Detection of Injury-Induced Vascular Remodeling by Targeting Activated αvβ3 Integrin In Vivo

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Background—The αvβ3 integrin plays a critical role in cell proliferation and migration. We hypothesized that vascular cell proliferation, a hallmark of injury-induced remodeling, can be tracked by targeting αvβ3 integrin expression in vivo.

Methods and Results—RP748, a novel 111In-labeled αvβ3-specific radiotracer, was evaluated for its cell-binding characteristics and ability to track injury-induced vascular proliferation in vivo. Three groups of experiments were performed. In cultured endothelial cells (ECs), TA145, a cy3-labeled homologue of RP748, localized to αvβ3 at focal contacts. Activation of αvβ3 by Mn2+ led to increased EC binding of TA145. Left common carotid artery wire injury in apolipoprotein E–/– mice led to vascular wall expansion over a period of 4 weeks. RP748 (7.4 MBq) was injected into groups of 9 mice at 1, 3, or 4 weeks after left carotid injury, and carotids were harvested for autoradiography. Relative autographic intensity, defined as counts/pixel of the injured left carotid area divided by counts/pixel of the uninjured right carotid area, was higher at 1 and 3 weeks (1.8±0.1 and 1.9±0.2, respectively) and decreased significantly by 4 weeks after injury (1.4±0.1, P<0.05). Carotid αv and β3 integrin expression was maximal at 1 week and decreased by 4 weeks after injury. The proliferation index, as determined by Ki67 staining, followed a temporal pattern similar to that of RP748 uptake. Dynamic gamma imaging demonstrated rapid renal clearance of RP748.

Conclusions—RP748 has preferential binding to activated αvβ3 integrin and can track the injury-induced vascular proliferative process in vivo. (Circulation. 2004;110:84-90.)

Key Words: imaging ■ nuclear medicine ■ arteries ■ restenosis

Integrins are a family of heterodimeric adhesion molecules involved in cell–cell and cell–matrix interactions.1,2 Through interactions with their specific counterreceptors, integrins play a central role in normal homeostasis and pathological states. Integrin function can be modulated by changes in the expression level and/or activation state of the integrin. These activation states are classically studied by use of activating antibodies and antibodies that recognize the activation epitopes of various integrins. The αvβ3 integrin is expressed on vascular endothelial cells (ECs)3 and smooth muscle cells (SMCs),4 as well as nonvascular cells such as osteoclasts.5 Classically described as the vitronectin receptor, αvβ3 integrin can also bind to other extracellular matrix proteins (eg, fibrinogen, fibronectin) as well as prothrombin. Modulation of αvβ3 expression by vascular cells plays a central role in vascular processes associated with cell proliferation and migration, such as angiogenesis6 and vascular remodeling.7 As such, αvβ3 integrin has emerged as a promising target for imaging tumor angiogenesis.8,9 αvβ3 Antagonists have been shown to limit neointimal hyperplasia and lumen stenosis in experimental models of vascular injury.10–12 Therefore, we hypothesized that injury-induced vascular remodeling/proliferative process could be imaged in vivo by targeting αvβ3 expression with a novel 111In-labeled αvβ3–specific small molecule, RP748.

Here we demonstrate that (1) RP748 and its homologues bind preferentially to activated αvβ3 on ECs in vitro and exhibit favorable binding characteristics for in vivo imaging, (2) RP748 uptake can track/detect the proliferative process associated with carotid artery injury by targeting activated αvβ3 integrin expression in vivo in apolipoprotein E–negative (apoE–/–) mice, and (3) pharmacokinetic characteristics are suitable for in vivo imaging.

Methods

Reagents

Reagents were from Sigma Chemical Co unless otherwise specified. TA138 and its cy3-labeled (TA145) and 111In-labeled (RP748)
homologues were provided by Bristol-Myers Squibb Medical Imaging. TA138 is a high-affinity, α3β3, integrin–specific small molecule ligand, originally selected from a combinatorial library on the basis of its ability to inhibit human umbilical vein EC (HUV EC) adhesion to fibrinogen (α3β3-dependent) with an IC50 of 52 ± 6 nmol/L.13

Cell Culture

Single-donor HUVECs were isolated and cultured as described previously.14 Murine lung ECs were kindly provided by Dr William Sessa (Yale University) and were grown in HUVEC medium.

