Oxidation and Endothelial Dysfunction Biomarkers of Atherosclerotic Plaque Instability. Studies of the Vascular Wall and Blood

Yu. I. Ragino, A. M. Chernjavski*, Ya. V. Polonskaya, A. M. Volkov*, E. V. Kashtanova, A. V. Tikhonov, and S. Yu. Tcimbal*

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 153, No. 3, pp. 308-312, March, 2012
Original article submitted October 27, 2010

The concentrations of LPO products (including those present in LDL), oxidative modification of proteins, paraoxonase activity, concentrations of antioxidants, lipid values and biomarkers of endothelial dysfunction were studied in the blood and coronary artery intima/media of male patients with coronary atherosclerosis without acute coronary syndrome. Blood levels of LDL oxidized apolipoproteins and lipoprotein (a) were higher, while the content of NO metabolites, sVCAM endothelial adhesion molecules, and LDL oxidation resistance were lower in men with mainly unstable atherosclerotic plaques in the coronary arteries in comparison with men with mainly stable plaques in the coronary arteries. Of these blood biomarkers, only NO metabolites, oxidized proteins, and sVCAM correlated with the presence of unstable atherosclerotic plaques. A significant correlation between the levels of biomarkers in the vascular wall and blood was detected only for LPO parameters.

Key Words: *oxidative and antioxidant, lipid biomarkers; endothelial dysfunction; stable and unstable atherosclerotic plaques; correlations*

The prevalence of and mortality from myocardial infarction, united together with unstable angina pectoris and sudden cardiac death by the term "acute coronary syndrome" (ACS), are high in Russia.

The clinical manifestations of ACS are triggered by violation of the endothelial integrity at the site of ulceration/destruction of the unstable atherosclerotic plaque cap, subsequent clotting and occlusion of the artery, myocardial ischemia and necrosis [9,11]. A stable plaque has a thick cap, homogeneous lipid core, and no inflammatory changes, while an unstable plaque is characterized by a thin cap (<65 μ) or a thinned cap site with focal destruction of the endothelium, inflammatory cell infiltration (more than 25 cells in a visual field 0.3 mm long), a loose lipid core (>40% of plaque volume) with a necrotic focus [14].

Modern views on the mechanisms of unstable plaque formation are contradictory. Inflammation plays an important role in the plaque destabilization, as significant infiltration of unstable plaques by macrophages and T lymphocytes has been shown. High levels of inflammatory cytokines and matrix metalloproteinases (MMP) have been found in atherosclerotic plaques and blood in ACS [5,8,11].

The contribution of oxidative stress factors and oxidized lipids and proteins to this process is less studied. Macrophages, T lymphocytes, and smooth muscle cells (SMC) produce active oxygen metabolites causing, along with MMP, SMC necrosis/apoptosis, leading to thinning of the fibrous cap and its ulceration [1,10,12]. Under conditions of endothelial dysfunction and oxidative/antioxidant disorders in the vascular wall, characteristic of atherogenesis, the stimulated macro-

Institute of Therapy, Siberian Division of the Russian Academy of Medical Sciences; *E. N. Meshalkin Institute of Circulatory Diseases, Ministry of Health and Social Development of the Russian Federation, Novosibirsk, Russia. ***Address for correspondence:*** ragino@mail.ru. Yu. I. Ragino

0007-4888/12/1533-0331

phages release chemoattractants, including cell-cell (sICAM-1) and endotheliocytic adhesion (sVCAM-1) molecules [1,11,13,15] potentiating the chemotaxis of new monocyte/macrophages into atheromas.

According to some findings, patients with ACS have high blood levels of inflammatory cytokines and biomarkers (IL-1β, IL-6, C-reactive protein, *etc.*); independent associations thereof with the development of atherosclerosis and ACS have been detected. High blood levels of MMP-1, MMP-3, and MMP-9 were found in the blood of patients with ACS [6,7]. Our knowledge of the blood levels of oxidation and antioxidant biomarkers and their correlations with inflammation markers in coronary atherosclerosis and ACS is insufficient.

