Optical Imaging of Hydroxyapatite in the Calcified Vasculature of Transgenic Animals

Atif Zaheer, Monzur Murshed, Alec M. De Grand, Timothy G. Morgan, Gerard Karsenty, John V. Frangioni

Objective—To detect the hydroxyapatite component of vascular calcification in vivo so that the process of calcium deposition can be studied in transgenic model systems.

Methods and Results—We have previously developed a near-infrared fluorescent bisphosphonate derivative that binds with high affinity and specificity to hydroxyapatite, and an intraoperative near-infrared fluorescence imaging system for small animals. Using these tools, and a transgenic mouse strain with homozygous deletion of the matrix GLA protein (Mgp−/−), we demonstrate that the hydroxyapatite component of vascular calcification can be detected in vivo with high sensitivity, specificity, and resolution.

Conclusions—The hydroxyapatite component of vascular calcification can be detected optically, in real-time, without sacrifice of the animal. It is now possible to study the earliest events associated with vascular mineralization, at the cell and organ level, and to monitor the process in living animals. (Arterioscler Thromb Vasc Biol. 2006;26:1132-1136.)

Key Words: bisphosphonates • hydroxyapatite • matrix GLA protein • near-infrared fluorescence imaging • vascular calcification

Although calcification is a prominent feature of atherosclerosis, the mechanisms underlying it are not understood fully. Calcification was previously viewed as an end-stage process of passive mineralization. However, there is increasing evidence that vascular calcification is an active process, with the deposition of hydroxyapatite occurring in a similar fashion to bone formation. Subpopulations of calcifying vascular cells in the artery wall undergo a similar sequence of events in vitro as do osteoblasts during differentiation. Recent data suggest that noninvasive detection of coronary calcification by computed tomography (CT) may be predictive of clinically important disease.

A transgenic mouse model useful for studying arterial calcification (in the absence of atherosclerosis) results from homozygous deletion of the matrix GLA protein (Mgp−/−). Mgp is a member of a family of mineral ion binding proteins characterized by γ-carboxylated glutamate residues. By 2 weeks of age, Mgp−/− mice have extensive calcification of the media of large and medium arteries, including the coronary arteries. By 5 to 6 weeks of age, the mice die secondary to rupture of ossified vessels. The medial calcification of Mgp−/− mice resembles the clinical phenomenon of Monckeberg’s sclerosis, which is seen with aging, diabetes, and renal failure. The calcification associated with atherosclerosis is typically in the intima rather than the media, and has been hypothesized to be the result of degeneration of elastic fibers in the media, active deposition from calcifying vascular cells, apoptosis, or other processes not yet discovered.

Common to all types of vascular calcification, and to bone formation itself, is deposition of the mineral hydroxyapatite (HA). Through complex processes involving ion substitution and adsorption, HA can be further “hardened” by the addition of other insoluble calcium salts. At present, there is no sensitive method to detect HA within vessels of living animals, or to distinguish HA from other calcium salts.

Near-infrared (NIR) (700 to 900 nm) light offers several advantages for the in vivo imaging of targets such as HA, including relatively deep photon penetration into tissue, low background autofluorescence, and relatively high photon yield from commercially available fluorophores. In fact, NIR fluorescence has recently been used to measure protease activity associated with atherosclerosis in vivo. We have already developed a NIR fluorescent derivative of pamidronate and have demonstrated its ability to image HA in the skeleton of living animals. We have also described an intraoperative NIR fluorescence imaging system for small animal surgery. In this study, we show that these tools can be used to image the HA component of vascular calcification in transgenic model systems.
Methods

Animals
Animal husbandry was provided by an Association for Assessment and Accreditation of Laboratory Animal Care–certified animal research facility, and animals were used in accordance with an approved institutional protocol. Four- to 5-week-old Mgp<sup>−/−</sup> (average weight 20 grams) or Mgp<sup>+/−</sup> control littermates<sup>6</sup> (average weight 25 grams), 5 of each strain and of random sex, were used for the experiments. Animals were anesthetized with 50 mg/kg intraperitoneal pentobarbital before imaging. Transgenic mice were derived at the animal facility of the Center for Comparative Medicine at the Baylor College of Medicine.

