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Immunocytochemical indicators of apoptosis in gingival tissues of patients with chronic periodontitis

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ABSTRACT

Introduction. Inflammatory mechanisms of chronic periodontitis (CP) may be linked to various forms of disturbances in apoptosis.
Aim. The study aimed at comparison of tissue expression of anti-apoptotic protein (Bcl-2) and proapoptotic proteins (p53, caspase-3) in gingival tissues of 30 patients with CP and of 15 with healthy periodontium.
Material and methods. Gingival samples (n = 68) were obtained during the open curettage procedure with gingivectomy of adult patients (18 women and 12 men) with CP. Classical immunocytochemical (IHC) method was used to detect apoptotic proteins, and the obtained expression was evaluated using semi-quantitative IRS scale.
Results. No differences could be revealed in expression intensity or reciprocal correlations between apoptotic proteins within the group of patients with CP. Greater expression of the two apoptotic proteins (Bcl-2 and p53) were detected in patients with CP than in control individuals. Moreover, a more pronounced expression of Bcl-2 was demonstrated in gingival samples of patients with localised form as compared to generalised form of CP. Expression of caspase-3 (effector phase of apoptosis) manifested no differences between CP and control individuals. Greater expression of the anti-apoptotic protein Bcl-2 and caspase-3 was detected in cells of inflammatory infiltrates in lamina propria than in keratinocytes.
Conclusions. In CP significant alterations developed in expression of indicators of apoptosis, with prevalence of Bcl-2 and p53 expression, as compared to the control. The localised form of CP was linked to higher proportion of Bcl-2-positive cells of inflammatory infiltrates, suggesting that apoptosis was inhibited mainly in this form of CP. The comparable expression of caspase-3 in gingival cells with CP and in control and absence of correlation with clinical data suggested that the process of apoptosis did not play a significant role in destruction of periodontium tissues in CP.

Key words: chronic periodontitis, apoptosis, immunocytochemistry.

Introduction

Chronic periodontitis (CP) develops due to bacterial and/or viral infection and is accompanied by a progressive loss of clinical attachment level/loss (CAL) and of alveolar bone. The process represents one of principal causes of dental loss in adult patients [1–3]. It is estimated that in various world populations 1–5% individuals suffer from aggressive periodontitis and 80% from chronic periodontitis, which allows to consider CP as representing a social problem [4]. Several hypotheses exist related to pathomechanisms of the disease [3–5]. Involvement of bacteria which are pathogenic for periodontal tissues (*A. actinomycetem-comitans, Porphyromonas gingivalis, Prevotella intermedia, Tannarella forsythensis*) in promotion of cell apoptosis was confirmed by numerous studies in the *in*

vitro models and in the *in vivo* conditions [6–12]. The most typical for apoptosis morphological lesions in cell nuclei (increased fragmentation of DNA, the typical DNA „ladder") were induced by *Tannarella forsythensis* (earlier termed *B. forsythus*) and serotype „a" of *A. Actinomycetemcomitans* [6]. *Porphyromonas gingivalis* induced a direct cytopathic effect [13, 14]. Apoptosis induced by infection with the bacteria was demonstrated in several cell types, *i.e.* in peripheral blood mononuclear cells (PBMCs) [13], lymphocytes [14], epithelial cells [9, 11, 12], fibroblasts and endothelial cells of gingival blood vessels [15–18] and in macrophages [6, 12]. The *in vitro* models explained role of bacterial factors engaged in the apoptotic process in periodontitis. A proportion of authors indicated that gingipains (Arg-gingipain, and Lys-gingipain) and endopeptidase O (PepO) may induce alterations in adhesion proteins and may promote apoptosis of fibroblasts and vascular endothelial cells [15–17]. A team of polish investigators demonstrated that gingipains influenced the immune system inducing degradation of TNF-α, which resulted in, *i.a.*, dysregulation of cytokine network and inhibition of activity manifested by transcription factor, NF-κB [19]. Other investigators showed that apoptosis in primary human gingival fibroblasts (HGF) in infection with *P. gingivalis* developed in two stages and involved a variable in time regulation of anti- and proapoptotic molecules [18]. The authors confirmed the earlier observations that gingipains may directly promote apoptosis through the intracellular proteolytic activation of caspases.

