Detection of Neovessels in Atherosclerotic Plaques of Rabbits Using Dynamic Contrast Enhanced MRI and 18F-FDG PET

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Objective—The association of inflammatory cells and neovessels in atherosclerosis is considered a histological hallmark of high-risk active lesions. Therefore, the development and validation of noninvasive imaging techniques that allow for the detection of inflammation and neangiogenesis in atherosclerosis would be of major clinical interest. Our aim was to test 2 techniques, black blood dynamic contrast enhanced MRI (DCE-MRI) and 18-fluorine-fluorodeoxyglucose (18F-FDG) PET, to quantify inflammation expressed as plaque neovessels content in a rabbit model of atherosclerosis.

Methods and Results—Atherosclerotic plaques were induced in the aorta of 10 rabbits by a combination of 2 endothelial abrasions and 4 months hyperlipidemic diet. Six rabbits underwent MRI during the injection of Gd-DTPA, whereas 4 rabbits were imaged after injection of 18F-FDG with PET. We found a positive correlation between neovessels count in atherosclerotic plaques and (1) Gd-DTPA uptake parameters evaluated by DCE-MRI ($r=0.89, P=0.016$) and (2) 18F-FDG uptake evaluated by PET ($r=0.5, P=0.103$ after clustered robust, Huber-White, standard errors analysis).

Conclusion—DCE-MRI and 18F-FDG PET may allow for the evaluation of inflammation in atherosclerotic plaques of rabbits. These noninvasive imaging modalities could be proposed as clinical tools in the evaluation of lesion prognosis and monitoring of anti–angiogenic therapies. (Arterioscler Thromb Vasc Biol. 2008;28:1311-1317)

Key Words: atherosclerosis ▪ inflammation ▪ neovessels ▪ MRI ▪ PET

Neovascularization is one of the hallmarks of high-risk/vulnerable atherosclerotic lesions. It is characterized by the formation of new capillaries in the atherosclerotic plaque, and it is usually considered a response to the hypoxic conditions within the vessel wall during plaque growth. However, more recent reports have identified hypoxia independent pathways of angiogenesis mediated primarily by inflammation; these studies have highlighted the link between the presence of neovessels, the extravasation and activation of inflammatory cells, and lipid deposition in the vessel wall. Neovessels seem to play a key role in the progression of atherosclerotic plaques and plaque rupture. From those reports it appears that the presence and extent of neovessels and inflammation in atherosclerotic plaques can be considered a marker of risk associated with the lesion. Therefore it would be of clinical relevance to develop techniques capable of quantifying the degree of plaque inflammation in a noninvasive and reliable manner.

DCE-MRI is an imaging technique extensively used to study the vascularity of tumors. This technique takes advantage of the administration of clinically-available contrast agents (ie, Gadolinium (Gd)-chelates) to quantify the extent of tumor blood supply and its associated physiological characteristics, such as permeability surface area product, extraction fraction, and blood flow. Recent studies on human carotid atherosclerotic plaques have shown that several gadolinium uptake parameters, evaluated by kinetic modeling of DCE-MRI bright blood acquisitions, correlate with the extent of plaque vascularity and macrophage burden (confirmed by staining of histological specimens). However, the use of the bright blood DCE technique makes it intrinsically difficult to reliably delineate the vessel lumen from the wall because of the bright signal coming from the blood.

PET uses an FDA-approved glucose analog, 18F-FDG, to provide a functional assessment of plaque inflammation. Once administered, 18F-FDG competes for uptake with glucose in metabolically active cells (such as active macrophages in inflamed atherosclerotic plaques) through the GLUT1 glucose transporter; once internalized, it is phosphorylated by the enzyme hexokinase but cannot be metabolized, and therefore accumulates in the cells in proportion to their energy demand. The accumulation of 18F-FDG in the tissue...
of interest can subsequently be measured by PET imaging. Recent studies have demonstrated that 18F-FDG accumulates in highly inflamed plaques of both cholesterol-fed rabbits and human patients. Its accumulation is believed to be attributable to the high glucose demand that follows the respiratory burst of inflammatory cells present in the atherosclerotic lesion. 18F-FDG uptake has furthermore been correlated with the number of active macrophages in the plaque and has thus been proposed as a mean to quantify the extent of inflammation.

