EXPERIMENTAL METHODS FOR CLINICAL PRACTICE

Blood Level of Osteonectin in Stenosing Atherosclerosis and Calcinosis of Coronary Arteries

Yu. I. Ragino, E. V. Kashtanova, A. M. Chernjavski*, A. M. Volkov*, Ya. V. Polonskaya, S. Yu. Tsimbal*, N. V. Eremenko, and M. V. Ivanova

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Blood levels of stem cell marker proteins CD34 and osteonectin were studied in male patients with coronary atherosclerosis by direct biomagnetic separation of proteins with magnetic microspheres using the PureProteome Protein A and Protein G Magnetic Beads proteomic technology. High concentration of osteonectin in the blood was detected, particularly in men with stenosing atherosclerosis and coronary artery calcinosis. Blood osteonectin concentration correlated significantly with some key biomarkers of atherosclerosis and with stenosing atherosclerosis and calcinosis of coronary arteries. The results indicate that osteonectin as a marker of stromal stem cells with osteogenic potential presumably plays an important role in atherogenesis and can serve as a new biomarker of stenosing atherosclerosis and calcinosis of coronary arteries.

Key Words: *osteonectin; CD34; stenosing atherosclerosis; coronary artery calcinosis; atherosclerosis biomarkers*

Coronary atherosclerosis (CA) is the pathomorphological basis of coronary disease; its prevalence and mortality from its complications remain high all over the world. The complex pattern and significance of such components of atherogenesis as dyslipoproteinemia, inflammation, oxidation, endothelial dysfunction, *etc.* were demonstrated [2,13,14]. Great attention is now paid to studies of stenosing atherosclerosis (SA) and calcinosis of the aorta and coronary arteries (CCA) [4,11], and active search for their new biomarkers is in progress.

It is known that bone marrow stem cells of hemopoietic and stromal differentiation lineages are involved in atherogenesis. Presumably, cells proliferating in the vascular intima are of bone marrow origin, and bone marrow colony-forming stem cells of hemopoietic and stromal differentiation lineages penetrate into the vascular intima in sites of lipid concentration and development of atherosclerotic foci [1,6,12]. CD34, highly glycosylated type 1 transmembrane protein (sialomucin) is a marker of hemopoietic stem cells, while stromal stem cells carry on their surface osteonectin, a non-collagen bone tissue glycoprotein selectively binding calcium and phosphorus salts to collagen. Osteonectin is intensely expressed by cells present in the vascular wall during atherosclerosis progress, specifically, during calcification of the atherosclerotic plaque. High level of osteonectin-positive cells in the peripheral blood can reflect the onset of the productive stage of inflammatory process in the vascular wall [7,8,15].

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

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Using direct method of biomagnetic separation of proteins with magnetic microspheres, we studied blood levels of stem cell marker proteins CD34 and osteonectin in men with CA and the relationship between the levels of these proteins and the presence of SA and CCA and some other atherosclerosis markers.

MATERIALS AND METHODS

The study was carried out within the framework of collaboration between Institute of Therapy and Institute of Circulatory System Diseases and was approved by the Ethic Committees of both institutes. The study was carried out in 80 men aged 42-72 years (mean age 56.9±4.8 years).

The main group consisted of 40 patients with verified CA without acute coronary syndrome with functional class II-IV stable effort angina. Selective coronarography was carried out on an Advantex LC/LP angiographic device (General Electric) on admission to Clinical Department of Institute of Diseases of the Circulatory System for aortocoronary bypass surgery. During the surgery, endarterectomy from the coronary artery was carried out, if intraoperative indications for it emerged during the intervention. Histological analysis of endarterectomy material containing the intima/media of the coronary artery was carried out after standard staining with hematoxylin and eosin and by van Gieson's method under an Axiostar Plus binocular microscope (Zeiss) with a digital photooutput.

The control group consisted of 40 sex- and age-matched male individuals examined at Clinical Department of Institute of Therapy, who had no CHD (according to the data of clinical and functional studies, including ECG recording by the Minnesota code deciphering). The groups were comparable by the major risk factors for coronary disease (dyslipoproteinemia, arterial hypertension, excessive body weight, type II diabetes mellitus, tobacco smoking).

The blood was collected from the ulnar vein after overnight (12 h) fasting in all men. Lipid profiles (total cholesterol (CH), triglycerides, HDL CH and LDL CH) and serum glucose levels were measured by enzymatic methods using Biocon Fluitest standard reagents on a Labsystem FP-901 biochemical analyzer.

The parameters of LPO processes activity, such as initial level of LPO products in LDL isolated from the blood (by the concentration of LPO end-product MDA) and LDL oxidation resistance (evaluated by the level of LPO products in LDL after 30-min incubation *in vitro* with oxidation catalysts, copper ions) were evaluated by fluorometry [3] on a Versafluor spectrofluorometer (Bio-Rad). Serum levels of C-peptide, highly sensitive C-reactive protein, TNF-α, IL-1β, IL-6, IL-8 were measured by ELISA using standard kits (Biomerica, BCM Diagnostics, DSL) on a Multiscan EX EIA analyzer.

