Integrin-Targeted Imaging of Inflammation in Vascular Remodeling

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Objective—Inflammation plays a key role in the development of vascular diseases. Monocytes and macrophages express \( \alpha_\beta_3 \) integrin. We used an \( \alpha_v \) integrin-specific tracer, \( \text{\(^{99m}\text{Tc}\)-NC100692} \), to investigate integrin-targeted imaging for detection of vessel wall inflammation.

Methods and Results—The binding of a fluorescent homologue of NC100692 to \( \alpha_\beta_3 \) on human monocytes and macrophages was shown by flow cytometry. Vessel wall inflammation and remodeling was induced in murine carotid arteries through adventitial exposure to \( \text{CaCl}_2 \). NC100692 microSPECT/CT imaging was performed after 2 and 4 weeks and showed significantly higher uptake of the tracer in \( \text{CaCl}_2 \)-exposed left carotids compared with sham-operated contralateral arteries. Histological analysis at 4 weeks demonstrated significant remodeling of left carotid arteries and considerable macrophage infiltration, which was confirmed by real-time polymerase chain reaction. There was no significant difference in normalized \( \alpha_v \), \( \beta_3 \), or \( \beta_5 \) mRNA expression between right and left carotid arteries. Finally, NC100692 uptake strongly correlated with macrophage marker expression in carotid arteries.

Conclusion—NC100692 imaging can detect vessel wall inflammation in vivo. If further validated, \( \alpha_v \)-targeted imaging may provide a noninvasive approach for identifying patients who are at high risk for vascular events and tracking the effect of antiinflammatory treatments. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: imaging agents ■ radionuclide imaging ■ vascular biology ■ integrins ■ inflammation

Inflammation is a common feature of many vascular diseases and plays a central role in their pathogenesis. Typical examples include atherosclerosis and aneurysm formation, where an inflammatory process is critical to the development of the disease and its complications. It is therefore not surprising that many therapeutic interventions aim at modulating vessel wall inflammation. One of the limitations of the modern approach to managing vascular diseases is the lack of reliable approaches to detecting and tracking the effect of interventions on vessel wall biology. This may be addressed by targeting molecular signatures of relevant processes by molecular imaging.

Endothelial activation, leukocyte recruitment and activation, and matrix remodeling are integral parts of inflammation, which is closely intertwined with vessel wall angiogenesis. \( \alpha_\beta_3 \) integrin-targeted imaging has been introduced for detection of angiogenesis associated with myocardial infarction,1 peripheral arterial disease,2 atherosclerosis,3 and neoplasm.4 In studies of \( \alpha_\beta_3 \)-targeted imaging of ischemia-induced angiogenesis, part of the tracer localized in what appeared to be the inflammatory infiltrate associated with angiogenesis.2 This led us to investigate whether \( \alpha_v \)-targeted imaging may be used for detection of vessel wall inflammation in vivo. Here, we demonstrate that peripheral blood monocytes and monocyte-derived macrophages express \( \alpha_\beta_3 \) integrin and bind to NC100692, a cyclic RGD peptide with specificity for activated \( \alpha_v \) integrins,2,5,6 to levels comparable to that of endothelial cells (ECs). In a mouse model of vessel wall inflammation, NC100692 uptake was clearly detectable by microSPECT/CT imaging in chemically injured carotid arteries, and the uptake correlated well with the presence of macrophages.

Materials and Methods

Materials
Materials were obtained from Sigma-Aldrich (St. Louis, MO), unless indicated otherwise. NC100692 precursor and its fluorescent-labeled homologue were provided by GE Healthcare. NC100692 radiolabeling with \( \text{\(^{99m}\text{Tc}\)} \) was performed using kits provided by GE Healthcare according to the manufacturer’s instructions.6 Each kit contained \( \approx 44 \) nmol of NC100692 (molecular weight 1697) and was labeled with 1.1 GBq sodium pertechnetate (\( \text{\(^{99m}\text{Tc}\)} \)).

