Detection of Vascular Adhesion Molecule-1 Expression Using a Novel Multimodal Nanoparticle

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Abstract—Endothelial vascular adhesion molecule-1 (VCAM-1) is a critical component of the leukocyte–endothelial adhesion cascade, and its strict temporal and spatial regulation make it an ideal target for imaging and therapy. The goal of this study was to develop novel VCAM-1–targeted imaging agents detectable by MRI and fluorescence imaging using phage display–derived peptide sequences and multimodal nanoparticles (NPs). We hypothesized that VCAM-1–mediated cell internalization of phage display–selected peptides could be harnessed as an amplification strategy to chaperone and trap imaging agents inside VCAM-1–expressing cells, thus improving target-to-background ratios. To accomplish our goal, iterative phage display was performed on murine endothelium under physiological flow conditions to identify a family of VCAM-1–mediated cell-internalizing peptides. One specific sequence, containing the VHSPNKK motif that has homology to the α-chain of very late antigen (a known ligand for VCAM-1), was shown to bind VCAM-1 and block leukocyte–endothelial interactions. Compared with VCAM-1 monoclonal antibody, the peptide showed 12-fold higher target-to-background ratios. A VHSPNKK-modified magnetofluorescent NP (VNP) showed high affinity for endothelial cells expressing VCAM-1 but surprisingly low affinity for macrophages. In contrast, a control NP without VCAM-1–targeting sequences showed no affinity for endothelial cells. In vivo, VNP successfully identified VCAM-1–expressing endothelial cells in a murine tumor necrosis factor-α–induced inflammatory model and colocalized with VCAM-1–expressing cells in atherosclerotic lesions present in cholesterol-fed apolipoprotein E apoE−/− mice. These results indicate that: (1) small peptide sequences can significantly alter targeting of NPs, (2) the used amplification strategy of internalization results in high target-to-background ratios, and (3) this technology is useful for in vivo imaging of endothelial markers. (Circ Res. 2005;96:327-336.)

Key Words: cardiovascular diseases ■ cell adhesion molecules ■ imaging ■ inflammation ■ vasculature

Cardiovascular diseases are the leading cause of death in the developed world and represent an immense clinical burden. Atherosclerosis is the major contributor to the pathogenesis of acute myocardial and cerebral thrombosis, with an estimated 500 000 deaths annually in the United States alone. Although significant advances have been made in the primary prevention of cardiovascular disease, risk stratification remains suboptimal in large segments of the population. It is now widely accepted that the degree of coronary artery stenosis is not predictive of the risk of plaque rupture and subsequent thrombosis at that location. Clinical risk scores, such as that derived from the Framingham Heart Study, are useful but may lose predictive value in the large segments of the population at intermediate risk. Despite the addition of new measurements to stratify clinical risk in individuals at intermediate risk, such as serum C-reactive protein or the detection of coronary calcification by computed tomography, there remains a need for novel molecular imaging probes to better define cardiovascular risk and guide therapy more rationally.

Atherosclerosis is a chronic, progressive inflammatory disease characterized by a specific series of cellular and molecular events, from early inflammatory lesions (“fatty streaks”), comprised of monocyte-derived macrophages and T lymphocytes, to development of mature plaque and potentially rupture and thrombosis. The earliest molecular changes occur on endothelial surfaces of the aorta and vasa vasorum and are key contributors to the initiation, progression, and thrombotic complications of atherosclerosis. Although lipid-lowering therapies have demonstrated direct clinical benefit, there remains a dramatic need for novel diagnostic tools to noninvasively assess the benefit of therapies and identify early molecular changes associated with atherosclerosis, and for new targeted and specific therapies.

