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Myeloid-related protein-8/14 in acute coronary syndrome

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ABSTRACT

Background: The alarmin family member myeloid-related protein (MRP)-14 (S100A9), which has been identified by platelet transcriptional profiling as an acute myocardial infarction gene, regulates vascular inflammation and thrombosis. Elevated plasma levels of MRP-8/14 (S100A8/A9) heterodimer predict first and recurrent cardiovascular events. The aim of this study was to elucidate pathophysiological roles of MRP-8/14 in acute coronary syndrome (ACS).

Methods and results: In 38 consecutive ACS patients, the MRP-8/14 level in coronary artery blood obtained at thrombus aspiration was higher in 23 patients, in whom aspirated thrombus was confirmed, compared to the 15 patients, in whom it was absent [4.86 (1.95, 8.29) vs 2.94 (1.31, 4.44), $P = 0.017$]. The MRP-8/14 level was correlated with myeloperoxidase (MPO) level ($R^2 = 0.52$), but not with soluble P-selectin level ($R^2 = 0.0002$) in the coronary artery blood. Immunohistochemistry of the aspirated thrombus exhibited that expression of MRP8/14 was co-localized with leukocytes positive for activated Mac-1. Finally, in cultured human umbilical vein endothelial cells, MRP-8/14 increased tissue factor expression.

Conclusions: Our findings indicate that MRP-8/14 concentration increases in coronary artery blood in association with thrombus formation in ACS, co-localizes with leukocytes, and is associated with leukocyte activation. MRP-8/14 is positioned as a unique biomarker at the interface of inflammation and thrombosis in ACS.

1. Introduction

Atherothrombosis, which results from vulnerable plaque disruption or superficial endothelial cell erosion [1], is a major cause of acute myocardial infarction (AMI) and cardiovascular death. Atherosclerosis represents a series of highly specific cellular and molecular responses that are commonly viewed as a chronic inflammatory disease [2]. The lesions associated with acute coronary syndrome (ACS) are characterized by mild-moderate stenosis, thin-cap fibroatheroma, a large plaque burden (≥70% in coronary vessel cross-sectional area), a minimal luminal area of ≤4 mm^2 or some combination of these characteristics, as determined by gray-scale and radiofrequency intravascular ultrasonography [3]. However, the pathological mechanisms responsible for plaque activation and thrombus formation are poorly understood. We utilized transcriptional profiling for platelets to identify novel regulators of vascular inflammation and thrombus formation in patients with acute ST-segment elevation myocardial infarction (STEMI) or stable coronary artery disease (CAD). Myeloid-related protein (MRP)-14, which is also known as S100A9 or calgranulin B, was identified as one of 54 STEMI-related genes [4]. MRP-14 and MRP-8 (S100A8), members of the alarmin family and S100 family of calcium-modulated proteins, are expressed in cells of myeloid origin, especially in monocytes and neutrophils [5]. In both mice and humans, MRP-8 and MRP-14 form the heterodimer MRP-8/14 (S100A8/A9), which is the predominant form and is far more abundant than MRP-8 or MRP-14 homodimers. Activated monocytes and neutrophils secrete MRP-8/14 [6], which regulates myeloid cell function by modulating calcium signaling [7] and cytoskeletal reorganization [8], by operating as a chemoattractant [9], and by binding to cell surface receptors, such as CD36 [10], toll-like receptor-4 (TLR-4) [11], and receptor for advanced glycation end products (RAGE) [12]. MRP-8/14 broadly regulates vascular inflammation. Deficiency of MRP-14 reduces neointimal formation after femoral artery wire injury, attenuates thrombohemorrhagic vasculitis, and atherosclerotic lesion formation and plaque inflammation in apo *E*-deficient mice [13].

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We reported previously that elevated plasma levels of MRP-8/14 predicted both first [4] and recurrent [14] cardiovascular events. MRP-8/14 was also shown to be a novel biomarker of ACS, capable of discriminating myocardial injury earlier than creatine kinase (CK)-MB, and cardiac troponin [15]. In addition, high levels of MRP-14 expressed in a subset of non-foam cell macrophages in human plaques, are strongly associated with histopathologic evidence for plaque activation [16]. These findings suggest that MRP8/14 broadly regulates vascular inflammation and may be a useful biomarker of ACS. However, a pathophysiological role for MRP-8/14 in ACS is incompletely characterized. Recently, we identified a new pathway of thrombosis distinct from hemostasis that involves platelet MRP-14 and the platelet receptor CD36, and suggested that targeting MRP-14 has potential for treating atherothrombotic disorders, including MI and stroke [17]. The aim of this study was to investigate the pathophysiological significance of MRP-8/14 in patients presenting with ACS.

