INTRODUCTION

Atherosclerosis is an inflammatory-forming process of the vascular wall. The participation of immune system cells and specific antibodies in atherogenesis has been widely described. Antibodies directed against heat shock proteins (HSP) are associated with atherosclerosis development (1, 2), as well as the occurrence of complications in the coronary circulation (3, 4) of vessels supplying the brain (5) and lower limb arteries (6, 7). Diabetes particularly predisposes to the development of atherosclerosis. However, the role of anti-HSP 60/65 antibodies in the development of diabetic macroangiopathy has not been extensively investigated.

Heat shock proteins constitute a phylogenetically old cellular protection system. Their expression in the cell increases as a result of factors that are potentially harmful to cells, for example, under the influence of elevated temperature. HSP do not only protect proteins from denaturation, they also have immune functions. This feature determines the high conservatism of the amino acid structure of these proteins, which indicates that the proteins of different organisms are similar. Amino acid sequence homology for proteins from the HSP 60 group reaches >70% between prokaryotic organisms and humans (8), which enables the possibility of cross-reactive immune response to bacterial antigens with antigenic HSP of human cells subjected to stress factors. This phenomenon, known as antigenic mimicry, was suggested by Iruna Cohen in 1990 (9) and Wick et al. (10) suggested the possibility of cross-reactivity between exogenous material (mHSP bacterial proteins -65 mycobacteria or GroEL and GroES Escherichia coli) and human HSP counterparts exposed by the endothelium subjected to stress factors, such as increased shear stress at vessel bifurcations, hypertension, and nicotine (10). Perschinski et al. (8) also confirmed the ability of antibodies directed against bacterial homologues of HSP 60/65 to recognize specific epitopes of human HSP 60 molecules. Mayr et al. experimentally demonstrated the cytotoxicity of such antibodies in respect to endothelial cells (11).

We analyzed a group of patients with type 2 diabetes complicated by peripheral macroangiopathy and chronic lower limb ischemia. Our aim was to determine the role of heat shock proteins in atherogenesis by measuring the levels of anti-HSP 60/65 antibodies in the serum of patients with diabetic macroangiopathy. The data were compared with a healthy control group.

MATERIALS AND METHODS

Patients

The Ethics Committee of the Wroclaw Medical University approved the study. Participants were informed of the aims of the study and gave their written consent.
The study included patients with type 2 diabetes complicated with macroangiopathy, treated at the Department of Angiology, Hypertension and Diabetology, Wroclaw Medical University. The group consisted of 30 patients with type 2 diabetes with impaired peripheral circulation; 10 women and 20 men, aged 48 to 60 years (mean age 54.73±3.35), with symptoms of intermittent claudication during walking less than 200 m, without necrotic lesions or rest pain (stage II B according to Fontaine). Ischemia was defined as a deficit in peripheral circulation with a reduction in the Ankle Brachial Index (ABI) <0.85 in segmental blood pressure measurement, in at least one lower limb. Diabetes was diagnosed according to the criteria of the Polish Diabetes Association guidelines (12). All patients were taking insulin or oral hypoglycemic preparations. Mean HbA1c was 8.1±1.9%.

Exclusion criteria were: persons who had symptoms of infection or inflammation in the last 3 months, or acute coronary syndrome in the last 6 months. We also excluded patients taking nonsteroidal anti-inflammatory drugs (except acetylsalicylic acid in a dose up to 325 mg/24 hours), patients diagnosed with cancer, liver failure or another serious concomitant disease.

The control group (n=18) consisted of healthy volunteers (9 women and 9 men), aged from 40 to 59 years (mean age 50.38±5.29), appropriately matched for sex and age to the studied group of patients.

Methods

Blood pressure was measured as the mean of three consecutive measurements at five-minute intervals, in the sitting position. In accordance with the recommendations made in the JNC VII report, hypertension was diagnosed in case of values above 140/90 mm Hg or protracted use of hypotensive drugs.

In the morning, venous blood was sampled from the elbow to assess: peripheral blood count using the 16-parameter ABX Mikros OT hematology analyzer, lipid metabolism determined by enzymatic method using the Analco Medical Trade kit, and concentrations of creatinine, urea, uric acid, and fibrinogen using the Behring Coagulation Timer. In addition, we measured HbA1c using the Unimate kit (Roche Diagnostics) in patients with a family history of diabetes.

