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Cell proliferation, apoptosis and apoptosis-related factors in odontogenic keratocysts and in dentigerous cysts

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BACKGROUND: The purpose of this study was to elucidate why odontogenic keratocysts (OKC) can form cystic lesions but not tumor masses, notwithstanding their prominent proliferative activity.
METHODS: We investigated cellular proliferation, cell death, and expression of apoptosis-related proteins in the lining cells of OKCs and of dentigerous cysts (DGCs).
RESULTS: TdT-mediated dUTP-biotin nick end labeling (TUNEL)-positive cells were observed in the surface layers of OKCs and of DGCs. However, no TUNEL-positive cells were seen in the basal or intermediate layers of both cysts. Ki67-positive ratio in the intermediate layer was the highest in OKCs. The p53-positive ratio of the intermediate layer was highest in OKCs. Bcl-2-positive cells were discernible exclusively in the basal layer of OKCs.
CONCLUSIONS: These results suggest that cellular proliferation and death is regulated in association with apoptosis-related proteins in the lining epithelia of OKCs, and subsequently those cysts are seen as cystic lesions but not as tumor masses.
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Keywords: apoptosis; apoptosis-related factors: bcl-2; cell proliferation; dentigerous cyst; Ki67; odontogenic keratocyst; p53

Introduction

Odontogenic keratocysts (OKCs) develop from the odontogenic epithelium or its remains (1). It is well known that the OKCs show an aggressive behavior, recurring at greater frequency than other types of odontogenic cysts (1–6). These clinical findings have been supported by numerous reports focusing on the greater proliferative potential of the epithelial lining of OKCs compared with other types of odontogenic cysts (7–10). Consequently, it has been proposed that OKCs should be regarded as benign neoplasms (11).

In relation to the proliferative activity of the lining epithelium of OKCs, a number of investigations have examined the expression of p53 (12–15), proliferating cell nuclear antigen (PCNA) (10, 16–18) and Ki67 (5, 13, 19) in OKCs. Those studies concluded that p53, PCNA and Ki67 are expressed more strongly in OKCs than in other types of odontogenic cysts (11).

p53 as well as bcl-2 are apoptosis-related factors which can modulate the apoptotic pathway (18, 20). p53 is a transcription factor that integrates cell responses to various cellular stresses. Adaptive responses elicited by p53 activation include growth arrest and apoptosis (21). The bcl-2 proto-oncogene is a member of a gene family that includes cell death suppressors and cell death promoters. Its gene product, the protein bcl-2, is a 26 kDa putative membrane-associated protein which acts as a cell death suppressor that facilitates cell survival by regulating apoptosis (22). Investigations on the immunoreactivities of bcl-2 protein have been demonstrated in tooth germs, ameloblastomas, OKCs and dentigerous cysts (DGCs) (18–20, 23–27). Although apoptosis-related factors have been compared between sporadic OKCs and OKCs associated with nevoid basal cell carcinoma (18–20), there have been no reports on the significance of apoptosis and apoptosis-related factors in sporadic OKCs. On the other hand, a few studies that TdT-mediated dUTP-biotin nick end labeling (TUNEL)-positive apoptotic cells are found in the superficial cells of the lining epithelia of OKCs have demonstrated (19, 28).

Thus, a number of immunohistochemical studies have examined OKCs employing various markers of proliferation and of apoptosis (11, 12, 14, 15–20, 28). However, the question remains open why OKCs form cysts but do not form tumor masses in spite of their high potential to proliferate. We have postulated that there is a regulated balance between cell proliferation, cell differentiation and cell death in this type of cyst. Few studies have reported on the pathogenesis of cysts,

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including OKCs and DGCs, or have examined the relationships between proliferation, apoptosis and apoptosis-related proteins.

The purpose of this study was to elucidate why OKCs, which possess a high proliferative activity, can form cystic lesions but not tumor masses. In particular, we observed precisely the immunohistochemical reactions in the lining cells of OKCs and of DGCs dividing their constitutive layers into basal, intermediate and surface layers.