2. Method

2.1. Clinical investigation

The subjects included 38 patients with ACS, who underwent PCI for a culprit lesion using radial approach. ACS consisted of unstable angina (UA) and AMI, including non-ST-segment elevation myocardial infarction (NSTEMI) and STEMI. UA was defined as typical chest pain at rest with the last episode occurring within 24 h of admission and either a history of CAD or ischemic changes on standard 12 lead electrocardiogram, but without elevation of cardiac biomarkers (i.e., creatine kinase-MB and/or cardiac troponins). AMI was defined as symptoms of ischemia and detection of a rise and/or fall of cardiac biomarker values with at least one value above the 99th percentile upper reference limit and new or presumably new significant ST-T changes or new left bundle branch block or Q waves on the 12 lead electrocardiogram and imaging evidence of new loss of viable myocardium or new regional wall motion abnormality [18]. All of the patients received 162 mg aspirin and 300 mg clopidogrel as loading doses prior to the PCI procedure, and 81 mg/day aspirin and 75 mg/day clopidogrel as maintenance doses for at least 12 months after the PCI. Intravenous heparin was administrated to maintain an adequate activated clotted time during PCI procedure.

Prior to PCI procedure, aortic blood was taken via the guiding catheter positioned in the aortic root after therapeutic anticoagulation with heparin. Prior to revascularization, thrombectomy was performed using a thrombus aspiration catheter (Thrombuster: Kaneka Medix Corp., Osaka, Japan). For "thrombus" aspiration, the coronary artery blood samples were aspirated with the catheter just distal to the site of the culprit lesion without regard to presence or absence of angiographically visible thrombus. If aspirated thrombus was identified after filtering, the thrombus was removed and fixed accordingly for standard immunohistochemical analysis. We used blood samples from the aorta and coronary artery distal to the culprit lesion for biomarker quantification (Fig. 1A). Routine balloon angioplasty and stent deployment were performed subsequently. The local institutional ethics committees approved the protocol. Written informed consent was obtained from all patients.

2.2. Sample preparation

Whole blood was immediately collected into tubes containing either serum separating medium or ethylene-diamine-tetraacetic acid (EDTA) for plasma separation. Blood was centrifuged at 1500 G for 15 min at room temperature, and the serum and plasma samples were frozen and stored at −80 °C until analysis. Serum samples were used for high sensitvity C-reactive protein (hsCRP) assay. EDTA plasma samples were used for enzyme-linked immunosorbent assay (ELISA). The aspirated thrombi obtained from the culprit lesion in the ACS patients were frozen at -80 °C and sliced 20 μm-thick sections to maintain enzymatically intact status for immunohistochemistry.

2.3. Biomarker assay

ELISA assays were conducted using commercially available kits for the measurement of myeloperoxidase (R&D Systems Inc., Minneapolis, MN, USA), soluble P-selectin (RayBiotech Inc., Norcross, GA, USA), and MRP-8/14 (Bühlmann Laboratories AG, Schönenbuch, Switzerland). Performance characteristics of the ELISA assay for MRP-8/14 includes intra-assay imprecision of 4.8% at 3.4 mg/L, inter-assay imprecision of 4.4% at 5.1 mg/L, minimal detectable concentration of 0.3 mg/L and functional sensitivity (level of 15% imprecision) of 0.56 mg/L. All ELISA biomarker assays were performed duplicated wells to confirm the reproducibility. The hsCRP level was also measured by particle-enhanced technology on the Behring BN II nephelometer (Dade Behring Inc., Newark, DE, USA), using monoclonal anti-CRP antibodies and a calibrator that was traceable to WHO Reference Material [19].

2.4. Immunohistochemistory

Aspirated thrombi were first stained by hematoxylin-eosin and then immunofluorescence staining was performed using standard avidin-biotin procedures for MRP-8/14 antibody (BMA Biomedicals, August, Switzerland) conjugated to Alexa-Fluor 488 (Thermo Fisher Scientific K.K., Yokohama, Japan), CBRM1/5 antibody (Santa Cruz Biotechnology, Dallas, TX, USA), an antibody against activation-dependent neoepitope of Mac-1 (CD11b/CD18) on the surface of leukocytes conjugated to Alexa-Fluor 647 (Thermo Fisher Scientific), and CD42a antibody, a platelet membrane glycoprotein (GP) IX (Bio—Rad Laboratories, Herculus, CA, USA) conjugated to Alexa-Fluor 647 (Thermo Fisher Scientific). Cells positive for immunofluorescence staining were observed using a confocal laser scanning microscope (LSM5 Pascal: Carl Zeiss A.G., Jena, Germany), in which Alexa-Fluor 488-positive cells were shown in green color and Alexa-Fluor 647-positive cells were in red color.

