

Cell Proliferation and Apoptosis in Ameloblastomas and Keratocystic Odontogenic Tumors

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A high proliferative activity of the odontogenic epithelium in ameloblastoma (AM) and keratocystic odontogenic tumor (KOT) has been demonstrated. However, no previous study has simultaneously evaluated cell proliferation and apoptotic indexes in AM and KOT, comparing both lesions. The aim of this study was to assess and compare cell proliferation and apoptotic rates between these two tumors. Specimens of 11 solid AM and 11 sporadic KOT were evaluated. The proliferation index (PI) was assessed by immunohistochemical detection of Ki-67 and the apoptotic index (AI) by methyl green-pyronine and *in situ* DNA nick end-labelling methods. KOT presented a higher PI than AM ($p < 0.05$). No statistically significant difference was found in the AI between AM and KOT. PI and AI were higher in the peripheral cells of AM and respectively in the suprabasal and superficial layers of KOT. In conclusion, KOT showed a higher cell proliferation than AM and the AI was similar between these tumors. These findings reinforce the classification of KOT as an odontogenic tumor and should contribute to its aggressive clinical behavior.

Key Words: cell proliferation, apoptosis, ameloblastoma, odontogenic keratocyst.

INTRODUCTION

Ameloblastoma (AM) is a locally invasive benign epithelial odontogenic tumor that may arise from rests of dental lamina, enamel organ rests, cell rests, the epithelial lining of an odontogenic cyst or from the basal cell layer of oral mucosa (1). Its occurrence varies from 10% to 45.2% of all odontogenic tumors and reaches approximately 1% of all oral cavity neoplasms (2). The usual clinical presentation includes a painless swelling with expansion of the jaws. It can be classified in 3 main groups: solid or multicystic ameloblastoma, unicystic and peripheral. Among these, the solid type is more common and histopathologically represented by follicular, plexiform, acanthomatous, desmoplastic, granular and basal cell patterns (3).

Keratocystic odontogenic tumor (KOT) is a benign cystic lesion originated from dental lamina rests and presents an aggressive clinical behavior. It tends to grow in an anteroposterior direction within the medullary cavity of the bone, not causing evident bone expansion (4). Its aggressive nature resulted in its classification as a benign epithelial odontogenic tumor in 2005 (5).

Since cell population and turnover are controlled by a balance between cell proliferation and programmed cell death, cell proliferation and apoptosis are fundamental events for the development and tissue homeostasis (6).

A higher proliferation activity in AM epithelium in relation to KOT epithelial lining has been demonstrated (7-9), even though opposite results have also been reported (10,11).

Several studies have evaluated apoptosis and related apoptotic factors in the odontogenic epithelium of AM and epithelial lining of KOT (10-19). Nevertheless, despite the fact that the cell population is controlled by the balance between cell proliferation and apoptosis, no study has evaluated simultaneously apoptotic and proliferation indexes in AMs and KOTs. Therefore, the aim of this study was to evaluate and compare the cell proliferation index (PI), and the apoptotic index (AI) in AM and KOT.

MATERIAL AND METHODS

Tissues and Samples

This study was approved by the Bioethics Committee of the Pontifical Catholic University of Minas Gerais, Brazil (CAAE 0025.0.213.000-06).

A total of 11 solid AM and 11 KOT from archival formalin-fixed, paraffin-embedded specimens were collected at two Oral Pathology Services: Pontifical Catholic University of Minas Gerais and Federal University of Minas Gerais, Brazil.

Assessment of Cell Proliferation Index

The PI was assessed by immunohistochemical detection of the cell proliferation marker Ki-67.

