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Clinical Significance of Leukotriene B₄ and Extracellular Matrix Metalloproteinase Inducer in Acute Coronary Syndrome

Abstract

Purpose: Leukotriene B₄ (LTB₄) and extracellular matrix metalloproteinase (EMMPRIN) have been suggested as modulators of atherosclerotic plaque instability. This study sought to evaluate the potential diagnostic implication of LTB₄ and EMMPRIN in patients with acute coronary syndrome (ACS).

Methods: Patients (n=153) who underwent coronary angiography, including 105 patients diagnosed with ACS, were divided into four groups: stable angina pectoris (SAP, n=19), unstable angina pectoris (UAP, n=39), acute myocardial infarction (AMI, n=66) and control (with normal coronary angiography, n=29). EMMPRIN expression in peripheral blood mononuclear cells was determined by flow cytometry and serum LTB₄ levels were measured by ELISA. To examine whether LTB₄ can regulate the expression of EMMPRIN and matrix metalloproteinases (MMPs) in macrophages, differentiated THP-1 macrophages were stimulated with different concentrations of LTB₄ (10⁻¹⁰-10⁻⁷mmol/L). Expression of EMMPRIN was evaluated by Western blotting. MMP-9 mRNA expression and enzymatic activity were determined by RT-PCR and SDS-PAGE gelatin zymography.

Result: Serum LTB₄ concentration was significantly higher in AMI and UAP groups, compared with control and SAP groups (p<0.01). Subgroups analysis showed that LTB₄ was significantly higher in the AMI<24h group, compared with the AMI>24h group. Expression of EMMPRIN on circulating monocytes was significantly higher in patients with UAP and AMI (>24h), compared with control, SAP and AMI (<24h) groups (p<0.05). In vitro study showed LTB₄ up-regulated the expression of EMMPRIN, as well as the expression and activity of MMP-9, in cultured THP-1-derived macrophages (p<0.05).

Conclusion: LTB₄ and EMMPRIN are associated with the pathogenesis of ACS and may be potential biomarkers for patients with ACS.

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Acute coronary syndrome (ACS) encompasses a continuum of conditions ranging from unstable angina (UAP) to non-ST-segment elevation myocardial infarction (NSTEMI) and ST-segment elevation myocardial infarction (STEMI), usually due to the acute rupture of a plaque. Although the mechanisms of unstable plaque rupture are complex and poorly understood, more and more studies have indicated a key role of leukotriene (LTs), products of the 5-lipoxygenase (5-LO) pathway, in the pathogenesis of ACS [1]. Matrix metalloproteinases (MMPs) are a family of zinc-containing enzymes that play an important role in a variety of physiological and pathological processes, including tissue repair, cardiac and vascular remodeling and atherosclerotic plaque instability [2-4]. In atherosclerosis, MMPs secreted by cells are able to digest the extracellular matrix and contribute to weakening and rupturing of plaque [5,6]. It has been reported that the enforced expression of an active form of MMP-9 in macrophages induced disruption of atherosclerosis plaques in apoE-deficient mice [7]. Given that MMP-9 was shown to co-localize with 5-LO in activated macrophages in atherosclerotic plaques [8], it was predicted that 5-LO in macrophages at atherosclerotic lesions may accelerate rupture of atherosclerotic plaques through enhanced production of MMP-9. Leukotriene B₄ (LTB₄), the most potent chemotactic substance ever described for leukocytes, can stimulate macrophages and smooth muscle cells to express MMPs [9-11]. Extracellular matrix metalloproteinase inducer (EMMPRIN), named from its function as an activator of MMPs, has been demonstrated to be involved in ACS as well [12-15]; however, there are limited studies about the circulating levels of LTB₄ and EMMPRIN and their potential relationship.

In this study, the potential diagnostic implications of LTB₄ and EMMPRIN were investigated in patients with ACS. The ability of LTB₄ to regulate the expression of EMMPRIN and MMP-9 in cultured macrophages was also evaluated.

