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Cellular proliferation markers in peripheral and central fibromas: a comparative study

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ABSTRACT

Objective: To perform a comparative study of the cellular proliferation in the peripheral and central fibromas. Material and Methods: Immunohistochemistry for PCNA and the AgNOR technique were performed in 9 cases of peripheral odontogenic fibroma (POF), in 4 cases of odontogenic fibroma (OdF), in 8 cases of peripheral ossifying fibroma (PEOF) and 7 cases of ossifying fibroma (OsF). The Kruskal-Wallis and Mann-Whitney tests were used for the statistical analyses. Results: Mesenchymal component of the central lesions presented a higher mean number of AgNOR *per* nucleus and PCNA index than did the peripheral lesions ($P \leq 0.05$). The mean number of AgNOR per nucleus in the epithelial component proved to be higher in the OdF than in the POF ($P \leq 0.05$). The mesenchymal and epithelial components presented similar mean numbers of AgNOR per nucleus and PCNA index in the OdF, as well as a similar mean number of AgNOR per nucleus in the POF. Conclusions: The mesenchymal component may well play a role in the differences between the biological behaviour of the central lesions as compared to the peripheral lesions. Moreover, considering that the epithelial and mesenchymal components in odontogenic fibromas presented a similar proliferation index, more research is warranted to understand the true role of the epithelial components, which are believed to be inactive in nature, as well as in the development and biological behaviour of these lesions.

Key words: AgNORs. Odontogenic tumours. Ossifying fibroma. PCNA.

INTRODUCTION

Odontogenic fibroma (OdF) is a rare odontogenic tumour classified by the World Health Organization[6] as a benign fibroblastic neoplasm containing a large amount of apparently inactive odontogenic epithelium. OdF presents a slow-growing, progressive but painless swelling, often with cortical expansion or tooth displacement. A recurrence rate of 13% after enucleation has been reported in the literature[6,18,22]. Peripheral odontogenic fibroma (POF) is the rare peripheral counterpart of OdF. POF is an apparently innocuous, elevated gingival lesion that has yet to produce conclusive data regarding its exact prognosis[1,11,12]. The ossifying fibroma (OsF) is a benign fibro-osseous neoplasm which consists of a benign connective-tissue matrix and islands, or trabeculae, of new bones[13,25]. Curettage or radical surgical resection is the most common treatment for OsF, depending on the lesion's size. Recurrence rates varied from 6% to 28% for mandible lesions, while the recurrence rates for maxillary lesions are unknown[18]. Nevertheless, the peripheral ossifying fibroma (PEOF) is a condition of the inflammatory reactive nature associated with mineralization and derived from the periodontal ligament cells.

Dental calculus, plaque, dental appliances, ill-fitting crows and rough restorations are considered to be local irritants[7,9]. Local surgical excision is the most common treatment for PEOF and the recurrence rate is approximately 16.0%[7].

Nucleolar organizer regions (NOR) represent the loops of DNA which actively transcribe to ribosomal RNA, thus transcribing to ribosomes and ultimately to protein. The NOR are associated with acidic argyrophilic non-histonic proteins which can be viewed through the AgNOR technique[20,24]. Studies have applied this technique as a useful method to evaluate the differences among cellular proliferation indexes in non-neoplastic reactive lesions, as well as in benign or malignant neoplasms[3,9,15,17,23].

The proliferating cellular nuclear antigen (PCNA) is a 36-kD acidic non-histone nuclear protein which acts as an auxiliary protein for DNA delta polymerase, which is an absolute requirement for DNA synthesis. Its distribution in the cell cycle increases through the G_1 phase, peaks at the G_1/S interphase and decreases in the G_2 phase. Immunohistochemical analysis of PCNA has also been used as an auxiliary tool in the evaluation of cellular proliferation indexes in lesions with variable biological behaviour[5,17]. Although PCNA is considered to be a cellular proliferation marker, it has been established that growth and technical factors, repair processes, biological half-lives of approximately 20 h and cytokines released by the tumour or by inflammatory cells may influence the PCNA immunoexpression[5,16].

OdF, POF, OsF and PEOF contain similar histomorphological features, but present different conceptions in nature and classifications[1,6,7,25]. Since the classification of odontogenic lesions is still a major theme of discussion, additional information concerning the proliferation indexes is warranted in an attempt to better clarify the differences in biological behaviour among OdF, POF, OsF and PEOF. Also, another important fact is the comparative analysis of cellular proliferation between the POF and PEOF, which were considered the same pathology in the past[12]. Therefore, the core aims of this study are: 1) to determine the cellular proliferation of OdF, POF, OsF and PEOF and 2) to compare the cellular proliferation indexes among these lesions.