Materials and methods

Samples

Twenty cases of OKCs and twenty cases of DGCs were selected from banked tissue specimen blocks, comprised of 249 cases of OKCs and 668 cases of DGCs, prepared by the Department of Pathology, Tokyo Dental College, from 1993 to 1999. All materials were previously fixed in 10% neutral buffered formalin solution, dehydrated by graded alcohol and embedded in paraffin. The paraffin sections were cut serially into approximately 5 μm thick sections.

TUNEL method

To detect apoptotic cells, TUNEL was carried out using the ApopTag Plus peroxidase *in situ* apoptosis detection kit (Intergen Company, Purchase, NY, USA). The sections were deparaffinized, washed for 5 min in phosphate buffered saline (PBS), and incubated in 20 μg/ml proteinase K (Roche Diagnostics GmbH, Mannheim, Germany) in PBS for 15 min and then washed in PBS. Endogenous peroxidase was inactivated with 3% hydrogen peroxide in PBS for 5 min. The sections were rinsed twice with PBS for 5 min, then immersed in equilibration buffer and incubated with TdT in a humidified chamber at 37°C for 60 min. The reaction was terminated by incubation in PBS. These sections were then incubated with ant-digoxigenin conjugate for 30 min, and were finally incubated with 3,3′-diaminobenzidine for 3–6 min and counterstained with Mayer's hematoxylin. For the positive control, specimens were treated for 10 min in DN buffer (30 mM Tris-HCl buffer, pH 7.2, 4 mM $MgCl_2$, 0.1 mM DTT) and DNase (Bovine pancreas, 0.1 μg/ml; Amresco Inc, Solon, OH, USA).

Immunohistochemistry

For immunohistochemistry, the streptavidin-biotin immuno-peroxidase method was employed using a Histofine SAB-PO (MULTI) kit (Nichirei Co., Ltd., Tokyo, Japan). Sections were deparaffinized with xylene, and washed with 100% alcohol and then distilled water. Endogenous peroxidase activity was blocked by incubating the sections with 3% H_2O_2 in methanol for 30 min. They were then microwaved for 20 min at 90°C in 0.01 M citrate buffer (pH 6.0), cooled to room temperature and then washed in PBS three times for 5 min each. To prevent non-specific reactions, sections were incubated with 10% serum for 10 min. Ki-67 antibody at a dilution of 1:50 (Dako A/S, Glostrup, Denmark), p53 antibody at a dilution of 1:100 (Dako) and bcl-2 antibody at a dilution of 1:40 (Dako) were used as primary antibodies. These were reacted at 4°C overnight. As a negative control, PBS was used instead of the primary antibody. After the primary antibody reaction, the sections were rinsed in PBS three times for 5 min each. The secondary antibody (biotinylated anti-mouse IgG or anti-rabbit IgG) was reacted at room temperature for 30 min. After washing in PBS three times for 5 min each, 3, 3′ diaminobenzidine-tetrahydrochloride Tris-HCl buffer (pH 7.6) was used to visualize the reaction. Finally, sections were counterstained in Mayer's hematoxylin. Specimens were examined by light microscopy (Axiophot 2, Carl Zeiss, Oberkochen, Germany) and photographed.

Evaluation methods

For histomorphometry, tissue sections were photographed randomly as 35 mm color slides. The color slides were enlarged and printed at a final magnification of ×930. To evaluate the TUNEL and immunohistochemical staining, the constituent cells of the lining epithelium were divided into basal, intermediate or surface layers in both types of cysts. We considered cuboidal cells located from one to three rows at or near the basement membrane as the basal layer. The surface layer signifies flattened or polygonal cells consisting of one to three layers localized just underneath the surface of the lining epithelium. The intermediate layer is composed of relatively large round cells between the basal and the surface layers. Ratios of cells stained positively for TUNEL, Ki67, p53 and bcl-2 per 500 cells in 10 randomly selected fields were calculated in the basal and intermediate layers of both types of cysts. We used 15 randomly selected fields to estimate the ratio of the surface layer because of the paucity of cells in that layer. These values are expressed as positive ratios, and were analyzed statistically using the Mann–Whitney *U*-test for non-paired observations.

