Human Coronary Thrombus Formation Is Associated With Degree of Plaque Disruption and Expression of Tissue Factor and Hexokinase II

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**Background:** Atherosclerotic plaque thrombogenicity is a critical factor that affects thrombus formation and the onset of acute myocardial infarction (AMI). The aim of this study was to identify the vascular factors involved in thrombus formation and AMI onset.

**Methods and Results:** Culprit lesions in 40 coronary arteries with thrombi at autopsy after lethal AMI and non-cardiac death (asymptomatic plaque disruption) were analyzed on histology. Thrombus size, ratio of thrombus to lumen area, length of plaque disruption, and immunopositive areas for tissue factor (TF) and hexokinase (HK)-II were significantly larger in coronary arteries with AMI than with asymptomatic plaque disruption. The size of coronary thrombus positively correlated with the length of plaque disruption (r=0.80) and with immunopositive areas for TF (r=0.38) and HK-II (r=0.40). Because both M1 and M2 macrophages express TF and HK-II in symptomatic plaques, we assessed TF and HK-II expression in M1- and M2-polarized macrophages. The expression of TF was increased and that of HK-II was decreased in M2-, compared with M1-polarized THP-1 macrophages. Inhibiting glycolysis enhanced TF expression in the macrophages partly via hypoxia inducible factor-1α.

**Conclusions:** The degree of plaque disruption and expression of TF and HK-II appear to be important vascular factors for AMI onset, and polarized macrophages make a distinct contribution to thrombogenicity and glucose metabolism. (Circ J 2015; 79: 2430–2438)

**Key Words:** Acute myocardial infarction; Glycolysis; Macrophage polarization; Pathology; Vascular thrombogenicity

Thrombus formation on disrupted atherosclerotic plaque is a critical event that leads to atherothrombosis, but it does not always result in complete thrombotic occlusion with subsequent acute symptomatic events. Clinical angioscopy has indicated that multiple plaque rupture is a frequent complication in patients with coronary atherothrombosis. Plaque disruption in various stages of healing is also occasionally evident on autopsy with or without coronary atherothrombosis. Therefore, plaque disruption is not the final step, whereas large thrombus formation is critical to the onset of clinical events. The thrombogenicity of exposed plaque contents, local hemorheology and blood factors are thought to affect atherothrombosis formation. Among them, atherosclerotic vascular change is essential for atherothrombosis. Determinants of thrombotic occlusion after plaque disruption remain unknown.

Tissue factor (TF) is an initiator of the coagulation cascade that is normally expressed in adventitia and to various degrees in the media of normal arteries. Atherosclerotic lesions express active TF, which is thus considered a major determinant of vascular wall thrombogenicity. Positron emission tomography (PET) using [18F]-fluorodeoxyglucose ([18F-FDG]) has been advocated as a means of evaluating arterial inflammation. The uptake of [18F-FDG] closely correlates with plaque macrophage contents in animal models and human carotid plaque. We recently found that arterial [18F-FDG] uptake reflects TF expression and vascular thrombogenicity in rabbits. This implies an association between arterial glucose metabolism and vascular thrombogenicity.

We investigated which vascular factors affect the size of coronary thrombus in autopsy cases of acute myocardial...
infarction (AMI) and non-cardiac death (asymptomatic plaque disruption).

**Methods**

**Coronary Arteries in Non-Cardiac Death and AMI**

The Ethics Committees at the participating institutions approved the study protocol (Approval No. 942). We assessed thrombotic coronary arteries from 35 individuals who had died of non-cardiac causes (20 coronary lesions from 15 patient) or AMI (20 coronary lesions from 19 patients).

The major epicardial coronary arteries (left main, left anterior descending, left circumflex, and right coronary arteries) isolated from formalin-fixed hearts as described were cut transversely at 3-mm intervals (after decalcification if necessary) and embedded in paraffin for histological evaluation. Sections (4 μm thick) were stained with hematoxylin and eosin for morphological evaluation. Consecutive sections were analyzed on immunohistochemistry.

