Immunotargeting of glucose oxidase to endothelium in vivo causes oxidative vascular injury in the lungs

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Immunotargeting of glucose oxidase to endothelium in vivo causes oxidative vascular injury in the lungs. Am J Physiol Lung Cell Mol Physiol 278: L794–L805, 2000.—Vascular immunotargeting is a novel approach for site-selective drug delivery to endothelium. To validate the strategy, we conjugated glucose oxidase (GOX) via streptavidin with antibodies to the endothelial cell surface antigen platelet endothelial cell adhesion molecule (PECAM). Previous work documented that 1) anti-PECAM-streptavidin carrier accumulates in the lungs after intravenous injection in animals and 2) anti-PECAM-GOX binds to, enters, and kills endothelium via intracellular H2O2 generation in cell culture. In the present work, we studied the targeting and effect of anti-PECAM-GOX in animals. Anti-PECAM-GOX, but not IgG-GOX, accumulated in the isolated rat lungs, produced H2O2 and caused endothelial injury manifested by a fourfold elevation of angiotensin-converting enzyme activity in the perfusate. In intact mice, anti-PECAM-GOX accumulated in the lungs (27 ± 9 vs. 2.4 ± 0.3% injected dose/g for IgG-GOX) and caused severe lung injury and 95% lethality within hours after intravenous injection. Endothelial disruption and blebbing, elevated lung wet-to-dry ratio, and interstitial and alveolar edema indicated that anti-PECAM-GOX damaged pulmonary endothelium. The vascular injury in the lungs was associated with positive immunostaining for iPF2-III isoprostane, a marker for oxidative stress. In contrast, IgG-GOX caused a minor lung injury and little (5%) lethality. Anti-PECAM conjugated with inert proteins induced no death or lung injury. None of the conjugates caused major injury to other internal organs. These results indicate that an immunotargeting strategy can deliver an active enzyme to selected target cells in intact animals. Anti-PECAM-GOX provides a novel model of oxidative injury to the pulmonary endothelium in vivo.

CONJUGATION (either chemically or genetically) of effector compounds with carrier antibodies directed against endothelial surface antigens promises a novel strategy for site-specific delivery of drugs to endothelium (vascular immunotargeting) (2, 17, 25, 28, 40, 42). The pulmonary vasculature contains roughly one-third of the endothelial cells in the body, receives all the cardiac blood output, and, therefore, represents a privileged target organ. Antibodies directed against endothelial antigens accumulate in the lungs after intravenous injection in animals and permit pulmonary accumulation of the radiolabeled conjugated enzymes (2, 8, 19, 26, 27, 29, 33). However, neither the functional activity of the targeted enzymes nor the consequences of the targeting of antibody-enzyme conjugates to the pulmonary vasculature has been documented in intact animals. Demonstration of delivery of an active enzyme to pulmonary endothelium would, therefore, provide the final data needed to validate the strategy.

To provide this proof of principle for immunotargeting to the pulmonary endothelium, we conjugated glucose oxidase (GOX) with an antibody directed against a highly expressed endothelial surface antigen. GOX is a relatively stable enzyme that generates H2O2 from glucose, inducing oxidative stress in cells and tissues (31, 35). For example, GOX conjugated with collagen antibody caused skin lesions after intradermal injection in rats (24). GOX conjugated with antibodies to endothelial antigens caused oxidative injury in endothelial cell culture (22, 23). 125I-GOX conjugated with monoclonal antibody against angiotensin-converting enzyme (ACE) displayed preferential accumulation in the rat lungs after intravenous injection (29). However, neither functional activity of antibody-conjugated GOX in the lung nor potential manifestation of this activity has been characterized in vivo.

In the present study, to deliver GOX to endothelium in vivo, we used antibodies to platelet endothelial cell adhesion molecule (PECAM)-1 (anti-PECAM). PECAM-1 or CD31 is highly expressed on pulmonary vascular endothelium, whereas platelets and leukocytes express this antigen at much lower levels (9, 32). In a recent study, we found that a biotinylated anti-PECAM-streptavidin carrier bound to and entered...
endothelium in cell culture and accumulated preferentially in the lungs after intravenous injection in intact animals (28). Furthermore, biotinylated GOX conjugated with an anti-PECAM-streptavidin carrier (i.e., anti-PECAM-GOX) bound specifically to endothelial cells in culture, entered the target cells, and produced H₂O₂ intracellularly leading to cellular oxidative stress and death (15).

Based on these results, we postulated that intravenously injected anti-PECAM-GOX would 1) accumulate in the pulmonary endothelium, 2) generate H₂O₂, 3) induce oxidative stress in endothelium, and 4) cause vascular pulmonary injury. In this paper, we describe the pulmonary targeting of anti-PECAM-GOX in isolated perfused rat lungs and in intact mice. We report that systemic administration of anti-PECAM-GOX provides organ-selective targeting of an active enzyme that results in oxidative injury to the pulmonary vasculature, thus validating a novel drug delivery strategy.

