Odontogenic Epithelium: Immunolabeling of Ki-67, EGFR and Survivin in Pericoronal Follicles, Dentigerous Cysts and Keratocystic Odontogenic Tumors

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Abstract The aim of this study was to evaluate the biological profile of odontogenic epithelium by immunolabeling of epidermal growth factor receptor (EGFR), Ki-67 and survivin in keratocystic odontogenic tumors (KOT), dentigerous cysts (DC), and pericoronal follicles (PF). Immunohistochemical analysis was performed in 13 KOTs, 14 DCs and 9 PFs. Immunolabeling was analyzed in the basal and suprabasal layers of KOTs and DCs, and in the islands of odontogenic epithelium and/or reduced enamel epithelium of PFs. KOTs showed the highest proliferation rate among the three groups, mainly in suprabasal layers. EGFR immunolabeling was observed mainly in the cytoplasm in basal and suprabasal layers of KOTs and in the suprabasal layer of DCs. Immunolabeling in both membrane and cytoplasm was greater in PFs. In PFs, membrane-only staining was observed. Survivin immunolabeling showed a greater percentage of positive cells (scoring ++++) in the suprabasal layer of KOTs. In DCs, both layers showed similar percentages of cells scoring ++++; PFs showed the highest percentage of these cells. In KOTs, epithelial cells showed stimulus-independent neoplastic proliferative characteristics, suggesting the presence of a suprabasal proliferative compartment, maintained by inhibition of apoptosis. In DCs, the basal layer seemed to proliferate in response to stimulus. Although PFs showed low proliferative activity, the expression of EGFR indicates that some cells have a high capacity to respond to stimuli,

which could probably explain the origin of odontogenic lesions.

Keywords Survivin · Ki-67 · EGFR · Odontogenic cysts · Odontogenic tumors · Odontogenic lesions · Epithelium · Immunohistochemistry · Pericoronal follicles · Dentigerous cyst · Keratocyst odontogenic tumor

Introduction

The epithelium involved in odontogenesis and/or its remnants are the origin of different odontogenic lesions, with distinct clinical behavior. Several studies have reported differences in the proliferative potential of odontogenic epithelial cells, with significant impacts on the formation of odontogenic cysts and tumors [1–5]. Tissue homeostasis is maintained by a regulated balance between cell proliferation and cell death, and changes in the regulation of these mechanisms are implicated in the pathogenesis of several lesions.

Baumgart et al. [3] assessed pericoronal follicles and normal oral mucosa and found that 7% of the islands of odontogenic epithelium presented immunolabeling of epidermal growth factor receptor (EGFR) compatible with neoplasia (oral squamous cell carcinoma) in the membrane only. Reduced enamel epithelium showed EGFR immunolabeling in 63.3% of the cells, a result that is similar to findings of normal oral mucosa, in both the membrane and the cytoplasm (membrane-only staining did not occur in these cells). On the other hand, in the study conducted by Payeras et al. [5], the behavior of odontogenic epithelium in ameloblastomas suggested differences in the proliferative potential of islands depending on their size. Membrane EGFR expression was usually observed in smaller islands,

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a pattern compatible with a more rapid response to proliferative stimuli and therefore with a higher proliferative rate. Based on these results, the motivation for our study was to further investigate the biological profile of odontogenic epithelium.

Pericoronal follicles (PF) are histologically characterized by fibrous connective tissue with variable amounts of reduced enamel epithelium and epithelial remnants of dental lamina [2–4]. They are present in unerupted teeth, but the proliferative potential of odontogenic epithelium in PFs of teeth in this condition has not been elucidated. Dentigerous cysts (DC) may also affect unerupted teeth, enclosing the crown and attaching to the neck of the unerupted tooth [6]. The origins of DCs have been attributed to odontogenic epithelium [1–4, 6, 7].