Biochemical studies of both blood and arterial wall of patients with coronary atherosclerosis are rare. We studied the oxidation and antioxidant, some lipid, and endothelial dysfunction biomarkers in the atherosclerotic plaques of the coronary arteries and in the blood of men suffering from coronary atherosclerosis without ACS in order to detect associations between the values in the coronary wall and blood and detect the significant biomarkers of the atherosclerotic focus instability.

MATERIALS AND METHODS

The study was carried out within the framework of Program of Collaborative Research of Institute of Therapy and E. N. Meshalkin Institute of Circulatory Diseases and was approved by Ethic Committees of both Institutes (protocols No. 1 of 14.09.2005 and No. 4 of 28.09.2005).

The study was carried out in 54 men aged 40-70 years with coronary atherosclerosis, verified by coronarography, without ACS, with stable angina of effort (functional class II-III). The patients were hospitalized at Clinical Department of E. N. Meshalkin Institute for coronary shunting. All patients gave written informed consent to participation in the study. Endarterectomy from the coronary artery (arteries) was carried out for intraoperative indications. The endarterectomy material containing the arterial intima/media was longitudinally and transversely symmetrically divided into fragments for histological and biochemical studies.

Histological analysis of fragments of the coronary artery intima/media after standard hematoxylin and eosin and Van-Gieson staining was carried out under an Axiostar Plus binocular microscope (Carl Zeiss) with a digital output. Only stable atherosclerotic plaques were found in 14 men, stable and unstable plaques in 22, and only unstable plaques in 18 patients. Stable atherosclerotic plaques were differentiated from unstable ones by the criteria described previously [14].

Fragments of the intima/media, frozen in liquid nitrogen, were homogenized in PBS (pH 7.4) for biochemical studies. Protein was measured in homogenous suspension by Lowry's method, biochemical values were calculated with reference to protein. The following oxidation and antioxidant, lipid, and endothelial dysfunction biomarkers were evaluated in the homogenated material: concentrations of LPO products (by MDA level) by fluorometry on Versafluor spectrofluorometer (Bio-Rad), α-tocopherol and retinol by fluorometry, β-carotene by photometric methods, oxidative modification of proteins by the photometric method after reaction with 2,4-dinitrophenylhydrazine (DNPG), initial level of paraoxonase (PON1) by photometry in Tris-HCl buffer with paraoxon (Sigma) [5], cholesterol (CH) by enzymatic method with Biocon Fluitest reagents, endothelin-1 and sVCAM by ELISA using standard ELISA kits (Biosource) on a Multiscan EX analyzer.

Venous blood was collected in all patients after overnight fasting before surgery. The following oxidation and antioxidant, lipid, and endothelial dysfunction biomarkers were measured in the serum: initial level of LPO products in LDL isolated from the blood and LDL oxidation resistance (duration of LDL oxidation lag phase, level of LPO products in LDL after 30-min *in vitro* incubation with oxidation catalysts, copper ions), LDL concentrations of α-tocopherol and retinol by fluorometric methods [3]; oxidative modification of LDL apolipoproteins (ox-apoLDL) by photometry [4]; PON1 activity by photometry in Tris-HCl buffer with paraoxon; oxidative modification of proteins by the photometry after reaction with 2,4-DNPG [5]; the concentrations of total CH, triglycerides, LDL-CH and HDL-CH by the enzymatic method with Biocon Fluitest reagents on a ConeLab 300i automated biochemical analyzer; lipoprotein (a) (LP(a)) by ELISA using standard ELISA test system (Mercodia); NO metabolites by photometry after deproteinization and NO3 reduction to NO2 by granulated cadmium [2]; homocysteine, endothelin-1, sICAM and sVCAM by ELISA using ELISA standard test systems (Axis-Shield and Biosource).

The results were statistically processed by licensed version of SPSS for Windows with analysis of correlations, linear analysis, regression analysis, and One-Way ANOVA using Dunnet's test for multiple comparison.

