cNGR: A Novel Homing Sequence for CD13/APN Targeted Molecular Imaging of Murine Cardiac Angiogenesis In Vivo


Objective—Previously, the peptide sequence cNGR has been shown to home specifically to CD13/APN (aminopeptidase N) on tumor endothelium. Here, we investigated the feasibility of selective imaging of cardiac angiogenesis using the cNGR-CD13/APN system.

Methods and Results—CD13/APN induction and cNGR homing were studied in the murine myocardial infarction (MI) model. By real-time polymerase chain reaction (PCR) at 7 days after MI, CD13/APN expression was 10- to 20-fold higher in the angiogenic infarct border zone and the MI area than in non-MI areas. In vivo fluorescence microscopy confirmed specific homing of fluorophore-tagged cNGR to the border zone and MI territory at 4 and 7 days after MI with a local advantage of 2.3, but not at 1 or 14 days after MI. Tissue residence half-life was 9.1±0.3 hours, whereas the half-life in plasma was 15.4±3.4 minutes. Pulse chase experiments confirmed reversible binding of cNGR in the infarct area. Fluorescent labeled cNGR conjugates or antibodies were injected in vivo, and their distribution was studied ex vivo by 2-photon laser scanning microscopy (TPLSM). cNGR co-localized exclusively with CD13/APN and the endothelial marker CD31 on vessels.

Conclusions—In cardiac angiogenesis endothelial CD13/APN is upregulated. It can be targeted specifically with cNGR conjugates. In the heart cNGR binds its endothelial target only in angiogenic areas. (Arterioscler Thromb Vasc Biol. 2006;26:2681-2687.)

Key Words: aminopeptidase N ■ angiogenesis ■ molecular imaging ■ NGR ■ vascular biology

Neovascularization through angiogenesis, arteriogenesis, or vasculogenesis is a natural adaptation to tissue hypoxia and inflammation. In the heart, the extent of spontaneous neovascularization through angiogenesis and arteriogenesis likely determines symptoms and prognosis in patients with coronary artery disease. Strategies to enhance neovascularization through exogenous supplementation of growth factors or fractions of monocytes have therefore been designed to improve the outcome of coronary artery disease. Unfortunately, in the clinical arena this has proven more difficult than anticipated. It is likely that improvements in growth factor selection, growth factor delivery, and perhaps combination treatment will move this field forward in small incremental steps. Recognizing these improvements requires sensitive and preferably noninvasive imaging techniques that allow monitoring of vessel development in response to therapy.

Molecular imaging, ie, using targeting antibody or nonantibody ligands to molecular markers is emerging as a useful technique that may provide the necessary sensitivity while also providing insight into the disease mechanisms. A critical component of molecular targeting is the identification of a marker, for instance angiogenic or “activated” endothelium. Novel markers of angiogenic endothelium and their ligands have been identified by differential phage display experiments in tumor models. Two peptide motifs, NGR (Asn-Gly-Arg) and RGD, have been shown to home specifically to tumor vessels in tumor xenograft bearing mice. NGR had a 3-fold higher homing efficacy than RGD, and it showed the greatest tumor selectivity of all peptides that were tested. In addition NGR conjugated doxorubicin, tumor necrosis factor-α, and pro-apoptotic peptides, had a greater anti-tumor effect than uncoupled drugs, probably because of local concentration. The vascular address for NGR has been identified as CD13/APN (aminopeptidase N, henceforth named CD13), a membrane bound glycoprotein that functions as an extracellular aminopeptidase. Antibodies against CD13 inhibited tumor-homing of NGR phage, whereas cells overexpressing CD13 bind the NGR phage specifically. CD13 is mainly expressed on tumor endothelium and new vessels in the corpus luteum, but not on quiescent vessels. In accordance, CD13 is transcriptionally activated by multiple angiogenic signals and ap-
pears to regulate angiogenesis in in vitro models.8,9 The expression of CD13 in adult ischemic tissues undergoing active neovascularization has not been studied yet.