Given the close relationship between neovessels and macrophages in atherosclerosis, in this study we explored the correlation between DCE-MRI, 18F-FDG PET, and neovessels count (a histological marker of inflammation) in aortic plaques of New Zealand White rabbits to evaluate their possible usage in the assessment of plaque associated risk. Furthermore, to overcome the difficulties in lesion delineation intrinsic to a bright blood acquisition, we propose to use a black blood turbo spin echo (TSE) sequence for DCE acquisition to improve plaque characterization by suppressing the blood signal coming from the vessel lumen.

Methods

Animal Protocol
Aortic atherosclerotic plaques were induced in the aorta of 10 New Zealand white (NZW) male rabbits (mean age, 4 months; mean weight=3.1±0.2 kg; Covance, Princeton, NJ) by a combination of 4 months of high cholesterol diet (4.7% palm oil and 0.3% [weeks 1 through 8] and 0.15% [weeks 9 through 16]; Research Diet Inc) and repeated balloon injury of the aorta (2 weeks and 6 weeks after starting the high-cholesterol diet; Figure 1). Aortic injury was performed from the aortic arch to the iliac bifurcation with a 4F Fogarty embolectomy catheter introduced through the femoral artery. All procedures were performed under general anesthesia by an intramuscular injection of ketamine (20 mg/kg; Ft Dodge Animal Health), xylazine (5 mg/kg; Bayer Corp), and acepromazine (0.5 mg/Kg; Boehringer Ingelheim Vetmedica Inc). The protocol was approved by the Mount Sinai School of Medicine Institute Animal Care and Use Committee.

MRI Protocol
Under anesthesia 6 rabbits were imaged using a 1.5 Tesla MRI system (Siemens Sonata, Siemens Medical Solutions) and a conventional knee coil. To locate atherosclerotic plaques, 3-mm thick sequential axial images of the aorta were obtained from the celiac trunk to the iliac bifurcation using a black blood T1-weighted fast spin-echo sequence with an in-plane resolution of 470 × 470 µm (TE=5.6ms, TR=800 ms, interslice gap=3 mm, field of view 12×12 cm, matrix 256×256, echo train length=7, and signal averages=4). Inferior and superior radiofrequency saturation pulses were added to null the signal from flowing blood in the inferior vena cava and aorta together with spectral fat suppression, to null the signal from the periadventitial fat. DCE-MRI was performed on one selected axial slice using a black blood turbo spin echo (TSE) sequence (slice thickness=3 mm, TE=5.6ms, TR=250ms, field of view 16×16 cm, matrix size 256×256, with an in-plane resolution of 630×630 µm, suitable for imaging of the rabbit aorta). A total of 150 images per rabbit were acquired, with a time resolution of 4.8 s. After the acquisition of the 5th image, 0.2 mmol/Kg of Gd-DTPA (Magnevist) was injected at a rate of 0.5 mL/s followed by a 10 mL saline flush, through a marginal ear vein.

DCE MRI Image Analysis
We studied the change of signal intensity in a region-of-interest (ROI) including the entire atherosclerotic plaque during the injection of the contrast agent with a custom made Matlab program. We calculated the area under the signal intensity versus time curve (AUC) at different time points (2 and 7 minutes after injection of contrast agent and AUC for the whole acquisition) by numeric integration via the trapezoidal rule of the time series using the following equation:

$$AUC(T) = \int_0^T (SI(t) - SI_{precontrast})dt,$$

where SI(t) represents the signal intensity in 1 given pixel at time t, SI_{precontrast} represents the average precontrast signal intensity value calculated as the pixel-by-pixel average intensity of the first 5 precontrast images and T=2 and 7 after injection and 12 minutes (whole acquisition). Measuring AUC at early time points after injection allowed us to exclude the wash-out portion of the enhancement.

