



Upregulation of Monocyte Tissue Factor Activity Is Significantly Associated With Low-Grade Chronic Inflammation and Insulin Resistance in Patients With Metabolic Syndrome

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Background: The metabolic syndrome (MetS) is a cluster of cardiovascular risk factors closely linked to inflammation and insulin resistance (IR). Tissue factor (TF) is an initiator of the extrinsic coagulation cascade and is expressed on peripheral blood monocytes and macrophages in atherosclerotic plaques. Monocytes are the principle cells capable of TF synthesis. Therefore, TF plays an important role in both thrombosis and atherosclerosis. Elevated levels of lipopolysaccharide (LPS), a strong stimulator of TF, have been observed in patients with MetS. No study has investigated the relationship between monocyte TF activity and inflammation, and IR in MetS.

Methods and Results: Peripheral blood mononuclear cells (PBMCs) were collected from 40 normal subjects and 77 patients with MetS. Mononuclear cell TF procoagulant activity (MPCA) was measured with and without 100pg/ml LPS stimulation using a 1-stage clotting assay and expressed as the mean \pm SD (mU TF/10⁶ PBMCs). MPCA in MetS was significantly greater than in normal subjects (without LPS: 88.0 \pm 74.8 vs 52.6 \pm 9.8 mU TF/10⁶ PBMCs, $P < 0.001$; with LPS: 269.6 \pm 165.6 vs 158.6 \pm 42.8 mU TF/10⁶ PBMCs, $P < 0.001$). The LPS-stimulated log MPCA in MetS patients was significantly associated with homeostasis model assessment of IR ($r = 0.256$, $P = 0.024$) and log high-sensitivity C-reactive protein ($r = 0.332$, $P = 0.003$).

Conclusions: Upregulation of monocyte TF is significantly associated with low-grade inflammation and IR in MetS. (*Circ J* 2010; **74**: 572–577)

Key Words: Inflammation; Insulin resistance; Metabolic syndrome; Tissue factor

Metabolic syndrome (MetS) has been identified as a clustering of cardiovascular risk factors, including abdominal obesity, hypertension and dyslipidemia with high triglycerides (TG), and low high-density lipoprotein-cholesterol (HDL-C). Recent evidence suggests that MetS is significantly associated with insulin resistance (IR) and low-grade chronic inflammation and an increased risk of cardiovascular disease (CVD), including acute coronary syndrome (ACS).^{1–4} Tissue factor (TF) is an initiator of the extrinsic coagulation cascade and is expressed in peripheral blood monocytes and on macrophages in atherosclerotic plaques. TF may play a significant role in both thrombus formation and the progression of atherosclerosis in patients with MetS.⁵

Bacterial lipopolysaccharide (LPS) constitutes the outer

cell wall membrane of Gram-negative bacteria and is a strong stimulator of TF. In a mouse model of MetS, the mean LPS level was 400pg/ml,⁶ and Wiedermann et al report that elevated LPS is associated with an increased risk of CVD.⁷ No previous studies have determined the relationship between monocyte TF and inflammation, and IR in patients with MetS.

Methods

Study Subjects

A total of 77 patients with MetS (52 males, 25 females; mean age, 66.7 \pm 11.2 years) and 40 normal subjects (23 males, 17 females; mean age, 67.2 \pm 10.5 years) were enrolled. All patients were recruited from Tama-Nagayama Hospital, Nippon

