Lung Endothelium Targeting for Pulmonary Embolism Thrombolysis

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Background—Pulmonary embolism occurs frequently in hospitalized patients. Thrombolytic therapy, currently used as the major treatment, has often been associated with severe bleeding complications and has thereby been life-threatening. We have developed a novel therapeutic method based on our newly created pulmonary endothelium-specific antibody.

Methods and Results—We isolated membrane proteins of rat pulmonary vascular luminal endothelium and obtained a monoclonal antibody, RE8F5, which antigen was uniquely expressed by the pulmonary capillary endothelium. In vivo biodistribution showed that RE8F5 and its urokinase conjugate were rapidly and specifically accumulated in lung. Urokinase and the conjugate were compared in rats with pulmonary, hepatic, and lower-limb embolus. In a pulmonary embolus model, the conjugate exhibited 12-fold enhanced thrombolytic potency over urokinase, whereas plasma fibrinogen and bleeding time were unaffected. In 2 other models, no significant thrombolysis was induced by the conjugate. In contrast, thrombolysis by urokinase was found to be comparable to the pulmonary embolus model. In addition, urokinase caused significant consumption of fibrinogen in all experiments.

Conclusions—These data show that urokinase equipped with lung endothelium-specific antibody is an ideal treatment for pulmonary embolism, with a high efficacy of thrombolysis and low risk of bleeding. (Circulation. 2003;108:2892-2898.)

Key Words: pulmonary embolism • endothelium • thrombolysis • urokinase
brane proteins are induced to express according to the specialized microenvironment. There is evidence in the literature supporting this concept. First, serial analysis of gene expression showed various patterns of gene expression to be clearly distinguishable from endothelial cells derived from blood vessels (capillaries, arteries, or veins) of normal or malignant tissues.15 Second, Rajotte and Ruoslahti16 and Pasqualini and Ruoslahti17 had discovered a range of peptides homing to vessels of particular tumors and organs, including one exclusive for the lung endothelium, using the organ targeting of peptide phage display. Third, many different efforts to generate tissue endothelium–specific mAbs have been made, with considerable success.18–20 Rooted in these previous findings, tissue-specific targeting of therapeutics at vascular endothelial heterogeneity should be possible.

In the present study, a novel approach has been developed and tested in rats, based on an original discovery of lung endothelium–specific antibody, which is used to specifically deliver thrombolysis to lung for the treatment of PE.

Methods

Animal Studies

All procedures in animal experiments were approved by the Animal Study Committee of Institute of Molecular Medicine, Nanjing University.

Isolation of Rat Pulmonary Vascular Endothelial Membrane Proteins and Antibody Generation

Female Sprague-Dawley (SD) rats (Qinglongshan, Inc, Nanjing, China) were anesthetized with pentobarbital sodium (50 mg/kg). After thoracotomy, membrane proteins of rat pulmonary endothelium were biotinylated by use of perfusate containing 20 mg sulfo-NHS-LC-biotin (Pierce) by pulmonary perfusion, which passed through the pulmonary vasculature bed. After one lobe was removed for histochemical analysis, the lung tissue was homogenized, and the proteins were solubilized with PBS containing 5% Triton X-100 and 1% SDS, followed by affinity chromatography of immobilized streptavidin (Pierce). The purified protein concentration was determined with the BCA kit (Pierce) after the sample was solubilized in 2% SDS, boiled, and centrifuged. BALB/c mice (bred in a pathogen-free environment at our institute) were immunized with purified proteins, and mAbs were developed by the standard somatic cell hybridization.

Histochemical Analysis

Cryostat sections (5 μm) were made with the biotinylated lung tissue. After they had been air-dried and fixed in cold acetone, sections were quenched in 2% BSA and then incubated with peroxidase-conjugated streptavidin (Pierce). The red color was developed with the AEC Substrate Kit (Zymed). To screen the antibodies generated, cryostat sections were prepared similarly with normal SD rat tissues and incubated with supernatants of positive antibodies generated, cryostat sections were prepared similarly with normal rat tissues and incubated with supernatants of positive antibodies generated, cryostat sections were prepared similarly with normal SD rat tissues and incubated with supernatants of positive antibodies generated, cryostat sections were prepared similarly with normal SD rat tissues and incubated with supernatants of positive antibodies generated.