In order to determine the concentrations of anti-HSP 60/65 antibodies, von Willebrand factor (vWF) and hsCRP in serum, blood was collected in the morning from the median cubital vein. Up to 30 minutes after collection, it was centrifuged at a temperature below -40°C until the determinations were performed. The determinations were made using: 1) Stressgen’s anti-human HSP 60/65 antibodies, 2) Diagnostica anti-human HSP 60 (total) ELISA kit (catalog No. EKS-650), concentration values were expressed in ng/ml; 3) high sensitivity C-reactive protein enzyme immunoassay test kits (catalog No. EIA-3954) from DRG International, Inc., USA, concentration values were expressed in mg/l.

Claudication distance was determined in a treadmill test on a Track Master at a constant speed of 3.2 k/h and slope of 12°.

Static effort based on isometric contraction lasting until full fatigue was assessed using the Microfet 2 manual muscle tester. It is a device that enables measurement in two ranges for small and large muscle groups. In this case, the assessment included quadriceps, triceps and tibialis anterior muscles. The measured values are given in Newtons and pounds (1 pound =4.448 N). Both lower limbs were evaluated.

Statistical analysis

The collected material was analyzed statistically in order to obtain descriptive statistics such as arithmetic mean, standard deviation, median, correlation coefficient, and linear regression analysis. For all tests, a level of p<0.05 was considered statistically significant. Because of the nonparametric distribution of the analyzed parameters in the groups, the Mann-Whitney test was used. Spearman’s rank correlation was used to verify correlation between the parameters. The correlational strength was expressed in the form of a correlation coefficient (denoted R). A positive value indicates a positive relationship, a negative value a negative relationship. All calculations were performed using the STATISTICA 7.1 software.

RESULTS

The characteristics of selected clinical parameters such as sex, age, smoking, presence of comorbidities such as hypertension, and weight are presented in Table 1. The analysis of laboratory parameters is illustrated in Table 2 and 3.

Smoking was common in both groups, but was significantly higher in patients with diabetes and peripheral ischemia than in the control group. Smoking is a strong risk factor for atherosclerosis, especially in the lower limbs, the increased frequency of this habit among diabetics could contribute to the enhanced presence of atherosclerosis in the patients. Most of this group had hypertension, another risk factor for atherosclerosis.

In the studied group of patients, we found statistically significantly higher ESR, WBC, fibrinogen, and creatinine values; however, the values remained within the normal range.

Table 1. Selected clinical parameters of the analyzed groups of patients.

<table>
<thead>
<tr>
<th>n</th>
<th>type 2 diabetes</th>
<th>control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>30</td>
<td>18</td>
</tr>
<tr>
<td>women/men</td>
<td>10/20</td>
<td>9/9</td>
</tr>
<tr>
<td>age [years]</td>
<td>54.73±3.55</td>
<td>50.38±5.29</td>
</tr>
<tr>
<td>smoking [%]</td>
<td>96%</td>
<td>58%</td>
</tr>
<tr>
<td>hypertension [%]</td>
<td>90%</td>
<td>-</td>
</tr>
<tr>
<td>BMI [kg/m²]</td>
<td>28.81±3.95</td>
<td>26.86±4.86</td>
</tr>
</tbody>
</table>

Table 2. Selected laboratory parameters of the investigated groups. The results are expressed as mean ±standard deviation (NS - not significant).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>diabetes</th>
<th>control group</th>
<th>significance of type 2 differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESR [mm/h]</td>
<td>17.43±13.76</td>
<td>8.22±5.61</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>WBC [10³/µl]</td>
<td>7.56±2.13</td>
<td>5.92±1.25</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>fibrinogen [g/l]</td>
<td>3.68±1.03</td>
<td>3.05±0.78</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>creatinine [mg/dl]</td>
<td>1.09±0.28</td>
<td>0.93±0.10</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>urea [mmol/l]</td>
<td>5.96±2.22</td>
<td>5.15±1.53</td>
<td>NS</td>
</tr>
</tbody>
</table>
except for ESR. This seems to result from the activation of inflammatory processes accompanying diabetes. In fact we concluded from the study persons who had symptoms of infection or inflammation in the last 3 months, or acute coronary syndrome in the last 6 months. We also excluded patients taking nonsteroidal anti-inflammatory drugs (except acetylsalicylic acid in a dose up to 325 mg/24 h), patients diagnosed with cancer, liver failure or another serious concomitant disease. The vWF and hsCPR results we obtained also seem to confirm this.

