Background: Imaging modalities to assess atherosclerotic plaque thrombogenicity have not been established, so in this study the relationship between [18F]-fluorodeoxyglucose (18F-FDG) uptake and thrombus formation was investigated in rabbit atherosclerotic arteries.

Methods and Results: Atherosclerotic plaque was induced in the iliacofemoral artery by balloon injury and a 0.5% cholesterol diet. At 3 weeks after the first balloon injury, the arteries were visualized by 18F-FDG positron emission tomography (PET) imaging 2 h after an 18F-FDG infusion, and then arterial thrombus was induced by a second balloon injury of both iliacofemoral arteries. Imaging with 18F-FDG-PET revealed significantly more radioactivity along the injured (0.63±0.12 SUVmax), than the contralateral non-injured artery (0.34±0.08 SUVmax, n=17, P<0.0001). Arterial radioactivity measured by autoradiography positively correlated with macrophage area, the number of nuclei that were immunopositive for nuclear factor κ B (NF-κB), and tissue factor (TF) expression. The immunopositive areas for glycoprotein IIb/IIIa and fibrin in thrombi were significantly larger in the atherosclerotic than in the contralateral arteries, and significantly correlated with radioactivity in PET (r=0.92, P<0.001, n=10) and autoradiography (r=0.73, P<0.0001, n=50) in the arteries. Inhibition of NF-κB significantly reduced TF expression in cultured atherosclerotic plaque.

Conclusions: Arterial 18F-FDG uptake reflects the thrombogenicity of atherosclerotic plaque following balloon injury. (Circ J 2013; 77: 2626–2635)

Key Words: Atherothrombosis; 18F-FDG; Nuclear factor-κB; Rabbits; Tissue factor

Thrombus formation is a major complication of atherosclerosis, but although disruption of atherosclerotic plaques is recognized as a trigger of atherothrombosis, not all thrombi result in complete luminal occlusion and symptomatic events. Therefore, thrombus size is critical to the onset of clinical events. Thrombus formation and propagation are regulated by many factors, such as vascular wall thrombogenicity, local hemorheology, blood thrombogenicity, and fibrinolytic activity. Among these, the thrombogenicity of atherosclerotic plaque is an essential factor in atherothrombosis. Tissue factor (TF) is an initiator of the coagulation cascade that is expressed in the adventitia and to various degrees in the media of normal arteries. Because atherosclerotic plaques express TF, the degree of TF expression is a crucial determinant of plaque thrombogenicity. Although current imaging modalities support high-quality plaque characterization, those that can assess atherosclerotic plaque thrombogenicity have not been established.

Received November 27, 2012; revised manuscript received April 22, 2013; accepted May 23, 2013; released online July 6, 2013 Time for primary review: 29 days
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Fluorodeoxyglucose (18F-FDG) has been advocated as a means of evaluating arterial inflammation.8 The uptake of 18F-FDG closely correlates with plaque macrophage contents in animal models,9–10 which suggests that the degree of 18F-FDG accumulation in the vessel wall reflects underlying levels of inflammation. Clinical studies have also identified a relationship between 18F-FDG uptake and numbers of cardiovascular risk factors, as well as risk for future events.11–13 The uptake of 18F-FDG is significantly higher in aortic segments with, than without thrombus in a rabbit model of advanced atherosclerosis.14 These lines of evidence imply an association between 18F-FDG uptake and arterial thrombus formation, although the underlying mechanism is unknown. We investigated this notion in a rabbit model of atherosclerosis under fluoroscopic guidance to induce an atherosclerotic lesion in the artery. The catheter was inflated to 1.5 atm (balloon-to-artery ratio; 1.1:1 to 1.2:1) and retracted 3 times to denude the endothelium.

After 18F-FDG-PET imaging, a 2F balloon catheter (Baxter Healthcare) was inserted via the anterior tibial arteries into both iliacofemoral arteries, inflated to 1.4 atm, and retracted twice to induce arterial thrombus;15 15 min later, the rabbits were injected with heparin (500 U/kg, i.v.) and then killed 5 min later with an overdose of pentobarbital (60 mg/kg, i.v.). The animals were perfused with 50 ml of cold 0.01 mol/L of phosphate-buffered saline to remove residual blood from the arteries for subsequent autoradiography and immunohistochemical evaluation.

