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THE POTENTIAL ROLE OF CD31 AND BCL-2 IN THE ETIOPATHOGENESIS OF ORAL PYOGENIC GRANULOMA

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ABSTRACT

Pyogenic granuloma (PG) is a vascular reactive lesion of the oral cavity which is caused by local trauma or irritation and has a distinct biological behavior. Up till now, the pathogenesis of PG is not adequately understood.

Aim: Assessment of the expression of CD31 and Bcl-2 in PG to clarify the etiopathogenesis of this lesion and to determine whether vascular proliferation in this lesion has a relation to its apoptotic status.

Materials and Methods: 4μm sections were prepared from paraffin-embedded blocks of PG (20 samples) and normal gingiva as a control (10 samples). Staining of each specimen with hematoxylin and eosin (H&E), CD31 and Bcl-2 was done.

Results: all lesions of PG showed positive CD31 and Bcl-2 immunostaining in the endothelial cells of blood vessels and in the stromal ovoid cells. Control group showed positive CD31expression in the endothelial cells of blood vessels while Bcl-2 expression revealed positive expression mainly in the basal cells of the epithelium. A significant higher expression of CD31 and Bcl-2 was detected in PG than normal gingiva. A strong positive correlation was found between CD31 and Bcl-2 area percent of immune-expression.

Conclusions: The data of the present study suggest a role of Bcl-2 overexpression in the potentiation of angiogenic response and support the role of CD31 and Bcl-2 in the etiopathogenesis of PG.

Keywords: Pyogenic Granuloma, CD31, Bcl-2, Immunohistochemistry

INTRODUCTION

Pyogenic granuloma (PG) is a relatively common reactive lesion which mostly affects the oral mucosa and skin. The term pyogenic granuloma was first introduced by Hartzell in 1904 and is still being used to represent this lesion.[1] But this term is confusing as neither it is caused by bacterial infection nor does it contain any pus. In addition, there is no granuloma formation histopathologically.[2] It is considered as an inflammatory hyperplasia.[3]

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PG arises as a painless nodular mass which may be rapidly growing in nature but rarely exceeding 2.5 cm.[4] It may have a smooth or lobulated surface and a sessile or pedunculated base. The lesion appears red due to the presence of numerous capillaries. Bleeding is a common finding.[5,6,7] Marked histopathological finding in pyogenic granulomas is a prominent capillary growth within a hyperplastic granulation reaction. A mixed inflammatory cell infiltrate of neutrophils, plasma cells, and lymphocytes is evident.[4,8]

A possible correlation has been found between PG and chronic physical irritations. These stimuli initiate the healing and repair response and may lead to inappropriate regulation of inflammatory reaction, hyperplasia, angiogenesis and hence, improper formation of granulation tissue which is the histopathological hallmark of PG. Though, PG is considered as a reactive lesion rather than a neoplastic one. Depending on these observations, it becomes obvious that pathogenesis of PG could be related to either inflammatory response or angiogenesis.[4,9] The most common etiologic factors are poor oral hygiene with accumulated plaque and calculus in the gingival crevice and overhanging restorations.[10,11] Hormonal disturbances could be also considered precipitating factors for pyogenic granuloma. The gingiva is the most frequently affected site.[12,13] Buccal mucosa, tongue and lips could also be affected. Incomplete surgical removal, failure of removing irritating factors and repeated trauma are considered reasons for recurrence.[10]

The pathogenesis of PG is not well established. Owing to the clinical behavior of this lesion as rapid growth rate and repeated recurrence some investigators consider it as a benign neoplasm but it is believed to be an inflammatory reactive lesion caused by different stimuli.[3,14,15]

The marked growth of capillaries in PGs indicates a potent angiogenic action (formation of new blood vessels) which may play a role in their etiopathogenesis.[8,9,16,17] Detection of blood vessels could be achieved by the utilization of antibodies that have an affinity for specific epitopes on the surface of endothelial cells as CD34, CD31 and factor VIII. [18]