**MATERIALS AND METHODS**

The following materials were used in the study: IODOGEN from Pierce (Rockford, IL); Na²¹²⁵ from Amersham (Arlington Heights, IL), fatty acid-free BSA from Boehringer Mannheim (Indianapolis, IN); dimethyl formamide, 30% aqueous solution of H₂O₂, mouse IgG, biotinylated glucose oxidase (b-GOX), biotinylated β-galactosidase, biotinylated ferritin, and components of buffer solutions from Sigma (St. Louis, MO); O-phenthaldehyde and Z-Phe-His-Leu from Serva (Heidelberg, Germany); bovine liver catalase (20,000 U/mg) from Fluka (Ronkonkoma, NY); and streptavidin (SA) and 6-biotinylaminocaproic acid N-hydroxysuccinimide ester (BxNHS) from Calbiochem (San Diego, CA). Protein concentration was determined by Bio-Rad microassay kit ( Hercules, CA). A monoclonal antibody to human PECAM-1 (Mab 62, a mouse monoclonal IgG reacting with the first Ig-like loop of human and rat PECAM-1) was kindly provided by Dr. Marian Nakada (Centocor, Malvern, PA). Monoclonal antibody MAB 390 is a monoclonal antibody produced in rat and reacting with murine PECAM-1 (45). A polyclonal rabbit antiserum directed against isoprostane, I⁴F₂₃-III, was a generous gift from Dr. Jacques Madlouf (INSERM Unit 398, Institut Curie, Hôpital Lariboisière, Paris, France).

Conjugation of glucose oxidase to anti-PECAM. Immunoglobinulins were biotinylated at a 10-fold molar excess of biotinylating reagent BxNHS as described previously (24, 26). In the following text, biotinylated proteins will be designated as b-1gG, b-Mab, or b-GOX. Biotinylated GOX was labeled with ¹²⁵I using IODOGEN-coated tubes, according to the manufacturer’s recommendation. To construct the trimolecular heteropolymer complex, b-anti-PECAM-SA-b-GOX or b-1gG-SA-b-GOX, as well as conjugates with other biotinylated enzymes (b-catalase), we used a two-step procedure established in our laboratory (24). Briefly, SA and b-GOX were mixed at a molar ratio of SA to b-GOX = 5 and incubated for 1 h on ice. This ratio is optimal for conjugation of SA-b-GOX complex with biotinylated immunoglobulins. The complex was then incubated with b-anti-PECAM or b-1gG to form b-anti-PECAM-SA-b-GOX conjugate or its nonimmune counterpart b-1gG-SA-b-GOX. These conjugates are indicated as anti-PECAM-GOX and IgG-GOX in the text. Enzymatic activity of GOX conjugated with either the immune or the nonimmune carrier did not differ from that of the initial preparation of biotinylated GOX (−100 U/mg).

Isolated perfused rat lungs. Isolated perfused rat lungs (IPL) were prepared as described (3). Briefly, male Sprague-Dawley rats (170–200 g) were anesthetized, the trachea was cannulated, and the lungs were ventilated with a humidified gas mixture containing 5% CO₂-95% air. Ventilation was achieved with an SAR-830 rodent ventilator (CWE) at 60 cycles/min, a tidal volume of 2 ml, and 2 cmH₂O end-expiratory pressure. After thoracotomy, the main pulmonary artery was cannulated through the transected heart. The lungs were then excised and transferred to the water-jacketed perfusion chamber maintained at 37°C. There was no interruption of ventilation during this transfer process, and interruption of lung perfusion was <5 s. Perfusion through the artery was maintained by a peristaltic pump at a constant flow rate of 10 ml/min. The perfusate (45 ml) was Krebs-Ringer buffer (KRB), pH 7.4, containing 10 mM glucose and 3% fatty acid-free BSA (KRB-BSA solution). The perfusate was filtered through a 0.4-μm filter before perfusion to eliminate particulates.

Determination of the pulmonary uptake of the conjugates in IPL. Perfusion of radiolabeled GOX conjugates was performed as previously described (2). Briefly, 1 µg of anti-PECAM-¹²⁵I-GOX or IgG-¹²⁵I-GOX was added to the perfusate and allowed to circulate for 1 h at 37°C. Biotinylated conjugates were eliminated by 5-min nonrecirculating perfusion with conjugate-free buffer. Radioactivity in the lung tissue was determined in a gamma counter and calculated as a percent of the injected dose per gram of lung tissue (%ID/g).

Determination of ACE activity in the perfusates. ACE activity in the perfusates was measured by the rate of generation of His-Leu formed from the ACE substrate Z-Phe-His-Leu using a fluorometric assay (12). Ten microliters of the perfusate were added to 200 µl of 50 mM Tris-HCl-0.15 M NaCl, pH 8.3, buffer containing 0.5 mM substrate. Samples of perfusate were incubated at 37°C for 120 min, then the reaction was terminated by the addition of 1.5 ml of 0.28 N NaOH. O-phenthaldehyde (1 mg in 100 µl of methanol) was added for 10 min before the reaction was stopped by 2 N HCl. His-Leu was measured with a fluorescence spectrophotometer at an excitation wavelength of 363 nm and an emission wavelength of 500 nm. Results were calculated as milliunits of ACE activity per total perfusate (45 ml), where 1 mU represents the generation of 1 nmol His-Leu/min.

