Endothelial Targeting of High-Affinity Multivalent Polymer Nanocarriers Directed to Intercellular Adhesion Molecule 1

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ABSTRACT

Targeting of diagnostic and therapeutic agents to endothelial cells (ECs) provides avenues to improve treatment of many maladies. For example, intercellular adhesion molecule 1 (ICAM-1), a constitutive endothelial cell adhesion molecule up-regulated in many diseases, is a good determinant for endothelial targeting of therapeutic enzymes and polymer nanocarriers (PNCs) conjugated with anti-ICAM (anti-ICAM/PNCs). However, intrinsic and extrinsic factors that control targeting of anti-ICAM/PNCs to ECs (e.g., anti-ICAM affinity and PNC valency and flow) have not been defined. In this study we tested in vitro and in vivo parameters of targeting to ECs of anti-ICAM/PNCs consisting of either prototype polystyrene or biodegradable poly(lactic-coglycolic) acid polymers (~200 nm diameter spheres carrying ~200 anti-ICAM molecules). Anti-ICAM/PNCs, but not control IgG/PNCs 1) rapidly (t1/2 ~5 min) and specifically bound to tumor necrosis factor-activated ECs in a dose-dependent manner (Bmax ~350 PNC/cell) at both static and physiological shear stress conditions and 2) bound to ECs and accumulated in the pulmonary vasculature after i.v. injection in mice. Anti-ICAM/PNCs displayed markedly higher EC affinity versus naked anti-ICAM (Kd ~80 pM versus ~8 nM) in cell culture and, probably because of this factor, higher value (185.3 ± 24.2 versus 50.5 ± 1.5% injected dose/g) and selectivity (lung/blood ratio 81.0 ± 10.9 versus 2.1 ± 0.2, in part due to faster blood clearance) of pulmonary targeting. These results 1) show that reformattining monomolecular anti-ICAM into high-affinity multivalent PNCs boosts their vascular immuno-targeting, which withstands physiological hydrodynamics and 2) support potential anti-ICAM/PNCs utility for medical applications.

Targeting of diagnostic and therapeutic agents to endothelial cell (ECs) provides avenues for improvement of specificity and effectiveness of treatment of many diseases (Moelma, 2002; Song et al., 2002; Oh et al., 2004; Muzykantov, 2005). Among other EC surface determinants, intercellular adhesion molecule-1 (CD54 or ICAM-1, a 110-kDa Ig-like transmembrane constitutive endothelial adhesion molecule) is a good candidate target for this goal. ICAM-1 is 1) exposed to blood primarily by ECs and readily accessible to the circulation, 2) up-regulated by pathological factors, and 3) implicated in pathogenesis of many disease conditions including inflammation, ischemia-reperfusion, atherosclerosis, thrombosis, and oxidative stress (Marlin and Springer, 1987; Jevnikar et al., 1990; Muro and Muzykantov, 2005). Because of the latter factor, blocking of leukocyte interaction with ICAM-1 provides beneficial anti-inflammatory effects (Hallahan and Virudachalam, 1997; Hopkins et al., 2004).

ICAM-1 targeting can be achieved by coupling ICAM-1 ligands to drugs or drug carriers [e.g., polymer nanocarriers (PNCs), ranging from <100 nm to <1 μm in diameter]. Such ligands include high-affinity monoclonal ICAM antibodies (anti-ICAM) or their Fab, and single chain Fv fragments, some of which undergo clinical testing (Takei et al., 1996; Luo et al., 2003; Muro and Muzykantov, 2005). Numerous studies have reported targeting to ECs in cell cultures, perfused organs, and in vivo using anti-ICAM-conjugated isostopes, ultrasound and magnetic resonance imaging contrast probes (Villanueva et al., 1998; Weiner et al., 2001; Weller et al., 2002), therapeutic agents (Murciano et al.,...
2003; Muro et al., 2005, 2006), and drug carriers including liposomes, PNCs, and micron-size polymer particles (Bloemen et al., 1995; Sakhalkar et al., 2003). Hence, ICAM targeting is a promising area of molecular diagnostics and drug delivery (Muro and Muzykantov, 2005).