CD31 or Platelet-endothelial cell adhesion molecule (PECAM-1) is a signaling receptor which is significantly expressed on the surface of the adult and embryonic endothelial cells. This molecule is a transmembrane glycoprotein that has a cytoplasmic domain. [19,20] CD31 is essential for angiogenesis due to its significant role in the adhesion between endothelial cells[21] and is considered one of the greatest valuable markers that detect endothelial cells.[22]

Apoptosis or programmed cell death is a physiologic normal function which maintains tissue homeostasis.[23] Consequently, disturbances in the function of the apoptotic system may lead to prolonged survival or excessive removal of cells and thus pathogenesis of different diseases. Various stimuli can modulate apoptosis such as growth factors, hormonal changes, cytokines, immunological responses and viral or bacterial infections.[24]

Bcl-2 protein is regarded as an apoptosis suppressor. It is a component of the outer mitochondrial membrane and a part of the endoplasmic reticulum.[25] This protein can interrupt apoptosis not only in the initial phase but also in the final one. That is because Bcl-2 stabilizes the mitochondrial membrane potential as well as it inhibits intracellular acidification and oxygen-reactive species formation.[26]

In the current work, the expression of CD31 and Bcl-2 in PG and normal gingiva (control) was investigated using immunohistochemical staining. The possible correlation between both markers was statistically analyzed. The aim is to gain a better understanding of the etiopathogenesis of PG that may have an impact on its biological behavior as

well as to determine whether vascular proliferation in this lesion has a relation to its apoptotic status.

MATERIALS AND METHODS

A total of twenty archival paraffin blocks of oral pyogenic granuloma were retrieved from:

- The pathological files of General Pathology Department, Nasser Institute for Research and Treatment, Ministry of health and population.
- The pathological files of General Pathology Department, Al Hussein Hospital, Al Azhar university.

Normal gingiva samples from 10 patients free of any systemic disorders were used as controls. Samples were taken after tooth extraction for orthodontic reasons. They were fixed in formalin and embedded in paraffin.

Section preparation

Three sections from each formalin-fixed, paraffin-embedded tissue block were cut into 4 μm thickness and stained with the following:

a) First section stained with hematoxylin and eosin to confirm the diagnosis of pyogenic granuloma.

b) Second section stained with CD31 polyclonal antibody.

c) Third section stained with Bcl-2 monoclonal antibody.

Immunohistochemical (IHC) staining procedure

The sections were deparaffinized with xylene and rehydrated in graded ethanol for IHC staining with CD31 and Bcl-2 antibodies. Heat mediated antigen retrieval was done using citrate buffer PH (6.0), then the sections were immersed in hydrogen peroxide (H2O2) to block the endogenous peroxidase activity, washed in phosphate-buffered saline (PBS), and then protein blocking reagent was added and incubated for 20 minutes at 37°C within humid chamber to reduce the non-specific staining. The primary antibodies used in the present study were as follows:

- Concentrated polyclonal rabbit antibody for CD31 (Code No. PA5-16301 at dilution 1:50, Thermo Fisher Scientific USA).
- Concentrated monoclonal mouse antibody for Bcl-2 (code No. M0887 at dilution 1:50, Dako, Denmark).

Sections were incubated with the primary antibody overnight.

The sections were then washed twice in PBS and treated with the labelled streptavidin biotin complex (LSAB + System-HRP, Dako) at room temperature to bind the primary antibodies. Peroxidase activity was visualized by immersing the tissue sections in diaminobenzidine (Liquid DAB+ Substrate, Dako), which resulted in a brown reaction product. Finally, the sections were counterstained with Mayer's hematoxylin and cover-slipped.

Immunohistochemical Interpretation

The immunoexpression of Bcl-2 was evaluated by presence of brown colored immunostaining reaction in the cytoplasm of the endothelial cells and stromal cells of the connective tissue, and in the basal cells of the epithelium.

Presence of brown colored immunostaining reaction in the cytoplasm and cell membrane of endothelial cells and stromal connective tissue cells was considered a positive reaction for CD31.

In each slide, 5 microscopic fields showing the highest immunopositivity were selected and photomicrographed using image analyzer.

Immunoreactivity, for CD 31 and Bcl-2 was assessed by estimating the area percentage of positive immunostained cells in relation to the area examined in each microscopic field using computerized image

analyzer (Leica Qwin - Germany) at research unit (Faculty of Dentistry Cairo University).