Western Blot Analysis

After normal rat tissues were homogenized on ice, total proteins were extracted. Tissue extracts (100 μg) were immunoblotted after separation by SDS-PAGE using hybridoma supernatants and then peroxidase-conjugated anti-mouse IgG (Pierce). Biotinylated membrane proteins isolated from 100 μg of lung extracts and the residual fractions were also immunoblotted with hybridoma supernatants.

Preparation and Purification of RE8F5 and UK/RE8F5

The pulmonary endothelium–specific mAb RE8F5 was purified from ascites by protein G chromatography (Pierce) according to the vendor’s instructions and then conjugated with 4-succinimidylisothiocyanate-methyl-a-(2-pyridyldithio)-toluene (SMPT) (Pierce). UK and reteplase (K,

In Vivo Biodistribution of Antibodies and UK/RE8F5

RE8F5 and UK/RE8F5 were biotinylated with sulfo-NHS-LC-biotin (Pierce). Rats were injected with different doses of labeled RE8F5 and normal mouse IgG (Pierce) via tail vein and killed at 1 hour after injection. Labeled UK/RE8F5 (150 μg) was also administered intravenously. Rats were killed at 15, 30, 60, and 120 minutes. Tissues and blood were collected and weighed. After the total proteins were extracted, the amount of biotinylated antibodies and conjugates accumulated in tissues was determined by a quantitative ELISA. In brief, dilutions of tissue lysates were incubated with 100 ng avidin–coated plates (Pierce). Peroxidase-conjugated anti-mouse IgG served as a reporter reagent. Standard curves were used to quantify the targeted antibodies or conjugates.

Rat Embolus Models

We made the clots as described previously,21 using 50 mL of rabbit plasma. The microclots were suspended in PBS and biotinylated. After washing 3 times, the labeled clots were resuspended in 100 mL PBS and divided into 400-μL aliquots. To determine the amount of initially injected clots, the total biotins of the clots were measured. The labeled clots were dissolved in 8 mol/L urea, 5% SDS, and 3% 2-mercaptoethanol; boiled for 20 minutes; diluted in PBS; and then digested by pronase (Sigma). After inactivation of pronase, the biotin concentration of the dissolved clots was measured with a competitive ELISA described previously.22

We injected 1 aliquot of clots into anesthetized female SD rats (180 to 200 g) via the exposed external jugular vein, hepatic portal vein, and femoral artery to produce pulmonary embolus, hepatic embolus, and lower-limb embolus models, respectively.21,23

Thrombolysis Assay

Five minutes after injection of clots, UK, K2PA, UK/RE8F5, and PBS were administered intravenously as a bolus injection. Rat plasma samples were collected during the experiment. Clot lysis and plasma residual fibrinogen were assayed at 1 hour after the injection of clots. The plasma concentration of biotin was determined as described above, which represented the plasma concentration of soluble fibrin fragments. The endogenous biotin was also examined in plasma from untreated rats. After compensation for extravascular distribution, the amount of circulating biotin was compared with the biotin initially injected to indicate the course of thrombolysis. If circulating biotins were >40% of the initially injected amount at 5 minutes after injection of clots, the rat was discarded because of the failure to achieve embolization. The concentration of fibrinogen was determined as described previously24 and expressed as a percentage of those of untreated rats. Bleeding times were measured at 1 hour before and after the treatment by a tail bleeding time method as previously described.25

Statistical Analyses

Bleeding times were compared using Student’s t test. Thrombolyses were compared by two-way ANOVA followed by Scheffé’s test.
Results

Isolation of Membrane Proteins of Rat Pulmonary Vascular Endothelium

Histochemical analysis of the biotinylated lung tissue showed that pulmonary perfusion with a water-soluble biotinylation reagent thoroughly labeled alveolar capillaries without affecting bronchial walls (Figure 1, A and B). The high purity of the isolated membrane proteins was examined by Western blot using the well-characterized endothelial membrane proteins (Flk-1, VE-cadherin, intercellular adhesion molecule-1) and intracellular proteins (histone, lamin A/C) as markers. Little contamination of intracellular proteins was found (data not shown).