In the current study, we aimed to investigate the utility of CD13 as a vascular address for targeted imaging of angiogenic vessels in the ischemic heart using cyclic NGR (cNGR) conjugates labeled with 2 different fluorescent tags (Oregon Green and Quantum dots [Qd]). Qd enhance the fluorescent signal and are very stable. We used the well-characterized angiogenic marker CD105 as a positive control. First, we studied the expression of CD13 in the angiogenic border zone of mouse cardiac infarcts. Using 2 complementary optical imaging methods, in vivo fluorescence imaging and ex vivo 2-photon laser scanning microscopy (TPLSM), we further documented specific homing of cNGR conjugates to CD13 on the newly formed vasculature in infarcted myocardium.

Vascular addresses as targets for molecular contrasts can serve as prognosticators and tools to monitor therapy in postmyocardial infarct remodeling including angiogenesis.

Materials and Methods
Please see http://atvb.ahajournals.org for further details concerning Materials and Methods.

All animal studies were approved by the Animal Welfare Committee of the University of Maastricht. Animals were handled in accordance with the American Physiological Society guidelines for animal welfare and the Guide for Care and Use of Laboratory Animals published by the United States National Institutes of Health.

Murine Myocardial Infarction
Myocardial infarction (MI) was induced in 10- to 12-week-old male Swiss mice by ligation of the left coronary artery, as previously described.10 The wound healing response leads to angiogenesis in the connective tissue and myocytes (purple, 0.25 μmol/L, 540 nm; Molecular Probes, Eugene, Ore). Eosin was used to visualize macrophage, neutrophil, and eosinophil staining (red, 0.25 μmol/L, 470 nm; Pharmingen, San Diego, Calif).

Real-Time Polymerase Chain Reaction
CD13 expression in the border zone (BZ) and in the infarct area (IA) was assayed quantitatively by real-time polymerase chain reaction (PCR). To measure the CD13 mRNA content, hearts were harvested at 4 days, 7 days, or 14 days after MI or sham surgery (n = 6 to 7). Quantities were determined by comparison with known quantities of cloned CD13 and 18S PCR products.

In Vivo Fluorescence Microscopy
Myocardial infarction or sham procedures in the mouse were performed as described earlier11 One, 4, 7, and 14 days after MI, cNGR-OG488 binding to the infarct zone was studied using in vivo fluorescence imaging (n = 2 to 5). Unconjugated OG488 was used as a control. Probes were injected before imaging through an indwelling catheter in the left jugular vein and were allowed to recirculate for 3 hours. Seven days after MI the pharmacokinetic properties of molecular contrast were determined by fluorescent imaging at 15 minutes, and 6, 12, and 24 hours after injection of 30 μg cNGR-OG488 or 12.5 μg OG488 in 150 μL PBS (n = 3 per time point, and per probe). Reversibility of cNGR binding was studied in a separate washout study in which a 20-fold excess of unlabeled cNGR was given 15 minutes after injection of the cNGR-OG488 (n = 3). No further postprocessing of images was performed. Mean fluorescent intensity (FI) after 15 minutes was set at 100% and local advantage (the ratio of target and background concentration of the fluorescent label) was calculated as the ratio of target to background concentration (right ventricle or atrium) of the fluorescent label.

TTC Protocol
After in vivo image acquisition the hearts were excised, frozen at −20°C for 15 minutes and subsequently cut into 1.5-mm-thick slices. To determine the infarction area and relate it to the area of contrast labeling, slices were incubated in 1.5% triphenyltetrazolium chloride (TTC) (Sigma; T8877) in PBS at 37°C for 20 minutes. Brightfield and fluorescent images were taken and areas of necrosis and of fluorescent labeling were determined as percentage of the total area.

2-Photon Laser Scanning Microscopy
To assess intra-myocardial localization of CD13 expression, and cNGR binding after intravenous administration, we applied 2-photon laser scanning microscopy (TPLSM)14 to intact excised heart tissue. To determine the infarction area and relate it to the area of contrast labeling, slices were incubated in 1.5% triphenyltetrazolium chloride (TTC) (Sigma; T8877) in PBS at 37°C for 20 minutes. Brightfield and fluorescent images were taken and areas of necrosis and of fluorescent labeling were determined as percentage of the total area.

