A Novel Monoclonal Antibody for RAGE-Directed Imaging Identifies Accelerated Atherosclerosis in Diabetes

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Diabetes has become a worldwide epidemic and is a major risk factor for coronary artery disease. Atherosclerosis in diabetic patients takes an accelerated course. Assessing total plaque burden is important in tailoring individual patient therapy. Advances in molecular biology over the past 10 years have identified potential sites in atherosclerotic plaque that can be targeted with probes producing signals that can be detected by external imaging. Experimental and clinical studies have reported the feasibility of detecting signals from atherosclerotic plaque using probes in nanomolar concentrations. Targets below the resolution of imaging devices can be detected as beacons if there are abundant binding sites and low background activity.

Receptor for advanced glycation end products (RAGE) is a member of an immunoglobulin superfamily expressed at low levels in adult tissues in homeostasis but highly expressed in atherosclerotic lesions (1–3). RAGE was initially identified and characterized as the cellular binding and interaction site for advanced glycation end products (AGEs) resulting from glycation or oxidation of proteins and lipids during normal aging and from the accelerated degree of hyperglycemia in diabetes (4,5). The ligand-enriched environment in diabetes increases the expression of RAGE. Binding of AGEs to RAGE induces multiple signaling pathways involved in plaque progression and inflammation. These receptors also bind the non–AGE-related proinflammatory markers S100/calgranulins, amphoterin, and extracellular newly identified RAGE-binding protein (6,7). Administration of RAGE antagonists to diabetic apolipoprotein E null (Apoe−/−) mice attenuates vascular injury and greatly attenuates the initiation and acceleration of atherosclerosis (8,9). These findings show that RAGE expression is greater in diabetic than nondiabetic atherosclerosis and plays an important role in accelerating the disease in diabetes.

In a proof-of-concept paper, we showed the ability to visualize uptake of an F(ab′)2 fragment of a 99mTc-labeled polyclonal antibody in atherosclerotic plaque in the proxim...
imal aortae of 20-wk-old nondiabetic ApoE<sup>−/−</sup> mice using planar imaging (10). The generation of monoclonal antibodies is now a standard and increasingly routine procedure. Because the hybridoma cell lines are immortal, there is an unlimited source of the monoclonal antibody. Compared with polyclonal antibodies, monoclonal antibodies show reduced nonspecific binding and reduced nonspecific background activity, and we therefore developed a monoclonal antibody directed against the same peptide sequence on the extracellular receptor domain of RAGE. The hypothesis of the present study was that in age-matched ApoE<sup>−/−</sup> mice, uptake of a radiolabeled fragment of the monoclonal anti-RAGE antibody would be greater in diabetic than in nondiabetic mice and would be a marker of the accelerated course of atherosclerosis in these diabetic animals.

**MATERIALS AND METHODS**

**Experimental Model**

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Columbia University. Male ApoE<sup>−/−</sup> mice that backcrossed more than 10 generations in the C57BL/6 background were obtained (Jackson Laboratories). The RAGE<sup>−/−</sup>/ApoE<sup>−/−</sup> mice (n = 6) were generated by backcrossing RAGE<sup>−/−</sup> mice on the C57BL/6 background into ApoE<sup>−/−</sup> mice of the Institutional Animal Care and Use Committee of Columbia University. Male ApoE<sup>−/−</sup> mice on the same background for 10 generations in the C57BL/6 background were obtained (Jackson Laboratories). The RAGE<sup>−/−</sup>/ApoE<sup>−/−</sup> mice (n = 6) were generated by backcrossing RAGE<sup>−/−</sup> mice on the C57BL/6 background into ApoE<sup>−/−</sup> mice of the same background for 10 generations (11). At age 6 wk, ApoE<sup>−/−</sup> mice (n = 8) were made diabetic via 5 daily intraperitoneal injection of streptozotocin (Sigma), 50 mg/kg in citrate buffer (0.05 mol/L, pH 4.5), resulting in insulin deficiency (12). Control ApoE<sup>−/−</sup> mice (n = 8) received citrate buffer only. All animals were studied at age 24 wk.