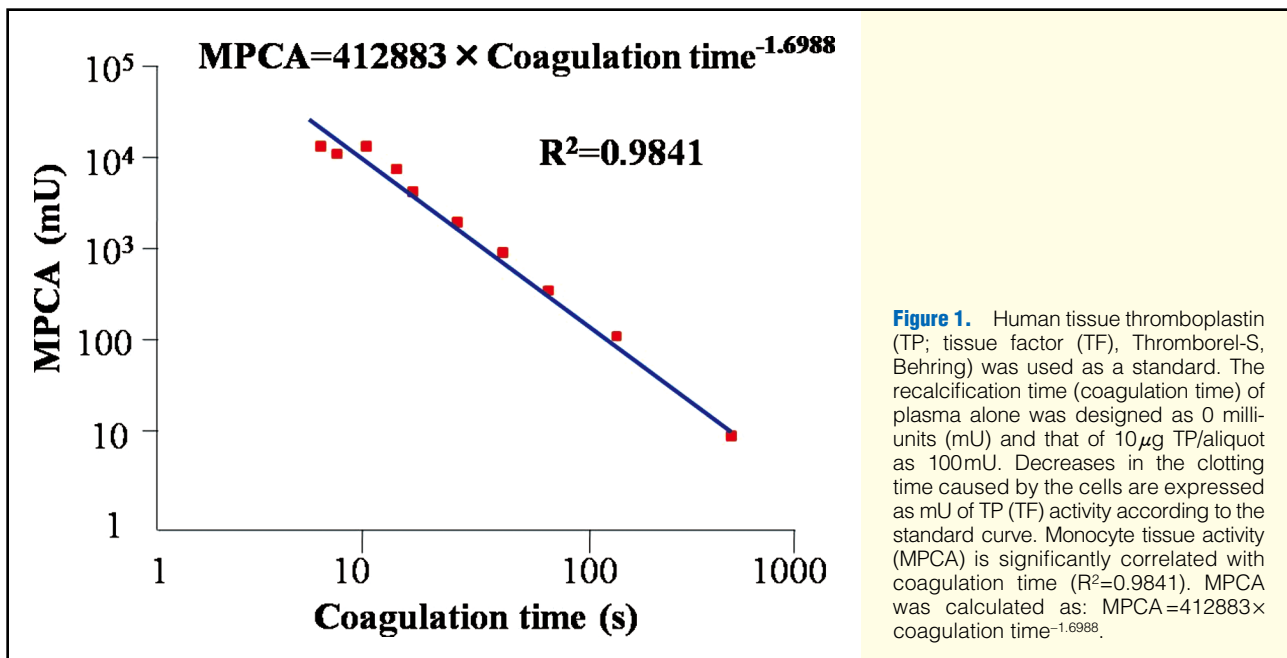
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Medical School at an outpatient clinic from January 2005 to December 2008.

MetS was diagnosed in accordance with the current Japanese criteria:⁸ the presence of visceral obesity defined as waist circumference (WC) ≥ 85 cm in males or ≥ 90 cm in females as an essential component combined with 2 or more of the following components: (1) serum TG ≥ 150 mg/dl, (2) HDL-C < 40 mg/dl in either males and females, (3) blood pressure (BP) $\geq 130/85$ mmHg, and (4) fasting plasma glucose (FPG) ≥ 110 mg/dl. Anyone with clinical signs of acute infection, autoimmune disorders, severe renal (serum creatinine level > 2.0 mg/dl) or hepatic disease or with a suspected malignancy was excluded. In addition, patients with a history of CVD, including coronary artery disease, cardiomyopathy and valvular heart disease, stroke and arteriosclerosis obliterans, were also excluded from this study. The investigation protocol was designed according to the guidelines of the institutional ethics committee. All subjects provided written informed consent to participate in the study.

Monocyte Isolation and Laboratory Measurements

Citrated venous peripheral blood (20 ml) was collected from normal subjects and the MetS patients. Peripheral blood mononuclear cells (PBMCs: monocytes and lymphocytes) were isolated as described previously.⁹ In brief, PBMCs were obtained by gradient centrifugation on Lymphoprep. PBMCs in serum-free RPMI were incubated at 37°C (in air containing 5% CO₂). The viability of the PBMCs was $> 99\%$, as determined by trypan blue exclusion. We usually got approximately 5×10^7 PBMCs from 20 ml of venous peripheral blood and counted the numbers of cells with a hemocytometer. PBMCs were then pipetted into 96-well plates, and 10^6 PBMCs was added per well. For most experiments, PBMCs isolated as described were used because other blood cells are not capable of expressing TF. However, in some experiments, the monocytes were enriched ($> 95\%$ pure by nonspecific esterase staining) by incubating PBMCs for 2 h at 37°C in serum-coated wells and then harvesting lymphocytes, washing the wells with warmed RPMI to remove

any nonadherent cells (lymphocytes) and then adding fresh RPMI and the experimental reagents. This procedure yields cells that consistently comprise more than 95% monocytes as determined by nonspecific esterase staining.