Keratocystic odontogenic tumors (KOT) arise from odontogenic epithelial cells and remnants of the dental lamina, and also from extensions of basal cells of the oral epithelium. Epithelial cells in KOTs seem to have a different proliferative potential from those of other odontogenic lesions [8–11].

Several markers have been used to assess the behavior or epithelial cells of odontogenic lesions. EGFR is involved in various cellular processes, including cell growth, motility, inhibition of apoptosis, and cell adhesion [12, 13]. Lin et al. [14] have demonstrated that the localization of EGFR is correlated with a highly proliferative status of tissues, and have provided evidence to support the potential roles of EGFR as a transcription factor or a coactivator, which might activate the genes required for its mitogenic effects. The location of this receptor within a cell may be related to its response to proliferative stimuli. Cells proliferating at a physiologic rate express this receptor in both membrane and cytoplasm. The immunolocation of EGFR in odontogenic epithelium may therefore be associated with the origin of odontogenic cysts and tumors [3].

Survivin (BIRC5) is an inhibitor of apoptosis protein, of the IAP gene family. This gene family acts on the regulation of apoptosis and on the promotion of mitosis and angiogenesis. Survivin plays an important role in the suppression of apoptosis by either directly or indirectly inhibiting caspase activity, and may be found in cells showing proliferative activity, such as epithelial basal cells. Great amounts of survivin are expressed in the G2/M phase, but very little expression is observed in the G1/S phase [15–17]. The expression of survivin in odontogenic epithelial cells has already been described and may be associated with changes in cell cycle caused by the pathogenesis of some lesions [18, 19].

Finally, Ki-67 has been employed as a marker of cell proliferation; the monoclonal antibody MIB-1 recognizes epitopes of Ki-67 antigen in all active cell cycle phases (G1, S, G2 and M). Immunohistochemical detection of

Ki-67 has been used to evaluate the proliferative potential of healthy cells as well as of preneoplastic and neoplastic lesions [8, 20–23].

Odontogenic cysts and tumors share the same source of odontogenic epithelium. In an attempt to better understand the mechanisms regulating odontogenic epithelium, this study was designed using DCs as representatives of odontogenic epithelium in cysts, KOTs as representatives of odontogenic epithelium in tumors, and PFs as the physiological pattern of odontogenic epithelium. Moreover, based on previous reports, three proteins were selected for the assessment of different aspects as follows: Ki-67, related to cell proliferation [8, 20–23]; EGFR, related to the capacity of cells to respond to stimuli [13, 24, 25]; and survivin, related to inhibition of apoptosis [26–29]. The aim of this study was to evaluate the biological profile of odontogenic epithelium by immunolabeling of EGFR, Ki-67 and survivin in a neoplasm (KOTs), a cystic lesion (DCs), and in PFs.

Materials and Methods

This study was approved by the Research Ethics Committee at the School of Dentistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, and by the National Committee on Ethics in Research (CONEP), under protocol number 1166/2003.

The sample comprised 13 KOTs, 14 DCs, and 9 PFs. All samples were fixed in 10% neutral buffered formalin, dehydrated in alcohol, cleared with xylene, and embedded in paraffin. A 4- μ m section was obtained from each specimen and stained with hematoxylin-eosin (HE). Slides were reviewed and diagnoses confirmed on the basis of clinical, radiographic and histopathological findings. The following criteria were used to define each type of lesion: KOTs, presence of a palisade basal layer and a corrugated parakeratin layer, with absent or no significant inflammatory infiltrate; DCs, stratified epithelium with more than one layer; and PFs, presence of islands of odontogenic epithelium and/or reduced enamel epithelium. Three additional 4- μ m sections were obtained from each block for immunolabeling.

Immunohistochemical Method

Immunohistochemical staining was performed at the Oral Pathology Laboratory of the School of Dentistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil. Sections were deparaffinized in xylene and rehydrated in alcohol. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol in two 15-min baths. Antigen retrieval was performed using a low-pH retrieval

solution (S1699, DakoCytomation, Carpinteria, CA, USA) previously heated to 90°C, in a 25-min steam bath (Acqua Timer, ARNO, São Paulo, SP, Brazil) at 96°C for EGFR and survivin, and in a microwave oven for 20 min (four 5-min cycles) for Ki-67.