Results

General histopathology

Each OKC was lined by a keratinized stratified squamous epithelium, approximately five to nine cell layers thick, without rete ridges. The form of keratinization was almost parakeratotic and the surface was corrugated. The number of cells in the surface layer was less than in the basal and in the intermediate layers. The basal layer consisted of columnar or cuboidal cells. The intermediate layer was composed of relatively large and polygonal-shaped cells (Figs 1a, 2a, 3a and 4a).

Each DGC consisted of three to seven layers of flat or cuboidal cells, which were undifferentiated cells. The epithelial lining was not keratinized. The constitutive cells were relatively large and round cells, and discriminating between basal and intermediate cells was not easy. A small number of epithelial cells could be observed in the surface layer (Figs 1b, 2b, 3b and 4b).

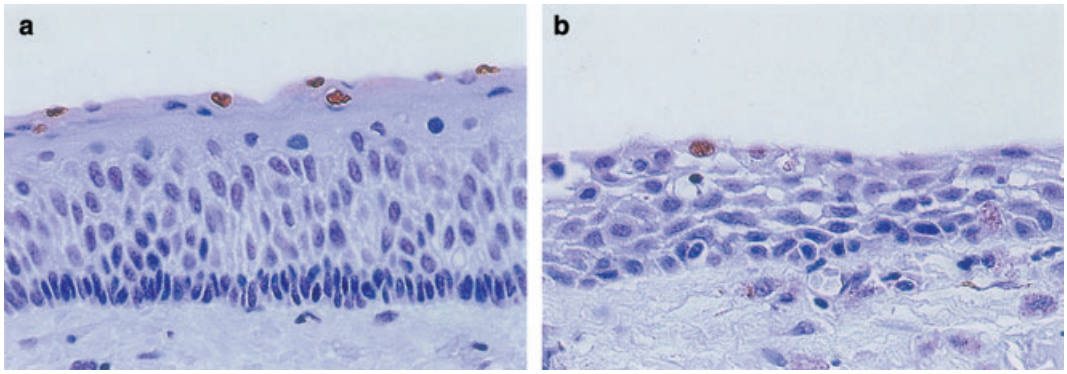

Figure 1 Photographs of TUNEL-positive cells in the lining epithelium. (a) In OKCs, the TUNEL-positive cells can be detected as brown-colored condensations and aggregates of nuclear chromatin in the surface layer. No TUNEL-positive cells are seen in the basal or intermediate layers. (b) TUNEL-positive cells are discernible in the lining epithelium of DGCs, but the number of TUNEL-positive cells is smaller than that in OKCs.

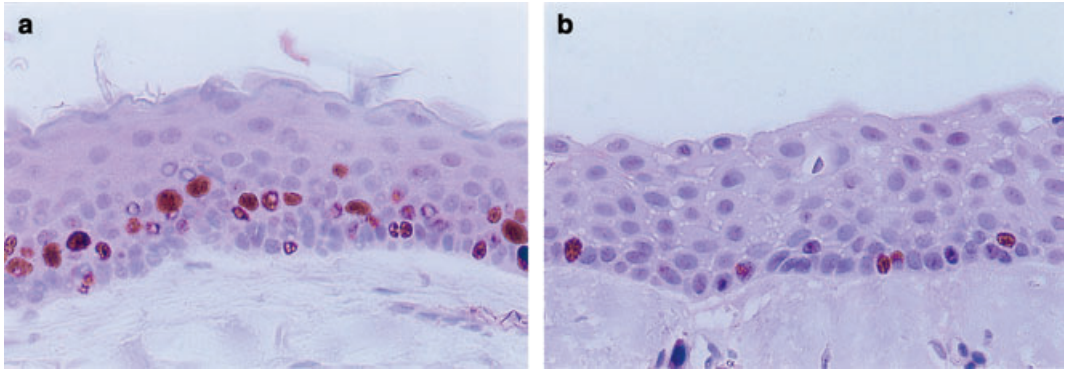

Figure 2 Immunostaining for Ki67. (a) The Ki67-immunopositive cells are distinct in the basal and in the intermediate layers of the lining epithelium of OKCs. A number of large cells are Ki-67-immunoreactive in the suprabasal layer. (b) In DGCs, a few Ki67-positive-cells can be found exclusively in the basal layer.