The strict temporal and spatial regulation of inducible endothelial adhesion molecules and their critical function in
atherosclerosis makes them ideal targets for the next generation of diagnostics and therapeutics. Vascular adhesion molecule-1 (VCAM-1) is upregulated on the endothelium under inflammatory conditions including atherosclerosis and cardiac allograft rejection\(^a\) and appears on the endothelial cell surface of atheroprone areas before the onset of visible disease.\(^b\) Its importance in the initiation of atherogenesis is demonstrated by delayed lesion development in mice carrying a mutation that hinders VCAM-1 function.\(^c\) Comparable to mouse atherosclerosis, VCAM-1 expression is induced early in human atheroma and is an important element in the inflammatory component of atherosclerosis, contributing to monocyte and lymphocyte recruitment from adventitial vessels and the arterial lumen.\(^d\)\(^e\)\(^f\)\(^g\) Although several approaches have been described to image VCAM-1 expression using radiolabeled antibodies (Abs),\(^12\)\(^13\) these agents usually result in modest target-to-background ratios, limiting their use for in vivo cardiovascular imaging.

We determined that additional amplification steps would be required to image VCAM-1 expression directly in the endothelium. Thus, we developed a modified phage display approach to screen for specific peptide sequences that would be internalized by VCAM-1-expressing endothelial cells under physiological flow conditions. One specific sequence (VHSNKPK), with homology to the α-chain of very late antigen (VLA\(_\alpha\); a known ligand for VCAM-1), was shown to bind VCAM-1 and to block leukocyte–endothelial interactions. A derived multivalent nanoparticle (NP) carrying the VHSNKPK peptide successfully identified VCAM-1-expressing endothelial cells in a murine tumor necrosis factor-α (TNF-α)-induced inflammatory model via confocal microscopy, and furthermore, it allowed in vivo detection of atherosclerotic lesions in apolipoprotein E\(^-\) mice via MRI.

**Materials and Methods**

**Antibodies**

- Anti-mouse CD31 (clone MEC 13.3), anti-CD16/CD32 (FcγR block), anti-mouse CD106 (VCAM-1, clone 429), and anti-mouse CD106-fluorescein isothiocyanate (FITC) VCAM-1, clone 429 were from BD Pharmingen (San Diego, Calif), and anti-mouse VCAM-1 (clone M/K 2.7) was from American Type Culture Collection (Manassas, Va). Biotinylated anti-rat and anti-rabbit IgG (heavy and light chain [H+L]) Texas Red or Cy3 anti-rat IgG (H+L) were from Jackson ImmunoResearch (West Grove, Pa), and anti-FITC/Oregon Green–horseradish peroxidase (HRP) was from Molecular Probes (Eugene, Ore). Biotin–anti-M13 rabbit IgG was from AbCam, and goat F(ab\(^\prime\))\(_2\) anti-rat IgG (H+L)-phycoerythrin and Texas Red were from Caltag Laboratories (Burlingame, Calif).

**Mice**

C57BL/6 wild-type mice (7 to 9 weeks of age) from Taconic and apoE\(^-\) (The Jackson Laboratory) were maintained in approved pathogen-free institutional housing facilities. All experiments were performed according to institutional guidelines. Animals were anesthetized for imaging procedures using isoflurane inhalation (1% to 2% isoﬂurane; 1 L O\(_2\)). Animals were euthanized by CO\(_2\) asphyxiation as approved by the panel on euthanasia at the American Veterinary Association.

**Endothelial Cell Culture**

Murine cardiac endothelial cells (MCECs) or murine lung endothelial cells (MLECs) were isolated using previously published methods\(^14\) and used at passages 1 to 3.

**Phage-Display Library Selection**

The parallel plate-flow chamber has been described in detail.\(^5\) Smith and Petrenko produced a detailed rationale of phage display selection and negative selection or depletion procedures.\(^6\) Phage selection and negative depletion were performed using strain-matched MCECs and MLECs.\(^7\) PFU of phage, displaying random disulfide-constrained 7-aa peptides (C7C-PhD; New England Biolabs), were drawn across MCEC monolayers at 37°C for 10 minutes at 0.52 mL/min (estimated wall shear stress 1.0 dyne/cm\(^2\)). MCECs were then incubated at 37°C for an additional 15 minutes to allow time for VCAM-1–mediated internalization.\(^8\) Extracellular-restricted phage were removed with 0.2 mol/L glycine, pH 2.2 (3×8 minutes). Internalized phage were recovered by lysis with 0.1% triethanolamine (Sigma) in PBS, pH 7.4, (4 minutes; room temperature). Extracts were neutralized with 500 μL of 0.5 mol/L Tris-HCl, pH 9.0. To deplete phage that bound to identical markers present on MCECs and MLECs, the phage pool isolated after one round of selection was subtracted by three rounds of successive incubation at 37°C for 30 minutes with confluent monolayers of strain-matched MLECs that do not express VCAM-1. The phage that were internalized by MCECs but not MLECs were amplified by *Escherichia coli*, titered, subjected to three additional rounds of positive selection, and individual clones selected for ELISA assay and sequencing.

