Original Article

Transcriptional Activity of Tumour Necrosis Factor α (TNF-α) in Patients with Subclinical Coronary Atherosclerosis – Preliminary Results

(tumour necrosis factor α / subclinical atherosclerosis)

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Abstract. The most frequent cause of ischaemic heart disease is coronary arteriosclerosis. This study was aimed at assessing gene expression of TNF4 and its two receptors (TNFR1, TNFR2), as well as determining coronary artery calcium score (CACS) in the context of occurrence of classical risk factors in patients with subclinical arteriosclerosis of coronary vessels. The study involved 47 subjects with complaints of chest pain and suspicion of acute coronary syndrome or stable coronary disease. Additionally, CACS was assessed by 64-slice computerized tomography. QRT-PCR molecular studies were performed using RNA isolated from peripheral blood mononuclear cells. Preliminary results of molecular studies on patients with subclinical coronary arteriosclerosis revealed a significantly lower numbers of TNFR1 and TNFR2 gene copies as compared with healthy subjects. In addition, it can be demonstrated that among classical risk factors hypertension is of substantial importance in the progression of coronary arteries’ calcification, and that in the examined group CACS increases together with the rising number of classical risk factors involved. No correlation was observed, however, between expression of TNF4, TNFR1 and TNFR2 genes and the value of CACS.

Conclusions: 1. The occurrence of hypertension facilitates initiation and progress of arteriosclerotic lesions in blood vessels including the coronary ones; the raised number of circulatory disease classical risk factors involved correlates with elevated calcification of coronary arteries as shown by 64-slice computerized tomography scans. 2. Significantly decreased numbers of TNFR1 and TNFR2 gene copies observed in the investigated group may play a significant role in initiation and progression of arteriosclerosis.

Introduction

Circulatory system diseases are the main cause of death in the developed countries (Saraste et al., 1997). The principal factor involved in their occurrence is atherosclerosis, the onset of which occurs already in the first years of life. Its progression, through many years, leads to several late complications including acute coronary syndrome. Among main risk factors present in atherogenesis are hypertension (characterized by non-laminar blood flow), decreased activity of nitrogen oxide synthase (NO) and disturbances of endothelium regeneration and vasodilation (Cunningham and Gotlieb, 2005). The inflammatory process begins in blood vessel wall with inflammatory cells’ migration to the site of lesion within the endothelium and subsequent adhesion and proliferation. At this stage, an important role is played by adhesion molecules: vascular adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) (Jang et al., 1994; Milioti et al., 2008). Disturbances of lipid metabolism (hypercholesterolaemia, hypertriglyceridaemia) are another risk factor for atherosclerotic plaque formation through increasing migration, accumulation and modification of lipid particles in blood vessel intima. Inflammatory cells accumulating lipoproteins become foam cells. Proliferation of vascular smooth muscle cells (VSMC) takes place along with a decrease of their contractibility. Ultimately, the process leads to the formation of fibroadipose atherosclerotic plaque.

Besides hypertension and lipid metabolism disturbances, classical risk factors in the development of atherosclerotic plaque include: diabetes, age, male sex, burdening family history, obesity and tobacco smoking. The INTER-HEART study assessing the influence of modifiable atherosclerosis risk factors upon myocardial
infarction found that they are responsible in nearly 90% for the occurrence of acute coronary syndrome (ACS) (Oumpuu et al., 2001).

The protracted inflammatory condition, such as atherosclerosis, involves activity of numerous pro-inflammatory agents. The key role in the course of inflammatory reaction leading to the appearance of atherosclerotic lesions is played by tumour necrosis factor α (TNF-α) and its two transmembrane receptors: TNFR1 and TNFR2. TNF-α is produced by macrophages in response to inflammatory processes. Studies on chronic inflammatory diseases have unequivocally pointed out the TNF-α family members as key mediators of inflammation. Currently, chronic inflammatory diseases, including atherosclerosis, are considered to be autoimmune diseases (Watts, 2005; Dinarello, 2009). TNF-α is thought to be involved in initiation and progression of atherosclerotic plaque (Xanthoulea et al., 2009). Survey of literature data reveals reports describing increased TNF-α concentration in both coronary disease patients with ACS and patients with asymptomatic coronary disease. Such reports suggest that elevated TNF-α correlates with risk of death due to cardiovascular causes, including cardiac insufficiency (Ridker et al., 2000; Heeschen et al., 2003; Secchiero et al., 2009). Jacobsson et al. (2005) noticed that blockade of the TNF-α pathway reduces cardiovascular incidents in rheumatoid arthritis patients. Most likely, such blockade directly inhibits the development of atherosclerosis. TNF-α also activates the transcription factor κ B (nuclear factor κ B – NFκB) which is involved at every stage of atherosclerosis progression, from the beginning to late complications (Lyon et al., 2003).