High-sensitivity C-reactive protein (hs-CRP) was used as the parameter of inflammation and the homeostasis model assessment of IR (HOMA-IR; score equals immunoreactive insulin (μ U/ml) \times FPG (mg/dl) divided by 405) was calculated and used as the marker of IR. Low-density lipoprotein-cholesterol (LDL-C) concentrations were measured by a Wako homogeneous LDL-C assay as described in elsewhere.¹⁰

Measurement of Procoagulant

The mononuclear cell TF procoagulant activity (MPCA) stimulated with and without 100 pg/ml LPS (*Escherichia coli* 055: B5) was obvious after 4 h and reached an optimal level between 8 h and 12 h, and thereafter declined slightly. Therefore, PBMCs were routinely cultured in 96-well plates for 16 h in the presence or absence of LPS, and then washed twice with RPMI to remove lymphocytes, and tested. MPCA was measured using a 1-stage plasma recalcification test with an automatic coagulometer (Diagnostica Stago), as previously described.⁹ MPCA was calculated from a standard curve using dilutions of human tissue thromboplastin (Thromborel-S, Behring) as a standard (Figure 1); the results are expressed as milliunits (mU) TF/ 10^6 PBMCs.

Endotoxin (LPS) Assay

Endotoxin (LPS) measurements were performed using the QCL-1000 Chromogenic Limulus Amebocyte Lysate (LAL) Endpoint Assay (Lonza, Japan) as described by Miller et al.¹¹ This assay is a quantitative test for Gram-negative bacterial endotoxin. Samples (in duplicate) were mixed with the LAL supplied in the test kit and incubated at 37°C for 10 min. Substrate solution was then mixed with the LAL-samples and incubated at 37°C for an additional 6 min. The reaction was stopped with stop reagent. The absorbance of the sample was determined by plate-reader at 405 nm by comparison with a standard curve (range 0–100 pg/ml). The intra-assay

Table. Clinical Characteristics and Blood Chemistry of the Patients With MetS and the Normal Subjects

	MetS (n=77)	Normal (n=40)	P value
Age (years)	67±11	67±10	0.802
Gender (M/F)	52/25	23/17	0.314
BMI (kg/m ²)	27±3	22±2	<0.001
WC (cm)	96±7	77±10	<0.001
SBP (mmHg)	132±8	118±7	<0.001
HR (beats/min)	72±4	70±4	0.386
TC (mg/dl)	210±30	196±12	0.004
TG (mg/dl)	151±81	102±21	<0.001
HDL-C (mg/dl)	55±12	59±7	0.037
LDL-C (mg/dl)	134±32	116±14	0.002
FPG (mg/dl)	134±32	96±8	<0.001
hs-CRP (mg/dl)	0.21±0.32	0.12±0.06	0.020

MetS, metabolic syndrome; BMI, body mass index; WC, waist circumference; SBP, systolic blood pressure; HR, heart rate; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; FPG, fasting plasma glucose; hs-CRP, high-sensitivity C-reactive protein.

coefficient of variance was 3.5%. Media, including RPMI, were tested for endotoxin levels, which demonstrated levels <5 pg/ml of endotoxin (LPS).

Statistical Analysis

The results are presented as the mean values±SD for continuous variables and as percentages of the total number of patients for categorical variables. Student's t-test for independent samples and the chi-square test were used for comparison of the continuous and categorical variables, respectively. MPCA and hs-CRP levels were not normally distributed, thus some MPCA (with and without LPS) and the hs-CRP data were log transformed (log₁₀), and the Mann-Whitney test was used for unpaired comparisons between the 2 groups, and Wilcoxon's signed rank test was used for paired comparisons within the 2 groups. The coefficients of correlation (r) were calculated using the Spearman test. The Statistical Package for Social Science (SPSS) for Windows, version 11.0 (Chicago, IL, USA) was used for all statistical analyses and P<0.05 was considered to be statistically significant.