PET Imaging and Analysis Protocol
Images were acquired using a combined PET/CT (16 slices multidetector CT) scanner (GE Discovery LS) in 4 rabbits. A noncontrast CT was used for anatomic coregistration and for attenuation correction of the PET dataset. Under anesthesia 7 PET slices for each rabbit were acquired over 10 minutes in 3D mode, 3 hours after injection (in the marginal ear vein) of 1mCi/Kg of 18F-FDG from the approximate level of the diaphragm to aortic bifurcation. One bed was used and the rabbit was placed in supine position on the table. A total of 27 PET images were acquired for each rabbit, with 3 cycles for each bed position. PET images were acquired using a 4D PET protocol that included 7 contiguous 3D slices covering the thoracic aorta and aortic arch (Siemens Medical Solutions, Siemens Medical Solutions, 1430.6 mCi, net activity 2600 MBq). A 4D image was created from each set of 7 slices. The 4D PET images were coregistered with the planning PET images using the GE Discovery PET/CT software (GE Healthcare, Waukesha, WI). A 4D image was then created from each set of 7 slices. The 4D PET images were coregistered with the planning PET images using the GE Discovery PET/CT software (GE Healthcare, Waukesha, WI).
position was used (FOV 15.5 cm). PET images were reconstructed using the FORE-IT algorithm. For image analysis, we drew regions of interest around the aorta on the PET/CT images on a GE Xeleris 2.0 workstation and calculated the mean slice-by-slice standard uptake value (SUV). SUV gives an estimate of 18F-FDG uptake after correction for injected 18F-FDG dose and body mass.\(^{10}\)

**Histology**

After imaging, rabbits were euthanized by an intravenous injection of 120 mg/kg of sodium pentobarbital (Sleepaway; Ft Dodge Animal Health). A bolus of heparin was injected before euthanasia to prevent clot formation. Aortas were excised, fixed for 24 hours in 4% paraformaldehyde, and embedded in paraffin. Five-\(\mu\)-m-thick slices were sectioned in the same direction as MR and PET slices and stained with Masson’s trichrome. Neovessels were detected on adjacent slices by immunohistochemistry. Aortic sections were incubated with 0.3% hydrogen peroxide to block endogenous peroxidase, then with a monoclonal mouse antibody directed against CD-31, a marker of endothelial cells (dilution 1:50, Dako). Biotinylated polyclonal antimouse secondary antibody and peroxidase-conjugated streptavidin were applied for 30 minutes each with the use of the ABC Kit (Dako). Peroxidase activity was visualized by diaminobenzidine to yield brown cytoplasmic reaction products. Sections were counterstained with hematoxylin. A magnification factor of 40 was used for analysis of neovessel count. On each section, plaque neovessels were counted manually by an experienced reader (J.C.C.).

**Statistical Methods**

Analyses were performed using Matlab software. A value of \(P<0.05\) was considered significant. After matching PET and DCE-MRI datasets with histology in the 2 groups of rabbits via anatomic fiducial markers such as the iliac bifurcation, the renal arteries, and the diaphragmatic crossing, we correlated the 2 imaging techniques to histology. Correlations between neovessels count and (1) DCE-MRI parameters and (2) 18F-FDG PET/CT uptake (SUV) were performed using Pearson test. To account for the correlation of measurements acquired from the same animal during PET imaging, robust (Huber-white) standard errors were calculated in the PET data analysis.

**Results**

**Correlation Between Area Under the Curve Calculated by DCE-MRI and Neovessel Count**

Six rabbits were imaged using DCE-MRI. We saw a strong enhancement in atherosclerotic plaques during the injection
of gadolinium (Figure 2). We then analyzed using a custom made Matlab (The MathWorks, Inc) program the change of signal intensity in atherosclerotic plaques over time (Figures 3 and 4a). We found a significant positive correlation between the AUC calculated from DCE-MRI at 2 and 7 minutes after injection and neovessel count in the intima (AUC – 2 minutes: \( r = 0.89, P = 0.016 \); AUC – 7 minutes: \( r = 0.91, P = 0.011 \)).

**Correlation Between 18F-FDG Uptake and Neovessel Count**

Four rabbits were imaged using 18F-FDG PET/CT (Figure 4c). Seven slices per animal were analyzed. We noted positive correlations between 18F-FDG uptake measured by PET and neovessel count within both the intima (\( r = 0.50, P = 0.103 \)) and across the whole plaque (intima and adventitia; \( r = 0.52, P = 0.128 \)) by using clustered robust standard error analysis (3 degrees of freedom).