The non-cardiac causes of death consisted of malignancy (n=8; 53%), pneumonia (n=3; 20%) and other causes, namely chronic renal failure, subarachnoid hemorrhage, and pulmonary hemorrhage (n=4; 27%). Hypertension was defined as blood pressure ≥140/90 mmHg or medication with anti-hypertensive agents. Diabetes mellitus was defined as fasting glucose ≥126 mg/dl, random non-fasting glucose ≥200 mg/dl hemoglobin A1c ≥6.5% or medication with anti-hyperglycemic agents. Hyperlipidemia was defined as total cholesterol ≥220 mg/dl, low-density lipoprotein (LDL) ≥140 mg/dl, triglycerides ≥150 mg/dl or medication with an oral lipid-lowering agent. Obesity was defined as body mass index ≥25 kg/m².

Table S1 lists the clinical characteristics of non-cardiac death and AMI at autopsy. The clinical background except for hypertension did not significantly differ between the groups.

**Immunohistochemistry**

Consecutive 4-μm slices were stained using antibodies against α-smooth muscle actin (SMA; 1A4, Dako, Glostrup, Denmark), CD68 (PGM-1; Dako), glucose transporter-1 (Glut-1; Acris, Herford, Germany), hexokinase-II (HK-II; Abcam, Cambridge, UK) and TF (Santa Cruz Biotechnology, Dallas, TX, USA). The sections were stained with Envision (Dako) or donkey anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, Herford, Germany) and Meyer’s hematoxylin counterstain. Immunostaining conditions were optimized using antibodies to CD68, α-smooth muscle actin, CD68, and TF were semiquantified using Win Roof (Nikon, Tokyo, Japan). The length of plaque disruption was defined as the extent of disrupted fibrous cap over a lipid core and/or the extent of damage beneath mural thrombi. Areas of SMA, CD68, and TF were semiquantified using Win Roof (Mitani, Fukui, Japan). These areas are expressed as a ratio of positively stained areas per total vascular area. Nucleated cells that were immunopositive for Glut-1 and HK-II in the coronary arterial wall were counted under 200× magnification, and cell density is expressed as the number of immunopositive cells per mm².

**Cell Culture**

Human THP-1 cells (Dainippon Sumitomo Pharm, Suita, Japan) were cultured in RPMI1640 (Dainippon Sumitomo Pharm) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). THP-1 cells (1.0×10⁵ cells/cm²) were differentiated into macrophages using 125 nmol/L phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO, USA), and polarized as described with modification. Cells were incubated with PMA for 6h and then cultured with PMA plus tumor necrosis factor (TNF-α; 10 ng/ml; Sigma-Aldrich) and interferon-γ (INF-γ; 20 ng/ml; R & D Systems, Minneapolis, MN, USA) for a further 42h to polarize them to the M1 phenotype. Cells were polarized to the M2 phenotype by incubation with PMA for 6h, followed by PMA and 20 ng/ml each of interleukin (IL)-4 and IL-13 (both Sigma-Aldrich) for a further 42h. Control macrophages consisted of THP-1 cells cultured with PMA for 48h. Appropriate induction of polarization was evaluated by measuring the expression of M1 and M2 marker genes, IL-6, TNF-α, and MRC-1. We assessed TF and HK-II mRNA and protein expression and procoagulant activity in these macrophages 42h later. We measured lactate level in the supernatant using a lactate oxidase-based method, and normalized values to total cellular protein or total RNA. The TF mRNA stability in polarized macrophages was assessed with actinomycin D, a transcriptional inhibitor (10 μg/ml; Sigma). The contribution of glycolysis to TF expression was evaluated by culturing THP-1 macrophages with or without the glycolysis inhibitor 2-deoxyglucose (2DG; 5 mmol/L; Sigma) and hypoxia inducible factor (HIF)-1α inhibitor (dimethyl bisphosphonate A; 100 μmol/L; Abcam).

**Quantitative Real-Time PCR**

Complementary DNA (cDNA) was prepared using Primerscript RT Master Mix kits (Takara Bio, Otsu, Japan), from cellular total RNA isolated using Trizol (Life Technologies, Carlsbad, CA, USA) and PureLink™ RNA mini kits (Life Technologies). The cDNA was assessed using quantitative PCR on a LightCycler 480 (Roche Applied Science, Penzberg, Germany) with SYBR Premix Ex Tag™ kits (Takara Bio) and the specific primers listed in Table S2. Amounts of messenger RNA (mRNA) were subsequently normalized to those of β-actin, and are shown as fold change relative to the mean of control or M1-polarized macrophages.