Four-micrometer-thick sections from the paraffin-embedded samples were used. Sections were deparaffined and immersed in methanol with 0.3% H₂O₂. The sections were heated in a 0.01 M citrate buffer (pH 6.0) for 30 min. The primary monoclonal antibody Ki-67 was used overnight (clone MM1, diluted 1:100; Novocastra Laboratories Ltd, Newcastle, UK). The LSAB kit (Dako Corporation, Carpinteria, CA, USA) was used for application of the biotinylated link antibody and peroxidase-labeled streptavidin, according to the manufacturer's instructions. The reactive products were able to be visualized due to the immersion of the sections for 3 min in 0.03% diaminobenzidine (DAB) solution, containing 2 mM H₂O₂. The sections were then counterstained with Mayer's hematoxylin, dehydrated and mounted. Sections of oral squamous cell carcinoma were used as a positive control.

Epithelial cells with distinct brown nuclear staining were regarded as Ki-67 positive. Cell counts were made at ×400 magnification, using an eyepiece grid in light microscopy, counting at least 10 fields.

The percentage of positive cells was then calculated to obtain the PI.

PI was evaluated in the whole epithelium of AM and KOT as well as independently in peripheral and central cells of the AM epithelium and in basal, suprabasal and superficial layers of KOT epithelial lining.

Assessment of Apoptosis Index

The AI was assessed quantitatively by morphological evaluation of sections stained by the methyl green-pyronine method. The TUNEL method was used qualitatively to confirm the occurrence of apoptosis in the odontogenic epithelium of AM and KOT.

The TUNEL method was performed using a commercial TdT-FragELTM DNA fragmentation detection kit (Calbiochem®; Oncogene Research Products, Cambridge, MA, USA) and the protocols were followed according to the manufacturer's instructions. Four-micrometer-thick sections from the paraffin-embedded samples were deparaffined. Afterwards, the specimens were treated with 20 mg/mL proteinase K for 5 min and with 0.6% H₂O₂ in methanol to eliminate endogenous peroxidase activity. Subsequently they were incubated with TDT together with a biotinylated nucleotides solution. Then, the sections were immersed in a streptavidin-peroxidase complex solution. The reactive products were able to be visualized by the immersion of the sections for 3 min in a 0.03% diaminobenzidine (DAB) solution, containing 2 mM H₂O₂. The sections were then counterstained with methyl green, dehydrated and mounted.

In the methyl green-pyronine method, 4-µm-thick sections from the paraffin-embedded samples were used. Tissue sections were deparaffined. The sections were stained for 5 min in a solution prepared with 2% methyl green and 1% pyronine (proportion 7:3). Afterwards, the sections were enveloped in a paper filter for 5 min and washed individually in distilled water. The sections were then evaluated using light microscopy and, if they were exceedingly red, they were washed with 80% ethanol at 5°C. The sections were then dehydrated and mounted.

Cell counts were made at ×1000 magnification, in the methyl green-pyronine sections using an eyepiece grid in light microscopy, counting at least 10 fields. The percentage of apoptotic cells was then calculated to obtain the AI in whole epithelium of AM and KOT as well as independently in peripheral and central cells

of the AM epithelium and in basal, suprabasal and superficial layers of KOT epithelial lining.

Statistical Analysis

Differences in PI and AI between AM and KOT were analyzed using the Mann-Whitney test. Differences in PI and AI between the epithelium lining layers of KOT as well as between peripheral and central cells of AM were analyzed using the Wilcoxon test. Data were analyzed using Biostat 4.0 software (Optical Digital Technology, Belém, PA, Brazil). Tests were considered

significant when their p-values were <0.05 .

RESULTS

PI and AI were higher in KOT than in AM ($p<0.05$) (Table 1 and Figs. 1 and 2).

In AM, PI in the peripheral cells was higher than in the central cells ($p<0.05$). No statistically significant difference was observed in AI of these two areas ($p<0.05$) (Table 2).