Detection of H₂O₂ generation in IPL. We used the oxidation-dependent fluorogen 2,7-dichlorofluorescin diacetate (DCF-DA; Molecular Probes, Eugene, OR) to detect generation of H₂O₂ in the lung tissue (1). This probe readily enters and becomes irreversibly entrapped intracellularly via cleavage by esterases. DCF-DA was perfused concomitantly with 100 µg of anti-PECAM-GOX or IgG-GOX in KRB-BSA for 1 h at 37°C to allow for accumulation of the conjugates and the probe. Glucose-free buffer was used to avoid massive formation of H₂O₂ in the perfusate by the circulating conjugates. Lungs were perfused for 2 h with glucose-containing KRB-BSA after elimination of nonbound material. DCF fluorescence (excitation 488 nm, emission 530 nm) was determined in the lung homogenates in a spectrofluorimeter and is expressed in arbitrary fluorescent units.

Evaluation of the pulmonary targeting and biodistribution of anti-PECAM-GOX in intact mice. Normal BALB/c mice were injected with 1 µg of anti-PECAM-¹²⁵I-GOX or IgG-¹²⁵I-GOX in 100 µl of physiological saline via the tail vein to characterize targeting of the conjugated GOX in vivo. One hour after injection, animals were killed, and the tissues were harvested. Radioactivity in the tissues was measured in a gamma counter. The tissue uptake of ¹²⁵I-GOX was calculated as a percent of the injected dose per gram of lung tissue.
Assessment of pathological effect(s) caused by anti-PECAM-GOX in mice. To study effects of the conjugates in intact animals, we injected anesthetized BALB/c mice with 25–100 µg of anti-PECAM-GOX or control counterparts (IgG-GOX, nonconjugated anti-PECAM, anti-PECAM-ferritin, or anti-PECAM-catalase) via the tail vein. Surviving animals were killed 4–24 h after injection. Internal organs were inspected, photographed, and processed for cryosectioning, routine histological processing, or electron microscopy.

Histological evaluation of mouse organs. Tissue evaluation after treatment with anti-PECAM-GOX or IgG-GOX was performed on paraffin-embedded tissues. The lungs were instilled with 0.75 ml of buffered Formalin intratracheally with 20-gauge angiocatheters (Fisher) before removal from the animal. After excision, all organs (kidneys, liver, spleen, heart, and lung) were immersed in buffered Formalin overnight and processed for conventional paraffin histology. The sections were stained with hematoxylin and eosin and evaluated by light microscopy.

Immunohistochemical evaluation of mouse tissues. Immunostaining was performed with 6-µm-thick frozen sections from optimal cutting temperature-embedded tissues or from 4-µm paraffin sections. The following antibodies were used: a rabbit polyclonal antibody directed against iPF2α, an isoprostane formerly known as 8-epi- or 8-iso-PGF2α, and the monoclonal antibody against GOX obtained from Sigma. Visualization was achieved by the use of Vectastain kit or by alkaline phosphatase kit (Vector Laboratories). For conventional light microscopy, the sections were viewed with an Olympus II photomicroscope and for immunofluorescence with a Zeiss fluorescent microscope.

Electron-microscopic processing of the lung tissue. This was performed on Epon-embedded tissues as described previously (7). For lung tissue, the trachea was exposed and cannulated with a 20-gauge angiocatheter. The lungs were instilled with cold 0.75 ml Karnowsky's fixative (2% paraformaldehyde and 2.5% glutaraldehyde; Electron Microscopy Sciences) in 0.1 M cacodylate buffer. They were immediately removed from the animal intact, the trachea was tied with a suture, and the entire tissue block was immersed in the same fixative for 30 min on ice. The lungs were then cut with a blade in 1-mm3 pieces, placed in fresh fixative, and left under vacuum (20 mmHg) overnight. After a rinse in cacodylate buffer, followed by a rinse in 0.05 M maleate buffer, pH 5.2, the tissue was postfixed with 1% osmium tetroxide in 0.7 M potassium ferrocyanide for 1 h on ice. En block staining was performed with uranyl acetate for 20 min, followed by dehydration in a graded series of alcohols and embedding in Poly/Bed 812 (Polysciences). Ultrathin sections (gold) were cut with a diamond knife (Diatome) on a Reichert-Jung (Vienna, Austria) ultramicrotome. Sections were collected on uncoated 150-mesh copper grids (Electron Microscopy Sciences) postcontrasted with 20% aqueous uranyl acetate (Amersham) for 3 min followed by aqueous 0.5% lead citrate for 3 min. The sections were viewed on a Hitachi H-600 transmission electron microscope (Nissey Sangyo) at 75 kV.

Statistical analysis. Statistical analysis was performed with a one-way ANOVA. When significant differences were found (P < 0.05), individual comparisons were made with the Bonferroni-Dunn test (Statview 4.0).