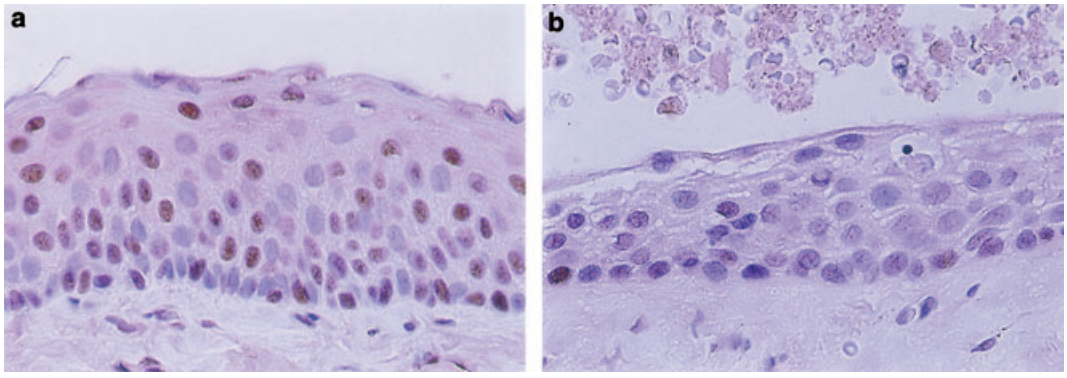

Figure 3 Immunohistochemistry of p53. (a) In OKCs, p53-positive cells are observed diffusely in all layers of the lining epithelium. (b) In DGCs, a small number of p53-immunopositive cells are obvious in the basal layer of the lining epithelium.

TUNEL staining

The TUNEL-positive cells were observed as brown-colored condensations and aggregates of nuclear chromatin in the surface layers of the lining epithelium in OKCs (Fig. 1a) and in DGCs (Fig. 1b). However, no TUNEL-positive cells were seen in the basal or intermediate layers of DGCs. The number of TUNEL-positive cells in OKCs was greater than in DGCs (Fig. 1a,b).

The ratio of TUNEL-positive cells in OKCs was approximately 25% in the surface layer, however, a ratio could not be calculated for the basal or intermediate layers. The TUNEL-positive ratio was about 8% in the entire layer of the lining epithelium. The

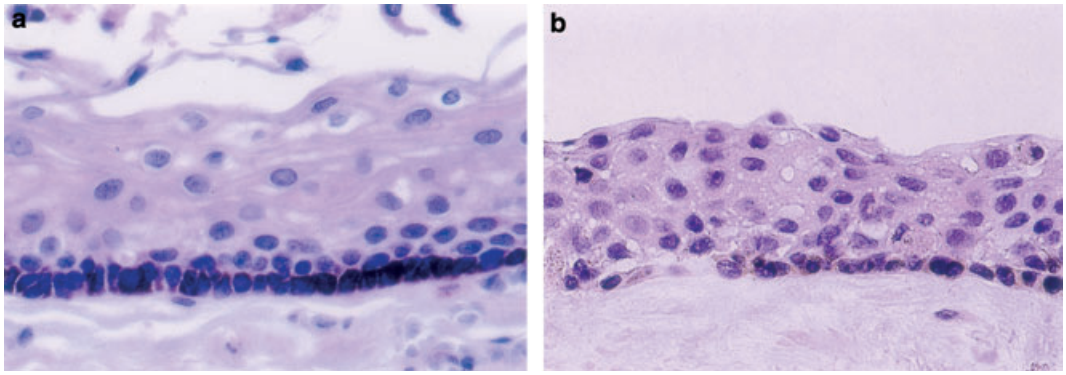

Figure 4 Immunohistochemical staining of bcl-2. (a) bcl-2-positive cells are recognized exclusively in the basal layer of the OKCs. Positive reaction for bcl-2 was distinct throughout the cytoplasm of the basal layer. (b) In DGCs, the bcl-2-immunopositive reaction was very weak and was observed in the basal layer.