In KOT, PI was higher in the suprabasal layer than in the basal layer ($p<0.05$). PI in these two layers was

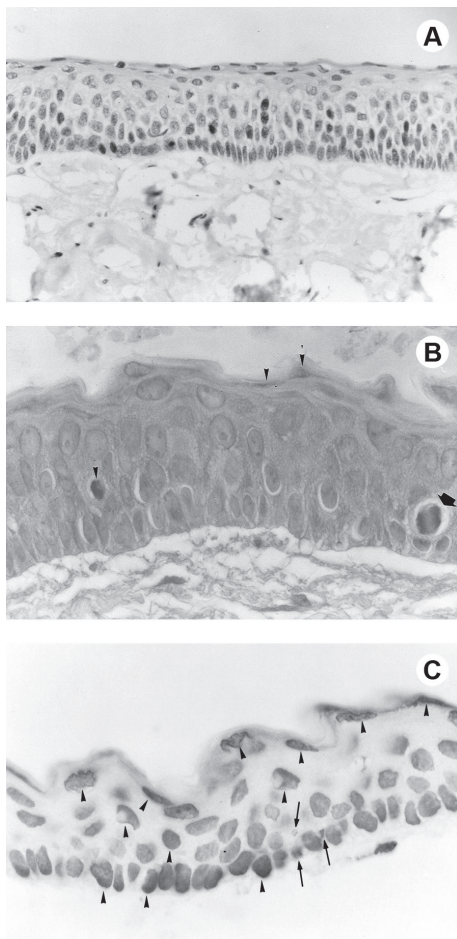


Figure 1. Keratocyst odontogenic tumor: A: Immunohistochemical reactivity for Ki-67. Odontogenic epithelium cells with distinct brown nuclear staining were regarded as Ki-67 positive (original magnification $\times 400$). B: Methyl green-pyronine staining. Apoptotic cells (arrowheads) and mitotic cell (large arrow) can be observed in the odontogenic epithelium (original magnification: $\times 1000$). C: TUNEL reaction. Apoptotic cells (arrowheads) and apoptotic bodies (arrows) can be observed in the odontogenic epithelium (original magnification $\times 1000$).

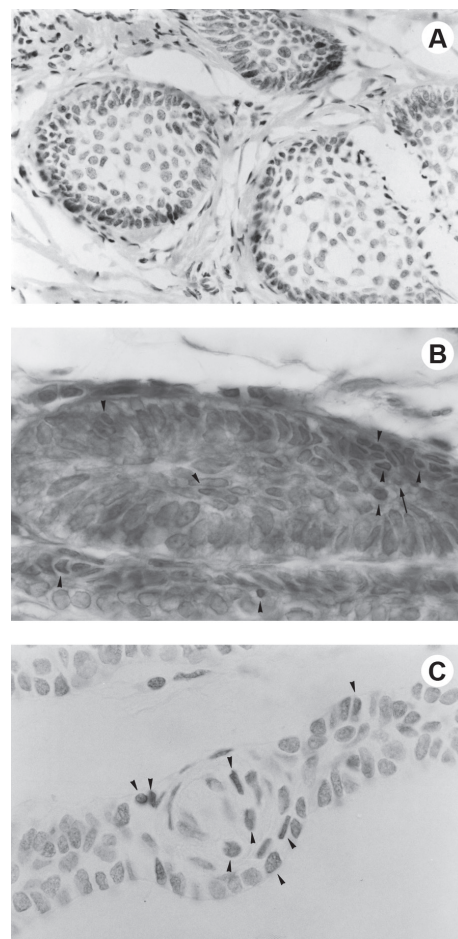


Figure 2. Ameloblastoma: A: Immunohistochemical reactivity for Ki-67. Odontogenic epithelium cells with distinct brown nuclear staining were regarded as Ki-67 positive (original magnification: $\times 400$). B: Methyl green-pyronine staining. Apoptotic cells (arrowheads) and apoptotic bodies (arrow) can be observed in the odontogenic epithelium (original magnification $\times 1000$). C: TUNEL reaction. Apoptotic cells (arrowheads) can be observed in the odontogenic epithelium (original magnification $\times 1000$).

higher than in the superficial layer ($p < 0.05$) (Table 3 and Fig. 1). In KOT, no statistically significant difference was observed in AI between the basal and suprabasal layers ($p > 0.05$). Nevertheless, AI in these two layers was lower than in the superficial layer ($p < 0.05$) (Table 3).

