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Comparative Ki-67 Expression and Apoptosis in the Odontogenic Keratocyst Associated with or without an Impacted Tooth in Addition to Unilocular and Multilocular Varieties

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It is not known whether the presence of an impacted tooth or the radiographic types in an odontogenic keratocyst (OKC) change the clinical biologic behavior and therapeutic approaches. This study evaluated the comparative proliferative activity and apoptosis in OKC associated with or without an impacted tooth, as well as between the unilocular and multilocular OKC varieties.

Immunohistochemical expression of Ki-67 as a proliferation marker and the apoptotic reactions were assessed by the TUNEL method for 32 cases of OKC (OKC with impacted tooth, n=16; OKC without impacted tooth, n=16) and 10 cases of dentigerous cyst (DC).

OKC showed a greater proliferative potential and more apoptotic reactions than DC. In particular, OKC contained proliferating and apoptotic cells situated predominantly in the suprabasal and superficial layers, respectively. However, no significant difference was found between OKC associated with or without impacted tooth, or between the unilocular and multilocular OKC varieties, in terms of proliferative activity or apoptosis.

In conclusion, OKC is characterized by an increase in both cell proliferation and apoptosis, suggesting a unique proliferative and differentiation process. It is believed that incomplete removal or other contributing factors, rather than intrinsic growth or apoptosis, may be the main reasons for the aggressive biologic behavior or recurrence in multilocular OKC.

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INTRODUCTION

The odontogenic keratocyst (OKC) is a relatively common developmental odontogenic cyst that arises from the dental lamina remnants.[1] The keratocyst demands special consideration because of its aggressive clinical behavior and propensity for recurrence.[2,3] Approximately 25 to 40% of OKCs are associated with an unerupted or impacted tooth. In such circumstances, the radiographic features are similar to those of a dentigerous cyst (DC).[1] OKC shows a different growth mechanism and biologic behavior from the more common DC.[1] In addition, the multilocular OKC type tend to recur with a greater frequency than the unilocular variant.[4,5]

The precise nature of OKC and the reasons for its high recurrence rate remain substantially unknown.[2-5] It is believed that OKC's proliferative activity is one of the factors playing a significant role in cyst expansion and possibly recurrence.[6,7] This hypothesis is based upon an immunohistochemical demonstration of the proliferation markers.[8-11] It is thought that this might support the view that OKC had a neoplastic potential.[12] The growth behavior of tumors has been analyzed mainly from the standpoint of their proliferation activity.[8-12] In addition, apoptosis is an important mechanism to achieve cellular homeostasis.[13] In

tumor biology, it has been reported that the growth rate of tissues is determined by both the proliferative capacity and apoptosis.[14,15] Currently, there is a lack of data on either the proliferative activity or apoptosis in OKC associated with or without an impacted tooth, as well as in the unilocular or multilocular OKC varieties.

The aim of this study was to evaluate OKC by assessing Ki-67 expression as a proliferative marker and the apoptotic reactions. The TUNEL method was used to determine whether an impacted tooth or the radiographic OKC types change the clinical biologic behavior and therapeutic approaches. The proliferative capacity and apoptotic reactions were compared with OKC associated with or without an impacted tooth, and between the unilocular and multilocular OKC varieties.

MATERIALS AND METHODS

Selection of cases

From files of the Department of Oral Pathology, Chosun University School of Dentistry, 32 OKC cases, 16 associated with an impacted tooth (hereafter referred to as "follicular OKC", n=16, mean age: 23.4 years; male/female ratio: 9/7) and 16 without an impacted tooth (hereafter "extrafollicular OKC", n=16, mean age: 34.2 years; male/female ratio: 9/7), were examined in this study. Orthokeratinized odontogenic cysts and OKCs associated with the basal cell nevus syndrome were not included. Ten DC cases (n=10, mean age: 28.4 years; male/female: 6/4) were included in this study as a comparison. All diagnoses were confirmed according to the 2nd WHO classification of odontogenic tumors.[16] All samples had been fixed in 10% neutral-buffered formalin and processed by a routine method, including decalcification when necessary, before being embedding in paraffin wax. Tissue blocks were sliced into 4 μm thickness and the tissue sections were mounted on 3-aminopropyl-triethoxy-silane coated glass slides.

