Immunohistochemical Expression of PCNA and Ki-67 in Periapical Granuloma and Radicular Cyst

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Abstract

Objectives: Periapical Granulomas (PGs) and Radicular Cysts (RCs), as the most common odontogenic lesions have yet unclear pathogenesis. This study was aimed to compare PCNA and Ki-67 expression in PGs and RCs and evaluate their possible relationship with two lesions.

Methods: In this cross-sectional descriptive study, twenty PGs and twenty RCs were evaluated immunohistochemically using an anti-PCNA and anti-Ki-67 polyclonal antibodies. $PCNA^+$ and $Ki\text{-}67^+$ cells were counted in connective tissue wall and epithelial lining (in RCs). Statistical analysis was performed by using Mann-Whitney U test and Spearman's rank correlation coefficient**.**

Results: In PGs, percentage of PCNA and Ki-67 expression were found 70% and 30%, respectively; In RCs, PCNA and Ki-67 expression were observed 90% and 55%, respectively. Additionally, in RCS, Immunoexpression of PCNA (85%) and Ki-67 (60%) were detected at epithelial lining area. The positive immunoexpression of PCNA in RCs was greater than PGs ($p<0.05$).

Conclusion: Immunoexpression of PCNA and Ki-67 were detected in both lesions which may be mentioned as valuable markers for the prediction of biologic behavior of PGs and RCs.

Key words: Immunohistochemistry, Ki-67, PCNA, Periapical Granuloma, Radicular Cyst

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Introduction

Periapical lesions are focal inflammatory conditions represents host defensive reaction in response to bacterial infection (toxins, metabolites) in root canal with spread into the apical area (1). Periapical lesions result in apical periodontitis, a disease of persistent microbial infection of the root canal system of the affected tooth (2). This inflammatory condition can lead formation of inflammatory fibro-vascular connective tissue as a result of bone resorption in Periapical lesion; therefore form a PG (3). Latter, the epithelial rest of malassez in PGs can be stimulated to proliferate by inflammatory stimuli to convert the lesion into the RCs, but the actual genesis of the cyst is not clear (4-7).

RCs is the most common odontogenic cyst with inflammatory origin which has unknown pathogenesis, found more in fourth and fifth decades of life and occurs more frequently in maxilla (8, 9). It has a thin, regular and atrophic layer of stratified squamous epithelium, which presents mild to moderate inflammatory activity and considers as the replication of its functional activity (10). RCs have some complications including pain, infection, root displacement and resorption of adjacent root as a result of cyst enlargement (11).

Several studied have reported the expression of growth factors such as PCNA and Ki-67 which are value markers in prediction of

biological behavior of cyst lesion (3, 12, 13).
PCNA is a nuclear non-hist one protein which is necessary for DNA synthesis and elevated during the G1/S phase of the cell cycle (14). PCNA expression may be used as a marker of cell proliferation because cells remained a longer time in the G1/S phase when proliferating and has an essential role in nucleic acid metabolism as a component of DNA replication and repair mechanism. Increased level of PCNA may be induced by growth factors or as a result of DNA damage in the absence of cell cycling (15).
Ki-67 antigen is the nuclear protein which is expressed by proliferating cells in nuclear of growing cells and in all phases of active cell cycle (G1, S, G2 and M phase) (16) which has the peak at G2 and M phase. One of the most applications of Ki-67 antibodies is establishing the cell growing rate in different neoplasms (17). As a fact, inflammation in PGs is the main reason for epithelial proliferation and RCs formation. However, some of them are not converting into the cyst. This study was aimed to assess the Immunohistochemical expression of PCNA and Ki-67 markers in PGs and RCs to assess if proliferation activity of inflammatory cells, especially chronic inflammatory cells play role as a causative factor to changing a PG to RC.

Methods

This cross- sectional study was performed on archival cases. Forty formalin fixed and paraffin embedded samples were obtained from Oral pathology department of Dental school, Shahid Beheshti University of Medical Sciences, Tehran, Iran for this cross-sectional descriptive study. All the samples were obtained from human teeth including PGs and RCs of twenty each.

Immunohistochemical procedures

For all the Sections, 4-μm thick sections were mounted for sialinized cated slides, deparaffinized in xylene and rehydrated through graded alcohol. To prevent endogenous peroxidase activity, they immersed in 0.3% hydrogen peroxidase over the slides for 10 minutes. An antigen retrieval procedure was performed by placing the sections in a citrate buffer solution in the microwave oven (700w, 15 min). The specimens were then treated with non-serum protein for 30 minutes to prevent unspecific staining. For immunostaining, sections were incubated overnight at 4°C with the primary antibodies as follows:
Anti PCNA that reacts with N1529 (ready to use, Dako, Denmark) and anti Ki-67 that reacts with N163330-2 (ready to use, Dako, Denmark). The day after, the excess antibodies were removed by washing with Tris Buffered Saline (TBS) for ten minutes. Next, the sections were treated with the secondary Biotinylated anti-mouse and anti-rabbit immunoglobulin in 1:200 dilution (E0354; Dako) for twenty minutes at 37°C followed by rinsed with TBS. Afterwards, sections were treated with streptavidin conjugated to horseradish peroxidase for 20 minutes followed by rinsed with TBS for 10 minutes and Visualizing by a 3, 3’diaminobenzidine hydrochloride (DAB; Sigma, St. Louis, MO, USA). The sections were incubated and remained in tap water for 10 minutes. The sectioned were counterstained by Meyer's hematoxylin, rehydrated in alcohol, cleared in xylene and mounted DPX and cover slipped.

