

WOJCIECH RZESZUTKO¹, TOMASZ KONOPKA², WACŁAW KOPEĆ³

Anti-Neutrophil Cytoplasmic Antibodies and Autoantibodies to Human Heat Shock Protein 60 in Periodontitis Patients

Autoprzeciwiała dla antygenów cytoplazmatycznych neutrofilii oraz ludzkiego białka szoku termicznego 60 u pacjentów z zapaleniem przyzębia

¹ Department of Pathological Anatomy, Silesian Piasts University of Medicine in Wrocław

² Department of Periodontology

³ Laboratory of Immunology, Department of Nephrology, Silesian Piasts University of Medicine in Wrocław, Poland

Abstract

Background. The autoimmune response may contribute to the pathogenesis of periodontitis. Changing levels of anti-neutrophil cytoplasmic autoantibodies (ANCA) are serologic markers for many infective and connective tissue diseases. Hsp 60 may also be an important factor in the initiation of an autoimmune response.

Objectives. Investigating the presence of ANCA (p-ANCA to myeloperoxidase and c-ANCA to proteinase 3) and antibodies to human heat shock protein 60 (Hsp 60) in the sera of 73 patients with periodontitis and 13 control subjects.

Material and Methods. The study group consisted of 73 patients aged 19–50 years: 31 with generalized aggressive periodontitis, 13 with localized aggressive periodontitis, and 29 with chronic periodontitis. The control group comprised 13 periodontally healthy subjects. The serum levels of p-ANCA, c-ANCA, and anti-Hsp 60 were detected by ELISA in each subject.

Results. Elevated levels of p-ANCA were demonstrated only in five patients with generalized aggressive periodontitis. Elevated levels of antibodies to Hsp 60 were found only in 12.9% of patients with generalized aggressive periodontitis, 7.6% of patients with localized aggressive periodontitis, and 13.7% of patients with chronic periodontitis.

Conclusions. There was no relationship between the significantly elevated levels of p-ANCA and antibodies to Hsp 60 and clinical measurements of periodontitis. These results do not support evidence for a primary role of autoimmunity in periodontal disease. It seems probable that the destruction of periodontal tissues may sometimes be the primary driving factor in the induction of the enhanced autoimmune response in periodontitis (*Adv Clin Exp Med* 2006, 15, 4, 599–605).

Key words: antibodies, anti-neutrophil cytoplasmic, heat shock protein 60, autoimmunity, periodontitis.

Streszczenie

Wprowadzenie. Odpowiedź autoodpornościowa może uczestniczyć w etiopatogenezie zapalenia przyzębia. Zmienne stężenia autoprzeciwciał dla cytoplazmy neutrofilii (ANCA) są markerami serologicznymi dla wielu chorób zapalnych i tkanki łącznej. Przeciwiała dla białka szoku termicznego 60 (HSP 60) mogą być również istotnym czynnikiem w inicjowaniu reakcji autoimmunologicznej.

Cel pracy. Zbadanie występowania ANCA (p-ANCA antymieloperoksydazie i c-ANCA antyproteinazie 3) oraz przeciwciał dla Hsp 60 w surowicy 73 pacjentów z zapaleniem przyzębia i u 13 osób z grupy kontrolnej.

Materiał i metody. Grupa badana składała się z 73 pacjentów w wieku 19–50 lat: 31 z uogólnionym agresywnym zapaleniem przyzębia, 13 z umiejscowionym agresywnym zapaleniem przyzębia i 29 z przewlekłym zapaleniem przyzębia. Grupa kontrolna liczyła 13 osób ze zdrowym klinicznie przyzęciem. Stężenia surowicze p-ANCA, c-ANCA i przeciwciał dla HSP 60 badano u każdego pacjenta metodą ELISA.

Wyniki. Podwyższone stężenia p-ANCA wykazano tylko u pięciu pacjentów z uogólnionym agresywnym zapaleniem przyzębia. Podwyższone stężenia przeciwciał dla HSP 60 stwierdzono tylko u 12,9% pacjentów z uogólnionym agresywnym zapaleniem przyzębia, 7,6% z umiejscowionym zapaleniem przyzębia i u 13% pacjentów z przewlekłym zapaleniem przyzębia.

