JBR Journal of Interdisciplinary Medicine and Dental Science

Mitic et al., J Interdiscipl Med Dent Sci 2014, 2:5
DOI: 10.4172/2376-032X.1000141

Research Article Open Access

Gingival Tissue and Apoptosis

Kristina Mitic[1*], **Mirjana Popovska**[1], **Ana Belazelkoska**[2] **and Rubens Jovanovic**[3]

[1]*Dental Clinical Center "St. Pantelejmon"- Clinic for Periodontology, Faculty of Dentistry , Skopje, R. Macedonia*

[2]*European University Dental Clinical Center, Skopje, R. Macedonia*

[3]*Institute of Pathology, Faculty of Medicine, Vodnjanska, Skopje, R. Macedonia*

[*]**Corresponding author:** Kristina Mitic, Dental Clinical Center "St. Pantelejmon"- Clinic for Periodontology, Faculty of Dentistry , Skopje 1000, R. Macedonia, Tel: 0038970350909; E-mail: 3mkristina@gmail.com

Received date: June 9, 2014, **Accepted date:** August 4, 2014, **Published date:** August 11, 2014

Abstract

Background: Several factors can influence gingival tissues. Among them: disrupting tissue homeostasis and occurrence of pathological conditions. The aim of our study was to investigate and compare the presence of apoptosis in patients treated with immunosuppressive therapy, patients with periodontitis and on the other hand, healthy patients, as well as to better understand the role of apoptosis in the these processes.

Methods: The first objective group consisted of 21 patients (10 males and 11 females; mean age 37.4 ± 10.2 years old) with neither kidney diseases nor treated with cyclosporine A (CsA) therapy, who had a verified periodontal disease. The second group consisted of 21 kidney-transplant patients (9 males and 12 females; mean age 36.2 ± 9.5 years old), with diagnosed gingival overgrowth (GO) subjected to 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 old) with plaque-induced gingivitis.

The following indexes were analyzed: plaque index (PI) Sillnes-Löe, index of gingival inflammation (GI) according to Löe-Sillnes, 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 semiquantitative analysis in order to determine the presence of apoptotic cells and immunohistochemical expression of the bcl-2 and p53 proteins.

Results: We found statistical differences in bcl-2 and apoptotic index, among the groups: greatest expression of bcl-2 and apoptotic index was registered in the group treated with CsA, and the lowest expression was noted in the gingivitis group($p<0.01$). There was a statistical significant positive correlation between bcl-2 and apoptotic index, PI, and GI index ($p<0,05$). There was no significant correlation between the blood concentration of CsA and apoptosis ($p>0,05$; $r = 0.187$).

Conclusion: Our findings suggest that increased apoptosis may have a role in the pathogenesis of CsA-induced gingival overgrowth in the cases of patients on high dose of CsA.

Keywords: Gingival overgrowth; Cyclosporine A; Gingival inflammation; Apoptosis; bcl-2; p53

Introduction

Tissue homeostasis is maintained by keeping a balance between cell proliferation and cell death. Inhibition of apoptosis is implicated in the pathogenesis of malignancy and autoimmune disorders and increased apoptosis is thought to be involved in acquired immunodeficiency syndrome and ischemic injury [1]. Several studies suggest that apoptosis plays an important role in the control of tissue overgrowth. Remodeling of granulation tissues occurs through apoptosis of fibroblasts. Wounds in which there is inadequate apoptosis may result in formation of fibrotic tissues [2-4]. This modulation of apoptosis may contribute to the etiology of the occurrence of fibrosis in gingival tissues.

Several studies indicate that bacterium-modulated apoptosis appears to be an important phenomenon in the pathogenesis of infectious diseases [5,6]. Specific pathogens or their exocellular products may directly induce apoptosis in host cell [7,8] and bacterial components such as lipopolysaccharide may delay programmed cell death of terminally differentiated polymorphonuclear leukocytes (PMN) [9,10].

The aim of our study was to investigate and compare the presence of apoptosis in patients treated with immunosuppressive therapy, patients with periodontitis and healthy patients, as well as to better understand the role of apoptosis in the these processes.

Materials and Methods

Patients and methods

The first group, subject to our study consisted of 21 patients (10 males and 11 females; mean age 37.4 ± 10.2 years old) with neither kidney diseases nor treated with CsA therapy, who had a verified periodontal disease where the average loss of attachment was 1.98. The

medical history of all patients excluded use of any medicament causing GO.

The second group consisted of 21 kidney-transplant patients (9 males and 12 females), diagnosed with gingival overgrowth (GO) and subjected to continuous immunosuppressive therapy (175 mg Cs / a day). The patients' mean age at the time of renal transplantation was 36.2 ± 9.5 years old. The mean duration of therapy was 42.4 ± 36.2 months. The post-transplant immunosuppressive therapy consisted of cyclosporine (Neoral®) reaching 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/den, 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 old) with plaque-induced gingivitis, but with no signs of periodontitis. The use of antibiotics, anti-inflammatory agents and the history of treatment with medicaments known to cause drug-induced GO were excluded of all patients of all three groups.

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

- Plaque Index (PI) according to Silness-Löe [11]; 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 deposits within the gingival pocket, or the tooth and gingival margin which can be seen with a naked eye; 3-Abundance of soft matter within the gingival pocket and/or on the tooth and gingival margin.
- Gingival Inflammation Index (GI) was estimated according to Löe-Silnes scale as [12]; grade 0-normal; grade1-mild inflammation, slight color change and edema, no bleeding; garse 2-moderate inflammation, redness, edema, bleeds 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 Pathological Anatomy at the Medical Faculty in Skopje.