Subsequently, sections were incubated for 60 min at room temperature with each marker as follows: Ki-67, 1:50 monoclonal anti-human antibody, clone MIB-1 (DakoCytomation, Glostrup, Denmark); EGFR, 1:30 monoclonal anti-human antibody, clone EGFR113 (Novocastra, Newcastle, UK); and survivin, 1:75 monoclonal anti-human antibody, clone D-8 (Santa Cruz Biotechnology, USA); and then washed in phosphate buffered saline (PBS). The detection system used was Envision +® (DakoCytomation, Carpinteria, CA, USA), and the chromogen was liquid DAB (DakoCytomation, Carpinteria, CA, USA). Sections were rinsed, counterstained with Harris hematoxylin, rehydrated and cleared in xylene; slides were mounted in Entellan® (Merck, Daarmstadt, Germany). Sections of oral squamous cell carcinoma were used as positive controls, and negative controls were obtained by omitting the primary antibodies.

Immunolabeling

All HE-stained slides were examined using an optical microscope at 400× magnification. Five representative fields of the lesion were selected and identified in each of the Ki-67, EGFR and survivin slides; images were recorded with a digital camera at a resolution of 2,048 × 1,536 pixels. Immunolabeling was analyzed in the basal and suprabasal layers of KOTs and DCs, and in the odontogenic epithelium of PFs (islands of odontogenic epithelium and/or reduced enamel epithelium). The criterion used to identify cells in the suprabasal layer was based on nuclear morphology: cells immediately above the basal layer (4–5 cells above the basal cell layer) and with a spherical nucleus were classified as suprabasal; cells with a flattened nucleus were classified as superficial and were not counted. The criterion for positivity was brown color regardless of staining intensity.

ImageTool for Windows 3.00 software was used to assess the expression of Ki-67; the total number of epithelial cells, including negative and positive cells, was counted, and the percentage of positive cells was calculated. EGFR analysis was based on the distribution of brown staining, as described by Baumgart et al. [3]: membrane-only staining, cytoplasm-only staining, or combined membrane and cytoplasmic staining, indicating the presence of this protein in each of these locations. Survivin immunolabeling was analyzed semiquantitatively using the following scores according to the percentage of positive cells: 0 (<5%), + (5–25%), ++ (26–50%), and +++ (>50%).

Calibration and Statistical Analysis

Intraobserver calibration was performed by reviewing the first of every five fields analyzed. Kappa agreement ranged from 0.7–1.00 and was considered to be acceptable.

The nonparametric Kolmogorov–Smirnov test was used to assess normality of data. The chi-square test was used to compare qualitative variables between groups, and Fisher's exact test was used to compare small samples. The Spearman coefficient was used to analyze correlations. The nonparametric Mann–Whitney test was used for comparisons between two or three independent groups, and the nonparametric Wilcoxon test was used to compare basal and suprabasal layers (paired data). Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 10.0.

Results

The percentages of cells immunolabeled with Ki-67, EGFR, and survivin, as well as the immunolocalization of EGFR, are presented in Table 1.

Values obtained for the basal and suprabasal layers of KOTs and DCs were combined and then compared with the results found for PFs. The analysis showed that KOTs presented significantly greater percentages of cells stained with Ki-67 when compared with DCs and PFs (Kruskal–Wallis test, $P \leq 0.01$) (data not shown).

