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COULD LYMPHOCYTE CASPASE-3 ACTIVITY PREDICT ATHEROSCLEROTIC PLAQUE VULNERABILITY?

MOGUĆNOST PREDVIĐANJA VULNERABILNOSTI ATEROSKLEROTSKOG PLAKA POMOĆU AKTIVNOSTI LIMFOCITNE KASPAZE-3

Tatjana Ristić[1], Vladan Ćosić[1], Predrag Vlahović[1], Marina Deljanin-Ilić[2], Vidosava B. Đorđević[3]

[1]Centre for Medical Biochemistry, Clinical Centre, Niš, Serbia
[2]Institute for Cardiovascular and Rheumatic Diseases, Niška Banja, Serbia
[3]Institute of Biochemistry, Medical Faculty, Niš, Serbia

Summary: Apoptotic cell death may play a critical role in a variety of cardiovascular diseases, especially in those developing on the basis of atherosclerosis. The goal of this study was to compare the activity of caspase-3 in different forms of ischemic heart disease and to correlate caspase-3 activity with inflammatory and lipid markers as well as risk factors. This enzyme activity was determined in peripheral blood mononuclear cells (PBMC) of 30 patients with stable angina pectoris (SAP), 27 with unstable angina (USAP), 39 with acute ST-elevation myocardial infarction (STEMI) and 27 healthy volunteers by a colorimetric commercially available ELISA method. In the SAP group caspase-3 activity was 0.093±0.036 μmol/mg protein, in patients with STEMI it was 0.110±0.062 μmol/mg protein, and both values were insignificantly higher in comparison with controls (0.092±0.022 μmol/mg protein). In PBMC of USAP patients caspase-3 activity (0.122±0.062 mmol/mg protein) was significantly higher ($p<0.05$) compared to the control group. In SAP patients caspase-3 activity showed a significant positive correlation with triglicerydes ($p<0.05$). Caspase-3 activity may be a valid parameter for assessing the atherosclerotic plaque activity, and a new target for therapeutic intervention.

Keywords: ischemic heart disease, caspase-3, inflammatory markers, lipid markers

Kratak sadržaj: Smrt ćelija apoptozom ima značajnu ulogu u različitim kardiovaskularnim oboljenjima, posebno u onim koja se razvijaju na temelju ateroskleroze. Cilj ove studije bilo je poređenje aktivnosti kaspaze-3 u različitim oblicima ishemijske bolesti srca i koreliranje njene aktivnosti sa inflamatornim, lipidnim markerima i faktorima rizika. Aktivnost kaspaze-3 određivana je u mononuklearnim ćelijama periferne krvi (MĆPK) kolorimetrijskim komercijalnim ELISA testom. Enzimska aktivnost određivana je kod 30 pacijenata sa stabilnom anginom pektoris (SAP), 27 sa nestabilnom anginom pektoris (NSAP), 39 sa akutnim infarktom miokarda sa elevacijom ST segmenta (STEMI) i 27 zdravih dobrovoljaca. U MĆPK pacijenata sa SAP aktivnost enzima bila je 0,093±0,036 μmol/mg proteina, a kod pacijenata sa STEMI 0,110±0,062 μmol/mg proteina, i obe vrednosti su bile statistički neznačajno više u poređenju sa kontrolnom grupom (0,092±0,022 μmol/mg proteina). U MĆPK pacijenata sa NSAP aktivnost kaspaze-3 (0,122±0,062 μmol/mg proteina) bila je statistički značajno viša ($p<0,05$) u poređenju sa kontrolnom grupom. U grupi pacijenata sa SAP postoji statistički značajna pozitivna korelacija kaspaze-3 i triglicerida ($p<0,05$). Aktivnost kaspaze-3 može biti validan parametar u praćenju aktivnosti aterosklerotičnog plaka i nova meta za terapijske intervencije.