The image analyzer consisted of a colored video camera, colored monitor, and hard disk of *hp* personal computer connected to the microscope and controlled by Leica Qwin 500 software. The image analyzer was calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. The area and area percentage reaction were measured using a magnification x200. Mean values were then obtained for each specimen.

Statistical analysis

Statistical analysis was then performed using a commercially available software program (SPSS 19; SPSS, Chicago, IL, USA).

As data was parametric, significance of the difference between both groups was evaluated using unpaired t test.

Pearson correlation test was used to study correlation between different parameters. The Pearson correlation coefficient is used to measure the strength of a linear association between two variables, where the value r = 1 means a perfect positive correlation and the value r = -1 means a perfect negative correlation. The level of significance was set at $P < 0.05$.

RESULTS

1-Heamatoxylin and eosin stain findings

Histologically PG revealed a hyperplastic keratinized stratified squamous epithelium. The underlying connective tissue stroma revealed collagen fibers with numerous dilated capillaries engorged with red blood cells, inflammatory cell infiltration and plump ovoid endothelial cells.

For the control (normal gingival tissue), the epithelium displayed a keratinized stratified squamous epithelium that covers a core of connective tissue. The epithelium revealed a normal arrangement of its layers (6–8 layers). The basement membrane was flat with no extended rete ridges. The underlying connective tissue revealed normal arrangement of its fibers, blood vessels, and few chronic inflammatory cells (Figure 1, A, B).

Immunohistochemical findings

CD31 immune-reactivity:

All lesions of PG showed CD31 positive immune-reactivity in the endothelial cells of blood vessels and in the stromal ovoid cells. The expression was cytoplasmic and membranous.

For normal gingival tissues, all specimens showed staining of endothelial cells of blood vessels (figure 1, C, D).

Bcl-2 immune-reactivity:

All lesions of PG showed cytoplasmic immunopositivity for Bcl-2 in the endothelial cells of blood vessels and in the stromal cells. Normal gingival tissues showed Bcl-2 expression mainly in the basal cells of the epithelium. (figure 1, E, F).

Results of the immunohistochemical staining:

1. Comparison between the expression of Bcl-2 and CD31 in pyogenic granuloma and normal gingiva

Regarding Bcl-2 area percent of immunoexpression, a higher mean value was recorded in pyogenic granuloma than the control group with a statistically significant difference ($p<0.0001$), (Table 1, Figure 2)

Regarding CD31 area percent of immunoexpression, a higher mean value was recorded in pyogenic granuloma than the control group with a statistically significant difference ($p<0.0001$), (Table1, Figure 2)