Methods

Patients

A total of 153 consecutive patients who underwent coronary angiography (CAG) for suspected coronary artery disease (CAD) were prospectively enrolled at Taizhou Hospital from March through September 2009. Baseline characteristics are provided in Table 1. There were 105 patients diagnosed as ACS, including 39 patients with unstable angina pectoris (UAP) and 66 with acute myocardial infarction (AMI). AMI was diagnosed based on a history of prolonged ischemic chest pain, elevation of troponin-I and creatine kinase more than three times the normal value, with a concomitant rise in

troponin-I and MB isoenzyme, and ST-segment elevations. Patients who met at least two of these criteria were included in the study. UAP was diagnosed in patients with typical angina pain at rest, combined with reversible or persistent ischemic EKG changes with or without changes in troponin-I and CK-MB activity below the decision limits for AMI. AMI patients were divided into two subgroups that were classified by the time of the latest onset of chest pain: the 32 AMI patients who had the most recent chest pain within 24 hours (AMI<24h) and the other 32 AMI patients who had the most recent chest pain more than 24 hours prior to AMI (AMI>24h). Fasting blood samples were obtained early on the second morning. Patients in the control group were confirmed to be free of coronary heart disease by coronary angiography. Patients with inflammatory disorders, cancer, asthma or infection were excluded. The protocols for this study were approved by the hospital ethics committee. Informed consent was provided to the patients according to the Declaration of Helsinki.

Enzyme linked immunosorbent assay (ELISA)

Peripheral blood samples were centrifuged to collect serum, which was dispensed in 50 µl aliquots and stored at -80°C until use. Human LTB₄ Immunoassay kit was purchased from R&D SYSTEMS, with a detectability of 5.63 ng/L. The experiments were performed according to manufacturer's instructions.

Flow cytometry

Expression of EMMPRIN in peripheral blood mononuclear cells (PBMCs) was determined by flow cytometry, according to the manufacturer's instructions. PBMC-surface EMMPRIN expression was detected by direct immunofluorescence. Whole blood cells were labeled with fluorescence in isothiocyanate (FITC)-conjugated anti-CD14 monoclonal antibody and phycoerythrin (PE)-conjugated anti-CD147 monoclonal antibody. Whole blood cells treated with fluorescein-labeled mouse IgG1 antibody as negative controls. The red cells in whole blood were lysed using a lysis reagent. CD14+ cells were gated and 10000 events were measured. Before cytometry, the cells were washed twice (800 rpm, 7 min) with PBS. Cells were analyzed on a Becton-Dickenson FACsCalibur flow cytometer; auto fluorescence was subtracted. Data were analyzed by CELLQuest software (Becton-Dickinson).

Cell cultures

THP-1 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum in cul-

TABLE 1. Demographic and Clinical Characteristics of Subjects.

	CON	SAP	UAP	AMI		P value
				<24h	>24h	
n	29	19	39	36	30	
Age	65 ± 11	67 ± 6	68 ± 11	64 ± 11	65 ± 15	>0.05
Gender (M/F)	15/14	12/7	31/8	27/9	20/10	>0.05
Smoking (%)	9 (31.0%)	11 (57.9%)	22(56.4%)	19 (52.8%)	15 (50.0%)	>0.05
Hypertension (%)	12 (41.4%)	8 (42.1%)	28 (72%)	20 (55.6%)	22 (73.3%)	>0.05
Diabetes (%)	1 (3.5%)	4 (21.1%)	12 (31%)	5 (13.9%)	8 (26.7%)	>0.05
TC (mmol/L)	4.66 ± 1.12	4.68 ± 1.85	4.43 ± 0.94	5.06 ± 0.85	4.49 ± 1.04	>0.05
TG (mmol/L)	1.62 ± 0.98	1.97 ± 1.65	1.45 ± 0.7	1.72 ± 1.04	1.41 ± 0.60	>0.05
HDL (mmol/L)	1.30 ± 0.26	1.31 ± 0.27	1.25 ± 0.22	1.30 ± 0.22	1.30 ± 0.27	>0.05
LDL (mmol/L)	2.60 ± 0.93	2.53 ± 1.50	2.52 ± 0.86	2.95 ± 0.77	2.50 ± 0.73	>0.05
Apo A (mmol/L)	1.01 ± 0.19	1.04 ± 0.18	1.00 ± 0.16	0.97 ± 0.13	0.98 ± 0.22	>0.05