Figure 1. CD13/APN mRNA expression by real time PCR after MI. Expression of CD13/APN (pg/g) in the infarct area (IA) and the border zone (BZ) peaked at 10 at 7 days after MI. CD13/APN levels in the right ventricle (RV), septum (S), and nonrisk area (NRA) remained at baseline. *MI vs RV, S, NRA, and sham; †MI vs RV, S, NRA, and sham; ‡MI vs RV, S, NRA, and sham; §7-day BZ vs 4-day and 14-day sham.
Statistical Analyses

All data are represented as mean\pm standard error of the mean (SEM). Differences between groups were analyzed with either ANOVA with Dunn’s post hoc analysis or a Kruskal Wallis (real-time PCR data). A \( P<0.05 \) was considered statistically significant.

Results

Please see http://atvb.ahajournals.org for additional results.

CD13 Is Preferentially Expressed in the Angiogenic Area After MI

By real-time PCR, CD13 mRNA expression was significantly upregulated in the IA and BZ, but not in other areas of the infarcted heart and the sham operated hearts (Figure 1). CD13 mRNA expression in IA and BZ peaked at 7 days after MI with a significant 10- to 20-fold increase, confirming that CD13 is induced in MI.

In Vivo Homing of cNGR to the Infarct Region

To study the homing of intravenously administered cNGR, in vivo fluorescence microscopy was applied 7 days after MI, at the peak of CD13 expression in the BZ and IA. Monovalent cNGR-OG488 showed a rapid and unequivocal above background fluorescence in the target areas that could not be detected in other myocardial regions or sham-operated animals (Figure 2A). FI peaked at 15 minutes after injection and this FI was taken as 100% \( (SD=14\%\), \( P<0.001 \) compared with background FI, \( n=7 \)). At 12 hours, FI was still significantly elevated (17.8\pm1.2\%, \( P<0.05 \), but at 24 hours fluorescence had decreased to background levels of \( \sim2\pm2\% \) (Figure 2B). Sham operated hearts showed non-specific binding (eg, FI of 8.7\pm0.4\% in LV, \( n=3 \)). Likewise, untargeted OG488 did not bind in infarcted hearts (FI of 7.8\pm1.5\% in IA, \( n=3 \)). Applying single-order kinetics, the residence half time \( (T_{1/2}) \) of cNGR-OG488 in the infarct area was 9.1\pm0.3 hours. Chase experiments with a 20-fold excess of unlabeled cNGR (\( n=3 \), showed rapid displacement of cNGR-OG488 and a decrease in \( T_{1/2} \) down to 1.3\pm0.2 hours, strongly suggesting reversible binding of cNGR. The
plasma T1/2 of cNGR-OG488 in plasma (please see http://atvb.ahajournals.org) was 15±3 minutes and 7.7±0.9 minutes for untargeted OG488. Rapid clearance of the conjugate from the bloodstream is in accordance with its small size and predominant clearance by the kidneys2 as attested by fluorescence in the bladder (not shown).

In accordance with the CD13 expression data, specific binding of the cNGR conjugated label to the infarct zone was enhanced at 4 and 7 days after induction of the infarct (Figure 3A), but not at 1 or 14 days. The local advantage of the cNGR probe was 2.28±0.14 at 4 days after MI and 2.26±0.3 at 7 days after MI. Fourteen days after MI an advantage of 1.37±0.49 was observed, which was similar to unconjugated OG488 at 1.33±0.08.

These results show that at 4 and 7 days after MI cNGR specifically targets to the BZ and IA. However, the limited resolution of in vivo fluorescence microscopy did not allow visualization of the exact target of cNGR at the cellular level.

Macroscopic Targeting to the Target Area
To study the relation between the necrotic area and cNGR labeling we compared macroscopic fluorescent labeling with TTC staining for the 4- and 7-day specimens. TTC staining did not influence the fluorescent intensity (data not shown). The areas showed an excellent match with a correlation coefficient of 1.01 with an R²=0.90, P<0.001 (Figure 3B).