RESULTS

Accumulation and effects of anti-PECAM-GOX in isolated rat lungs. In the first series of experiments, we studied the uptake and effects of anti-PECAM-GOX in isolated rat lungs perfused with blood-free buffer. This approach allowed us to examine the effects of conjugates in a simplified “ex vivo” system without confounding effects due to the conjugate action in other organs or its interactions with blood elements.

Pulmonary uptake of anti-PECAM-125I-GOX in the isolated rat lungs attained 20%ID/g, whereas that of IgG-125I-GOX did not exceed 0.5%ID/g (Fig. 1A). To detect H2O2 generated by anti-PECAM-GOX accumulated in the lung tissue, we used DCF-DA, a probe that forms a fluorescent dye in reaction with H2O2. Fluorescence in the tissue homogenates was markedly higher after perfusion of anti-PECAM-GOX than that after perfusion of IgG-GOX (Fig. 1B). We measured activity of ACE in the perfusate to characterize specifically endothelial injury by the conjugates. Release/shedding of ACE to the perfusate is a sensitive and cell-specific marker of endothelial injury in the lungs (3, 12). Anti-PECAM-GOX caused a marked elevation of ACE activity in the perfusate, whereas IgG-GOX had no effect (Fig. 1C) compared with the level of ACE activity in the perfusates of control lungs perfused with the buffer (shown as a dashed line in Fig. 1C).

These results indicate that, in the absence of blood components, anti-PECAM-GOX (1) accumulates in the lung tissue via specific binding to PECAM localized in the pulmonary vasculature, 2) is enzymatically active and generates H2O2 in the lung, and 3) causes endothelial injury in the pulmonary vasculature.

Biodistribution and pulmonary uptake of anti-PECAM-GOX in intact animals. Based on the positive results obtained in an ex vivo model, we initiated a series of studies in intact animals. A trace amount of anti-PECAM-125I-GOX or IgG-125I-GOX was injected in the tail veins of BALB/c mice to characterize the immuno-targeting of anti-PECAM-GOX in vivo. The aims of this study were to 1) compare the carrier, anti-PECAM-streptavidin, with other known carriers and 2) draw a correlation between uptake and the effect of the conjugate in different organs.

One hour after intravenous injection, the blood level of anti-PECAM-125I-GOX was similar to that of IgG-125I-GOX (2.9 ± 0.2 vs. 2.7 ± 0.1%ID/g). In contrast, pulmonary uptake of anti-PECAM-125I-GOX achieved 30%ID/g and was 10 times higher than that of IgG-125I-GOX (Fig. 2). The lung-to-blood ratio was 10.6 ± 1.6 for anti-PECAM-125I-GOX vs. 0.9 ± 0.1 for IgG-125I-GOX.

Tissue uptake of anti-PECAM-125I-GOX and IgG-125I-GOX after intravenous injection in mice is shown in Table 1. Both conjugates displayed similar levels in plasma, significantly exceeding those in blood. This result implies that anti-PECAM-GOX does not interact specifically with blood cells. Anti-PECAM-125I-GOX and IgG-125I-GOX showed similar levels of uptake in all organs but the lungs. Therefore, the immunospecificity index was close to 1 in all tissues except the lungs. Both anti-PECAM-125I-GOX and IgG-125I-GOX displayed relatively low cardiac (1%ID/g), modest renal (2–3%ID/g), and high hepatic and splenic (30–50%ID/g) uptake.

We visualized GOX in the lung tissue using a commercially available antibody directed against glucose oxidase (anti-GOX) and a secondary FITC-labeled anti-
In isolated rat lungs. A: pulmonary uptake of anti-PECAM-125I-GOX-1 (cross hatched bars) and IgG-125I-GOX (solid bars) in the perfused rat lung. One microgram of the conjugates was perfused for 1 h in blood-free buffer in the isolated rat lungs via the pulmonary artery. Radioactivity in the lungs was determined after elimination of nonbound material and expressed as percent of injected dose per gram of tissue (%ID/g). Ab, antibody. B: generation of reactive oxygen species (ROS) in the isolated rat lungs perfused with anti-PECAM-GOX. Isolated rat lungs were perfused for 1 h with glucose-containing buffer, and DCF fluorescence in the homogenates was determined. The data are shown as arbitrary fluorescent units (AFU)/mg tissue protein in the lung homogenates. Dashed line indicates the background level of fluorescence in the homogenates obtained from control lungs perfused with the conjugate-free buffer containing DCF diacetate. C: endothelial injury in the isolated rat lungs perfused with anti-PECAM-GOX. Isolated rat lungs were perfused for anti-PECAM-GOX or IgG-GOX in glucose-free buffer. After elimination of nonbound material, lungs were perfused for 2 h with glucose-containing buffer, and angiotensin-converting enzyme (ACE) activity was measured in the perfusate. Elevation of ACE activity in the perfusate (a marker of endothelial injury in the lung) is expressed in mU/ml. Dashed line indicates the background level of ACE activity in the perfusates obtained from control lungs perfused with the conjugate-free buffer. All data are shown as means ± SE (n = 3 or 4 lungs). Significant difference from control value: *P < 0.05; **P < 0.01.

These results indicate that intravenous injection of anti-PECAM-GOX provides tissue-selective immunotargeting of GOX to the pulmonary endothelium in intact animals compared with the nonimmune conjugate.