**Development of Monoclonal Anti-RAGE Antibody**

We developed a novel antibody in rabbits against the V-domain of RAGE designed to display immunoreactivity in mice, pigs, and humans. Based on Genbank sequences of human, murine, and porcine RAGE, the following sequence alignment was determined and peptide prepared (13): human, 103-NRRGKETKSNYRVRVYQIP-121; murine, 102-NRRGKVEKSNYRVRVYQIP-120; and peptide, 1-NRRGKVEKSNYRVRVYQC-19.

For hybridoma development, 20 mg of the peptide Ac-NRRGKVEKSNYRVRVYQC-amide was produced (Quality Controlled Biochemicals). The peptide was conjugated to carrier molecule keyhole limpet hemocyanin, and 15 BALB/c mice were immunized with the conjugated peptide (Strategic BioSolution). Test bleeds were obtained to evaluate polyclonal antisera binding by screening enzyme-linked immunosorbent assay. Fusion was successful and supernatants selected to test the hybridomas. The hybridomas were subcloned and the best clones selected. Monoclonal antibodies were produced in vitro and purified by protein A and low endotoxin units (less than 3 endotoxin units/mg of purified antibody).

The immunoreactivity of diethyleneetriaminepentaacetic acid (DTPA)–modified antibody was tested by enzyme-linked immunosorbent assay using soluble RAGE antigen-coated microtiter plates. Binding of the anti-RAGE F(ab′)<sub>2</sub> to the receptor was compared with that of unmodified anti-RAGE IgG using horse-radish peroxidase–conjugated secondary antirabbit IgG. The antibody concentration, which gave 50% of maximum binding with anti-RAGE F(ab′)<sub>2</sub>, was 0.2 μg/mL, which is equivalent to 2 × 10⁻⁹ mol/L or apparent affinity of 0.5 × 10⁶ L/mol. The 50% of maximum binding concentration of unmodified anti-RAGE IgG was 0.4 μg/mL, which is equivalent to 2 × 10⁻⁹ mol/L or apparent affinity of 0.5 × 10⁶ L/mol.

**Preparation of F(ab′)<sub>2</sub> Fragments and Radiolabeling**

F(ab′)<sub>2</sub> fragments of the purified anti-RAGE antibody were prepared as previously described (10). These fragments have more antigen binding sites available than Fab and faster blood-pool and renal clearance than whole antibody. Direct coupling of anti-RAGE F(ab′)<sub>2</sub> antibodies to DTPA (Sigma) for ⁹⁹mTc labeling was performed as previously described (10,14). The mean specific activity was 6.58 ± 0.68 MBq/μg of protein, and the mean radiopurity was 98% ± 0.54% by instant thin-layer chromatography. The mean injected ⁹⁹mTc dose was 15.14 ± 1.23 MBq.

For the first 3 mice, serial drops of blood were obtained from tail vein nicking at 5, 10, 30, 60, 120, 180, and 360 min and 24 h and counted to measure blood-pool clearance and determine the optimal time for imaging after tracer injection.

**SPECT**

Whole-body in vivo SPECT (HiSPECT; BioScan) was performed on all mice 4 h after injection of radiolabeled anti-RAGE antibody fragment. SPECT images were acquired with specially designed multipinhole collimators with 1.0-mm pinhole tungsten apertures mounted on a triple-detector γ-camera mounted on a triple-detector γ-camera (Prism 3000XP; Picker). The acquisition was set up for the photopeak for ⁹⁹mTc imaging (140 keV, 10% energy window) with the following parameters: step-and-shoot rotation, 90 s per step, 30° step in 360° rotation, and 15.3-cm radius of rotation. We acquired images with a 256 × 256 matrix and a reconstructed voxel size of 0.125 mm³.