TPLSM: Cellular Localization of cNGR After In Vivo Injection
TPLSM was used to identify the cellular target of cNGR. Intravenous injection of OG488 (n=2) did not result in tissue staining, neither in the BZ and IA nor in the control areas (not shown). Likewise, in non-infarcted hearts or control tissues of infarcted hearts, we were unable to detect any cNGR binding after intravenous injection of cNGR, linked to OG488, QD525, or QD585 (n=3). Postmortem staining of tissue with
eosin and Syto44 resulted in clear visualization of myocyte cytoplasm and cell-nuclei (of both myocytes and vascular cells; Figure 4A), respectively. After injection of anti-CD31-PE (n=2) networks of interconnected small vessels surrounding myocytes and networks of larger vessels were distinguishable (Figure 4B). Additional staining with Syto44 showed myocytes with one or more nuclei, surrounded by multiple capillaries (Figure 4C). After co-injection of anti-CD31-PE and anti-CD13-fluorescein isothiocyanate (FITC), noninfarct heart tissue showed presence of endothelial CD31 in vascular patterns with occasional co-localization of CD13 at the cellular level (Figure 4D; please see http://atvb.ahajournals.org for a movie of these measurements). Subcellular co-localization was not always complete as judged by the absence of yellow hue typical of red and green overlay. This is in accordance with the predominant basolateral location of CD3112 and the apical location of CD13.

The findings in the IA and BZ are summarized in Figure 5. In these areas autofluorescence was prominent (n=4). Injection of cNGR-QD585, cNGR-QD525, or cNGR-OG488 (n=4) all resulted in similar labeling patterns, although fluorescence of QD was brighter and less bleachable than that of OG488. Further-
more, 2 hours after excision of the hearts, cNGR-OG488 fluorescence had disappeared, even in parts of the hearts that had not been illuminated before. In contrast, the fluorescence of cNGR-QD525 or cNGR-QD585 remained present for hours. This indicates more stable binding of QD to the tissue, probably attributable to the multivalency of QD. Because of the irregular pattern of fluorescent spots, the exact cellular location of the cNGR binding site could not be discerned. However, co-injection of anti-CD31-PE with cNGR conjugates revealed preferential localization of cNGR to endothelial cells of capillaries and larger vessels with an inner diameter up to 15 μm (Figure 5A and 5B). Although many such small vessels exhibit cNGR staining, we want to stress that not all do. CD13 presence on endothelium of small and large vessels was confirmed by double labeling with anti-CD13-FITC and anti-CD31-PE (n=2, Figure 5C). Whereas myocytes were scarce, anti-CD13–FITC staining showed that CD13-positive macrophages were abundant. cNGR-OG488 did not stain these macrophages (data not shown). Injection of cNGR-QD585, immediately followed by injection with anti-CD13–FITC showed that cNGR binds only to a subset of CD13-positive endothelial cells (Figure 5D).

To test whether cNGR specifically binds to angiogenic endothelial cells, experiments using anti-CD105-FITC were performed, both in healthy (n=3) and infarcted (n=4) tissue. In noninfarcted tissue of both MI and healthy mice CD13 had low expression in the microvascular endothelium illustrated by co-localization with CD31 (Figure 5E). However, in IA or BZ tissue, CD105 expression was much higher (Figure 5F) and also present in larger vessels. Co-labeling of CD105 (FITC) with cNGR (QD585) showed strong co-localization of both probes in IA and BZ (Figure 5G). In control tissue of MI mice only CD105 was stained, with staining pattern similar as in Figure 5E. cNGR staining was not observed (not shown).

In summary, intravenously injected cNGR binds specifically to the endothelium of small vessels in the BZ and IA and not in normal myocardium. cNGR specifically targets cells that over-express CD105, ie, endothelial cells that are actively involved in angiogenesis.