**PET Imaging and Analysis**

18F-FDG-PET imaging proceeded as described with minor modifications.16 The rabbits (n=5) were fasted for 4 h prior to PET scanning, infused with 18F-FDG (average, 193 MBq/rabbit) 2 h prior to imaging, and anesthetized by 1.5–2.0% isoflurane inhalation. The injected dose of 18F-FDG was determined in consideration of the half-life of 18F (110 min). After urinary bladder lavage with warmed saline, the rabbits were placed supine on a customized acrylic bed in a small animal PET scanner (Inveon, Siemens Medical Solutions USA, Knoxville, TN, USA) and scanned (list-mode acquisition) for 40 min. Body temperature (37.4±0.2°C) was maintained using cotton bedding prepared during the PET scanning. The data were reconstructed and corrected for attenuation and scatter using 2D filtered back-projection (FBP) and maximum a posteriori (MAP) algorithm. The spatial resolution was 1.63 mm full-width at half-maximum (FWHM) in FBP and 1.05 mm FWHM in MAP. The former was used to quantify the radioactivity and the latter for visual observations. The image matrix was 256×256×159, resulting in a voxel size of 0.385×0.385×0.796 mm. Coronal, axial and sagittal images were visually graded. Coronal images were quantified by centering regions of interest (ROIs) over the iliacofemoral arteries, then 3 or 4 circular ROIs (10 pixels in diameter) in the FBP images were placed on each injured and contralateral non-injured artery. The results are expressed as standardized uptake value (SUV). The maximum values of SUV (SUVmax) for the bilateral arteries were confirmed and represented the 18F-FDG uptake in the PET images.

**Radioactivity of Tissue and Arterial Slices**

The amount of radioactivity in the iliacofemoral arteries, skel-

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**Figure 1.** Experimental protocol for the animal studies of [18F]-fluorodeoxyglucose ([18F]-FDG) uptake and thrombus formation in rabbit atherosclerotic arteries (n=5 Japanese white rabbits). ARG, autoradiography; IHC, immunohistochemistry.
The data were normalized with the animal’s body weight (%ID/kg) and then converted to SUV. The arteries were cut into 5 sections that were embedded in Tissue-Tek (Sakura, Tokyo, Japan) and frozen. Consecutive 10- or 5-μm slices were prepared for autoradiographic or histologic analysis, respectively. The 10-μm cryostat sections were exposed to phosphor imaging plates (Fuji Imaging Plate BAS-SR 2025, Fuji Photo Film Co Ltd, Tokyo, Japan) for 12 h, together with a set of calibrated standards. The imaging plates were then scanned using a Fuji Bio-imaging Analyzer BAS-5000 with an internal resolution of 25 μm (Fuji Photo Film Co Ltd) and the images were examined using image analysis software (Multi Gauge Ver. 3.0, Fuji Photo Film Co Ltd). The amount of radioactivity in each image is expressed as photo-stimulated luminescence per unit area (PSL=α×D×t, where α is a constant, D is the amount of radioactivity exposed on the image plate, and t is exposure time). Each PSL value/mm² of arterial tissue was recorded and converted to a ratio (% of the activity of the standard injected dose/mm² of lesion area (% ID/mm²). The data were normalized with the animal’s body weight (%ID/kg/mm²) and then converted to SUV.

Statistical Analysis
All data are presented as means and standard deviation. Differences between individual groups were tested using the Mann-Whitney U-test (GraphPad Prizm 4.03, GraphPad Software Inc, San Diego, CA, USA). Relationships between factors were evaluated using Spearman’s rank correlation coefficient and P<0.05 was considered statistically significant.

Results

PET Imaging and Radioactivity of Arteries
Figure 2 shows the ¹⁸F-FDG-PET coronal MAP image and radioactivity accumulation before arterial thrombus formation. The accumulation of ¹⁸F-FDG is greater along the injured than the non-injured artery (Figure 2A). We compared ¹⁸F-FDG uptake between the injured and non-injured arteries by analyzing coronal FBP images after drawing ROIs centered over the iliacofemoral arteries. The maximal amount of radioactivity in the ROIs was significantly higher in the injured than in the non-injured arteries (0.63±0.12 vs. 0.34±0.08 SUVmax; n=17, P<0.0001; Figure 2B). Significantly more radioactivity was found in the excised injured but not the non-injured iliacofemoral artery (1.32±0.55 vs. 0.35±0.11 SUVmean; n=5, P<0.01; Figure 2C). Figure 2D shows that level of ¹⁸F-FDG uptake correlated between PET image and tissue (r=0.71, P<0.05; n=10). Levels of ¹⁸F-FDG uptake were similar among the non-injured artery, blood and skeletal muscle tissue (0.35±0.11, 0.28±0.05 and 0.37±0.24 SUV, respectively; mean, n=5).