**Table 1. Biodistribution of anti-PECAM-125I-GOX or IgG-125I-GOX in intact BALB/c mice**

<table>
<thead>
<tr>
<th>%ID/g</th>
<th>MAb-GOX</th>
<th>IgG-GOX</th>
<th>ISI</th>
<th>Loc Ratio</th>
<th>MAb-GOX</th>
<th>IgG-GOX</th>
<th>ISI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>2.95 ± 0.24</td>
<td>2.73 ± 0.13</td>
<td>1.1</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
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<tr>
<td>Lung</td>
<td>30.98 ± 4.95</td>
<td>2.41 ± 0.11</td>
<td>12.9</td>
<td>10.64 ± 1.63</td>
<td>0.90 ± 0.07</td>
<td>11.8</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>36.73 ± 3.05</td>
<td>35.72 ± 1.71</td>
<td>1.0</td>
<td>12.75 ± 1.22</td>
<td>13.16 ± 0.50</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>2.71 ± 0.39</td>
<td>2.02 ± 0.21</td>
<td>1.3</td>
<td>2.71 ± 0.11</td>
<td>0.74 ± 0.6</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>47.66 ± 9.02</td>
<td>38.81 ± 6.76</td>
<td>1.2</td>
<td>16.19 ± 2.99</td>
<td>14.56 ± 2.88</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>1.29 ± 0.11</td>
<td>1.21 ± 0.24</td>
<td>1.1</td>
<td>0.45 ± 0.05</td>
<td>0.43 ± 0.07</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>3.89 ± 0.21</td>
<td>3.72 ± 0.18</td>
<td>1.1</td>
<td>1.33 ± 0.04</td>
<td>1.37 ± 0.03</td>
<td>1.0</td>
<td></td>
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</table>

Values are means ± SE. Intact mice were injected with 3 µg of either IgG-125I-labeled glucose oxidase (GOX) (n = 5) or anti-platelet endothelial cell adhesion molecule (PECAM)-125I-GOX (n = 7). Biodistribution of 125I-GOX 1 h after intravenous injection is shown. Data are shown as a percent of injected dose per gram of tissue (%ID/g) and as localization ratio (Loc. ratio), which is equal to ratio of the radioactivity per gram of a tissue to that of blood. Immunospecificity index (ISI) was calculated as ratio between anti-PECAM-125I-GOX and IgG-125I-GOX using either percent of injected dose per gram (column 4) or Loc. ratio (column 7). The latter method of ISI calculation allows one to compensate for a difference in blood level of the immune and nonimmune conjugates. Both methods of ISI calculation give similar results.
Effect of systemic administration of anti-PECAM-GOX in intact mice. In the next series of experiments, we injected mice with 25–100 µg of anti-PECAM-GOX or IgG-GOX to determine the physiological effects of conjugate injections. Mice injected with saline served as negative controls for this study. Because anti-endothelial antibodies or conjugates could cause GOX-independent lung injury via complement- and/or leukocyte-mediated pathways, we also studied the effects of nonconjugated anti-PECAM as well as anti-PECAM conjugated to inert proteins (catalase, β-galactosidase, or ferritin). The goals of these experiments were to 1) determine whether intravenous injection of anti-PECAM-GOX causes lung injury, 2) characterize the specificity of the injury (by comparing anti-PECAM-GOX with control counterparts), 3) evaluate tissue selectivity of the injury (by comparing lung with other organs), and 4) determine the lethality, kinetics, and dose dependence of the effect of anti-PECAM-GOX.

In intact BALB/c mice, injection of anti-PECAM-GOX (100 µg GOX/mouse) caused acute pulmonary injury manifested grossly by diffuse hemorrhages as early as several hours after intravenous injection (Fig. 4C). Animals became cyanotic 2–4 h after injection. In contrast, IgG-GOX caused minimal gross lung injury (Fig. 4B). The control injections, including saline (Fig. 4A) as well as nonconjugated anti-PECAM, anti-PECAM-ferritin, anti-PECAM-β-galactosidase, or anti-PECAM-catalase conjugates, did not cause detectable lung injury (data not shown).

Lung injury caused by this dose of anti-PECAM-GOX was so severe that it led to a very high mortality in mice. Ninety-five percent of the animals died within the first day after injection of 100 µg of anti-PECAM-GOX (Fig. 5A). In sharp contrast, 5% of animals died after injection of 100 µg of IgG-GOX. At higher doses, both anti-PECAM-GOX and IgG-GOX caused significant hemolysis. No lethality was observed after injection of 100 µg of anti-PECAM, anti-PECAM-β-galactosidase, anti-PECAM-ferritin, or anti-PECAM-catalase. The toxic effect of anti-PECAM-GOX was dose dependent, with a 50% lethal dose of ~30–40 µg (Fig. 5B).