These empirical successes justify analysis of factors that control ICAM-1 targeting including anti-ICAM/PNC affinity, valency, size, shape, plasticity, and biological factors such as ICAM-1 expression and organization in the EC plasma membrane, and hydrodynamic conditions. For example, effects of flow and levels of endothelial ICAM-1 expression on binding of micron-size particles coated with ICAM-1 ligands have been studied by several groups to test their targeting potential and obtain numerical data for simplified models of leukocyte adhesion (Sakhalkar et al., 2003; Eniola et al., 2005).

Conceivably, internalizable nanoscale carriers (e.g., PNCs or liposomes) may be more suitable for vascular targeting versus micron-scale carriers (Bronich et al., 2002; Davda and Labhasetwar, 2002), yet parameters of anti-ICAM/PNC binding to ECs have not been characterized. Changing carrier size and anti-ICAM affinity and density on the carrier surface may profoundly alter targeting. For example, modulation of valency and size of anti-ICAM-based targeting systems alters their internalization by ECs: monomeric anti-ICAM and micron-size anti-ICAM conjugates bind to ECs without internalization, whereas 100- to 500-nm diameter anti-ICAM/PNCs are internalized by ECs via a unique pathway, cell adhesion molecule-mediated endocytosis (Muro et al., 2003). Multivalent delivery systems targeting ICAM-1 provide intracellular delivery of antioxidant and lysosomal enzymes, optimal for their therapeutic effects (Muro et al., 2005, 2006). These experimental results imply potential medical utility of anti-ICAM/PNCs.

In this study, we used isotope tracing and fluorescence microscopy to determine and visualize EC targeting in vitro, ex vivo, and in vivo of multivalent spherical anti-ICAM/PNCs carrying 200 anti-ICAM molecules per carrier (final diameter ~200 nm) based on prototype polystyrene or biodegradable polymers. For the latter iteration, we produced anti-ICAM/PNCs using biocompatible poly(lactic-co-glycolic) acid (PLGA), a Food and Drug Administration-approved candidate material (Davda and Labhasetwar, 2002; Dziubla and Muzykantov, 2006).

We determined amplitude and kinetics of anti-ICAM/PNC binding to ECs under static and flow conditions in cell cultures and their biodistribution and pulmonary uptake, reflecting endothelial targeting after intravenous injection in animals. Of particular interest, comparison of anti-ICAM/PNCs versus naked maternal anti-ICAM showed that reformatting monomolecular anti-ICAM into nanoscale multivalent PNCs iteration greatly enhances affinity and targeting to ECs in vitro and in vivo.

**Materials and Methods**

**Antibodies and Reagents.** The monoclonal antibodies against human, murine, and rat ICAM-1 were R6.5 (Marlin and Springer, 1987), VN1 (Jevnikar et al., 1990), and 1A29 (Christensen et al., 1993), respectively. Secondary antibodies were from Molecular Probes (Eugene, OR). Green fluorescent polystyrene latex beads, 100 nm in diameter, were from Polysciences (Warrington, PA). Poly(lactic-co-glycolic) acid (50:50 monomer ratio, 11.2 kDa mol. wt.) was obtained from Alkermes (Cambridge, MA). Na<sup>252</sup>I was obtained from Perkin Elmer (Wellesley, MA), and Iodogen was purchased from Pierce Biotechnology (Rockford, IL). Unless otherwise stated, all other reagents were from Sigma Chemical (St. Louis, MO).

**Synthesis of PLGA Nanocarriers.** A solvent extraction emulsification procedure was used to form PLGA nanoparticles. In brief, 25 mg of polymer was dissolved in dry acetone for a final concentration of 10 mg/ml (1 wt% of 125<sup>1</sup>-labeled polytyrosine (10–40 kDa mol. wt.) was added for in vivo tracing studies). This solution was then slowly pipetted into 100 ml of PBS solution containing 3 wt% Pluronic P88 [polyethylene glycol (PEG)-polypropylene glycol-PEG triblock copolymer]. The acetone was evaporated off at room temperature overnight. After this time, particles were collected by centrifuging at 20,000 g for 25 min and then were washed twice more in 40 ml of pure PBS solution. Purified particles were stored in PBS. Final particle concentration was determined using an enzymatic assay for lactic acid as previously published, and residual surfactant content was evaluated by a spectrophotometer PEG assay (Dziubla et al., 2005). Size and ζ potential of uncoated PLGA nanoparticles were determined on a Brookhaven 90Plus ζ potential and dynamic light scattering apparatus.