MATERIAL AND METHODS

Ethics statement

The present study's protocol was approved by the Committee of Bioethics in Research from the Universidade Federal de Minas Gerais (UFMG, COEP – 124/07).

Specimens

Specimens of the OdF (4 cases), POF (9 cases), OsF (7 cases) and PEOF (8 cases) were retrieved from the files of the Oral Pathology Service of the UFMG (Belo Horizonte, Brazil) and from the Oral Pathology Service of the University of São Paulo (São Paulo, Brazil). The criteria for diagnosis of OdF, POF and OsF were in accordance with the WHO 2005 Classification[6]. OdF can appear in two patterns: the epithelium-poor type and the epithelium-rich type[6]. In this study, two cases of OdF were of the epithelium-rich type and two cases were of the epithelium-poor type. Criteria to identify the PEOF were in accordance with Buchner and Hansen[7] (1987).

AgNOR technique

The AgNOR technique was performed according to the standardized method of Trerè[24] (2000). Sections of 3 µm from routinely processed paraffin-embedded blocks were de-waxed and hydrated. The sections were immersed in a sodium citrate buffer (10 mM, pH 6.0) and boiled at 120°C for 20 min. These were then cooled down to room temperature and washed with distilled water. The sections were immersed in a gelatine and silver nitrate solution in the dark at room temperature for 25 min.

Immunohistochemistry

Immunohistochemistry was performed using the streptavidin-biotin standard protocol. Sections of 3 µm from routinely processed paraffin-embedded blocks were de-waxed and hydrated. The specimens were immersed in a 10 mM citrate buffer (pH 6.0, 20 min at 98°C) for antigen retrieval. The endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. Sections were incubated with primary antibody PCNA (PC10, MO879, Dako Corporation, Carpinteria CA, USA) for 18 hours at room temperature. Next, the sections were treated with LSAB^{â}+system, HRP Peroxidase Kit (KO675, Dako Corporation, Carpinteria CA, USA) and 3.3'-diaminobenzidine tetrahydrochloride chromogen (D5637, DAB; Sigma Chemical, St Louis MO, USA). Sections of oral squamous cell carcinoma were used as the positive controls.

Analysis of AgNOR and PCNA indexes

Fibroblasts (mesenchymal component) were evaluated in four groups of lesions. The epithelial cells of the islands or strands of odontogenic epithelium in the POF and OdF were also evaluated. Inflammatory cells and the cells lining calcified materials presented in the POF and PEOF were not included in this analysis.

AgNOR parameters were established in 100 cells for each case using KS300 software coupled to a Carl Zeiss Image Analyzer (Carl Zeiss, Oberkochen,

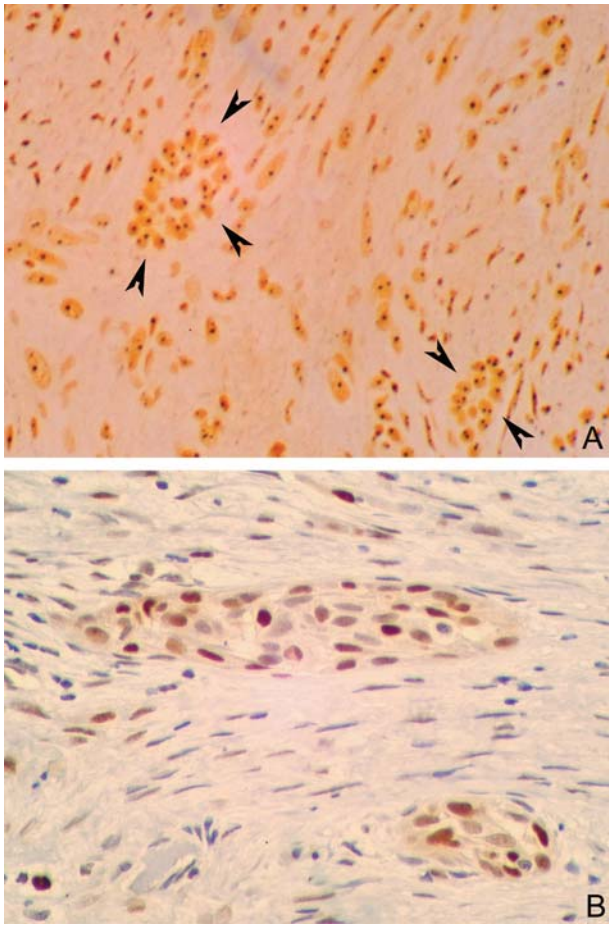

Figure 1- AgNOR and proliferating cellular nuclear antigen (PCNA) staining. A- AgNOR in the peripheral odontogenic fibroma can be seen as black, well-defined, intra-nuclear homogeneous dots in the epithelial and mesenchymal components. The arrow heads identify islands of odontogenic epithelium (AgNOR technique, 400x). B- PCNA-positive cells are identified as brown nuclei in the epithelial and mesenchymal components of the odontogenic fibroma (Streptavidin-biotin standard protocol, 400x)

Baden-Württemberg, Germany). The number, area and contour index of AgNOR were obtained from digital images taken by a JVC TK-1270/RGB micro camera, at 400x magnification. The AgNOR were viewed as black, well-defined, intra-nuclear homogeneous dots (Figure 1A). The mean numbers of AgNOR *per* nucleus, area and contour index for all cases were determined. The contour index varied from 0.76 to 0.92. Values near 1 corresponded to a round structure with a regular contour and a value distant from 1 indicated an irregular structure.