Tissue biopsies from the second group (periodontal disease) and the third group (control group), were obtained during routine dental treatment (gingivoplasty and tooth extraction from orthodontic reasons or any other indication). All patients enrolled into the study were informed and gave their consent for participation, in accordance to the Helsinki Declaration of 1975, revised in 2000.

Furthermore, the tissue samples were placed into 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 antigene 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. Secondary antibody was then added, which was connected with biotin. The Avidin-Biotin Complex contains HRP enzyme (Horse radish peroxidase), which bonds with the biotin molecule of the secondary antibody, and that 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 involved adding AVS reagent and rinsing with phosphate buffer. The final result as a positive antigene antibody reaction was followed by forming 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 semiquantitative 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 periodontitis group, the CsA-treated group and the control group with respect to the clinical parameters and the histopathological findings were analyzed by using the Student 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.

Results

The immunohistochemical findings of the examined groups are shown in Table 1.There was no statistical differences between the groups for p53. Statistical differences were found in the levels of bcl-2 among the groups: between the first (group with periodontitis) and the second (group with CsA therapy), the first and the third group(group with gingivitis), and between the second and third group ($p<0.01$), respectively. Greatest expression of bcl-2 was registered with the second group treated with CsA, a bit lower expression in the group with periodontal disease, and lowest expression was noted in the gingivitis group. Identical results were found when comparing the apoptotic index, which was highest in the CsA group ($p<0.01$) when compared with the other groups.

Patients of the CsA group had significantly higher PI and GI values than the patients in the other groups. There were statistical differences for PI and GI among the groups, between the first (group with periodontitis) and the second (CsA group), the first and the third group (group with gingivitis), and between the second and third group ($p<0.01$), respectively (Table 2).

However, there was no correlation between p53 and the other parameters between the groups. The findings of our research showed 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).

Groups	Index	N	Percent %
Paradontitis	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
Paradontitis	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
Paradontitis	apoptoza-0	9	2
	apoptoza-1	16	76
	apoptoza-2	3	15
Cs group	apoptoza-2∗	9	43
	apoptoza-3∗	12	67
Gingivitis	apoptoza-0	2	9
	apoptoza-1	11	52

Table 1: Distribution of immunohistohemical findings in Parodontitis group, Cs group, and Gingivitis group (∗Significantly higher than the gingivitis and parodontitis grops ($p<0.01$)).

Discussion

Although the presence of bacterial pathogens is necessary for the initiation of periodontal diseases, numerous inflammatory and immune reactions also play a critical role in their progression [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 phenomenon 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.

Group	Index	N	%
Periodontitis Group	IDP-1	15	71
	IDP-2	6	29
CsA Group	IDP-2	5	24
	IDP-3	16	76∗
Gingivitis Group	IDP-0	16	76
	IDP-1	5	24
Periodontitis Group	IGI-1	16	76
	IGI-2	5	24
CsA Group	IGI-2	11	52∗
	IGI-3	10	48
Gingivitis Group	IGI-0	20	95
	IGI-1	1	5

Table 2: Distribution of clinical findings in various study groups (∗ Significantly higher than the gingivitis and parodontitis grops ($p<0.01$))

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 concentrac of CyA			0.187	

Table 3: Correlations between the different parameters (∗∗Correlation is significant at the 0.01 level)

Gamonal et al. [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 ≥ 5mm and attachment of 3mm.

Furthermore, it has been well documented that a variety of bacterial pathogens are able to induce apoptosis in the infected cells. For instance the leukotoxin from Actinobacillus actinomycetemcomitans has been reported to induce apoptosis on 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 the group with periodontal disease due to the presence of dental plaque and the consequent inflammatory changes,

thereby leading prevention of apoptotic dying of the cells. Our results also correspond with the results of Bulut et al. [17], where the frequency of grade 3+ expression of bcl-2 was found to be significantly higher in the group with generalized aggressive periodontitis (GAP) than that of the control group.

According to the results of Pandilova [18], further progressions of loss of attachment results in increased inflammation and consequently decrease of expression of bcl-2. Identical results were reported by Ellis et al. [19]. 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. [20] did not find 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, the analysis of apoptosis in our study took into account the possibility that the antiocogen 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 make its expression almost undetectable in normal healthy tissues. Upon activation, p53 stabilizes so that its expression can be detected with anti-p53 antibodies using standard immunohistochemicals techniques [21].

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 [22]. Expression of p53 protein has been noticed in the histollogically 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 [23].

Bulut et al. [24] found no significant differences between CsA and gingivitis group with respect to immunolocalization of p53 and bcl-2. Furthermore, Saito et al. [25] evaluated the expression p53 immunohistochemically in overgrown 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 impared 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. [26] demonstrated that the apoptotic process is involved in chronic, bacterially induced gingival inflammation. Analysis of data showed that inflammation leads 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 due to the fact that inflammation of the gingival tissue is greatest in the CsA tissues, and therefore apoptosis is greater. In parallel with the apoptotic processes proliferate activity also occurs in the gingival tissue as a compensatory mechanism. 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.

Alaaddinoğlu et al. [27] observed 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 of the other groups, as a result of the pseudopockets, which had a lot more plaque and at the same time difficult possibilities to maintain good oral hygiene.

The absence of any correlation of p53 with all analyzed parameters, as well as the participation in control of the cell-cycle, restricts us from any conclusion about its connection with apoptosis during peridontitis.

The findings of our research showed 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 overgrown gingiva is a result of the compensational epithelium proliferation. Similar results were reported by Minovska et al. [28], 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. [29] supported the idea about the participation of inflammation and local immune stimuli in the development of GO, which nonetheless remains a multi-factorial 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.

Greatest expression of apoptosis was registered in the group treated with highest dose of cyclosporine, most probably because of the action of cyclosporine which increases the appearance of 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, 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.

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