Other studies related to the role of apoptosis in periodontitis posed the question related to the role played by inhibition of apoptotic process in chronic transformation of pathology in CP. According to certain authors, the inhibition of apoptosis in, *e.g.*, macrophages may reflect a decreased production of interleukin 4 (IL-4) by cells of inflammatory infiltrates [20]. Other authors described the phenomenon of resistance to apoptosis manifested by infiltrating lymphocytes (absence of an appropriate expression of Fas ligand at the cell surface with preserved function of Fas receptor) [21]. Subsequent studies demonstrated induction of the anti-apoptotic Bcl-2 protein in gingival keratinocytes under effect of *P. gingivalis* [22]. Yilmaz et al. provided the observation that *P. gingivalis* at first triggered surface phosphatidylserine exposure through a mechanism requiring caspase activation, but after 1 day of infection protected infected cells against apoptosis [23].

Using immunocytochemical (IHC) techniques apoptotic proteins in CP were demonstrated both in epithelial keratinocytes and in cells of inflammatory infiltrates in *lamina propria* [8–10, 14, 24]. A more pronounced expression of proapoptotic proteins was manifested in gingival samples of CP patients than in a control group [8, 14]. Studies related to the active phase of apoptosis suggested that activation of caspases (-3, -7 and PARP) represents the principal trait of tissue injury in the course of periodontitis and it affects both keratinocytes and connective tissue cells [10].

This study aimed at comparison of expression manifested by key apoptotic proteins, *i.e.*, the anti-apoptotic Bcl-2 protein and the two pro-apoptotic proteins, p53 (early phase of apoptosis) and caspase-3 (effector phase of apoptosis) in gingival samples obtained from patients with CP and from persons with clinically healthy periodontium, as related to selected clinical data.

Material and methods

Clinical data of CP patients and of control individuals are given in Tables 1 and 2. The studies were conducted on the total of 17 patients with generalised form of CP and 13 patients with localised CP. The parameter defining clinical advancement of the disease involved clinical attachment level/loss (CAL), estimated using Florida Probe®. The system consists of a periodontological optical probe PD/CEJ and a computer software FP32 v.6. The PD/CEJ (10.8x0.45 mm STANDARD probe) manifests the accuracy of 0.2 mm at the pressure corresponding to weight of 15 g. The values of CAL I, II and III were demonstrated in, respectively, 7, 19 and 42 fragments of gingivae obtained from CP patients (Table 1).

Tissue material

Gingival samples (n = 68) from 30 adult patients (18 women and 12 men; mean age: 45 ± 2 years) were isolated using a scalpel from various sextants, from proximity of various maxillary and mandibular teeth (mainly incisors, canines and premolars) during the open curettage with gingivectomy, following a successful completion of hygienic phase of the periodontal therapy (API index <15%) (Table 1). In every patient 1–4 fragments of periodontal tissues were sampled from proximity of various teeth with various values of CAL: CAL I ⩽ 3 mm, CAL II 4–6 mm, CAL III ⩾7 mm. The control material (negative control) included gingival samples isolated from 15 patients with clinically healthy periodontium, the gingival fragments of which were sampled during procedures of esthetic periosur-

Table 1. Selected clinical data on patients with chronic periodontitis (CP) and semiquantitative evaluation of tissue expression of apoptotic proteins (Bcl-2, p53, caspase-3) in gingival samples

Patient	Age (years); sex	CP form	Proximity of tooth no.	CAL	Grading	Bcl-2*	p53*	caspase-3*
1.	47; M	G	21	III	3	0/6	nt	0/3
			11	III	3	0/6	nt	0/3
			12	III	3	0/9	6/3	0/6
2.	52; F	L	33	I	2	nt	nt	nt
			31	III	1	0/6	4/3	3/3
			41	III	1	nt	6/6	6/9
3.	47; M	G	22	II	3	0/3	nt	0/9
			11	III	3	0/3	0/2	0/6
			14	III	2	0/4	nt	0/3
			33	III	3	0/3	6/2	0/3
			41	III	3	3/4	6/0	0/9
			43	III	3	0/12	0/3	2/4
4.	49; F	G	42	II	1	0/0	nt	nt
			32	III	3	0/6	nt	0/3
			31	III	3	0/8	3/3	0/3
			41	III	2	nt	0/0	0/2
5.	20; F	L	22	I	2	6/8	0/3	0/6
			11	II	2	0/2	nt	0/0
			13	II	2	3/3	3/3	0/0
6.	49; M	L	31	III	3	2/6	6/3	2/2
			33	I	3	0/4	6/4	0/3
			41	III	3	0/6	6/0	0/9
7.	63; F	G	44	I	2	4/0	0/3	0/9
			43	III	3	0/9	0/3	0/3
			42	III	2	0/0	3/0	6/6
			33	III	2	0/0	0/3	0/8
8.	28; F	L	37	II	1	0/8	0/0	0/3
9.	58; F	G	41	I	2	0/4	0/3	0/0
			43	III	3	0/4	6/6	0/8
			45	II	2	0/0	3/3	0/2
10.	49; F	G	14	III	3	0/3	nt	0/9
11.	31; M	G	23	II	2	0/3	nt	0/3
			24	II	1	0/3	6/3	0/6
12.	47; M	G	22	III	2	6/0	4/3	0/0
			11	III	3	0/6	6/6	0/6
			12	III	3	0/0	0/3	6/6
13.	53; F	G	22	III	3	0/3	nt	0/6
14.	54; F	G	13	III	3	0/3	8/8	0/3
			14	III	3	0/9	0/3	0/6
15.	56; M	G	21	III	2	0/6	nt	0/3
			11	III	2	0/3	0/3	0/3
			23	II	1	0/3	3/0	0/3
16.	43; F	L	17	II	3	0/4	4/0	nt
17.	32; M	L	33	II	3	0/8	3/0	0/2
			41	II	3	0/2	0/2	0/0
			42	II	3	0/12	6/6	0/0
18.	43; F	G	21	II	2	0/3	nt	0/6
			27	III	3	0/6	6/3	0/3
			25	III	3	0/6	nt	0/8
19.	59; F	L	17	II	2	0/4	4/0	0/3