**Image Analysis and Ex Vivo Counting**

At the end of imaging, the animals were sacrificed, the organs were removed and weighed, and organ-associated radioactivity was counted in a γ-counter (Wallac Wizard 1470; PerkinElmer) for determination of the percentage injected dose (%ID) of radiotracer per gram of tissue. Absolute quantification of the reconstructed SPECT images was performed in Interview XP software (Mediso) using region-of-interest drawings in the transaxial plane. The system is calibrated for absolute quantification using specially designed rat- and mouse-shaped phantoms filled with known levels of ⁹⁹mTc imaged with the same protocol used for the animal studies. This quantification technique has been shown to be better than 4% accurate when performed with the same methodology (HiSPECT and Mediso software) on a comparable imaging system (15). The quantitative uptake in the aorta, in megabecquerels, was divided by total injected dose to obtain %ID.

**Histology**

The proximal aortae were harvested by perfusion fixation for 10 min with 10% neutral buffered formalin. Tissues were fixed for 24 h in 10% formalin, followed by paraffin embedding. A 400-μm section of the proximal aorta from the aortic valve leaflets was excised. Serial 5-μm-thick sections were cut and stained with hematoxylin–eosin for morphology and for immunohistochemistry.

For cellular characterization of atherosclerotic lesions, immunohistochemical staining was performed as previously described (10). Briefly, tissue sections were deparaffinized in xylene, and endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide.
peroxide. Tissue sections were then incubated in protein-free block (Dako) for 10 min to inhibit the nonspecific binding of primary antibody. Staining for RAGE was performed using antibody against RAGE (50 μg/mL). Macrophages were identified using the marker Mac-3 (1:20; BD Pharmingen). Smooth muscle cells were identified using a primary antibody HHHF-35 against α-actin (1:250; Sigma). Control immunostaining was performed using the respective nonspecific IgG. Detection was performed with horseradish peroxidase-conjugated respective secondary antibody. Color was developed with 3,3′-diaminobenzidine (DAB substrate kit; Vector Laboratories) and counterstaining with the Gill hematoxylin solution. Slides were photographed and saved as digital files.

Morphometric analyses of the arterial segments were performed using a Nikon microscope and Image Pro Plus software (Media Cybernetics Inc.). The amount of aortic lesion formation in each animal was measured as percentage lesion area per total area of the aorta (16). Cells staining positively for a particular chromogen were identified using a color recognition method, and the number of nuclei from positively staining cells was divided by the total number of cells in the tissue cross section.

Dual-label confocal microscopy studies were performed to determine the cell types expressing RAGE in atherosclerotic lesions. Aortic sections were subjected to immunofluorescent colocalization of RAGE with macrophages and smooth muscle cells by overnight incubation at 4°C with the respective antisera as described above. The sections were then incubated with fluorescent conjugated secondary antibodies (Texas Red antirabbit, fluorescein isothiocyanate antismooth muscle and antirat; Vector Laboratories) at 1:200 at 4°C for 2 h. When the Texas Red and fluorescein isothiocyanate signals colocalize, a yellow signal is seen.

RESULTS

Blood-Pool Clearance

Blood-pool clearance of the 99mTc-labeled monoclonal F(ab′)_2 was measured, to determine the optimal time for imaging. The clearance is biexponential, with the half-time equal to 20 min for the first (fast) component and 7 h for the second (slower) component. From these data, we determined that the optimal time for imaging is 4 h after tracer injection.

Detection of RAGE in Atherosclerotic Plaques

In vivo imaging of diabetic ApoE−/− mice showed focal accumulation of the tracer in the thorax corresponding to the location of the proximal aortic atherosclerotic lesion. An example from one experiment is shown in Figure 1A. [Fig. 1] The nondiabetic ApoE−/− mice also showed tracer uptake in the thorax corresponding to the proximal aorta, but the uptake was less intense (Fig. 1B). Control ApoE−/−/RAGE−/− mice showed no uptake of the radiotracer in the aortic region of the thorax, and histologic examination of the aorta revealed minimal lesions (Fig. 1C). Atherosclerotic ApoE−/− mice injected with a control—irrelevant 99mTc-DTPA–labeled antibody fragment—showed no tracer uptake in the thorax although the in situ dissection of the aortic arch showed extensive atherosclerotic plaque (Fig. 1D). From regions of interest drawn on the scans around the focal uptake in the thorax, uptake in the diabetic ApoE−/− group (1.39 ± 0.16 × 10−2 %ID) was significantly greater than that in corresponding locations in the nondiabetic ApoE−/− group (0.48 ± 0.27 × 10−2; P < 0.0001).