Results

Patients with MetS showed a significantly higher body mass index (BMI: 27±3 vs 22±2 kg/m², P<0.001), WC (96.1±6.6 vs 76.7±9.6 cm, P<0.001), systolic BP (132±8 vs 118±7 mmHg, P<0.001), hs-CRP (0.21±0.32 vs 0.12±0.06 mg/dl, P=0.020), FPG (134±32 vs 96±7 mg/dl, P<0.001), LDL-C (133±32 vs 116±14 mg/dl, P=0.002) and TG (151±81 vs 102±21 mg/dl, P<0.001), and had lower HDL-C (55±12 vs 59±7 mg/dl, P=0.037) compared with the normal subjects. However, there were no significant differences in age or sex between the 2 groups (Table).

Comparison of TF Activity on PBMCs and Monocytes Alone

The MPCA stimulated with and without 100 pg/ml LPS was compared between from PBMCs and purified monocytes to examine the possibility of TF activity by other blood cells such as lymphocytes. Preliminary studies from 10 patients with MetS showed that MPCA stimulated with and without LPS was slightly higher than that of monocytes alone; however, the differences did not reach statistical significance (without LPS: 77.8±38.9 vs 66.9±33.5, P=0.172; with LPS: 232.2±116.1 vs 225.6±112.8 mU TF, P=0.105). These data indicate that monocytes are the principle cells capable of TF synthesis, so PBMCs were routinely cultured for this study.

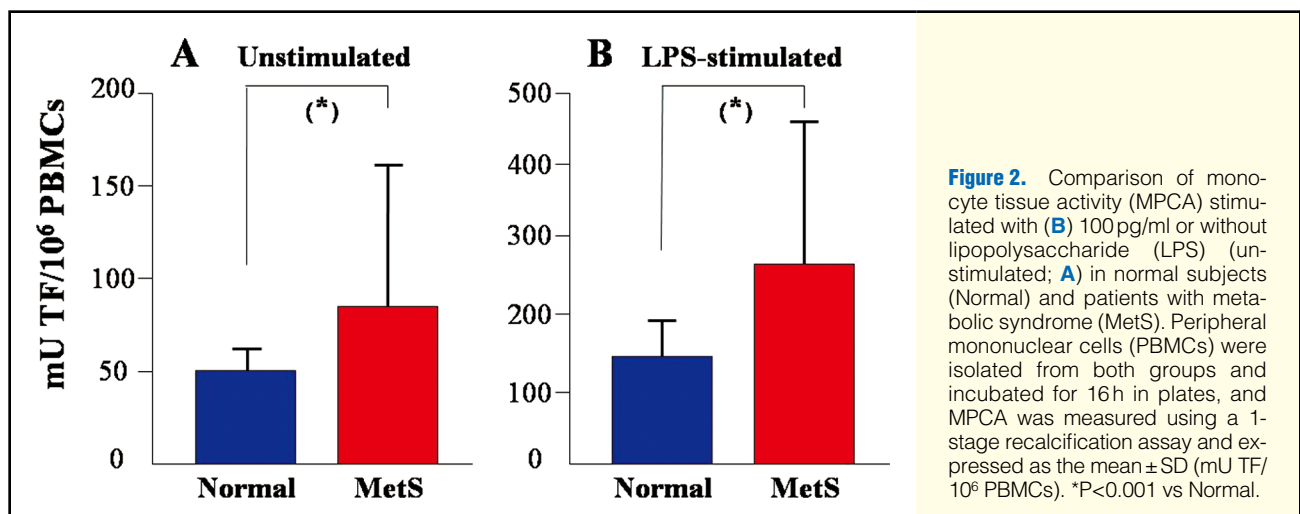
MPCA in Normal Subjects and Patients With MetS