Ključne reči: ishemijska bolest srca, kaspaza-3, inflamatorni markeri, lipidni markeri

Introduction

Ischemic heart disease occurs on the basis of atherosclerosis (1). Atherosclerosis is a chronic inflammatory disease characterized by endothelial dysfunction and lipid and monocyte-derived macrophages accumulation within the vessel wall (2). Both the vessel wall and blood derived cells may undergo necrotic or apoptotic cell death during atherogenesis.

Address for correspondence:

Tatjana Ristić
Center for Medical Biochemistry, Clinical Center, Niš
Bulevar dr Zorana Đinđića 48, 18000 Niš, Serbia
Phone: +381 18 4232210, +381 18 217261
e-mail: ristict@bankerinter.net

Apoptosis occurs in atherosclerotic coronary arteries, and the significance of apoptosis depends on the stage of the plaque, localization and the cell type involved. In initial lesions, only a few cells undergo apoptosis. In advanced lesions, many cells die by programmed cell death (PCD).

Apoptosis can be initiated by one of two pathways: the death receptor (extrinsic) pathway or mitochondrial activation of cytochrome c (intrinsic) pathway (3). In both pathways caspase-3 is activated as an executor molecule of apoptosis leading to cleavage of DNA and cell death (4). Caspases are expressed as proenzymes (5) and are activated following proteolytic processing and association of the large and small subunits (6). The activation of these enzymes may occur autocatalytically or in a cascade (7). The distribution of caspase-3 is consistent with its role as a downstream caspase that targets proteins including lamin A and B in the nuclear lamina, poly (ADP-ribose) polymerase and nuclear endonucleases (8) and actin as a cytoskeletal protein. Caspase-3 can cleave both the antiapoptotic protein bcl-2, releasing a fragment that promotes apoptosis, and ICAD (inhibitor of caspase-activated deoxyribonuclease), releasing CAD, the nuclease that induces DNA fragmentation. In addition, caspase-3 also cleaves gelsolin, an actin-associated protein that may have influence on DNase activity (9).

Caspase-3 is a cysteine protease located in both the cytoplasm and mitochondrial intermembrane space. In resting cells, a subset of caspase-3 zymogens is S-nitrosylated at the active site cysteine thus inhibiting enzyme activity. The majority of mitochondrial, but not cytoplasmatic, caspase-3 zymogens contain this inhibitory modification (10). During Fas-induced apoptosis, caspases are denitrosylated, allowing the catalytic site to function. Therefore, apoptosis is regulated by intracellular nitric oxide (NO) production. Since endothelial dysfunction may be caused by an accelerated inactivation of NO by reactive oxygen species (11), this may lead to the increased caspase-3 activation and cell death.

The role of apoptosis in atherosclerotic plaque has been studied in animal and human tissue specimens (12). Caspase-3 activity and apoptosis rates are low in the normal vasculature (13). Apoptosis rate in atherosclerotic lesions is higher than in normal vasculature and correlates with disease activity: higher levels of apoptosis were seen in atherectomy specimens from patients with unstable angina compared to those with stable angina and among those with symptomatic vs. asymptomatic carotid plaques (14). Fas/APO-1 is documented in foam cells whose source are macrophages or smooth muscle cells (SMC). Also, CD3-positive T lymphocytes found around foam cells have been found to express Fas/APO-1 (15).

The goal of this study was to compare the activity of caspase-3 in different forms of ischemic heart disease and to correlate caspase-3 activity with inflammatory and lipid markers as well as risk factors.

