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Abstract

Introduction. Several factors can influence the gingival tissues such as disrupting tissue homeostasis and the occurrence of pathological conditions. The aim of our study was to investigate and compare the presence of apoptosis in patients undergoing immunosuppressive therapy, patients with periodontitis and healthy patients, as well as to achieve better understanding of the role of apotosis in the same processes.

Methods. The first examined group consisted of 21 patients (10 males and 11 females; mean age 37.4±10.2 years) with neither kidney diseases nor cyclosporine A (CsA) therapy, who had a verified periodontal disease. The second group consisted of 21 kidney-transplant patients (9 males and 12 females), with diagnosed gingival overgrowth (GO) undergoing continuous immunosuppressive therapy. The control group consisted of the same number of patients, clinically healthy subjects (15 males and 6 females; mean age 29±14.0 years) with plaque-induced gingivitis. The following indexes were analyzed: plaque index (PI), index of gingival inflammation (GI) according to Loe-Silnes, and gingival overgrowth index (GOI) according to MacGaw, *et al*. The determination of CsA in blood was performed by a fluorescence polarised immunoassay (FPIA). The tissue samples were estimated by semi-quantitative analysis in order to determine the presence of apoptotic cells and imunohistochemical expression of the bcl-2 and p53 proteins

The second group consisted of 21 kidney-transplant patients (9 males and 12 females), with diagnosed GO and subjected on continuous immunosuppressive therapy (175 mg Cs/day). Patients' mean age at time of renal transplantation was 36.2±9.5 years. The mean duration of therapy was 42.4±36.2 months. The post-transplant immunosuppressive therapy consisted of cyclosporine (Neoral®) reaching a satisfactory C2 level (concentration in serum 2 hours after administration of the medicament), prednizolon (0.1 mg/kg/den, Merck), and mycophenolate mofetil (Cellcept 1.5-2g/day, Roche).
The control group consisted of the same number of patients, clinically healthy subjects (15 males and 6 females; mean age 29±14.0 years) with plaque-induced gingivitis, but with no signs of periodontitis. In all patients included in the three groups in our study the use of antibiotics, anti-inflammatory agents and the history of treatment with medicaments known to cause drug-induced GO were excluded.
The patients included in our study underwent the same clinical and para-clinical examinations.

Clinical examinations

The clinical examinations were made by applying the following indexes:

1. Plaque Index (PI) according to Silness-Loe [11];
2. Each of the four surfaces of the teeth (buccal, lingual, mesial and distal) was scored from 0-3. The scores from the four areas of the tooth are added and divided by four in order to show the plaque index for the tooth with the following scores and criteria: 0-No plaque; 1-A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may be seen in situ only after application of disclosing solution or by using the probe on the tooth surface; 2-Moderate accumulation of soft deposit within the gingival pocket, or the tooth and gingival margin which can be seen with the naked eye; 3-Abundance of soft matter within the gingival pocket and/or on the tooth and gingival margin;
3. Gingival Inflammation Index (GI) was estimated according to Loe-Silnes scale [12] as: grade 0- normal; grade 1-mild inflammation, slight color change and edema, no bleeding; grade 2-moderate inflammation, redness, edema, bleeding on probing and grade 3-severe inflammation, marked redness and edema, ulceration and spontaneous bleeding.

Tissue Processing and Histochemistry

In the first phase of the periodontal treatment, bioptical material from the overgrown interdental papilla (patients treated with Cs) was taken during gingivectomy procedure under infiltrated anesthesia and was fixed in 10% neutral formalin, while a standard patohistologic processing was made at the Institute for Pathology at the Medical Faculty in Skopje.
Tissue biopsies from the second group (periodontal disease) and the third group (control group) were obtained during the routine dental treatment (gingivoplasty and tooth extraction from orthodontic reasons or any other indication). All patients enrolled in the study gave their informed consent for participation, according to Helsinki Declaration in 1975, revised in 2000.
Furthermore, the tissue samples were placed into the paraffin moulds, out of which tissue cross-sections with 4-6 μm thickness were obtained. These tissue cross-sections were placed on glasses by a standardized manner and colored with hematoxylin eosin (HE), while the cross-sections for the immunohistochemical coloring were placed on silane glasses and colored with ABC-Avidin Biotin Complex method, LSAB + variant.