**Preparation of Anti-ICAM Nanocarriers.** Anti-ICAM nanocarriers (anti-ICAM/PNCs) and control IgG/PNCs were prepared by coating green fluorescent polystyrene beads with either anti-human, anti-murine, or anti-rat ICAM-1, or nonspecific IgG, respectively (Muro et al., 2003). Radiolabeled PNCs contained a mix of anti-ICAM and 125<sup>1</sup>-IgG at 95:5 M ratio (Muro et al., 2005). After separation of the free anti-ICAM or IgG by centrifugation, the amount of 125<sup>1</sup>-tracer coated onto the nanocarriers was determined in a gamma counter. A saturating antibody density on the PNC surface (~320 ± 10 molecules/PNC, ~7000 antibody molecules/μm<sup>2</sup>) was used in the study. The same procedure was used to prepare coated PLGA nanocarriers. The diameter of the final anti-ICAM/PNCs and IgG/PNCs ranged from 150 to 300 nm, as determined by dynamic light scattering.

**Cell Culture.** Pooled human umbilical vein endothelial cells (HUVECs) (Clonetics, San Diego, CA) were cultured at 37°C, 5% CO<sub>2</sub> and 95% relative humidity in supplemented M199 medium (Gibco BRL, Grand Island, NY) and used at passage 4 to 5. For experiments under static conditions, cells were seeded onto 12 mm<sup>2</sup> gelatin-coated coverslips in 24-well plates. For experiments under flow conditions, cells seeded in gelatin-coated 22 × 40 mm coverslips were grown to confluence, then placed in a parallel plate flow chamber (RC-30HV, Warner Instruments Inc., Hamden, CT) and perfused overnight at 9 dynes/cm<sup>2</sup> using a peristaltic pump (Pharmacia Biotech AB, Appalsa, Sweden). Cells were always treated overnight with TNF-α before experiments.

**Binding of Anti-ICAM and Anti-ICAM Nanocarriers to Endothelial Cells.** Varying concentrations of 125<sup>1</sup>-labeled free anti-ICAM or green fluorescent multivalent anti-ICAM/PNCs were incubated with activated HUVECs for 1 h at 4°C. In the case of free antibody, the cells were washed and lysed with 1% Triton X-100 in 1 M NaOH, and the amount of 125<sup>1</sup>-anti-ICAM was determined in a gamma counter. In the case of multivalent anti-ICAM/PNCs, the cells were washed, fixed in cold with 2% paraformaldehyde, and analyzed by phase-contrast and fluorescence microscopy (Eclipse TE2000-U, Nikon, Melville, NY) using a 4×/NA1.4 PlanApo objective (Nikon). PLGA anti-ICAM/PNCs were visualized by staining fixed cells with fluorescein isothiocyanate-labeled goat anti-mouse IgG. For experiments under flow conditions, green fluorescent anti-ICAM/PNCs were perfused at 9 dynes/cm<sup>2</sup> shear stress at 37°C in a flow chamber with TNF-activated HUVEC adapted to these flow conditions for 24 h. The amount of nanocarriers bound per cell was determined from micrographs obtained with an Orca-1 charge-coupled device camera (Hamamatsu, Bridgewater, NJ) and analyzed using ImagePro 3.0 software (Media Cybernetics, Silver Spring, MD).

**Targeting to Endothelium in Isolated Vasculature.** The mesentery was isolated from anesthetized 200-g male Sprague-Dawley...
rats and perfused at 1.5 ml/min using a peristaltic pump with Krebs-Ringer buffer containing green fluorescent anti-ICAM/PNCs at 37°C. Fluorescent microographs were collected at varying periods of time during 30 min to detect accumulation of anti-ICAM/PNCs in the mesenteric vessels.