Table 1 cont.

Patient	Age (years); sex	CP form	Proximity of tooth no.	CAL	Grading	Bcl-2*	p53*	caspase-3*
20.	43; M	L	11	III	3	0/9	0/9	0/12
			15	III	3	nt	6/0	0/3
			23	I	2	0/4	6/3	0/3
21.	29; M	L	32	II	2	0/6	0/0	0/6
			42	II	3	0/9	nt	0/3
22.	47; F	G	31	III	2	0/0	nt	0/0
			41	III	3	0/0	0/2	0/0
23.	39; F	L	31	II	3	0/3	nt	0/3
			42	III	2	0/12	2/0	0/0
24.	50; F	G	13	III	2	0/0	6/3	0/3
25.	53; F	L	31	III	3	0/0	0/3	6/3
			41	III	2	0/6	0/3	0/2
26.	51; M	G	16	I	1	0/4	0/3	0/3
27.	28; M	L	21	III	3	0/6	0/2	0/9
			12	III	3	0/8	nt	9/0
28.	21; F	L	13	II	2	3/4	4/0	0/6
29.	58; M	G	31	III	0	0/0	nt	0/3
30.	56; F	G	15	III	3	0/12	3/3	0/2

*F – Female; M – male; G – generalised type of CP; L – localized type of CP; nt – not tested; * – expression of apoptosis proteins in 12 point semi-quantitative IRS scale (see Material and Methods); the first number indicates intraepithelial localisation, the second in lamina propria ; CAL – clinical attachment level/loss*

gery, uncovering of the retained teeth and/or dental extraction. The tissue material related to the studied group (CP) and control group with respective clinical data was obtained from the Private Dental Practice in Ostrów Wlkp (MH). Written informed consent was obtained from every patient before gingivectomy,

Table 2. Semiquantitative analysis of tissue expression of apoptosis protein in control group

Patient/age (years), sex	Bcl-2*	p53*	Caspase-3*
1. 29; F	0/0	0/0	0/0
2. 15; M	0/0	3/0	6/0
3. 24; F	0/3	3/3	9/0
4. 65; M	0/6	0/0	2/0
5. 45; F	0/0	4/2	9/0
6. 36; M	0/4	0/0	3/0
7. 25; F	0/0	2/2	0/6
8. 36; F	0/0	0/0	3/0
9. 24; F	2/0	0/2	nt
10. 21; F	0/3	3/3	6/8
11. 29; F	0/3	6/6	6/6
12. 28; M	0/3	3/3	6/0
13. 23; F	0/3	6/2	3/0
14. 16; M	0/3	0/2	3/0
15. 16; M	0/0	Nt	3/0

*F – female; M – male; nt – not tested; * – expression of apoptosis proteins in 12 point semiquantitative IRS scale (see Material and Methods); the first number indicates intraepithelial localisation, the second in lamina propria*

and approval for the study was granted by the institution's Ethical Committee (no 695/05). The entire tissue material was fixed for 24 hours in 4% buffered formalin and, then, embedded in paraffin. The mean size of the tissue material was 4x2x2mm. Serial 5μm thick sections were deposited onto SuperFrost/Plus microscope glasses.