Flow Cytometry

Cells were stained and analyzed on a FACSort (Becton Dickinson) as described previously.15 Cells were suspended in calcium/magnesium-free PBS. The primary mouse monoclonal antibodies used, anti-α3β3 (LM609, Chemicon), or the irrelevant isotype-matched control antibody (MOPC-21), were followed by staining with FITC-conjugated goat anti-mouse IgG secondary antibody. TA145, a cys3-labeled TA138, was used directly. Inhibition assays were performed in the presence of 50-fold excess unlabeled antibody. To study the effect of integrin activation on TA145 binding, cells were treated with Mn2+ or EDTA (1 mmol/L) for 10 minutes before staining. Five thousand cells were analyzed per sample.

Affinity Determination

HUVECs were exposed to serial dilutions of RP748 with or without Mn2+ for 30 minutes at 4°C. After 4 washes, the remaining activity was measured in a gamma counter (Packard Biosciences Co). Nonisotypic binding was calculated with 100-fold excess unlabeled ligand, assuming a linear relation with the labeled ligand concentrations. Saturation binding was determined and Scatchard analysis was performed with GraphPad PRISM software.

Animal Model

Left common carotid artery injury was induced in apoE−/− mice as described previously16 with minor modifications. Six- to 8-week-old female apoE−/− mice (Jackson Laboratory, Bar Harbor, Me) were fed high-cholesterol (1.25% cholesterol, Harlan) chow ad libitum. After 85 days, mice were fed normal saline (or 3% paraformaldehyde for morphometric analysis) at 37°C until further use. For imaging experiments, groups of 3 animals at each time point were perfused with normal saline (or 3% paraformaldehyde for morphometric analysis) at 37°C for 5 minutes under physiological pressure through an intracardiac puncture. Carotid arteries were dissected, embedded in OCT compound, snap-frozen, and stored at −80°C until further use. For imaging experiments, groups of 9 animals at each time point were used. RP748 (7.4 MBq) was administered through an intravenous catheter. Blood samples were obtained at various time points after tracer administration and weighed, and activity was quantified in a gamma-well counter. Animals were killed after 2.5 hours, and total and Ki67-positive nuclei in 4 random sections along the common carotid artery were counted, and the maximal ratio of Ki67 antibody–positive nuclei (in percents) was used as index of cell proliferation.

Autoradiography

Samples were exposed to x-radiographic X-OMAT Kodak Scientific Imaging Film (Eastman Kodak) for various times to optimize detection. Signal intensity in the regions of interest was measured on high-resolution images, and background noise was subtracted using ID Image Analysis Software (Eastman Kodak Co). Relative autoradiographic intensity was defined as the average autoradiographic counts/pixel in the left common carotid divided by that of the uninjured right common carotid artery.

Serial Gamma Imaging for Pharmacokinetics

RP748 (7.4 MBq) was injected through an intravenous catheter. Serial 30-second planar high-resolution images were obtained using a 1-mm aperture pinhole collimator and a SPECT camera (GE Millennium VG) with a 256 × 256 matrix for 15 minutes, followed by a 30-minute late image at 2 hours. This system provides much higher resolution than can be obtained with the standard parallel-hole collimators. At 4-cm distance, using radioisotope-filled capillary tubes, the discrimination for the GE camera is found to be ~1.5 mm for 111In, with a count-sensitivity in the range of 1 to 2 counts per second/μCi. Images were acquired with a 10% window centered on photopeak of the radioisotope (174 and 242 keV for 111In). Time-activity curves were derived for various regions of interest.

Statistical Analysis

All data are presented as mean ± SEM. Multiple groups were compared by use of ANOVA with Tukey’s test for multiple post hoc comparisons. Significance was set at the 0.05 level.