RESULTS

Some of previously studied oxidation and antioxidant biomarkers were found significant for the unstable atheroma stage [5]. The highest concentrations of LPO

products (2.0±0.3 nmol MDA/mg protein) and PON1 activity (72.9±20.7 μg/mg protein) and the least level of retinol (4.3±0.5 μg/mg protein) were characteristic of unstable plaques in comparison with other stages of atherosclerotic focus development (lipid spot, stable young and fibrous plaques).

The aim of our study was to detect the most significant blood biomarkers of atherosclerotic foci instability in the coronary arteries and hence, we carried out a two-staged statistical analysis of the results. In order to detect the associations between the oxidation and antioxidant, lipid and endothelial dysfunctional disorders in the arterial wall and blood, all the patients were divided into 3 subgroups by the results of histological studies. Subgroup 1 included men without unstable atherosclerotic plaques, only stable ones in fragments of the coronary artery intima/media; group 2 were men with stable and unstable plaques; and group 3 patients had exclusively unstable plaques (Table 1).

Patients of subgroup 3 (only unstable plaques in fragments of the coronary artery intima/media) had lower LDL oxidation resistance in comparison with patients of subgroups 2 and 1 (1.3 and 1.2 times lower, respectively) and higher levels of ox-apoLDL (1.7 and 1.3 times, respectively). These data were in line with our previous results [5] on oxidative biomarkers at various stages of atheroma development and indicated an important role of high oxidative modification of lipids and proteins in the formation of the plaque instability.

Of the lipid biomarkers, higher (1.8 times) blood concentrations of LP(a) were found in patients of subgroups 2 and 3 with unstable plaques in the coronary arteries in comparison with patients without unstable plaques in the coronary artery. This result did not contradict previous data on the important pathophysiological role of LP(a) in the development of ACS.

Of the studied endothelial dysfunction biomarkers, the concentrations of NO metabolites were low in subgroup 3 patients (without stable plaques in the coronary artery) in comparison with subgroup 2 and subgroup 1 patients (1.30 and 1.45 times lower, respectively). This fact indicated more pronounced endotheliocyte dysfunction under conditions of atherosclerotic focus instability. Blood concentration of sVCAM was the highest in subgroup 1 patients (without unstable plaques) 1.1 and 1.2 times higher than in subgroups 2 and 3, respectively. Importantly that adhesive molecules play a significant role at the early stages of the plaque formation (chemotaxis, adhesion and migration of monocytes to the subendothelium) but not at the plaque destabilization stage [15].

Stage 2 of statistical processing of the results con-

TABLE 1. Levels of Blood Biomarkers in Men with Atherosclerotic Plaques of Different Types in the Coronary Arteries ($M\pm\sigma$)

Parameter	Subgroup 1 (no unstable plaques; N=14)	Subgroup 2 (stable and unstable plaques; N=26)	Subgroup 3 (no stable plaques; N=14)
Initial LDL LPO level, nM MDA/mg LDL protein	3.7±0.5	4.0±0.4	3.4±0.5
Lag phase of LDL oxidation, nmol MDA/mg LDL protein	2.3±0.3	2.4±0.3	2.1±0.3
LDL oxidation resistance, nmol MDA/mg LDL protein	23.3±1.5	25.3±1.6	30.1±2.0*+
Oxidized apoLDL, unit/mg LDL protein	26.5±2.6	35.5±3.1	45.7±5.5*+
Oxidized proteins, unit/ml	5.1±0.7	4.6±0.8	4.8±0.9
PON1 activity, nmol/min/ml	328.2±25.7	301.4±24.6	291.1±22.7
Total CH, mmol/liter	5.4±0.4	6.1±0.4	5.8±0.5
LDL CH, mmol/liter	3.7±0.3	4.2±0.4	4.0±0.3
HDL CH, mmol/liter	0.8±0.1	0.9±0.1	0.9±0.1
TG, mmol/liter	2.0±0.2	2.1±0.3	2.0±0.3
LP(a), mg/dl	8.7±0.5	15.6±1.4*	15.5±1.5*
Homocysteine, μmol/liter	19.0±1.5	20.9±1.9	18.5±2.0
NO metabolites, μmol/liter	4.5±0.3	4.0±0.3	3.1±0.3*+
sVCAM, ng/ml	915.1±57.8+o	782.6±48.3	750.7±61.5

Note. TG: triglycerides. p<0.05 in comparison with: *subgroup 1, +subgroup 2, osubgroup 3.