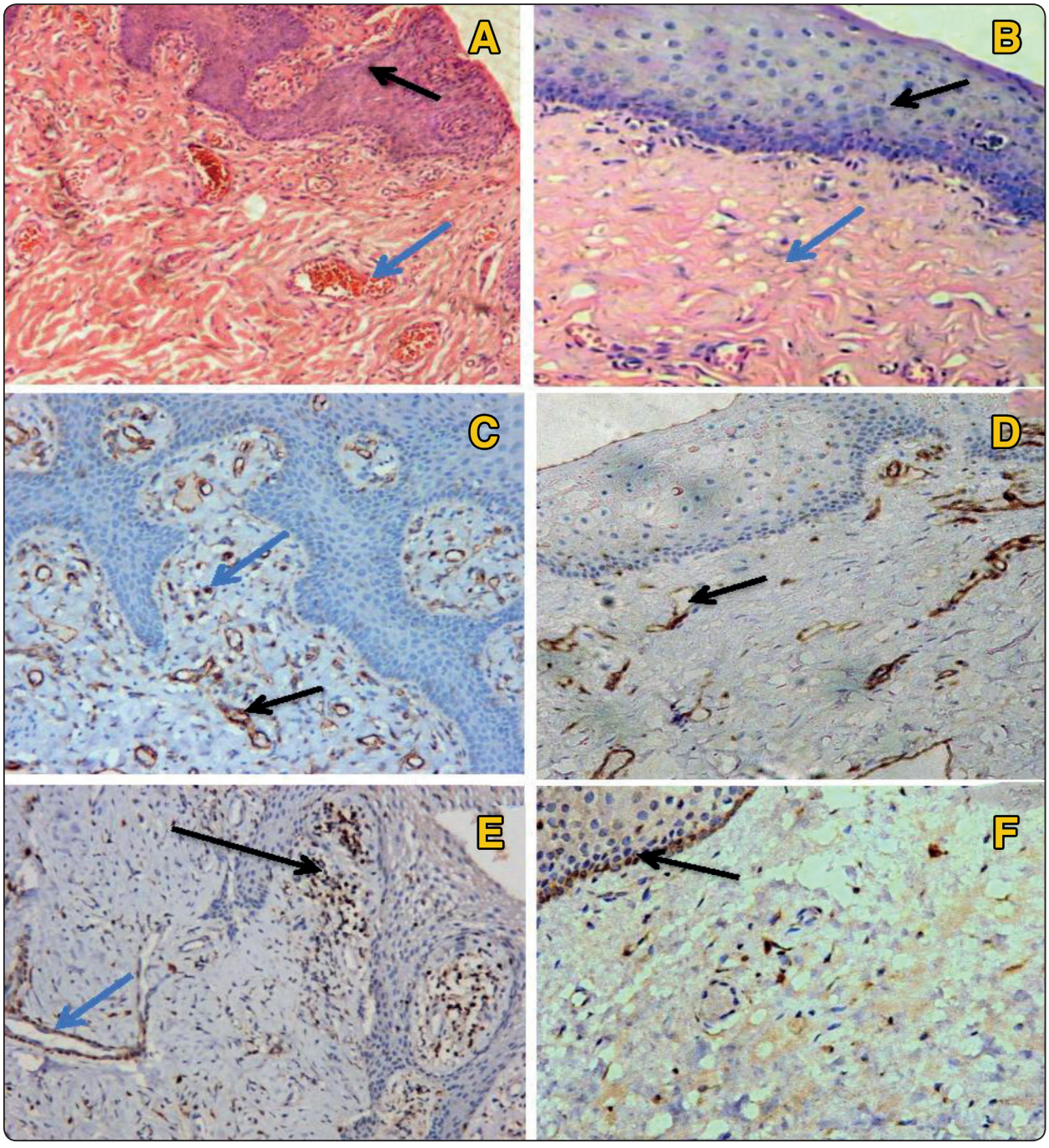

Fig. 1 (A): A photomicrograph of PG showing a hyperplastic keratinized stratified squamous epithelium (black arrow). The underlying connective tissue stroma revealed numerous dilated capillaries engorged with red blood cells (blue arrow) (H&E x100). (B): A photomicrograph of normal gingival tissue (control) showing a keratinized stratified squamous epithelium (black arrow) covering a core of connective tissue (blue arrow). The epithelium reveals normal arrangement of its layers (H&E x100). (C): A photomicrograph of PG showing immunopositivity for CD31 in the endothelial cells of the blood vessels (black arrow) and in the stromal ovoid cells (blue arrow) (CD31 x200). (D): A photomicrograph of normal oral epithelium showing CD31 expression in the endothelial cells of blood vessels (black arrow) (CD31 x100). (E): A photomicrograph of PG showing immunopositivity for Bcl-2 in the endothelial cells of blood vessels (blue arrow) and in the stromal cells (black arrow) (Bcl-2 x200). (F): A photomicrograph of normal gingival tissue showing Bcl-2 expression mainly in the basal cells of the epithelium (black arrow) (Bcl-2 x200).

TABLE (1) Comparison between the expression of Bcl-2 and CD31 in pyogenic granuloma and normal gingiva (unpaired t test)

	bcl-2 Area percent		CD31 Area percent	
	Pyogenic Granuloma	Normal gingiva	Pyogenic granuloma	Normal gingiva
Mean	19.719	0.752	5.302	0.61
Std Dev	1.795	0.363	0.73	0.225
Max	22.714	1.222	6.4	1.299
Min	17.974	0.333	4.92	0.119
T	32.75		3.22	
P value	<0.0001*		<0.0001*	

Significance level p<0.05, *significant, ns=non-significant

2- Correlation between expression of Bcl2 and CD31 in pyogenic granuloma.