We confirmed tracer uptake in the proximal aorta by ex vivo radioactivity counting of aortic tissues. There was a significant difference between the diabetic and nondiabetic ApoE−/− mice (0.36 ± 0.19 vs. 0.052 ± 0.028 %ID/g; P = 0.002) (Fig. 2). The radiotracer uptake in the aorta of nondiabetic mice was greater than in ApoE−/−/RAGE−/−
mice, but the significance was borderline ($P = 0.05$) by 2-tailed testing.

Biodistribution studies of radiolabeled anti-RAGE F(ab')$_2$ in nontarget organs of diabetic and nondiabetic mice were performed (Fig. 3). The highest radiotracer uptake in both groups was in the liver and spleen.

**Histologic Characterization of Atherosclerotic Lesions**

Aortic sections from diabetic ApoE$^{-/-}$ mice showed mainly American Heart Association class III lesions, whereas nondiabetic mice showed mainly American Heart Association class II lesions. The mean cross-sectional area of the aortic lesions, expressed as percentage lesion area of total aortic area, was 37.1% ± 16.1% (range, 16.4%–66.5%) in diabetic ApoE$^{-/-}$ mice and 20.7% ± 9.5% (range, 8.9–33.5) in nondiabetic ApoE$^{-/-}$ mice ($P = 0.04$).

Immunohistochemical staining of the proximal aorta identified higher expressions of macrophages and RAGE in diabetic ApoE$^{-/-}$ mice than in nondiabetic mice (Fig. 4A). Total RAGE and macrophage burden in the atherosclerotic lesions was quantified. The percentage of RAGE-positive cells in the lesions in diabetic ApoE$^{-/-}$ mice (37.7% ± 6.9%) was increased more than 2-fold over the percentage in nondiabetic mice (15.9% ± 4.7%, $P = 0.0008$). Similarly, total macrophage burden was significantly increased in the lesions in diabetic mice (28.2% ± 7.6%), compared with nondiabetic mice (13.4% ± 4.8%, $P = 0.0004$). The specificity of anti-RAGE antibody was confirmed by lack of staining of aortic lesions in control ApoE$^{-/-}$/RAGE$^{-/-}$ mice (Fig. 4B). Dual immunofluorescent staining for RAGE-expressing cells showed RAGE to colocalize predominantly with macrophages (Fig. 5).

**Quantitative In Vivo RAGE Uptake Versus Quantitative Histomorphometry**

For regression analysis, we plotted individual values for radiotracer uptake in the proximal aorta against percentages...
of RAGE-positive cells and macrophage-positive cells. Radiotracer uptake correlated strongly with RAGE expression \((r = 0.82, P = 0.002)\) (Fig. 6A) and with macrophages \((r = 0.86, P < 0.0001)\) (Fig. 6B). There was also a significant positive correlation between radiotracer uptake in the proximal aorta from scans and ex vivo radioactivity counts of the proximal aorta \((r = 0.82, P = 0.0001)\).

**FIGURE 5.** Immunofluorescence double staining for cells expressing RAGE in aortic lesion in ApoE \(^{-/-}\) mouse. Shown is confocal microscopy of serial sections double-immunofluorescently labeled for RAGE (anti-RAGE, Texas Red), for endothelial cells (FVIII, fluorescein isothiocyanate), for smooth muscle cells \((\alpha\text{-actin, fluorescein isothiocyanate})\), and for macrophages (Mac-3, fluorescein isothiocyanate). Areas in yellow represent colocalization. SMC = smooth muscle cell; EC = endothelial cells. (x200)

**DISCUSSION**

The report describes follow-up data of a previously published proof-of-concept study with a polyclonal anti-RAGE antibody. We subsequently developed a novel monoclonal antibody directed against the V-domain of RAGE designed to display immunoreactivity in mice, pigs, and humans and radiolabel the \(\text{F(ab')}_2\) fragments. This present study compared uptake of the monoclonal antibody in atherosclerotic lesions of the proximal aorta in ApoE \(^{-/-}\) mice with and without diabetes. The quantitative uptake of radioactivity in the target lesion correlated both with RAGE expression and with macrophages by quantitative histomorphometry.