Results

TA138 Interacts With α3β3 Integrin on Human and Murine ECs

We first determined whether TA138 can detect α3β3 integrin expressed on cells of human and murine origin. In HUVECs, LM609 (an anti-human α3β3 integrin antibody) and TA145 colocalized to α3β3 integrin in focal contacts (Figure 1, a, b, and c). The specificity of staining was demonstrated by lack of staining with an isotype-matched control antibody, MOPC-21 (data not shown). Similar results were obtained with murine ECs. In the absence of a specific anti-murine α3β3 antibody, staining with anti-murine α3 and β3 antibodies as well as TA145 localized to focal contacts (Figure 1, d, e, and f). Specificity of binding was further assessed by flow cytometry. LM609 but not MOPC-21 inhibited TA145 binding to HUVECs (Figure 2a), demonstrating the α3β3 specificity of TA145 binding to live ECs.

TA138 Recognition of α3β3 Integrin Is Activation Dependent

The binding of monovalent reagents, such as TA138, is not affected by clustering of target molecules. Therefore, TA138 may be able to detect α3β3 conformational changes indepen-
dently of changes in integrin avidity. To test this hypothesis, we assessed the ability of TA145 to bind to αβ3 integrin on Mn2+-treated HUVECs by use of flow cytometry. Presence of Mn2+, a universal activator of integrins through binding to metal ion–dependent adhesion sites and consequent conformational changes, increased the binding of TA145 to HUVECs in a concentration-dependent manner, with the maximal binding observed at 20 μmol/L (Figure 2b). This increased binding was not a result of increased surface expression of αβ3 integrin because LM609 antibody binding was unaffected by the presence of Mn2+ (Figure 2c). Chelation of divalent ions by EDTA (1 mmol/L) reduced the baseline binding of TA145 (in the absence of Mn2+) to the level of unstained cells and resulted in a complete inhibition of the effect of Mn2+ (Figure 2d). Conversely, integrin activation by the protein kinase C activator phorbol 12-myristate 13-acetate (PMA, 200 nmol/L), which exerts its effects primarily through changes in avidity rather than conformation, did not affect the TA145 binding to HUVECs (data not shown).

**RP748 Binding Characteristics to Activated αβ3 Integrin Are Favorable for In Vivo Imaging**

The binding characteristics of RP748, the 111In-labeled homologue of TA138, to HUVECs and the effect of integrin activation were assessed by saturation binding and Scatchard analysis (Figure 3). There was an approximately 15-fold increase in RP748 affinity for αβ3 integrin on ECs in suspension in the presence of Mn2+ (2 mmol/L), compared with nonactivated cells, with the Kd decreasing from 374±44 nmol/L in the absence of Mn2+ to 21±7 nmol/L in the presence of Mn2+ (2 mmol/L). Integrin activation with Mn2+ also led to an increase in maximum binding (Bmax) from 130 000±5000 molecules/EC at baseline to 230 000±16 000 molecules/EC in the presence of Mn2+.

![Figure 1](image1.png)

**Figure 1.** Detection of integrin expression in human (a, b, c) and murine (d, e, f) ECs by immunofluorescent staining. Anti-αβ3 antibody (a) and TA145 (b) colocalize (c) to focal contacts in human ECs. Similarly, in murine ECs, anti-α3 (d) and anti-β3 (e) antibodies, as well as TA145 (f), localize to focal contacts.

![Figure 2](image2.png)

**Figure 2.** Flow cytometric evaluation of TA145 binding to endothelial cells (ECs). A specific anti-αβ3 antibody (LM609) but not control antibody (MOPC-21) inhibits TA145 binding (a). Presence of Mn2+ increases TA145 binding to ECs in a concentration-dependent manner (b) without affecting αβ3 expression (c). Conversely, TA145 binding to ECs is inhibited by EDTA (d). Figure is representative of 4 independent experiments.

![Figure 3](image3.png)

**Figure 3.** RP748 saturation binding to endothelial cells. Various amounts of RP748 were added to HUVECs (in triplicates) in absence or presence of Mn2+, and specific binding was calculated. Bmax and Kd (230 000±16 000 binding sites/cell and 21±7 nmol/L, respectively, with Mn2+ and 130 000±5000 binding sites/cell and 374±44 nmol/L without Mn2+) were derived by Scatchard analysis. Figure is representative of 3 independent experiments.
Injury-Induced Vascular Remodeling Is Associated With Increased Expression of $\alpha_\text{v}$ and $\beta_3$ Integrins

Expression patterns of $\alpha_\text{v}$ and $\beta_3$ in the vessel wall were studied by immunohistochemistry at different times after carotid wire injury in apoE$^{-/-}$ mice. Very low levels of $\alpha_\text{v}$ and $\beta_3$ staining could be detected in uninjured carotid arteries. On injury, and in conjunction with vascular wall expansion, higher levels of both the $\alpha_\text{v}$ (Figure 4) and $\beta_3$ (not shown) integrins could be detected in the media and neointima. Expression of $\alpha_\text{v}$ and $\beta_3$ integrin was maximal at 1 to 3 weeks after injury and decreased toward baseline levels by 4 weeks.