**Discussion**

Neovascularization is a histological hallmark of high-risk/vulnerable atherosclerotic lesions. It is characterized by the formation of new capillaries in the atherosclerotic plaque attributable to both hypoxia-dependent and independent pathways. Recent reports have shown that hypoxia-independent pathways of angiogenesis are primarily mediated by inflammation. Neovessels play a key role in the progression of atherosclerotic plaques from fatty streaks into more advanced and vulnerable lesions, because they are considered a major route of leukocyte recruitment into the fibrous cap and plaque shoulder. Stemming from this observation, several studies reported a higher neovessel count in highly inflamed macrophage-rich atherosclerotic plaques compared to fibrocalcific lesions. Once recruited in the atherosclerotic plaque, active inflammatory cells, such as macrophages, secrete lytic enzymes (in particular metalloproteinases) that are known to contribute to the rupture of the internal elastic lamina and to the collagenolysis of the fibrous cap, increasing the risk of plaque rupture and arterial thrombosis.

From those reports, it appears that the presence and extent of inflammation in atherosclerotic plaques can be considered a marker of risk associated with the lesion. Therefore, it would be of clinical relevance to develop techniques capable of quantifying the degree of plaque inflammation in a noninvasive and reliable manner. Among the several techniques used in imaging of atherosclerosis, 18F-FDG PET and DCE-MRI are of interest for their noninvasiveness and their capability to provide quantification of plaque physiological and activity parameters.

In this study, we aimed to compare these 2 independent imaging techniques and to validate them against neovessel count (a histological marker of inflammation) to evaluate their possible usage in the assessment of plaque associated risk. 18F-FDG has been extensively used to quantify myocardial glucose consumption, to identify tumors, and to monitor systemic vasculitis. Recently several studies have demonstrated that 18F-FDG accumulates in highly inflamed plaques of both cholesterol-fed rabbits and human patients.
its accumulation is believed to be attributable to the high glucose demand that follows the respiratory burst of inflammatory cells present in the atherosclerotic lesion. 18F-FDG uptake has furthermore been correlated with the number of active macrophages in the plaque and has been proposed as a mean to quantify the extent of inflammation. Given the close relationship between neovessels and macrophages in atherosclerosis, in this study we explored the correlation between 18F-FDG PET and neovessel count in atherosclerotic plaques. To take into account for possible dependence between different slices acquired in the same animal, we analyzed the correlation between 18F-FDG PET uptake and neovessel count in the intima by using clustered robust standard error analysis. Our results indicate a positive correlation between 18F-FDG PET uptake and neovessel count in the intima of atherosclerotic plaques ($r=0.50$, $P=0.103$).

DCE-MRI is a technique extensively used in the quantification and in the study of the physiological characteristics of tumor vascularization where it has been shown to correlate with several prognostic parameters, such as tumor grade, neovessel count, and expression of vascular endothelial growth factor (VEGF). It has furthermore been used in phase 1 trials of tumoral antiangiogenic therapies to investigate the relationship between tumor vascularity in response to the administration of antiangiogenic drugs, such as anti-VEGF agents and tyrosine-kinase inhibitors. Those reports showed the potential of this technique to become an imaging marker for the evaluation and follow-up of patients with cancer. More recently DCE-MRI has been applied to the study of atherosclerotic plaques in human patients by using a bright-blood SPGR sequence for DCE acquisition, Kerwin et al used kinetic modeling of contrast agent uptake to investigate the relationship between kinetic parameters, such as Ktrans and blood volume, and plaque histological parameters, such as neovessel density and macrophages count. These reports showed a positive linear correlation between