DISCUSSION

AM and KOT are epithelial odontogenic tumors with aggressive clinical behavior. Since KOT had been classified as an odontogenic cyst, several authors have compared cell proliferation between KOT and

odontogenic cysts (8,12,13). Although several authors have assessed cell proliferation and apoptosis in the odontogenic epithelium in AM and in the epithelial lining in KOT (7-22), few studies have compared cell proliferation between these tumors (7,8,11) and no previous study has simultaneously evaluated cell proliferation and apoptotic indexes in AM and KOT, comparing both lesions. Therefore, this is the first study assessing comparatively cell proliferation and apoptosis indexes in AM and KOT, although tissue cell population is controlled by a balance among these processes.

In the present study, PI was assessed by the immunohistochemical detection of Ki-67, which is one of the most widely used and trustworthy proliferation markers (23). AI was assessed by morphological evaluation of sections stained by methyl green-pyronine method, a histochemical staining with an affinity for the DNA molecule. Through this method, apoptotic cells with a characteristic morphology can be easily detected. The TUNEL method was also used to confirm the occurrence of apoptosis in each layer of the epithelium (Figs. 1 and 2).

PI was found to be higher in KOT than in AM (Table 1; $p < 0.05$). This is in accordance with previous reports (10,11). In contrast, studies have also demonstrated a higher cell proliferation in AM in relation to KOT (7-8). All these authors used different methods to evaluate PI. In some studies (7,11), the proliferative activity was assessed by PCNA labelling. PCNA expression is positive in all the cell cycle phases and in G0. In G0, PCNA is known to be associated with DNA repair process. Therefore, PCNA may be expressed in cells not synthesizing DNA. The IPO-38, another marker of proliferating cells, was evaluated in odontogenic keratocysts (8). IPO-38 expression is constant through most stages of the cell cycle. However,

during mitosis, it presents a 400-fold increase in its concentration. In the present study, Ki-67, an antigen whose expression occurs in the G1, S and G2 stages as well as during mitosis, was used. This antigen is considered to be a better specific marker of proliferating cells (23). Although several authors have used Ki-67 to assess cell proliferation in KOT (12,13) and AM (19,20), few studies have compared cell proliferation between AM and KOT using the Ki-67 (10).

In the AM, PI in the peripheral cells was higher than in the central cells (Table

Table 1. Median and range of proliferation index (PI) and apoptotic index (AI) in ameloblastomas (AM) and keratocystic odontogenic tumors (KOT).

Index	AM	KOT	p-value
PI median	3.42	9.83	<0.05
PI range	1.18-5.63	7.19-19.78	
AI median	14.61	18.35	n.s.
AI range	10.61-23.06	13.23-29.99	

p-value was obtained by the Mann-Whitney test; n.s. - not significant.

Table 2. Median and range of proliferation index (PI) and apoptotic index (AI) in each cell layer of ameloblastoma.

Index	Peripheral cells	Central cells	p-value
PI median	3.80	3.00	<0.05
PI range	1.59-5.97	1.07-5.24	
AI median	16.80	13.30	<0.05
AI range	11.64-25.96	8.55-21.10	

p-value was obtained by the Wilcoxon test.

Table 3. Median and range of proliferation index (PI) and apoptotic index (AI) in each cell layer of keratocystic odontogenic tumor.

Index	Basal	Suprabasal	Superficial	p-value
PI median	3.52	16.57	0	<0.05 ^a
PI range	0.92-27.25	10.93-34.09	0	<0.05 ^b
AI median	9.41	10.25	99.31	n.s. ^a
AI range	4.96-10.98	6.78-17.62	79.37-100	<0.05 ^b

p-value was obtained by the Wilcoxon test; ^aBasal vs. Suprabasal; ^bBasal vs. Superficial; ^cSuprabasal vs. Superficial; n.s. not significant.