Generation and Characterization of mAbs

In a panel of mAbs generated against the pulmonary endothelium, mAb RE8F5 was found specifically binding to the alveolar capillary vessels but not to the large blood vessels or the bronchial epithelium, on the basis of the immunostaining (Figure 1C). The frozen sections from other tissues were negative. The immunostaining results of antibodies identified on various tissue sections are summarized in Table 1, illustrating the biochemical distinction of blood vessels in individual tissues. The Western blot with the tissue lysates revealed that RE8F5 recognized a protein with a molecular weight of 79 kDa expressed only in the lung tissue (Figure 2A). As a comparison, another protein with a molecular weight of 50 kDa recognized by mAb RE9B5 was expressed in most tissues tested. Immunoblotting with isolated endothelial membrane proteins and with lung extracts after depletion of biotinylated proteins revealed that the antigen of RE8F5 was significantly enriched in the isolated membrane proteins (Figure 2B).

Preparation and Characterization of the Conjugate

Eighteen milligrams of RE8F5 modified with SMPT was obtained after dialysis. The SMPT derivative was then mixed with equimolar partially reduced UK (6 mg) before another protein G affinity chromatography. After conjugation and isolation, the flow-through contained 2.4 mg protein but little

Table 1. Immunoreactivity Pattern of mAbs in Different Frozen Tissue Sections

<table>
<thead>
<tr>
<th></th>
<th>RE8F5</th>
<th>RE2C12</th>
<th>RE14A2</th>
<th>RE9B5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>LV, SV, PC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>--</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>LV, SV</td>
<td>LV, SV</td>
<td>LV, SV</td>
<td></td>
</tr>
<tr>
<td>Intestine</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EP (capsule membrane)</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SV</td>
<td>LV, SV, EP (tubular)</td>
</tr>
<tr>
<td>Liver</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LV (portal)</td>
<td>LV (portal)</td>
</tr>
<tr>
<td>Lung</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>SV</td>
<td>LV, SV</td>
<td>LV, SV</td>
<td>LV, SV, EP</td>
</tr>
<tr>
<td>Muscle</td>
<td>--</td>
<td>++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>LV, SV</td>
<td>LV, SV</td>
<td></td>
<td>LV, SV</td>
</tr>
<tr>
<td>Spleen</td>
<td>--</td>
<td>--</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LV</td>
<td>LV (central artery), EP</td>
</tr>
<tr>
<td>Stomach</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ep</td>
</tr>
</tbody>
</table>

LV indicates large vessels; SV, small vessels; EP, epithelium; and PC, parenchymal cells. The extent of immunoreactivity was scored from − (negative) to +++ (strongest).
activity of UK. It was found that the conventional condition (0.1 mol/L glycine, pH 2.8) eluted only nonconjugated antibody (3.3 mg) rather than the conjugate. The conjugate was eluted with 0.1 mol/L glycine (pH 2.8) containing 0.5 mol/L NaCl and appeared as a major band with a molecular weight of 190 kDa by a nonreduced SDS-PAGE (data not shown). The enzyme activity assay showed that the conjugate contained 5.1 mg of UK (85% recovery of the initial UK activity), suggesting that 0.2 mmol/L 2-mercaptoethanol reduced only the interchain disulfide bond of UK to generate a reactive sulfhydryl group, leaving most intrachain disulfide bonds unaffected. The catalytic unit of UK was preferentially coupled to antibody molecules. Protein concentration assay showed that 18 mg of the conjugate was obtained after purification. Thus, the SMPT-modified RE8F5 molecule formed a 1:1 stoichiometric complex with the catalytic unit of UK.