The correlation between markers revealed a significant direct correlation only between Ki-67 and survivin in the PF group (Spearman correlation, $r_s = 0.481$; $P \leq 0.01$) (data not shown). The correlation of EGFR and survivin for the basal layer of DCs revealed statistically significant associations: score + was associated with staining in cytoplasm, whereas score +++ was associated with staining in both the membrane and cytoplasm (Fisher exact test; $\chi^2 = 11.51$, $P \leq 0.01$) (data not shown). The same association was observed in PFs (Fisher exact test; $\chi^2 = 12.04$, $P \leq 0.01$) (data not shown). In contrast, the comparison of Ki-67 and EGFR results in epithelial cells of PFs revealed that the values obtained with EGFR staining in both membrane and cytoplasm were significantly greater than those obtained with Ki-67 staining (Kruskal–Wallis; $P \leq 0.01$) (data not shown).

Discussion

The behavior of odontogenic epithelium and its remnants and their potential to develop odontogenic cysts and tumors has not been completely elucidated. Based on reports found in the literature [3–5, 11, 20], this study was designed to

Table 1 Percentages of immunolabeled cells and immunolocalization of epidermal growth factor receptor (EGFR) in the study groups

Lesion	No. of fields	Layer	Ki-67		EGFR			Survivin				
			Mean %	SD	M	C (%)	M + C (%)	– (%)	0 (%)	+	++ (%)	+++ (%)
KOT	65	Basal	11.82	8.97	0	84.6	15.4	0	0	32.3	41.5	26.1
		Suprabasal	28.14 ^a	13.84	0	96.9	0	3.1	0	0	3.1	96.9
DC	70	Basal	16.06 ^a	9.12	0	57.1	42.8	0	1.4	11.4	27.1	60
		Suprabasal	3.28	3.11	0	82.8	17.14	0	0	21.4	17.1	61.4
PF	45		7.88	6.94	22.2	20	57.8	0	0	22.2	22.2	55.5

SD standard deviation, M staining in membrane, C staining in cytoplasm, M + C staining in both membrane and cytoplasm

For the survivin data: – means negative, 0 implies <5% of cells stained, + implies 5–25% of cells stained, ++ implies 26–50% of cells stained, +++ implies more than 50% of cells stained

^a Wilcoxon test, $P \leq 0.01$

investigate the immunohistochemical profile of three proteins involved in cell proliferation, cell response to proliferative stimuli, and inhibition of apoptosis in the odontogenic epithelium of PFs, DCs, and KOTs.

Ki-67, or clone MIB-1, is an already consolidated marker of cell proliferation, and can be identified in all active cell cycle phases. The average number of cells positive for Ki-67 in KOTs, DCs and PFs in this study was in line with values reported in the literature. KOTs presented the greatest proliferation rate among the three groups, with Ki-67 immunolabeling found mainly in the suprabasal layer, suggesting an altered cell cycle and indicating the presence of a suprabasal proliferative compartment [7, 22, 30–34]. In DCs, the basal layer was found to be the proliferative compartment [34, 35]. In PFs, Ki-67 immunolabeling revealed a lower proliferative activity (7.88%) [2, 4], an expected finding for resting cells.

EGFR is a transmembrane protein that is present both in normal epithelium and in benign and malignant neoplasms. It is expressed mostly in basal e proliferating cells. Cells proliferating at a physiologic rate express this receptor in both the membrane and the cytoplasm. When EGFR is located only in the membrane, the speed of cell response to proliferative stimuli may be increased; on the other hand, when EGFR is found only in the cytoplasm (i.e., internalized or inactive), a slower response may be observed [24–26]. In the sample assessed in our study, EGFR immunolabeling was observed mainly in the cytoplasm in basal and suprabasal layers of KOTs, and in the suprabasal layer of DCs. Immunolabeling in both membrane and cytoplasm was higher in PFs. Membrane-only staining was observed exclusively in PFs.

According to previous studies, survivin is detected as a cytoplasmic and nuclear protein [36, 37]. This finding is in line with the role played by survivin in the regulation of cell viability and cell division [38]. Li et al. [38] have suggested that nuclear survivin is involved in the promotion of cell proliferation, whereas cytoplasmic survivin

may help control cell survival. The samples of our three groups presented nuclear and cytoplasmic labeling. DCs showed a higher number of cells with nuclear labeling in the basal layer. In KOTs, on the other hand, the highest percentage of positive cells scoring as +++ was associated with the suprabasal layer. In DCs, both layers showed similar percentages of cells scoring as +++. In PFs, score +++ was the most frequent finding.

