Immunohistochemical evaluation of cell proliferation antigen Ki-67 and apoptosis-related proteins Bcl-2 and caspase-3 in oral granular cell tumor

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Purpose. We sought to evaluate the cell proliferation activity and immunohistochemical expression of proteins that promote or inhibit apoptosis in oral granular cell tumor (GCT).
Study design. Immunohistochemistry for Ki-67, a cell proliferation marker; Bcl-2, an anti-apoptotic protein; and caspase-3, a protein implicated in the execution of apoptosis, was performed on tissues from 12 patients with GCT of the tongue.
Results. Nuclear immunostaining for Ki-67 was observed only in isolated GCs (less than 2%). All patients exhibited cytoplasmic immunoreactivity for Bcl-2 in the majority of tumor cells. Cytoplasmic staining for caspase-3 was also present in more than 50% of GCs in all tumors.
Conclusions. GCT cells display low proliferation activity, a finding possibly related to their benign behavior. Caspase-3 expression suggests activation of the apoptotic cascade in the GCs, but persistence of the cells in the tissues could be attributed to the expression of Bcl-2 protein, a molecule that functions as a survival factor.
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The granular cell tumor (GCT) is an infrequently occurring benign soft tissue tumor with a well-known predilection for the oral cavity, especially the tongue.[1] Many studies have been performed to address the controversial issue of the histogenesis of this lesion. Although the histopathologic, immunohistochemical, and ultrastructural findings have been, to some extent, contradictory, the prevailing theory supports a neural origin.[2-7] Moreover, the nature of GCT—whether it represents a reactive lesion or a true neoplasm—has not yet been established conclusively.[6] Nonetheless, its biological behavior usually makes for a favorable prognosis.[8]

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A GCT is not encapsulated and is often characterized by a pseudoinvasive growth pattern, on the basis of the tendency of tumor cells to infiltrate between adjacent connective tissue elements, especially muscle fibers and nerve bundles.[1] A characteristic microscopic finding in approximately 50% of GCTs is the presence of varying degrees of pseudoepitheliomatous hyperplasia (PEH) of the overlying epithelium.[1,3] In these cases, diagnostic difficulties may arise, because PEH is occasionally so pronounced that it may be mistaken for squamous cell carcinoma, especially in superficial biopsy specimens.[1]

Dysregulations in the mechanisms and rates of cell proliferation and apoptosis are decisive factors for tumorigenesis in both benign and malignant neoplasms. Research on these cellular processes in recent years has been facilitated considerably through the use of numerous immunohistochemical markers. Among these, Ki-67, Bcl-2, and caspase-3 have been extensively studied in a variety of neoplasms.[9-13] Ki-67, a nuclear antigen expressed throughout the cell cycle but not in the G0 phase, has been used mainly as a cell proliferation marker.[14] Bcl-2 is an oncogene with well-characterized antiapoptotic properties. Bcl-2 overexpression has been implicated in neoplastic transformation because it contributes to the prevention of apoptotic cell death.[15,16] In contrast, caspase-3 (CPP32) is involved in the early phase of apoptosis. The active form is composed of 2 subunits with relative molecular weights of 17 kd and 12 kd that are derived from the cleavage of a 32-kd