Table 1 Positive ratios of TUNEL, Ki-67, p53 and bcl-2 in the lining epithelium of odontogenic keratocysts and dentigerous cysts

	TUNEL		*Ki-67*		*p53*		*bcl-2*	
	Odontogenic keratocyst	*Dentigerous cyst*	*Odontogenic keratocyst*	*Dentigerous cyst*	*Odontogenic keratocyst*	*Dentigerous cyst*	*Odontogenic keratocyst*	*Dentigerous cyst*
Total	8.2 ± 0.9	1.8 ± 0.6	18.5 ± 1.5	6.7 ± 0.7	60.9 ± 1.9	7.8 ± 1.7	32.2 ± 0.2	1.2 ± 0.8
Surface layer	24.5 ± 2.1	5.5 ± 1.8	3.6 ± 1.8	0.8 ± 0.4	44.5 ± 3.1	4.5 ± 2.6	0	0
Intermediate layer	0	0	36.4 ± 2.1	3.5 ± 1.8	72.4 ± 2.7	9.0 ± 2.5	0	0
Basal layer	0	0	15.6 ± 1.9	15.7 ± 2.0	65.8 ± 3.2	9.9 ± 2.4	99.6 ± 0.5	3.4 ± 2.2

*$P < 0.001$.
Values are given as mean ± SD (%).

TUNEL-positive ratio in DGCs was around 6% in the surface layer, but again it was impossible to calculate that ratio for the basal or intermediate layers. The TUNEL-positive ratio of the entire layer was about 2% (Table 1).

There was a statistically significant difference in the ratio of TUNEL-positive cells in the entire and in the surface layers of the lining epithelium between OKCs and DGCs ($P < 0.001$; Table 1).

Ki67

Immunopositive cells stained for Ki67 could be detected in the basal and in the intermediate layers of the lining epithelium of OKCs. Ki67 staining was especially noticeable in the nuclei of large cells in the suprabasal layer (Fig. 2a). In contrast, only a few cells that reacted positively for Ki67 could be found in the basal layer in DGCs (Fig. 2b). No Ki67-positive cells were discernible in the surface layer in either type of cyst (Fig. 2a,b).

The ratios of Ki67-immunopositive cells in OKCs were around 16% in the basal layer, 36% in the intermediate layer, and 4% in the surface layer. The ratio was approximately 19% in the whole layer of the lining epithelium in OKCs. The Ki67-positive ratios in DGCs were almost 16% in the basal layer, 4% in the intermediate layer, and 1% in the surface layer. The Ki67-positive ratio in all layers was about 7% (Table 1).

There was a statistically significant difference in the ratio of Ki67-positive cells in the total, surface and intermediate layers between OKCs and DGCs ($P < 0.001$). Significant differences were evident between each layer in both types of cyst ($P < 0.001$; Table 1).

p53

Immunopositive reactions for p53 were observed in the nuclei of cells localized diffusely in the entire layer of the lining epithelium of OKCs. The majority of cells which reacted positively for p53, was discernible in the intermediate layer, however p53-immunoreactive cells could also be detected in the basal and in the surface layers (Fig. 3a). In contrast, a small number of p53-immunopositive cells were seen in the basal layer of the lining epithelium of DGCs (Fig. 3b).

The ratios of p53-positive cells in OKCs were around 66% in the basal layer, 72% in the intermediate layer, and 45% in the surface layer. The p53-positive ratio in the entire layer of the lining epithelium was about 61% in OKCs. The p53-positive ratios in DGCs were 10% in the basal layer, 9% in the intermediate layer, and 5% in the surface layer. The p53-positive ratio in the entire layer was approximately 8% (Table 1).