Cell Culture
Human umbilical vein ECs were isolated and cultured as described.7 Peripheral blood mononuclear cells were isolated under protocols approved by the Yale Human Investigation Committee from normal anonymous donors’ leukapheresis product by gradient density centrifugation following standard procedures. Monocytes were isolated
to high purity from peripheral blood mononuclear cells by magnetic cell sorting using anti-CD14-coated beads according to the manufacturer’s instructions (StemCell Technologies, Vancouver, British Columbia, Canada). Monocyte purity was verified by flow cytometry and was found to be >85%. Purified monocytes were cultured for 10 days in RPMI plus 10% fetal bovine serum (Lonza, Walkersville, MD), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in the presence of recombinant granulocyte macrophage–colony-stimulating factor (50 ng/mL, PeproTech, Rocky Hill, NJ) to generate type 1 macrophages.8

Flow Cytometry
Expression of surface proteins was analyzed by staining live cells with conjugated anti-CD14 (BD Pharmingen), αβ1 integrin (LM609, Millipore Corp, Temecula, CA) antibody, the corresponding isotype control antibodies, or a fluorescent RGD peptide homologue of NC100692 (GE Healthcare). To investigate the effect of integrin activation, cells were exposed to MnCl2 (0.2 mmol/L) in calcium- and magnesium-free phosphate-buffered saline for 10 minutes before staining. MnCl2 was kept in all buffers during staining and flow cytometry. At least 2500 cells that satisfied a gate on forward and side scatter were acquired using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Data analysis was performed using CellQuest software (San Jose, CA).

Animal Model
Seventeen animals underwent surgery to induced carotid artery inflammation as described9 (Supplemental Figure I, available online at http://atvb.ahajournals.org). Briefly, in 8- to 10-week-old female apolipoprotein E (apoE)7/7 mice (Jackson Laboratory, Bar Harbor, ME) fed a high-cholesterol chow (1.25% cholesterol, Harlan Teklad, Madison, WI) for 1 week, the carotid arteries were surgically exposed under anesthesia (100 mg/kg ketamine and 10 mg/kg xylazine, IP). The left common carotid artery just below carotid bifurcation was adventitiously painted with a 10% solution of CaCl2 for 20 minutes. The opposite carotid artery was exposed to normal saline and served as control for imaging studies. Ibuprofen (0.11 mg/kg per day, PO) was used for postoperative antiplatelet therapy, and aspirin (2 mg/kg/day per os) was administered through a right jugular vein intravenous catheter placed under anesthesia (1%–3% isoflurane). Animals were imaged after 2 hours on a high-resolution small animal imaging system (X-SPECT, Gamma Medica-Ideas, Northridge, CA) with 1-mm low-energy pinhole collimators. The following acquisition parameters were used for microSPECT imaging: 360 degrees, 128 projections, 30 seconds/projection (~80-minute image acquisition), with 140 keV photopeaks ±10% window. After completion of microSPECT imaging, animals were injected with a continuous infusion of iotinated CT contrast (iohexol 100 μL/min) over 2 minutes or Fenestra (200 μL, ART Advanced Research Technologies, Montreal, Quebec, Canada), and CT imaging was performed (energy 75 kVp/280 μA, 512×512 matrix) to identify anatomic structure. To avoid tissue damage, we did not perform any additional ex vivo imaging and preserved the tissue immediately for mRNA and immunohistological analysis. To establish imaging specificity, 3 animals were injected with a 50-fold excess of unlabeled precursor before NC100692 imaging at 2 weeks after surgery. For quantitative analysis of tracer uptake, cylindrical regions of interest were drawn at the level of carotid artery bifurcation (2×2×2 mm). A region of interest immediately posterior to both carotids was used to calculate the background activity. Data were expressed as background-corrected counts per voxel (cpv)/MBq injected.

Morphometric Analysis and Immunostaining
After imaging, carotid arteries were harvested, embedded in OCT compound, snap-frozen, and stored at −80°C. Hematoxylin and eosin immunostaining were performed according to standard protocols on 5-μm-thick cryostat sections. Morphometric analysis was performed on cryostat sections with ImageJ software (National Institutes of Health, Bethesda, MD), as previously described.11 The area within the external elastic lamina representing total vessel area was calculated by averaging measurements from serial sections at 200-μm intervals from 200 to 2000 μm below carotid bifurcation. For immunostaining, primary antibodies were anti-mouse αα, integrin (Millipore), anti-smooth muscle α-actin (Sigma-Aldrich), anti-CD31 (BD Pharmingen, San Jose, CA), and F4/80 (Invitrogen, Carlsbad, CA). Isotype-matched antibodies were used as controls. Nuclei were detected with 4′,6-diamidino-2-phenylindole.
integrin activation in monocytes from different donors. To address the effect of monocyte differentiation into macrophages on $\alpha_\beta$ expression and NC100692 binding, purified monocytes were differentiated into type I macrophages. Macrophage differentiation was confirmed by their distinct morphology. Similar to resting monocytes, macrophages expressed high levels of $\alpha_\beta_3$ integrin and bound to NC100692. MnCl$_2$ enhanced fluorescent NC100692 binding to macrophages (Figure 1a) without altering the integrin expression level. Because of the time required for macrophage differentiation, monocytes and macrophages from the same donor were stained on different days. The changes in integrin expression and RGD peptide binding observed in the course of monocyte to macrophage differentiation were not consistent and varied from experiment to experiment.