Staining evaluation

For analysis of PCNA and Ki67 positive cells, the specimens were evaluated by two operators using optical microscope (OLYMPUS model CHK) at X400 magnification with a double blinded system.

The inflammatory cells (and epithelial cell in RCs) were considered to be positive if there was any staining of the nucleus, regardless of staining intensity. The average number of positively stained nuclei of connective tissue inflammatory cells in both lesions and in epithelial lining of RCs were calculated per specimen and the intensity of immune staining was qualitatively assessed as: weak, less than 10%; moderate, between 10 to 50% and strong, greater than 50% based on previous study (11) and assess the current samples in our study. Cells with non-stained nuclei were considered as false positive and were not counted.

The data were recorded in SPSS (Statistical Package for the Social Sciences, 17 Inc., Chicago, IL, USA). Statistical analysis was performed using Mann-Whitney U test and Spearman's rank correlation coefficient. The level of significantly was set at $P \leq 0.05$.

Result

Expression according to tissue

The Immunohistochemical examination of PGs and RCs revealed that PCNA and Ki-67 markers were found to be positive in 40 specimens of two lesions. The degree of immunostaining is presented in Table 1.

According to the data, mean percentages of PCNA positive cells showed that RCs had the significant (P=0.31) higher number of positive cells comparing with PGs (Figures 1, 2). In addition, the positive ratio of Ki67 cells didn't show significant differences between RCs and PGs (Figures 3, 4) (P=0.31).

Table 1- Comparison of staining degree of positive PCNA and Ki-67 cells (n=20, %) in each lesion

marker	Staining degree	PGs	RCs
PCNA	weak	6(30)	2(10)
	Moderate	10(50)	8(40)
	strong	4(20)	10(50)
Ki-67	weak	14(70)	9(45)
	Moderate	4(20)	11(55)
	strong	2(10)	0(0)

Expression according to epithelium

The PCNA and Ki-67 found to be positive in RCs (Table 2). The Ki-67 ratio of weak to moderate inflammatory changes was 40-45% and of the moderate to strong changes was 45-15%, respectively. The PCNA positive cells of the weak to moderate inflammatory changes was 15-45% and of the moderate to strong was 45-40%, respectively (Figures 5, 6). The correlation of positive PCNA cells in epithelial cells and tissue presented direct significant correlation (P=0.046). Furthermore, direct significant correlation (P=0.000) was detected in evaluating the correlation of positive Ki-67 cells in epithelial cells and tissue.

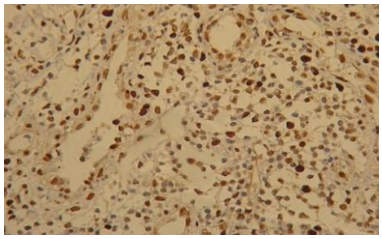

Figure1- Photomicrograph of PG showing PCNA Immunostainnig (Magnification X400)

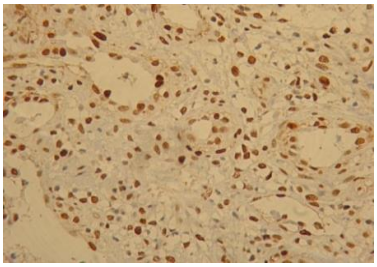

Figure 2- Photomicrograph of PGs showing Ki67 Immunostainig (Magnification X400)

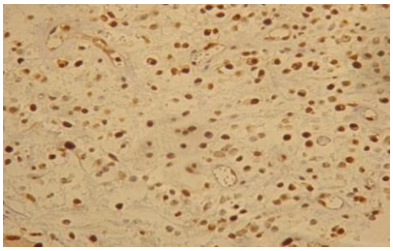

Figure 3- PCNA Immunostainig in the cyst wall of RC (Magnification X400)

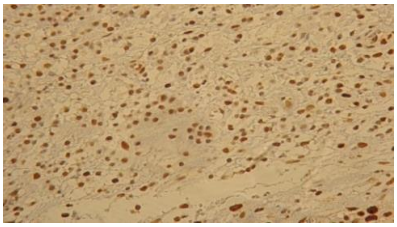

Figure 4- Ki67 Immunostainig in cyst wall of RC (Magnification X400)

Table 2- Staining degree (n, %) in RCs.