Immunohistochemistry (ABC-Avidin Biotin Complex method)

Primary antibody, with a determined antigenic determinant, was added to the tissue sample, and thereafter incubated at room temperature (30 min). Later on the samples were rinsed with phosphate buffer, then deluded into 10% normal serum and rinsed again with phosphate buffer. The secondary antibody, which is coupled with biotin was added further on. The Avidin-Biotin Complex contains HRP enzyme (Horse radish peroxidase), which bonds with the biotin molecule of the secondary antibody, and which bonds with determinants of the primary antibody. The samples were incubated at room temperature for 30 minutes and then rinsed with phosphate buffer. The next step involves adding AVS reagent and rinsing with phosphate buffer. The final result as a positive antigenic antibody reaction was followed by forming of a brown precipitate from the polymerized substrate.
Following the immunohistochemical coloring by applying a light microscope on the tissue cross-sections, detection and counting of the apoptotic cells was done, bcl-2 and p53, expressed as average number of cells on ten visual fields (X 400). The level of expression of p53 and bcl-2 as well as the apoptotic cells of each slide was graded on a semi-quantitative manner using a graduation of 0-3+; (0)=no staining; (1+)=stained cells comprising >10% of the inflammatory infiltrate; (2+)=stained cells comprising up to 30% of the inflammatory infiltrate; (3+)= stained cells comprising >30% of the inflammatory infiltrate. The obtained results were photo-documented.

Statistical analysis

Differences between the parodontitis group, CsA-treated group and the control group with respect to the clinical parameters and the histopathological findings were analyzed by using the Student's t-test, Kruskal-Wallis test and Mann-Whitney U test of inversion. Statistical

significance was defined as p<0,05. Correlations between histopathological findings and clinical parameters were tested using Spearman's rank correlation coefficient.
The immunohistochemical findings of the examined groups are shown in Table 1. There was no statistical difference between the groups for p53. In our study statistical differences were found in the levels of bcl-2 among the groups, between the first (group with parodontitis) and the second (group with CsA therapy), the first and the third group (group with gingivitis), and between the second and the third group (p<0,01), respectively. The greatest expression of bcl-2 was registered in the second group, treated with CsA, a bit lower expression in the group with periodontal disease, and thelowest expression was noted in the gingivitis group. Identical results were found when compared the apoptotic index, which was highest in the CsA group (p<0.01), when compared to the other groups.
Patients in the CsA group had significantly higher PI and GI values than did patients in the other groups. There was statistical differences for PI and GI among the groups, between the first (group with parodontitis) and the second (CsA group), the first and the third group (group with gingivitis), and between the second and the third group (p<0.01), respectively (Table 2).

Table1. Distribution of immunohistohemical findings in Parodontitis group, Cs group, and Gingivitis group

Groups	Index	N	Percent %
Parodontitis	p53-1	16	76
	p53-2	5	24
Cs group	p53-1	16	76
	p53-2	5	2
Gingivitis	p53-1	19	91
	p53-2	2	9
Parodontitis	bcl2-1	16	76
	bcl2-2	5	24
Cs group	bcl2-1	5	24
	bcl2-2*	16	76
Gingivitis	bcl2-0	8	38
	bcl2-1	13	62
Parodontitis	apoptosis-0	9	2
	apoptosis -1	16	76
	apoptosis -2	3	15
Cs group	apoptosis -2*	9	43
	apoptosis -3*	12	67
Gingivitis	apoptosis -0	2	9
	apoptosis -1	11	52

*Significantly higher than the gingivitis and parodontitis grops (p<0.01)

Table 2. Distribution of clinical findings in various study groups

Group	Index	N	%
Paraodontitis Group	PI-1	15	71
	PI-2	6	29
CsA Group	PI-2	5	24
	PI -3	16	76*
Gingivitis Group	PI -0	16	76
	PI -1	5	24
Paraodontitis Group	GI-1	16	76
	GI-2	5	24
CsA Group	GI-2	11	52*
	GI-3	10	48
Gingivitis Group	GI-0	20	95
	GI-1	1	5

*Significantly higher PI and GI than in the the other 2 grops (p<0.05)

However, there was no correlation between p53 and the other parameters, between the groups. The findings of our research showed a positive significant correlation between bcl-2 and apoptotic index, PI, and GI index (p<0.01). There was no significant correlation between the blood concentration of CsA and apoptosis (p>0.05; r=0.187) (Table 3).