Serum levels of CD34 and osteonectin were measured using proteomic technology PureProteome Protein A and Protein G Magnetic Beads (Millipore). Direct biomagnetic separation of proteins by Protein A and Protein G Magnetic Beads (microspheres) on a Magna GrIPTM Rack magnetic separator was carried out.

For binding of antibodies to specific antigens with magnetic beads, 50 µl suspension with magnetic beads was transferred into Eppendorf tubes, placed into a magnetic separator, and after 2 min the buffer was removed. The magnetic beads were washed in 500 µl phosphate buffer (pH 7.4) with 0.1% Twin 20, the tubes were shaken (10 sec), placed into a magnetic separator, and after 2 min the buffer was removed. After washout (2×100 µl phosphate buffer), antibodies (25 µl) were added to magnetic beads. When measuring CD34 protein concentration, mouse anti-human monoclonal antibodies to class II CD34, clone QBEND/10 (Chemicon) were added. For measuring osteonectin concentration, rabbit polyclonal antibodies to human osteonectin (Chemicon) were added. The samples were incubated at ambient temperature for 10 min at constant stirring on a Bio RS-24 minirotator with a PRS-22 platform (Biosan). The resultant complexes were washed 3 times in phosphate buffer. The tubes were then removed from the magnetic separator and 1 ml test serum was added. The samples were incubated for 1 h at 4°C and continuous mixing on a Bio RS-24 minirotator for the formation of immunomagnetosensitive beads complex with CD34 or human osteonectin receptors. The tubes were placed into a magnetic separator, the supernatant was removed, the samples were washed 3 times in phosphate buffer, the tubes were removed from the magnetic separator, and elution buffer (60 µl 0.2 M glycine, pH 2.5) was added. Incubation was carried out for 2 min at ambient temperature, after which the tubes were placed into a magnetic separator, the supernatants were transferred into new tubes, and 5 µl 1 M Tris (pH 8.5) was added to each tube.

Quantitative assay of the isolated proteins (CD34 and osteonectin) in the supernatants was carried out by the method of Lowry and electrophoresis in 6% PAAG on a SE 600 Ruby Standard Dual Cooled Vertical (GE Healthcare) for 1.5 h with subsequent staining of the gel with Coomassie Blue R. Densitometry of the gels was carried out using GelDoc transilluminator system (BioRad). A Sigma protein calibration kit (High Range, 36-200 kDa) containing rabbit myosin 200 kDa, *E. coli* β-galactosidase 116 kDa, rabbit phosphorylase b 97 kDa, BSA 66 kDa, liver glutamine dehydrogenase 55 kDa, egg ovalbumin 45 kDa, and rabbit glyceraldehyde-3-phosphate dehydrogenase

TABLE 1. Serum Levels (mg/ml) of CD34 and Osteonectin in Men with CA (*N*=40) and Controls (*N*=40) according to Measurements by Lowry's Method

Protein	Group	Mean	Error of the mean	Standard deviation	Minimum–maximum	*p*
CD34	CA	4.14	0.21	0.81	2.94-6.87	0.365
	control	4.38	0.24	0.69	3.48-5.43	
Osteonectin	CA	6.45*	0.51	1.23	3.75-12.63	0.002
	control	4.11	0.27	0.84	2.11-6.03	

TABLE 2. Serum Levels (mg/ml) of CD34 and Osteonectin in Men with CA (*N*=40) and Controls (*N*=40) according to Measurements by Electrophoresis

Protein	Group	Mean	Error of the mean	Standard deviation	Minimum–maximum	*p*
CD34	CA	3.57*	0.29	0.91	2.28-5.77	0.047
	control	4.51	0.41	1.74	2.89-7.24	
Osteonectin	CA	5.71*	0.47	1.43	2.24-8.97	0.000
	control	2.18	0.37	1.21	0.88-3.52	

36 kDa was used as the reference samples. Molecular weights of osteonectin and CD34 are 43 and 105-120 kDa, respectively.

The results were statistically processed, the differences were considered significant at $p<0.05$.

RESULTS

Evaluation of serum CD34 content by the method of Lowry revealed no differences between the two groups of men (Table 1). Osteonectin assay by the same method detected a difference: serum concentration of this protein in patients with CA was 1.57 times higher than in the control.

Measurements of serum CD34 and osteonectin by electrophoresis showed significant differences between the groups (Table 2). Osteonectin concentrations in men with CA were 2.62 times higher than in controls. The concentrations of CD34 in them were 1.26 times lower than in the control.