Wnioski. Nie wykazano związku między istotnie podwyższonymi stężeniami p-NCA i przeciwciałami dla HSP 60 a wskaźnikami klinicznymi zapalenia przyzębia. Wyniki te nie potwierdzają pierwotnej roli procesów autoodpornościowych w zapaleniach przyzębia. Wydaje się, że zniszczenie tkanek przyzębia może być niekiedy czynnikiem wywołującym reakcję autoimmunologiczną w zapaleniu przyzębia (*Adv Clin Exp Med* 2006, 15, 4, 599–605).

Słowa kluczowe: przeciwciała, anty cytoplazmie neutrofilii, białko szoku termicznego 60, autoimmunizacja, zapalenie przyzębia.

Research on autoimmunity in periodontal disease was initiated by Fits et al. [1], who discovered autoantibodies to type I collagen in the sera of patients with adult periodontitis (AP) in 1986. In later experiments, the role of collagen-I was confirmed as the main autoantibody in periodontitis [2–7]. Higher levels of IgG and IgA anti-collagen-I antibodies were found in the inflamed gingival tissue than in serum, which was explained by the endogenous synthesis of these autoantibodies in the periods of gingival tissues damage [4] and that collagen type I-specific T-cell clones could be identified in the inflamed gingival tissue of periodontitis patients [6]. CD5⁺ B cells seem to be the main source of anti-collagen antibodies, and with the increase in inflamed gingival tissue a switch occurs from natural IgM to IgG and IgA [7]. Govze and Herzberg [8] reported the presence of antibodies of subclass IgG anti-desmosomal proteins (desmoplakin 1 and 3) and also glycoproteins (desmoglein 1, 2, and 3) in 90% of sera and in all analyzed samples of gingival crevicular fluid from patients with periodontitis. In view of these observations it was suggested that some of the already discovered autoantigens, for example collagen I and desmosomal proteins, and glycoproteins uncovered or drawn from inflamed gingival tissues, may initiate polyclonal activation of populations of B lymphocytes and the synthesis of antibodies typical to these fragments [3, 5–8]. These findings point to a secondary character of autoimmunity caused by the destruction of gingival tissues.

Other autoantibodies in the sera of patients with AP were found by Novo and Viera [9]. They discovered the presence of anti-neutrophil cytoplasmic antibodies (ANCA) directed against “a granular extract” of granulocytes in 75% of analyzed patients and, to a lesser degree, against lactoferrin. The increased levels of ANCA are a useful diagnostic serologic marker for vasculitis, glomerulonephritis, pulmonary renal syndrome, Wegener’s granulomatosis, and connective tissue diseases. In the course of these diseases, activation of neutrophils occurs, with the release of the contents of the granules (myeloperoxidase-MPO, proteinase 3-PR3, elastase, lactoferrin, cathepsin G, eosinophil peroxidase) which are antigenic [10]. The main neutrophil cytoplasmic antigens for ANCA are MPO (p-ANCA) and PR3 (c-ANCA).

Novo and Viera [9] suggested an important role of autoimmunity in the pathogenesis of periodontitis, manifesting itself in the form of anti-MPO-induced complement cell lysis.

The heat shock proteins (Hsp) have gained wide interest as potential autoantigens due to their high degree of amino-acid sequence identity in eukaryotic and prokaryotic cells [11–19]. This molecular mimicry may be particularly important for the initiation of an autoimmune response. Hsp 60 may be especially important in the causal relationship between microbial infections and autoimmunity [20]. In juvenile chronic arthritis, rheumatoid arthritis, and Crohn’s disease, a specific anti-Hsp antibody is presented on the cells of the host (endogenous Hsp) or on the infectious microorganisms (exogenous Hsp) [11, 13, 16]. Hsp are found in bacteria that are generally considered pathogenic to periodontium (*Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Treponema denticola*) [20–23]. They have also been identified in the basal cells of inflamed gingivae of periodontitis patients [24, 25]. The homology between the *A. actinomycetemcomitans* 64-kDa protein and human Hsp 60 is 48% [22]. It is possible that antibodies to the exogenous Hsp of periodontal pathogens may be cross-reacted and directed towards the endogenous Hsp of host cells.

The purpose of this study was to investigate the levels of p-ANCA, c-ANCA, and antibodies to human Hsp 60 in the sera of patients with periodontitis. This study was undertaken to evaluate the role of these autoantibodies in immune mechanisms in periodontitis.

