Independent Roles of Monocyte Chemoattractant Protein-1, Regulated on Activation, Normal T-Cell Expressed and Secreted and Fractalkine in the Vulnerability of Coronary Atherosclerotic Plaques

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Background: Monocyte chemotactic factors contribute to the formation of atherosclerotic plaques. The present study aimed to elucidate the roles of monocyte chemoattractant protein-1 (MCP-1), Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES) and fractalkine on the vulnerability of atherosclerotic plaques in patients with acute myocardial infarction (AMI) or unstable angina pectoris (UAP).

Methods and Results: Sixty patients with AMI, 60 patients with UAP, 60 patients with stable angina pectoris (SAP) and 40 patients without coronary heart disease comprised the study group. Quantitative coronary angiography and intravascular ultrasound (IVUS) were performed. Concentrations and mRNA expression levels of high-sensitivity C-reactive protein, MCP-1, RANTES and fractalkine were measured by ELISA and RT-PCR, respectively. IVUS found that 51.3% of the AMI patients and 47.7% of the UAP patients had soft lipid plaques. Among the SAP patients, 52.4% had fibrous plaques and only 17.1% had soft plaques. AMI and UAP patients had larger plaque burden and vascular remodeling index than did the SAP patients ($P<0.01$). The averaged number of migrated monocytes was higher in AMI and UAP patients. Concentrations and mRNA expression levels of MCP-1, RANTES and fractalkine were significantly higher in AMI and UAP patients than in SAP patients ($P<0.05$ – 0.01). The plaque burden in UAP patients as measured with IVUS correlated well with monocytes chemotaxis ($r=0.56$, $P<0.01$).

Conclusions: MCP-1, RANTES and fractalkine independently participate in the pathogenesis of plaque vulnerability and subsequent plaque rupture. (*Circ J* 2012; **76**: 2167–2173)

Key Words: Acute coronary syndrome; Atherosclerosis; Monocyte chemotactic factors; Vulnerable plaque

Vulnerable atherosclerotic plaque is a major pathological lesion leading to plaque rupture and thrombosis in patients with acute coronary syndrome (ACS).^{1,2} Studies have demonstrated that atherosclerosis is a complex and perpetuating inflammatory disease and active inflammation is an important feature of vulnerable plaques. Monocyte infiltration of the arterial intima is essential for inflammation and contributes to the formation of atherosclerotic plaques. Leukocyte migration from blood into tissues is controlled by endothelial leukocyte adhesion molecules and multiple chemokines.

Chemokines constitute a family of structurally related and secreted basic chemotactic cytokines in both human and ex-

perimental atherosclerotic disease. Chemokines are classified into 4 subgroups (CXC, CC, C, CX3C) according to the position of the N-terminal cysteines.^{3–5} As the first and prototypic CC chemokine, monocyte chemoattractant protein-1 (MCP-1) has high expression in human atherosclerotic lesions and in animal models of atherosclerosis with dietary-induced hypercholesterolemia. The intensive infiltration of macrophages into plaques with active inflammation could be, to a large extent, due to the chemotactic effects of MCP-1.^{6–8} Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES) also belongs to CC family of chemokines, and studies in humans and murine models of atherosclerosis demonstrate a central role

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Table 1. Clinical and Biochemical Characteristics of the 4 Study Groups