Immunohistochemistry

All sections for immunohistochemistry were deparaffinized and treated with a boiling solution of a freshly prepared, citrate/HCL buffer (10 mmol) pH 6.0 for 15 min on an electric hotplate. After cooling to room temperature, sections were rinsed in phosphate buffered saline (PBS) (5 min $\times$ 3), and incubated with 0.3% hydrogen peroxide for 20 min to block the endogenous peroxidase activity. The tissue sections were then washed in PBS, and 10% normal goat serum was applied for 20 min to reduce the nonspecific antibody binding. Ki-67 staining was performed using mouse monoclonal antibody (MM1, Novocastra, Newcastle, UK), which was diluted to 1:100 and was applied to the tissue sections overnight in a moist chamber at 4℃. After incubation with the primary antibody, the tissue sections were washed in PBS and treated with the biotin-streptavidin method (LASB2 Kit Peroxidase, Dako, Carpinteria, CA, USA). The peroxidase activity was developed by 3,3 diaminobenzidine hydrochloride (DAB, Sigma), resulting in a brown reaction product. The sections were finally counterstained with Mayer's hematoxylin and mounted. Tissue sections of the oral squamous cell carcinoma were also stained as positive controls. For negative control, PBS was applied to substitute for the specific antibody.

In situ DNA nick end-labeling method (TUNEL assay)

In order to detect apoptotic cells, a terminal deoxynucleotidyl, transferase mediated, nick end-labeling (TUNEL) method was performed using an ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Integran, NY, USA). Briefly, the sections were deparaffinized and treated with 20 μg/ml proteinase K (Boehringer-Mannheim, Indianapolis, IN, USA) solution for 15 min to enhance the staining. After immersing in 3% hydrogen peroxide to block the endogenous peroxidase, the sections were incubated with TdT together with digoxigenin-labelled dUTP and deoxyadenosine triphosphate (dATP) to bind the fragmented DNA ends of the apoptotic cells in a moist chamber at 37℃ for 60 min. Peroxidase-conjugated anti-digoxigenin antibody was then applied to the sections at room temperature for 30 min, and the reaction products were visualized by 0.03% DAB solution containing 2 mM hydrogen peroxide. Counter

staining was achieved with 0.5% methyl green. The negative control sections were treated with substituting TdT with dUTP and dATP for PBS, which were confirmed to be non-reactive.

Evaluation

Tissue sections for Ki-67 and TUNEL staining were examined for the presence of brown stained nuclei and evaluated by locating the epithelial linings most heavily labeled by scanning the sections at a 200 × magnification.[17] The Ki-67 labeling or apoptosis index (AI) was calculated as the mean number of cells with unequivocal positive nuclear staining in 5 randomly selected fields.[15] The results are presented as mean ± the standard deviation (SD). Statistically significance was analyzed using Mann-Whitney U test for groups. $p<0.05$ was considered to indicate statistical significance.

RESULTS

Ki-67 immunoreactivity

In OKC, the distribution of Ki-67 positive cells was mainly confined to the suprabasal location with a positive basal cell only rarely observed (Fig. 1). In DC, the positive nuclei were mainly confined to the basal cell layer, with only a few in the suprabasal position. The number of Ki-67 positive cells in follicular OKC (17.75 ± 6.47) was similar to that in the extrafollicular type (19.06 ± 9.99). Although no significant difference was found between follicular and extrafollicular OKC, the number of Ki-67 immunoreactive cells in follicular OKC was significantly greater than that in DC (6.60 ± 2.01, $p<0.05$, Fig. 2). In addition, there was no correlation between the unilocular (18.44 ± 9.23) and multilocular (17.86 ± 7.84) OKC types with regard to the radiographic varieties.

In situ DNA nick end-labeling

The apoptotic bodies visualized by in situ DNA nick end-labeling were detected as brownish round dots on the nuclei of the lining epithelial cells, and positive cells were recognized as being apoptotic cells. Nuclear staining for the TUNEL method was detected in 29 of the 32 OKC cases and in 4 of the 10 DC cases examined. There were a few scattered apoptotic cells in the DC cases, while TUNEL positive cells were found among the superficial parakeratotic or orthokeratotic cells in the lining of the OKCs (Fig. 3). The mean AI values were 4.78 ± 1.94 in follicular OKC, 4.56 ± 1.99 in extrafollicular OKC, and 2.30 ± 0.48 in DC. The mean AI value in follicular OKC was significantly higher than that in DC ($p<0.05$). However, no correlation between the OKC type and the presence of labeled cells was apparent (Fig. 4). In addition, the apoptotic reactions showed no