2; $p < 0.05$). This is in agreement to previous literature (11,18,19). The peripheral cells reflect the growth of the AM. Our results and those of other studies (11,19) reinforce this finding.

No significant difference in AI was observed between AM and KOT (Table 1; $p > 0.05$). The literature has evaluated apoptosis independently in AM or KOT (15,16,18,19). However, to the best of our knowledge, no previous study has compared AI between AM and KOT.

In the AM, AI in the peripheral cells was higher than in the central cells (Table 2; $p < 0.05$). In contrast to our results, other authors (18,19) have demonstrated more apoptotic cells in central areas than in peripheral areas of AM. In follicular AM, the cystic degeneration is a common feature (1-3). In these lesions, the cystic change within the tumor islands may be associated with the apoptotic process (18). In the present study, there were specimens with formed cavity, which may justify the lower number of apoptotic cells in central areas. This fact might suggest that apoptosis had already occurred and in the moment of the evaluation. Anti-apoptotic factors such as bcl-2 and bcl-X proteins have been detected in the peripheral cells in AM (18,19). Nonetheless, pro-apoptotic factors such as fas, fas-L, caspases, cytochrome C, apoptotic protease-activating factor-I (APAF-I), apoptosis-inducing factor (AIF), TNF α and TNF-related apoptosis inducing ligand (TRAIL) have also been demonstrated in these peripheral epithelial cells (15,16). In KOT, there were no differences in AI between basal and suprabasal epithelial layers. However, AI was significantly higher in the superficial layer. Possible explanations for this finding include the physiological occurrence of apoptosis in the superficial layer of the keratinized epithelium of the keratocystic odontogenic tumor lining and the contribution of apoptosis in eliminating mutated cells at the G1-S checkpoint. Although previous studies have observed apoptotic cells only in the superficial layer of KOT (12-14), this study showed apoptotic cells in all epithelial layers. These differences might have occurred due to the misinterpretation of the TUNEL method in apoptosis evaluation. In this method, apoptotic cells should be detected not only by the diaminobenzidine marker, but also by the cell morphologic alterations such as those found in the methyl green pyronine method (24).

The present study demonstrated that cell proliferation and apoptosis occurred in distinct patterns in AM and KOT. Although AM and KOT are classified as epithelial odontogenic tumors and show some common

clinical and radiographic characteristics, PI was higher in KOT. Nevertheless, no significant difference in AI was observed between the two lesions.

In conclusion, KOT showed a higher cell proliferation than AM and apoptosis was similar between these tumors. These findings reinforce the classification of KOT as an odontogenic tumor and should contribute to its aggressive clinical behavior.

RESUMO

Uma elevada atividade proliferativa do epitélio odontogênico em ameloblastoma (AM) e tumor odontogênico ceratocístico (TOC) tem sido demonstrada. Entretanto, não há estudos prévios avaliando simultaneamente os índices de proliferação celular e apoptótico em AM e TOC, comparando ambas as lesões. O objetivo desse estudo foi avaliar e comparar os índices de proliferação celular e apoptótico entre esses dois tumores. Onze amostras de AM sólido e 11 amostras de TOC esporádico foram avaliadas. O índice de proliferação celular foi avaliado por meio da imunomarcagem para o antígeno Ki-67 e o índice apoptótico pelas técnicas de metil-verde-pironina e TUNEL. O TOC apresentou um índice de proliferação celular maior que o AM ($p < 0,05$). Nenhuma diferença estatística foi encontrada no índice apoptótico entre AM e TOC. Os índices de proliferação celular e apoptótico foram maiores nas células da camada periférica do AM e, respectivamente, nas camadas suprabasal e superficial do TOC. Em conclusão, o TOC apresentou proliferação celular maior que o AM e o índice apoptótico foi similar entre estes tumores. Estes achados reforçam a classificação do TOC como um tumor odontogênico e podem contribuir para o seu comportamento clínico agressivo.

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