Imaging $\alpha_\beta$ Integrin Activation in Vessel Wall Inflammation

To investigate $\alpha_\beta$-targeted imaging for detection of vessel wall inflammation in vivo, we used an established model of vascular inflammation. In this model, adventitial application of CaCl$_2$ to common carotid arteries of high-fat-fed apoE$^{-/-}$ mice triggers an inflammatory response that leads to aneurysmal dilatation of the artery over a period of 4 weeks. ApoE$^{-/-}$ mice underwent $^{99m}$Tc-NC100692 microSPECT imaging at 2 or 4 weeks after surgery. NC100692 signal was

Figure 1. Flow cytometric assessment of $\alpha_\beta_3$ expression and activation in monocytes and macrophages. a, Representative histograms of $\alpha_\beta_3$ (top row) and RGD (bottom row) immunostaining of monocytes and monocyte-derived macrophages. b, Representative contour plots demonstrating costaining of monocytes with anti-$\alpha_\beta_3$ antibody and RGD peptide. ctrl indicates control; ab, antibody; RGD, fluorescent homologue of NC100691.
readily visible on inflamed left, but not sham-operated right, carotid arteries identified by CT angiography at either time point after surgery (Figure 2). Quantitative analysis of NC100692 uptake from in vivo images showed significantly higher tracer uptake in the left, as compared with control right, carotid arteries (0.52 ± 0.09 versus 0.08 ± 0.02 cpv/MBq injected, n = 9, P < 0.001 at 2 weeks, and 0.42 ± 0.04 versus 0.13 ± 0.02 cpv/MBq injected, n = 8, P < 0.001 at 4 weeks, Figure 2b). As expected, considerable NC100692 uptake was also present in the surgical wound. Tracer uptake specificity in inflamed arteries was investigated in a group of animals at 2 weeks after surgery that were pretreated with a 50-fold excess of nonlabeled precursor before 99mTc-NC100692 administration. Left carotid (as well as surgical wound) uptake was significantly reduced following administration of excess unlabeled precursor (0.16 ± 0.03 cpv/MBq, n = 3 versus 0.52 ± 0.09 cpv/MBq without blocking, n = 9, P = 0.004), establishing specificity of NC100692 uptake (Figure 3).

**Biological Correlate of NC100692 Uptake in Carotid Arteries**

A number of cells in the vessel wall, including ECs, vascular smooth muscle cells, and monocyte-derived macrophages, express αv integrins and may bind to NC100692 in vivo. The expression levels of cell-specific markers assessed by real-time

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**Figure 2.** MicroSPECT/CT imaging of αvβ3 activation in vascular inflammation. **a**, Examples of contrast-enhanced computed tomography and NC100692 microSPECT/CT fused images of an apolipoprotein E−/− mouse 4 weeks after surgery to induce left common carotid artery vascular inflammation. Arrows point to common carotid arteries. Tracer uptake was also detected in the surgical wound (arrowheads). **b**, MicroSPECT-derived quantification of NC100692 uptake in remodeling left and sham-operated right common carotid artery at 2 (n = 9) and 4 (n = 8) weeks after surgery. *P < 0.001. R indicates right; L, left; T, transverse; C, coronal; S, sagittal; cpv, counts per voxel.

**Figure 3.** NC100692 (NC) uptake specificity in vascular inflammation. MicroSPECT-derived quantification of NC100692 signal in remodeling carotid artery in animals without (n = 8) or with (n = 3) injection with 50-fold excess unlabeled precursor before tracer administration. *P = 0.004. cpv indicates counts per voxel.

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**Ex Vivo Analysis of Integrin Expression and Inflammation**