TABLE 2. Correlations of Blood and Vascular Wall Levels of the Studied Biomarkers in Men with Coronary Atherosclerosis

Plaque	NO metabolites	LDL LPO	LDL oxidation lag phase	LDL oxidation resistance	LDL CH	HDL CH	PON1 activity	LP(a)	Oxidized proteins	sVCAM
Unstable plaque	-0.292*	$p>0.05$	$p>0.05$	$p>0.05$	0.246**	-0.235**	$p>0.05$	$p>0.05$	0.261**	-0.282**
CH	$p>0.05$	$p>0.05$	$p>0.05$	0.315**	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	0.256**	$p>0.05$
Level of LPO products	$p>0.05$	0.280*	$p>0.05$	0.298*	$p>0.05$	$p>0.05$	0.241*	$p>0.05$	0.237*	$p>0.05$
Oxidized proteins	-0.324**	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$	0.596**	$p>0.05$	0.270*
PON1 activity	$p>0.05$	$p>0.05$	$p>0.05$	0.336**	-0.283*	0.230*	$p>0.05$	$p>0.05$	$p>0.05$	-0.461**
α-Tocopherol	$p>0.05$	$p>0.05$	0.361**	0.365**	$p>0.05$	$p>0.05$	0.334*	$p>0.05$	$p>0.05$	$p>0.05$
Retinol	$p>0.05$	$p>0.05$	$p>0.05$	0.384**	$p>0.05$	0.318*	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$
Endothelin-1	-0.277*	$p>0.05$	0.373**	0.357**	$p>0.05$	$p>0.05$	$p>0.05$	0.278*	$p>0.05$	$p>0.05$
sVCAM	0.353**	$p>0.05$	$p>0.05$	$p>0.05$	-0.189*	$p>0.05$	0.243*	0.480**	$p>0.05$	0.198*

Note. *$p<0.05$, **$p<0.01$.

sisted in analysis of correlations of the vascular wall and blood biomarkers with consideration for nonparametrical distribution of signs (Table 2).

Of the entire spectrum of the vascular wall and blood biomarkers, a significant correlation has been detected only for LPO activity parameters (level of LPO products in atheromatous plaques and LDL LPO products' level and oxidation resistance in the blood). Therefore, oxidation-modified LDL play the key role in triggering of atherogenesis and formation of lipid spots in the arterial wall [1,13] and make an important contribution to the formation of unstable atherosclerotic plaques. Presumably, oxidation-modified LDL with low oxidation resistance stimulate macrophages and T lymphocytes to production of active oxygen metabolites, causing SMC necrosis/apoptosis and thinning of the plaque cap [1,10,12] and are cytotoxic towards the cap endotheliocytes.

Instability of atheromatous plaques in the coronary arteries correlated with not only concentrations of oxidation-modified proteins, sVCAM, and NO metabolites, but also with LDL CH and HDL CH. Correlation with this latter parameter could be attributed to antioxidant activity of LDL at the expense of HDL-bound PON1 (antioxidant enzyme) and its close pathophysiological relationship with LDL lipophilic antioxidants. A correlation between blood LP(a) and vascular wall oxidative and endothelial dysfunctional disorders was detected.

Hence, blood levels of ox-apoLDL and LP(a) were higher and levels of NO metabolites and sVCAM and LDL oxidation resistance were lower in men with mainly unstable atheromatous plaques in the coronary artery than in patients with mainly stable plaques. Three of these blood biomarkers (NO metabolites, oxidized proteins, and endotheliocyte adhesion molecules) correlated with the atheromatous plaque instability in the coronary arteries. Of all studied biomarkers, a significant correlation between the vascular wall and blood values was detected only for LPO values.

Studies of the oxidation and antioxidant, lipid and endothelial dysfunction biomarkers of coronary atherosclerosis without ACS indicated a relationship between LPO values in the coronary arteries and blood.

The study was supported by the Russian Foundation for Basic Research (Grant No. 09-04-00374a).

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