Reagents
Pam78<sup>15</sup>, the conjugate of pamidronate with the N-Hydroxysuccinimide ester of IRDye78 (LI-COR, Lincoln, Ne), and the carboxylic acid form of IRDye78<sup>16</sup> (IRDye78-CA) were prepared as described previously. HA (Catalog #391947) was from Calbiochem (La Jolla, Calif), Calcium carbonate (Catalog #C-6763) was from Sigma (St. Louis, Mich). All other reagents were from Fisher Scientific (Hanover Park, Ill). Fluorophores were stored as a dry powder and desiccated at −80°C in the dark before resuspension in phosphate-buffered saline (PBS), pH 7.4, for injection.

Small Animal NIR Fluorescence Imaging System
Details of the small animal NIR fluorescence imaging system have been described in detail.<sup>14</sup> Briefly, NIR excitation fluence rate (725 to 775 nm) was 8 mW/cm² over a 10-cm diameter field of view. White light (400 to 700 nm) fluence rate was 1 mW/cm². Color video images were acquired with a model HV-D27 (Hitachi, Tarrytown, NY) camera and NIR fluorescence images were acquired with an Orca-ER (Hamamatsu Photonic Systems, Bridgewater, NJ) camera. The Orca-ER was set for no binning. As indicated, exposure times were 25 to 500 ms and camera gain was between 50% and 100%. Computer control was via IPLab software (Scanalytics, Fairfax, Va). To create merged images, the NIR fluorescence image was pseudocolored in lime green and overlaid with the anatomic (color video) image as described previously.<sup>14</sup>

In Vivo Imaging
The lateral tail vein was injected with 0.1 μmol/kg (2.6 nmol into a 25-gram mouse) of Pam78 diluted into 80 μL of PBS. This dose was previously optimized for in vivo imaging.<sup>15</sup> Images were acquired at 4 hours after injection. For imaging cutaneous vessels, fur was removed with the depilatory Nair (Carter-Horner, Montreal, Quebec) 4 hours after injection. For imaging cutaneous vessels, fur was removed with the depilatory Nair (Carter-Horner, Montreal, Quebec) 4 hours after injection. Animals were anesthetized with 50 mg/kg intraperitoneal pentobarbital before imaging. Transgenic mice were derived at the animal facility of the Center for Comparative Medicine at the Baylor College of Medicine.

Calcium Salt Specificity Experiments
HA and the oxalate, phosphate, pyrophosphate, and carbonate salts of calcium were incubated with 100 nM Pam78 for 30 minutes at room temperature with constant motion, then washed 4 times with a 100-fold excess of PBS before NIR fluorescence and microCT measurements.

Histological Analysis
Specimens for histological analysis were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into consecutive 5-μm sections, all without decalcification. Von Kossa staining was performed as described.<sup>18</sup> Crystal binding experiments and the NIR fluorescence microscope have been described previously.<sup>15</sup>

MicroCT Analysis
An eXplore Locus microCT system (GE Healthcare Biosciences, Waukesha, Wis) was used for measurement of calcium salt radiodensity, and correlation with Pam78-based NIR fluorescence measurements. The microCT was set to 80 keV, 450 μA, 100 ms exposure time, 4×4 binning, and a 95-μm final resolution. Calcium density, and correlation with Pam78-based NIR fluorescence measurements. The microCT was set to 80 keV, 450 μA, 100 ms exposure time, 4×4 binning, and a 95-μm final resolution. Calcium carbonate (Catalog #C-6763) was from Sigma (St. Louis, Mich). All other reagents were from Fisher Scientific (Hanover Park, Ill). Fluorophores were stored as a dry powder and desiccated at −80°C in the dark before resuspension in phosphate-buffered saline (PBS), pH 7.4, for injection.

Results
Chemical Synthesis of Pam78, HA Specificity, and Correlation With CT Radiodensity
The conjugation, purification, and optical properties of Pam78 have been described previously.<sup>15</sup> The specificity of Pam78 for HA over other calcium salts is shown in Figure 1A and quantified in Figure 1B. Pam78, but not its parent fluorophore IRDye78-CA (data not shown), bound specifically to HA, and not to other calcium salts, even though CT radiodensity for all calcium salts varied little (Figure 1B). Pam78-induced NIR fluorescence of HA was linearly correlated with CT radiodensity (Figure 1C).