Abbreviation: CON=Control; SAP=stable angina pectoris; UAP=unstable angina pectoris; AMI=acute myocardial infarction; TC=total cholesterol; TG= triglyceride; HDL=high density lipoprotein; LDL=low density lipoprotein; Apo A= Apolipoprotein A.

ture flasks. Cells were maintained at 37 °C in a humidified 5% CO₂ atmosphere. Experiments were routinely carried out using cells in the log phase of growth. PMA was dissolved in dehydrated alcohol to obtain a 1 mmol/L stock solution and further diluted with PBS before use. For all experiments, THP-1 cells were cultured at an initial density of 5×10⁸ cells/L and treated with PMA at a final concentration of 40 µg/L for 24 h. After being washed three times with complete medium, macrophages were treated with vehicle or LTB₄ (10⁻¹⁰-10⁻⁷ mmol/l) for 24 h. Cells and supernatants were collected for further assay.

Western blot analyses

After washed by PBS for 3 times, the THP-1 derived macrophages harvested were lysed in RIPA lysis buffer (150 mM NaCl,50 mM Tris pH 8.0,1% Nonidet P-40,0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (1 mM PMSF,10 µM E64,1 mM EDTA,1 µg/ml pepstatin A). Proteins (10 µg) were resolved by 10% SDS/PAGE and transferred to PVDF membranes. Membranes were blocked with 1% bovine serum albumin (BSA) in PBS at room temperature for 1 hour and incubated overnight with polyclonal rabbit anti-human EMMPRIN antibody (Abcam, 1:50) or mouse anti-human β-actin antibody (Santa Cruz, 1:1000). For signal detection, goat anti-rabbit (Zhongshan Goldenbridge Biotechnology) or goat anti-mouse (Zhongshan Goldenbridge Biotechnology) IgG coupled to horseradish peroxidase and the enhanced chemiluminescence (ECL) detection system ECL Plus were used.

Isolation of total RNA and RT-PCR

Total RNA was extracted from THP-1 derived macrophages treated with LTB₄ (0, 10⁻¹⁰, 10⁻⁹, 10⁻⁸ and 10⁻⁷ mol/L) for 24 h (5×10⁸ cells/L) using Trizol reagent (Generay) according to the manufacturer's instructions. Reverse-transcription reaction components were as follows: total RNA (2 µg), random primer (5 pmol/L), M-MLV reverse transcriptase (0.5 U: 1 µL), dNTP (1 mmol/L), and 1×RT buffer. The reaction volume was brought up to 20 µL with nuclease-free water. All components were mixed on ice and then reacted for 60 min at 37 °C and 15 min at 72 °C.

PCR reaction components were as follows: RT product mix (4 µL), primers (0.5 µmol/L of each, for MMP-9 sequences F:5'-GATGCGTGGAGAGTCGAAATC-3', R: 5'-CACCAAACCTGGATGACGATG-3', 337bp), Taq polymerase (0.1 U: 1 µL), MgCl₂ (1.5 mmol/L), dNTP (0.125 mmol/L of each), and 1×PCR buffer. PCR was carried out with the temperatures: 2 min at 95 °C, then 45 s at 94 °C, 1.5 min at 52°C, and 2 min at 72°C for 32 cycles, followed by a final 10 min extension at 72°C.

The mixture of PCR products was analyzed by electrophoresis in 1.5% agarose gels stained with ethidium bromide. The ratio between the sample RNA and human β-actin (F: 5'-CGCTGCGCTGGTCGTCGACA-3'; R: 5'-GTCAGGCACGATTTCCCGCT-3', 619bp) was calculated to normalize for initial variations in the sample concentration and as a control for reaction efficiency. Images of the RT-PCR ethidium bromide-stained agarose gels were acquired with a digital Ko-

dak camera. Quantification of the bands was analyzed by imaging analysis software Quantity One.