**ELISA and Western Blot**

Cellular proteins were extracted using a mammalian protein extraction reagent (Thermo Fisher Scientific, Waltham, MA, USA) with 1% protease inhibitor cocktail (Thermo Fisher). Protein concentrations were determined using DC protein assay kits (Biorad, Hercules, CA, USA) and TF protein expression in M1- and M2-polarized macrophages was measured using Quantikine ELISA for human TF kits (R & D Systems).

The expression of HK-II in M1- and M2-polarized macrophages was analyzed on sodium dodecyl sulfate-polyacrylamide
macrophages in 100\(\mu\)l of Tris-buffered saline (pH 7.4) containing 5 mmol/L CaCl\(_2\) and 20 mmol/L CaCl\(_2\) (100\(\mu\)l). A standard curve was constructed from serial dilutions of TF derived from human placenta (Thromborel S; Sysmex, Kobe, Japan) and procoagulant activity is expressed as arbitrary units.

**Statistical Analysis**

All data are presented as mean±SD. Categorical variables were compared using Fisher’s exact test. Differences between groups were analyzed by the Mann-Whitney U-test or unpaired t-test. Differences among individual groups were analyzed using one-way ANOVA with the Bonferroni multiple comparison test or two-way ANOVA (GraphPad Prizm 6; GraphPad Software, San Diego, CA, USA). Relationships between factors were evaluated using Spearman’s rank correlation coefficient, and P<0.05 was considered significant.

**Results**

Large Thrombus Formation and Thrombotic Occlusion on Symptomatic Plaque

Vascular factors at autopsy associated with symptomatic and asymptomatic coronary thrombi were examined on histology. The size of the thrombus, ratio of thrombus to lumen area, degree of luminal narrowing, ratio of plaque to media area, area of necrotic core, and length of plaque disruption were measured at arterial sections with thrombi. The length of plaque disruption was defined as the extent of a disrupted fibrous cap over a lipid core and/or the extent of damage beneath a mural thrombus (Figure 1). The thrombus size, ratio of thrombus to lumen area, length of plaque disruption, ratio of intima and media area, stenotic ratio, and necrotic core area were larger in symptomatic, than in asymptomatic thrombi (Figure 2A).

Figure 2B shows histological and immunohistochemical findings for CD68 (macrophage) and SMA (SMC) in symptomatic and asymptomatic macrophage coronary thrombi. Both types of arteries have plaque disruption and thrombus formation, as well as macrophage accumulation and loss of SMC at the disrupted site. Areas immunopositive for SMC and macrophages were smaller and larger in symptomatic disrupted, than in asymptomatic plaque, respectively, but the difference did not reach statistical significance (Figure 2A). TF was localized along the rim of the necrotic core or in the superficial portions of disrupted plaque (Figure 2C). The TF immunopositive area was larger in symptomatic, than in asymptomatic thrombotic plaque (Figure 2A). Figure 2D shows histological and immunohistochemical findings for CD68, Glut-1 and HK-II. Plaque cells expressed Glut-1 and HK-II in macrophage-rich areas, and 10-fold more nucleated cells were immunopositive for HK-II than for Glut-1 (Figure 2A). Erythrocytes in thrombus and plaques were also immunopositive for Glut-1. We excluded them, because this does not always reflect vascular metabolism. More cells were immunopositive for Glut-1 and HK-II cells in symptomatic, than in asymptomatic thrombotic plaques (Figure 2A). The ratios of Glut-1 and of HK-II immunopositive cells to CD68 immunopositive area were higher in symptomatic, than in asymptomatic thrombotic plaques (Figure 2A).

Double immunohistochemical staining showed that the cells expressing TF and HK-II in symptomatic thrombotic plaques were predominantly macrophages. Therefore, we examined TF and HK-II expression by macrophages that were iNOS (a M1 marker) or MRC-1 (a M2 marker) positive in plaques. Both, but not all, iNOS- and MRC-1-immunopositive cells expressed TF and HK-II (Figure 3).
Determinants of Coronary Thrombus Size

M1- and M2-Polarized Macrophages

TF and HK-II Expression  Because both M1 and M2 macrophages expressed TF and HK-II in symptomatic plaque, we compared TF and HK-II expression in THP-1-derived macrophages that were polarized to the M1 phenotype using TNF-α and INF-γ, or to the M2 phenotype using IL-4 and IL-13. Polarization of THP-1 derived macrophages to the M1 and M2 type was confirmed by the mRNA expression of TNF-α, IL-6,
activity was significantly enhanced in M2 macrophages (Figure 4E) and M1 macrophages produced more lactate (Figure 4E).