**Discussion**

In this study, in a murine model of cardiac angiogenesis, we show for the first time to our knowledge that the cNGR peptide homing sequence co-localizes with its presumed vascular address CD13, by using 2 complementary optical imaging techniques, in vivo fluorescence imaging and ex vivo TPLSM. In our studies, we lend further support to the notion that cNGR binds to CD13 on vessels in angiogenic areas. First, we demonstrated that cNGR-OG488 labeling was restricted to the BZ and IA at 4 and 7 days after MI with a local advantage of 2.3. Earlier histological observations in this model\(^{10}\) that are supported by the current TPLSM data showed that the infarct and border zone were highly vascularized at this time. Second, as opposed to the plasma T\(_{1/2}\) of the cNGR label, the tissue residence time of cNGR label was 9.1 hours, and this could be competed out with unlabeled cNGR, showing reversibility of binding and specificity of the cNGR moiety. Third, cNGR only bind to cells that express CD13 in vitro. Fourth, spatial co-localization and correlation between the necrotic infarct area and the area covered by the cNGR label was extremely high. Finally, TPLSM clearly established co-localization of cNGR, both as a monoconjugate, and as a multivalent particle, and CD13 on the endothelium of blood vessels in angiogenic areas after MI.

Previous immunohistochemical studies show that CD13 is preferentially expressed on angiogenic vessels in tumor bearing mice and on vessels in human cancer samples.\(^{7,14}\) In this study we extended these observations to ischemia and inflammation driven neovascularization in the heart. We established that CD13 is expressed in the infarct area and border zone by real-time PCR. No CD13 upregulation was observed in non risk areas, in accordance with earlier findings that adaptation of capillary microstructure fails to occur in viable tissue after MI.\(^{15}\) As a next step, we took advantage of optical sectioning, high-resolution (0.5×0.5×1 μm) and large penetration depth of TPLSM to obtain a more comprehensive picture of CD13 distribution on angiogenic and quiescent vessels in this model.\(^{16}\) Indeed, TPLSM allowed us to generate 3D- images of the vasculature in the target areas (see supplemental data).\(^{17}\) By combining multiple probes in vivo and with additional staining ex vivo, we were able to show that CD13 is expressed on vessels in healthy myocardium, and is abundantly present on endothelium of capillaries and larger vessels in infarct areas and their border zones. In accordance to the known expression pattern of CD13 we also found CD13 labeling on macrophages.\(^{18}\) Remarkably, however, cNGR binding was limited to endothelial CD13 in angiogenic infarct and border zone areas, based on co-localization with CD105 and CD31 and absence of binding to CD13-positive vessels in the healthy myocardium. Also, cNGR bound preferentially (≥70%) to vessels with an inner diameter below 15 μm and not to larger CD13 was positive vessels. It has been suggested that cNGR targets a subset of posttranslationally modified CD13 that might be specific to smaller and perhaps newly formed vessels. For instance, earlier work in human tumor specimens indicated that different antigenic forms of CD13 were expressed on normal epithelial cells in the brush border of renal proximal tubules and vascular endothelium in renal carcinoma, and that cNGR only bind to tumor vessels.\(^{14}\) The observed co-localization with CD105 suggests that cNGR binds to activated, not quiescent, endothelium.

RGD is another known tripeptide that labels activated endothelium by binding to the integrin α\(_5\)β\(_3\). Several studies in different angiogenic vascular beds have been performed using an RGD conjugated contrast agent. Both radiolabeled RGD in a canine MI model\(^{19}\) and magnetic resonance-focused paramagnetic-tagged RGD in a mouse tumor model\(^{20}\) have been used to image neovascularization noninvasively in vivo.

In phage display selective for tumor vessels, both RGD and RGD were found to bind to these vessels.\(^{4}\) However, competition studies with NGR and RGD phage showed that they both home to different targets in tumor endothelium, and that the homing ratio (tumor/control organ) for NGR was 3-fold greater than for RGD.\(^{4}\) We therefore chose to pursue the NGR peptide and used the cyclic form that has a 10-fold greater targeting efficacy than linear modalities.\(^{21}\)

In this study we used a monovalent contrast label, OG488, and a multivalent label, Quantum Dots. QD are decorated with up to 20 cNGR moieties and have a higher intrinsic fluorescence energy with lower bleaching rates than OG-488. Multivalent targeting particles have been proposed to bind with higher...
avidity than a monovalent homing peptide.\(^{22}\) In accordance with these observations, we were more successful with Quantum Dots than with cNGR-OG488 to show robust differences between CD13-poor and CD13-rich cells. Also in vivo, the QD probes displayed greater signal intensity and longer persistence of fluorescence than cNGR-OG488, despite \(\geq 400\)-fold lower dose. Conjugating QDs to gadolinium renders them useful in magnetic resonance imaging.\(^{20}\) Hence, we show that QD decorated with peptide homing sequences are a useful tool for obtaining proof of principle targeted optical imaging. In the near future cNGR-binding to angiogenic vessels might be shown noninvasively by conjugating cNGR to either radiophores or paramagnetic particles such as gadolinium.