Table 3. Correlation between different parameters

Spearman Rank Correlations p<0,01	p53	bcl-2	apoptosis	PI
bcl-2	0.041	1.000	0.448**	0.692**
apoptosis	0.045	0.448**	1.000	0.560**
PI	0.108	0.692**	0.560**	1.000
GI	0.082	0.693**	0.553**	0.900**
blood concentr. of CyA			0.187	

**Correlation is significant at the p<0.01 level

Discussion

Although the presence of bacterial pathogens is necessary for the initiation of periodontal diseases, inflammatory and immune responses also play a critical role in the progression of gingival tissue disease [13,14]. The presence of DNA damage-positive cells associated with the expression of the wild type p53 apoptosis-inducing protein in the subepithelial inflammatory infiltrate suggests that apoptotic cell death may be an important phenolmenon in the regulation of the inflammatory response to a chronic bacterial challenge. About 4% of the cells present in the subepithelial mononuclear inflammatory infiltrate displayed apoptosis-associated changes.
Gamonal and coworkers [15] detected presence of p53, Fas, FasL and active caspase-3 in the inflammatory infiltrates only in biopsies performed in the cases with chronic periodontitis, whereas Bcl-2 positive cells were reported to be present in the tissues from the healthy controls and gingival tissues from patients with chronic periodontitis. They also reported presence of apoptotic cells

in the deep area of biopsies taken from sites with probing deep of ≥5 mm and attachment of 3 mm.

Furthermore, it has been well-documented that a variety of bacterial pathogens are able to induce apoptosis in the infected cells. Leukotoxin of a periodontal pathogen and Actinobacillus actinomycetemcomitans have been shown to induce apoptosis in human T cell. Another study reported that bacterial products isolated from different strains Porphyromonas gingivalis may delay neutrophil apoptosis in a dose-dependent fashion [16].

In our study, the value of bcl-2 was highest in the group treated with cyclosporine and in the group with periodontal disease, due to the presence of dental plaque and the consequent inflammatory changes, thereby leading to prevention of dead cell apoptosis. Our results also correspond with the results of Bulut, *et al*. [17], where the frequency of grade3+ expression of bcl-2 was found to be significantly higher in the group with generalized aggressive periodontitis (GAP) than that in the control group. According to the results of Pandilova [18], further progressions of loss of attachment result in increased inflammation and consequently decrease of expression of bcl-2. Identical results were reported by Ellis, *et al.* [14]. Namely, they confirmed the association of decrease of bcl-2 expression in parallel with the greater loss of attachment. On the other hand, Gamonall, *et al.* [19] found no statistical significance between the different amount of bcl-2 in healthy gingiva and in gingiva of patients with periodontal disease.

The presence of functional p53 protein is necessary for certain activators of apoptosis, and therefore, when analysing apoptosis in our study we took into account the possibility that the antioncogen protein p53 takes part in the apoptotic processes of the gingiva.

P53 is a tumor suppressive protein, which in the active phase participates in the regulation of the cell cycle, promotes the reparatory mechanisms of the DNA and in case no reparation takes place, apoptosis occurs.

Although p53 is present in normal tissues and cells, its short half-life makes its expression almost undetectable in healthy normal tissues. Upon activation, p53 stabilizes and hence its expression can be detected with anti-p53 antibodies using standard immunohistochemical techniques [16].

There were no significant differences between the groups regarding the rate of p53 protein expression (Table 2). According to immunolocalization of p53 protein in the epithelia of hyperplastic gingival tissues, these may be in part explained by undergoing DNA damage and by the genotoxic stress of the Cs.