Parameter	Gr AMI (n=60)	UAP (n=60)	Gr SAP (n=60)	Control (n=40)
Age (years)	55.4±5.3	57.9±7.2	57.3±5.7	57.0±7.1
Male	39	33	35	37
Systolic BP (mmHg)	139.4±11.9	136.0±11.1	136.7±12.3	130.1±9.2
Diastolic BP (mmHg)	82.3±12.7	80.1±12.5	81.2±15.0	78.4±9.9
BMI (kg/m ²)	27.6±3.1	26.9±2.5	27.1±2.7	25.0±2.2
Diabetes mellitus	23 (38.3%)	18 (30.0%)	16 (26.7%)	10 (25.0%)
Dyslipidemia	26 (43.3%)	20 (33.3%)	19 (31.7%)	12 (30.0%)
Hypertension	22 (36.7%)	19 (31.7%)	18 (30.0%)	11 (27.5%)
Current smoker	21 (35.0%)	21 (35.0%)	20 (33.3%)	12 (30.0%)
Glucose (mg/dl)	115.0±31.0	116.1±36.5	114.7±30.2	103.3±23.0
Total cholesterol (mg/dl)	226.6±31.3	229.3±38.7	213.5±38.3	211.6±29.8
LDL (mg/dl)	151.2±34.0	155.1±37.5	133.0±33.6	130.7±22.4
HDL (mg/dl)	40.2±5.8	41.0±6.2	39.2±5.4	42.9±8.9
Triglycerides (mg/dl)	139.1±54.0	142.6±54.9	140.8±57.6	137.3±50.5
Leucocytes (×10 ⁹ /L)	8.2±3.1*†	8.0±3.0*†	5.2±2.2	4.9±2.0
hsCRP (mg/L)	6.9±2.7**††	5.7±2.3**††	2.9±1.2	2.7±1.4
MCP-1 (pg/ml)	91.2±9.7**††	87.3±9.1**††	37.0±6.6†	23.7±7.1
RANTES (pg/ml)	62.7±8.2*††	61.8±7.9*††	27.3±4.5†	15.8±5.1
Fractalkine (pg/ml)	65.1±9.7*††	62.3±10.5*††	34.0±4.7†	19.9±6.0

*P<0.05, **P<0.01 vs. SAP; †P<0.05, ††P<0.01 vs. Control.

AMI, acute myocardial infarction; UAP, unstable angina pectoris; SAP, stable angina pectoris; BP, blood pressure; BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; MCP, monocyte chemoattractant protein-1; RANTES, Regulated on Activation, Normal T-cell Expressed and Secreted.

Table 2. Primers for Real-Time RT-PCR

Gene	Primer sequence
β-actin	F:5' CATGTACGTTGCTATCCAGGC 3' R:5' CTCCTTAATGTCACGCACGAT 3'
MCP-1	F:5' CAGCCAGATGCAATCAATGCC 3' R:5' TGAATCCTGAACCCACTTCT 3'
RANTES	F:5' ATCCTCATTGCTACTGCCCTC 3' R:5' GCCACTGGTGTAGAAATACTCC 3'
Fractalkine	F:5' ACCACGGTGTGACGAAATG 3' R:5' CTCCAAGATGATTGCGCGTTT 3'

RT-PCR, reverse transcription polymerase chain reaction. Other abbreviations as in Table 1.

of RANTES in the initiation of atherosclerotic plaque and in promoting late-stage atherosclerosis.^{9,10} Fractalkine (CX3CL1) is a unique member of the CX3C subfamily and is expressed in both a soluble and membrane-bound form on the surface of the inflamed endothelium.^{11,12} Combadière et al found that the CX3CR1–CX3CL1 pathway plays a direct and major role in the development of macrophage-rich atherosclerotic lesions and that CX3CR1/apolipoprotein E double knockout mice show decreased atherosclerotic lesion formation.¹³ Fractalkine is reportedly associated with atherogenesis and 2 specific mutations of the CX3CR1 gene have been shown to reduce the risk for future coronary events.¹⁴ However, whether MCP-1, RANTES and fractalkine prompt plaque vulnerability and contribute to ACS is still unknown. In the present study, we assessed the hypothesis that the expression of MCP-1, RANTES and fractalkine is higher in patients with acute myocardial infarction (AMI) or unstable angina pectoris (UAP) and is closely associated with plaque vulnerability.

Methods

Study Population

All 220 patients underwent selective coronary angiography (CAG; 127 men, 93 women; age 59.8±8.2 years, range 38–84 years) and were divided into 4 groups according to the guidelines of the American College of Cardiology and American Heart Association.^{15,16} Group A comprised 60 patients with AMI and group B included 60 patients with UAP. There were 60 patients with stable angina pectoris (SAP) in group C and 40 patients without coronary heart disease in group D (control group). The baseline characteristics of the 4 groups are shown in Table 1. All patients in the AMI, UAP and SAP groups were treated after admission with antiplatelet and other therapies including angiotensin-converting enzyme inhibitors/angiotensin II receptor blockers, β-blockers and statins. All patients gave their informed consent to this study and the protocol was approved by the Ethics Committee of Qilu Hospital of Shandong University.