Detection and studies on cellular localisation of Bcl-2, p53 and active caspase-3 in gingival samples took advantage of the classic ABC (streptavidin-biotinylated peroxidase complex) immunocytochemical technique with LSAB methodology (LSAB+ System HRP, Dako, Glostrup, Denmark), described earlier [25]. Mouse or rabbit anti-human monoclonal antibodies (mAbs) were employed, directed against Bcl-2 protein (ready to use) (Dako) and p53 protein (in dilution 1:50) (Dako) and against active form of caspase-3 protein (in dilution of 1:50) (R&D Systems). The sections were incubated with these primary mAbs at night at 4^0C, and afterwards were incubated with the LSAB+ System HRP (Dako, Glostrup, Denmark). Following deparaffination and rehydration, the preparations were additionally boiled in 10mM citrate buffer in a 700 W microwave oven for 18 min (in case of anti-Bcl-2 and anti-p53), washed in PBS and, then, subjected to the reaction according to the standard procedure. Every test was accompanied by a negative control, in which specific antibodies were supplemented by a normal serum of a respective spe-

cies in 0.05M Tris-HCl, pH~7.6, supplemented with 0.1% bovine serum albumin (BSA) and 15mM sodium azide.

The results were evaluated using semi-quantitative 12-points immunoreactive score (IRS) scale [26]. The preparations were examined under a light microscope, at 400x magnification. In each section of gingiva, five fields in epithelium and five of *lamina propria* were examined.

Statistical analysis

The descriptive statistics included calculation of mean, mediane values, standard deviation and standard error of the mean for quantitative traits. For qualitative variables, a distribution of patient numbers in individual categories of a trait was analysed.

Statistical analysis took advantage of the Mann-Whitney U test for nonparametric independent data and the Wilcoxon test for nonparametric dependent data. Correlations between data rows were determined employing Spearman's rank correlation index.

For comparisons of three independent samples the nonparametric Kruskal-Wallis test was used. Test for two structural indices was used to evaluate differences

between groups related to percentage detectability of selected traits.

Probability values less than 0.05 were considered significant.

Results

Principal clinical data and intensity of expression manifested by apoptotic proteins in gingival samples of CP patients and control individuals are presented in Tables 1 and 2. In tissue material (68 gingival fragments) of CP patients the highest inflammatory activity (grading 2 and 3, G2, G3) was detected in, respectively, 24 (G2) and 36 gingival fragments (G3) (Table 1).

Expression of the anti-apoptotic Bcl-2 protein in patients with CP

Tissue expression of the protein was demonstrated in gingival samples of 90% patients with CP and it was significantly more pronounced than in the control (60%) (Figure 1). In semi-quantitative evaluation of Bcl-2 expression also significantly higher amounts of the protein were detected in patients with CP (4.75 ± 0.41) than in the control (2.00 ± 0.49) (Fig-
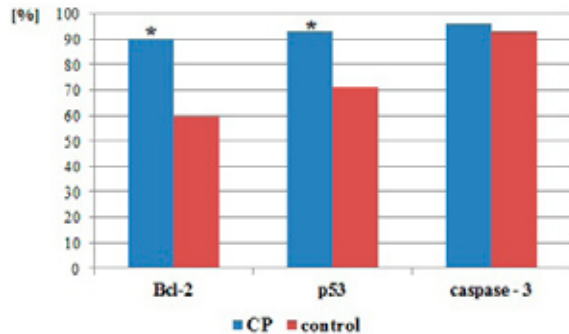

Figure 1. Comparison of detectability of apoptotic proteins (%) in gingival samples of the patients with chronic periodontitis (CP) and in the control; *p < 0.05
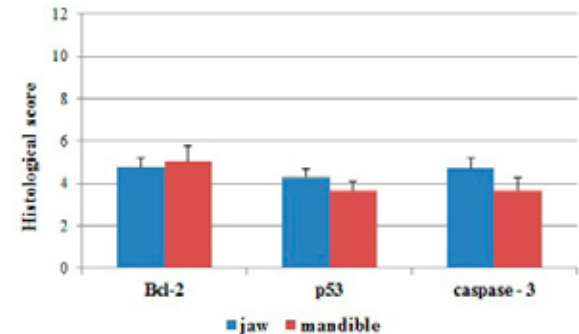

Figure 3. Comparison of tissue expression of apoptotic proteins (IRS scale) in gingival samples between maxillary and mandibular localisation of chronic periodontitis
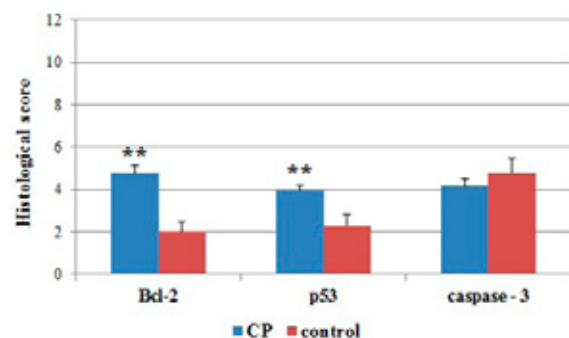