The Hydroxyapatite Component of Vascular Calcification
We next asked whether Pam78 could be used as a histological stain to label the HA component of vascular calcification. The
homozygous \( Mgp^{-/-} \) mouse develops widespread calcification of the media of large and medium arteries by 3 to 4 weeks of age. Resection of the thoracic aorta and calcium staining by the method of von Kossa confirmed dense medial calcification in a band-like pattern (Figure 2). However, staining of a consecutive section using Pam78 revealed a more restricted pattern of HA deposition within the arterial wall, and specifically absent were the more amorphous areas of calcification seen with the method of von Kossa. Wild-type \( Mgp^{+/+} \) littermates did not exhibit any vascular staining with Pam78 (data not shown). Also, control staining with the parent fluorophore of Pam78 (IRDye78-CA) was negative (Figure 2). The difference in staining pattern between Pam78 and the method of von Kossa is best understood in terms of specificity. The former is specific for apatite-type crystals, whereas the latter stains a wide variety of calcium salts, regardless of their phosphate content18 (Figure 1A and 1B). Hence, Pam78 detects, specifically, the HA component of vascular calcification when used as a histological stain.

Detection of HA Deposition in the Living Animal

When injected intravenously, the low-molecular-weight ligand Pam78 (1388 Da) distributes freely throughout the body and binds tightly to sites of exposed HA.15 Blood clearance is rapid, with detectable signal in bone detectable within 15 minutes, and near complete clearance by 4 to 6 hours.15 When injected into 4-week-old \( Mgp^{-/-} \) mice and allowed to clear for 4 hours, Pam78 displayed a bright signal in medium to large arteries, in addition to a bright signal in bone. Calcified arteries of the skin could be visualized in the intact animal (Figure 3A) or after a surgical flap was prepared (Figure 3B). The signal to background ratio (SBR) of the NIR fluorescence signal in these calcified vessels was typically between 1.7 and 1.8 for a 125-ms exposure time at a fluence rate of 8 mW/cm². Interestingly, the veins (Figure 3A) were completely devoid of HA deposition, and some arteries demonstrated clear “skip” lesions of HA deposition (Figure 3B). Injection of the parent fluorophore (IRDye78-CA) did not show any NIR fluorescence in either bone or the vasculature, and injection of Pam78 into \( Mgp^{+/+} \) littermates showed only bone, but not vascular NIR fluorescence signal (data not shown). Hence, Pam78 can be used to detect the HA component of vascular calcification in vivo, without sacrifice of the animal.

Coronary Artery Calcification In Vivo

Given the known association of vascular calcification with coronary artery disease, we next asked whether Pam78 could be used to detect HA deposition in the coronary arteries. Four hours after intravenous injection of Pam78 into a 4-week-old \( Mgp^{-/-} \) mouse, and after exposure via thoracotomy, there was intense staining of the coronary arteries, with a SBR of \( \approx 1.6 \) for a 100-ms exposure (Figure 4). HA deposition was restricted to larger diameter arteries and ended abruptly after the second-order branch. Once again, there was absence of HA deposition in the coronary veins.

Hydroxyapatite Deposition in Visceral Arteries

Because aging and certain disease processes are associated with calcification of visceral arteries, we asked whether Pam78 could be used to image HA deposition in vital organs.

Figure 2. The thoracic aorta from a 3.5 week old \( Mgp^{-/-} \) mouse was cryo-preserved and consecutive sections stained using hematoxylin and eosin (H&E), the method of von Kossa, Pam78, or the parent NIR fluorophore IRDye78-CA. NIR fluorescent micrographs have identical exposure times and normalizations. The results are representative of three independent experiments.

Figure 3. In vivo imaging of vascular calcification in living \( Mgp^{-/-} \) mice. A, After depilatory treatment, veins (V) of the skin become apparent in the color video image. NIR fluorescence imaging 4 hours after intravenous injection of Pam78 reveals a highly calcified artery (A) running along the vein. SBR of the calcified artery in the dotted circle is 1.7 using a 125-ms exposure and 78% camera gain. Also visible in the NIR fluorescent image is the thoracic spine, as well as micro-hemorrhages in the skin caused by depilatory treatment. The results are representative of three independent experiments. B, Surgical flap of the skin reveals multiple arterial calcifications (arrows) with some vessels exhibiting skip lesions (arrowhead). SBR of the calcified artery in the dotted circle is 1.8 using a 125-ms exposure and 78% camera gain. The results are representative of 3 independent experiments.
Figure 4. Coronary calcification in transgenic Mgp<sup>+/−</sup> mice. Four hours after intravenous injection of Pam78, the heart was surgically exposed and imaged. Shown are the color video image, NIR fluorescence image, and a pseudo-colored (lime green) merge of the two. Rib (R) staining by Pam78 is also indicated. SBR of the calcified artery in the dotted circle is 1.6 using a 100-ms exposure and 100% camera gain. A hematoxylin and eosin (H+E) section through the heart (oriented by dotted line in NIR fluorescence image) is also shown, with position of the coronary arteries (C) indicated. The results are representative of 3 independent experiments.