Brown nuclei, regardless of staining intensity, were considered PCNA-positive cells (Figure 1B). An index (IP) was determined considering the number of PCNA positive cells *per* 500 cells in each case. This count was performed at 400x magnification using optical microscopy (Carl Zeiss – Axiostar 1122-100, Carl Zeiss, Oberkochen, Baden-Württemberg, Germany).

The Kruskal-Wallis and the Mann-Whitney tests were used to compare the AgNOR and IP data. The statistical analysis was performed by the BioEstat® software and the alpha level was set at 0.05[4].

RESULTS

The AgNOR and IP mean in the mesenchymal component of OdF and OsF proved to be significantly higher than in the POF and PEOF, respectively. The AgNOR area of the central lesions was statistically smaller than those found in the peripheral lesions (P≤0.05, Table 1). The mean number of AgNOR *per* nucleus, area and IP in the mesenchymal component between the POF and PEOF presented no statistically significant difference. An identical result was observed between the OdF and OsF (P>0.05, Table 2).

The mean number of AgNOR *per* nucleus in the epithelial component was significantly higher in the COF than in the POF, while the AgNOR area of the POF was statistically higher than those

Table 1- Comparative analysis of data concerning the mean number of AgNOR *per* nucleus, area and proliferating cellular nuclear antigen (PCNA) index (IP) in the mesenchymal component between peripheral odontogenig fibroma (POF) and odontogenic fibroma (OdF) and peripheral ossifying fibroma (PEOF) and ossifying fibroma (OsF)

	POF mean (min/max)	OdF mean (min/max)	P value[a]	PEOF mean (min/max)	OsF mean (min/max)	P value[a]
Mean number of AgNOR *per* nucleus[b]	1.26 (1.10/1.50)	1.49 (1.40/1.70)	0.0136*	1.25 (1.10/1.40)	1.48 (1.30/1.70)	0.0428*
AgNOR area (µm2)[c]	1.46 (1.40/1.90)	1.21 (1.10/1.40)	0.0308*	1.62 (1.39/1.86)	1.25 (1.00/1.53)	0.0128*
IP[d]	43.6 (11.6/58.2)	61.2 (56.0/67.6)	0.0087*	47.6 (40.2/55.6)	57.1 (50.8/62.8)	0.0038*

[a] Mann-Whitney test. * Statistically significant values (P≤0.05)
[b] Kruskal-Wallis test: H=113880, p=0.0098
[c] Kruskal-Wallis test: H=115380 p=0.0091
[d] Kruskal-Wallis test: H=162612 p=0.0010

Table 2- Comparative analysis of data concerning the mean number of AgNOR *per* nucleus, area and proliferating cellular nuclear antigen (PCNA) index (IP) in the mesenchymal component between peripheral odontogenic (POF) and peripheral ossifying (PEOF) fibromas and odontogenic (OdF) and ossifying (OsF) fibromas

	POF mean (min/max)	PEOF mean (min/max)	P value[a]	OdF mean (min/max)	OsF mean (min/max)	P value[a]
Mean number of AgNOR *per* nucleus	1.26 (1.10/1.50)	1.25 (1.10/1.40)	0.9233	1.49 (1.40/1.70)	1.48 (1.30/1.70)	0.5708
AgNOR area (μm²)	1.46 (1.40/1.90)	1.62 (1.39/1.86)	0.0922	1.21 (1.10/1.40)	1.25 (1.00/1.53)	0.8501
IP	43.6 (11.6/58.2)	47.6 (40.2/55.6)	0.8099	61.2 (56.0/67.6)	57.1 (50.8/62.8)	0.2193

[a] Mann-Whitney test.