Up to now diagnostics of ischaemic heart disease have involved electrocardiographic-type exercise tests (treadmill or exercise bicycle). Recently, a new non-invasive method of diagnosing coronary disease has been introduced which involves assessment of the coronary artery calcification status (coronary artery calcium score – CACS) using a 64-slice computerized tomography approach. Sensitivity and specificity of CACS is similar to other non-invasive methods used so far (Oudkerk et al., 2008; Javadrashid et al., 2010). It is a valuable tool allowing assessment of atherosclerosis progression and choice of suitable action, especially in the group of asymptomatic patients (Sharma et al., 2010; Fernandez-Friera et al., 2011).

The goal of this study was to assess expression of the TNFA gene and its receptors (TNFR1 and TNFR2) in mononuclear cells isolated from peripheral blood collected from patients with subclinical atherosclerosis that had been confirmed by positive CACS in 64-slice computerized tomography test, as well as analysis of CACS in the context of the occurrence of classical risk factors for cardiovascular diseases in these patients.

Material and Methods

The study involved 47 patients admitted to the Cardiology Clinic with suspected ACS or stable angina. Within this group 23 patients had positive CACS in 64-lead CT (CACS > 0 Agatston units), whereas the remaining 24 patients had normal CACS (0 Agatston units). The latter 24-member group with correct picture of coronary vessels, in both coronaryography and 64-lead CT (CACS = 0), served as control.

Study inclusion criteria were as follows:

- Patients with CCS II-IV angina class and/or positive result of ECG stress test
- No lesions narrowing coronary arteries, as revealed by coronaryography
- Voluntary and conscious consent
- Study exclusion criteria were as follows:
  - Non-heart-related causes of chest pain
  - Chronic inflammatory diseases
  - Lack of consent

Each individual qualified into the experimental group underwent interview, physical examination and stationary 12-lead ECG, transthoracic echocardiogram, as well as laboratory tests (blood morphology, ESR, lipidogram, serum concentration of electrolytes, creatinine and glucose). Additionally, patients with suspected ACS had serum concentration of myocardial necrosis markers determined (troponin I and creatine kinase CK-MB isoenzyme). The whole 47-member group was qualified to participate in the QRT-PCR-based molecular study. Study material was isolated using mononuclear cells from peripheral blood collected during the first 24 h of hospitalization. Using Ficol Paque-Plus (GE Healthcare, formerly Amersham Biosciences, Bath, UK) mononuclear cells were separated from the collected blood samples and ultimately RNA was isolated from these cells using a modified Chomczynski-Sacchi method.

Assessment of transcriptional activity of the TNFA gene and receptors TNFR1 and TNFR2 was carried out using a commercial kit (Qiagen, Hilden, Germany) including QuantiTect SYBR Green RT-PCR Master Mix; reaction mix conditions were as recommended by the manufacturer: 50 μl reaction mix volume containing 25 μl 2× QuantiTect SYBR Green RT-PCR Master Mix (DNA HotStarTaq polymerase, QuantiTect SYBR Green RT-PCR buffer (Tris-Cl, KCl, (NH₄)₂SO₄, 5 mM MgCl₂, pH = 8.7)), dNTP mix, SYBR Green I intercalating dye, ROX reference dye, 5 μl F and R starters (10 μM each), 5 μl template and 10 μl pyrogen-free water. QRT-PCR control for all investigated samples was the commercially available β-actin gene. Reverse transcription reaction was carried out as follows: 30 min at 50 °C, 15 min at 95 °C and 45 cycles (15 s at 94 °C and 30 s at 60 °C). Final elongation of amplification products was carried out for 10 min at 72 °C. Together with the genes investigated, commercially available DNA standards of β-actin gene were amplified (Applied Biosystems, Carlsbad, CA).