As expected, total vessel area at 4 weeks after surgery was ~2-fold higher in CaCl2-treated left carotid arteries compared with NaCl-treated right carotid arteries (0.192 ± 0.010 and 0.093 ± 0.007 mm², respectively; n = 8, P < 0.0001, Figure 4). Immunostaining with a macrophage-specific marker, F4/80, showed the presence of a large number of macrophages in the vessel wall in injured arteries (Figure 5). CD31 (EC) and smooth muscle α-actin (vascular smooth muscle cells) staining demonstrated the presence of small blood vessels in the vessel wall (Supplemental Figure III). αv integrin immunostaining was detected in the intima and media of injured arteries, and its distribution resembled macrophage staining (Figure 5). Macrophage content of the vessel wall was quantified by real-time reverse transcription–polymerase chain reaction, which showed significantly higher levels of GAPDH-normalized CD68 and EMR1 mRNA expression in injured arteries, as compared with sham-operated arteries (n = 8, P = 0.03 for CD68 and 0.003 for EMR1, Figure 6a). Interestingly, although there was no difference in CD31 mRNA expression between right and left carotid arteries, smooth muscle α-actin mRNA expression was significantly reduced in aneurismal arteries, indicating loss of vascular smooth muscle cells (n = 8, P = 0.045). There was no significant difference in GAPDH-normalized αv, β3, or β5 expression between control right and aneurismal left carotid arteries (Figure 6b).
reverse transcription–polymerase chain reaction were used to define the biological correlates of NC100692 uptake in carotid arteries. NC100692 uptake significantly correlated with CD68 expression, predominantly in angiogenesis.17–19 In general, the binding motif in these probes is structured based on RGD tripeptide, and they show broader specificity for $\alpha_v$ integrins. We have previously shown that RP748, a peptidomimetic quinolone, preferentially interacts with the active conformation of the integrin.11 Here, we showed a similar preferential binding to the integrin active conformation for NC100692, a cyclic RGD peptide. In monocytes and macrophages, Mn-binding to the integrin active conformation for NC100692, a low-affinity (off) state, and on activation, whether through outside-in or inside-out signals, they convert to a high-affinity (on) state that can bind specific ligands and trigger signaling.13 Integrin activation state is cell-dependent. For example, $\alpha_v\beta_3$ integrin is mostly in a low-affinity state in JY lymphoblastoid cells, whereas in melanoma cell lines it is present in an active conformation.14 In resting ECs, $\alpha_v\beta_3$ is mostly in a low-affinity state, and EC activation (eg, with shear stress) increases the level of high-affinity integrin.12

Integrin $\alpha_v\beta_3$ is expressed at a high density on proliferating ECs, and $\alpha_v\beta_3$-targeted imaging appears to be a promising approach for detection of angiogenesis associated with tumors5 and myocardial or hindlimb ischemia.1,2 Expression of $\alpha_v\beta_3$ integrins by other cells raises the possibility that this integrin may be targeted for imaging other processes where cell proliferation and integrin-mediated cell-cell and cell-matrix interactions are critically involved. Indeed, RP748, an $^{111}$In-labeled, $\alpha_v\beta_3$-targeted tracer, localizes in murine or human arteries following mechanical or immune injury in parallel with changes in cell proliferation.7,11 In a previous study of targeted imaging of angiogenesis, NC100692 appeared to localize in part in the inflammatory infiltrate in addition to ECs.2 This, in conjunction with the reports on integrin expression in monocyte-macrophages,15,16 led us to investigate NC100692 for imaging vascular inflammation in vivo.

Several tracers have been developed for imaging $\alpha_v\beta_3$ expression, predominantly in angiogenesis.17–19 In general, the binding motif in these probes is structured based on RGD tripeptide, and they show broader specificity for $\alpha_v$ integrins. We have previously shown that RP748, a peptidomimetic quinolone, preferentially interacts with the active conformation of the integrin.11 Here, we showed a similar preferential binding to the integrin active conformation for NC100692, a cyclic RGD peptide. In monocytes and macrophages, Mn-induced integrin activation enhanced RGD peptide binding, indicating that similar to ECs, $\alpha_v$ integrins in resting monocytes and macrophages are not in a fully activated state.