Tissue Culture of Rabbit Atherosclerotic Plaque
Atherosclerotic lesions were induced as described. The rabbits were injected with heparin (500 U/kg, i.v.), killed 5 min later with an overdose of pentobarbital (60 mg/kg, i.v.) and perfused with 50ml of 0.01 mol/L of phosphate-buffered saline and Dulbecco’s modified Eagle’s medium. The iliacofemoral artery was excised and the atherosclerotic plaque was separated from the media and adventitia. The plaque was divided into 3-mm segments and placed in incubation medium containing 10% fetal bovine serum in multidishes for 6h. Cell viability was evaluated in plaque segments stained with nigroniumicide adenine.
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Figure 5 shows the correlation between 18F-FDG uptake and histologic findings. Autoradiographic 18F-FDG uptake positively correlated with areas that were immunopositive (r=0.71, P<0.0001, n=50), TF (r=0.64, P<0.0001, n=50), and the number of NF-κB (r=0.85, P<0.0001, n=50), and negatively with SMCs (r=–0.44, P<0.01, n=50) (Figure 5A). The 18F-FDG uptake also positively correlated with areas that were immunopositive for GPIIb/IIIa (r=0.77, P<0.0001, n=50), fibrin (r=0.66, P<0.0001, n=50), GPIIb/IIIa and fibrin (r=0.73, P<0.0001, n=50; Figure 5B). In addition, maximal 18F-FDG uptake in PET images positively correlated with areas that were immunopositive for GPIIb/IIIa (r=0.82, P<0.01, n=10), fibrin (r=0.95, P<0.001, n=10), GPIIb/IIIa and fibrin (r=0.92, P<0.001, n=10; Figure 5C).

Correlation Between 18F-FDG Uptake and Histologic Findings

Figure 5 shows the correlation between 18F-FDG uptake and vascular/thrombus factors. Autoradiographic 18F-FDG uptake positively correlated with areas that were immunopositive (%) for macrophages (r=0.71, P<0.0001, n=50), TF (r=0.64, P<0.0001, n=50), and the number of NF-κB (r=0.85, P<0.0001, n=50), and negatively with SMCs (r=–0.44, P<0.01, n=50) (Figure 5A). The 18F-FDG uptake also positively correlated with areas that were immunopositive for GPIIb/IIIa (r=0.77, P<0.0001, n=50), fibrin (r=0.66, P<0.0001, n=50), GPIIb/IIIa and fibrin (r=0.73, P<0.0001, n=50; Figure 5B). In addition, maximal 18F-FDG uptake in PET images positively correlated with areas that were immunopositive for GPIIb/IIIa (r=0.82, P<0.01, n=10), fibrin (r=0.95, P<0.001, n=10), GPIIb/IIIa and fibrin (r=0.92, P<0.001, n=10; Figure 5C).