	Staining Degree	
Stain	**PCNA**	**Ki-67**
Weak	3 (15)	8 (40)
Moderate	9 (45)	9 (45)
strong	8 (40)	3 (15)

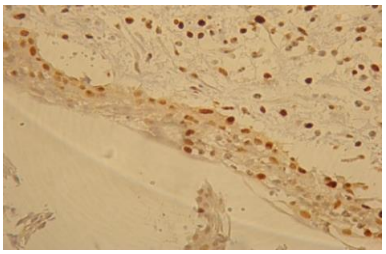

Figure 5- PCNA Expression in epithlial lining of RC (Magnification X400)

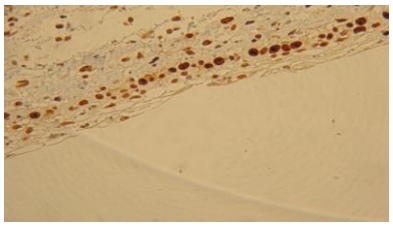

Figure 6- Ki-67 expression in epithelial lining of RC (Magnification X400)

Discussion

Periapical lesions develop in response to the inflammatory stimuli originate from bacterial infection of root canals with production of growth factors, inflammatory cytokines (interleukin-1, interleukin-6 and TNF) which result in cell proliferation (1, 4). In addition, releasing inflammatory cytokines could result in cell stress, which causes Immunoexpression of PCNA and Ki-67 markers in PGs (18). The epithelial cell proliferation in PGs results in RCs formation. Many studies have reported the Immunoexpression of PCNA and Ki-67 in premalignant and malignant lesions in oral cavity (3, 11, 12). However, limited studies have focused in the role of mentioned markers in Odontogenic cysts and neoplasms (19, 20, 21). On the other hand, most studies focused on inflammatory pathway in response to antigenic irritation, bone resorption or cellular or hormonal response with focusing on interleukins (interleukin-21 and interleukin-33) (22, 23) or degranulation of mast cells (24) which explains some differences between PGs and RCs and between heavily inflamed and slightly inflamed RCs and between fibrous and inflamed area of RCs (22-23). Moreover, few studies assess the role of connective tissue and CD-10 expression in RCs comparing with other odontogenic cysts (24). Although, the key point which start the RC formation from pre-existing PG is not clearly defined (25-30). Studies have mentioned the presence of inflammation as an important factor in increasing proliferating activity in the epithelial lining of non-inflammatory Odontogenic cyst especially in Odontogenic Keratocyst OKC (19-31). The first hypothesis was introduced by Rodu *et al.* (1987) which suggested inflammation in the cyst wall of OKC when produces a change in epithelial lining influence on biologic behavior (32). This theory is confirmed by De

Palma *et al.* (2010) which showed PCNA and Ki-67 expression in inflammatory OKCs which is higher than non-inflammatory OKCs (33). Although, OKS showed the highest proliferation activity due to the expression of PCNA and RCs was the second among all Odontogenic cysts, which has the highest epithelial proliferation activity and PCNA expression in basal layer compared with OKC, Gorlin Cyst and dentigerous cyst (15, 31). These data suggests that inflammation is not only a critical point for converting PGs to RCs, but also may play important role in cyst aggressiveness and biological behavior especially if inflammatory stimuli persist. Similar finding were observed for K-i67 expression in OKC comparing with RCs (19).

The present study investigated the expression of PCNA and Ki67 staining in PGs and RCs. The result exhibited the significant ratio of PCNA and Ki-67 markers in both lesions under studied. According to the data, RCs had greater ratio of Ki-67 following PCNA (90% and 55%, respectively) comparing with PGs (70% and 30%, respectively). RCs had greater mean percentage of Ki-67 in basal layer, which indicated a natural proliferation activity. However, some RCs had greater mean percentage of Ki-67 in suprabasal layers which may indicated the increased proliferative or aggressive potential of epithelial cells in cyst walls. Slootweg (1995) showed that RCs had greater positive immunostaining of Ki-67 in basal layer comparing with OKC which showed the suprabasal layer had greater Ki-67 expression that explained their different behavioral biology (34). Moreover, Shahela *et al*. (2013) findings showed 100% of RCs had positive PCNA staining of epithelial lining, and in all cases both basal and parabasal layers were positive (35). This findings show some differences with our findings, which shows 85% positivity of PCNA staining in epithelial lining of RCs and higher percentage of basal layer staining comparing with suprabasal layer. In our study, the epithelial lining of RCs had greater PCNA values (85%) comparing with Ki-67, which are in agreement with finding of Tripi *et al*. (2003) (36). However, the differences between two lesions were not determined. Ørstavik and Pitt Ford (1998) reported that cysts with smooth and constant thickness of epithelial lining reached the maximum growth and maintained a constant thickness (37). This is in contrast with our study that revealed greater Immunoexpression of Ki-67 and PCNA in RCs with variable thickness of epithelial lining. Our results strengthen the theory of the role of stimuli in connective tissue for epithelial proliferation and explain the lack of differentiation of some PGs into RCs in correlation with growth factors including Ki-67 and PCNA (6, 8). However, further studies utilizing a larger sample size and more aggressive lesions such as large RCs are recommended.

Conclusion

The present results revealed that the positive ratio of PCNA and Ki-67 markers was detected in PGs and with a greater percentage in RCs; which shows more proliferative activity in RCs maybe due to connective tissue intermediates and immunopatholgic reactions.

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Conflict of Interest: "None Declared"

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