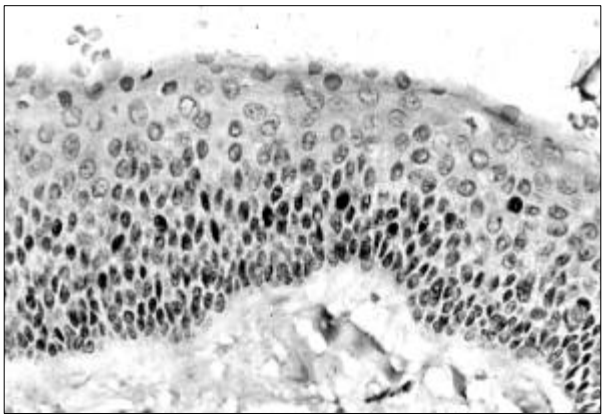

Fig. 1. Ki-67 immunoreactive cells are mainly observed in the parabasal cell layer of an odontogenic keratocyst (DAB and counterstaining with Mayer's hematoxylin, ×200).

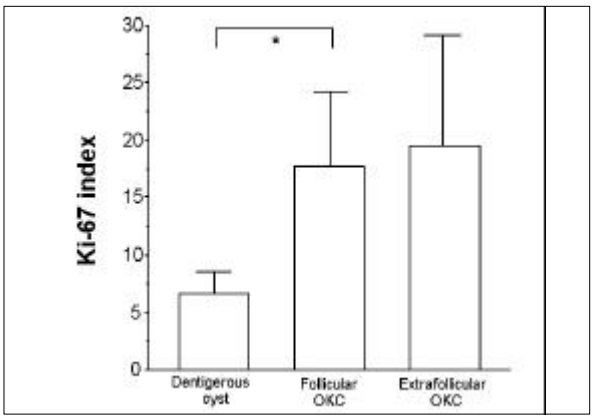

Fig. 2. Ki-67 labeling index of a dentigerous cyst and an odontogenic keratocyst.
OKC, odontogenic keratocyst; Values were compared by Mann-Whiney test; Statistical difference, $*p<0.05$.

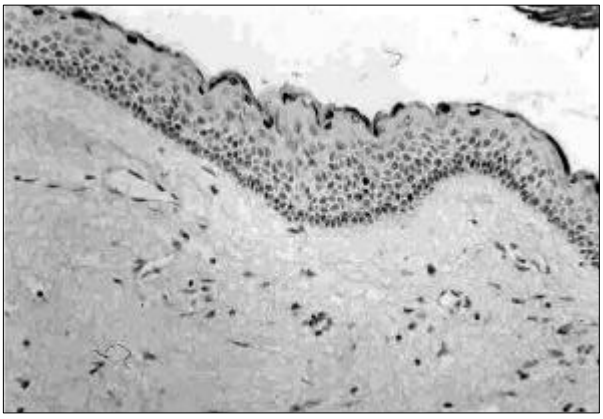

Fig. 3. The parakeratotic cells in an odontogenic keratocyst show a positive reaction in TUNEL assay (DAB and counterstaining with methyl green, × 200).

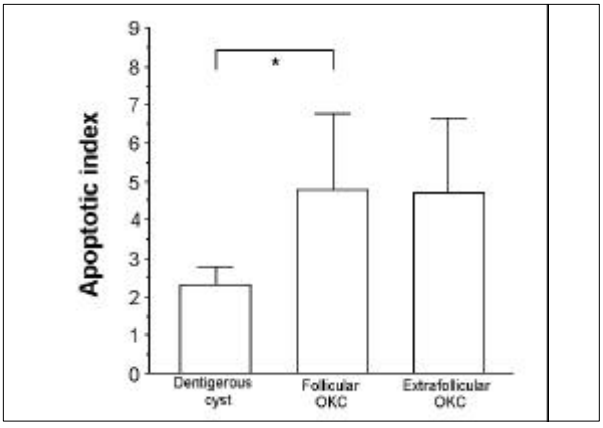

Fig. 4. Apoptotic index of a dentigerous cyst and an odontogenic keratocyst.
OKC, odontogenic keratocyst; Values were compared by Mann-Whiney test; Statistical difference, $*p<0.05$.

significant difference between the unilocular (4.18 ± 1.55) and multilocular (5.02 ± 2.22) OKC types with regard to the radiographic varieties.