Patients and Methods

Among 96 observed patients who were admitted to the Institute for Cardiovascular and Rheumathic Diseases »Niška Banja«, 30 patients had chronic stable angina pectoris (SAP), 27 had unstable angina pectoris (USAP) and 39 had acute ST-elevation myocardial infarction (STEMI). SAP was diagnosed by typical chest pain on effort lasting from 1 to 15 minutes mitigated by glyceryl trinitrate, electrocardiogram (ECG) changes (depression or elevation of ST-segment) in angina attack or with positive responses to exercise electrocardiogram and/or positive stress echocardiography testing. USAP was defined by angina chest pain at rest within the previous 48 hours (class IIIB) (16), typical ECG changes (ST-segment changes, T-wave changes), negative cardiac enzymes and negative troponin I. Acute myocardial infarction (AMI) patients had chest pain with duration longer than 30 minutes, typical ECG changes at admission and elevated troponin I levels. All of these patients had STEMI, which was defined as significant ST-elevation according to the current Guidelines of the European Society of Cardiology (17).

All patients provided the data about age, sex, risk factors (hypertension, diabetes mellitus (DM), smoking, obesity, family history, physical inactivity, cholesterol and triglyceride levels) and current therapy just after admission.

Control group included 27 healthy volunteers. They did not have any history of hypertension, diabetes or ischemic heart disease. The patients and volunteers gave written infomed consent before the study entry and the study was ratified by the local Ethic Committee.

Caspase-3 activity was determined in peripheral blood mononuclear cells (PBMCs) isolated using lymphocyte separation medium. This enzyme activity was measured by a colorimetric commercially available ELISA kit (ApoTarget Caspase-3 Protease Assay, BioSource, Nivelles, Belgium) based on the degradation of synthetic tetrapeptide DEVD-pNa. The substrate, DEVD is composed of the chromophore, p--nitroanilide (pNA), and a synthetic tetrapeptide, DEVD (Asp-Glue-Val-Asp), which is the upstream amino acid sequence of the caspase-3 cleavage site in poly (ADP-ribose) polymerase (PARP). Upon cleavage of the substrate by caspase-3 or related caspases, free pNA light absorbance can be quantified using a spectrophotometer or a microplate reader at 400 or 405 nm.

All other biochemical parameters were estimated by an autoanalyzer Olympus AU 400 (Olympus, Tokio, Japan), and troponin I concentration by AxSYM analyzer (Abbott Laboratories, Abbott Park IL, USA).

Obtained data were tested using analyses of descriptive (average, standard deviation) and analytical (Dunnett' test – for multiple comparisons; Student's non-paired t-test) statistics. Linear regression

analysis was used to assess the relationships between the studied apoptotic and biochemical markers as well as the risk factors. Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) 15.0 computer program (SPCC Inc., Chicago, IL, USA).

Results

Baseline characteristics and risk factors are shown in *Table I*. Average age in the STEMI group was 64.87±9.03 years, in the USAP group 68.33± 8.78 years, in the SAP group it was 60.17± 11.78 years. The control group average age was 58.52± 5.60 years. USAP patients had the highest prevalence of physical inactivity (100%) and hypertension (81.48 %), higher than SAP and STEMI patients. SAP patients had a higher prevalence of smoking habits (50 %) and most STEMI patients had familial history of coronary heart disease (51.28 %).

Lipid markers and markers of inflammation in patients with ishemic heart disease are shown in *Table II*. In STEMI and USAP patients total cholesterol levels (5.74±1.06 mmol/L, 5.76±1.26 mmol/L, respectively) were significantly higher while HDL-cholesterol (1.18±0.29 mmol/L, 1.22±0.28 mmol/L, respectively) and LDL-cholesterol (3.66±0.90 mmol/L, 3.73± 1.12 mmol/L, respectively) values were significantly lower than those in healthy persons. At the same time, hsCRP values (17.90 (0.56–270.29) mg/L) and total number of leukocytes (10.76±5.01x10^9/L) were significantly higher in STEMI group than values of these parameters in other groups. In USAP patients erythrocyte sedimentation rate values (25.00 (2.00–86.00) arb.units) were significantly higher than in SAP patients and controls. SAP patients had a significantly higher leukocyte count than controls, and the highest, but not significantly different values of serum triglyceride concentrations (2.76±3.84 mmol/L) in comparison with the other three groups.