Figure 2. Comparison of tissue expression of apoptotic proteins (12-points IRS scale) in gingival samples of the patients with chronic periodontitis (CP) and in the control; **p < 0.01
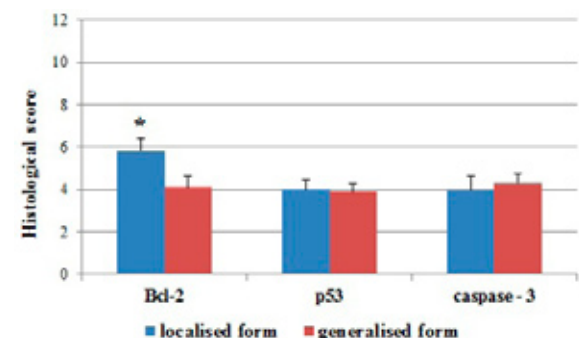

Figure 4. Comparison of tissue expression of apoptotic proteins (IRS scale) in gingival samples between localised and generalised form of chronic periodontitis

ure 2). No quantitative differences were disclosed in Bcl-2 expression which would be related to the location of the tooth (maxilla vs mandible) (4.76 ± 0.45 vs 5.04 ± 0.74) (Figure 3). A more pronounced expression of Bcl-2 protein was observed in the localised form of CP (5.83 ± 0.62) than in its generalised form (4.10 ± 0.51) (Figure 4).

Product of IHC reaction was present mainly in perinuclear space of numerous mononuclear cells of inflammatory infiltrates (mainly lymphocytes) (Figure 5A) and, sporadically, in the cell nuclei themselves. Significantly more cells with expression of Bcl-2 were demonstrated in cells located in *lamina propria* (4.59 ± 0.41) as compared to epithelial cells (0.42 ± 0.16) (Figure 6).

Analysis of proapoptotic protein expression in patients with CP

p53 protein

The protein was detected in samples of 93% patients with CP and in 71% control patients, which involved a statistically significant difference (Figure 1). Semiquantitative analysis demonstrated significantly higher amounts of p53 protein in patients with CP (3.93 ± 0.28) as compared to control patients (2.28 ± 0.53) (Figure 2). On the other hand, no significant differences in expression of the protein were detected between maxilla and mandible (4.30 ± 0.40 and 3.67 ± 0.43, respectively) (Figure 3) or depending upon the form of the disease (localised or generalised form) (4.00 ± 0.47 vs 3.89 ± 0.35) (Figure 4).

p53 protein was detected in cell nuclei in cells of inflammatory infiltrate (mainly lymphocytes) and in keratinocytes of gingival epithelium and/or of periodontal pocket (Figure 5B). Individual cells of inflammatory infiltrates with cytoplasmic expression of p53 protein were found to be enlarged (macrophages, dendritic cells). No quantitative differences could be demonstrated in cellular location of the protein (*lamina propria* vs keratinocytes of epithelium) (2.59 ± 0.30 vs 2.88 ± 0.38) (Figure 6).