Material and Methods

Experimental and Control Subjects

The study group comprised 73 patients aged 19–50 years (mean age: 35.1, male to female ratio: 26 : 47), referred to the Department of Periodontology, Silesian Piasts University of Medicine in Wrocław. All participants in the study signed an informed consent form. The patients were system-

ically healthy, with no signs of symptomatic infection. None of the patients had been taking any medication during the three months before the examination which could cause any changes in the microflora, immunological system, or inflammatory response. This study group consisted of 31 patients (aged 19–39 years, mean age: 28.3, 19 women) with generalized aggressive periodontitis (GAP), 13 patients (aged 19–29 years, mean age: 24.3, 10 women) with localized aggressive periodontitis (LAP), and 29 patients (aged 35–50 years, mean age: 40.7, 18 women) with chronic periodontitis (CP) (Table 1). The diagnoses were determined on the basis of clinical and radiographic criteria according to the International Workshop for the Classification of Periodontal Diseases and Conditions in 1999 [26]. The control group consisted of 13 periodontally healthy subjects aged 24 to 45 years (mean age: 32.3), eight of whom were women.

The clinical state of the periodontium was indicated by means of the Silness and Løe (1964) Plaque Index (PI), the Løe and Silness (1963) Gingival Index (GI), the Saxer et al. (1977) Papilla Bleeding Index (PBI), and the Sulcus Fluid Rate (SFR) according Løe and Holm-Pedersen (1965), and the pocket depth (PD) was measured using a periodontal probe from four surfaces of each tooth.

Sera

Five milliliters of blood were taken from the elbow vein. It was incubated at room temperature for 30 minutes to coagulate it, then it was centrifuged for 10 minutes at $1000 \times g$. Sera were stored at -70°C until used.

Measurement of ANCA

Levels of p-ANCA and c-ANCA were detected by means of enzyme-linked immunosorbent assay (ELISA) from Millenia Diagnostic Products

Table 1. Division of the examined groups according to diagnosis

Tabela 1. Podział badanych grup pod względem rozpoznania

Examined group (Grupa badana)	Control group (Grupa kontrolna)
31 patients with GAP	–
13 patients with LAP	–
29 patients with CP	–
Total: 73 patients	Total: 13 persons
Total: 86 subjects	

Corporation (Los Angeles, CA, USA) test sets. The investigation was performed according to the recommendations of the manufacturer of the antibody. The results of the test were expressed in units per milliliter (U/ml). The norm for p-ANCA is less than 8 U/ml and for c-ANCA less than 5 U/ml.

Measurement of Hsp 60 Antibodies

ELISA was also used to mark the antibody anti-heat shock protein 60 (Hsp 60, human recombinant, Stressgen Biotechnology Co., Victoria, Canada). The recombinant Hsp was used in the lipophilated form. According to the manufacturer it had a purity above 90% (SDS-PAGE). In the preliminary tests, the optimal concentration of protein on the polystyrene plates and the optimal dilution of the tested sera were determined in the sera of 12 patients with periodontitis. On the basis of these tests, 5 μg of heat shock proteins and a dilution of 1 : 5 of the tested sera were considered as the optimal concentration and dilution in the ELISA test, respectively.

Experimental Procedures in the ELISA Test

Ninety-six polystyrene plates were used (Nunc-Immuno Plate, Poly Sorp). Each of the plates was filled with 100 μl phosphate buffer, pH 7.5, each containing 5 μg of heat shock protein. The plates were then incubated overnight at 4°C , then washed three times using phosphate-buffered saline (PBS) to which 0.05% Tween 20 was added. The buffer was also used to wash the plates in the successive steps of the ELISA test. The test sera were diluted with 1 : 5 PBS containing 1% bovine serum albumin and 0.05% Tween 20.

One hundred μl of the test sera was added to each of the plates and incubated for two hours at 37°C , after which they were washed three times with PBS. To each of the plates was added 100 μl of rabbit antibody anti-human IgG-alkaline phosphatase (DAKO, A/S, Glostrup, Denmark; a 1 : 500 dilution in PBS with 0.05% Tween 20) and it was incubated for 2 hours at 37°C . The plates were then washed three times with PBS and 100 μl of substrate (p-nitrophenyl phosphate, Sigma Chemical Co., St. Louis, USA; diluted with 1 M diethanolamine buffer, pH 9.8, containing 0.05 mM magnesium chloride) was added to each plate. The plates were incubated for 1 hour at 37°C , after which the absorbance was measured at 450 nm using an automated Stat Fax 2100 reader.