The expression of p53 gene has been confirmed as critical processes in tumorogenesis [20]. Expression of p53 protein has been noticed in the histologically normal epithelia adjacent to oral carcinomas and other carcinomas. It has been reported that normal epidermis, when exposed to UV radiation, results in DNA damage and shows sporadic patterns of p53 protein expression and mutations of the p53 gene [21].

Bulut, *et al*. [22] found no significant differences between CsA and gingivitis group with respect to immunolocalization of p53 and bcl-2. Furthermore, Saito, *et al.* [23] evaluated the expression of p53 immunohistochemically in overgrowth tissues induced by nifedipine and phenitoin. They observed positive expression of p53 protein in the nuclei of epithelial cells in overgrowth tissues, while no expression was found to be evident in non-overgrowth control tissues. It was suggested that bcl-2 may lead to cell accumulation, leading to acanthosis and that p53 may be implicated in the pathogenesis of nifedipine- and phenition-induced gingival overgrowth through impaired DNA.

The number of apoptotic cells was significantly higher in the CsA-treated group compared to the group with periodontitis and the inflamed gingiva of healthy individuals. Tonetti, *et al*. [24] demonstrated that the apoptotic process is involved in chronic, bacterially induced gingival inflammation. Analysis of data showed that inflammation led to an increase in apoptosis in "no overgrowth" control gingiva, and inflammation similarly appeared to stimulate apoptosis within the context of gingival overgrowth, but to a lower degree. We believe that the obtained results are mainly due to the fact that inflammation of the gingival tissue is greatest in the CsA tissues, and hence the activation of apoptosis with a consequently proliferative activite as a compensatory mechanism occurs. At the same time, the effect of cyclosporine on the gingival tissue was also present, which cannot be neglected, despite the fact that its influence was not a subject of interest in our study.

Alaaddinoglu, *et al.* [25] demonstrated that keratinocyte apoptosis in the gingiva of kidney recipients with CsA-induced GO is similar to that observed in inflamed gingiva of healthy individuals.

The patients in the CsA group had significantly higher PI and GI values than those in the other groups, as a result of the pseudopockets, which maintain more plaque and at the same time create difficulties in maintaining good oral hygiene.

The absence of any correlation of p53 with all of the analyzed parameters, as well as the participation in the cell-cycle control, is a limitation to connect it with apoptosis during peridontitis.

Our findings showed a positive significant correlation between bcl-2 and apoptotic index, PI, and GI index ($p<0.05$) (Table 3). These results support the idea that inflammation causes apoptosis, and that relatively overgrowth gingiva is a result of the compensational epithetlium proliferation. Similar results were reported by Minovska, *et al.* [26], who proved the existence of a strong positive correlation between inflammation and apoptosis, which participates in the loss of attachment, but does not participate in the recession occurrence and progression. Taking into account the obtained results for the increased mononuclear cell infiltrate in tissues treated with CsA, compared to the non-treated ones, Bulut, *et*

al. [27] supported the idea about the participation of inflammation and local immune stimuli in the development of GO, which nonetheless remains a multifactorial process. Cell composition and the existence of inflammatory cells reflect its chronic nature, which may result in a long-term local stimulation process leading to GO. The largest degree of expression of apoptosis was registered in the group treated with the highest dose of cyclosporine, most probably due to the cyclosporine action which increases the apoptosis, and at the same time this effect is strengthened by the level of inflammatory infiltrate in the fibrous tissue. The level of inflammation in the gingival tissues and the action of cyclosporine are indisputable and important factors in triggering apoptosis. In the group with gingivitis, a greater presence of apoptosis was registered, compared to the group with periodontitis, which we believe is due to the inflammatory changes (acute stage) of the gingival tissue, accompanied with a present enlargement which influences the interrupted homeostasis.

In conclusion, our findings suggest that increased apoptosis may have a role in the pathogenesis of CsA-induced gingival overgrowth in the case of high dose of CsA**.**

Conflict of interest statement. None declared.

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