RESEARCH LETTER

Transcriptional activity of nuclear factor-κB genes in peripheral blood mononuclear cells in patients with early atherosclerosis: preliminary findings

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Introduction Atherosclerosis and its complications, including the development of coronary artery disease (CAD) and heart failure, are the primary cause of morbidity and mortality. Cardiovascular diseases are the most frequent cause of death, and coronary heart disease accounts for 46% of deaths.[1] Subclinical atherosclerosis is the early stage in the development of atherosclerosis, and there are only minor changes in the arterial wall before the onset of clinical symptoms. The incidence of subclinical atherosclerosis is estimated at 36% in women and 38.7% in men, and increases with age. Atherosclerosis can be diagnosed by the assessment of coronary artery calcium score (CACS) and coronary angiography in the asymptomatic phase. An angiographic definition of CAD is as follows: coronary lumen stenosis exceeding 50%, significant stenosis exceeding 70%, and insignificant lesions of less than 50%.[2,3]

Nuclear factor κB (NFκB) plays an important role in an inflammatory process underlying the development of atherosclerosis. NFκB constitutes a family of transcriptional factors: NFκB1 (protein p50 and its precursor p105), NFκB2 (protein p52 and its precursor p100), c-Rel, RelB, and RelA (p65).[4] NFκB is considered to be the key transcription factor regulating survival, growth, and differentiation of cells. After activation and dissociation from its inhibitor (IκB), NFκB enters the nucleus and binds to the corresponding sequence in the promoters and enhancers of transcription of the genes encoding cytokines, growth factors, adhesion molecules (eg, selectin P), and regulators of the cell cycle and apoptosis (eg, activation and proliferation of smooth muscle cells). Under normal conditions, NFκB is inactivated by IκB.[5,6]

The main aim of this study was to evaluate transcripitional activity of genes encoding proteins of the NFκB (p50, p52, p65, c-Rel) and its inhibitor (IκBα) in peripheral blood mononuclear cells (PBMCs) in patients with early stages of atherosclerosis in the coronary arteries in comparison with a control group. An additional aim was to analyze transcriptional activity of the NFκB gene and IκBα in patients with CAD in terms of cardiovascular risk factors.

Patients and methods The study protocol was approved by an institutional review board (KNW/0022/KB1/17/I/12). We enrolled consecutive patients with suspected CAD, who were admitted to the Department of Cardiology, Medical University of Silesia, Katowice, Poland. In all patients, a 64-row multidetector computed tomography scan was performed for calcium scoring and coronary angiography. From this group, 83 patients with no abnormalities on coronary angiography or with only minimal vascular lesions (<10%) were selected for further investigation. There were only a few patients with minimal vascular lesions.

The inclusion criteria were age over 18 years and normal coronary angiography results, while the exclusion criteria were advanced CAD, advanced heart failure, creatinine level exceeding 2.5 mg/dl, active inflammation, chronic inflammatory diseases, and neoplasm.

Depending on the results of the CACS, the study group was divided into 2 subgroups: 1) the control group including participants with normal coronary angiography results and a CACS of 0; and 2) the patient group including individuals with early stages of atherosclerosis in