Table 4 shows the ratio and percentage of drugs used in the studied group of patients. Diabetic patients were characterized by a higher BMI and a more frequent co-occurrence of hypertension. The group of patients with diabetes had leukocytosis and higher triglyceride levels and lower HDL cholesterol levels than the control group. Patients with symptoms of lower limb ischemia with concomitant diabetes were characterized by higher concentrations of uric acid.

Patients with lower limb ischemia in diabetic macroangiopathy had higher concentrations of anti-HSP 60/65 antibodies than the control group, but the difference was not statistically significant (44.77±55.00 vs. 26.09±13.85, NS). We found a positive correlation between anti-HSP 60/65 antibodies and von Willebrand factor levels in blood serum (R=0.543, p<0.05) (Fig. 1).

Patients with lower limb ischemia in diabetic macroangiopathy had significantly higher levels of vWF than the control group (145.34±42.12 vs. 97.28±19.85; p<0.05) (Table 4). Patients with diabetic macroangiopathy had higher serum hsCRP concentrations than healthy volunteers in the control group (7.17±6.49 vs. 2.59±3.34, p<0.005). We did not find a significant correlation between hsCRP and the other analyzed parameters in the studied groups of patients.

The broad spectrum of the atherosclerosis process, which affects many areas of life, was reflected in the undertaken physical activity. So, we assessed the maximal lower limb muscle strength during isometric contraction in all the studied groups. We found significantly lower muscle strength values in the group with macroangiopathy. The large disparity in the results between the group of patients and the control indicates the enormous impact of the ongoing disease process and its stage on the muscle strength of lower limbs. The results pertaining to the strength of the quadriceps muscle of the thigh (21.47 vs. 27.82

![Fig. 1. Correlation of level of anti-HSP 60/65 antibodies with vWF concentration in the serum of the group of patients with chronic lower limb ischemia in diabetic macroangiopathy.](image-url)
between coronary heart disease and myocardial infarction and the was published in 2006 (19). The authors proved a relationship pathogenesis of coronary heart disease in patients with diabetes were not analyzed as a separate group.

Although the patients in the cited studies also included people macroangiopathy had not yet been extensively investigated.

The first report regarding the participation of HSP 60/65 in the pathogenesis of coronary heart disease in patients with diabetes was published in 2006 (19). The authors proved a relationship between coronary heart disease and myocardial infarction and the presence of HSP 60/65 in the serum of patients with diabetes. The role of anti-HSP 60/65 immunoglobulin in atherogenesis was confirmed in another study conducted on animals, where mice intravascularly received anti-HSP 60 antibodies isolated from the serum of patients with coronary heart disease (20).

The present results showed the presence of anti-HSP60/65 antibodies in the serum of patients with diabetes and lower limb ischemia in higher concentrations than in the control group of healthy volunteers, but the difference was not statistically significant. The lack of significance may be due to the presence of additional unanalyzed factors in the study, such as the type of treatment regiment used. The patients received oral antidiabetic drugs, almost half of the cases received insulin (alone or in combination with oral drugs). Chen HS et al. (21) suggested the possibility of insulin modulating the expression of HSP 60 in cardiac muscle cells. The present study group was too small and not enough heterogeneous (duration of diabetes, type of treatment) to assess the impact of antidiabetic therapy on changes in the levels of anti-HSP 60/65 antibodies. The metabolic factors affecting the obtained results of anti-HSP 60/65 antibody levels should include: lipid metabolism disorders (22), BMI (23), as well as other parameters, such as the amount of antioxidants and fats in the diet (24). Past acute coronary syndromes significantly affect the serum levels of the investigated antibodies (25).

The lack of statistical significance in the concentrations of HSP 60/65 in the analyzed groups may also result from smoking, common in the analyzed group. Studies conducted among patients with hypertension (26) demonstrated an interesting, not entirely explained observation. Although smoking is recognized as a one of the better-documented risk factors for atherosclerosis in patients with confirmed carotid atherosclerosis and hypertension, HSP 65 antibodies were found in lower titers than in normotensive smoking subjects free of hypertension. When comparing similar groups of non-smokers, there was no such relationship. The authors suggest that the observed phenomenon may be based on the endothelial cells' reduced ability to respond to adverse conditions. While in the physiological environment the expression of HSP molecules on the cell surface occurs after the

Table 5. Level of vWF in peripheral blood of the studied groups.