**In Vivo Biodistribution of RE8F5**

The in vivo biodistributions of RE8F5 and normal mouse IgG were clearly distinct (Figure 3, A and B). One hour after intravenous injection, RE8F5 had accumulated substantially in the lung tissue. At the dose of 20 μg, ~40.5 ± 3.4% of the injected dose per gram tissue (%ID/g) of RE8F5 was detected in the lung tissue and 1%ID/g in blood. In contrast, only 0.26 ± 0.13%ID/g of normal mouse IgG was detected in the lung tissue and 2.7%ID/g in blood. Injection with 100 μg of antibody caused a similar biodistribution. Lung tissue retained 28.1 ± 2.9%ID/g RE8F5, whereas the accumulation level in other tissues did not exceed 5%. Pulmonary uptake of normal IgG was negligible (0.55%ID/g). The binding curve of RE8F5 in lung tissue suggested that the pulmonary uptake of RE8F5 injected intravenously was dose-dependent (Figure 3C). This result implied that the antigen was specifically distributed on pulmonary endothelium and that RE8F5 possessed a high binding capacity.

**Tissue Uptake of UK/RE8F5**

Because higher doses may be required for therapeutic purposes, we injected the conjugate of UK/RE8F5 at a dose of 150 μg. Lung uptake was found to plateau at 30 minutes after injection (Figure 4A). UK/RE8F5 manifested an in vivo biodistribution profile similar to that of RE8F5 (Figure 4B). Lung uptake was 23%ID/g at 1 hour after administration. As a conclusion, RE8F5 could efficiently carry thrombolysis to pulmonary vasculature. Regarding the PE treatment, the rapid lung uptake of the urokinase conjugate afforded us an expeditious thrombolytic therapy.

**Thrombolysis in Rats With Pulmonary Embolus**

To determine the thrombolytic activity of the conjugate, a rat PE model was developed. The rats injected intravenously

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**Figure 2.** A, Western blot analysis on rat tissue lysates using identified antibodies. Antigen recognized by RE8F5 was expressed only in lung tissue, migrating at 79 kDa. In contrast, 50-kDa protein recognized by RE9B5 can be detected in multiple tissues. B, Purified endothelial membrane proteins and residual lung extracts after depletion were subjected to immunoblotting with RE8F5. This antigen was concentrated in purified endothelial membrane proteins. PMP indicates purified membrane proteins; LE-PMP, residual lung extracts after avidin affinity chromatography.

**Figure 3.** In vivo biodistributions of RE8F5 and normal mouse IgG in rats at 1 hour after injection of antibodies. Tissue uptakes of RE8F5 (solid bars) and normal IgG (open bars) were expressed as %ID/g. A, At a dose of 20 μg, RE8F5 was 156-fold enriched over normal IgG. In other tissues, it was <6% of RE8F5 injected. B, RE8F5 displayed a similar in vivo biodistribution when antibodies (100 μg) were injected. C, Binding curve of RE8F5 with lung tissue. Lung uptake levels (μg/g) of RE8F5 were plotted against injected doses. Antibody accumulated in a dose-dependent manner.
with PBS had 30±6% clot lysis, on the basis of the blood concentration of biotin after correction for extravascular distribution (Table 2). Within 1 hour, UK at doses of 20 000, 60 000, and 100 000 IU yielded 51±3%, 68±2%, and 92±3% clot lysis, respectively. Likewise, K2tPA at doses of 100 000 and 150 000 IU correspondingly caused 85±6% and 97±6% clot lysis. In contrast, 90±4% clot lysis was obtained with 8000 IU of UK/RE8F5. The residual fibrinogen was 76±5% at 92% clot lysis by UK, 74±8% at 97% clot lysis by K2tPA, and 113±11% at 90% clot lysis by the conjugate. The rate of clot lysis between UK, K2tPA, and the conjugate was compared (Figure 5A). The conjugate was 12-fold more effective than UK and 16-fold more effective than K2tPA in rat PE thrombolysis without affecting fibrinogen in blood, indicating that the enhanced thrombolytic potency by the conjugate was achieved locally while avoiding systemic plasminogen activation. Moreover, injection with 8000 IU of the conjugate caused little increase in the tail bleeding time, whereas administration of 100 000 IU UK and 150 000 IU K2tPA both markedly prolonged bleeding time of the rats (P<0.05, n=4) (Figure 5B). This result indicated that systemic hemorrhage could be minimized by selective targeting to the alveolar capillary.