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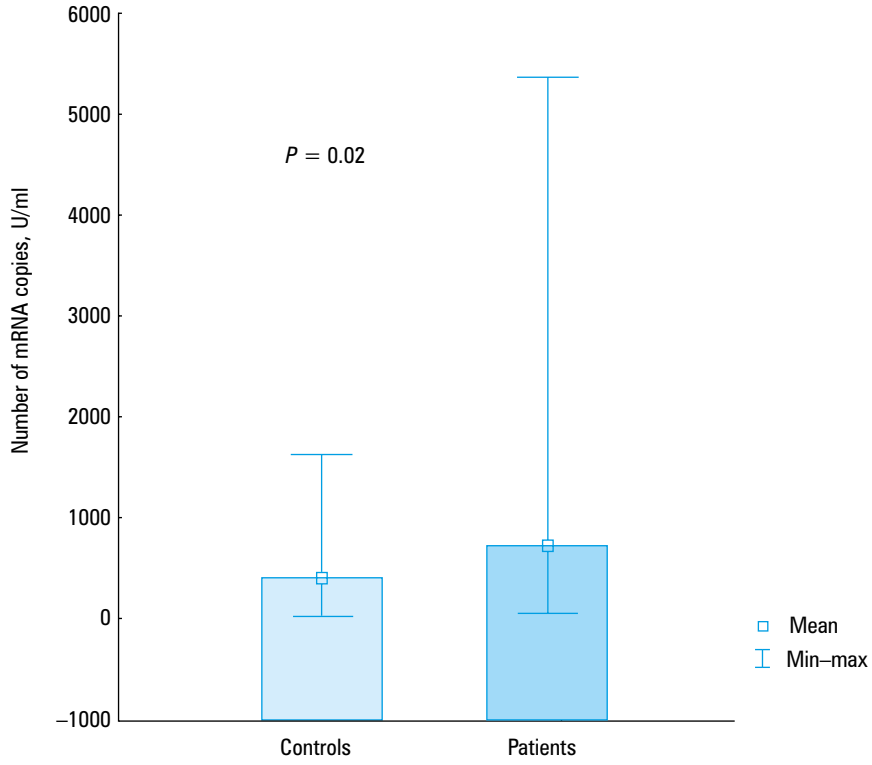

FIGURE 1
Transcriptional activity of the p50 gene in patients with early stages of atherosclerosis of the coronary arteries and in controls assessed by real-time quantitative polymerase chain reaction in peripheral blood mononuclear cells (Mann–Whitney test)

the coronary arteries, that is, with normal coronary angiography and a CACS exceeding 0.

PBMCs were separated from blood samples in a density gradient of Ficcol during centrifugation. Total RNA was extracted from PBMCs using TRIzol Reagent (Invitrogen™, Carlsbad, California, United States), according to the modified method by Chomczynski and Sacchi.[7] The RNA extracts were qualitatively evaluated by electrophoresis in 1% agarose gel with ethidium bromide (0.5 mg/ml) in a SUBMINI device (Kucharczyk, Warsaw, Poland). Total RNA extracts were quantified by a spectrophotometric measurement of the RNA concentration (Gene Quant II, ALT, Cambridge, England).

RNA obtained during extraction and purification was used to determine the number of copies of selected mRNA of genes in real-time quantitative polymerase chain reaction (RT-qPCR), using QuantiTect SYBR Green RT-PCR Master Mix and a sequence detector DNA Engine Opticon (Bio-Rad, California, United States) with fluorescent SYBR Green I stain. The procedure and reaction conditions were performed according to the manufacturer's recommendations (Qiagen, Düsseldorf, Germany). The primers and probes for RT-qPCR were designed using the Primer ExpressTM Version 1.0 computer program, ThermoFisher Scientific, Waltham, Massachusetts, United States) on the basis of the sequence homologies from the GenBank database. Their specificity was tested in the online database BLAST.

The number of copies of cDNA of the analyzed genes was determined using the established standard curve. Transcriptional activity of the genes of NFκB and IκBα and 2 genes of endogenous control, glyceraldehyde-3-phosphate dehydrogenase and β-actin, was evaluated. The gene expression was assessed on the basis of the amount of mRNA copies per 1 μg of total RNA.

Statistical analysis The obtained data were exported to STATISTICA PL v 6.0, (StatSoft, Kraków, Poland). The mean and SD were calculated. To compare the parameters of the control and patient groups, the Mann–Whitney test was used. The results were considered significant at a P value of less than 0.05.

Results There were 38 participants in the control group and 45 in the patient group. The mean (SD) age in the control group was 52.29 (11.05) years, and in the patient group—62.84 (9.62) years. The difference in age between the groups was nonsignificant. Arterial hypertension was more common in the patient group than in the control group (P = 0.01). More patients with premature CAD took statins and β-blockers (P <0.05). The mean (SD) serum creatinine level was 0.82 (0.16) mg/dl in the control group and 0.89 (0.24) mg/dl in the patient group (P = 0.1).