Pearson's correlation test revealed a strong positive correlation between CD31 and Bcl-2 area percent of immunoexpression (Table 2, Figure 3).

TABLE (2) Correlation between Bcl-2 and CD31 area percent of immunoexpression in PG

R	R^2	Interpretation	P value
0.9683	0.9375	strong positive	<0.0001* (significant)

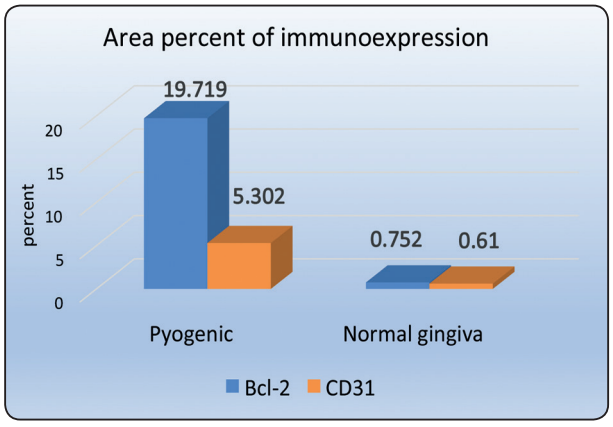

Fig. (2) Column chart showing mean Bcl-2 and CD31 area percent of immunoexpression in pyogenic granuloma and normal gingiva

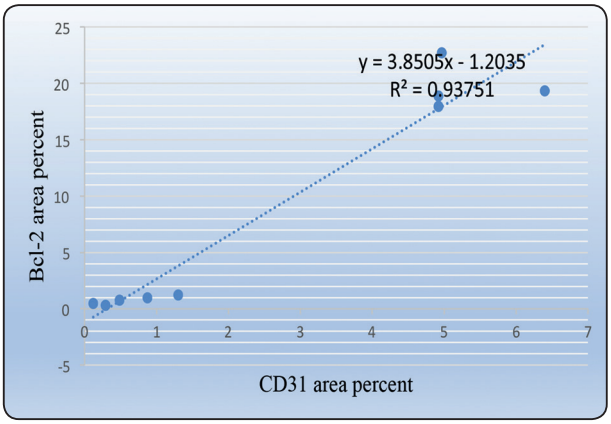

Fig. (3) Scatter plot showing correlation between CD31 and Bcl-2 area percent of immunoexpression in PG

DISCUSSION

Angiogenesis is controlled by the interaction between angiogenesis activators and inhibitors which are secreted by tumor cells and stromal cells. Inflammatory cells as macrophages and mast cells have an important role in angiogenesis and development of PG through the production of inflammatory mediators. Though the changes in the balance between angiogenesis stimulators and inhibitors can lead to the formation or regression of the blood vessels. [27]

The aim of the present study is to gain a better understanding of the etiopathogenesis of PG that may have an impact on its biological behavior as well as to determine whether vascular proliferation in this lesion is related to its apoptotic status.

PG may be regarded as an enormous expression of granulation tissue formation during an extended healing procedure after trauma or inflammation.[28,29] Local inflammation or trauma activates the release of cytokines (as angiogenic factors and basic fibroblast growth factor) from the macrophages and mast cells at the site of the injury. These cytokines stimulate angiogenesis process and neovascularization.[30]

CD31 immune-reactivity in the present study was detected in the endothelial cells of the blood vessels and in the ovoid stromal cells of PG. Similar

expression pattern was obtained in the previous studies of Saghafi et al., 2011, Freitas et al., 2005 and Rezvani et al., 2010.[9, 31,32] Normal gingival tissues revealed CD31 expression mainly in the endothelial cells of blood vessels which meet the results of the previous study of Kasprzak et al., 2012. [33]

