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Local immune activity in acute coronary syndrome: oxLDL abrogates LPS-tolerance in mononuclear cells isolated from culprit lesion

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ABSTRACT

Background: OxLDL plays a major role in the initiation and progression of atherosclerotic lesions even though further factors are needed to promote fibrous cap rupture and thrombotic occlusion of the arterial lumen. Pathogens have been implicated in this process but it remains unclear how they can cooperate with oxLDL in amplifying the destructive inflammatory response.
Objective: To phenotypically analyze culprit coronary inflammatory cells, evaluate their responsiveness to endotoxins and ascertain whether oxLDL alters the sensitivity of coronary mononuclear cells to bacterial components.
Methods: Mononuclear cells isolated from culprit and non-culprit coronary blood samples of patients with ST-segment elevation myocardial infarction (STEMI) and controls were analyzed for cell-specific surface markers and cytokines by flow-cytometry.
Results and conclusions: CD14+ cells contained elevated levels of TLR4, expressed high CD80, and produced huge amounts of inflammatory cytokines in response to LPS. Using a well-established model of endotoxin tolerance, we next showed that mononuclear cells isolated from control coronary artery, but not from culprit coronary artery, were tolerant to LPS, but pre-treatment of such cells with oxLDL abrogated LPS tolerance. Flow-cytometry analysis also showed that IL-17A, IL-21 and IFN-γ were over-produced by CD4+ and CD56+ cells isolated from the culprit coronary artery. All this data indicate that monocytes circulating in the culprit coronary artery of patients with STEMI are primed to synthesize high levels of inflammatory cytokines and suggest that oxLDL can amplify the inflammatory response of such cells to endotoxins.

1. Introduction

Cardiovascular disease, the leading cause of death worldwide, is generated by atherosclerotic lesion, which leads to unstable angina, heart attacks, sudden cardiac death and stroke [1]. Atherosclerosis represents a state of inflammation and increased oxidative stress characterized by the accumulation of macrophages and other inflammatory cells and products of lipid and protein oxidation in endothelial space, or intima, of the blood vessel wall [2–4]. The most important risk factors for atherosclerosis, such as elevated low-density lipoproteins (LDL), smoking, diabetes, hypertension, infections, or combinations of these factors, alter normal homeostasis of endothelium, causing endothelial dysfunction and production of reactive oxygen species [5,6]. The pro-atherosclerotic potential of LDL is increased after oxidative modification to oxidized LDL (oxLDL), whose uptake by macrophage scavenger receptors, as LOX-1, is thought to be a key process in the formation of foam cells, the hallmark of atherosclerotic lesions [7–10]. OxLDL changes the homeostasis of endothelium causing its dysfunction, the "primummovens" in the pathogenesis of atherosclerosis [7]. By inhibiting the expression of endothelial nitric-oxide synthase and inducing the expression of adhesion molecules on the endothelium, oxLDL stimulates monocytes and T cells to adhere to the dysfunctional endothelium, thereby promoting their migration to the sub-endothelial region [11]. Although adhesion of monocytes and T cells to the luminal endothelium is a hallmark of the early stages of the development of atherosclerotic lesions [5,12,13], this process alone does not cause acute cardiovascular events, because blood flow is preserved through outward remodeling of the arterial wall or, in the setting of gradual luminal encroachment, new vessel formation [14]. Nevertheless, in a small percentage of lesions, an occlusive luminal thrombosis is triggered after rupture of a lipid rich atheroma, leading to ischemia or cardiac death and stroke [15]. Therefore, understanding the mechanisms of plaque unstabilization could help to identify patients at high risk of thrombotic complications. In this context, several epidemiological studies have suggested that chronic infectious diseases, such as periodontitis and chlamydial infection, accelerate atherosclerosis process and exacerbate its clinical manifestations, leading to acute cardiovascular events [16–18]. These observations raise the possibility that oxLDL and