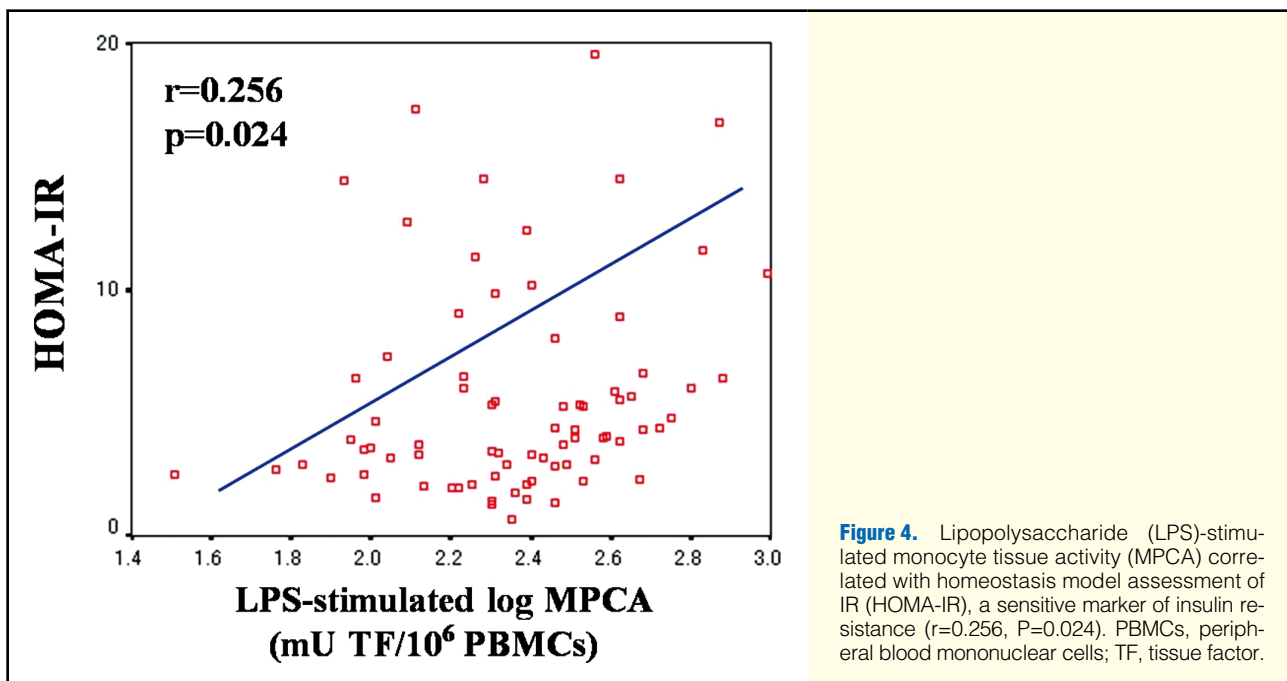
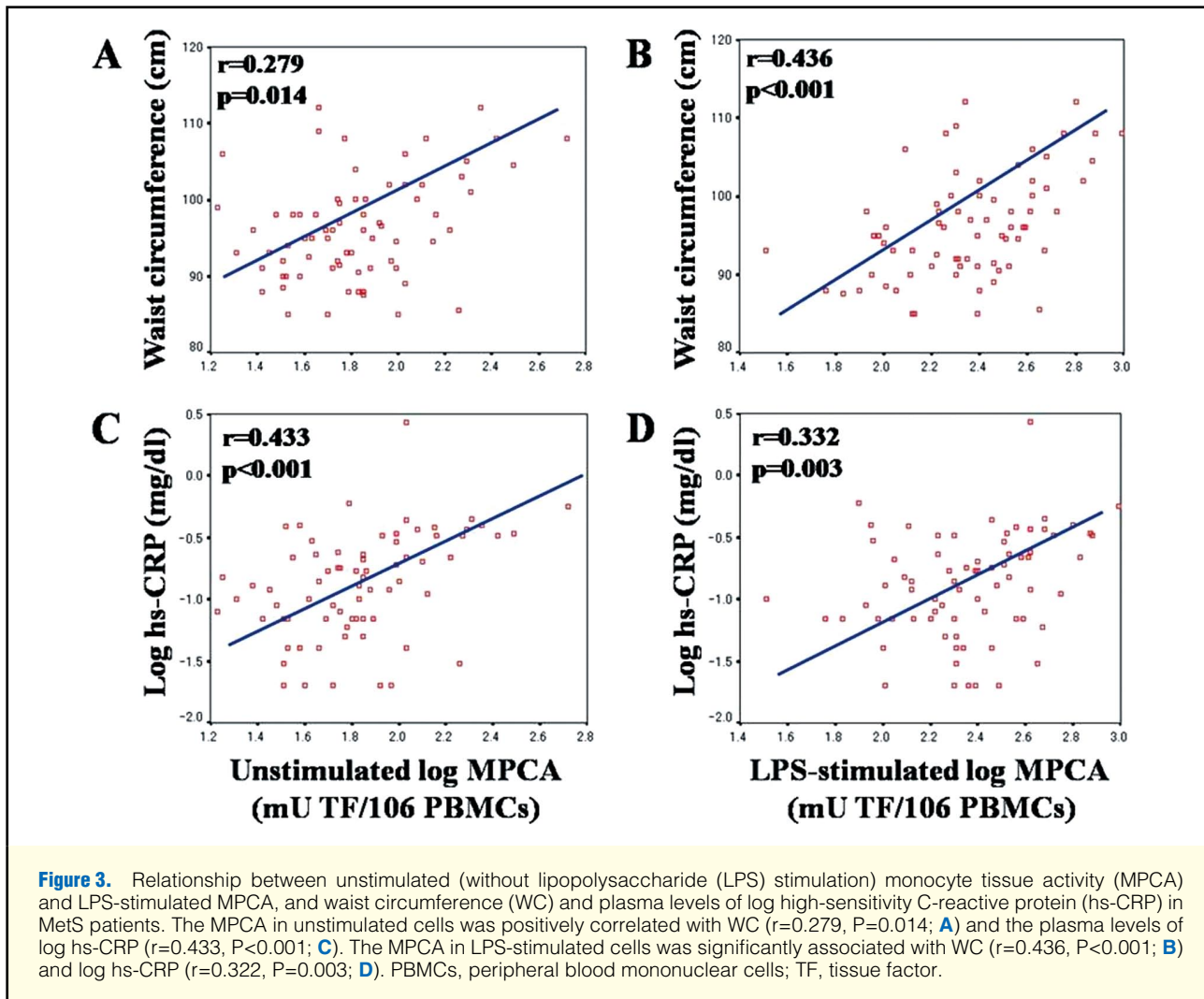
MPCA stimulated with LPS (Figure 2B) and without LPS (unstimulated cells; Figure 2A) in patients with MetS was significantly greater than in normal subjects (without LPS: 88.0±74.8 vs 52.6±9.8 mU TF/10⁶ PBMCs, P<0.001; with LPS: 269.6±165.6 vs 158.6±42.8 mU TF/10⁶ PBMCs, P<0.001). These data indicate that MPCA was upregulated in patients with MetS.