The results of the current work showed a higher mean area percentage of CD31 immunoexpression in pyogenic granuloma in comparison with normal gingiva with a statistically significant difference that are in accordance with the results of the study of Seyedmajidi et al., 2015[34] and could be explained by the results of the study of Rezvani et al., 2010. They found CD31immunoreactivity in the lining endothelial cells and the proliferating stromal cells of PG and suggested that these stromal ovoid cells are mostly immature endothelial cells.[32] Hematopoietic stem cells or monocytic endothelial cell progenitors could be the origin of this immature cells in PG. The endothelial cells that derive from monocytes express endothelial cell markers including CD31.[35]

Moreover, the study of Blackwell et al., 2016 confirmed the presence of immature cells in PG. It was the first study that demonstrates embryonic stem cell (ESC) markers expression in PG and revealed the presence of hematopoietic stem cells in this lesion. They displayed the expression of the ESC markers SOX2, pSTAT3 and NANOG on the lining endothelial cells and the stromal endothelial cells.[36]

One of the regulators of endothelial cell progenitors is vascular endothelial growth factor (VEGF). It can stimulate the transformation of the progenitor cells to endothelial cells (both hematopoietic stem cell and monocytic endothelial cell progenitors). VEGF mediated this action by increasing both cell differentiation and proliferation.[37] It also acts as a chemotactic factor for endothelial cell progenitors and stimulates vascular permeability, assisting the entrance and migration of progenitor cells at the injured site.[38,39]

The most common cells that synthesize and secrete VEGF are macrophages, fibroblasts, mast cells and epithelial cells. Their role is manifested in case of chronic inflammation.[40,41]

Taken together, the results obtained in the previous studies and the data reported in the current work suggest that angiogenesis has an essential role in the etiopathogenesis of pyogenic granuloma. The increased expression of CD31 in PG may correspond to the reactive or inflammatory character of this lesion.[32] Though, estimation of the expression of angiogenesis markers can assist the diagnosis and treatment of vascular lesions and may play a role in the production of new therapies that depend on anti-angiogenic drugs.[17,34]

Bcl-2 is an apoptosis suppressor protein.[25] It is a part of the outer mitochondrial membrane and the endoplasmic reticulum.[42]

Studies that had been done on the expression of Bcl-2 in oral reactive lesions are scarce. So, in the current work, Bcl-2 expression in oral pyogenic granuloma and normal gingiva was assessed. The study of Nakamura, 2000 is the only study that had been done to investigate the expression of Bcl-2 marker in PG.[14]

In the present study, PG showed increased expression of Bcl-2 than normal gingiva. Cytoplasmic immunopositivity for Bcl-2 was detected in the endothelial cells of blood vessels and in the stromal cells. Similar results were obtained in the study of Nakamura, 2000. He reported that PG showed less apoptosis and higher expression of Bcl-2 in comparison to conventional granulation tissue (GT) and suggested that suppression of apoptosis in PG played a role in its rapid growth.[14] The difference in the behavior between PG and GT could be related to apoptosis regulation. Granulation tissue in wound healing process shows regression to scar tissue due to apoptosis of endothelial cells and fibroblasts.[43,44]

All the samples of normal gingiva of the present work revealed positive staining with Bcl-2 that was detected in the basal cells of the epithelium and are consistent with previous studies.[23,45] This pattern of expression in normal epithelium revealed the role of the progenitor basal cells, which need protection against apoptosis to maintain the survival of the whole epithelium.[46]

Bcl-2 area percent of immunoexpression, showed a higher mean value in pyogenic granuloma than normal gingiva, with a statistically significant difference.

Previous studies revealed that decreased apoptosis of endothelial cells may have a role in the rapid growth of PG. [8,14] The increased expression of Bcl-2 in the endothelial cells can facilitate the generation of a sustained potent angiogenesis through the expression of angiogenic stimulators as VEGF.[47] In addition, Bcl-2 may act to enhance the maturation of the newly developed vessels.[48]

Altogether these data explain the strong positive correlation between CD31 and Bcl-2 area percent of immunoexpression in the present study and suggest that Bcl-2 overexpression may have a role in angiogenesis process.

CONCLUSION

The results of the current work are suggestive of a role of Bcl-2 overexpression in the potentiation of angiogenic response. They also support the reactive nature of pyogenic granuloma and the role of both angiogenesis and apoptosis in the etiopathogenesis of this lesion.

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