<table>
<thead>
<tr>
<th>parameter</th>
<th>Type 2 diabetes</th>
<th>control group</th>
<th>significance of differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWF [%]</td>
<td>145.34±42.12</td>
<td>97.28±19.85</td>
<td>p&lt;0.00005</td>
</tr>
<tr>
<td>anti-HSP 60/65 [ng/ml]</td>
<td>44.77±55.00</td>
<td>26.09±13.85</td>
<td>NS</td>
</tr>
<tr>
<td>hsCRP [mg/l]</td>
<td>7.17±6.49</td>
<td>2.59±3.34</td>
<td>p&lt;0.005</td>
</tr>
</tbody>
</table>

Table 6. Mean values of muscle strength in the groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Skeletal</th>
<th>Mean (pound)</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 2 diabetes</td>
<td>right thigh quadriceps muscle</td>
<td>21.47</td>
<td>5.80</td>
</tr>
<tr>
<td></td>
<td>left thigh quadriceps muscle</td>
<td>20.27</td>
<td>5.17</td>
</tr>
<tr>
<td></td>
<td>right calf triceps muscle</td>
<td>19.86</td>
<td>3.88</td>
</tr>
<tr>
<td></td>
<td>left calf triceps muscle</td>
<td>19.02</td>
<td>3.63</td>
</tr>
<tr>
<td></td>
<td>right tibialis anterior muscle</td>
<td>14.77</td>
<td>5.20</td>
</tr>
<tr>
<td></td>
<td>left tibialis anterior muscle</td>
<td>14.21</td>
<td>4.42</td>
</tr>
<tr>
<td>Control group</td>
<td>right thigh quadriceps muscle</td>
<td>27.82</td>
<td>6.70</td>
</tr>
<tr>
<td></td>
<td>left thigh quadriceps muscle</td>
<td>28.33</td>
<td>7.86</td>
</tr>
<tr>
<td></td>
<td>right calf triceps muscle</td>
<td>22.23</td>
<td>4.99</td>
</tr>
<tr>
<td></td>
<td>left calf triceps muscle</td>
<td>22.35</td>
<td>5.34</td>
</tr>
<tr>
<td></td>
<td>right tibialis anterior muscle</td>
<td>18.45</td>
<td>5.20</td>
</tr>
<tr>
<td></td>
<td>left tibialis anterior muscle</td>
<td>17.28</td>
<td>4.33</td>
</tr>
</tbody>
</table>

DISCUSSION

The participation of HSP in atherogenesis was confirmed in the experiment conducted in an animal model and described by Xu et al. in 1992 (13). The results of epidemiological studies conducted among 867 residents of South Tyrol (14) comparing the titers of anti-HSP 65 antibodies with presence of atherosclerosis in carotid arteries, assessed by Doppler ultrasound of the area, confirmed a relationship between the investigated proteins and atherogenesis in humans. Subsequent studies demonstrated a relationship between high titers of anti-HSP 65 antibodies and the occurrence of apoplexy (15). A similar relationship was confirmed for lower extremity atherosclerosis (16). Zhu et al. (17) collected the vastest research material on the participation of anti-HSP 60/65 antibodies in the development of coronary atherosclerosis. The authors also demonstrated a correlation between increasing titers of the investigated antibodies and the number of coronary arteries affected by atherosclerosis. In another study, the same group of authors showed a correlation between the presence of anti-HSP 65 antibodies and the degree of coronary calcification, a state which corresponds to pre-clinical coronary heart disease (18).

Diabetes promotes atherogenesis in a specific way, however the role of heat shock proteins in the development of diabetic macroangiopathy had not yet been extensively investigated. Although the patients in the cited studies also included people with diabetes (5), the disease was treated merely as an additional strong risk factor for atherosclerosis, and patients with diabetes were not analyzed as a separate group.

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action of the damaging agent, in smokers this process is inhibited. Reduction of heat shock protein expression would result in greater susceptibility to endothelial cell damage. The information available on this topic is scarce and requires further research and analysis. So far, we do not know whether cigarette smoke contains substances responsible for the inhibition of an important system for the protection of cells against stress factors.