Inflammation plays a key role in the pathogenesis of several vasculopathies, including atherosclerosis and aneurysm. In atherosclerosis, vessel wall inflammation has been linked to plaque vulnerability, and imaging vessel wall inflammation may help identify patients at high risk for acute coronary syndromes and stroke.20 Similarly, vessel wall inflammation is linked to aortic aneurysm expansion and rupture, and detection of vessel wall inflammation in vivo may help stratify patients based on their risk of rupture.21 As such, imaging vessel wall inflammation can potentially transform the clinical care of patients with atherosclerosis, aneurysm and other vascular pathologies. This is especially true for noninvasive imaging modalities, such as SPECT and PET, which are routinely used in humans. Potential applications of such imaging approaches include identification of patients at high risk for morbid events who may benefit from early treatment and tracking and optimizing therapeutic interventions. A number of tracers, predominantly those targeting endothelial adhesion molecules (vascular cell adhesion molecule-122), cellular metabolism (with $^{18}$F-fluorodeoxy glucose23,24), and protease activity (matrix metalloprotei-nases,25–28 cathepsins29,30) have been studied for their ability
to track vessel wall inflammation in vivo. Recently, ex vivo studies have raised the possibility of \( \alpha_\beta_3 \)-targeted imaging of vessel wall inflammation. Using autoradiography, \(^{18}\)F-galacto-RGD was shown to localize in atherosclerotic lesions, and RGD uptake correlated with the density of nuclei and \(^3\)H-fluorodeoxy glucose uptake. Similarly, RGD-Cy5.5 localized in the arterial wall following carotid ligation in the mouse, and the uptake was detectable by ex vivo near-infrared fluorescence reflectance imaging. Here, we demonstrated that NC100692 microSPECT/CT imaging can detect remodeling carotid arteries in a prototypic model of vascular inflammation in apoE \(^{-/-} \) mice in vivo. Blocking with excess unlabeled precursor confirmed the specificity of NC100692 signal. A similar protocol was used to image matrix metalloproteinase activation in vascular remodeling, where the approach to in vivo quantification of carotid signal was validated with ex vivo measures of tracer uptake.

\( \alpha_\beta_3 \) targeted paramagnetic nanoparticles have been used to image atherosclerotic plaque angiogenesis by magnetic resonance imaging. Unlike these nanoparticles, smaller probes, such as NC100692, are not confined to intravascular space, and any \( \alpha_\beta_3 \) integrin expressing vascular cell may be target for NC100692 binding in vivo. In our experiments, the intense autofluorescence of elastic laminae did not permit direct colocalization of fluorescent RGD peptide with specific vascular cells. However, \( \alpha_\beta_3 \) expressing proliferating ECs and inflammatory cells are both components of the inflammatory response in the vessel wall. Therefore, by targeting multiple cellular events linked to inflammation, our imaging approach may prove to be highly effective for detection of inflammation. Cellular content and target expression in the vessel wall is often measured by immunostaining. Because immunostaining is at best a semiquantitative technique and only a limited number of histological sections (5–7\( \mu \)m) are evaluated, such data may not reliably relate to imaging data obtained from much larger segments (≈2 mm) of the artery. Because the small size of murine carotid arteries prohibits the use of a more quantitative approach (eg, Western blotting) for protein measurement, we relied on mRNA analysis to quantify macrophage content and integrin expression in carotid arteries. Using this approach, we found a strong correlation between NC100692 uptake in vivo and GAPDH-normalized CD68 (macrophage marker) expression in the vessel wall, validating \( \alpha_\beta_3 \) targeted imaging for detection of vessel wall inflammation in vivo. Importantly, despite the marked difference in NC100692 uptake, there was no significant difference in normalized \( \alpha_\beta_3 \), \( \beta_3 \), or \( \beta_5 \) integrin expression between control and remodeling carotid arteries. This, in conjunction with the preferential binding of NC100692 to active conformation of integrins, may indicate that \( \alpha_\beta_3 \) integrins are in an active state in remodeling arteries.

In conclusion, we demonstrated that NC100692, a tracer with preferential binding to active conformation of \( \alpha_\beta_3 \) integrins, specifically localizes in inflamed carotid arteries of apoE \(^{-/-} \) mice and provides a signal that is detectable by microSPECT/CT imaging in vivo. NC100692 uptake in the artery correlates well with macrophage content of the vessel wall, indicating that this RGD peptide may be used to image...
vascular inflammation in vivo. Further validation of our observations in other models of vessel wall inflammation may lead to the development of a novel imaging approach for identifying patients who are at high risk for vascular events and tracking the effect of antiinflammatory treatments.

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Disclosures

Albert J. Sinusas and Mehran M. Sadeghi received experimental tracers from GE Healthcare.

References


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Supplement Material.

Supplemental Figure I. Schematic presentation of the experimental design

Supplemental Figure II. Flow cytometric assessment of αvβ3 activation in endothelial cells. RGD: fluorescent homologue of NC100691.

Supplemental Figure III. Representative examples of CD31 (EC) and smooth muscle α-actin immunostaining (in red) of control right and remodeling left carotid arteries at 4 weeks after surgery. Nuclei are stained with DAPI in blue and elastic membrane autofluorescence is seen in green. L: lumen. Scale bar: 20 µm.
Supplemental Figure I

- High fat diet (n=17)
- Cardiomyoplasty
- Imaging (n=9) Tissue harvesting (n=5)
- Imaging (n=5) Tissue harvesting (n=5)
- Imaging with excess precursor (n=3)

Time scale (weeks): -1 0 +1 +2 +3 +4
Supplemental Figure II