Caspase-3

Detectability of the protein in CP patients amounted to 96% and manifested no significant difference as compared to the control (93%) (Figure 1). Surprisingly, no quantitative differences between CP patients
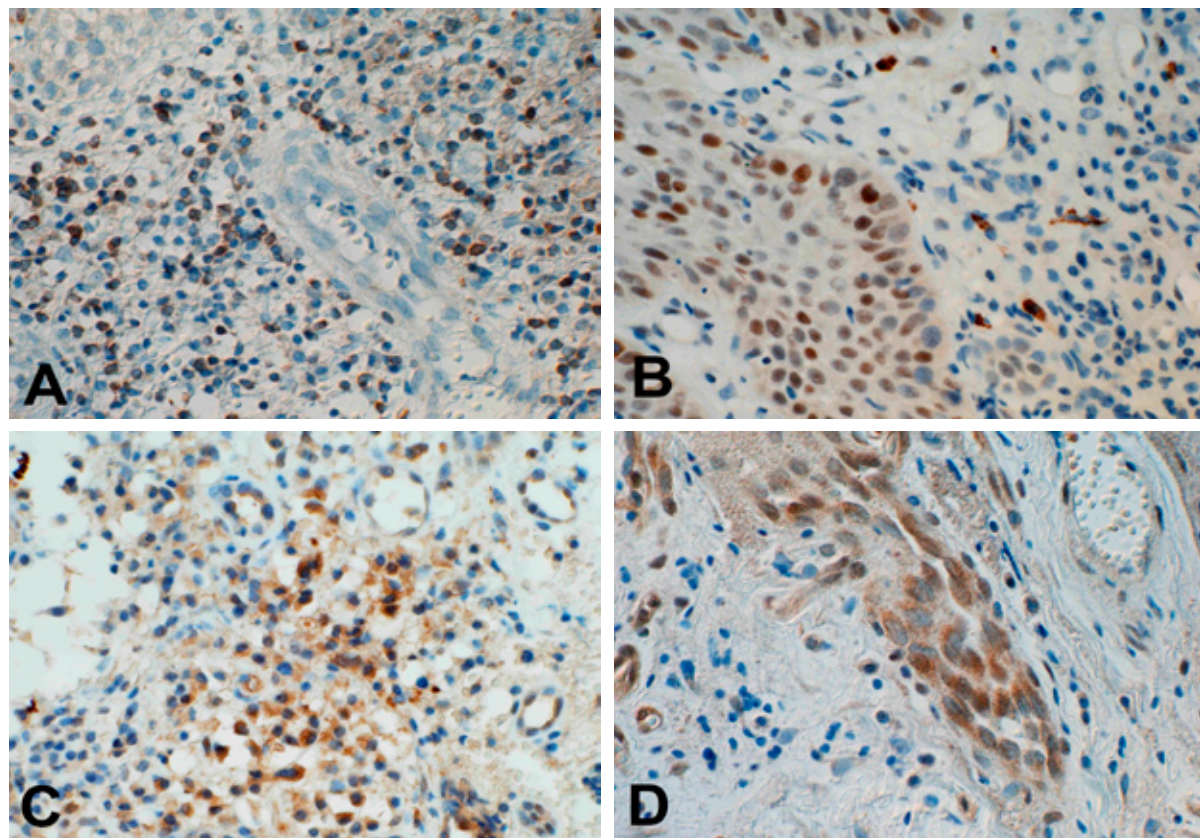

Figure 5. Immunocytochemical localisation of apoptotic proteins in gingival samples of the patients with chronic periodontitis. (A) perinuclear/nuclear localisation of Bcl-2 protein in inflammatory cells in *lamina propria;* (B) nuclear (in keratinocytes) and cytoplasmic (mostly in *lamina propria* cells) localisation of p53 protein; (C) cytoplasmic localisation of caspase-3 in inflammatory cells in *lamina propria;* (D) cytoplasmic localisation of caspase-3 mostly in keratinocytes of gingival sample. ABC technique. Hematoxylin counterstained. Objective magnification x40

(4.17 ± 0.36) and the control (4.78 ± 0.73) were detected also in expression of caspase-3 (Figure 2). In contrast to expectations no significant differences in expression of the protein of effector phase of apoptosis were noted between generalised form of CP (4.30 ± 0.42) and its localised form (3.96 ± 0.68) (Figure 4), nor between maxillary and mandibular localisation (4.70 ± 0.49 and 3.68 ± 0.58, respectively) (Figure 3). As compared to epithelial keratinocytes (0.62 ± 0.23), a significantly higher expression of the protein was detected in cells of inflammatory infiltrate (mainly lymphocytes) in *lamina propria* (4.03 ± 0.36) (Figure 6).

Microscope examination demonstrated presence of caspase-3 in cell cytoplasm of cells in inflammatory infiltrates, epithelial cells, individual cells of vascular endothelium and fibroblasts (Figure 5C and D). Very frequently, the IHC reaction was very intense.

Reciprocal correlations between expression of apoptosis proteins in gingival samples of CP patients

No significant relationships were detected between reciprocal expression of individual apoptosis proteins (p > 0.05) in patients with CP (Table 3).

Reciprocal correlations between expression of apoptosis proteins in control gingival samples

In control group the only significant and direct relationship was detected between expression of two pro-apoptotic proteins (p53 and caspase-3) (r = 0.547; p < 0.05) (data not shown).

Expression of apoptosis proteins in gingival samples of CP patients and selected clinical data

In tissue material obtained from patients with CP no significant differences were detected in intensity of expression of apoptosis proteins which would depend on activity of inflammatory lesions (*grading)* or degree of CAL (Tables 4 and 5).
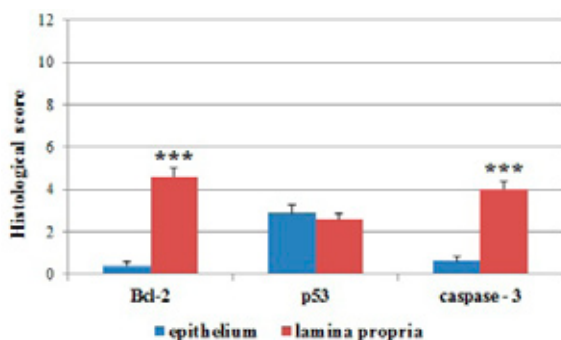