Thrombolysis in Rats With Hepatic Embolus and Lower-Limb Embolus

To confirm the tissue-specific thrombolytic activity of the conjugate, 2 other models were developed. A dose of 100 000 IU of UK induced a similar degree of clot lysis (91±7% and 96±6%) and greater loss in residual fibrinogen (68±3% and 50±5%) in these 2 models in comparison with the rat PE (92±6% of clot lysis, 76% of fibrinogen) (Table 3). In contrast, 8000 IU of the conjugate, which had 90% clot lysis in the rat PE, induced clot lysis (48±3% and 24±4%), similar to the PBS controls (45±2% and 26±4%). The blood fibrinogen was unaffected with 8000 IU of the conjugate in both models.

Discussion

Tissue-specific vascular targeting is a promising innovation for many therapeutic purposes. mAb exhibits apparent advantages over other carriers because of its high specificity for targeted antigens and long half-life in vivo. Nevertheless,
TABLE 3. Thrombolysis of Pulmonary, Hepatic, and Lower-Limb Embolus in Rats

<table>
<thead>
<tr>
<th>Thrombolytic/Model</th>
<th>UK Dose, IU</th>
<th>Clot Lysis, %</th>
<th>Residual Fibrinogen, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>100 000</td>
<td>92±3</td>
<td>76±5</td>
</tr>
<tr>
<td>HE</td>
<td>8000</td>
<td>48±3</td>
<td>98±10</td>
</tr>
<tr>
<td>LE</td>
<td>3</td>
<td>24±4</td>
<td>103±12</td>
</tr>
<tr>
<td>PBS</td>
<td>4</td>
<td>30±6</td>
<td>111±12</td>
</tr>
<tr>
<td>UK/RE8F5</td>
<td>8000</td>
<td>45±2</td>
<td>104±5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>26±4</td>
<td>96±11</td>
</tr>
</tbody>
</table>

HE indicates hepatic embolus; LE, lower-limb embolus. Data are expressed as mean±SEM; n, No. of experiments.

Although UK and RE8F5 showed similar thrombolytic potencies in the PE model, Scheffé's test suggested that thrombolyses caused by UK and UK/RE8F5 were statistically different (P<0.001).

The discovery of a suitable antibody for vascular targeting has been greatly hindered by lack of appropriate antigens. In the present study, using in situ labeling and specific affinity adsorption, highly purified endothelial membrane proteins were obtained from rat alveolar capillaries as the antigens. Therefore, the notorious instability of cultured endothelial cells was averted. As a result, a series of mAbs recognizing distinct antigens have been identified, including one (RE8F5) with high in vitro specificity for pulmonary capillary. Moreover, this in vitro specificity was well translated into selective accumulation of the antibody and its urokinase conjugate in lung tissue in vivo. Because capillaries represent the sites with the greatest potential for vascular targeting, the specificity of RE8F5 for alveolar capillary has afforded us a more efficient approach for lung-specific vascular targeting.