**Targeting to Vascular Endothelium in Mice.** Anesthetized C57BL/6 male mice were injected intravenously with 125I-labeled anti-ICAM or control IgG (~0.12 mg/kg b.w.t.), polystyrene anti-ICAM/PNCs or IgG/PNCs (~2.8 mg of IgG and 5.2 × 10^13 PNCs/kg b.w.t.), PLGA anti-ICAM/PNCs, or untargeted PLGA nanocarriers (~10 mg of polymer/kg b.w.t.). Blood samples were collected from the retro-orbital plexus at 1, 15, and 30 min postinjection, and organs (heart, kidneys, liver, spleen, and lungs) were collected at 30 min postinjection. The radioactivity and weight of the samples were determined to calculate nanocarrier targeting parameters, including percentage of injected dose (ID) per gram, localization ratio (LR) (organ/blood ratio of percentage of ID per gram), and immunospecificity index (ratio of LR targeted versus nontargeted formulation in an organ) (Murciano et al., 2003). In parallel experiments, accumulation of green fluorescent anti-ICAM/PNCs or IgG/PNCs in mice organs was visualized by fluorescence microscopy from 10-μm-thick frozen sections. These studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

**Statistics.** Unless otherwise stated, the data were calculated as means ± S.D., where statistical significance was determined by Student’s t test.

### Results

**Antibody Surface Density and Binding of Anti-ICAM/PNCs to Endothelial Cells.** We used two types of PNCs to study ICAM-directed endothelial targeting. Green fluorescent polystyrene beads (200-nm diameter) were used as homogeneous prototype “carriers,” permitting visualization and radioisotope tracing of anti-ICAM/PNC targeting in culture of HUVECs, ex vivo in perfused rat mesentery and in vivo. In addition, anti-ICAM/PNCs based on clinically used biodegradable PLGA copolymer were produced in the laboratory to validate prototype polystyrene anti-ICAM/PNCs data (see the last section).

First, we determined PNC coating by radiolabeled anti-ICAM or control IgG and found that a saturating concentration (~800 antibody molecules added/PNC) provides noncovalent coupling of ~250 IgG molecules/200 nm of PNCs (Fig. 1A). Hence, surface density of anti-ICAM on PNCs prepared at a saturating anti-ICAM concentration was 7000 molecules/μm².

Fluorescence microscopy showed that anti-ICAM/PNCs, but not IgG/PNCs, bound to TNF-α-activated HUVECs (Fig. 1B). Anti-ICAM/PNCs bound to the apical surface of cells with relatively uniform distribution. Computer analysis of images showed binding of 174 ± 6 anti-ICAM/PNCs per TNF-activated EC, which exceeded IgG/PNC binding by ~90 times (2 ± 2 IgG/PNCs per cell).

**Targeting of anti-ICAM/PNCs to Endothelial Cells under Flow.** The EC phenotype degenerates in static culture. On the other hand, PNC binding to and detachment from ECs in vivo may be also affected by hydrodynamic conditions. To test the effects of these factors, we perfused anti-ICAM/PNCs in a flow chamber containing a monolayer of HUVECs adapted for 24 h to a physiological level of shear stress, 9 dynes/cm². Anti-ICAM/PNC binding to flow-adapted cells (note cell alignment to flow direction in Fig. 2A) under flow was indistinguishable from that to static cells (138.7 ± 6.5 versus 121.6 ± 10.1 after a 60-min incubation). The kinetics of anti-ICAM/PNC binding to HUVECs was very rapid (t_{1/2} = 5 min) under both static and flow conditions (Fig. 2B).

To test binding to ECs in intact vasculature under physiological flow conditions, we perfused anti-ICAM/PNCs ex vivo through the blood vessels in isolated rat mesentery (Fig. 3). Bright punctate fluorescence associated with the luminal surface of either large (Fig. 3, A–C) and small (Fig. 3D) vessels revealed anti-ICAM/PNC binding to ECs in this model. In agreement with cell culture data, endothelial targeting was rapid and clearly detectable as early as 5 min after the start of perfusion (Fig. 3B).

**Targeting of Anti-ICAM/PNCs to Vascular Endothelium in Vivo.** Tracing of radiolabeled anti-ICAM/PNCs injected in vivo provides an ultimate proof and the most physiological model of endothelial targeting. The lung is a privileged target for circulating antibodies to pan-endothelial
antigens (e.g., cell adhesion molecules including ICAM-1), because lungs contain ~30% of total endothelial surface, represent the first major vascular network after i.v. injection, and receive 100% of venous cardiac output of blood flowing via a high-capacity, low-resistance pulmonary vasculature at a relatively slow rate, which favors binding to endothelium (Muzykantov, 2005). ICAM antibodies and anti-ICAM conjugates bind to ECs and accumulate in lungs after i.v. injection in naive animals, due to a high constitutive level of ICAM-1 expression by ECs in vivo (Murciano et al., 2003; Muro et al., 2005). Thus, pulmonary uptake serves as an indicator of endothelial targeting of anti-ICAM/PNCs after systemic intravascular administration.