For all organs examined, large and medium arteries demonstrated significant HA deposition, which was easily seen to be distinct from the venous system using the color/NIR fluorescence merged images of Figure 5. As shown, there was striking restriction of HA deposition to certain sizes of vessels and skip areas within some vessels.

Discussion

There has been much interest recently in osteoblast-like cells of the vasculature, and their participation in disease processes such as atherosclerosis. Our data suggest that it is possible to detect the earliest product of these cells, HA, either on histological sections or in the living animal, using Pam78 and NIR fluorescence. We have already described a simple fluorescence microscopy system that permits four color (blue, green, red, and NIR) imaging. After intravenous injection of Pam78, tissues could be frozen-sectioned, and the same section visualized with 3 different protein or genetic markers in addition to the NIR fluorescence signal of HA. Such a system could be used to correlate, directly, which cells in the vasculature deposit HA, how nearby cells influence the process, and if other molecules participate.

Optical detection of HA should prove extremely useful since standard decalcification techniques destroy this critical information. Calcification stains such as von Kossa overestimate HA deposition, and the sectioning process itself will often result in loss of calcium crystals. Pam78 provides sensitive and specific detection of HA, a critical early component of vascular calcification, and the primary mineral in medial calcification in humans. CT, ultrasound, and optical coherence tomography (OCT), however, generate contrast based primarily on object density, and cannot differentiate the type of calcium salt present. This distinction might be especially important in studies of older individuals, where HA deposition may have already occurred, and the “calcification” being imaged is from the deposition of non-HA calcium salts.

Our data show striking “skip” areas of HA deposition within the same artery, even in the absence of bifurcations or other obvious alteration in blood flow. Although we have no explanation for this phenomenon at present, it is possible that some type of vascular heterogeneity is playing a role. Understanding this heterogeneity at a cellular and molecular level may lead to therapeutic strategies to limit or reverse HA deposition.

As shown in Figure 1C, there is a linear relationship between Pam78-induced NIR fluorescence and CT radiodensity, permitting, if needed, absolute calibration of the NIR fluorescence signal. However, in most situations, qualitative or semi-quantitative analysis will be sufficient. For example, Pam78 could be used to compare HA deposition in one vessel to another in the same animal, or to monitor the effect of drug therapy on calcification initiation and/or progress over time. With respect to sensitivity and resolution, Pam78 detected calcification in 50 to 100 μm arteries after short (∼100 ms) exposure times with no camera binning, and with SBRs that suggest it could easily detect HA deposition at the limit of resolution of the imaging system (25 μm) if such calcification in smaller arteries and capillaries were actually present. The importance of using NIR fluorescence as the detection method is also highlighted by the fact that the arterial calcification of 4-week-old Mgp<sup>+/−</sup> mice could not be imaged using soft x-rays because of the extremely small size of the
vessels and the poor sensitivity of this method (data not shown).

Pam78 and NIR fluorescent light might be useful for screening vascular calcification in transgenic animals having complex genotypes, such as crosses of apoE and eNOS knockouts.\textsuperscript{13,14} We have previously demonstrated the use of reflectance NIR fluorescence imaging for noninvasive screening of modulators of adaptive thermogenesis in transgenic animals.\textsuperscript{13} The Weissleder group has previously demonstrated the use of tomographic NIR fluorescence imaging for noninvasive imaging of internal vasculature and vital organs in transgenic animals.\textsuperscript{17} Taken together, routine noninvasive detection of HA in animal models of disease should now be possible.

We have previously described an intraoperative NIR fluorescence imaging system for cardiac and other surgeries.\textsuperscript{14} In this study we show how such a system can be used to identify coronary calcification. Such identification may prove important for cardiac surgeons, who could use this information to avoid grafting directly onto calcified native vessels. Cardiac surgeons could also use the technology to assess heart valve calcification, especially given its sensitivity relative to x-ray imaging. Additionally, Pam78 could be used in conjunction with fluorescence angioscopy to detect coronary artery and/or valvular calcification during cardiac catheterization.

In summary, we have demonstrated that the HA component of arterial calcification can be detected optically and in real-time using NIR fluorescence and a targeted fluorophore. Such a system can now be used to study the earliest events associated with vascular calcification at the molecular, cellular, and animal scale.

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**References**

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