Benefits of Thrombolysis Delivered to Pulmonary Endothelium

As the most practical illustration, RE8F5 was used to target thrombolytic to lung for the PE thrombolysis. Because the interchain disulfide bond of UK can be reduced selectively to generate a reactive sulphydryl group with no effect on its activity, UK was chosen for the chemical conjugation. Conversely, streptokinase and tPA are not suitable for this purpose, because all the disulfide bonds are required for their function. The thrombolytic potency of UK/RE8F5 was found to be 12-fold enhanced over native UK and 16-fold over KtPA in the PE rats without triggering any significant systemic plasminogen activation. Apparently, the conjugate could bind to pulmonary capillaries and induce local thrombolysis. Most importantly, the other 2 animal models of thrombosis lent further credence to its tissue specificity. As for UK/RE8F5, at the dose yielding nearly maximal clot lysis in PE, it resulted in only a background level of thrombolysis in other 2 models. This suggested that a vast majority of the therapeutic agent was enriched in the lung tissue. In addition, this tissue-specific thrombolytic approach ostensibly minimized consumption of fibrinogen. The improvement of the thrombolytic effectiveness was unlikely to be attributable to the change in the pharmacokinetics of the conjugate, because plasminogen activator–antibody conjugate that possessed no specificity led to no augmentation of fibrinolytic efficiency, as evidenced by previous reports.9

Apparentiy, pulmonary capillary–specific thrombolysis manifested advantages over traditional therapeutic agents, including fibrin-specific immunoconjugates. Because fibrin-specific immunoconjugates bind specifically to fibrin, their limitations remain inevitable. First, fibrin-specific immunoconjugates cannot discriminate between protective hemostatic thrombi and occlusive thrombi. Second, fibrin fragments containing the targeted epitope will be broken down from thrombi and released to the circulation during thrombolysis, which may cause unwanted plasminogen activation in the circulation. Finally, the spatial hindrance may disable the plasminogen activation and the proteolytic activity of plasmin on fibrin, considering that the conjugates are relatively large molecules. Therefore, the fibrin-specific immunotargeting produced only trivial improvement for in vivo thrombolysis. In a sharp contrast, our work made a significant departure from the status of current thrombolytics. Because the lung vasculature–specific conjugate can be enriched in pulmonary capillary, plasminogen would be locally activated and specifically dissolve clots in the lung. In addition, no systemic fibrinogen breakdown or hemorrhage was observed. This suggested that plasmin was inactivated by inhibitors after it went into the circulation and that plasminogen activation was retained locally. Furthermore, no UK activity was observed in rat lung lysates at 24 hours after injection of conjugate, indicating that inhibitors of UK in blood contributed to inactivate UK (data not shown). This was consistent with the complete preservation of fibrinogen in UK/RE8F5–induced thrombolysis. Accordingly, tissue-specific thrombolysis is presenting a novel method to prevent unwanted plasminogen activation somewhere else during thrombolytic therapy.

Measurement of Thrombolysis

Technically, sulfo-NHS-LC-biotin was used to label fibrin in the clots instead of the traditional radiolabeling method.21 The amount of soluble biotin in plasma was assayed to monitor the process of clot lysis. Endogenous biotin was undetected in the control rat plasma in our method. For the controls treated with PBS, spontaneous lysis occurred that was comparable to those previously reported.21,23 Because sulfo-NHS-LC-biotin reacts with amino groups of fibrin including lysine residues, its effect on thrombolysis was also studied. Biotin hydrazide (Pierce) that reacts with the carboxyl groups was used to biotinylate fibrin clots, and no significant difference was found in thrombolysis between 2 agents labeled fibrin. Therefore, using biotinylated fibrin to determine clot lysis is evidently compelling.

Conclusions

Equipping UK, a non–fibrin-specific plasminogen activator, with tissue specificity evidently promoted local thrombolysis,
which was accompanied by little systemic fibrinogen break-
down. To the best of our knowledge, this was the first
successful lung-specific PE thrombolysis reported. It indi-
cated that tissue-specific immunotargeting has great potential
in the treatment of cancer, edema, or tuberculosis to avoid
harmful side effects.

Acknowledgments
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