RP748 Uptake Is Increased After Carotid Wire Injury

To assess the ability of RP748 to track activated $\alpha_\text{v}\beta_3$ integrin expression in vivo, RP748 (7.4 MBq) was injected into apoE$^{-/-}$ mice at 1, 3, and 4 weeks after left common carotid injury. Animals were killed at 2.5 hours, and the aortic arch and carotids were harvested for autoradiography. Despite some variability, there was significantly higher uptake of the tracer in the left injured common carotid artery compared with the contralateral uninjured artery (Figure 5a). Relative autoradiographic intensity was highest at 1 to 3 weeks and decreased significantly by 4 weeks after injury (1.8 ± 0.8, 62.7 ± 8.3, and 132.4 ± 12.1 mm, n = 3, $P < 0.0001^\dagger$ for uninjured control right carotid and left carotid at 1, 3, and 4 weeks after injury, respectively) (Figure 6b). The proliferative process was quantified by Ki67 staining, a marker of cell proliferation. In uninjured arteries, Ki67 staining was minimal (proliferation index < 1) at all time points. The left carotid proliferation index was maximal at 1 to 3 weeks and decreased significantly by 4 weeks after injury (18.6 ± 0.3 and 21.4 ± 2.4 versus 5.3 ± 0.3, n = 3, $P < 0.01$, at 1, 3, and 4 weeks after injury, respectively). As such, the temporal pattern of RP748 relative carotid intensity closely paralleled that of the proliferation index (Figure 7).

RP748 Exhibits Favorable Pharmacokinetics for In Vivo Imaging

Dynamic high-resolution planar images demonstrated rapid renal clearance of RP748 in apoE$^{-/-}$ mice. Figure 8a represents a typical time-activity curve. RP748 could be detected in the kidneys as early as 3 minutes after intravenous administration. By 2.5 hours, most of the activity was detected in the bladder. RP748 clearance was quantified by serial blood sampling. In concordance with imaging data, RP748 rapidly cleared from the circulation. Less than 0.5% of the injected dose was detectable in the blood pool at 2.5 hours (Figure 8b).

Discussion

In this series of experiments, we demonstrate the following: (1) RP748 binds to activated $\alpha_\text{v}\beta_3$ integrin with favorable characteristics for in vivo imaging, (2) RP748 uptake tracks the injury-induced carotid artery proliferative process in apoE$^{-/-}$ mice, and (3) RP748 pharmacokinetics are suitable for in vivo imaging. These findings serve as a foundation for the development of noninvasive imaging modalities directed at vascular proliferation and remodeling.