Progress in the field of vascular biology coupled with pathologic studies of human autopsy material has delineated the course of plaque evolution and signals of plaque vulnerability. The latter include increased lesional macrophages indicating inflammation, apoptosis of macrophages in the shoulder regions of thin-cap fibroatheromas, and activation of metalloproteinases in the connective tissue of plaques \((11)\). Imaging studies have targeted inflammation with \(^{18}\text{F-FDG}\) \((17)\), apoptosis in the plaque with annexin-V \((18)\), and metalloproteinase expression with radiolabeled broad-based matrix metalloproteinase inhibitor \((19)\). This study extended targeted imaging of atherosclerosis to imaging RAGE.

Binding of AGEs to RAGE induces multiple signaling pathways involved in plaque progression. RAGE is highly conserved across species. Several reports using human material have studied RAGE expression in atherosclerotic plaques \((4,5)\). One study used plaques obtained from patients undergoing carotid endarterectomy, and the other used coronary arteries from subjects who had sudden cardiac death. Both studies showed greater immunoreactivity for RAGE in atherosclerotic tissue from diabetic patients than from nondiabetic patients. In addition, there were greater numbers of inflammatory cells (macrophages, T lymphocytes) in the plaques, and the cells stained positively for RAGE. As a marker of inflammation, RAGE
is similar to other targeted radiotracers that colocalize with macrophages. It is unique because it also represents a marker of prolonged hyperglycemia and plays a role in complications of diabetes, including atherosclerosis, limb ischemia, and neurologic disorders.

This study had several limitations: the choice of DTPA as chelator, the relatively large size of the antibody fragment, and the lack of hybrid SPECT/CT. DTPA has proven to be a useful chelator for binding radiotracers to antibody fragments, but the binding kinetics of other chelators such as hydrazinonicotinamide have proven to be superior and are largely replacing DTPA for reliable labeling of proteins with radionuclides. The low %ID in the aortic lesions could partially be explained by loss of radionuclide signal through dissociation from the antibody fragment. We plan to explore other chemical approaches to labeling the antibody fragment with $^{99m}$Tc. The slow blood-pool clearance of the F(ab$'$)$_2$ fragment due to its size is a drawback for in vivo imaging. However, for maximal labeling of targets in the vascular wall, there is some advantage to the longer exposure of the receptors to the ligands afforded by residual blood activity. We plan to develop the single-chain variable fragment to the monoclonal anti-RAGE antibody in the hopes that this smaller fragment with more rapid blood-pool clearance will still have an adequate exposure time to the binding sites. Hybrid imaging with SPECT and contrast CT would provide the opportunity to localize the focal uptake of the radiotracer in the thorax to vascular structures. We now have the capability to perform hybrid imaging of atherosclerosis in mice.

**CONCLUSION**

In a previous publication, our group documented in vivo uptake of radiolabeled polyclonal anti-RAGE antibodies on planar scans in the proximal aortae of 20-wk-old ApoE$^{-/-}$ mice fed a Western-type diet. This present report extends our previous work in several important ways. We used a newly developed monoclonal anti-RAGE antibody that shows improved specificity, compared with the polyclonal antibody. We compared uptake in proximal aortic atherosclerotic lesions in ApoE$^{-/-}$ mice both with and without streptozotocin-induced diabetes. Most importantly, we performed SPECT and correlated the radiotracer uptake in the lesions from the scans with quantitative staining both for RAGE and for macrophages. There were excellent correlations with both variables across a range of lesion sizes and histologic types, including both diabetic and non-diabetic mice. In mice genetically prone to atherosclerosis, $^{99m}$Tc-labeled anti-RAGE F(ab$'$)$_2$ SPECT identified early accelerated lesions in diabetic, compared with non-diabetic, animals. This technique for in vivo nondestructive quantification of RAGE expression may have applications in assessing novel therapies in experimental animals and possibly in humans.

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