The ultimate goal is to translate this principle into a clinical imaging technique such as MRI. By combining targeted vascular imaging with cNGR-labeled magnetic resonance imaging contrast agents and perfusion-sensitive imaging techniques that we have developed,\(^{23}\) we will be able to acquire signals from the neovascu-
lar changes in combination with measurements of myocardial perfusion, cardiac function, and extent of myocardial scar. The biggest challenge is to concentrate sufficient label at the target to boost the inherently low signal to noise ratio of magnetic resonance imaging.\(^{3}\) QDs might be a useful tool to achieve this.

Thus, we show that, in the heart, CD13 is preferentially upregulated in angiogenic areas, and that it can be targeted effectively with monovalent and multivalent cNGR conjugates. Importantly, we demonstrate that the homing sequence binds its endothelial target only in angiogenic areas of the heart, and for the first time to our knowledge we show co-localization of a peptide homing sequence with its target after injection in vivo. Nanoparticles decorated with cNGR are effective optical imaging agents, and in a translational step can be further developed into particles for targeted magnetic resonance imaging of vessel growth and targeted delivery of agents that stimulate this process. The concept of treating and monitoring therapeutic effect at the same target holds great promise for the development of clinically successful strategies that promote new vessel growth.

Acknowledgments

The authors thank Dr Wim Engels for his technical assistance with the TPLSM, Dr Rob Hermans’ contribution to the pharmacokinetic experiments, Dr Ben Janssen and their group for their help with the surgical procedures, and Dr Johannes Waltenberger for critically reviewing the manuscript. Additionally, we thank Denny Sonnemans, Niek van Stipdonk, Maria Vroomen, and Anique Janssen for their technical assistance.

Sources of Funding

This study was supported from a grant from the Dutch Heart Foundation (NHS 2001-B047) and by the Interuniversity Cardiology Institute of the Netherlands (ICIN 33) and the Dutch Scientific Organization (NWO 902-16-276).

Disclosures

None.

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Arterioscler Thromb Vasc Biol. 2006;26:2681-2687; originally published online September 21, 2006;
doi: 10.1161/01.ATV.0000245807.65714.0b
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/26/12/2681

Data Supplement (unedited) at:
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Materials and Methods

_Synthesis of cyclic NGR Peptides (cNGR) and cNGR-coated quantum dots_

It has previously been shown that the cyclic form of NGR has a ten-fold greater targeting efficacy than linear configurations of the same peptide sequence. Therefore, cyclic NAc-Cys-Asn-Gly-Arg-Cys-Gly-Gly-Lys(Ac)-NH$_2$ (cNGR) peptides were synthesized using tBoc-solid-phase peptide synthesis. The peptides were either acetylated, or labeled on the resin with Oregon Green 488 (OG488) or Biotin (Molecular Probes, Eugene, OR) through the $\varepsilon$-amino group of the lysine. After hydrogen fluoride (HF) -cleavage of the peptide-resin and oxidative folding of the crude product to obtain the internal disulfide bond, peptides were purified using High-Pressure-Liquid-Chromatography (HPLC) and then lyophilized.