Determination of the Specificity of Anti-Hsp 60 Antibodies

Test sera taken from four patients with periodontitis, which was highly reactive with Hsp 60 in ELISA (high absorbance), were incubated with Hsp60 (80 µg Hsp 60 + 120 µl patient sera, 120 µg Hsp 60 + 120 µl patient sera) for one night at 4°C, after which ELISA of Hsp 60 antigen was repeated. The braking percent in the ELISA test was calculated from the difference in the optical densities of the serum before and after incubation with antigen Hsp 60.

Statistical Analysis

Serum antibody levels between two groups and means of the clinical measurement were evaluated using the Mann-Whitney test. A *p*-value of < 0.05 was considered to indicate a statistically significant difference.

Results

Of the 73 patients with periodontitis, a significant level of p-ANCA was observed only in 5 subjects (6.8%). These were exclusively patients with GAP (significant antibody levels of anti-MPO neutrophils were found in 16.1% of the tested patients with GAP). It was observed that the clinical state of the periodontium in the GAP group, where the level of the p-ANCA antibody was higher than 8 U/ml, was not worse compared with patients with GAP in whom the level of this antibody was not of a notable value (Table 2). A significant level of p-ANCA in the blood sera was not noted in any of the subjects of the control group. In the patients with periodontitis, the level of c-ANCA was not higher than the noted values.

The presence of anti-HSP 60 antibody higher

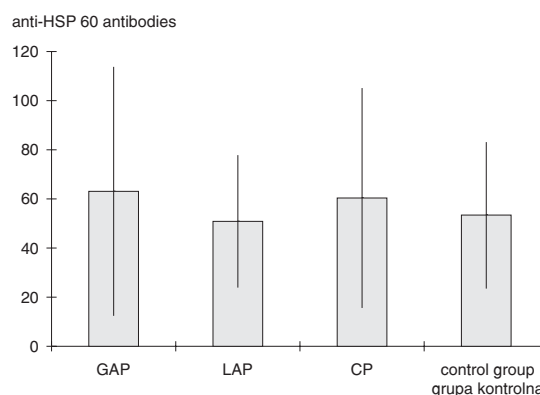


Fig. 1. Levels of antibodies to human Hsp 60.

Antibody levels are expressed as absorbance values for each group. There were no significant differences in the levels of antibodies to Hsp 60 between periodontitis patients and control group (*p* > 0.05) in Mann-Whitney test

Ryc. 1. Stężenie przeciwciała dla ludzkiego Hsp 60

than 2 *SD* above the mean control value was shown in 12.9% of the patients with GAP (4 patients), 7.6% with LAP (1 patient), and 13.7% with CP (4 patients). The levels of anti-HSP 60 antibodies in patients with periodontitis and in the control group are shown in Fig. 1. No significant difference was found in the levels of these antibodies between the analyzed groups. It was not established whether the clinical state of the periodontal tissues in patients with anti-HSP 60 antibodies levels more than 2 *SD* above the mean control value differed greatly from patients in whom the level of this antibody was less than this limit (Table 3).

Discussion

Described results reveal a higher level than the established norms for p-ANCA in only 5 of the 73 patients using commercially available tests for

Table 2. Means of the clinical measurements in GAP patients with blood serum p-ANCA levels above and below 8 U/ml

Tabela 2. Średnie wskaźników klinicznych u pacjentów chorych na uogólnione agresywne zapalenie przyzębia, u których stężenie p-ANCA w surowicy krwi > i < 8 U/ml

Clinical measurement (Pomiar kliniczny)	ANCA > 8 U/ml (n = 5)	ANCA < 8 U/ml (n = 26)	Z value in Mann-Whitney test (Wartości Z w teście Manna-Whitneya)	<i>p</i>
PI	1.11 ± 0.53	0.98 ± 0.45	0.8	0.42
GI	2.06 ± 0.72	1.82 ± 0.64	0.91	0.36
PBI	2.38 ± 1.09	2.21 ± 0.96	0.37	0.7
SFR (mm)	5.8 ± 0.75	6.2 ± 2.65	0.18	0.85
PD (mm)	5.19 ± 1.12	4.44 ± 1.16	1.5	0.13

Table 3. Means of the clinical measurements in periodontitis patients with blood serum levels of antibodies to Hsp 60 above and below the mean control value + 2 SD**Tabela 3.** Średnie wskaźników klinicznych u pacjentów chorych na zapalenie przyzębia, u których stężenie przeciwciał Hsp 60 w surowicy krwi > i < wartości średniej w grupie kontrolnej + 2 SD