Effects of Glycolysis Inhibition on TF Expression and Procoagulant Activity Macrophages were cultured for 42 h with or without the glycolysis inhibitor 2DG to assess the relationship between glycolysis and TF expression in polarized macrophages. The glycolysis inhibitor reduced lactate production approximately 30% in both M1 and M2 macrophages. The inhibition of glycolysis enhanced the expression of TF mRNA and protein in M1 macrophage, TF mRNA in M2 macrophage and procoagulant activity in both M1- and M2-polarized macrophages (Figure 5A).

Enhancement of HIF-1α Expression and Nuclear Translocation by Glycolysis Inhibition Because 2DG is a pseudo-hypoxic state-inducing agent, we examined the effect of 2DG on HIF-1α mRNA and protein expression and TF mRNA stability in M1- and M2-polarized macrophages. The inhibition of glycolysis enhanced the expression of TF mRNA and protein in both macrophages (Figure 5A). The enhanced TF mRNA expression...
Determination of Coronary Thrombus Size

focused on determinants of coronary thrombus size and thrombotic occlusion. As previously reported, TF was localized in the rim of necrotic cores and expressed in the superficial portion of disrupted plaque. The development of thrombus aspiration has enabled the evaluation of coronary artery thrombi at the onset of AMI. Nishihira et al examined aspirated coronary thrombi obtained from 264 patients within 24 h of AMI onset on pathology, and identified plaque components in 177 (44%) of them. TF co-localizes with fibrin in coronary thrombi. Exposing blood to materials containing thrombogenic TF might contribute to coronary thrombosis. Disturbed blood flow induces erosive damage to neointima, and induces thrombus formation in rabbits. The progression of neointimal damage caused by disturbed blood flow was associated with thrombus growth. The present autopsy findings agree with these experimental findings. Therefore, the degree of and procoagulant activity by glycolysis inhibition was significantly suppressed by a HIF-1α inhibition in both M1- and M2-polarized macrophages (Figure 5C).

Discussion

The present study found that thrombotic occlusion in human coronary arteries is associated with length of plaque disruption, stenosis, necrotic core size and TF and HK-II expression in atherosclerotic plaque, and that procoagulant activity and lactate production differ in polarized macrophages. In addition, glycolytic flux negatively affected TF expression and procoagulant activity in such macrophages.

Recent findings have indicated that plaque disruption is not the final step, and that occlusive thrombus formation is critical to the onset of clinical events. Therefore, the present study focused on determinants of coronary thrombus size and thrombotic occlusion. As previously reported, TF was localized in the rim of necrotic cores and expressed in the superficial portion of disrupted plaque. The development of thrombus aspiration has enabled the evaluation of coronary artery thrombi at the onset of AMI. Nishihira et al examined aspirated coronary thrombi obtained from 264 patients within 24 h of AMI onset on pathology, and identified plaque components in 177 (44%) of them. TF co-localizes with fibrin in coronary thrombi. Exposing blood to materials containing thrombogenic TF might contribute to coronary thrombosis. Disturbed blood flow induces erosive damage to neointima, and induces thrombus formation in rabbits. The progression of neointimal damage caused by disturbed blood flow was associated with thrombus growth. The present autopsy findings agree with these experimental findings. Therefore, the degree of
Figure 5. Effect of glycolysis on tissue factor (TF) expression in polarized macrophages derived from THP-1 cells. (A) Expression of TF mRNA (n=6 each), TF protein (n=6 each), and procoagulant activity (PCA; n=7 each) in polarized macrophages with or without 2-deoxyglucose (2DG, 5 mmol/L; unpaired t-test). AU, arbitrary units. (B) Expression of hypoxia inducible factor (HIF)-1α mRNA (n=6 each, unpaired t-test), HIF-1α protein in nuclear protein (n=4 each, Mann-Whitney U-test), and TF mRNA stability in polarized macrophages with or without 2DG (5 mmol/L; n=6 each, two-way ANOVA). Expressions of HIF-1α mRNA or protein were measured on real-time PCR or ELISA, and shown as fold change relative to mean control. (C) TF mRNA expression (n=6 each) and PCA (n=5 each) in polarized macrophage with or without 2DG (5 mmol/L) and HIF-1α inhibitor (HIF-1 inh, 100 μmol/L), one-way ANOVA.
plaque disruption and subsequent exposure of TF might be the main determinant of coronary thrombotic occlusion.