TF Expression in Tissue Cultures of Atherosclerotic Plaque

We assayed cultured plaques to determine the associations between NF-κB and TF expression in rabbit atherosclerotic arterial 18 F-FDG uptake and thrombogenicity.
Figure 3. Autoradiographic (ARG) and histologic findings of atherosclerotic (injured) and contralateral (non-injured) arteries with thrombus in rabbits fed with 0.5% cholesterol diet. (A) Representative autoradiogram (HE stain), and immunohistochemistry for muscle actin, rabbit macrophage, GPIIb/IIIa, and fibrin. Immunoreaction is visualized by 3,3′-diaminobenzidine tetrahydrochloride (brown). Uptake of 18F-FDG is low in the contralateral artery (Upper) comprising smooth muscle cells (SMCs) and some macrophages and a thin layer of mural thrombus comprising platelets and fibrin (arrowheads) covers the luminal surface. Uptake of 18F-FDG is high in atherosclerotic artery (lower rows) that is rich in macrophages. A thicker layer of mural thrombus comprising platelets and fibrin (arrows) covers the luminal surface. Immunohistochemical images of GPIIb/IIIa and fibrin correspond to square frames in the HE images. Bars=500 μm (ARG, HE, SMC, Macrophage) or 100 μm (GPIIb/IIIa and fibrin). (B) Uptake of 18F-FDG and immunopositive areas for muscle actin, macrophages, GPIIb/IIIa, fibrin, and GPIIb/IIIa and fibrin in arterial sections (n=25 each). 18F-FDG, [18F]-fluorodeoxyglucose.
lesions. Atherosclerotic lesions were cut into 2-mm sections and cultured in conditioning medium with 10% FBS. The number of NF-κB-immunopositive nuclei and the TF protein levels in the plaques were significantly increased 6h later, compared with before plaque culture. Inhibiting NF-κB using Bay 11-7055, an inhibitor of inhibitor of κB kinase, significantly reduced both the number of NF-κB-immunopositive nuclei (Figure 6A) and TF protein production (Figure 6B). Staining

for NADH did not significantly differ among the groups (before culture, 40.4±8.7%; 6h after culture with DMSO and with Bay 11-7055, 43.2±10.6% and 46.8±5.0%, respectively; n=5 each).

Discussion

The main findings of the present study are presented. Signifi-
Figure 5. Correlations between 18F-FDG uptake and vascular/thrombus components. Correlation with vascular (A) and thrombus (B) components in arterial sections. Correlation between 18F-FDG uptake in PET image and thrombus components in corresponding arterial sections (C). 18F-FDG, [18F]-fluorodeoxyglucose; ARG, autoradiography; NF-κB, nuclear factor κB; PET, positron emission tomography; SUV, standardized uptake value.
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Figure 6. Numbers of nuclear factor κ B (NF-κB)-immunopositive nuclei and tissue factor (TF) protein levels in cultured atherosclerotic plaque. Atherosclerotic lesions (2-mm long) were cultured in conditioning medium with 10% fetal bovine serum. Numbers of NF-κB-immunopositive nuclei (A) and TF protein level (B) in plaques were measured before and 6 h after plaque culture with and without Bay 11–7055, an inhibitor of κB kinase (n=5 each).

Arterial ¹⁸F-FDG uptake significantly correlated with the size of thrombus induced by balloon injury of the rabbit iliacofemoral artery (Figure 5B). A clinical study of patients with atherosclerosis has demonstrated higher ¹⁸F-FDG uptake in culprit lesions associated with acute coronary thrombosis than in lesions stented for stable angina.²⁰ Azis et al found significantly more ¹⁸F-FDG uptake in rabbit atherosclerotic aortic segments with than without thrombus formation triggered by Russell’s viper venom and histamine.¹⁴ Patel et al reported that lowering cholesterol absorption reduces plaque burden.¹⁹ ¹⁸F-FDG uptake and thrombus formation in rabbit atherosclerotic aortas, whereas ¹⁸F-FDG uptake and macrophage content in the aortas do not correlate.²¹ Those results imply an association between ¹⁸F-FDG uptake and thrombogenic factors in the arteries, but the macrophage content does not necessarily correspond to thrombus formation. The present study identified a positive correlation between ¹⁸F-FDG uptake and thrombus size, as well as TF expression. In addition, not only the areas of fibrin but also those with platelets correlated with ¹⁸F-FDG uptake (Figures 5A,B). Because thrombin generated via the TF pathway accelerates platelet activation, as well as fibrin formation,²² evidence of a positive correlation between ¹⁸F-FDG uptake and TF expression would support previous findings indicating that ¹⁸F-FDG uptake is associated with plaque thrombogenicity.

Although radioactivity in thrombus might affect the correlation between thrombus size and ¹⁸F-FDG uptake measured autoradiographically, the amount of radioactivity in the blood and non-injured artery was equally low. Moreover, thrombus size also correlated with ¹⁸F-FDG uptake measured by PET imaging (Figure 5C). These results suggest that FDG uptake reflects the thrombogenic potential of the arterial wall following balloon injury.