Relationship Between MPCA and Inflammation, and IR

The log MPCA in unstimulated PBMCs was positively correlated with WC (r=0.279, P=0.014; Figure 3A) and log hs-CRP (r=0.433, P<0.001; Figure 3C); however, log MPCA was not related to HOMA-IR (r=0.209, P=0.068).

The log MPCA stimulated with 100 pg/ml LPS was positively correlated with WC (r=0.436, P<0.001; Figure 3B) and log hs-CRP (r=0.332, P=0.003; Figure 3D), and log MPCA was significantly, but weakly, correlated with systolic BP (r=





0.268, $P=0.019$) and HOMA-IR ($r=0.256$, $P=0.024$; **Figure 4**). However, MPCA with and without LPS was not related to FPG, TG, HDL-C or LDL-C.

Endotoxin (LPS) Levels in Normal Subjects and Patients With MetS

Endotoxin (LPS) levels were significantly higher in the 40 patients with MetS (median 19.4 pg/ml, range 10.3–68.2 pg/ml) than in the 20 normal subjects (median 11.8 pg/ml, range 10.2–18.9 pg/ml; $P<0.001$). Unstimulated MPCA and LPS-stimulated MPCA in patients with MetS were significantly associated with plasma levels of endotoxin (unstimulated: $r=0.576$, $P<0.001$; LPS-stimulated: $r=0.739$, $P<0.001$).

These data indicate that MPCA, particularly MPCA stimulated with LPS, is significantly associated with low-grade chronic inflammation and IR in patients with MetS. In addition, our preliminary studies showed that the TF procoagulant activity stimulated with and without LPS was similar between PBMCs and monocytes alone. We have also previously shown that TF antigen on monocytes from PBMCs can be detected by immunofluorescence.¹² Therefore, monocytes are the principle cells capable of TF synthesis. TF plays a significant role in both thrombosis and atherosclerosis. Taken together, upregulation of monocyte TF activity may contribute to the pathogenesis of thrombosis and to progression of atherosclerosis in patients with MetS.

Discussion

Monocytes Are the Principle Cells Capable of TF Synthesis

We showed that MPCA stimulated with or without LPS is similar between PBMCs and monocytes alone, thus indicating that monocytes are the principle cells capable of TF synthesis. Immunohistochemistry confirmed TF antigen levels on monocytes from the PBMCs.¹²

PBMCs From Patients With MetS Are Pre-Activated

MetS is associated with abdominal obesity, blood lipid disorders, inflammation and IR, and an increased risk of developing CVD, including ACS.^{1–4} Recent studies have shown that low-grade chronic inflammation associated with visceral obesity leads to the onset of IR and type 2 diabetes mellitus.^{3,4} Obesity associated with inflammation is characterized by an increased abundance of macrophages from peripheral blood monocytes in adipose tissue, together with production of proinflammatory cytokines, including tumor necrosis factor (TNF)- α and plasminogen activator inhibitor-1 (PAI-1), an inhibitor of the fibrinolytic system.^{4,13,14} Ghanim et al showed elevated TNF- α and interleukin (IL)-6 mRNA expression on PBMCs in obese subjects compared with normal subjects, thus indicating that PBMCs from obese subjects were in a proinflammatory state.¹⁵ In addition, we previously showed that monocytes from patients with unstable angina are pre-activated and express TF.¹² Here, we have shown that PBMCs and monocytes from patients with MetS are more responsive to LPS than cells from normal subjects, thus suggesting that cells from MetS patients have undergone pre-activation. Therefore, pre-activated monocytes may infiltrate adipose tissue and contribute to the pathogenesis of inflammation and IR in patients with MetS.

MetS and Hypercoagulability

Carter et al showed that plasma from patients with MetS formed denser clots compared with that from subjects free of MetS.¹⁶ In addition, subjects with MetS had prolonged clot

lysis times compared with those without MetS, partly because of increased circulating levels of PAI-1. TF, a potent activator of the extrinsic coagulation cascade is widely expressed in atherosclerotic plaques in ACS and found in macrophages and peripheral monocytes.^{17,18} TF binds factor VIIa, resulting in activation of factors IX and X, ultimately leading to fibrin formation. TF overexpression in a rat model accelerated neointimal development and thrombus formation,¹⁹ indicating that TF plays a significant role in the pathogenesis of thrombosis and in the development of atherosclerosis.

Monocyte-Derived TF Activity in MetS Patients

Diamant et al⁵ measured the number of microparticles (small membrane vesicles from activated blood cells and endothelial cells) in plasma and compared the number of TF-positive microparticles between normal subjects ($n=16$) and MetS patients ($n=18$). They reported that the median number of TF-positive microparticles analyzed by flow cytometry was twice as high in the MetS patients than in the normal subjects; however, they did not show the number of monocyte-derived microparticles. From that study, we cannot ascertain which cells produced TF.