Figure 6. Comparison of tissue expression of apoptotic proteins (IRS scale) in epithelial cells and *lamina propria* cells in gingival samples of the CP patients; ***p < 0.001

Table 3. Coefficients of Spearman's correlation between reciprocal tissue expression of apoptosis proteins within the patients with chronic periodontitis

	r	p
Expressions of Bcl-2 and p53	0.012	0.934
Expressions of Bcl-2 and caspase-3	0.049	0.705
Expressions of p53 and caspase-3	0.216	0.140

r – Spearman's correlation coefficient; p – level of significance

Table 4. Comparison of expression manifested by apoptosis proteins depending on intensity of inflammatory lesions (grading) in gingival tissues with chronic periodontitis (Kruskal-Wallis test)

	p
Expression of Bcl-2 and grading	0.067
Expression of p53 and grading	0.168
Expression of caspase-3 and grading	0.229

p – level of significance

Table 5. Comparison of expression of apoptosis proteins depending on clinical attachment level/loss (CAL) in gingival tissues with chronic periodontitis (Kruskal-Wallis test).

	p
Expression of Bcl-2 and CAL	0.427
Expression of p53 and CAL	0.549
Expression of caspase-3 and CAL	0.305

CAL – clinical attachment level/loss; p – level of significance

Discussion

Bcl-2 is a protein of 25 kDa molecular mass and is involved in control of cell programmed death. Expression or overexpression of the protein may protect the cell from death, preventing or delaying apoptotic process [27]. Both detectability of Bcl-2 in patients with CP and its expression intensity have been significantly higher in patients with CP than in healthy gingival tissues, which is consistent with results obtained by other authors in case of generalised aggressive periodontitis (AP) [28]. Our studies have demonstrated slightly higher amounts of Bcl-2 protein in the localised form of CP as compared to the generalised form of CP. Microscopic analysis has documented presence of IHC reaction product in numerous mononuclear cells of inflammatory infiltrate (mainly lymphocytes) within *lamina propria*. On the other hand, poor expression of the protein in lymphocytes of inflammatory infiltrate in CP was shown by Sawa et al. [21]. Our studies have confirmed the perinuclear location of Bcl-2 protein [29]. In cultured human gingival fibroblasts infected with *P. gingivalis* activation of Bcl-2 was observed in early stages of the infection (between second and 12th hour)

[18]. Other investigations demonstrated an increase in Bcl-2 expression and inhibition of apoptosis by *P. gingivalis* also in cells of gingival epithelium but failed to described time-dependent alterations in expression of the protein [22]. Carvalho-Filho et al. hypothesize that *P. gingivalis* HmuY protein plays a role in the pathogenesis of CP, possibly by reducing or delaying apoptosis in T cells through a pathway involving the Bcl-2 protein [30].

The higher expression of Bcl-2 anti-apoptotic protein detected in gingival samples (in addition in cells of *lamina propria)* of our patients with CP than in control group may suggest inhibition of apoptosis in cells of inflammatory infiltrate (mainly lymphocytes), their longer survival and, as the result, their destructive effect of periodontal tissues. However, we have failed to note significant relationships between presence of Bcl-2 and clinical indices such as grading and CAL values. Possibly, this might have reflected prevalence in the material of highly intense inflammatory lesions (grading 3) and high values of CAL parameter (CAL III) in most of the sampled fragments of gingiva with CP. On the other hand, a significantly higher expression of Bcl-2 protein has been detected in the localised as compared to generalised form of CP, which would suggest that the former form of CP is accompanied by a higher proportion of cells in inflammatory infiltrate with an extended life span (extended inhibition of apoptosis).

p53 protein, of 53 kDa molecular mass, termed the genome guardian, is responsible for DNA repair and, if the lesions cannot be repaired, for induction of programmed death of the cell [31]. It leads to apoptosis of terminally differentiated cells, including cells of inflammatory infiltrate [28]. IHC techniques permit to detect only the protein of an elongated half-life (a mutated one or manifesting an increased synthesis coefficient) [31].