Zymographic analyses

The supernatants of cultured THP-1 derived macrophages were collected, centrifuged to remove debris and analyzed by zymography. Gelatinolytic activities were assessed under non-reducing conditions using modified sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This technique was performed on a 8.0% polyacrylamide gel copolymerized with 0.1% gelatin type I and 10 μ L samples mixed with 10 μ L of loading buffer were run under non-reducing conditions without prior boiling. After electrophoresis, gels were washed two times with 2.5% Triton X-100 to remove SDS and allow proteins to renature and then immersed in buffer (50 mmol/L Tris, pH 7.5, 5 mmol/L CaCl₂, 1 μ mol/L ZnCl₂ and 0.01% NaN₃) for 18 h at 37 °C. Gels were stained with 0.2% Coomassie (0.25 % brilliant blue, 40% methanol, 7.5% acetic acid) for 4 h at room temperature, followed by treatment with destaining buffer (30% methanol, 10% acetic acid) until the desired contrast was achieved. A clear white band showing proteolytic activity was detected against a blue background of undigested gelatin. Gels were documented using an Epi ChemII darkroom (UVP Inc, Upland, CA) documentation system, and the intensity of the bands was quantified using imaging analysis software Quantity One.

Statistical analysis

Continuous variables are expressed as mean $\pm$ SD and one way analysis of variance (ANOVA) was used to determine the statistical differences between groups. Chi-square test was used for categorical variables. Data were presented as mean $\pm$ S.E.M. $P < 0.05$ was considered statistically significant.

Results

Serum LTB₄ and EMMPRIN levels in ACS

As shown in Figure 1, serum LTB₄ concentration was significantly higher in AMI and UAP groups, compared with control and SAP groups ($p < 0.01$). Subgroups analysis showed that LTB₄ was significantly higher in the AMI<24h group, compared with the AMI>24h group.

Monocyte expressions of EMMPRIN were determined by flow cytometric analysis. EMMPRIN expressed at significantly higher levels in patients with UAP and AMI (>24h), compared with control, SAP and AMI<24h groups ($p < 0.05$) (Figure 2).

FIGURE 1. Serum concentration of LTB₄.

* $P < 0.05$ compared with the control or SAP group. # $P < 0.05$ compared to UAP group. & $P < 0.05$ compared with AMI>24hrs group. SAP: stable angina pectoris; UAP: unstable angina pectoris; AMI: acute myocardial infarction.

LTB₄ up-regulated EMMPRIN and MMP-9 in macrophages

THP-1 derived macrophages were treated with various concentrations of LTB₄ for 24 h. LTB₄-mediated stimulation of EMMPRIN expression was dose-dependent, with the maximal effects occurred at the 10⁻⁷ mol/L LTB₄ treatment group ($p < 0.01$). A significant stimulation of EMMPRIN expression was also observed in the 10⁻¹⁰ mol/L LTB₄ treatment group ($p < 0.05$) (Figure 3). These data indicated that LTB₄ could increase EMMPRIN protein levels in human macrophages.

In order to study the effect of LTB₄ on MMP-9 production at the transcriptional level, THP-1 derived macrophages were maintained in culture in the presence of LTB₄ for 24 h. The total RNA of cells was isolated and RT-PCR was performed. As shown in Figure 4A, the level of MMP-9 mRNA was dose-dependently upregulated by LTB₄. A significant up-regulation was observed in the 10⁻¹⁰ - 10⁻⁷ mol/L LTB₄ treatment groups ($p < 0.05$).

Gelatinolytic activities of MMP-9 (92 kDa) in the conditioned medium were detected by electrophoresis. As shown in Figure 4B, the up-regulation of enzyme activity of MMP-9 was more pronounced in the groups treated with LTB₄ when compared with the control group.

Discussion

The inflammatory mediator LTB₄ plays an important role in the development of atherosclerosis; possibly through stimulating the expression of MMPs. EMMPRIN is an important modulator of MMPs activity in cardiovascular cells. The main

FIGURE 2. Expression of EMMPRIN on circulating monocytes. A. Flow cytometric analysis of surface expression of EMMPRIN on monocytes in patients with ACS or the controls. The letter R1 in the left panel represents for the monocytes in the peripheral blood. B. Subgroup comparisons.