Table 3- Comparative analysis of data concerning the mean number of AgNOR *per* nucleus, area and proliferating cellular nuclear antigen (PCNA) index (IP) in the epithelial (e) component between peripheral odontogenic (POF) and odontogenic (OdF) fibromas

	ePOF mean (min/max)	eOdF mean (min/max)	P value[a]
Mean number of AgNOR *per* nucleus	1.25 (1.07/1.51)	1.45 (1.40/1.51)	0.0372*
AgNOR area (μm²)	1.47 (1.30/1.65)	1.23 (1.07/1.48)	0.0449*
IP	78.5 (34.2/99.1)	78.45 (45.8/94.6)	0.7576

[a]Mann-Whitney test. * Statistically significant values (P≤0.05)

Table 4- Comparative analysis of data concerning the mean number of AgNOR *per* nucleus, area and proliferating cellular nuclear antigen (PCNA) index (IP) between mesenchymal (m) and epithelial (e) components in the peripheral odontogenic (POF) and odontogenic (OdF) fibromas

	mPOF mean (min/max)	ePOF mean (min/max)	P value[a]	mOdF mean (min/max)	eOdF mean (min/max)	P value[a]
Mean number of AgNOR *per* nucleus	1.26 (1.10/1.50)	1.25 (1.07/1.51)	0.8946	1.49 (1.40/1.70)	1.45 (1.40/1.51)	0.5637
AgNOR area (μm²)	1.46 (1.40/1.90)	1.47 (1.30/1.65)	0.7573	1.21 (1.10/1.40)	1.23 (1.07/1.48)	0.5637
IP	43.6 (11.6/58.2)	78.5 (34.2/99.1)	0.0071*	61.2 (56.0/67.6)	78.45 (45.8/94.6)	0.2482

[a]Mann-Whitney test. * Statistically significant values (P≤0.05)

found in the OdF (P≤0.05, Table 3). The IP mean presented similar data in the epithelial components of the POF and OdF (P>0.05, Table 3). Epithelial and mesenchymal components of the POF and OdF presented no statistically significant differences, except for the IP mean in the POF, in which the epithelial component presented a higher IP (Table 4).

DISCUSSION

Although the lesions evaluated in this study exhibited similar histomorphological features, the appropriate diagnosis and differentiation amongst them is possible. Therefore, the current study serves to provide information on cellular proliferation. The low number of cases in this study is due to the rarity of these lesions, especially the OdF and POF, which are rare benign odontogenic tumours[6,18]. The current study demonstrated differences in the cellular proliferation of these four lesions, which is important in understanding their biological behaviour.

The morphometric study of AgNOR is related to the degree of cellular proliferation in non-neoplastic reactive and neoplastic lesions[2]. Benign neoplasms and non-neoplastic reactive lesions are characterized by low numbers of AgNOR *per* nucleus, large areas and regular shapes or contour indexes[8,10,17]. These features of the mean number of AgNOR *per* nucleus could be observed in all evaluated lesions, defending the concept that these lesions do in fact present a profile of benign lesions.

Investigations of cellular proliferation using PCNA

in oral diseases may well bring about information concerning proliferative activity. Mesquita, et al.[17] (1998) and Ono, et al.[19] (2007) demonstrated, by means of the AgNOR and IP analyses, that the cellular proliferation in the OsF was higher than in the PEOF. These results were re-shown in the current study, emphasizing the non-neoplastic reactive nature of the PEOF. Other studies have demonstrated that non-neoplastic reactive lesions, *e.g*. inflammatory fibrous hyperplasia and reactive mesothelium, present lower AgNOR counts than do benign neoplasms with the same cell components[10]. It could be observed that the cellular proliferation of the POF proved to be less than the OdF but similar to the PEOF. These data suggest a similar cellular proliferation for both peripheral lesions, even though POF is in fact considered to be a neoplastic lesion[6].

AgNOR analysis and IP have been performed on odontogenic cysts and tumours[15,16]. Martins, et al.[15] (2001) evaluated AgNOR in amelobastic fibromas, which demonstrated that the epithelial and the mesenchymal components present a similar cellular proliferation. This fact is in accordance with the nature of ameloblastic fibromas in which both the epithelial and the mesenchymal components are considered neoplastic[21]. In the current study, it could be verified that both epithelial and the mesenchymal components presented similar AgNOR and IP values in the OdF and similar mean number of AgNOR *per* nucleus in the POF. This indicates that both the epithelial and mesenchymal components in the POF and OdF present similar cellular proliferation.

One expected finding in the current study was the higher IP, in contrast to the smaller AgNOR values, in the epithelial component of the POF. Cytokines released by inflammatory cells present in the POF may be responsible for this observation[14]. Therefore, it is important to evaluate the true role of cytokines in this type of lesion, as well as the utility of PCNA analysis as a cellular proliferation marker in the inflammatory lesions.

In conclusion, the mesenchymal component may well play a role in the differences between the biological behaviour of central lesions, as compared to peripheral lesions. Moreover, considering that the epithelial and mesenchymal components in odontogenic fibromas presented a similar proliferation index, further research is warranted to understand the true role of epithelial components, which are believed to be inactive in nature, as well as in the development and biological behaviour of these lesions.

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