Statistical analysis

The QRT-PCR-determined number of mRNA copies per 1 μg of total RNA was used as the measure of TNFA, TNFR1, TNFR2, β-actin and GAPDH gene expression.
Mann-Whitney U test served to statistically analyse the obtained results. They were judged statistically significant when $P < 0.05$. Correlation between the values of $TNF\alpha$, $TNFRI$ and $TNFR2$ gene expression and CACS for the investigated group was verified by calculating Spearman’s $r$ correlation coefficient.

Results

I. Clinical characteristics of the study group

The whole study group (N = 47) included 23 subjects (experimental) with subclinical atherosclerosis of coronary vessels (CACS > 0 Agatston units) and 24 subjects (control) with normal picture of coronary vessels in coronaryography and 64-lead CT (CACS = 0). The experimental group included 8 women (34.8 %) and 15 men (65.2 %) aged 44–78 (average 60.4 ± 9.8), whereas the control group consisted of 14 women (58.3 %) and 10 men (41.7 %) aged 42–77 (average 55.0 ± 9.2). The study group in terms of CACS values is shown in Fig. 1.

II. Molecular characteristics of the experimental group

The results of molecular studies performed on RNA samples generated from patients with subclinical atherosclerosis of coronary arteries have revealed a significantly lower number of copies for both $TNF\alpha$ receptor genes ($TNFRI$ and $TNFR2$), as compared to the number of copies for these genes in the control group. No statistically significant differences between the two groups could be demonstrated for gene copy numbers of the $TNF\alpha$ itself. The results are shown in Fig. 2.

III. Characteristics of the experimental group in terms of expression of the investigated genes and CACS

No correlation was found for the experimental group between gene expression of $TNF\alpha$, its receptors $TNFRI$ and $TNFR2$ on the one hand, and CACS on the other (Fig. 3).

IV. Characteristics of the studied group in terms of the occurrence of classical risk factors for cardiovascular diseases

Analysis of the occurrence of classical risk factors for cardiovascular disease in the studied group has shown a significant effect of arterial hypertension on the progression of calcification processes in coronary arteries.

Additionally, it was demonstrated that the increased number of risk factors in operation correlated with the increased value of CACS. The occurrence of classical risk factors for cardiovascular diseases in the studied group and relationship to CACS is shown in Fig. 4.

Discussion

Frequent occurrence and high costs of treating cardiovascular diseases both prompt continued effort in searching novel, increasingly sensitive and reproducible methods of detecting these diseases at early stages of atherosclerosis progression.

Three independent studies involving use of animals devoid of $TNF\alpha$ family genes have clearly demonstrated that $TNF\alpha$ is of great significance for the development of atherosclerosis.

![CACS values in the study group](image1.png)

![Gene expression and CACS](image2.png)

![Correlations between gene expression](image3.png)
of atherosclerosis, its early stages included (Branen et al., 2004; Boesten et al., 2005; Ohta et al., 2005). TNF-α exerts control over atherosclerotic plaque formation through activation of transmembrane receptors TNFR1 and TNFR2.

The proatherosclerotic signalling pathway appears to be largely understood, although Schreyer et al. (1996) have reported intensified atherosclerosis in animals devoid of TNFR1, as compared with wild-type mice. Similar data were obtained by Blessing et al. (2004). These data suggest that TNFR1 receptors found within macrophages can inhibit atherosclerosis, whereas those located in the vessel wall (in endothelial cells or in smooth muscle cells) can stimulate it. TNFRI expression in the blood vessel wall significantly affects aggregation and adhesion of cells, which may contribute to atherosclerosis development in both early and late stages (Mackay et al., 1993).

The results of our own molecular studies carried out in a group of patients presenting subclinical coronary artery atherosclerosis show significantly lower copy numbers of genes encoding TNFR1 and TNFR2 (TNF-α receptors), as compared with such copy numbers in the control group. The material used for molecular studies was RNA isolated from peripheral blood mononuclear cells, and the number of gene copies found could pertain to receptors localized within macrophages.

It was also reported that blockage of both receptors (TNFR1 and TNFR2) results in decreased tissue inflammation and lowered synthesis of TNF-α-dependent pro-inflammatory factors such as interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), or monocyte chemotactic protein (MCP 1). Such blockage also down-regulates production of metalloproteinases (MMPs), which are involved, to a substantial degree, in the process of atherosclerotic plaque destabilization (Aikawa et al., 2001; Catrina et al., 2002). Several reports have corroborated substantial contribution of classical risk factors (including arterial hypertension and diabetes) to atherosclerosis development, especially at its early stages (Bartoli et al., 2010; Papafaklis et al., 2010).