**ELISA Assay of VCAM-1 Binding**

Protein A (20 μg/mL) on Nunc Maxisorp plates (Fisher; 4°C overnight) was incubated sequentially at room temperature with Fc–VCAM-1 (10 μg/mL; 1 hour) and phase clones (10\(^9\) PFU; 1 hour), washed with PBS containing 0.1% Tween-20, incubated with biotinylated anti-M13 Ab (1:40; 1 hour), detected with streptavidin–HRP (1:500), and developed with tetra methyl benzidine, and absorbance was determined (Emax; Molecular Devices).

**Synthesis of FITC Peptides and Fluorescent Magnetic NP Conjugates**

The identified peptide sequence “CVHSPNKKC” and corresponding magnetofluorescent NPs were synthesized as described in the online data supplement (available at http://circres.ahajournals.org). The following compounds were synthesized in bulk: VCAM-1 peptide (VP); CVHSPNKKC/CGGSF/FITC/GK; control peptide (CP); CPKNVSKHCGGSK(FITC)GK; VCAM-1 NP (VNP); CVHSPNKKC/CGGSK(FITC)GK(CLIO-Cy5.5); control NP (CPNP): CPKNVSKHCGGSK(FITC)GK(CLIO-Cy5.5); and CLIO-Cy5.5 NP: CLIO-Cy5.5 backbone (no peptide).

**Fluorescence Microscopy and Flow Cytometry**

MCECs were incubated with 1 μmol/L of either VP or CP (1 hour; 37°C), washed 3× with PBS, and visualized by fluorescence microscopy (Nikon Eclipse TE2000-S; Insight QE; ×40 objective). For confocal microscopy, MCECs were incubated with 1 μmol/L of VP as above, fixed with 2% paraformaldehyde, stained with anti–VCAM-1 (10 μg/mL; 1 hour), and detected with Texas Red–conjugated secondary Ab (1:100; 30 minutes). Stained cells were analyzed on an LSM 5 PASCAL confocal microscope (Zeiss). For competition experiments, peptides were preincubated (30 minutes) with 5× murine Fc–VCAM-1 or Fc–ICAM-1 (R & D Systems). MCECs were then detached, stained for cell surface VCAM-1, and analyzed via flow cytometry (10 000 cells/sample) on a Becton Dickinson FACSCalibur. For comparison of VP with anti–VCAM-1–FITC, cells were incubated with equimolar concentrations (0.5 μmol/L) of VP or anti–VCAM-1–FITC for 2 hours at 37°C and analyzed by flow cytometry.
In Vitro Blocking Studies Under Flow Conditions
MCECs were activated for 5 hours with murine TNF-α (mTNF-α; R & D Systems) and incubated for 2 hours with either 25 μg/mL M/K 2.7 anti–VCAM-1 Ab, 100 μmol/L VP, or vehicle control. Strain-matched mononuclear cells (1 × 10^6 cells/mL in DPBS−0.1% BSA) were drawn across MCECs for 5 minutes at 0.52 mL/min. Leukocyte–endothelial interactions were determined from 6 to 8 high-power fields at 5 minutes.

Targeting of VNP to Endothelial Cells
The isolation of primary MCECs was performed as described previously. Primary mouse macrophages were isolated as presented in the online data supplement. Cells were incubated with either VNP or NP (4.5 μmol/L Cy5.5; 4 hours; 37°C) and analyzed by flow cytometry as described previously.