Clinical measurement (Pomiar kliniczny)	Hsp 60 > control mean + 2 SD (n = 9)	Anti-Hsp 60 < control mean + 2 SD (n = 64)	Z value in Mann-Whitney test (Wartości Z w teście Manna-Whitneya)	p
PI	1.34 ± 0.71	1.08 ± 0.69	1.15	0.24
GI	1.89 ± 0.58	1.61 ± 0.68	1.15	0.24
PBI	2.01 ± 0.83	1.97 ± 0.99	0.12	0.96
SFR (mm)	5.42 ± 1.73	5.12 ± 2.33	0.91	0.35
PD (mm)	4.16 ± 0.75	4.09 ± 1.09	0.62	0.55

determining p-ANCA and c-ANCA. In the control tests in these 5 patients, one had a negative test and the other four subjects revealed a significant drop from the values at the beginning. It is important to note that positive p-ANCA tests was found in patients with GAP, i.e. those with the most progressive periodontitis. Hence the question whether these autoantibodies are of significant importance in the pathogenesis of periodontal diseases. In presented observations only the anti-MPO antibodies were identified in several cases and in amounts just slightly exceeding the established norms. The presence of these autoantibodies can be explained by the immunological response to the myeloperoxidase of neutrophils, which intensively infiltrate the inflamed periodontal tissues. Novo et al. [27] recently found ANCA-positive sera in patients with systemic lupus erythematosus, rheumatoid arthritis, and periodontitis. This result confirms a well-known association between ANCA and these systemic conditions.

Presented results demonstrate that ANCA does not play an important role in the etiopathogenesis of periodontitis, as was suggested by Novo and Viera [9]. It must be taken into consideration that these authors used a neutrophil granular extract as the antigen in ELISA, omitting many controls which in this type of study are the basis for excluding non-specific reactions. Presented studies confirm that the autoimmune response is not a primary etiological factor in periodontitis. In some patients (mainly, it seems, in the generalized and progressive form of periodontitis) the presence of translocation of ANCA-reactive proteins to the neutrophil surface and degranulation can be detected, which may be one of the secondary mechanisms enhancing the vicious circle of this pathology.

Presented studies did not reveal a major difference in the levels of antibody to human Hsp 60 between patients with different types of periodon-

titis and the control group. This finding indicates that periodontitis does not have any effect on the production of specific autoantibodies to Hsp 60. This could be due to intraepithelial localization in the gingival tissue, the Hsp 60 epitopes not being sufficiently available to cause specific humoral immunological response. Presented results can be interpreted as another suggestion for the lack of an important component of the autoimmunological process in the initiation and support of the gingival tissue damage in periodontal diseases. Elevated levels of antibodies to human Hsp 60 in the sera of periodontitis patients compared with controls have been reported by Tabeta et al. [28]. They also suggested the cross-reactivity of antibodies to human Hsp 60 (a self antigen) and *P. gingivalis* GroEL (an exogenous bacterial Hsp 60 antigen). Significant associations between the concentration of antibodies to human Hsp and clinical indices of periodontitis were described by Lopatin et al. [29]. These authors also suggested that anti-Hsp antibodies may play a protective role in the development of periodontal disease and their levels are lowest in more advanced periodontitis. The differences between these results, also own observations, may be attributable to differences in periodontal disease advancement and activity at the time of sampling these antibodies.

In light of today's knowledge, the authors expect that in some periodontitis cases (especially in GAP), the autoimmunological process may take part in tissue destruction, but only as a secondary process in relation to earlier destruction of the attachment apparatus and the presence of periodontopathic bacteria as a consequence of periodontal diseases. Perhaps in refractory forms of periodontitis the autoimmunological mechanisms replace the dental plaque in the stimulation of disease-causing changes. Predominantly type I collagen and desmosomal proteins in the junctional epithelium were indicated as potential autoanti-

genes in periodontitis [3, 4, 8]. Described examinations do not confirm that, apart from special cases, the role of autoantigens in periodontitis could be fulfilled by myeloperoxidase and proteinase 3 from the azurophilic granules of neutrophils. The possi-

bility of occasional participation of antibodies to human Hsp 60 in a secondary autoimmune response in periodontitis cannot be excluded, although presented studies did not provide sufficient evidence of this.

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Address for correspondence:

Tomasz Konopka
Department of Periodontology
Silesian Piasts University of Medicine in Wrocław
Krakowska 26
50-425 Wrocław
Poland
Phone/fax: +48 71 784 03 81
E-mail: tkonopka@stom.am.wroc.pl

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