Ragino Yu. I.[1], Kashtanova E. V.[1], Murashov I. S.[2], Volkov A. M.[2], Kurguzov A. V.[2], Sadovski E. V.[1], Maslatsov N. A.[1], Scherbakova L. V.[1], Chernjavskii A. M.[2], Polonskaya Ya. V.[1]

[1] Research Institute of Internal and Preventive Medicine – Branch of the Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences, Novosibirsk, Russia

[2] The Federal State Budgetary Institution «National Medical Research Center named after academician E.N. Meshalkin «of the Ministry of Health of the Russian Federation, Novosibirsk, Russia

The Study of Biochemical Factors of Calcification of Stable and Unstable Plaques in the Coronary Arteries of Man

Objective	The aim of the study was to study biochemical factors of calcification in stable and unstable plaques of coronary arteries and in the blood of patients with severe coronary atherosclerosis, to find associations of biochemical factors of calcification with the development of unstable atherosclerotic plaque.
Materials and Methods	The study included 25 men aged 60,4±6,8 years who received coronary bypass surgery. In the course of the operation intraoperative indications in men were from coronary endarteriectomy (s) artery (a–d) and histological and biochemical analyses of the samples of the intima/media. Out of 85 fragments of intima/media of coronary arteries, 15 fragments of unchanged intima/media, 39 fragments of stable atheromatous plaque and 31 fragments of unstable plaque were determined. In homogenates of samples of intima/media (after measurement of protein by the method of Lowry) and in blood by ELISA were determined by biochemical factors of calcification: osteoprotegerin, osteocalcin, an osteopontin, osteonectin, as well as inflammatory factors (cytokines, chemokines).
Results	A significant direct correlation (Spearman coefficient =0.607, p<0.01) between the stages of atherosclerotic focus development to unstable plaque and the degree of calcification of atherosclerotic focus development samples was found. There was an increased content of osteocalcin in stable and unstable plaques by 3.3 times in comparison with the unchanged tissue of intima/media of coronary arteries, as well as in samples with small and dust-like, with coarse-grained calcifications in comparison with samples without calcifications by 2.8 and 2.1 times, respectively. According to multivariate logistic regression analysis, the relative risk of unstable atherosclerotic plaque in the coronary artery is associated with a reduced content of osteocalcin (OR=0.988, 95% CI 0.978–0.999, p=0.028). Also, the relative risk of calcifications in the atherosclerotic plaque in the coronary artery is associated with an increased content of osteocalcin (OR=1,008, 95% CI 1,001–1,015, p=0,035). In men with severe coronary atherosclerosis, a significant inverse correlation was found (Spearman coefficient –0.386, p=0.022) between the content of osteoprotegerin in the vascular wall and in the blood.
Conclusion	Atherosclerotic plaques have a higher level of osteocalcin compared to samples without atherosclerotic lesions. The risk of developing unstable atherosclerotic plaques in the SC is associated with a reduced content of osteocalcin in it. The risk of calcifications in ASB in CA is associated with an increased content of osteocalcin in it.

Keywords	Coronary atherosclerosis; stable and unstable atherosclerotic plaques; plaque calcification; osteocalcin; osteoprotegerin
For citation	Ragino Yu.I., Kashtanova E.V., Murashov I.S., Volkov A.M., Kurguzov A.V., Sadovski E.V., Maslatsov N.A., Scherbakova L.V., Chernjavskii A.M., Polonskaya Ya.V. The Study of Biochemical Factors of Calcification of Stable and Unstable Plaques in the Coronary Arteries of Man. Kardiologiia. 2020;60(2):83–88. [Russian: Рагино Ю.И., Каштанова Е.В., Мурашов И.С., Волков А.М., Кургузов А.В., Садовский Е.В., Маслацов Н.А., Щербакова Л.В., Чернявский А.М., Полонская Я.В. Исследование биохимических факторов кальцификации стабильных и нестабильных бляшек в коронарных артериях человека. Кардиология. 2020;60(2):83–88]

Corresponding author	Polonskaya Yana. E-mail: yana-polonskaya@yandex.ru

The study of etiological and pathophysiological mechanisms of vascular calcification, atherosclerosis, and their complications is a relevant, fundamental issue in medicine. It is highly controversial whether vascular calcification, particularly its severe form, activates or inhibits the formation of unstable atherosclerotic plaques that are the pathogenetic basis for the risk of acute coronary syndrome (ACS) and myocardial infarction (MI). It is traditionally believed that in most cases, unstable atherosclerotic plaques, which are

responsible for the development of ACS, are lipid-rich, noncalcified plaques with a soft, thin fibrous cap. It is also believed that severe coronary calcification indicates that atherosclerotic plaques are stable and not prone to rupture. However, several studies demonstrated that coronary calcification is an independent and reliable predictor for the development of unstable plaque, complications of atherosclerosis, and long-term cardiovascular events (CVEs) [1-8].