Immunohistochemistry and Intravital Confocal Microscopy
C57BL/6 mice (n=10) were injected subcutaneously in the right ear with 5 ng/50 μL mTNF-α in normal saline. After 24 hours, animals were anesthetized via inhaled isofluorane and injected intravenously (tail vein) with 10 nmol/L fluorochrome of VP (n=2), VNP (n=5), or CNP (n=3). Perfused vessels were located using bright-field illumination and fluorescence signal because of circulating peptide/conjugate. Intravital confocal microscopy was performed using a Nikon EF600N equipped with a BioRad Radiance 2100 confocal and X40 water immersion objective at 0 hours, 4 hours, and 24 hours after agent administration. Image acquisition was performed simultaneously in two fluorescence channels (photo multiplier tube [PMT1]: peptide-FITC; PMT3: peptide-NP) in several fields from both ears of each animal using built-in compensation routines to exclude channel bleed-through (Laser Sharp software; BioRad), and 3D reconstructions were performed using Amira (TGS). Subsequently, ears were removed for histological analysis. Adjacent serial frozen sections were stained for the presence of CD31, VCAM-1, or injected agent. Digital images were taken using a Nikon Eclipse E400 upright microscope (X40 objective) equipped with an Insight color camera.

MRI of VCAM-1 Expression
We performed in vivo MRI of 1-year-old apoE−/− mice with extensive lesion burden that had been cholesterol-fed for 3 months (n=3). T1- (repetition time [TR] 50; echo time [TE] 2; 20 number of excitations [NEX] 10 minutes) and T2-weighted (TR 2000; TE 20 to 200; NEX 6 minutes) spin echo images and gradient echo sequences were acquired before and 24 hours after intravenous administration of VNP or NP to apoE−/− mice or VNP to C57BL/6 mice (5 mg Fe/kg body weight). In addition, we obtained one bright-blood angiographic sequence (TR 50; TE 2; NEX 10 minutes) at the end of the imaging session using gadolinium-protected graph copolymer (Gd-PGC)19 (0.02 mmol/L Gd/kg body weight) to better outline the vascular lumen. The blood pressure and heart rate of animals before injection and after injection were monitored. Subsequently, aortas were removed for histological analysis. Serial frozen sections were stained for the presence of VCAM-1 (green), nuclei (4′,6-diamidino-2-phenylindole [DAPI]; blue), or injected agent (red).

Results
Selection of Phage Display Library Clones
In cell culture, MCECs exhibit high constitutive expression of VCAM-1 compared with strain-matched MLECs,19 making this an ideal target for positive selection of VCAM-1–targeted phage. Phage selection (4 rounds) resulted in a 330-fold increase in the ratio of cells internalized to extracellular bound phages (Figure 1A). Sixty clones were sequenced and screened for VCAM-1 binding. Phage clone 9 exhibited highest binding to VCAM-1 (Figure 1B). The peptide sequences of VCAM-1–binding clones were used to search the online National Center for Biotechnology Information database using the basic local alignment search tool (BLAST) algorithm (Table). Interestingly, clones 9 and 20 share sequence homology to the α-chain of VLA4, the VCAM-1 ligand expressed on circulating hematopoietic cells.20 We synthesized the corresponding peptide (termed VP) for clone 9, “CVHSPNKKC,” incorporating a C-terminal GGSKGK peptide extension for fluorescein attachment and NP conjugation.

Specificity of VCAM-1–Targeting VP
Fluorescent VP was rapidly internalized by target MCECs when incubated at 37°C (but not at 4°C), showing punctate intracellular staining (Figure 2A). Confocal microscopy of MCECs incubated with VP (green) and stained for the presence of VCAM-1 (red) demonstrated intracellular accumulation of VP (Figure 2C). Unlike VP, the scrambled CP did not bind to MCECs, confirming the specificity of the peptide (Figure 2B). When cells were preincubated with Fc–VCAM-1, no binding of either VP (Figure 2D) or CP (Figure 2E) could be observed. In subsequent quantitative flow cytometry analysis, >95% of MCECs were labeled with VP and cellular uptake correlated with VCAM-1 expression.
In functional analysis, preincubation of TNF-α–activated MCECs with either function-blocking monoclonal Ab (mAb) M/K 2.7 or VP significantly inhibited mononuclear cell recruitment under flow conditions. VP reduced mononuclear cell accumulation by 68%, compared with 52%, via mAb blocking, suggesting that the VP is as effective at preventing VCAM-1–mediated leukocyte–endothelial interactions (Figure 2H). These data suggest that VP is effective at blocking leukocyte–endothelial interactions and the mechanism may be attributable to binding of the peptide to the VLA-4 adhesion domain of VCAM-1 or may be the result of cell surface depletion of VCAM-1 by peptide binding.