Figure 6 shows typical results of histological examination of the internal organs. After injection of anti-PECAM-GOX, lungs displayed signs of severe vascular congestion, fluid exudation, intravascular accumulation, and margination of white blood cells. IgG-GOX injection did not cause major abnormalities in the lung. No significant cardiac injury was detected after injection of either conjugate, consistent with low uptake of either conjugate in the heart. Glomerular injury consisting of proteinaceous fluid accumulation in Bowman's capsule.
space was noted in the kidney after injection of either the immune or the nonimmune conjugate. There was no significant morphological signs of injury in liver and spleen.

Because liver displayed high uptake of both anti-PECAM-GOX and IgG-GOX (Table 1), we assessed for hepatic injury more carefully, using transmission electron microscopy. The results revealed that hepatocytes looked normal after injection of either saline or anti-PECAM-GOX, with normal-appearing endothelium and with intact mitochondrial membranes and cristae, nuclear membranes, and endoplasmic reticulum (Fig. 7). We also assessed the level of serum markers of hepatic injury in mice. Anti-PECAM-GOX at a dose of 75 µg/mouse (the dose which caused severe lung injury and 70% mortality) induced no elevation of serum alkaline phosphatase [96 ± 34 vs. 102 ± 14 (SE) IU/l], for anti-PECAM-GOX (n = 4) vs. saline (n = 5)]. There was a threefold elevation of serum bilirubin (0.53 ± 0.03 vs. 0.17 ± 0.03 mg/dl). However, this level was barely above the top normal value (0.5 mg/dl). Both aspartate aminotransferase (AST; 300 ± 18 vs. 74 ± 9 IU/l) and alanine aminotransferase (ALT; 71 ± 10 vs. 20 ± 2 IU/l) increased about threefold. Although this increase is statistically significant, this level of increase of these serum markers of hepatic injury is quite modest (for example, ALT increases by 1,000 in hepatic ischemia-reperfusion at the same time points). Overall, experiments with anti-PECAM-GOX showed a very minor degree of liver toxicity. In addition, bearing in mind the extreme severity of the pulmonary injury caused by anti-PECAM-GOX, it is difficult to rule out the possibility that the modest elevation of these markers we observed reflects secondary hepatic injury due to direct lung tissue injury.

These results indicate that anti-PECAM-GOX, but none of the control conjugates, causes severe lung injury and high mortality after intravenous injection in intact mice.

Characterization of the lung injury caused by anti-PECAM-GOX. The data shown above indicate that lung was the only organ where major differences between anti-PECAM-GOX and IgG-GOX occurred, both in terms of uptake and effect in the tissue. Based on these results, we focused on the lungs harvested from the mice injected with 100 µg of anti-PECAM-GOX or IgG-GOX to characterize more precisely the lung injury. The goals were to determine whether the endothelium was the primary site of anti-PECAM-GOX-mediated injury in vivo and whether the injury was associated with pulmonary oxidative stress.

First, we determined the lung wet-to-dry weight ratio. Anti-PECAM-GOX caused a significant elevation of the lung wet-to-dry weight ratio to 7.7 ± 0.7 (n = 8) vs. 4.5 ± 0.2 (n = 5) in the saline-injected control group (P < 0.05). IgG-GOX caused no elevation of the lung wet-to-dry weight ratio (4.5 ± 0.3, n = 5). Thus anti-PECAM-GOX caused an elevation of vascular permeability, an abnormality consistent with endothelial injury.

Second, we used transmission electron microscopy to characterize the lung injury at cellular and subcellular levels. IgG-GOX induced no marked pathological alterations in the lungs (Fig. 8, panels 1–3). In contrast, severe vascular injury was present in the lungs after injection of anti-PECAM-GOX. Although alveolar injury was also noted, the pulmonary microvascular endothelium appeared to be the major target of injury. This conclusion is based on the following findings. First, anti-PECAM-GOX caused massive interstitial injury and alveolar edema (Fig. 9, panel 1). This finding is consistent with an elevated lung wet-to-dry ratio and implies that the conjugate increases vascular permeability by disruption of endothelial lining (Fig. 9, panel 2). Second, endothelial blebbing (microvesiculation), disruption of mitochondrial cristae, and swelling of their matrix were noted in the endothelium (Fig. 9, panel 3), consistent with oxidative cellular injury (20).

We stained the lung tissue sections with an antibody against iPF_2-I, an F_2 isoprostane, to determine whether anti-PECAM-GOX causes oxidative stress in the lung in vivo. Isoprostanes are chemically stable, free radical-catalyzed products of arachidonic acid that reflect lipid peroxidation in the isolated lungs (4) and in vivo (34, 37–39). Figure 10 shows that injection of anti-PECAM-GOX, but not of IgG-GOX, resulted in
positive immunostaining of isoprostane in the lung tissue. This result is consistent with the ability of anti-PECAM-GOX to generate H$_2$O$_2$ in the endothelial cells in vitro (15) and in the perfused rat lungs (Fig. 1B). Thus lung injury is associated with oxidative stress caused by enzymatic activity of anti-PECAM-GOX delivered to the pulmonary vascular endothelium.

These results indicate that 1) the endothelium is the major target for action of anti-PECAM-GOX in vivo and 2) vascular injury is associated with oxidative stress in the lungs.