Interpretation of coronary calcification in patients with confirmed coronary atherosclerosis is based on the ultrastructure of the calcified lesion. Thick calcification may indicate stabilization of the atherosclerotic plaque. In contrast, disseminated microcalcifications may indicate the presence of unstable atherosclerotic lesions and a high risk of rupture with development of complications associated with atherothrombosis [9]. Davaine et al. found that the prevalence of coronary artery calcifications with atherosclerotic lesions can be as high as 90% and is an independent risk factor for CVEs [10].

Osteoblast-like cells have been identified in the calcified atherosclerotic plaque, and active resorption of ectopic vascular calcifications has been detected [11, 12]. Osteoblasts are known to be involved in the synthesis of essential components of intercellular substances, e.g., collagen type I, osteocalcin, osteonectin, etc. [13]. Vascular calcifications contain substances characteristic of bone tissue, e.g., calcium salts, phosphates, osteonectin, osteopontin, osteoprotegerin, osteocalcin, collagen type I, and other compounds. The process of vascular calcification, which is largely comparable to osteogenesis, is regulated by the same proteins [14, 15].

This study investigated biochemical factors of calcification of stable and unstable plaques in CAs and blood of patients with severe coronary atherosclerosis and searched for associations between biochemical factors of calcification and the development of unstable atherosclerotic plaque.

Materials and Methods

The study was carried out under the Program of Joint Research Projects of Research Institute for Internal and Preventive Medicine and E.N. Meshalkin National Medical Research Center in 2017-2020 and was approved by the Ethics Committees of both facilities. The study included 25 male patients, mean age 60.4±6.8 years old, with functional class III, stable exertional angina. All patients had excessive body weight with body mass index >25 kg/m2. All patients included in the study underwent coronary bypass surgery in the clinical hospital of E.N. Meshalkin National Medical Research Center. The exclusion criteria were myocardial infarction within the previous six months, acute or exacerbation of chronic infectious and inflammatory diseases, renal insufficiency, severe liver diseases, cancer, and hyperparathyroidism. Before surgery, all patients signed an informed consent form to participate in the study.

Blood samples were taken at admission before surgery. During surgery, endarterectomy of CAs was performed if indicated intraoperatively. Each sample of the postoperative material was longitudinally and transversely divided symmetrically into several fragments under visual control for histological and biochemical examinations. Macroscopic descriptions and histological analysis of 85 CA samples were made using an Axiostar Plus binocular microscope. Histological analysis provided a detailed description of an atherosclerotic cap, e.g., thick, thin, thinning, fibrous, loose, dense, hyalinized, thinned area, hemorrhages, rupture, ulceration, calcification, and atherocalcinosis. This analysis also described its endothelial surface, plaque cores, and plaque/lesion periphery. The histological analysis revealed 15 fragments without atherosclerotic lesions, 39 fragments of stable atheromatous plaques, and 31 fragments of unstable plaques. An unstable plaque was considered to be a damaged plaque with a fibrous cap thickness <65 μm, infiltrated with macrophages and T-lymphocytes, more than 25 cells 0.3 mm in diameter per field of view, and with a large lipid core >40%. The CA samples were categorized into three types: 1) without calcification; 2) with small and microcalcifications; 3) with macrocalcifications.

All patients were divided into two subgroups according to the histological results: 1) without unstable plaques in CAs (n=12); 2) with unstable plaques in CAs (n=13). Between the subgroups, there were no significant differences in age, presence of hypertension, abdominal obesity, diabetes, smoking, history of myocardial infarction, exertional angina functional classes, or drug treatment.

For the biochemical examinations, vessel samples frozen in liquid nitrogen were homogenized in phosphate-buffered saline solution to obtain 1% homogenates, which were aliquoted for further biochemical analyzes. Biochemical factors of calcification, i.e., osteoprotegerin, osteocalcin, osteopontin, osteonectin, and inflammation factors, i.e., cytokines and chemokines, were analyzed in the tissue homogenates, after protein quantification by the Lowry method, and in blood samples by ELISA using a Multiscan microplate reader.