Our study of subclinical atherosclerosis patients has demonstrated calcification of coronary arteries (using 64-slice CT). Also, significant impact of arterial hypertension was observed on the progression of coronary calcifications.

The pathomechanisms of arterial hypertension development are closely related. Long-term elevation of blood pressure causes mechanical damage to blood vessels’ endothelium. Cunningham and Gotlieb (2005) have reported that disturbances in shear stress (due to non-laminar blood flow which is characteristic of arterial hypertension) in consequence activate the inflammatory processes enhancing atherosclerosis. Additionally, arterial hypertension is accompanied by elevated activity of the renin-angiotensin-aldosterone (RAA) system. Acting via several factors, angiotensin II inhibits production of nitric oxide synthase, thus impairing vasodilatory functions. It also affects migration and proliferation of smooth muscle cells, stimulating them to synthesize collagen and causing loss of vessel elasticity. The angiotensin-converting enzyme degrades bradykinin, thus eliminating its favourable effect on the endothelium.

Our results demonstrate a significant impact of hypertension on atherosclerosis development. The results also show an increase in CACS that accompanies a higher number of cardiovascular disease classical risk factors involved. A study by Ketonen et al. (2005) has shown that one of the major risk factors leading to the development of atherosclerotic plaque is hypertension.

Studies by Weiss and Taylor (2008) carried out on an animal model have confirmed a substantial impact of hypertension on the development of atherosclerosis. Coutinho et al. (1999), who investigated the relationship between serum glucose concentration and the occurrence of cardiovascular incidents, have stressed, in turn, the role of diabetes in atherosclerosis development.

Merry et al. have shown the importance of the remaining classical risk factors (lifestyle, tobacco smoking, alcohol consumption, burdening family history, physical activity) to the development of coronary heart diseases (CHD) and the occurrence of complications such as ACSs (Merry et al., 2011).

During subsequent stages of atherosclerosis the plaque becomes calcified. CACS, which is the indicator of coronary arteries’ calcification, allows fast non-invasive assessment of the state of these arteries (Meij et al., 2009). Brown et al. (2008) have reported that CACS becomes elevated with the occurrence of atherosclerosis classical risk factors such as hypertension, diabetes and obesity. Similar results, based on CACS values and Framingham risk scores, were obtained by Greenland et al. (2004), who investigated the risk of coronary disease in asymptomatic subjects.

It is worth stressing that numerous reports do consider CACS as important for identifying persons at low risk, as well as asymptomatic individuals at risk for the occurrence of acute coronary syndrome (Arad et al., 2005). Analysis of correlation between gene expression values for TNFα and its receptors (TNFR1 and TNFR2), on the one hand, and CACS on the other, did not reveal...
any relationship between these parameters, which probably reflects very early stage of atherosclerosis among patients forming the studied group.

The rising number of investigations targeting the TNF-α family members makes it likely in the future to interfere with its signalling pathway and introduce tar-
currence of life-threatening complications.

Conclusions

1. The occurrence of arterial hypertension favours initiation and progression of atherosclerotic lesions in blood vessels, including the coronary ones; the occurrence of an increased number of classical risk factors for cardiovascular disease correlates with enhanced calcification of coronary arteries as demonstrated by results of 64-slice CT.

2. The significantly lower copy numbers of genes encoding receptors (TNFR1 and TNFR2) of tumour necrosis factor α (TNF-α), observed in the experimental group of patients, can play a significant role in the initiation as well as further progression of atherosclerosis.

3. The lack of statistically significant correlation between the gene expression level of TNFα and its receptors TNFR1 and TNFR2 on the one hand, and the degree of coronary arteries’ calcification status (as revealed by CACS) on the other, may be the result of very early stages of atherosclerosis and relatively small size of the investigated group of patients.

Acknowledgements

The research project entitled “Oligonucleotide micro-
array study of proinflammatory genes expression profile in mononuclear peripheral blood cells and right atrium cells obtained from atherosclerotic and ACS (unstable coronary disease, myocardial infarction) patients” and which was designed according to Declaration of Helsinki-based GCP guidelines was positively endorsed by the Bioethics Commission of the Medical University of Silesia in Katowice on December 21, 2004 (resolution NN-6501-223/1/04).

The experimental part of this study was performed at the laboratories of the Department of Molecular Biology, the Silesian Medical University.

No conflicts of interest.

References