**DISCUSSION**

Site-selective delivery of effector compounds (such as drugs, oligonucleotides, genes, toxins, or enzymes) to the vascular endothelium represents an important goal (2, 17, 25, 28, 40, 42). Enzymes are good candidates for immunotargeting for use in either experimental or therapeutic settings, since in contrast to other agents, even small amounts of active enzymes may produce an effect via generation of biologically active products when delivered to the target (36). Importantly, enzymes may work immediately after delivery, in contrast to genes, which require significant time for protein synthesis. Thus enzyme immunotargeting may afford a mechanism for effective, rapid, and site-selective interventions in endothelial cell function.

To our knowledge, results documented in this paper represent the first direct experimental evidence that systemic vascular administration of an enzyme conjugated with an antibody to endothelial surface antigen was able to provide site-selective delivery of an active enzyme to the pulmonary endothelium, causing a specific local effect in intact animals. Thus the present study verifies the validity and potential applicability of the strategy of vascular immunotargeting. In addition, this approach provides a novel model of oxidative vascular injury, a key component in pathogenesis of many pulmonary and cardiovascular diseases.

Although a number of endothelial antigens have been proposed for immunotargeting, our study focused on PECAM-1. In terms of the effectiveness (uptake of 30% D/g), tissue selectivity (lung-to-blood ratio of 10), and immunospecificity (ratio between uptake values of
the immune and nonimmune conjugates of 13), pulmonary targeting of anti-PECAM-GOX is similar to that of other radiolabeled compounds conjugated with other antiendothelial carrier antibodies, such as anti-ACE (26, 27) and anti-ICAM-1 (2). Immunotargeting to PECAM, however, offers potential advantages over other targeting systems. First, endothelial cells possess extremely high binding capacity for PECAM antibodies, and PECAM expression is not suppressed upon inflammation (32). Second, we have shown that enzymes conjugated with anti-PECAM enter endothelial cells, escape rapid intracellular degradation, and are

Fig. 7. Electron-microscopic evaluation of mouse liver harvested 4 h after injection of 100 µg of anti-PECAM-GOX (A) or saline (B). Note well-preserved cytoplasmic organelles (nuclear membrane, slightly dilated endoplasmic reticulum, mitochondria with dense matrix and well-preserved cristae) in both panels. Magnification, ×32,400. Nu, nucleus; mit, mitochondria; arrowheads, mitochondrial membrane.

Fig. 8. Electron-microscopic evaluation of mouse lungs harvested 4 h after injection of 100 µg of IgG-GOX. Panel 1: overview of the lung tissue (magnification, ×6,250). Alveolar spaces (*) are clear from plasma proteins. Capillaries (c), interstitial spaces (★), and epithelium appear normal. Panel 2: a focused view of a pulmonary capillary (magnification, ×70,000). RBC, red blood cell. Notice the absence of endothelial damage or edema. Panel 3: detail of the alveolar-capillary barrier (magnification, ×105,000). There is no edema, and the endothelial lining (arrowheads) of the capillary and the junctional region (arrows), endothelium (ec), basement membrane (bm), and epithelial cells (epi) appear normal.
enzymatically active (15, 28). Third, PECAM antibodies have been shown to inhibit transendothelial migration of leukocytes and thus may be advantageous when used in an anti-inflammatory therapeutic context (43). Therefore, PECAM-directed immunotargeting may be useful for the intracellular delivery of enzymes such as catalase or superoxide dismutase (for antioxidant protection), antisense oligonucleotides (for gene-selective suppression of protein synthesis), or plasmids (for specific stimulation of protein synthesis in the endothelial cells).

An important issue in immunotargeting relates to selectivity of delivery. Anti-PECAM-GOX caused primarily lung damage, consistent with the high pulmonary uptake of the conjugate. Injury was minimal in other organs (i.e., heart), consistent with the low tissue

Fig. 9. Electron-microscopic evaluation of mouse lungs after treatment with anti-PECAM-GOX. Panel 1: overview of the lung tissue (magnification, ×7,000). Note the widening of the interstitium and accumulation of electron-dense granular osmophilic material that indicate interstitial (●) and alveolar (*) edema in the lung, lifting of the endothelium, congestion in the vessels (V), and endothelial cytoplasmic gaps (arrowheads). Panel 2: a focused view of a representative pulmonary capillary (magnification, ×7,000). Notice blebbing of the endothelium (small arrows) and swelling of the endothelial mitochondrion (long arrow). Panel 3: detailed view of the pulmonary vessel shown in panel 2 (magnification: ×70,000). Note extensive blebbing of the endothelium, endothelial gap formation (arrows), and swelling of the type I epithelial cells.