Table I Baseline characteristics and risk factors in patients with ischemic heart disease.

	STEMI patients (n=39)	USAP patients (n=27)	SAP patients (n=30)	Controls (n=27)
Age, years	64.87±9.03[a]	68.33±8.75[c]	60.17±11.78	58.52±5.60
Sex (male/female), n	27/12	12/15	19/11	12/15
Coronary risk factors				
Hypertension (%)	76.62[c]	81.48[c]	66.67[c]	0
Diabetes mellitus (%)	30.77[c]	18.52	26.67[a]	0
Smoking (%)	41.03	29.63	50.00	25.6
Obesity (%)	17.95[a]	3.70[b]	26.67[a]	0
Family history (%)	51.28[a]	48.15	26.67	22
Physical inactivity (%)	92.31[a]	100.00[c]	96.67[c]	39.5

The results are presented as $\bar{x}$±SD.
[a]p<0.05 vs. controls, [b]p<0.05 vs. SAP, [c]p<0.001 vs. controls

Table II Lipid markers and markers of inflammation in patients with ischemic heart disease.

	STEMI patients (n=39)	USAP patients (n=27)	SAP patients (n=30)	Controls (n=27)
Lipid biomarkers				
Total cholesterol, mmol/L	5.74±1.06***	5.76±1.26**	5.62±2.12**	5.69±0.61
HDL-cholesterol, mmol/L	1.18±0.29***	1.22±0.28***	1.20±0.33***	1.72±0.36
LDL-cholesterol, mmol/L	3.66±0.90**	3.73±1.12*	3.17±1.10***	4.47±1.11
Triglycerides, mmol/L	2.00±0.79	1.68±0.70	2.76±3.84	1.65±0.68
Biomarkers of inflammation				
hs CRP, mg/L	17.90 (0.56–270.29)***,b	3.56 (0.43–122.90)	2.86 (0.74–84.35)	1.55 (0.32–9.61)
SE, arb.units.	14.00 (2.00–98.00)	25.00 (2.00–86.00)***,a	14.50 (2.00–48.00)	13.00 (2.00–36.00)
LE, x10^9/L	10.76±5.01***,b,c	7.91±2.14	7.61±1.37**	6.49±1.60

The results are presented as $\bar{x}$±SD, and Median (MinMax).
*p<0.05 vs. controls, ** p<0.01 vs. controls, ***p<0.001vs. controls, [a]p<0.05 vs. SAP patients, [b]p<0.01 vs. SAP patients, [c]p<0.01 vs. USAP, [d]p<0.001 vs. SAP patients, [e]p<0.001 vs. USAP patients

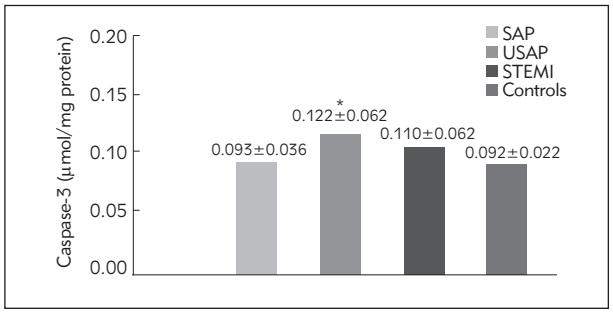

Figure 1 Caspase-3 activity in lymphocytes of patients with ischemic heart disease.
*p<0.05 vs. controls

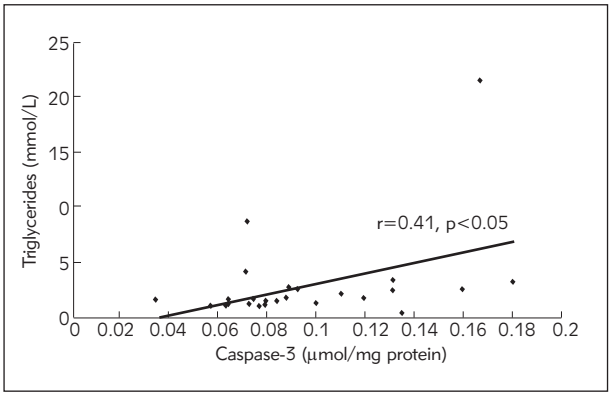

Figure 2 Correlation between caspase-3 and triglycerides in SAP patients.