Recently, Boden et al²⁰ measured circulating TF activity in whole blood, but not monocytes, from 22 normal subjects and 18 patients with type 2 diabetes. They reported that circulating TF activity in whole blood from patients with type 2 diabetes was significantly higher than in blood from normal subjects. They measured monocyte TF mRNA, but did not show monocyte-derived TF activity. Because monocytes are the principle cells capable of TF synthesis, it is very important to measure monocyte-derived TF activity. At the moment, there is scant data regarding monocyte-derived TF activity in MetS, and there are no data regarding the relationship between monocyte TF activity and inflammation, and IR. The current study shows for the first time that monocyte TF activity is upregulated and significantly associated with low-grade inflammation and IR in patients with MetS.

Potential Role of LPS as a Proinflammatory Mediator in MetS

It has been speculated that an elevated TF level might result from various stimulants such as oxidized LDL, hyperglycemia, angiotensin II, and proinflammatory cytokines.^{21,22} However, the pathophysiological mechanisms of elevated TF activity remain to be established in MetS.

Endotoxin (ie, LPS) is a unique glycolipid that comprises most of the outer leaflet of the outer wall of Gram-negative bacteria. LPS activates inflammatory cells, increases oxidative stress and modifies lipoprotein metabolism, thus possibly triggering or accelerating atherosclerosis.²³ LPS at levels as low as 50 pg/ml constitutes a strong risk factor for the development of atherosclerosis.⁷ In addition, increased endotoxemia was observed in an experimental model of MetS, with a mean LPS concentration of 400 pg/ml, and metabolic endotoxemia initiates obesity and IR.⁶ Recently, Miller et al¹¹ showed a graded increase in endotoxin level from black Africans to whites to South Asians. Here, we show that endotoxin (LPS) levels in patients with MetS were elevated, with a median level of 19.4 pg/ml, and that unstimulated and LPS-stimulated MPCA were positively correlated with the plasma level of endotoxin.

Monocytes are the principle cells capable of TF synthesis and the current study showed that monocyte TF activity in the presence or absence of LPS-stimulation was significantly upregulated in patients with MetS compared with normal

subjects, and that LPS-stimulated monocyte TF activity was significantly associated with the hs-CRP level and HOMA-IR, a sensitive marker of IR. These data suggest that upregulation of monocyte TF activity is, at least in part, related to LPS stimulation in the plasma of patients with MetS.

Relationship Between Monocyte TF Activity and IR in MetS

The mechanisms of IR are complicated and complex, but increased release of TNF- α , IL-6, monocyte chemoattractant protein (MCP)-1 and additional products of the monocytes and macrophages that populate adipose tissue might have a role in the development of IR.²⁴ Recently, we investigated monocyte-derived TNF- α and MCP-1 production stimulated with or without LPS in normal subjects and MetS patients. We found that monocyte-derived TNF- α and MCP-1 production with or without LPS stimulation were significantly greater in the patients than in the normal subjects (data not shown).

Nutrient excess is also linked to IR via activation of nuclear factor (NF)- κ B, a key transcriptional mediator of inflammation.²⁵ Toll-like receptor (TLR)-4, an endogenous ligand for bacterial LPS, has been implicated as a mediator of IR.²⁶ Bacterial LPS is a potent activator of NF- κ B in monocytes. In addition, Kim et al²⁷ showed that TLR-4 mediated vascular inflammation and IR in a mouse model of diet-induced obesity. Once again, LPS was a strong stimulator of TF via the TLR-4 pathway. Therefore, proinflammatory cytokines and chemokines, including TNF- α and MCP-1, LPS and their combination via the TLR-4 pathway, may be involved in the upregulation of monocyte TF activity and IR in MetS patients.

Study Limitations

This study had a small population, so future additional studies are necessary to confirm these findings in large-scale clinical trials.

Conclusion

Monocyte TF activity is significantly upregulated in patients with MetS and this is significantly associated with low-grade chronic inflammation and IR. Further studies are needed to confirm these preliminary findings.

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