Both radiolabeled anti-ICAM/PNCs and control IgG/PNCs were rapidly eliminated from the circulation (inset in Fig. 4A). The total amount of PNC-coupled antibody in circulation was $4.7 \pm 0.8$ versus $22.3 \pm 1.2$ µg at 1 min after injection and $3.4 \pm 0.7$ versus $2.6 \pm 0.6$ µg at 15 min after injection, for anti-ICAM/PNCs versus control IgG/PNCs, respectively. Blood clearance reflects uptake of anti-ICAM/PNC and IgG/PNCs in the reticuloendothelial system in liver and spleen (Fig. 4, A and B), at least in part due to Fc fragment-mediated phagocytosis.

However, anti-ICAM/PNCs, but not IgG/PNCs, showed pulmonary targeting that, when calculated as a percentage of the injected dose per gram of tissue, markedly exceeded uptake in liver and spleen (Fig. 4A). In fact, $33.65 \pm 4.4$% of the total ID of anti-ICAM/PNCs accumulated in the lungs versus $3.23 \pm 0.1$% ID of IgG/PNCs. Low uptake of IgG/PNCs indicates that anti-ICAM/PNC targeting is due to binding to ICAM-1, not PNC aggregation and mechanical retention in the pulmonary capillaries.

Visualization of PNCs in organs by fluorescence microscopy confirmed isotope tracing results. Both anti-ICAM/PNCs and IgG/PNCs showed high levels of uptake and similar tissue distribution in the liver (Fig. 4B) and spleen (not shown). In contrast, microscopy showed highly specific pulmonary targeting anti-ICAM/PNCs and revealed their relatively uniform distribution in the alveolar capillaries (Fig. 4C) and their presence in the luminal surface of larger vessels (not shown). Specific targeting of anti-ICAM/PNCs was also seen in the kidneys, albeit to a significant lesser extent (Fig. 4D).

High Affinity and Targeting Effectiveness of Multimeric Anti-ICAM/PNCs. We reported that reformatting a monomolecular anti-ICAM into multimeric conjugates or anti-ICAM/PNCs alters their subcellular targeting: ECs did not internalize anti-ICAM, yet internalized anti-ICAM conju-
gates and anti-ICAM/PNCs, due to clustering of ICAM-1 in the EC plasmalemma (Murciano et al., 2003; Muro et al., 2003). In this study we tested the role of the multivalent nature of anti-ICAM/PNCs in binding to ECs, the first step of targeting.

Both anti-ICAM and anti-ICAM/PNCs displayed dose-dependent binding to TNF-activated HUVECs (Fig. 5). Scatchard analysis revealed $1.6 \times 10^6$ binding sites for anti-ICAMs (which agrees well with previous reports by other groups (Remy et al., 1999)) and $3 \times 10^5$ binding sites for anti-ICAM/PNCs per cell (insets in Fig. 5). However, the affinity of anti-ICAM/PNC binding to cells calculated as an effective $K_d$ was practically two orders of magnitude higher than that of anti-ICAM ($77 \text{ pM}$ versus $8.5 \text{ nM}$), reflecting the multivalent character of anti-ICAM/PNC binding involving many copies of ICAM-1.

Because of higher affinity, $^{125}$I-labeled multivalent anti-ICAM/PNCs exerted enhanced in vivo targeting versus the maternal antibody. In fact, pulmonary uptake of anti-ICAM/PNCs tripled that of anti-ICAMs ($185.3 \pm 24.2$ versus $50.5 \pm 1.5\% \text{ ID/g}$, $p < 0.01$) (Fig. 6A). Furthermore, the lung/blood ratio of anti-ICAM/PNCs was $40$ fold higher versus that of anti-ICAMs (Fig. 6B). This was probably due to higher effectiveness both of targeting and blood clearance of anti-ICAM/PNCs. The pulmonary immunospecificity index (ISI), the ratio of levels of immune and nonimmune counterparts in an organ corrected for their blood levels, of anti-ICAM/PNC doubled this parameter of maternal anti-ICAM.