Targeting of Magnetofluorescent NPs to VCAM-1

Whereas Abs have been used to image VCAM-1 expression, the modest target-to-background ratios obtained using these agents often limit their use in vivo for cardiovascular imaging. To test the comparative amplification potentially afforded by cell-internalizing affinity ligands versus cell surface labeling with specific mAb, we directly compared FITC-labeled αVCAM-1 and VP by FACS analysis using equimolar concentrations. VP demonstrated a >10-fold higher uptake compared with the Ab (Figure 2I).

To permit evaluation of VCAM-1 expression via fluorescence or MRI and to develop a VCAM-1–targeted imaging agent, we coupled VP multivalently to a superparamagnetic fluorescent NP termed VNP. In vitro flow cytometric analysis demonstrated that VNP retained the ability of the peptide to bind specifically to MCECs (Figure 3A) and in fact showed an ~10-fold higher uptake by target cells compared with uptake of the negative control particle (CNP).

Because an NP-based in vivo imaging agent will contact multiple cell types, we also sought to determine the specificity of VNP for endothelial cells as opposed to macrophages, which have been shown to accumulate iron oxide NPs. In vitro flow cytometric analysis demonstrated that VNP preferentially accumulated in endothelial cells (Figure 3B). Furthermore, VNP had an 11-fold higher accumulation in endothelial cells compared with its accumulation in macrophages, consistent with the known pattern of VCAM-1 expression (Figure 3B). In contrast, endothelial cells lacked NP accumulation, suggesting VHS peptide labeling of NP changes the cellular specificity of the NP.

Intravital Confocal Microscopy of VCAM-1–Expressing Microvasculature

To create a biologically monovariant in vivo mouse model, we next induced acute inflammation (24 hours) in mouse ears by injecting TNF-α to upregulate VCAM-1.9 When VP was injected intravenously into this mouse model of inflammation, it exhibited very fast kinetics (as determined via intravital confocal microscopy), with a vascular T1/2 of 110 seconds, rapid extravasation into the interstitium, and near-complete renal clearance within 10 minutes. In contrast, injection of VNP, CNP, or NP resulted in no apparent extravasation of the agents within 20 minutes after injection, as observed via intravital confocal microscopy. VNP, CNP, and NP were cleared from the circulation within 4 hours after injection, with only VNP remaining bound to the vasculature. At 4 hours after VNP injection, near-infrared fluorescence was highly elevated in the vasculature of the inflamed ear, whereas no significant background fluorescence was observed (Figure 4A). In the control ear of the same animal, there was little detectable signal (Figure 4B). In control animals injected with CNP, an initial fluorescence signal throughout the circulation immediately after administration was observed, but no significant fluorescence in either ear after 4 hours was determined. Control animals that received NP alone had cleared the agent from the circulation by 4 hours after injection (data not shown). At 24 hours, significant amounts of VNP could still be visualized in the vessel walls of mTNF-α-treated ears (Figure 4C and 4E), whereas it was absent in the untreated ears (Figure 4D). In further time-lapse analysis (Figure 4F), the fluorescence profile of VNP did not change during a 5-minute observation, confirming that the probe was indeed retained at specific locations within the vessel wall and not free flowing in the circulation.