* $P < 0.05$ compared with control or SAP group. & $P < 0.05$ compared with AMI <24hrs group.

ACS: acute coronary syndrome; SAP: stable angina pectoris; UAP: unstable angina pectoris; AMI: acute myocardial infarction.

FIGURE 3. Effect of LTB₄ on EMMPRIN expression in THP-1-derived macrophages. A. Representative Western blot showing the expression of EMMPRIN in cultured macrophages stimulated with LTB₄. B. Quantification of the specific Western blot signals. Data was presented as means $\pm$ SEM (n = 4 per group).

* P<0.05 compared with the control group.

finding of the current study is that there were significantly higher levels of circulating LTB₄ and EMMPRIN in patients with AMI and UAP, compared with control and SAP groups. Furthermore, an *in vitro* study showed LTB₄ up-regulated the expression of EMMPRIN in macrophages, as well as the expression and activation of MMP-9.

The 5-LO pathway has previously been linked to atherosclerosis in humans [8]. Histological analyses revealed products of the 5-LO pathway were detectable in atherosclerotic lesions [16,17]. Levels of LTB₄, one of the 5-LO pathway components, has been reported to be increased in serum of patients undergoing carotid endarterectomy [18]. Our results showed that LTB₄ serum levels were increased in AMI patients.

The role of EMMPRIN in MMP activation was described originally in tumor cells [19]. It has been demonstrated *in vitro* that EMMPRIN is essential for MMPs activation in monocytes and in vascular SMCs. Thus, MMPs make an essential contribution to the pathophysiology of atherosclerosis [20-22]. Specifically, the presence of MMP-9 within vascular walls has been linked to an unstable plaque phenotype that is prone to

FIGURE 4. Effect of LTB₄ on (A) MMP-9 mRNA expression and (B) MMP-9 activity in THP-1 derived macrophages. The bands are representative findings. Graphs represent quantification of relative band intensities from RT-PCR assay and zymography. Data was presented as means $\pm$ SEM (n = 4 per group).

* P<0.05 compared with the control group.

rupture [23-26]. In this study, EMMPRIN was found to be increased in patients with unstable angina pectoris. Surprisingly, in subgroups of AMI, which were classified by intermediate stage of the latest chest pain onset, significantly higher levels of EMMPRIN expression were seen in AMI>24h groups but not in AMI<24h. These findings indicated that a period of

time might be needed for the monocytes in blood to activate the expression of EMMPRIN after the pain onset.

It has been reported that LTB₄ can stimulate macrophages and smooth muscle cells to express MMP-9: LTB₄- and cysLTs-enhanced MMP-9 production in murine macrophages was attenuated by inhibition of p38 MAPK and ERK pathways [27]. In agreement with the report by Lee *et al*, our study found that LTB₄ up-regulated EMMPRIN expression in PMA-induced THP-1 cells, as well as the expression and activation of MMP-9. Other researchers have found that activation of both the ERK1/2 and p38 signal pathways is necessary for the up-regulation of EMMPRIN in PMA-induced THP-1 cells [28]. Since EMMPRIN is essential for MMP activation in monocytes, including MMP-9, it was hypothesized that LTB₄ could stimulate PMA-induced THP-1 cells to express EMMPRIN. In our study, LTB₄ was found to directly increase EMMPRIN production and MMP-9 expression and activation. Thus, our study suggests that LTB₄ is involved in the formation and rupture of unstable plaque by up-regulation of EMMPRIN and MMP-9.

In summary, this study provides evidence showing that blood levels of LTB₄ and EMMPRIN are up-regulated during a certain time window in patients with ACS. Furthermore, EMMPRIN and MMP-9 are significantly activated in LTB₄-stimulated macrophages. Our results indicate that LTB₄ and EMMPRIN might be associated with the development of unstable plaque and they are potential biomarkers for patients with ACS.

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