Statistical processing was performed with an SPSS v13.0 software package. The data are presented as means and standard deviations (M±SD) or as medians and percentiles (Me [25%; 75%]). The significance of

ISSN 0022-9040. Kardiologiia. 2020;60(2). DOI: 10.18087/cardio.2020.2.n775

Table 1. Presence of calcifications in atherosclerotic plaques of coronary arteries according to histological analysis

Plaque calcifications	Stable plaques (n=39)	Unstable plaque (n=31)	p
None	12 (31%)	9 (29%)	>0.05
Small and microcalcifications	23 (59%)	18 (58%)	>0.05
Macrocalcifications	4 (10%)	4 (13%)	>0.05

Table 2. Changes in the calcification factors at different stages of development of atherosclerotic lesions

Variable	Intact intima-media (n=15)	Stable plaque (n=39)	Unstable plaque (n=31)	p
	1	2	3	
Osteoprotegerin, pg/mg protein	167.9±99.3	225.8±102.4	176.4±101.6	p>0.05
Osteopontin, ng/mg protein	6.9±4.0	7.3±4.1	4.0±2.2	p>0.05
Osteocalcin, ng/mg protein	23.7 (10,7;	82.4±23.9*	83.2±29.3*	p_{1-2} =0.011 p_{1-3} = 0.013
Osteonectin, μg/mg protein	7.1±3.1	3.4±3.5	3.4±3.0	p>0.05

The data are presented as the mean and standard deviation (M ± SD).

differences was evaluated using the Student's t-test or the Mann-Whitney test, depending on the type of data distribution. Multiple intergroup comparisons were performed by one-way ANOVA using the Bonferroni test for normal distribution or the Kruskal-Wallis criterion for non-normal distribution. Spearman correlation analysis and multivariate logistic regression analysis were also utilized. Differences were considered to be significant if p<0.05.

Results and discussion

Histological analysis identified no difference between stable and unstable atherosclerotic plaques in the presence of calcifications (Table 1). However, correlation analysis revealed a significant direct correlation of average power (rs=0.607; p <0.01) between stages of the development of atherosclerotic lesion into unstable plaque and the degree of sample calcification.

Biochemical factors of calcification at different stages of atherosclerotic lesion development are listed in Table. 2. The amount of osteocalcin in both stable and unstable plaques was 3.3-fold higher (p<0.05) than in CA samples without atherosclerotic lesions.

Concentrations of biochemical factors of calcification in SC sample homogenates were evaluated depending on the histological determination of the degree of calcification (Table 3). Compared to samples without calcifications, osteocalcin was increased 2.8-fold in samples with small and microcalcifications and 2.1-fold in samples with macrocalcifications.

Multivariate logistic regression analysis and the construction of multivariate models identified a significant relationship only between osteocalcin concentrations in CA samples and either a) stage of development of an atherosclerotic lesion into unstable plaque or b) the development of calcification in the atherosclerotic plaque. The results showed that the risk of formation of unstable atherosclerotic plaque in CA is associated with its reduced concentration of osteocalcin (odds ratio [OR] 0.99, 95% confidence interval [CI] 0.978–0.999; p=0028). The risk of calcification of unstable atherosclerotic plaques in CAs is also associated

Table 3. Calcification factors in coronary artery samples according to histological evaluation of the degree of calcification

Variable	Without calcifications (n = 36)	Small and microcalcifications (n=41)	Macrocalcifications (n=8)	p
	1	2	3	
Osteoprotegerin, pg/mg protein	134.9±100.3	204.7±109.0	197.4±111.3	p>0.05
Osteopontin, ng/mg protein	5.3±3.3	7.1±4.0	3.6±2.1	p>0.05
Osteocalcin, ng/mg protein	39.2±20.1	108.3±25.2*	83.3±28.0*	p_{1-2} = 0.045 p_{1-3} = 0.049
Osteonectin, μg/mg protein	4.3±3.0	4.0±3.1	1.8±1.7	p>0.05

The data are presented as the mean and standard deviation (M ± SD).

Table 4. Correlations between concentrations of biochemical factors of calcification and certain inflammatory factors and chemokines in the coronary artery samples (Spearman's coefficient)

Variable	MCP-1	sVCAM	S-selectine	MMP-9	IL-18
Osteoprotegerin	0.783	0.505	–	0.477	0.515
Osteopontin	0.830	0.673	0.524	0.607	0.636
Osteocalcin	0.426	0.512	–	0.290	0.460
Osteonectin	0.704	0.692	0.444	0.554	0.633

p<0.01 for all comparisons.

with increased osteocalcin (OR 1.01, 95% CI 1.001–1.015; p=0.035).

Vascular calcifications contain calcium salts, phosphates, osteopontin, osteonectin, osteoprotegerin, osteocalcin, and other components characteristic of bone tissue. They are expressed in atherosclerotic plaques mainly within vascular smooth muscle cells and serve as markers of osteoblastic differentiation. In this study, of the four biochemical factors of calcification of atherosclerotic lesions, only osteocalcin showed significant changes. The amounts of this factor in stable and unstable plaques were significantly higher than in samples without atherosclerotic lesions and calcified plaques. These findings do not contradict other studies. Along with the primary expression of osteocalcin by vascular smooth muscle cells, it is expressed in plaques by other cells. Thus, Zhang et al. [12] demonstrated a positive correlation between the number of osteocalcin carrying endothelial progenitor cells and the calcification of CA in patients with coronary artery disease. Foresta et al. [16] concluded that platelets contained in the atherosclerotic plaque area release additional osteocalcin.