Histological analysis of mTNF-α–treated ears (Figure 5A through 5F) revealed the upregulation of VCAM-1 in endothelial cells (Figure 5B and 5E). In contrast, endothelial cells from untreated ears did not show appreciable VCAM-1 staining (Figure 5H). Anti-FITC Ab staining revealed the presence of the VNP agent in endothelial cells from inflamed ears (Figure 5C) but not in control ears (Figure 5I). Endothe-
lial cells from CNP-injected animals were also devoid of anti-FITC staining (Figure 5F), confirming the specificity of VNP. Together, these data confirm the in vivo delivery and accumulation of VNP, but not CNP or NP, in the endothelium of acutely inflamed tissue.

MRI of Atherosclerotic Lesions in ApoE<sup>−/−</sup> Mice
To determine whether VCAM-1 expression could be detected in vivo in atherosclerotic lesions, we injected VNP into cholesterol-fed apoE<sup>−/−</sup> mice. In this model, there is extensive neovascularization and VCAM-1 expression in vast areas
throughout the lesion (Figure 6G). Injection of agents did not result in an increase of blood pressure or heart rate. After intravenous administration of VNP, we detected extensive signal intensity decreases associated with iron oxide NP accumulation in atherosclerotic lesions (Figure 6A and 6C), in particular in the aortic arch and around the take-off of large vessels. Using a spin echo sequence with a TE of 20 milliseconds, the signal-to-noise ratio of plaques decreased from 20.1 ± 1.1 to 9.1 ± 0.9 (aorta/muscle ratio before VNP administration 0.82 versus post-VNP administration 0.35). In areas where lesion development caused crescent-shaped aortic wall thickening, postadministration images showed darkening of the affected aortic wall (Figure 6B and 6C). These in vivo changes in magnetic resonance (MR) signal correlated with ex vivo MRI and macroscopic fluorescence imaging of the excised aortas (Figure 6D and 6E). NP administered to apoE−/− animals at equimolar amounts failed to accumulate in the aorta (Figure 7). Further, injection of VNP into C57BL/6 animals (wild type) also did not accumulate in a significant amount in the aorta (Figure 7). To confirm the presence of atherosclerotic lesions and to corroborate VNP colocalization with VCAM-1 expression, we performed histological evaluation (Figure 6F) and multicolor immunohistochemistry. Fluorescence microscopy confirmed extensive colocalization of

Figure 3. MCEC uptake of VNP A, MCECs were incubated with 1 μmol/L of VNP (filled graph), CNP (gray graph), or vehicle control (open graph) for 1 hour at 37°C, washed 3 × and analyzed by flow cytometry. VNP was >95% positive compared with CNP or control. B, MCECs or primary mouse macrophages were incubated with either VNP or NP for 4 hours at 37°C then analyzed by flow cytometry. Data presented are the mean percentage of positive cells ± SD and are the result of two experiments performed in triplicate (n = 6).

Figure 4. Intravital confocal microscopy. Animals with mTNF-α–induced inflammation in the right ear were injected with VNP (red) and then imaged via intravital confocal microscopy (green; tissue autofluorescence). Confocal projections of Z stacks from an mTNF-α–treated ear at 4 hours after VNP injection (A) and 24 hours after VNP injection (C); and from the contralateral control ear at 4 hours (B) and 24 hours (D). Images are representative of four different animals in each case and three different fields per ear. E, 3D reconstruction image of the Z series from C, showing the vascular network in the mTNF-α–treated ear stained with VNP. F, Time series of VNP staining within the vessels of a mTNF-α–treated ear. Each time point is given as a separate slice in the Z direction. The degree of VNP staining (low to high) is indicated by the color scale (blue to green). Note that there is no difference in staining between different time points.
VNP (Cy.5.5 channel; Figure 6H) and VCAM-1 expression (anti–VCAM-1–FITC channel (Figure 6G). In contrast, there was little accumulation of VNP in macrophages, reminiscent of the in vitro data shown in Figure 3B.

**Discussion**

Pathophysiologically inducible endothelial targets provide an attractive paradigm for the development of targeted and disease-specific molecular imaging agents, attributable largely to their immediate accessibility via the circulation. The strict temporal and spatial regulation of VCAM-1 in the development and progression of atherosclerosis\(^5\)–\(^9\) makes it a prime candidate for development of the next generation of targeted diagnostics and therapeutics.