Arterial ¹⁸F-FDG uptake significantly correlated with macrophage content (Figure 5A). The finding that ¹⁸F-FDG uptake positively correlated with this, but negatively with that of SMC, is compatible with those of previous studies.⁵⁻¹⁰,²³ Macrophages and granulocytes express glucose transporter 1 and hexokinase II in lung inflammatory lesions.¹⁸F-FDG uptake correlates with the degree of inflammation²⁴ and lipopolysaccharide enhances ¹⁸F-FDG uptake by isolated human monocyte-macrophages.²⁵ Therefore, it is considered that ¹⁸F-FDG uptake by macrophages is a hallmark of active inflammation in atherosclerotic plaques. However, clinical and experimental studies have failed to show a correlation between ¹⁸F-FDG uptake and macrophage content in atherosclerotic lesions.²¹,²⁶ The evidence suggests that the presence of macrophages does not always indicate active inflammation. Recent studies have revealed macrophage heterogeneity in atherosclerotic lesions,²⁷ and approximately 25% of macrophages in coronary atherosclerotic plaques have an anti-inflammatory phenotype.²⁸ Moreover, classical but not innate activation significantly increases 2-deoxy-D-glucose uptake, accompanied by glycolytic pathway activation.²⁹ In the present study, CD163-positive cells accounted for only 1% of macrophages in rabbit atherosclerotic artery. The positive relation between ¹⁸F-FDG uptake and macrophage content in this model may be related to a paucity of macrophages with an anti-inflammatory phenotype (ie, CD163-positive cells) in the rabbit atherosclerotic artery.

The nuclear transcription factor, NF-κB, affects the inflammatory response, cell proliferation and survival, and angiogenesis in atherosclerotic lesions.³⁰ Activated NF-κB has been identified in atherosclerotic plaques from humans and in animal models.³¹⁻³³ The expression of NF-κB in coronary atherosclerotic plaque is more enhanced in patients with unstable than with stable angina pectoris.³⁴ In the present study, immunoreactivity for NF-κB was distributed predominantly in macrophages and less so in SMCs in atherosclerotic lesions (Figure 4B), and there was significant correlation among ¹⁸F-FDG uptake, macrophage content, NF-κB, and TF. These results are compatible with those from a previous report showing colocalization of deoxyglucose and annexin A5, a marker of thrombogenic cell surface, in macrophage-rich atherosclerotic lesions in mice.⁸ These results also suggest that metabolically active cells in the arterial wall have a high-thrombogenic potential in lesions.
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These results indicated that NF-κB suggests that 18F-FDG-PET can identify patients at risk for future cardiovascular events and the present results support this notion. The present study did not show balloon catheter-induced thrombus formation in the normal artery of rabbits fed with conventional diet, because we have previously examined balloon catheter-induced thrombus formation in rabbit femoral arteries. The thrombus size in the normal femoral artery was significantly smaller than that in the injured (atherosclerotic) artery in rabbits fed with 0.5% cholesterol diet, and did not differ from that in the femoral artery of rabbits fed with 0.5% cholesterol diet.15

Study Limitations
The thrombus mechanically induced by a balloon catheter does not replicate spontaneous plaque rupture in human atherothrombosis. Although a recent in vitro study demonstrated that hypoxia augments 18F-FDG uptake in cultured macrophages,38 we did not assess the contribution of hypoxia to 18F-FDG uptake in our model. Because thrombus size was evaluated 15 min after its induction, it was not possible to show thrombus persistence and growth rates, both of which are important factors in human atherothrombosis.

Conclusions
Arterial 18F-FDG uptake is associated with thrombogenic factors and thrombus formation in rabbit atherosclerotic lesions, and arterial 18F-FDG uptake appears to reflect thrombotic risk of atherosclerotic plaques following balloon injury.

Acknowledgments
The work was supported in part by Grants-in-Aid for Scientific Research in Japan (Nos.23790410 A.Y., 23659573 Y.K., 23390084 Y.A.), Mitsubishi Pharma Research Foundation (A.Y.), and Integrated Research Project for Human and Veterinary Medicine, University of Miyazaki (A.Y., Y.A.), and Project for Developing Innovation Systems from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government (N.T.).

Disclosures
Conflict of Interest: We have no conflicts of interest to declare.

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