Blood Biomarker Measurements

Blood samples from every patient were collected on admission to hospital. Serum levels of total cholesterol, triglycerides, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol were measured by enzymatic assays. The C-reactive protein level (CRP) was assayed using a highly sensitive enzyme-linked immunosorbent assay kit (Diagnostic System Laboratory, Chicago, IL, USA). Levels of MCP-1, RANTES and fractalkine were measured using enzyme-linked immunosorbent assay kits (You er Biochemistry Company, Wuhan, China).

Quantitative CAG (QCA)

All patients underwent CAG. Percent diameter stenosis, percent area stenosis, culprit lesion length, and reference diameter were assessed by QCA, which was performed using the Digital Cardiac Imaging system. Coronary stenosis was defined if

Table 3. Measurements of Intravascular Ultrasound Imaging in the 3 Patient Groups			
Parameter	Gr AMI (n=60)	UAP (n=60)	Gr SAP (n=60)
Plaque	117	111	105
Lipid plaque	60 (51.3%)**	53 (47.7%)**	18 (17.1%)
Fibrous plaque	21 (17.9%)**	23 (20.7%)**	55 (52.4%)
Mixed plaque	19 (16.2%)	15 (13.5%)	13 (12.4%)
Calcified plaque	18 (15.4%)	19 (17.1%)	18 (17.1%)
Patients with ruptured plaque	81 (69.2%)**	59 (53.2%)**	27 (25.7%)
Minimum lumen area (mm ²)	3.3±0.9	3.3±0.8	3.8±0.9
External elastic membrane area (mm ²)	12.8±1.3**	12.6±1.8**	11.4±1.3
Plaque area (mm ²)	9.5±2.6**	9.3±2.0**	7.6±2.8
Plaque burden (%)	74.2±10.3**	73.8±8.9**	66.7±8.3
Eccentric index	0.7±0.2*	0.6±0.2*	0.5±0.2
Remodeling index	1.1±0.1**	1.1±0.1**	1.0±0.1

*P<0.05, **P<0.01 vs. SAP.

AMI, acute myocardial infarction; SAP, stable angina pectoris; UAP, unstable angina pectoris.

Figure 1. Intravascular ultrasound imaging of the coronary arteries. (A) Plaque with a large lipid core in the coronary artery of a patient with acute myocardial infarction. (B) Lipid plaque in the coronary artery of a patient with unstable angina. (C) Fibrous plaque in the coronary artery of a patient with stable angina.

there was a more than 50% decrease in diameter compared with an adjacent reference segment and the number of stenotic lesions in each coronary artery was recorded.

Intravascular Ultrasound (IVUS) Studies

Three-coronary IVUS (iLab, Boston Scientific Corporation,

USA) was carried out in all patients as described previously and the following parameters were measured: external elastic membrane area (EEMA), minimum lumen area, plaque area (PA), plaque burden (PB), plaque eccentricity index, and remodeling index (RI). RI >1.05 was regarded as positive remodeling, 0.95–1.05 as intermediate remodeling, and <0.95 as

negative remodeling. Coronary plaque composition was assessed visually according to plaque echogenicity.^{17,18} Plaque rupture was defined as a plaque containing a cavity that communicated with the lumen and had an overlying residual fibrous cap fragment or was wedged by newly-formed thrombus, no matter whether plaque rupture occurred in culprit or non-culprit lesions.¹⁹ The IVUS images were reviewed by 2 independent observers and the final consensus values were used for data analysis.

Chemotaxis Test

Monocytes were extracted by centrifugation of the blood sam-

ples, and 0.1 ml of the monocyte suspension ($4 \times 10^4/\text{ml}$) was instilled into the transwell chamber to assay monocyte chemotaxis. Monocytes were stimulated by the patient's own serum as described previously.²⁰ Next, the number of migrated monocytes and the distance traveled by monocytes in 10 high-power microscopic fields were measured and the values averaged.