Experiments in an animal model found that intravascular administration of mycobacterial antigen mHSP 65 promotes the development of atherosclerosis (27), and Maron et al. (28) confirmed the role of HSP 60/65 in atherogenesis and suggested the possibility of control of the progression of atherosclerosis through the induction of immune tolerance to these proteins. The potential proatherosclerotic properties of heat shock proteins are still to be found. Among the proposed mechanisms, stimulation of the immune response to produce anti-HSP 60/65 has been considered. Autologous heat shock protein may become an immunogenic antigen in the course of structural changes and potential modifications as a result of oxidative stress or metabolic disorders (29), forming complexes with other foreign antigens (29).

Some authors believe that intracellular protein - when found in the intercellular space - sHSP (soluble HSP) may not be recognized by the immune system as its own (30). A genetic basis for increased expression of anti-HSP 60/65 antibodies has also been suggested. Evidence of this is the dependence of the concentration of these antibodies on the polymorphism of the IL-6 gene (31).

Patients with symptoms of peripheral macroangiopathy had higher von Willebrand factor concentrations in serum. Von Willebrand factor is a glycoprotein produced by the endothelium and thrombocytes involved in adhesion and aggregation processes. Increased vWF level is a recognized marker of endothelial injury in the course of vascular diseases. Elevated levels of vWF are observed in diabetes, and have been related to cardiovascular risk (32). Several cytokines such as TNF-α, IL-6 are considered to stimulate vWF expression and may be related to inflammation associated with diabetes. The association between CRP and vWF has been demonstrated.

Authors of present study did not find any correlation with hsCRP. hsCRP demonstrated not as inflammatory factor but as inflammatory marker with atherogenesis. Therefore, it is an important predictor of cardiovascular diseases, particularly coronary heart disease and acute coronary syndromes. According to some authors, CRP is associated with impairment of endothelial function expressed as impaired vasorelaxation (33). In the analyzed groups of patients, we observed comparable increases in hsCRP; however, we did not find a significant correlation between CRP levels and the other evaluated parameters. In this study, high values of vWF were in significant correlation with the levels of anti-HSP 60/65 antibodies. A similar relationship was demonstrated in an experiment conducted on rabbits (34). According to our knowledge, this is the first report of correlation between vWF and anti-HSP60/65 antibodies in humans.

Recent years of research point to the tremendous value and transferability of the results of experimental studies in animals to humans. Jawien et al. (35, 36) focus on the process of translation of the performed experiments, as well as their importance in the process of atherogenesis.

The importance of HSP-60/65 in the process of atherogenesis is only one of the areas which should be searched to find predictors of atherosclerosis. It should be noted that the Smith’s et al. (37) study revealed a significant effect of ApoE (apolipoprotein E-null) on development of atherogenesis in females when compared to males. The researchers pointed that, there is only limited information on the regulation of gene expression and its effect on metabolic products. Korbut et al. (38) pay particular attention to 5-lipoxygenase (5-LO) as a factor modifying pathogenesis of atherosclerosis and the differences in 5-LO expression between the human body and mice. Pawlowska et al. (39) in turn, analyzed the inhibitory action of tetracycline on MMPs (matrix metalloproteinases) and their impact on the atherogenic process.

Patients with type 2 diabetes complicated with atherosclerosis are often forced to limit their daily physical activity. As time passes, this is reflected in decreased lower limb muscle strength. Our research confirms a statistically significant decline in muscle strength of the studied group compared with the control. Without a doubt, proper physical activity can prevent the development of vascular changes. Its effect on the body is multidirectional. It contributes to an advantageous modification of risk factors for cardiovascular diseases such as hypertension, obesity, diabetes and lipid disorders (40). Planned physical activity affects the strength and mass of skeletal muscle increasing their efficiency and activating adaptive mechanisms. It modifies lipid metabolism, thus reducing total cholesterol and triglyceride levels, which is important in patients suffering from type 2 diabetes.

In summary, patients with lower limb ischemia in diabetic macroangiopathy had significantly higher levels of vWF than the control group. High values of vWF significantly correlated with anti-HSP 60/65 antibodies suggesting their possible role in disease pathogenesis.

Conflict of interests: None declared.

REFERENCES


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