We identified significant, direct correlations of average power between concentrations of biochemical factors of calcification and certain inflammatory factors and chemokines in CA samples (Table 4).

On the one hand, severe coronary calcification is known to be a strong and independent predictor for long-term CVEs [17], but on the other hand, regression of the atherosclerotic plaque under statin therapy and its strengthening due to increasing amount of deposited calcium may be a favorable prognostic factor. Apparently, it is essential to not only detect vascular calcifications, but also to assess related factors. These include the activity of vascular inflammatory factors, and in particular, microscopic features of calcification, including their location within the plaque and their prevalence [7, 8]. Several publications suggest that atherosclerotic calcifications are closely associated with the inflammatory processes. There is evidence that vascular calcification may initiate inflammation and, thus, further progression of calcification [18, 19]. Chatrou et al. [20] demonstrated an increase in inflammatory processes at the stage of microcalcification.

Analyses of blood concentrations of biochemical factors were performed in male patients with atherosclerotic calcification of CAs (Table 5). The blood concentration of osteonectin in patients with unstable atherosclerotic CA plaques was 1.4-fold greater (p<0.05) than in patients without unstable plaques.

Correlation analysis revealed a significant inverse correlation of average power (rs=-0.386; p =0.022) between vascular wall and blood concentrations of osteoprotegerin. There were no other correlations between these concentrations of biochemical factors of calcification.

When discussing this result, it is essential to note that osteoprotegerin is a modulator of vessel wall calcification. This was demonstrated by the formation of calcification in relevant arteries of mice with osteoprotegerin gene deletion [10].

Table 5. Blood levels of biochemical factors of calcification in male patients with coronary atherosclerosis

Variable	No unstable plaques in coronary arteries (n=12)	Unstable plaques in coronary arteries (n=13)	p
Osteoprotegerin, pg/ml	66.2 [54.5; 78.2]	62.1 [43.9; 80.3]	p>0.05
Osteopontin, ng/ml	28.9 [5.4; 50.1]	24.4 [19.3; 34.2]	p>0.05
Osteocalcin, ng/ml	13.2 [9.0; 23.4]	14.3 [9.9; 16.2]	p>0.05
Osteonectin, μg/ml	6.5 [3.4; 7.9]	8.9 [7.5; 9.8]	p=0.034

The data are presented as the median and percentiles [25%; 75%].

ISSN 0022-9040. Kardiologiia. 2020;60(2). DOI: 10.18087/cardio.2020.2.n775

Krzanowski et al. [21] concluded that increased blood concentration of osteoprotegerin may be a marker of CA calcification and is associated with risk of CVEs in patients with severe arterial calcification. The mechanism of osteoprotegerin regulation of arterial calcification is very complex and not fully understood.

Conclusion

1. A significant positive correlation (rs=0.607; p<0.01) was observed between the stages of the development of atherosclerotic lesion to unstable plaque and the degree of calcification of atherosclerotic samples.
2. Compared to coronary arteries without atherosclerotic lesions, the amount of osteocalcin in stable and unstable plaques was 3.3-fold greater. It was also 2.8-fold greater in samples with small/microcalcifications and 2 1-fold greater in samples with macrocalcifications.
3. Multivariate logistical regression analysis showed that the risk of formation of unstable atherosclerotic plaque in the coronary artery is associated with reduced levels of osteocalcin (odds ratio 0.99, 95% confidence interval [0.978–0.999; p=0.028). The risk of calcification of atherosclerotic plaque in the coronary artery is also associated with the increased levels of osteocalcin (odds ratio 1.01, 95% confidence interval 1.001–1.015; p=0.035).
4. Male patients with severe coronary atherosclerosis had a significant inverse correlation (rs=-0.386; p=0.022) between the vascular wall and blood concentrations of osteoprotegerin.

The study was carried out under the state-financed research project on State Assignment #0324-2018-0001 and with the financial support through grant #19-015-00055a of the Russian Fund of Fundamental Investigations (RFFI) "The role of vascular calcification in the stability and instability of atherosclerotic plaques." Biological material from the collection of Research Institute for Internal and Preventive Medicine was used in the study.

No conflict of interest is reported.

The article was received on 18/09/19

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ISSN 0022-9040. Kardiologiia. 2020;60(2). DOI: 10.18087/cardio.2020.2.n775