Molecular Biological Studies

Total RNA of the monocytes was extracted, and the mRNA expression of MCP-1, RANTES and fractalkine was examined by quantitative reverse transcription polymerase chain reaction (RT-PCR) using a LightCycler (Roche Applied Science, USA) following the manufacturer's instructions. The mRNA sequences were obtained from GenBank (Table 2). Quantitative values were obtained from the threshold cycle value (Ct).²¹ The transcript number of glyceraldehyde 3-phosphate dehydrogenase was quantified as an internal control. The results of RT-PCR were confirmed by gel electrophoresis.

Statistical Analysis

All statistical analyses were performed with SPSS16.0 software package (SPSS Inc, Chicago, IL, USA). Values are expressed as mean $\pm$ SD. Comparison of continuous variables among multiple groups was performed by analysis of variance with ANOVA and post hoc comparisons were made using the lysergic acid diethylamide test. Chi-squared analysis was applied to compare categorical data. Linear regression analyses were performed to evaluate the relationship between the results of IVUS and chemotaxis and expression of MCP-1, RANTES and fractalkine. A 2-tailed $P < 0.05$ was considered statistically significant.

Results

Base Line Characteristics and Blood Biomarker Measurements

As shown in Table 1, there were no significant differences among the 4 groups regarding age, sex, blood pressure, body weight, blood sugar and lipid profile. Leucocyte counts and concentrations of high-sensitivity CRP (hsCRP), MCP-1, RANTES and fractalkine in groups A and B were significantly higher than those in groups C and D. However, there was no significant difference between groups A and B.

QCA

CAG studies demonstrated a total of 117 and 111 coronary stenotic lesions in the AMI and UAP groups, respectively, while in the SAP group there were 105 coronary lesions; 76 lesions (65%) in the AMI, 77 lesions (69%) in the UAP and 69 lesions (66%) in the SAP group had stenosis $\geq 70\%$. QCA results did not show significant differences regarding percent diameter stenosis and area stenosis among the AMI, UAP and SAP groups (Table 4).

IVUS Measurements

IVUS detected 117 plaques in group A, 111 plaques in group B and 105 plaques in group C. Patients in group A (51.3%) and group B (47.7%) had more lipid plaques than those in group C (17.1%) (all $P < 0.01$, respectively), while patients in group C had more fibrous plaques (52.4%) than those in group A (17.9%) and group B (20.7%) ($P < 0.01$, respectively). There were more ruptured plaques in groups A and B than those in group C ($P < 0.01$, respectively). However, there was no significant difference between group A and group B. There were more eccentric plaques in groups A and B, and the values of EEMA, PA, PB and RI in groups A and B were significantly larger than those in group C ($P < 0.05$ – 0.01) (Table 3, Figure 1).

Table 4. Measurements of Quantitative Coronary Angiography in 3 Patient Groups

Parameter	Gr AMI (n=60)	UAP (n=60)	Gr SAP (n=60)
Percent diameter stenosis (%)	78.8±11.3	76.8±9.1	72.4±8.0
Percent area stenosis (%)	95.5±5.4	94.6±5.2	92.4±4.9
Culprit lesion length (mm)	15.5±4.8	18.0±5.0	19.4±5.2
Reference lumen diameter (mm)	3.1±0.40	3.0±0.4	3.0±0.4
Reference lumen area (mm ²)	7.3±1.9	7.3±1.8	7.3±1.9

Abbreviations as in Table 1.

Table 5. mRNA Expression (%) of MCP-1, RANTES and Fractalkine in the 4 Study Groups

Parameter	Gr AMI (n=60)	UAP (n=60)	Gr SAP (n=60)	Control (n=40)
MCP-1	41.4±9.3 ^{*,††}	38.9±8.7 ^{*,††}	25.0±7.1 [†]	12.5±4.4
RANTES	42.8±7.1 ^{*,††}	35.2±6.1 ^{*,††}	23.4±5.2	15.3±3.9
Fractalkine	37.7±6.1 ^{*,††}	29.3±4.0 ^{*,†}	21.6±3.2	19.1±2.2

*P<0.05, **P<0.01 vs. SAP; †P<0.05, ††P<0.01 vs. Control.