DISCUSSION

In view of the fact that the growth rate of tissues is determined by proliferative activity and cell death,[8-15] the Ki-67 immunoreactivity as well as the apoptotic reactions as assessed by the TUNEL assay were examined. Among the monoclonal antibodies used in the study of the cellular kinetics there are those that recognize the proliferating cell nuclear antigen (PCNA) and the Ki-67 antigen. However, PCNA is not expressed throughout the whole cell cycle. Moreover, using immunohistochemical methods it is only detectable during the S or G1/S phase of the cell cycle, as it is impossible to detect during the G2 and M phases.[9,11,17] On the other hand, the Ki-67 antigen is expressed in proliferative cells throughout the G1, S, G2, and M phases, and immunohistochemically provides a reliable index of cellular proliferation.[18] In addition, the TUNEL assay is a simple and sensitive means of detecting apoptotic cells in tissue sections.[19] Several studies have revealed that the odontogenic epithelium of the tooth germ exhibits apoptotic reactions visible by this method.[20] Accordingly, Ki-67 monoclonal antibody was used as a proliferation marker and the TUNEL method was used for apoptotic reactions in OKC, as previously described.

In this study, the Ki-67 labeling index was significantly higher in follicular OKC than in DC. In addition, Ki-67 immunoreactivity was predominantly within the suprabasal layer. However, most of the Ki-67 positive cells in DC were located in the basal layer. The results of this study are in agreement with those of previous reports regarding the striking suprabasal location of PCNA and Ki-67 staining in OKC.[9,11,15,17] The highly significant correlation between PCNA and Ki-67 shows that Ki-67 positivity reflects the cell proliferative activity.[12] The greater proliferative activity confirmed by this study appears to indicate an epithelial lining with an intrinsic growth potential. This correlates well with the different clinical behavior of these two groups of lesions, and suggests that the more aggressive behavior observed in OKC might be due to the higher proliferation rate of its epithelial lining.[9,12,17] The predominant suprabasal location of the Ki-67 positive cells, together with the increased EGFR and p53 expression shown in previous studies, suggest that a unique proliferative and differentiation process occurs in the OKC lining.[9,12] However, this study showed that there was no correlation between Ki-67 immunoreactivity and the follicular or extrafollicular OKC types. These results indicate that follicular OKCs might arise following an eruption of a tooth into a pre-existing keratocyst cavity in the same way as a tooth erupts into the mouth.[21] However, the present

study demonstrated that there was no correlation between the unilocular and multilocular OKC varieties in terms of the proliferative activity.

Apoptosis plays a wide range of roles in maintaining normal homeostasis as well as in oncogenesis, and it has been proven to be a multistage process of DNA fragmentation by endogenous endonucleases.[13,15,19,20] This study confirmed the apoptotic reactions by TUNEL assay. In this study, the apoptotic reactions in OKC tended to be more numerous than in DC, and apoptosis was accelerated in OKC. Moreover, apoptotic cells were expressed in the nuclei of superficial cells in the OKC, while the TUNEL positive cells were a fewer and scattered randomly in DC. The results are also in agreement with a recent study of OKC.[15] These results suggest that the OKC epithelium is characterized by an increase in both cell proliferation and apoptosis in comparison with that of DC. However, no correlation between OKC type and the presence of labeled cells was apparent. In addition, the apoptotic reactions of the unilocular and multilocular OKC types were similar in terms of the radiographic varieties.

Previous studies revealed that the multilocular type tended to recur with a greater frequency than the unilocular OKC variant.[4,5] However, in this study, there were no significant differences in cell proliferation, as assessed by Ki-67 reactivity, or apoptosis between the unilocular and multilocular OKC varieties. Therefore, we consider that failure during surgery to completely remove the remnants of dental lamina, satellite cysts, and portions of the epithelial lining, rather than intrinsic growth or apoptosis, is likely to be responsible for this feature.[12] Similarly, Li, et al.[9] also demonstrated that Ki-67 expression is not related to OKC recurrence. An alternative explanation would be that the release of collagenase, prostaglandin, and highly active oxidative enzymes might contribute to the recurrence.[6,22,23] It has been shown that the growth factors and cytokines released by the inflammatory infiltrates in OKC might be responsible for the greater proliferative activity.[11] However, this relationship was not demonstrated in this study. Clearly, further research is necessary in order to establish the relationship between these proteins or enzymes with regard to the radiographic varieties in OKC.

In conclusion, the results showed that the proliferative capacity and apoptosis of OKC with an impacted or unerupted tooth was higher than those of DC. In particular, the OKC lining epithelium that contained proliferating or apoptotic cells was situated predominantly in the suprabasal or superficial layers, respectively. OKC showed a greater proliferative potential and more apoptotic reactions than DC. However, there was no correlation between the unilocular and multilocular OKC varieties in terms of their proliferative activity or apoptosis. It is believed that incomplete removal or other contributing factors, rather than intrinsic growth or apoptosis, may be the main reasons for the aggressive biologic behavior or recurrence in multilocular OKC. The reasons underlying these events should be delineated further.

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