Several approaches have been described previously using radiolabeled Abs to detect VCAM-1 expression;\(^{12,13}\) however, this approach has resulted in very modest target-to-background ratios, limiting its use for in vivo cardiovascular imaging. To overcome these limitations, we commenced development of an agent that not only decorated the endothelial surface but was internalized by endothelial cells ("biological amplification through intracellular trapping").\(^{21}\) We used phage display to identify peptide sequences that bound under physiological flow conditions and were capable of VCAM-1–mediated endothelial cell internalization. Using this approach, we identified a novel VCAM-1–specific cell internalizing peptide that results in higher target-to-background ratios than monoclonal Abs and allows in vivo imaging of inflammation and cardiovascular disease.

Our unique approach resulted in the identification of a small panel of peptides forming 42 consensus groups, with a subset that bound specifically to the extracellular domain of VCAM-1 (Table). One of these peptides, VHSPNKK, with the highest binding to VCAM-1, was homologous to VLA4, the ligand for VCAM-1. Direct intravenous injection of the fluorescently labeled selected peptide (VP), resulted in rapid leakage into the interstitium, and the resulting high background fluorescence in the surrounding tissue eliminated the possibility of using the ligand for intravital work, although it remains a highly specific and valuable agent for in vitro assays. The derived multivalent (chemical amplification) magnetofluorescent imaging agent (VNP) exhibited improved in vivo pharmacokinetics, retained specificity for VCAM-1, and in vivo accumulated in the vessel wall, remaining detectable for at least 24 hours by noninvasive optical and MRI modalities, confirming the ability of the

**Figure 5.** Immunohistochemical analysis of in vivo delivery of VNP to endothelial cells. Adjacent serial sections of ears taken from the in vivo intravital experiments were stained for CD31 (A, D, and G), VCAM-1 (B, E, and H), or FITC (C, F, and I). Results are representative of six sections in each case, taken from at least three animals.
peptide to shuttle large molecules across the plasma membrane. A number of previous publications have identified peptide sequences that bind to atherosclerotic plaque or are potentially relevant to targeting cell surface receptors. In particular, Liu et al. used phage display to interrogate the surface of atherosclerotic lesions in apoE mice and identified consensus sequences that bound to endothelial cell surface glucose-regulated protein 78 and a homologue of tissue inhibitor of metalloproteinase-2 (TIMP-2) that bound to mature lesions in vivo. In the present study, we identified three peptide sequences with similarity to TIMP-2; however, no other similar sequences were isolated. The difference in these results could be explained by the fact that Liu et al. examined the binding of phage in vivo in apoE mice with advanced lesion development, where proteins other than VCAM-1 may be more abundant on the endothelial cell surface.

The data presented here demonstrate for the first time that endothelial targets can be visualized by in vivo MRI without the use of monoclonal Abs. Our approach has far-reaching implications in the design of future imaging agents by allowing the visualization of nonabundant targets through intracellular accumulation. The first generation of nontargeted magnetic NPs are in clinical use, and thus, it is conceivable that targeted NPs could provide significant benefits in specificity for imaging and therapeutics. Our in vitro and in vivo results suggest that conjugation of the magnetofluorescent NP with phage display–derived VCAM-1–specific peptides changes the cellular specificity of the parent NP and allows the targeted imaging of activated endothelium. Indeed, we demonstrate that VCAM-1 expression can be visualized by high-resolution intravital confocal microscopy and MRI. This could have a number of important clinical implications. For example, VNP could be used to noninvasively monitor the efficacy of anti-inflammatory or lipid-lowering drugs by examining the level of VCAM-1 in vessels. Furthermore, the agent or similarly developed probes could be used for combined or simultaneous imaging and