Abbreviations as in Table 1.

Chemotaxis Test

The averaged number of migrated monocytes measured in 10 high-power microscopic fields in groups A and B was more than that in group C (162 ± 16 , 155 ± 15 vs. 46 ± 9 , $P < 0.01$, respectively; **Figure 2**). The averaged distance traveled by monocytes measured in 10 high-power microscopic fields in groups A and B was longer than that in group C ($117.8 \pm 17.9 \mu\text{m}$, $113.3 \pm 16.1 \mu\text{m}$ vs. $60.1 \pm 8.3 \mu\text{m}$, $P < 0.05$, respectively). Results of linear regression analyses showed that PB measured with IVUS correlated well with monocyte chemotaxis in group B ($r = 0.56$, $P < 0.01$, **Figure 3**).

Real-Time RT-PCR

The levels of mRNA expression of MCP-1, RANTES and fractalkine in groups A and B were significantly increased compared with groups C and D ($P < 0.05$, respectively). However, there was no significant difference between group A and B (**Table 5**).

Discussion

Atherosclerotic plaque rupture is the major cause of ACS and stable plaques may become vulnerable to rupture or erosion once they develop active inflammation. Inflammation has emerged as a crucial force driving the initiation and progression of atherosclerotic lesion formation.^{22,23} Accumulation of monocytes/macrophages has been noted in the intima of early atherosclerotic lesions and these inflammatory cells may promote progression of atherosclerosis by producing and releasing various cytokines and chemokines. Studies have demonstrated that the chemokines MCP-1, RANTES and fractalkine are potent activators and chemoattractant signals of inflammatory and vascular cells.^{6,9,12,24} In the present study, we have shown higher expressions of MCP-1, RANTES and fractalkine in patients with AMI or UAP. Our results demonstrate that MCP-1, RANTES and fractalkine independently partici-

pate in the pathogenesis of plaque vulnerability and subsequent plaque rupture.

Once combined with its receptor CCR2, MCP-1 activates monocytes/macrophages by promoting leukocyte-endothelium binding and migration to sites of inflammation. MCP-1 as an upstream chemokine in the atherosclerotic inflammatory pathway has been consistently detected in human atherosclerotic lesions, as well as in animal models of atherosclerosis with dietary-induced hypercholesterolemia, and it is a logical reasoning that the intensive infiltration of macrophages into plaques with active inflammation is, to a large extent, due to the chemotactic effects of MCP-1. Aiello et al reported that MCP-1 accelerated atherosclerosis in apolipoprotein E-deficient mice and was extensively expressed in macrophages in atherosclerotic plaques.²⁵ MCP-1-deficient mice also had less prominent aortic accumulation of macrophages, reflecting the relative paucity of MCP-1-derived monocyte chemoattraction.²⁶ Gosling et al found that the absence of MCP-1 appeared to confer dramatic protection against macrophage recruitment and atherosclerotic lesion formation without affecting lipoprotein metabolism in MCP-1-deficient mice.²⁷ Zhong et al in our laboratory found that dominant-negative mutation of MCP-1 prevented vulnerable plaques from rupture in rabbits, independent of serum lipid levels.⁶

The CC chemokine RANTES and its receptors CCR1 and CCR5 in atherosclerosis have been addressed in a number of studies. RANTES has been implicated in cardiac inflammatory disorders after organ transplantation or arterial injury. In addition, RANTES has been detected in plasma samples of patients suffering from coronary heart disease. Mice deficient in the RANTES gene show impaired recruitment of T cells and monocytes to inflammatory foci.²⁸ Barcelos et al found that antagonism of RANTES receptors with Met-RANTES reduced atherosclerotic plaque formation in mice.²⁹ Braunersreuther et al reported that RANTES plays a central role in promoting late-stage atherosclerosis in the context of accelerated atherosclerosis in ApoE^{-/-} mice promoted by high-fat diet.³⁰