Caspase-3 activity in PBMC of SAP patients was 0.093±0.036 μmol/mg protein, and in patients with STEMI it was 0.110±0.062 μmol/mg protein, and both values were insignificantly higher in comparison with controls (0.092±0.022 μmol/mg protein). In USAP patients PBMC caspase-3 activity (0.122± 0.062 μmol/mg protein) was significantly higher (p<0.05) compared to control *(Figure 1)*.

The correlation between caspase-3 activity and inflammatory and lipid markers was studied for each patient group. Significant correlation was observed only in SAP patients. Caspase-3 activity showed a significant positive correlation with triglycerides (r=0.41, p<0.05) *(Figure 2)*. No correlations were found either between caspase-3 and inflammatory markers or between caspase-3 and risk factors in any patient group.

Discussion

This study showed that caspase-3 activity was significantly increased in patients with USAP in comparison with healthy individuals. These results are in agreement with the recently published data showing a dysregulated apoptotic process in asymptomatic plaque and pointing to the significance of caspase-3 as a new target for therapeutic intervention. A univariable analysis of plasma caspase-3 activity and its relationship with coronary calcium, abdominal aortic wall thickness and aortic compliance done in 3221 patients from Dallas Heart Study showed that caspase-3 is independently associated with these parameters. These results suggest a link between apoptosis and atherosclerosis (18).

Tissue expression of caspase-3 correlates with TUNEL staining in a atherosclerotic plaque. Caspase-3 is found in variety of tissues and its levels are likely to rise when apoptosis increases in many different organ beds (19). As a downstream marker of apoptosis caspase-3 captures extrinsic and intrinsic pathways, and since it is involved only in apoptosis, if may be a good biomarker of vascular apoptosis and endothelial dysfunction.

There is evidence that plasma caspase-3 is significantly associated with traditional cardiovascular risk factors (diabetes, hypertension, current smoking, hypercholesterolemia, low HDL-c) as well as several biomarkers implicated in atherosclerosis, including hsCRP, sCD40 and monocyte chemoattractant protein-1 (MCP-1) (20). The data obtained from other studies show that caspase-3 activity is associated with several distinct subclinical atherosclerotic phenotypes, including coronary calcium, abdominal aortic wall thickness and aortic compliance, suggesting that apoptosis plays an important role in atherosclerosis development and progression (22–24). In our study caspase-3 activity showed a significant positive correlation with triglycerides (p<0.05) in SAP patients (21–23).

Caspase inhibitors represent potential novel therapeutic agents that can suppress atherosclerosis progression. In an animal model of transplant vasculopathy, treatment with a caspase-3 inhibitor decreased progression of coronary artery disease (24). Lower cholesterol diet and statin therapy have been associated with less macrophage apoptosis and plaque stabilization in atherosclerotic rabbits (25). In humans, higher expression of inhibitor of apoptosis proteins (IAPs) has been found in asymptomatic compared to symptomatic carotid plaques (26, 27), supporting the idea that the suppression of apoptosis may be a potential therapeutic target. The results obtained from the study with human coronary artery endothelial cells (HCAECs) have shown that epinephrine-induced apoptosis of HCAECs is associated with activation of caspase-3 by about 1.5 fold. Carvedilol completely blocked epinephrine-induced activation of caspase-3 and simultaneously totally inhibited apoptosis (28). In conclusion, caspase-3 activity may be a valid parameter for assessing the atherosclerotic plaque activity, and a new target for therapeutic intervention.

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