Figure 4. a, Representative color-coded AUC map by DCE-MRI. b, Representative histology slice showing CD31 neovessels staining. Magenta arrow indicates neovessel rich area. c, Representative FDG-PET image, showing 18F-FDG uptake along the whole abdominal aorta, with one focal spot of higher uptake (red markers, lower panel).
contrast agent uptake parameters and histological markers of plaque inflammation, thus suggesting the future clinical application of DCE-MRI in the evaluation of plaque associated risk and in the assessment of the lesion response to treatment. However, while allowing to extract kinetics parameter of contrast agent uptake by measuring the blood signal in the vessel lumen (ie, arterial input function, AIF), the use of bright blood techniques makes it intrinsically difficult to delineate the vessel wall and plaque for future analyses. In this study, we tested a black blood TSE sequence for DCE acquisition to improve the characterization of the atherosclerotic plaque by suppressing the signal coming from blood in the vessel lumen. Because evaluation of the AIF is not possible in this scenario, we use the area under the signal intensity curve (AUC) evaluated at different time points during acquisition as a measure of contrast agent uptake in the plaque. We further validate this parameter against neovessel counts measured by CD31 staining in corresponding atherosclerotic plaques. Our results indicate that AUC evaluated at 2 and 7 minutes after contrast agent injection show a positive significant correlation with neovessels count in the intima ($P<0.05$).

By studying the correlation of 18F-FDG PET and DCE MRI to the same histological marker of inflammation, we hope to exploit a correlation between DCE-MRI contrast agent uptake and 18F-FDG PET uptake in the plaque. Our results indicate that both AUC evaluated by DCE-MRI and 18F-FDG PET SUV show a positive correlation with plaque neovessel count. These results may indicate that these 2 techniques may allow for a noninvasive combined approach to assessment of plaque rupture risk.

**Study Limitations**

One of the shortcomings of the present study is that the correlation between the 2 imaging techniques and neovessel count were evaluated in 2 different groups of animals. We designed the experiments this way because there was no Institutional Animal Care and Use Committee (IACUC) approval to perform both experiments in the same animals. We would however expect the relationships between FDG PET, DCE MRI, and neovascularization of plaque to still apply, although a combined experiment is still needed to verify this. Furthermore, probably because of the small sample size of the PET experiments, the correlation between 18F-FDG uptake and neovessels count did not reach significance at the $P<0.05$ level after correcting for dependence between slices acquired in the same animal using clustered robust standard error analysis. We hypothesize that a larger number of animal would be required to better investigate the relationship between SUV and plaque neovascularization in this scenario.

A second relevant discussion point is the complex relationship between the AUC and standard physiological parameters commonly evaluated by kinetic modeling of DCE acquisitions. AUC values can be influenced not only by neovessel count, but also by their permeability and by the fraction of extravascular extracellular space within the lesion, which both influence the wash-out kinetics of the contrast agent from the plaque.$^{14}$ As previously mentioned, we chose the AUC parameter as a measure of the uptake of contrast agent because of the difficulty of getting an accurate arterial input function while using a black blood sequence for DCE MRI acquisition. To overcome this drawback we chose to evaluate the AUC at different time points, thus excluding the wash-out portion of the curve in some of the calculations. As expected AUC measured at earlier time points (2 minutes and 7 minutes after injection) when no significant wash-out has occurred (Figure 3), correlated better with neovessels count in
the plaque. We further recognize the need to develop and use relative model based approaches that would allow us to extract kinetic parameters from black blood DCE acquisitions without an arterial input function.15–18

Conclusions
In conclusion, we demonstrated in this study the correlation between the area under the signal intensity curve (AUC) evaluated by dynamic contrast enhanced MRI with Gd-DTPA, 18F-FDG PET uptake, and plaque neovascularization (defined by histology) in atherosclerotic rabbits. Our results show a significant positive correlation between AUC evaluated by DCE-MRI and histological markers of neovascularization in aortic plaque of New Zealand White rabbits. Furthermore we showed a positive correlation between 18F-FDG plaque uptake and plaque neovessels count. By correlating both imaging techniques to the same histological marker of inflammation, we hope to exploit their potential to identify high-risk/vulnerable lesions and quantify plaque inflammatory activity. Our results furthermore indicate that a correlation between DCE-MRI contrast agent uptake parameters and 18F-FDG PET uptake may be possible, thus envisaging a scenario in which the 2 imaging techniques could be used in a clinical setting to complementarily evaluate prognosis and treatment of atherosclerotic plaques.

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Disclosures
None.

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