Our results showed that the epithelium of KOTs presents a higher number of cells with proliferative activity when compared with DCs and PFs. The percentage of cells positive for survivin (scoring as +++) in the suprabasal layer was 96.9%, whereas in the basal layer it was 26.1%. The predominantly cytoplasmic EGFR staining found in basal and suprabasal layers of KOTs shows that the receptors are internalized in these cells (Fig. 1). We interpret these findings as cells cycling, demonstrating stimulus-independent proliferation, a characteristic of neoplastic cells. The suprabasal proliferative compartment seems to be maintained as a result of the significant expression of survivin, thus inhibiting apoptosis.

In DCs, immunolocalization of EGFR in the basal layer was similar in the cytoplasm (57%) and in the membrane and cytoplasm combined (42%), a finding that contrasts with the suprabasal layer, in which staining was predominantly observed in the cytoplasm (82.8%). The frequency of survivin class +++ was greater in both layers (Fig. 2). Analyzing these findings in association with Ki-67 immunolabeling, it is possible to state that cells of the basal layer in DCs are proliferating in response to stimuli, whereas almost all cells of the suprabasal layer are no longer proliferating, i.e., the EGFR is inactivated in the cytoplasm. DCs were the group that presented the highest number of cells positive for nuclear survivin, with a similar frequency in the two layers assessed for this marker. These findings suggest that, in the basal layer, survivin is promoting cell proliferation, whereas in the suprabasal layer, it is inhibiting apoptosis.

Fig. 1 Keratocystic odontogenic tumor: Ki-67 immunolabeling predominantly in the suprabasal layer (a); epidermal growth factor receptor (EGFR) immunolabeling mainly in the cytoplasm (b); survivin immunolabeling predominantly scoring as +++ in the suprabasal layer (c) (400× magnification)

Finally, PFs showed a lower proliferative activity, and the greatest percentage of cells stained for EGFR was found in both the membrane and the cytoplasm. Moreover, this was the only group presenting membrane-only staining, suggesting a high capacity of some cells to respond to stimuli, potentially leading to odontogenic

Fig. 2 Dentigerous cyst: Ki-67 immunolabeling in the basal layer (a); epidermal growth factor receptor (EGFR) immunolocalization predominantly in the cytoplasm in the suprabasal layers (b); survivin immunolabeling predominantly scoring as +++ in both layers (c) (400× magnification)

lesions, as also reported by Baumgart et al. [3]. Survivin class +++ was the most frequent finding in this group (Fig. 3). According to our understanding, the EGFR findings of our study indicate that, when stimulated, the epithelium of PFs may respond with proliferation. Taking into account the significant direct correlation between Ki-67 and survivin, the role of the latter seems to be its pro-mitotic function.

Fig. 3 Pericoronal follicles: Ki-67 immunolabeling in some epithelial cells (a); EGFR immunolabeling in the membrane of reduced enamel epithelium (b); survivin immunolabeling predominantly scoring as +++ (c) (400× magnification)

In summary, our findings confirm the neoplastic characteristic of epithelial cells in KOTs, i.e., their capacity to proliferate independently of the presence of stimuli, which could probably explain the high rates of relapse associated

with this type of lesion. Also, our data suggest that the proliferation of cells in DCs probably takes place based on a signal of mesenchymal origin, and that epithelial cells in PFs are embryonic cells which inhibit apoptosis, however with receptors that are expressed and ready to proliferate. These findings add to the current literature, corroborating the potential of odontogenic epithelial cells to develop odontogenic cysts and tumors, and allowing the recommendation of complete removal of these lesions during extraction of an unerupted tooth.

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