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endotoxins can cooperate to amplify the inflammatory process which promotes the atherosclerotic plaque rupture [19,20]. The exact mechanism underlying the interaction between oxLDL and bacterial products/components is not fully understood, but recent studies have shown that cholesterol may trigger the release of inflammatory cytokines from macrophages by activating the NLRP3 inflammasome, a subfamily of receptors which recognize a wide variety of pathogen-derived molecules [21,22]. In this context, it is also conceivable that oxLDL may make coronary monocytes/macrophages sensitive to lipopolysaccharide (LPS), a phenomenon which is known to associate with enhanced production of inflammatory molecules. We here show that monocytes isolated from culprit coronary artery are activated and produce huge amounts of inflammatory cytokines in response to LPS stimulation and oxLDL abrogates tolerance of monocytes isolated from peripheral blood and normal coronary artery to LPS. Altogether these novel data delineate a scenario whereby oxLDL and bacterial products/components cooperate to trigger an exaggerated inflammatory response which could accelerate the atherosclerotic progression, fibrous cap rupture and subsequent thrombotic occlusion of the arterial lumen.

2. Methods

2.1. Patients

Eighteen consecutive patients (14 M/4 F; mean age 57 ± 13) affected by ST-segment elevation myocardial infarction (STEMI) treated with primary percutaneous coronary intervention (PCI) and stent implantation were included in the study. Inclusion criteria for STEMI were the followings: continuous chest pain lasting < 30 min; ST segment elevation >/= 0.1 mV in 2 or more contiguous leads on 12-lead ECG; angiographic identification of culprit lesion with Thrombolysis in Myocardial Infarction (TIMI) flow grade </= 2; Troponin T > 0.1 ng/ml and an increase in Creatine kinase-MB (CK-MB) greater than 2 times the normal value; door to balloon time < 6 h. Exclusion criteria were as follows: culprit lesions in common trunk; heart failure, cardiogenic shock; chronic ischemic heart disease (previous MI); infectious disease, autoimmune, neoplastic; liver failure, chronic renal failure. Blood samples were collected at the site of occlusion using aspiration catheterization (Pronto V3; Vascular Solutions, Minneapolis, MN) after crossing the occlusive lesion. Clinical characteristics of the patients are shown in Table 1. Additionally, samples were collected from non-culprit coronary artery and peripheral blood of the same patients. Controls included peripheral and coronary blood samples taken from 12 patients with valvular desease(5 M/7 F; mean age 63 ± 10) undergoing coronary angiography before valve replacement surgery, with no angiographic evidence of coronary artery disease.The blood samples were analyzed within one hour of collection. The study protocol was approved by local Ethics Committee and informed consent was obtained from all subjects.

Table 1
Baseline and clinical characteristics of study populations.

Characteristic	Patients with AMI	Control
Age (years)	18	12
	57 (±13)	63 (±10)
Male gender (n)	14 (78%)	3 (26%)
Risk factors (n)		
Hypertension	10 (55%)	9 (75%)
Hypercholesterolaemia	7 (39%)	8 (67%)
Diabetes	1 (5%)	3 (25%)
Smoking	14 (78%)	4 (33%)
CAD Familiarity	5 (18%)	-
HDL-cholesterol (mg/dl)	36 (±11)	46 (±16)
LDL-cholesterol (mg/dl)	126 (±43)	112 (±24)
Total cholesterol (mg/dl)	182 (±49)	180 (±36)
Statins use at onset of sympotons	8 (44%)	6 (50%)
Ejection fraction admission FE%	52%	55%
Angiographic degree of CAD (n)		
One-vessel disease	13 (72%)	–
Two-vessel disease	4 (22%)	–
Three-vessel disease	1 (6%)	–
Culprit vessel (n)		
Left anterior descending artery	7 (39%)	–
Left circumflex artery	1 (6%)	–
Right coronary artery	10 (55%)	–
Initial TIMI flow grade (n)		
0	10 (55%)	–
1	7 (39%)	–
2	1 (6%)	–
3	–	
Final TIMI flow grade (n)		
0	–	–
1	–	–
2	1 (6%)	–
3	17 (94%)	–
Final blush flow grade (n)		
0	–	–
1	1 (6%)	–
2	4 (22%)	–
3	13 (72%)	–

Values are means ± S.D., or numbers (percentage). CAD, coronary artery disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

2.2. oxLDL preparation

Human LDL (density 1.019–1.063) was isolated from fresh plasma by ultracentrifugation and oxidative modification of LDL was performed with Cu2 + as indicated elsewhere [10]. Oxidation was monitored by measuring the amount of thiobarbituric acid-reactive substances (10.7 nmol malondialdehyde equivalent for mg protein in oxLDL). Agarose gel electrophoresis showed increase electrophoretic mobility and minimal aggregation of oxLDL particles.