The morphometric features differed between symptomatic and asymptomatic thrombotic plaques. The findings suggested that at least regional factors influence coronary thrombus size after plaque disruption. Luminal stenosis per se is not considered critical to AMI onset because of compensatory expansive remodeling of thin fibrous cap atheroma and severe luminal stenosis in patients with stable angina. Coronary occlusions that induce M1 evolve most frequently in segments without severe stenosis according to an angiographic study, but a more recent study has shown that plaque stenosis and size measured on intravascular ultrasound are related to clinical events. The expansive remodeling and differences in mathematical dimension might affect the interpretation. Mauriello et al showed that symptomatic thrombotic carotid plaques have long plaque disruption, severe stenosis, abundant macrophages and a large necrotic core, compared with asymptomatic thrombotic plaques. The present results are compatible with these features except for the macrophage content, which in the present study tended to be larger in symptomatic, than in asymptomatic thrombotic plaques. The difference, however, did not reach statistical significance, because only a few asymptomatic thrombotic plaques in the present study had abundant macrophages. Although further studies are required, the difference in macrophage content could be due to anatomical and rheological differences between carotid and coronary arteries.

Both Glut-1 and HK-II were upregulated in symptomatic thrombotic plaque. The expression of Glut-1 and HK-II in the carotid artery was enhanced in advanced atherosclerotic plaques compared with early lesions. More FDG accumulated in culprit coronary lesions from patients with AMI than in those with stable angina pectoris. We recently showed that arterial F-FDG uptake reflects TF expression and vascular thrombogenicity in rabbits. These lines of evidence and the present results suggest that arterial glucose metabolism reflects the thrombogenicity of coronary atherosclerotic plaques. Because more cells were immunopositive for HK-II than for Glut-1 and this correlated with coronary thrombus formation, HK-II expression might be a marker of coronary plaque thrombogenicity.

Macrophages comprise a heterogeneous cell population with functional variability in atherosclerotic plaques. Although the precise role of polarized macrophages in atherosclerotic lesion remains undefined, M1 and M2 macrophages are generally considered atherogenic and atheroprotective, respectively. The NF-κB pathway is important in M1 macrophages, and the myeloid deletion of IκB kinase results in decreased inflammation and reduced lesion formation in mice deficient in LDL receptor. M2 macrophages express abundant IL-10, which protected against atherogenesis by modulating macrophage functions. TF expression and procoagulant activity in polarized macrophages, however, remain unknown. We found more TF expression and procoagulant activity in RAW 264.7 macrophage identified sedoheptulose kinase, an enzyme of the pentose phosphate pathway, as a possible repressor of LPS-induced TNF-α production. Sedoheptulose kinase affects macrophage polarization through the control of glucose metabolism. The overexpression of glucose-6-phosphate dehydrogenase, the first and rate-limiting enzyme of the pentose phosphate pathway, affects the expression of pro-inflammatory cytokine genes in RAW 264.7 macrophages. Therefore, the present findings support the notion that glucose metabolism affects macrophage function. Glycolysis inhibition may induce a pseudohypoxic state, and low glucose concentration enhanced HIF-1α expression in non-neoplastic cells. The present results suggest that the enhanced TF mRNA expression and procoagulant activity caused by glycolysis inhibition is partly dependent on HIF-1α-mediated responses in M1- and M2-polarized macrophages.

This study has several limitations. Autopsy samples represent one feature of the dynamic process of thrombus formation and thrombolysis. We measured the length of plaque disruption in a single histological section and thus could not address the 3-D extent. Nevertheless, the close correlation suggested that the extent of plaque injury is a major determinant of coronary thrombus size and the onset of coronary events.

Conclusions

The degree of plaque disruption and expression of TF and HK-II appear to be important vascular factors for AMI onset; and polarized macrophages make a distinct contribution to thrombogenicity and glucose metabolism.

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Disclosures

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