Significant differences were seen in the ratio of p53-positive cells in the total, surface, intermediate and

basal layers between OKCs and DGCs ($P < 0.001$). There was a statistically significant difference between in each layer of both types of cyst ($P < 0.001$), except between the basal and intermediate layers in DGCs (Table 1).

bcl-2

Cells positive for bcl-2 were recognized exclusively in the basal layer of the lining epithelium of OKCs. Positive reactions for bcl-2 were distinct throughout the cytoplasm of basal cells in the lining epithelium. No bcl-2-immunoreactivity was evident in the intermediate or in the surface layers (Fig. 4a). In DGCs, bcl-2-positive cells could be detected in only four of the 20 cases examined. bcl-2-immunopositive reactions were very weak and were observed in the basal layer (Fig. 4b).

The ratio of cells immunopositive for bcl-2 in OKCs was about 97% in the basal layer. It was impossible to estimate the number of bcl-2-positive cells in either the intermediate or the surface layers not only in OKCs but also in DGCs. The bcl-2-positive ratio in the entire layer of the lining epithelium was approximately 32% in OKCs. The bcl-2-positive ratio in DGCs was 3% in the basal layer, and in the entire layer was 1% (Table 1).

There was a statistically significant difference in the ratio of bcl-2-positive cells in the entire and in the basal layers between OKCs and DGCs ($P < 0.001$). Significant differences are obvious between the basal and the intermediate or surface layers in both types of cysts ($P < 0.001$; Table 1).

Discussion

In this study, we employed the TUNEL method and immunohistochemistry to elucidate details of cell death and proliferation for cells constituting the lining epithelia of OKCs and DGCs, particularly with respect to the aggressive clinical behavior of OKCs. Antibodies used in immunohistochemical studies recognized Ki67, p53 and bcl-2, Ki67 being regarded as a marker for cellular proliferation, bcl-2 as an apoptosis-related protein and p53 as both markers (5, 13, 18–24).

Apoptosis

The TUNEL method, which is based on the addition of labeled UTP to the 3′ ends of fragmented DNA by TdT, has been reported as an *in situ* labeling method for detecting apoptotic cells (29). Our results demonstrate that TUNEL-positive cells are detected exclusively in the surface layer in both types of cyst. In OKCs, about 25% of the lining cells in the surface layer were TUNEL-positive, indicating that marked levels of apoptosis occurred in these cysts. It was clear that apoptosis also took place in DGCs, although the TUNEL-positive ratio of DGCs was about five times less than that of OKCs.

It has been surmised that homeostasis in renewing tissues is maintained by a regulated balance between cell proliferation, cell differentiation, and cell death (30). Apoptosis occurs at the end of differentiation in the skin and in oral keratinocytes (30–32). Therefore, our results suggest that apoptosis is significantly involved in the cyst formation and in maintaining a fixed thickness of the lining epithelium in cooperation with cellular proliferation in both types of cyst. This would explain why OKCs form cysts but do not form tumor masses in spite of their prominent proliferative potential.

In DGCs, apoptosis also seems to participate in maintaining a regular thickness of the cyst wall, although the TUNEL-positive ratio was relatively low in the surface layer (about 5.5%).

Proliferation

In the OKCs, the Ki67-positive ratio was about 15% in the basal layer, about 36% in the intermediate layer, and about 3.5% in the surface layer. These results were consistent with a study by Li et al. (5) who demonstrated that most Ki67-positive cells were detected in the suprabasal layer. These results imply that cells constituting the intermediate or suprabasal layers possess the highest proliferative activity in the OKCs.