Figure 6. MRI of atherosclerotic lesions with VNP. A, Cholesterol-fed apoE mice were imaged via MRI using Gd-PGC to delineate vascular lumen and structural aortic abnormalities such as narrowing (arrows). Axial images (at the level of the green line) were then obtained before (B) and 24 hours after (C) administration of the magnetic VNP. Note the decrease in signal intensity of the eccentrically thickened aortic wall between the two arrows. D, Ex vivo MRI confirms extensive low signal changes in the aortic wall induced by VNP accumulation (arrows), further corroborated by macroscopic epifluorescence imaging of Cy 5.5 in VNP (E). F through H, Histological validation of MR findings. F, Hematoxylin/eosin section of the aorta image in B and C confirms eccentric wall thickening (×2). G and H, Comparative immunofluorescence of VCAM-1 expression (G; green) and VNP accumulation (H; red). Nuclei in G and H are counterstained with DAPI (blue). Note that there is extensive colocalization of VCAM-1 expression and VNP. Bars = 10 μmol/L.
targeted drug delivery. An intriguing application would be to use the VP domain to shuttle larger therapeutic proteins to sites of inflammation in a targeted fashion. For example, targeting of doxorubicin to angiogenic cells through peptide conjugation increases the efficacy-to-toxicity ratio by 14-fold.27 Ultimately, the developed molecular framework could be applied to other targets, making it a useful scaffold for the development of novel targeted therapeutics or diagnostic tools.

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References

Figure 7. MRI and fluorescence imaging of aortas. A, Ex vivo MRI confirms extensive low signal changes in the aortic wall induced by VNP accumulation in plaques of apoE−/− mice (arrows). B, Further corroborated by macroscopic epifluorescence imaging of Cy 5.5 in VNP. C, NP administered to apoE−/− mice failed to highlight the aortic arch via ex vivo MRI or macroscopic epifluorescence imaging of Cy5.5 (D). E, Similarly, VNP failed to highlight the aortic arch in C57BL/6 mice via ex vivo MRI or macroscopic epifluorescence imaging (F).
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Synthesis of FITC peptides and fluorescent magnetic nanoparticle conjugates

The identified peptide sequence ‘CVHSPNKKC’ was synthesized on an ACT APEX 396 peptide synthesizer using standard FMOC chemistry and extended at the C-terminus by the sequence ‘GGSKGK’ for attachment to CLIO-Cy5.5, a cross-linked iron oxide nanoparticle. This nanoparticle is similar in physicochemical and biological properties to monocrystalline iron oxide nanoparticles (MION). Lysines in the binding sequence were orthogonally protected. Peptides were cleaved with reagent L, purified by HPLC, cyclized via S-S linkage, lyophilized, and verified by mass spectral analysis (MALDI). CLIO-Cy5.5 was synthesized in bulk using established procedures and aliquots used for the synthesis of the various nanoparticle conjugates. CLIO-Cy5.5 had the following physical properties: a) size 38.7 nm, b) relaxation time constants R1=21.1 and R2=62.6 and c) an average of 2.3 Cy5.5 per CLIO particle based on 2600 Fe/CLIO. Suberic acid bis (N-hydroxy-succinimide ester) derivatized CLIO-Cy5.5 were reacted with 100 uL of 1mM FITC-labeled VCAM-1 peptide (VNP) or control peptide (CNP). VNP was purified on a Sephadex G-25 M gel chromatography column equilibrated with PBS, and concentrated on a Microcon YM-50 ultracentrifugation filter device (Millipore). A typical experiment would result in the attachment of 6 peptides per NP. To ensure no cross-linking of NPs occurred during the bioconjugation procedure, light scattering was performed following DSS labeling and peptide conjugation.

Isolation of Primary Mouse Macrophages
Primary mouse macrophages were isolated as follows: peripheral blood was obtained by cardiac puncture, (15 mice) mixed with sodium citrate (100 mM), dextrose (pH 6.5, 136 mM), and 100 mL of H2O and centrifuged for 4 minutes at 1000 xg. Pellet was resuspended in HBSS, layered onto Ficoll (Histopaque-1083, Sigma, St. Louis, MO) then centrifuged at 1600 RPM for 30 minutes at room temperature. After centrifugation, two distinct bands were present. The top band was removed and plated onto gelatin coated wells in RPMI containing 10% FBS, 40 nM phorbol myristate acetate (Sigma), and 1% pen/strep. Non-adherent cells were removed 24 hours after plating.