The analysis revealed a significant increase in the transcriptional activity of the p50 gene in patients with early stages of atherosclerosis in the coronary arteries, as compared with the control group (FIGURE 1). However, we did not observe significant differences in the transcriptional activity of p52, p65, c-Rel, and IκBα genes between patients and controls.

For further statistical analysis, patients with early stages of atherosclerosis were divided into 2 subgroups (with or without the risk factor). We analyzed the transcriptional activity of the NFκB and IκBα genes as well as the following

risk factors: sex, family history of CAD, arterial hypertension, diabetes, obesity, smoking, presence of lipid disorders (patients with known lipid abnormalities treated or not treated with statins), abnormal levels of total cholesterol (threshold <5.172 mmol/l), high-density lipoprotein cholesterol (threshold >0.9051 mmol/l), low-density lipoprotein cholesterol (threshold <3.3618 mmol/l), and triglycerides (threshold <1.6935 mmol/l). Transcripitional activity of the p65 gene was increased in men (P = 0.01) and in patients with arterial hypertension (P = 0.00001). Similarly, transcriptional activity of the c-Rel gene was increased in patients with lipid disorders (P = 0.01), and of the IκBα gene—in men (P = 0.046), in patients with arterial hypertension (P = 0.01), and in smokers (P = 0.01).

Discussion Our study revealed a significant increase in the transcriptional activity of the p50 gene in patients with early stages of atherosclerosis in the coronary arteries (CACS >0), as compared with controls (CACS = 0). There were no significant differences in the expression of the p52, p65, c-Rel, and IκBα genes. Brand et al[8] demonstrated an increased transcripitional activity of the p65, p50, and c-Rel genes, but not of the p52 and RelB genes, in atherosclerotic lesions.

Our study suggests that the presence of cardiovascular risk factors (eg, male sex, arterial hypertension, smoking, lipid disorders) may be associated with changes in the transcriptional activity of the NFκB family genes. Arterial hypertension, especially if uncontrolled, may harm target organs by activating NFκB pathways.[9] Diabetes is associated with inflammation caused by the activation and changes in the immune system.[10] Our study did not reveal significant differences in the expression of NFκB family genes and IκBα in patients with a CACS exceeding 0 and diabetes mellitus and obesity. However, it was reported that patients with type 2 diabetes with or without obesity had increased expression of NFκB (p65), compared with nondiabetic individuals.[11]

Smokers with early stages of atherosclerosis had a significantly increased expression of the IκBα gene. Its expression may also be enhanced by the use of drugs, such as statins or angiotensin-converting enzyme inhibitors.

The presence of lipid disorders in the study group was associated with increased transcriptional activity of c-Rel. Animal studies showed that selective inhibition of c-Rel reduces stress-induced atherosclerotic lesions.[12]

In conclusion, it should be emphasized that the differences in the gene activity of the NFκB family genes in patients with a CACS of 0 and those with a CACS higher than 0 suggest that the inflammatory process underlying atherosclerosis is systemic and not restricted only to atherosclerotic plaque. It is probable that in atherosclerosis a vicious circle develops, whereby the local inflammatory process activates additional immune cells, which in turn circulate, spread, and result in systemic inflammation.

A significant increase in the transcriptional activity of the NFκB gene (p50), with no significant difference in the gene expression of IκBα in patients with early stages of atherosclerosis in the coronary arteries (CACS >0), indicates a key role of the NFκB gene (p50) and its signaling pathway in inflammatory processes associated with the development of atherosclerosis. Our results also revealed changes in the transcriptional activity of the NFκB family genes associated with risk factors such as male sex, hypertension, smoking, and lipid disorders in this patient group, which may partially explain the possible involvement of those risk factors in the development of atherosclerosis.

Evaluation of the transcriptional activity of NFκB genes may be useful in the diagnosis and prognosis of patients with atherosclerosis, even in early stages, and may allow early implementation of preventive measures in clinical practice.

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