In DGCs, however, the Ki67-positive ratios decreased in the intermediate and in the surface layers (about 3.5 and 1%, respectively), although the Ki67-positive ratio in the basal layer was similar (about 15%) to that of the OKCs. These results indicate that the relatively high values of proliferation in the basal layer of DCGs may participate in maintaining a regular thickness of the lining epithelium in partnership with the apoptosis that occurs in the surface layer.

p53 protein is a product of the tumor suppressor gene *p53*, which functions in G1 arrest to allow the repair of DNA damage and to prevent the cell from entering the S phase of the cell cycle, or alternatively to guide the damaged cells to apoptosis. The *p53* gene has a short half-life in normal cells and cannot be detected immunohistochemically, but when mutated, the p53 protein product is more stable and can be detected using immunohistochemistry. Many studies have now demonstrated that p53 protein is detectable in a wide range of malignant lesions but not in normal cells. Therefore, p53 protein is expressed in actively proliferating cells, particularly in neoplasms (11, 33, 34).

In the OKCs, the p53-positive ratios of cells in the lining epithelium revealed remarkably high values; about 66% in the basal, 72% in the intermediate, 45% in the surface, and 61% in all layers. Although these results cannot be compared directly with previously reported data because the methods used were different (12, 18, 19), our finding that the highest p53-positive ratio was seen in the intermediate layer was in accord with previous reports (12, 19).

In the present study, a relatively low p53-positive ratio and a high TUNEL-positive ratio were evident exclusively in the surface layer. This probably surmises that the decrease in p53-reactivity correlates with apoptosis in the surface layer. It has been postulated that p53 transmits apoptotic signals via a complicated mechanism, and DNA strand breaks are sensed by kinases leading to the phosphorylation and activation

of p53 (35). Hence, we are tempted to suggest that p53 functions not only as an apoptosis-related protein but also as a marker of cellular proliferation in OKCs (11). The diagnosis of OKCs are often confused with ameloblastoma because of their aggressive nature (11). We have previously shown that p53 mutation in ameloblastoma is detected by using a newly developed assay (36). The long half-life of mutant p53 leads to accumulate amount of the protein, showing a strong immunohistochemical reaction on the tissue (37). It cannot be, therefore, ruled out that mutant p53 is involved in the high positive ratios in the epithelium of the OKCs.

Apoptosis-related protein

Among all proto-oncogenes, bcl-2 is characteristically able to stop programmed cell death (apoptosis) without promoting cell proliferation. Its gene product, the bcl-2 protein, acts as a cell death suppressor that facilitates cell survival by regulating apoptosis (22). This inhibition of apoptosis constitutes one of the most common pathways of tumorigenesis (38, 39). Bcl-2 immunoreactivity has been shown in OKCs and in DGCs (18, 19, 23, 24). However, no study has reported the bcl-2-positive ratios in basal, intermediate and surface layers as we measured in this study.

The bcl-2-positive ratio was 96% in the basal layer of the lining epithelium of OKCs in the present study, although it was only about 3% in the DGCs. These results indicate that apoptosis does not occur in the basal cells of the lining epithelium of the OKCs (18, 19). The result in the DGCs was consistent with the report by Tosios et al. (24), which demonstrated that immunostaining for bcl-2 in DGCs was weak and restricted to the basal layer.

As bcl-2 is an apoptosis inhibiting protein, our results that bcl-2-positive cells were detected exclusively in the basal layer, and that TUNEL-positive cells were found only in the surface layers of both types of cysts, are reasonable. Namely, bcl-2 inhibits apoptosis to facilitate cellular proliferation in the basal layer, whereas apoptosis maintains the homeostasis of the thickness of the lining epithelium and allows the synthesis of large amounts of keratin in the surface layer of OKCs. This ingenious system to keep the balance between cellular proliferation and cell death in the lining epithelium can be detected in DGCs, but its degree is lower than that found in OKCs.

In conclusion, the reasons why OKCs are observed as cystic lesions but not as tumor masses in spite of their high potential to proliferate seem to be that: (i) cells constituting the lining epithelium have prominent proliferative activity in the basal and intermediate layers; (ii) apoptosis is inhibited by the expression of bcl-2 protein in the basal layer; (iii) apoptosis occurs in the surface layer to regulate the thickness of the lining epithelium; (iv) p53 contributes probably as an apoptosis-related protein as well as a marker of cellular proliferation; and (v) abundant keratin produced by apoptosis and other exudates from cyst wall may facilitate an expansion of cyst cavity.

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