**Endothelial Targeting of PLGA-Based Anti-ICAM/PNCs.** To evaluate the potential medical utility of anti-ICAM/PNC targeting to ECs, we used the Food and Drug Administration-approved copolymer PLGA to synthesize bio-

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**Fig. 4.** Specific targeting of anti-ICAM/PNCs to pulmonary endothelium in mice. A, biodistribution of $^{125}$I-labeled anti-ICAM/PNCs (black bars) versus control IgG/PNCs (gray bars) 30 min after i.v. injection in mice, calculated as a percentage of the ID per gram of organ. The inset shows PNC clearance from blood, calculated as a percentage of ID. Data are shown as means ± S.E.M., $n = 3–5$, $*, p < 0.05$, $**, p < 0.01$ by Student’s $t$ test. B–D, fluorescence micrographs showing accumulation of green fluorescent anti-ICAM/PNCs (right panels) versus IgG/PNCs (left panels) in liver (B), alveolar capillaries (C), and kidney (D). Original magnification, 200×.

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**Fig. 5.** Multivalency of nanocarriers markedly enhances affinity of anti-ICAM to endothelial cells in vitro. A, $^{125}$I-labeled monomolecular anti-ICAM was incubated at varying concentrations with TNF-activated HUVECs for 1 h at 4°C. The cells were washed and lysed with 1% Triton X-100 in 1 M NaOH, and the amount of bound $^{125}$I-anti-ICAM was determined in a gamma counter. Data are shown as means ± S.D. ($n = 4$). B, fluorescence anti-ICAM/PNCs were incubated at varying concentrations with activated HUVECs for 1 h at 4°C. Cells were then washed and fixed, and the number of particles bound per cell was quantified by fluorescence microscopy. Data are shown as means ± S.D. ($n = 50$ cells). Insets in A and B show Scatchard analyses of the data. Ab, antibody.
PLGA-based anti-ICAM/PNCs, but not IgG/PNCs, bound to TNF-activated HUVECs (Fig. 7A), reproducing and validating data obtained with prototype polystyrene carriers (Fig. 1B). Furthermore, PLGA-based anti-ICAM/PNCs, but not the nontargeted counterpart, accumulated in the lungs after intravenous injection in mice, with amplitude, selectivity, and specificity parameters similar to those of polystyrene anti-ICAM/PNCs (Fig. 7, B–D).

**Discussion**

The aim of vascular immunotargeting is to improve the safety, effectiveness, and specificity of diagnosis and treatment of a plethora of cardiovascular, pulmonary, genetic, oncological, and other disease conditions (McIntosh et al., 2002; Song et al., 2002; Oh et al., 2004; Kelly et al., 2005; Muzykantov, 2005; Muro et al., 2006). Caveolar antigens, eceptopeptides, receptors for hormones, transferrin, and albumin, and markers of pathological endothelia provide targets for drug delivery to, into, and across ECs (Danilov et al., 2001; McIntosh et al., 2002; Song et al., 2002; Muzykantov, 2005). Adhesion molecules represent an attractive class of targets including inducible selectins and vascular cellular adhesion molecule-1, useful for specific recognition of pathological endothelia (Lindner et al., 2001; Everts et al., 2002; Kelly, 2005), and constitutively expressed adhesion molecules ICAM-1 and platelet cellular adhesion molecule-1, useful for both prophylactic and therapeutic endothelial drug delivery (Muro and Muzykantov, 2005).

In particular, vascular immunotargeting of ICAM-1 provides delivery of imaging and therapeutic agents to ECs (Danilov et al., 2001; Murciano et al., 2003; Sakhalkar et al., 2003; Muzykantov, 2005; Muro et al., 2006). Anti-ICAM-targeted ultrasound contrasts bind to activated ECs, facilitating imaging of acute cardiac transplant rejection (Villanueva et al., 1998). ICAM-targeted isotopes detect acute inflammation sites (Weiner et al., 2001). Anti-ICAM immunoliposomes bind to ECs in vitro and in animal studies (Bloemen et al., 1995). Anti-ICAM delivers conjugated therapeutic and reporter enzymes to ECs in vitro and in vivo (Murciano et al., 2003).