Fractalkine (CX3CL1) is a unique member of the CX3C chemokine subfamily. Accumulating evidence indicates CX3CL1/CX3CR1 involvement in the pathogenesis of atherosclerosis.^{31,32} Teupser et al reported that the targeted deletion of CX3CL1 decreased atherosclerotic lesion formation in the brachiocephalic artery but not the aortic root, with fewer macrophages in the lesion area of atherosclerosis in CX3CL1^{-/-} ApoE^{-/-} mice, implicating a role of this chemokine system in atherogenesis.³³ Furthermore, Lucas et al found expression of CX3CL1 and CX3CR1 in human coronary atherosclerotic plaques, and that CX3CL1/CX3CR1 may contribute to atherogenesis and plaque destabilization in human coronary artery disease.³⁴ Cheng et al found that blockade of CX3CL1 inhibited plaque growth and resulted in striking differences in plaque composition in low shear stress regions of the carotid arteries.³⁵

Studies have also shown that MCP-1, RANTES and fractalkine play independent roles in atherogenesis. Digby et al reported that the anti-inflammatory effects of nicotinic acid in adipocytes contribute to its suppression of fractalkine, RANTES, and MCP-1 and upregulation of adiponectin.³⁶ Saederup et al reported that fractalkine deficiency markedly reduced macrophage accumulation and atherosclerotic lesion formation in CCR2^{-/-} mice, providing the first in vivo evidence of independent roles for CX3CL1 and CCR2 in direct monocyte recruitment to atherosclerotic lesions.³⁷ Combined inhibition of CCL2, CCR5, and CX3CR1 in ApoE^{-/-} mice abrogated bone marrow monocytoysis and additively reduced circulating monocytes and atherosclerosis, despite persistent hypercholesterolemia. More-

over, the inhibition of CCL2, CX3CR1, and CCR5 in ApoE^{-/-} mice almost abolished atherosclerosis, suggesting that CX3CR1-, CCL2-, and CCR5-mediated signals play independent and additive roles in atherogenesis.²⁴

Many investigators have reported that certain inflammatory markers, such as hsCRP, have the potential to predict the risk of future cardiovascular events.^{38,39} Liuzzo et al found that elevated CRP concentration (>3 mg/L) at the time of hospital admission predicted a poor outcome in patients with UAP.⁴⁰ In the present study, we found that the hsCRP, MCP-1, RANTES and fractalkine levels were significantly higher in patients with AMI and UAP than in patients with SAP, suggesting that serum levels of MCP-1, RANTES and fractalkine may be good markers of plaque vulnerability.

In the present study, we tested the monocyte chemotaxis in patients with ACS and found that the averaged number of migrated monocytes and the averaged distance traveled by monocytes in patients with AMI or UAP were higher than those of SAP patients, which reflects the close association of monocyte chemotaxis with the vulnerability of atherosclerotic plaques.

In order to show the expression levels of MCP-1, RANTES and fractalkine that modulate monocyte chemotaxis, we measured their mRNA expression levels in the monocytes of patients with ACS or SAP and found that mRNA expression levels of MCP-1, RANTES and fractalkine were higher in patients with ACS than in those with SAP. All these results reveal that MCP-1, RANTES and fractalkine are the main chemokines contributing to atherosclerotic plaque vulnerability.

Among the many plaque imaging techniques, IVUS has been advocated as assessing the morphological changes of vulnerable plaque in vivo.^{18,19,41-43} Our current study clearly demonstrated that patients with AMI or UAP have more vulnerable plaques characterized by a more eccentric distribution, a larger EEM area, a bigger PA and a larger PB. Correlation analysis found that PB measured with IVUS correlated well with monocyte chemotaxis, implying a role of monocytes chemotaxis and the related chemokines MCP-1, RANTES and fractalkine in the development of atherosclerotic plaques.

In conclusion, MCP-1, RANTES and fractalkine independently participate in the pathogenesis of coronary plaque vulnerability and subsequent plaque rupture.

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