In this study p53 protein has been detected mostly in cell nuclei, both in cells of inflammatory infiltrate (mostly lymphocytes) and in keratinocytes. Detection of the protein both in the tissue material from patients with CP and in the control may point to activation of p53 production under effect of stress factors (*e.g.*, inflammation, permanent exposure to bacterial infection in oral cavity). The more pronounced expression of p53 in patients with CP than in control individuals may indicate potential role of the protein in initiation of apoptosis (at least in some cells of inflammatory infiltrate) or may provide proof for an augmented reparative role of cell nucleus structures (mainly DNA), injured due to chronic inflammation. Other

authors could not demonstrate quantitative differences in expression of the protein between patients with aggressive periodontitis and control individuals [28]. The data of Ghosh et al. demonstrated that a proapoptotic fibronectin matrix induces the degradation of ubiquitinated p53 *via* proteasomes in periodontal ligament cells. The authors concluded that is may be a novel mechanism of apoptosis associated with inflammatory diseases [32]. In this study we have not been able to demonstrate a relationship between expression of p53 protein and form of the disease or remaining clinical data.

Caspases represent a family of cysteine proteases which enzymatically split various substrates contain in the cell [33]. Activation of caspase-3 starts the effector apoptosis phase, leading to a cascade of event terminated by cell death and its elimination. Detection in a tissue of cells immunopositive for the so called active caspase-3 form is thought to be a significant trait of apoptosis activation [34]. It is suggested that activation of caspases represents the main character of tissue injury in the course of periodontitis. In tissues of patients with CP activation of caspase-3, caspase-7 and splitting PARP, the caspase-3 substrate was demonstrated both in keratinocytes and in connective tissue cells [10]. According to the authors, detection of high number of cells containing active forms of caspases proves that early stages of apoptosis are detected.

In our study the cytoplasmic product of IHC reaction for caspase-3 has been detected in cytoplasm of inflammatory infiltrate cells and in epithelium of gingiva and/or periodontal pocket. A significantly higher expression has been noted in cells of inflammatory infiltrate (mainly lymphocytes) than in keratinocytes. No differences have been detected in detectability and intensity of caspase-3 expression between tissues obtained from CP patients and control individuals, consistent with the data of Bantel et al. [10]. A similar absence of differences was demonstrated in cases of AP and control [28]. Other studies demonstrated that fibroblasts represent the principal apoptosis-affected cell population in CP, apart from only small proportion of leukocytes. The authors suggested that apoptosis of fibroblasts represented a normal phenomenon in cases of remodelling of tissues such as periodontium and that it indicated presence of an equilibrium between fibroblast proliferation and their apoptosis [24]. The results of Lucas et al. indicated that apoptosis in periodontitis may be inhibited by elevated expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) decoy receptors and cleaved caspase-3 inhibitors [35].

In contrast to expectations, our investigations have failed to demonstrate significant differences in expression of effector phase apoptotic proteins between the localised and generalised forms of CP. Both detectability of the protein in patients with CP and intensity of the reaction (semiquantitative evaluation) have not significantly differed from the control. Gamonal et al. demonstrated cells with active caspase-3 and other apoptotic proteins only in patients with chronic periodontitis [8]. In their studies numerous cells in apoptosis were detected also in biopsies of gingival samples with PD≥5 mm and CAL≥3 mm. The principal cells in apoptosis involved neutrophils even if the process affected also other cells of inflammatory infiltrate [8].

In our studies we have not been able to document significant relationships between expression of caspase-3 and clinical data. Also, no reciprocal correlations have been observed in expression of apoptosis proteins within the group of patients with CP.

Results of our investigations on apoptosis markers in gingival cells of clinically healthy periodontium (lower expression of Bcl-2 and p53, but similar expression of caspase-3 as compared to CP patients, positive correlation between the two pro-apoptotic proteins) is consistent with the suggestion that periodontium tissues, continuously exposed to low degree bacterial infections may contain cells with DNA damage and a variable expression of apoptotic proteins. Tonetti et al. examining control gingival samples demonstrated apoptotic cells in particularly high numbers in subepithelial clumps of inflammatory infiltrates and within joining epithelium [36]. The caspase-3 dependent mechanism of apoptosis in experimental periodontitis in diabetic rats inoculated with *A. actinomycetemcomitans* showed increasing the inflammatory response with enhancing apoptosis of gingival epithelial and connective tissue cells [37].

Summary and conclusions

1. In chronic periodontitis significant alterations develop in expression of immunocytochemical exponents of apoptosis, with prevalence of Bcl-2 and p53 proteins, as compared to the control.
2. The localised but not the generalised form of CP is accompanied by higher proportion of Bcl-2 positive cells of inflammatory infiltrates, which suggests inhibition of apoptosis in this form of chronic periodontitis.
3. A comparable expression of caspase-3 (effector phase of apoptosis) in samples of CP patients and in clinically healthy periodontium and absence of correlation with advancement of clinical lesions in chronic periodontitis suggests that apoptotic process plays no significant role in destruction of periodontal tissue in CP.

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