Fig. 10. Immunohistochemical evaluation of lipid peroxidation in the lungs of mice injected with anti-PECAM-GOX. Paraffin sections were stained with either control IgG (A and B) or with a polyclonal antibody to 8-epi-isoprostane (C and D). Animals have been injected with 100 µg of either anti-PECAM-GOX (B and D) or IgG-GOX (A and C). Immunostaining was revealed with a secondary antibody conjugated with alkaline phosphatase. Positive reaction product is shown in blue color, and red color is a basic counterstain.
uptake of the conjugate in these organs. Similarly, the results of the survival study (Fig. 5) demonstrated that the injection of anti-PECAM-GOX is lethal, whereas IgG-GOX is well tolerated. However, the results of the biodistribution of anti-PECAM-125I-Go and IgG-125I-GoX (Table 1) demonstrate that liver and spleen collect equally large doses of GOX after injection of either conjugate. This high hepatic and splenic uptake is similar to that observed with radiolabeled catalase, superoxide dismutase, or urokinase conjugated with carrier antibodies directed against PECAM, ACE, or intracellular adhesion molecule-1, as well as with their nonimmune counterparts (2, 26, 27). Interestingly, although subclinical damage may have occurred, data from postmortem gross examination, light microscopy, and electron microscopy showed no significant injury in liver and spleen despite a very high uptake of the conjugates. This high hepatic and splenic uptake of IgG-GoX taken together with a low lethality of mice injected with IgG-GoX indicates that the liver and spleen are resistant to GOX. This is likely due to the fact that most of the uptake in these organs is probably by Fc receptor-bearing macrophages, Kupffer cells, or other phagocytic cells. The phagocytes may degrade the conjugates and/or be resistant to oxidants. Accordingly, we view our results as showing lung “selective” (rather than “specific”) delivery of an active enzyme, GOX.

We are currently performing studies with anti-PECAM-β-galactosidase conjugate, which causes no injury in animals but allows one to trace directly the enzymatic activity of the conjugates in vivo (A. Sherperel, R. Wiewrodt, M. Christofidou-Solomidou, R. Gervais, S. Albelda, and V. Muzykantov, unpublished data). We detected significant uptake and activity of β-galactosidase in the liver 1 h after injection of either IgG-β-galactosidase or anti-PECAM-β-galactosidase. Interestingly, β-galactosidase activity in the liver declined extremely rapidly and was indistinguishable from the background level as soon as 4 h after injection. In sharp contrast, 4 h after injection of anti-PECAM-β-galactosidase, the enzyme activity in the lungs declined by only 30% and was still 10 times higher than the background level. These new data show that the anti-PECAM-β-galactosidase conjugate undergoes extremely rapid degradation in the liver (probably within Kupffer cells), but not in the lungs. Therefore, we believe that the lack of anti-PECAM-GOX hepatotoxicity can be explained by different metabolism/resistance of the anti-PECAM-GOX in the lung and liver.

In this study, we used anti-PECAM antibodies to target a H₂O₂-generating enzyme, GOX. H₂O₂ forms the strong oxidants hydroxyl anion (in reactions with metals or with superoxide anion) and hypochlorous acid (in reaction catalyzed by myeloperoxidase), which cause oxidative stress in the cells and tissues (16). GOX has been used in previous in vitro and in vivo studies, where local administration of enzyme led to oxidant generation (21, 31, 35). The morphological characteristics of the lung injury caused by anti-PECAM-GOX (endothelial blebbing and disruption), elevation of the pulmonary vascular permeability, and release of ACE imply that the pulmonary endothelium is the primary site of injury. Elevated fluorescence of a H₂O₂-sensitive probe and positive immunostaining for iPF2-III isoprostane in the lung tissue indicate that oxidative injury to endothelium caused by H₂O₂ is likely a mechanism for the injury.

In addition to demonstrating the validity of vascular immunotargeting, anti-PECAM-GOX offers a new model of endothelial oxidative stress. Endothelial oxidative injury has been implicated in many types of lung pathology including ischemia-reperfusion injury, postcardiopulmonary bypass lung injury, sepsis, and the adult respiratory distress syndrome (1, 6, 14, 41). Studies in cell cultures, in isolated organs, and in vivo indicate that H₂O₂ is one of the key reactive oxygen species involved both in cell signaling, host defense, and oxidative injury in inflammation, infarction, and vascular injury (11, 16, 30). Endothelial oxidative stress caused by either extracellular reactive oxygen species (e.g., released from activated neutrophils) or intracellular reactive oxygen species (e.g., generated within endothelium upon ischemia-reperfusion) plays an important role in pathogenesis of cardiovascular and lung disease conditions (5, 6, 11, 18). Anti-PECAM-GOX could be useful to address mechanisms of pulmonary and vascular oxidative injury caused by or associated with a site-specific intracellular generation of the well-defined oxidant H₂O₂ in models ranging from cell culture to experiments in laboratory animals. Animal models based on GOX immunotargeting may help to dissect the primary (initiating) role of reactive oxygen species generated in endothelium and secondary (augmenting) role of oxidants released by leukocytes attracted to the site of the vascular injury. This area is under active investigation in our laboratory.

In conclusion, our results provide direct experimental evidence that vascular immunotargeting of enzymes conjugated with antibodies directed against surface endothelial antigens provides a mechanism for site-selective interventions in endothelial functions. Immunotargeting of glucose oxidase may be useful for investigation of the oxidative vascular injury and for tumor eradication. Conjugation of other potentially therapeutic compounds (such as antioxidant enzymes) with anti-endothelial antibodies could be useful in the treatment of a variety of pulmonary and vascular diseases caused by or associated with endothelial cell dysfunction.

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