Molecular masses were determined by electrospray ionization quadrupole mass spectrometry (ESI-Q-MS) and compared to the theoretical calculated masses based on average isotope composition. The molecular masses were as follows (theoretical between brackets): cNGR 874.3 (875.0), cNGR-OG488 1226.3 (1227.4) and cNGR-Biotin 1284.3 (1285.6). To obtain multivalent cNGR constructs, biotinylated cNGR peptides were bound to streptavidin-conjugated Quantum Dots (QD; Quantum Dot Corp., Hayward, CA). One of two QD variants were used, QD525 ($\lambda_{em}$=525 nm) and QD585 ($\lambda_{em}$=585 nm), depending on the emission wavelength of co-stains. cNGR coated Quantum Dots (cNGR-QD) were generated by mixing
60 µl of QD-streptavidin stock solution (2 µM), 125 µl PBS, and 15 µl cNGR-biotin (779 µM stock solution). Each QD contains approximately 20 streptavidin moieties, and each streptavidin can bind 4 biotin molecules. Since the ratio of cNGR-biotin to QD was >100 high loading of each QD with cNGR is ensured.

Real-Time Polymerase Chain Reaction

The hearts were cut in half along the long axis. One half was snap-frozen in liquid nitrogen; the other half was embedded in Tissue Tec®. Infarct size was measured on AZAN stained sections. Infarcts smaller than 25% of the left ventricular wall were excluded.

Total RNA was isolated from snap-frozen tissue, using the guanidine isothiocyanate method and purified by cesium chloride (CsCl) gradient. Mouse CD13 (Forward: 5’-ACACCACCTCCACCATCATC-3’; Reverse: 5’-TCCTCCACCGTAATTCTCA AAC-3’) and ribosomal 18S (Forward: 5’-TCAACACGGGAAACCTCAC-3’; Reverse:5’-ACCAGACAAATCGCTCCAC-3’) primers were designed using the prime program at the Center for Molecular and Biomolecular Informatics, Nijmegen, the Netherlands.

First-strand cDNAs (Ready-To-Go You-Prime First-Strand Beads, Amersham Pharmacia Biotech, Piscataway, NJ) were amplified in the I-cycler iQ™ Real Time PCR (Biorad Research Laboratories, Hercules, CA) in duplo. Each reaction tube (16.1 µl) contained 14 µl diluted cDNA, 200 µM dNTP, 1x reaction buffer (Biorad) containing 1:80,000 Cybergreen (Biorad), 2,5 U Taq DNA polymerase (Biorad) and 1 µM of each primer. The PCR started with 2 min at 94°C followed by 40 cycles of: 30 sec at 94°C, 30 sec at 55°C (CD13) or 65°C (18S) and 30 sec at 72°C.

Assessment of cNGR binding in cell cultures

A murine endothelial cell line that constitutively expresses CD13 (2F-2B) was used as positive control, and a murine hemangioendothelioma (EOMA) cell line served as negative
control. All cells were maintained in modified essential medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml).

Cells were seeded in a 24 wells-plate. Each well, containing $2.0 \times 10^4$ cells, was filled with growth medium (1 mL). Before labeling, the growth medium was removed followed by three washes of PBS over 2 hrs in an incubator. Just before labeling, new growth medium (0.5 mL) was added.

For staining with OG488 tagged cNGR, the buffer was replaced with a 10 µM solution of cNGR-OG488. As a control, untargeted OG488 (10 µM) was used. For staining with QD’s coated with cNGR, the buffer was replaced by a 20 nM solution of QD585-cNGR. Negative controls used buffer with or without streptavidin-coated QD585’s.

**In Vivo Fluorescence Microscopy**

The heart was exposed by thoracotomy and continuously moisturized at controlled temperature.

OG488 was visualized using a 470/525 filter (GFP-3, Omega Optics, Brattleboro, VT) and fluorescence was recorded with a CCD camera (C4742-95, Hamamatsu Photonics Systems, Hamamatsu City, Japan). Images were processed and analyzed using Hipic and AquaCosmos software (Hamamatsu Photonics Systems, Hamamatsu City, Japan). 3.