ECs bind and internalize multimeric anti-ICAM conjugates and polymer nanocarriers (anti-ICAM/PNCs, 100–500 nm) providing intracellular delivery of antioxidant enzymes for containment of vascular oxidative stress (Muro et al., 2005). Lysosomal trafficking of anti-ICAM/PNCs offers a new effective pathway for delivery of enzyme replacement therapies for lysosomal storage disorders, obviating defunct native delivery pathways (Muro et al., 2006).

Anti-ICAM-blocking leukocyte adhesion showed anti-inflammatory and protective effects (Kavanaugh et al., 1997) in animal models of stroke, cardiac ischemia-reperfusion, lung injury, and organ transplantation and in clinical trials (for a review see Muro and Muzykantov, 2005). Thus, ICAM-1 targeting may provide secondary anti-inflammatory benefits.

Yet, despite these empirical successes, our understanding of factors regulating targeting carriers to ICAM-1 is deficient. Anti-ICAM affinity and surface density on carriers have not been determined in many studies (Table 1), which in most cases utilized micron-scale, not nano-scale, carriers (Weller et al., 2002; Sakhalkar et al., 2003; Eniola et al., 2005). Studies with anti-ICAM/liposomes, a nano-scale drug.
delivery system, confirm endothelial targeting, but provide limited quantitative analyses (Bloemen et al., 1995). However, targeting of liposomes versus PNCs may differ. Lateral diffusion of antibody molecules in liposome membrane can facilitate multivalent binding. Liposomes can also flatten on the cell surface, minimizing the detaching force and anchoring additional binding sites. These advantages are unlikely for PNCs.

However, there are no data on ICAM-targeted PNC binding characteristics. Furthermore, no studies quantitatively analyzed PNC targeting in vitro and in vivo to any endothelial antigen. The goal of this study was to define and test factors that control binding of ICAM-targeted PNCs to ECs using tracing of anti-ICAM/PNCs in static and flow-perfused EC cultures (Figs. 1 and 2) and vascular perfusion ex vivo (Fig. 3) and in vivo (Figs. 4–7).

Because of the size difference with free anti-ICAMs, anti-ICAM/PNCs diffuse slower, which lowers the potential number of cell collisions necessary for binding to ECs. Yet, this feature may be offset by the greater momentum of PNCs, which may allow for more frequent wall collisions in vasculature. It is likely that the detaching force applied toward anti-ICAM/PNCs anchored on the EC is higher versus that of anti-ICAMs. This parameter is likely to depend on the shear stress in the binding system, which varies from a relatively low 1 dyne in capillaries to 100 dynes in large arteries. However, the multivalent character of the anti-ICAM/PNC interaction with ECs facilitates anchoring with a very high affinity (70 pM) and binding capacity ($B_{\text{max}} = 300 \text{ PNCs/cell}$) for 200 nm diameter PNCs possessing $250 \text{ antibodies/PNC}$ ($7000 \text{ antibodies/} \mu{\text{m}}^2$). As expected, the maximum binding sites available to PNC-coupled anti-ICAMs was lower versus that for naked anti-ICAMs ($7.5 \times 10^4$ versus $1.6 \times 10^6$ per cell). Free antibody can potentially interact with every ICAM-1 molecule in the plasma membrane. Yet, only those antibodies properly oriented onto the surface of PNCs facing the EC plasma membrane can engage ICAM-1. PNCs bulk bound to the EC surface may impair (due to its size) the access of additional PNCs to nonengaged ICAM-1 molecules. Access of PNC to ICAM-1 located in certain plasma membrane furrows may also be impaired due to steric hindrance. However, the effective affinity of anti-ICAM/PNCs exceeded that of naked anti-ICAM by $\sim 100$ times, probably due to the strong multivalent binding to ECs.

The time to reach equilibrium and equilibrium binding efficiency (Fig. 2) was identical for stagnant versus flow-adapted ECs under shear stress (9 dynes/cm$^2$, $130 \text{ l/s shear rate}$). Because this shear rate would be expected to provide a significant detachment force, the lack of appreciable negative effect of flow further emphasizes the importance of the high affinity of the multivalent PNCs in targeting. In fact, anti-ICAM/PNCs greatly outperformed pulmonary targeting of monomolecular anti-ICAM in mice. Therefore, reformatting of anti-ICAMs into nanoscale multivalent PNCs enhances targeting. This outcome correlates with a recent report that multivalent particles directed to cell adhesion molecules block leukocyte adhesion more effectively then their monomolecular counterparts (Ali et al., 2004).