2.3. Cells isolation and stimulation

All reagents were from Sigma-Aldrich (Milan, Italy) unless specified. Peripheral blood and coronary artery blood was extracted 6 hours after the symptom onset and mononuclear cells were separated by Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation and used for RNA extraction or flow-cytometry analysis. Additionally, mononuclear cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Lonza, Milan, Italy), penicillin (100 U/mL), and streptomycin (100 U/mL) with LPS (10 ng/ml) or medium alone for 24 h. To examine whether coronary mononuclear cells are tolerant to bacterial products/components, we used a well-established in vitro model in which cells pre-cultured with low doses of LPS are analyzed for their ability to synthesize cytokine in response to a subsequent stimulation with higher dose of LPS. For this purpose, cells isolated from culprit and non-culprit coronary of patients and controls were treated with medium alone or LPS (100 pg/ml) for 48 h (primary culture), then washed and re-stimulated with medium alone or LPS (100 ng/ml) for a further 24 h (secondary culture). Moreover, to investigate whether oxLDL abrogates tolerance to LPS, cells isolated from non-culprit coronary of patients were cultured in the presence or absence of LPS (100 pg/ml) or oxLDL (1 μg/ml) or LPS and oxLDL for 48 h (primary culture), then washed and re-stimulated again with medium alone or 100 ng/ml of LPS for a further 24 h (secondary culture). At the end, cell-free supernatants were harvested and stored at −80 °C until tested.

2.4. Flow cytometry

Cells were stained with the following Abs: anti-CD4-PerCP (1:50, final dilution, BD Biosciences, San Jose, CA), anti-CD8-APC (1:50, final dilution, BD Biosciences), anti-CD56-PE or APC (1:50 final dilution, BD Biosciences), anti-CD14-FITC (1:50 final dilution,Immunotools, Friesoythe, Germany), anti-TLR2-PE (1:50 final dilution, eBioscience, San Diego, CA), Anti-TLR4-PE (1:50 final dilution, eBioscience). To assess cytokine expression cells were fixed with 1% formaldehyde for 20 minutes and subsequently permeabilized with 0.5% saponin in 1% BSA FACS buffer and stained intracellularly with the following Abs: anti-IFN-γ-FITC (1:50 final dilution, eBioscience), and anti-IL-21-PE (1:50, finaldilution,eBioscience) and anti-IL-17A-PE(1:50 final dilution, eBioscience). Appropriate isotype-matched controls (BD Biosciences) were included in all experiments. Cells were analyzed using aFACSCalibur cytometer and Cell-QuestPro software.

2.5. RNA extraction and real-time PCR

RNA was extracted by using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). A constant amount of RNA (1 μg per sample) was reverse-transcribed into cDNA, and PCR was performed using a syber green-based PCR (Bio-Rad, Hercules, CA). cDNA was amplified using the following conditions: denaturation 1 min at 95 °C, annealing 30s at 60 °C for TNF-α and β-actin followed by 30s extension at 72 °C. Primer sequences were as follows: human TNF-α forward: 5′-AGGCGGTGCTTGTT CCTCAG-3′, reverse: 5′-GGCTACAGGCTTGTCACTCG-3′ and β-actin forward: 5′-AAGATGA CCCAGATCATGTTTGAGACC-′3 and reverse: 5′-AGCCAGTCCAGACGCAGGAT-′3, was used as a housekeeping gene. Gene expression was calculated using the ΔΔCt algorithm.

2.6. Enzyme-linked immunosorbent assay (ELISA)

TNF-α and IL-12 were measured in the cell culture supernatants using sensitive enzyme-linked immunosorbent assay kits according to the manufacturer's protocol (R&D System, Minneapolis, MN).