The activation state(s) of integrins is (are) associated with changes in affinity (conformational changes) and in avidity (lateral mobility and clustering) of the molecule. Analysis of $\alpha_\text{v}\beta_3$ integrin crystal structure has led to description of 3 distinct molecular conformations.$^{17}$ Mn$^{2+}$ is the prototype activator of integrins. Other stimuli, such as phorbol esters and ADP,$^{18}$ can similarly modulate the activation state of the integrins, although their effect on $\alpha_\text{v}\beta_3$ integrin conformation is less well studied. Recently, a synthetic antibody (WOW-1) was shown to specifically interact with activated $\alpha_\text{v}\beta_3$ integrin.$^{19}$ Here, we demonstrated that both the cy3- and $^{111}$In-labeled TA138 (ie, TA145 and RP748) bind preferentially to Mn$^{2+}$-activated $\alpha_\text{v}\beta_3$ integrin on ECs. Saturation binding assays demonstrated a higher apparent affinity and number of binding sites per EC for RP748 in the presence of Mn$^{2+}$. These binding parameters provide favorable conditions for in vivo imaging of activated $\alpha_\text{v}\beta_3$ integrin by RP748.$^{20}$ A similar number of $\alpha_\text{v}\beta_3$ binding sites per Mn$^{2+}$-activated EC was observed with another activation-dependent ligand of $\alpha_\text{v}\beta_3$ integrin, prothrombin.$^{18}$ Both the baseline and Mn$^{2+}$-induced binding to ECs were inhibited by EDTA. Of note is that the focal contact–localized $\alpha_\text{v}\beta_3$ molecules in adherent ECs are...
apparently in the active conformation, as demonstrated by TA145 and LM609 costaining (Figure 1). Thus, a component of \(\alpha_\beta_3\) integrin on resting ECs is in the activated state, also seen in flow cytometric analysis (Figure 2) and therefore recognized by TA138 homologues. Interestingly, and contrary to WOW-1,\(^19\) we did not observe any increased binding of TA145 to PMA-treated ECs, suggesting that TA138 homologues may discriminate between the PMA- and Mn\(^{2+}\)-activated forms of \(\alpha_\beta_3\) integrin. The effect of PMA on \(\beta_3\)-dependent leukocyte adhesion is mainly due to integrin clustering (ie, changes in avidity), without significantly affecting the affinity of the receptor for the ligand.\(^{21}\) A similar pattern may prove to be true for \(\alpha_\beta_3\) integrin.

Carotid wire injury in apoE\(^{-/-}\) mice led to significant neointima formation and media thickening over a period of 4 weeks. There was concomitant upregulation of \(\alpha_\beta_3\) integrins in the remodeling vessel wall. Maximal expression of both integrins was observed at 1 week after injury and preceded maximal morphometric changes. In the absence of specific anti-murine \(\alpha_\beta_3\) integrin antibodies, evaluation of \(\alpha_\beta_3\) and \(\alpha_\beta_3\) integrins constituted a reasonable substitute. Although we did not specifically study the cellular localization of the observed \(\alpha_\beta_3\) integrin expression, the predominance of immunostaining in the media and neointima strongly implicates both SMCs and ECs.

We observed a higher uptake of RP748 in the injured left carotid artery compared with the uninjured contralateral artery, with a time course following that of the proliferative process. Specificity of uptake was demonstrated by the inhibitory effect of excess nonlabeled tracer. Similar to the \(\alpha_\beta_3\) expression pattern, the maximal proliferative process preceded the maximal morphometric changes. Interestingly, RP748 uptake seems to track the proliferative process more...
closely than the immunohistochemical evaluation of α, and β3 integrin expression. Immunohistochemical detection of αvβ3 integrin by anti-α, and -β3 antibodies does not necessarily correlate with expression of the activated form of the integrin. The superior performance of RP748 may be due to its preferential binding to the activated form of αvβ3 integrin. There is limited information available on the physiopathological importance of αvβ3 activation in vivo. TA138 and its labeled homologues may provide a unique opportunity for studying these processes, both in vitro and in vivo. The rapid renal clearance of RP748, limited liver uptake, and its high affinity for activated αvβ3 integrin favorably affect its suitability for in vivo imaging in a number of biological processes. As such, high levels of RP748 uptake have been observed in αvβ3-expressing tumors. The dose of RP748 used in this study was selected on the basis of preliminary imaging experiments to allow for reasonable analysis of tracer biodistribution/kinetics by in vivo gamma imaging. The optimal dose and imaging parameters will need to be further defined. Future experiments will be directed at imaging injury-induced vascular remodeling in both normal and atherosclerotic arteries in larger animals.

In conclusion, we have demonstrated that RP748 exhibits selective binding to activated αvβ3 integrin and can track the injury-induced arterial proliferative process. These findings will potentially lead to the development of noninvasive imaging strategies for vascular cell proliferation–associated states, whether focal, as in postangioplasty restenosis, or diffuse, as in pulmonary hypertension. As such, the ability to track/predict the proliferative process in vivo can markedly affect the management of patients with graft vasculopathy, in which noninvasive imaging techniques are lacking. Moreover, RP748 provides investigators with a new experimental tool to study αvβ3 integrin activation in various biological processes in vivo. This and other molecular imaging–based approaches should lead to better understanding of pathophysiology and development of novel paradigms for management of cardiovascular disease.

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