2.5. Tissue factor release by MRP-14

Tissue factor (TF) expression and release were investigated in human umbilical vein endothelial cells (HUVEC) stimulated with recombinant MRP-14. HUVEC (2×10^5, Lonza Japan Ltd., Chiba, Japan) were cultured until semi-confluent in 6 cm dish containing Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich Corp., St. Louis, MO, USA), and 10% heat-inactivated fetal bovine serum (Biowest Inc., Neaillé, France). The cells were then synchronized by overnight culture in 0.5% serum-containing media. The medium was replaced with 10% serum-containing DMEM or 10% serum-containing DMEM together with recombinant MRP-14 (1 and 5 μg/mL, Giotto Biotech S.r.l., Sesto Fiorentino, Italy), angiotensin II (1 and 5 μg/mL, Sigma-Aldrich Corp.) as a positive control, or phosphate buffered saline (PBS) as a negative control. Cell culture supernatants were prepared at 2 and 4 h after stimulation by MRP-14, angiotensin II or PBS. The supernatants were assayed directly for TF level using a commercially available Tissue Factor (Human) ELISA kit (Abnova Corp., Taipei, Taiwan).

2.6. Statistical analysis

Data were expressed as mean ± SD or median and interquartile range. Normality for distribution of continuous variables was assessed using Shapiro-Wilk test. Intergroup comparisons were performed using the Chi-square test for categorical variables and Student unpaired *t*-test for normally distributed continuous variables. Intragroup comparisons for normally distributed continuous variables were performed using Student paired t-test. If the continuous variables were not normally distributed, Mann-Whitney *U* tests for intra-group comparisons and Wilcoxon Rank Sum test for intergroup comparisons were used. Serial changes in the variables were evaluated by repeated measures analysis of variance followed by post-hoc Tukey test for intra- and intergroup comparisons. Correlations between two parameters were assessed using simple liner regression. All statistical analyses were performed using the statistical package for the Social Science (Dr. SPSS II for Windows, SPSS Inc., Tokyo, Japan). $P < 0.05$ was considered significant.

3. Results

3.1. Plasma MRP-8/14 level in ACS patients

In total cohort ($n = 38$), there were no significant differences in the plasma level of MRP-8/14 [4.42 (2.98, 6.71) vs 3.56 (1.89, 6.77) μg/mL, $P = 0.578$] as well as serum level of hsCRP [2.06 (0.39, 6.52) vs 1.94 (0.42, 7.76) μg/mL, $P = 0.675$] between aortic blood samples and coronary artery blood samples distal to the culprit ACS lesion (Fig. 1B). Visible thrombi were present in aspirated coronary artery blood in 23 patients, with no visible thrombi in 15 patients. Baseline characteristics were similar between the 2 patient groups with and without visible thrombus (Table 1). We then compared the levels of MRP-8/14 and hsCRP between patients with and without aspirated thrombus. The MRP-8/14 level in the aortic samples tended to be higher in patients with visible thrombus compared to the patients without thrombus [5.37 (3.38, 7.65) vs 3.75 (2.26, 4.54) μg/mL, $P = 0.084$]. Interestingly, MRP-8/14 concentration in coronary artery blood distal to the culprit lesion was significantly higher in patients with thrombus, compared to patients without thrombus [4.86 (1.95, 8.29) vs 2.94 (1.31, 4.44) μg/mL, $P = 0.017$]. The hsCRP level in the aortic samples [1.94 (0.41, 6.89) vs 2.18 (0.35, 6.09) μg/mL, $P = 0.758$] and coronary artery samples [2.68 (0.58, 7.99) vs 1.68 (0.35, 3.75) μg/mL, $P = 0.572$] were both similar between patients with and without thrombus (Fig. 1C). Next we assessed potential correlations between MRP8/14 and leukocyte-secreted myeloperoxidase as well as between MRP-8/14 and soluble P-selectin, a platelet and endothelial cell activation marker in patients with and without thrombus using the coronary artery blood samples. In the patients with thrombus, MRP-8/14 concentration was significantly correlated with the myeloperoxidase level ($R^2 = 0.525$), but not with soluble P-selectin level ($R^2 = 0.0002$). On the other hand, in patients without thrombus,

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