The results of proteomic studies indicated high level of stromal stem cell marker osteonectin in the blood of CA patients, which was in line with published data [1,6-9]. High level of osteonectin-positive cells was suggested as an indicator of the presence and progress of atherosclerotic involvement of the vessels in humans [1]. Low level of CD34 stem cells in patients with CHD and CA in comparison with healthy individuals can be due to intensive infuzation of these cells in zones of endothelial destruction and atherosclerotic foci [9].

Our histological studies of endarterectomy material detected SA and CCA in 29 (72.5%) of 40 patients with CA; in 2 of these, signs of ossification and cartilage tissue development were found. Similar histological findings in atherosclerotic plaques were reported previously [10]. In addition, these two patients had the highest serum levels of osteonectin (10.45 and 12.63 mg/ml).

TABLE 3. Correlations between Serum Osteonectin Level and Atherosclerosis and Metabolic Syndrome Markers in Examined Men (*N*=80)

Biomarkers	Serum osteonectin level	
	Pearson *r*	Spearman *R*
Total CH	0.172	0.161
HDL CH	-0.556**	-0.568*
LDL CH	0.192	0.203
Triglycerides	0.356*	0.377
Plasma glucose	0.474**	0.489*
C-peptide	0.472**	0.478**
Highly sensitive C-reactive protein	0.216	0.228
TNF-α	0.379**	0.389**
IL-1β	0.209	0.218
IL-6	0.278*	0.264
IL-8	0.543**	0.562**
LPO products in LDL	0.574**	0.561*
LDL oxidation resistance	-0.483*	-0.477
SA and CCA (*n*=29)	0.388**	0.398**

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

Analysis of correlations (Table 3) showed that osteonectin content directly correlated with blood levels of triglycerides, glucose, C-peptide, TNF-α, IL-6, IL-8, and LDL LPO products and negatively correlated with blood HDL CH level and LDL oxidation resistance. In addition, significant correlations were detected between blood level of osteonectin and the presence of SA and CCA, which was in agreement with high concentrations of some bone tissue proteins, such as osteocalcin, bone morphogenic proteins, osteonectin, osteopontin, *etc.*, in the blood and in atherosclerotic plaques [5].

Hence, studies by proteomic technologies showed that osteonectin as a marker of stromal stem cells with osteogenic potential presumably plays an important role in atherogenesis and can serve as a new biomarker of SA and CCA. This is shown by its high concentrations in the blood, particularly in patients with SA and CCA, and its significant correlations with some key biomarkers of atherosclerosis, SA, and CCA.

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REFERENCES

1. Z. A. Gabbasov, A. A. Agapov, O. S. Saburova, *et al.*, *Byull. Eksp. Biol. Med.*, **139**, No. 2, 237-240 (2005).
2. *Diagnostics and Correction of Lipid Metabolism Disorders for Prevention and Therapy of Atherosclerosis. Russian Recommendations. 4th Revision*, Moscow, *Kardiovask. Ter. Prof.*, No. 6, Suppl. 3 (2009).
3. Yu. I. Ragino, M. I. Voevoda, M. I. Dushkin, *et al.*, *Klin. Lab. Diagn.*, No. 4, 11-15 (2005).
4. M. Bhakta, C. Bruce, D. Messika-Zeitoun, *et al.*, *J. Am. Board Fam. Med.*, **22**, No. 6, 610-616 (2009).
5. P. Collin-Osdoby, *Circ. Res.*, **95**, No. 11, 1046-1057 (2004).
6. Z. A. Gabbasov, A. A. Agapov, O. S. Saburova, *et al.*, *Can. J. Physiol. Pharmacol.*, **85**, Nos. 3-4, 295-300 (2007).
7. A. P. Gadeau, H. Chaulet, D. Daret, *et al.*, *J. Histochem. Cytochem.*, **49**, 79-86 (2001).
8. M. Gossl, U. I. Modder, E. J. Atkinson, *et al.*, *J. Am. Coll. Cardiol.*, **52**, No. 16, 1314-1325 (2008).
9. M. Hristov, W. Erl, and P. C. Weber, *Arterioscler. Thromb. Vasc. Biol.*, **23**, No. 7, 1185-1189 (2003).
10. J. L. Hunt, R. Fairman, M. E. Mitchell, *et al.*, *Stroke*, **33**, 1214-1219 (2002).
11. D. Messika-Zeitoun, L. F. Bielak, P. A. Peyser, *et al.*, *Arterioscler. Thromb. Vasc. Biol.*, **27**, 642-648 (2007).
12. E. L. Soboleva, Z. A. Gabbasov, A. A. Agapov, *et al.*, *Exp. Clin. Cardiol.*, **10**, No. 3, 184-188 (2005).
13. D. Steinberg, *J. Lipid Res.*, Suppl., S376-S381 (2009).
14. F. Tabet and K. A. Rye, *Clin. Sci.* (Lond.), **116**, No. 2, 87-98 (2009).
15. A. Trion and A. Van der Laarse, *Am. Heart J.*, **147**, No. 5, 808-814 (2004).