**Pharmacokinetics of cNGR-OG488 and OG488**

To study the pharmacokinetics of cNGR-OG488 and OG488 in male Swiss mice, plasma clearance of the intravenously administered label was determined. cNGR-OG488 (0.75µg/g) or OG488 (0.31µg/g) was injected through a catheter in the left jugular vein and plasma was sampled prior to and 5, 10, 20, 40, 60 min, 2, 4, 8, 12, 24, and 48 hrs after injection. OG488 fluorescence intensity in the samples ($\lambda_{ex}=496$nm, $\lambda_{em}=520$nm) was measured using an SLM Aminco fluorometer model 8100 (Spectronic Instruments, Rochester, NY). The plasma concentrations of OG488 or cNGR-OG488 at the various time-points were
calculated from the fluorescence intensities by comparing the plasma values to values obtained from a standard dilution curve of cNGR-OG488 up to 0.1 µg/ml PBS, which was generated in triplicate. Blood clearance parameters and distribution volumes were then calculated using inplot4 software (GraphPAD, San Diego, USA).

**TTC protocol**

After freezing, slicing and subsequent incubation of the tissue, both fluorescent and brightfield images were taken. Necrotic area and fluorescent area included areas with intensities exceeding the threshold level. The threshold was the sum of the mean intensity of the total slice and the standard deviation. Next, the size of the necrotic area and the fluorescent area relative to the area of the entire slice were determined and compared.

**Two-Photon Laser Scanning Microscopy (TPLSM)**

The cNGR-conjugates (cNGR-OG488 or cNGR-QD) were injected alone or in combination with anti-CD13 labeled with FITC (100 nm in 200 µl, green, λ<sub>em</sub>=515 nm, BD Biosciences, Palo Alto, CA), anti-CD31 labeled with PE (50 µg in 25 µl, red, endothelial cells, λ<sub>em</sub>=580 nm, Pharmingen, San Diego, CA) or anti-CD105-FITC (Endoglin; 55 µg in 25 µl, green, active endothelial cells, λ<sub>em</sub>=515 nm, BD Biosciences).

A BioRad 2100MP laser scanning microscope was used in standard multi-photon configuration fitted with an upright Nikon E600FN microscope (Nikon-Uvikon, Bunnik, the Netherlands). For excitation a Tsunami Ti:Sapphire laser (800 nm, 100 fsec pulse width, 82 MHz repetition rate, SpectraPhysics, Eindhoven, the Netherlands) was used. The tissue was scanned with a 60x water dipping objective (numerical aperture 1.00, working distance 2 mm) that had additional optical zoom in the scan head. Three separate photomultipliers accepting different wavelength spectra (410-490 nm, 508-523 nm, >570 nm) were used in open pinhole
mode. No more than 3 probes were used simultaneously to allow clean filter separation of fluorescence. Since no attempt was made to quantify fluorescence, spillover of fluorescence in adjacent channels was inconsequential. Due to such spillover eosin appears as purple in the final images. Kalman filtering was used on each color image before combining them into one multicolor image. An imaging speed of 1 Hz with a pixel dwell-time of 3.9 µs, and power at the sample of maximally 40 mW was used for all TPLSM imaging.
References Online


Results Online

cNGR binds to endothelial CD13 in vitro

To study specificity of cNGR binding to CD13, *in vitro* studies were carried out. The mono-valent cNGR-OG488 conjugates did not bind to CD13 expressing cells (not shown). However, cNGR-coated QD585 showed robust staining of CD13-positive 2F-2B cells (fig. 1A, in red), and did not bind to CD13-negative EOMA cells (fig. 1B). Upon exposure of either cell type to a solution without QD or QD585 without cNGR, only very low non-specific fluorescence was observed. These data show that cNGR coated QD bind specifically and with a stronger fluorescent signal to CD13 positive cells.

Macroscopic targeting to the target area

To study the relation between necrosis and cNGR labeling we compared macroscopic fluorescent labeling with TTC staining (Fig. 2) for the 4 and 7 days specimens. The areas showed an excellent match, confirming that the cNGR-probe bound in the infarcted area.
Fig. 1 Positive 2F-2B (A, in red) and negative EOMA (B) cells after incubation for 2 hours in buffer with QD585-cNGR. Incubation of 2F2B cells in buffer without QD’s or with QD585-streptavidin resulted in images similar to (B). Scale bar = 25 µm
Fig. 2

Fig. 2 Brightfield (BF) image of a TTC treated slice (left) and the corresponding fluorescent image (right). Note the similarity in infarct area, indicating that cNGR bound only in the infarcted area of the heart.