is in contradiction to several recent studies that have suggested distinct storage compartments for t-PA and vWF. In
Figure 8. Localization of insulin and vWF in human umbilical vein endothelial cells after adenovirus-mediated proinsulin gene transfer. Forty-eight hours after infection, cells were processed for double-label indirect immunofluorescence analysis. Staining for insulin (upper panel) colocalized with staining vWF (lower panel) in Weibel-Palade bodies. Arrows point to Weibel-Palade bodies that are positive for both insulin and vWF. The same field is shown in the upper and in the lower panel. Scale bar=20 μm.

1 study using butyrate to increase t-PA expression, subtle differences in calcium requirement and pertussis toxin sensitivity were observed between t-PA and vWF release. However, in our hands, treatment of HUVEC with butyrate increased t-PA expression in only a fraction of the cells devoid of vWF (Rosnolet et al, unpublished observations, 1996), and it cannot be excluded that butyrate has modified the secretory response of these cells. Another study reported that the density of t-PA storage granules was the same as that of Weibel-Palade bodies when analyzed in Nycodenz gradients but different in sucrose gradients. Furthermore, an immunoelectron microscopy analysis of HUVEC and of murine capillary EC detected t-PA in small electron dense granules distinct from Weibel-Palade bodies. To what extent the heterogeneity of t-PA expression in EC in culture (this study) and in vivo may explain these differences is not yet known.

The sorting of t-PA into Weibel-Palade bodies may be an intrinsic property of t-PA or be due to a noncovalent interaction with vWF. The relevance of this question is strengthened by the recent observation that transfected vWF can function as a targeting chaperone that diverts transfected coagulation factor VIII from a constitutive to a regulated secretory pathway in AtT-20 cells. Sorting of endogenous or transfected t-PA to the secretory granules of PC12 cells suggests that t-PA storage can occur independently of an interaction with vWF. In the present study, we expressed t-PA in the ACTH-producing AtT-20 cells, a model cell system that expresses neither t-PA nor vWF and that has been widely used to study the targeting of exogenous proteins toward constitutive or regulated secretory pathways. t-PA was found in round granules in colocalization with endogenous ACTH. This confirms that targeting of t-PA to the regulated pathway is not dependent on an interaction with vWF and strongly suggests that t-PA contains its own cis-acting sorting signal.

Coexpression of AtT-20 cells with adenoviral vectors expressing t-PA and proinsulin resulted in storage of the 2 proteins within the same granules. Although ACTH, t-PA, and proinsulin share no obvious structural homology, they probably each contain a signal that is appropriately recognized by the sorting machinery of the pituitary cells. The ability of different adenoviral vectors to infect the same cell is in keeping with our previous findings and suggests possibilities to study the elements that play a critical role in the process of storage and secretion. Indeed, complementation experiments in model cell systems may be achieved more easily by using simultaneous infection with different recombinant adenoviruses than by performing successive stable transfection experiments.

Weibel-Palade bodies are regulated secretory granules not only for vWF but also for EC proteins like vasoactive peptides and t-PA (this report). In addition, transfection of EC with coagulation factor VIII has demonstrated that factor VIII can be stored in Weibel-Palade bodies in a vWF-dependent fashion. The ability of Weibel-Palade bodies to function as storage granules for non-EC proteins with regulated secretion is illustrated by our finding that insulin is found in these organelles after adenovirus-mediated proinsulin gene transfer. This suggests that the mechanisms of sorting of proteins to Weibel-Palade bodies are not fundamentally different from those in other cells possessing regulated secretory mechanisms.

In conclusion, the present study demonstrates that EC are able to target t-PA to Weibel-Palade bodies. This finding may explain the acute coordinated secretion of t-PA and vWF observed in numerous in vitro and in vivo studies. The storage of t-PA within secretory granules after adenovirus-mediated t-PA gene transfer in AtT-20 cells that do not express vWF suggests that t-PA contains its own sorting signal. Adenovirus-mediated expression of insulin in HUVEC and storage of this protein in Weibel-Palade bodies suggests similar storage mechanisms for endothelial and neuroendocrine cells.

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