Table 1 shows that the value of anti-ICAM/PNC binding to ECs is more than 100 time higher than that of micron-scale anti-ICAM/carriers (Kiani et al., 2002; Weller et al., 2002; Sakhalkar et al., 2003; Eniola et al., 2005). It is likely that
size governs the effectiveness of EC targeting. Yet, there are too many variables between these studies (antibody affinity and surface density, carrier concentration, and shear rate). To fully elucidate the effects of size upon binding, one must compare carriers of variable sizes coated by antibodies with known affinity at known density. This aim is beyond the scope of this article and represents the subject of ongoing work.

Most of our experiments used prototype polystyrene PNCs, which can be replaced by PNCs made of biodegradable, biocompatible materials (Fig. 7). To exclude targeting variability due to different mode of anti-ICAM coating (which can, for example, alter steric freedom or orientation of carrier-coupled anti-ICAM), we used the same antibody-coupling technique as for polystyrene carriers, hydrophobic absorption of anti-ICAM. PNC materials (PLGA and Pluronic F68 surfactant) and synthesis were chosen to obtain PNCs with surface hydrophobicity similar to that of polystyrene PNCs. Other groups have demonstrated that surfactants (in these cases, poly(vinyl alcohol) can be irreversibly adsorbed to PNC during formulation (Scholes et al., 1999). In our study, F68 was shown to account for a very low residual mass (<0.4 wt%) after three washings. Furthermore, particle stability was maintained by a slight negative surface charge, which, along with the hydrophobic surface, permitted antibody coating via surface adsorption. As a result, targeting of PLGA PNCs in vitro and in vivo was comparable with that for polystyrene PNCs, supporting the utility of the targeting parameters of prototype PNCs to PLGA PNCs, more compatible with medical use.

Translation of multivalent ICAM-targeted PNCs into the medical domain will require rigorous testing of the safety of this approach. ICAM-1 blocking by antibodies and their fragments is well tolerated by laboratory animals and has been utilized to attenuate inflammation and infections (Hallahan and Virudachalam, 1997; Hopkins et al., 2004). However, EC response(s) to ICAM-1 clustering induced by multivalent anti-ICAM/PNCs may differ from those to the maternal antibody. ICAM-1 engagement may cause cytosolic Ca$^{2+}$ elevation and cytoskeletal remodeling (Etienne-Manneville et al., 2000). Thus, in theory, cytoskeletal reorganization in response to ICAM-1 engagement may modulate vascular permeability (Stevens et al., 2000). ICAM-1 clustering in ECs may also induce reactive oxygen species production, cytokine generation, and expression of cell adhesion molecules (vascular cellular adhesion molecule-1 and ICAM-1 itself) (Hubbard and Rothlein, 2000). Although these effects may vary among ECs from different vascular beds (Hubbard and Rothlein, 2000; Stevens et al., 2000; Wang et al., 2002), it is believed that these responses to ICAM-1 engagement serve as a physiological, transient signal for EC activation (Hubbard and Rothlein, 2000). Whether ECs respond to ICAM-1 clustering by nanocarriers in a similar manner remains to be determined.

However, anti-ICAM/PNCs did not induce systemic release of pro-inflammatory factors after i.v. injection in mice (Muro et al., 2005) and neither did they affect endothelial endocytosis and intracellular trafficking of fluid phase markers or HUVEC monolayer morphology or viability (Muro et al., 2005). Furthermore, cytoskeletal reorganizations in cultured ECs induced by ICAM-1 clustering by anti-ICAM/PNCs are transient and do not cause changes in vascular permeability in vitro and in vivo (S. Muro and V. Muzykantov, unpublished observations). These data corroborate with a recent report that uptake of PLGA nanocarriers did not induce endothelial damage (Davda and Labhasetwar, 2002) and imply that the side effects of multivalent anti-ICAM/PNCs may be benign, transient, and well tolerated.

In summary, reformatting monomolecular anti-ICAM into multivalently coupled anti-ICAM/PNCs, markedly increasing endothelial affinity, highlights the potential utility of this platform for diagnostic and therapeutic applications, for example, for the delivery of enzyme replacement or antioxidant therapies into endothelial cells (Dziubla et al., 2005; Muro et al., 2006; Dziubla and Muzykantov, 2006).

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References