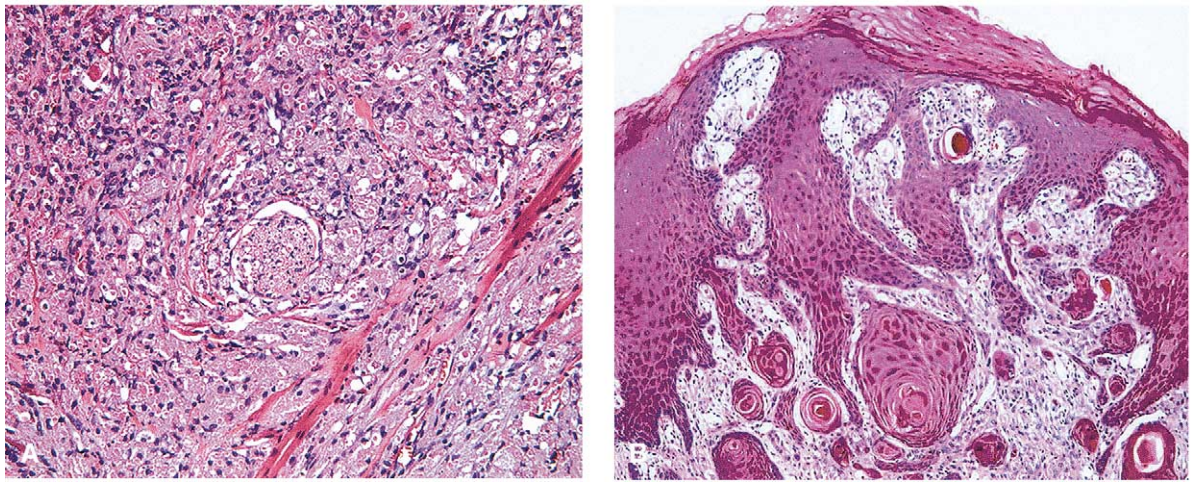

Fig 1. Histopathologic appearance of a granular cell tumor. **A,** Large, polygonal cells with abundant eosinophilic granular cytoplasm infiltrate between residual muscle fibers. Note the nerve bundle in the center, which is enveloped by granular cells (hematoxylin-eosin, original magnification ×200). **B,** Overlying epithelium with pronounced pseudoepitheliomatous hyperplasia (hematoxylin-eosin, original magnification ×100).

proenzyme.[17] Caspase-3 has a proapoptotic effect, participating in a cascade of events culminating in apoptotic cell death.[17,18] Despite the well-established contribution of these molecules to the pathogenesis of various neoplasms, their role in the development of oral GCT has not been investigated.

It was the purpose of this study to assess the cell proliferation activity of oral GCT by means of Ki-67 immunoreactivity testing and to evaluate the immunohistochemical expression of the apoptosis-related proteins Bcl-2 and caspase-3 in this tumor.

MATERIAL AND METHODS

Patients

The files of the Department of Oral Pathology and Surgery, University of Athens, Greece, encompassing a 26-year period (1974-2000) were reviewed. Fourteen patients with GCTs, all located in the tongue, were identified. Lesional tissue from 12 of 14 patients were selected for immunohistochemical analysis on the basis of tissue availability in paraffin blocks. The mean age of the 12 patients at diagnosis was 38.8 years; the age range was from 23 to 50 years. Seven patients were female and 5 were male. According to information obtained from the records of these patients, all GCTs exhibited benign biological behavior characteristic of this tumor. None of the tumors recurred over follow-up periods ranging from 6 months to 7 years.

During histopathologic analysis of hematoxylin and eosin–stained sections, all specimens were found to exhibit features typical of GCT, including large, polygonal cells with granular eosinophilic cytoplasm and small vesicular nuclei arranged in sheets, cords, and nests. The GCs were positive for S-100 in all patients. Three tumors were well circumscribed; however, 9 tumors infiltrated adjacent connective tissue, muscle fibers, and nerve bundles (Fig 1, *A*). PEH of different degrees was present in 8 patients, along with a close juxtaposition of GCs to the overlying epithelia (Fig 1, *B*). In the other 4 patients, GCs were more deeply situated in the connective tissue, thus separated from the oral surface epithelia.

Neither necrosis, spindling of GCs, vesicular nuclei with large nucleoli, increased mitotic activity, high nucleus-to-cytoplasm ratio, cellular atypia, pleomorphism nor other features suggestive of malignancy were identified.[19]

Immunohistochemical analysis

Five-micron-thick sections of formalin-fixed, paraffin-embedded tissue were deparaffinized and rehydrated. An antigen-retrieval procedure was performed by placing the sections in Citra-solution (HK086-9K; Biogenex, San Ramon, Calif) inside a plastic pressure cooker, which was positioned in a microwave oven (Kenmore; Sears, Chicago, Ill). The specimens were treated over 2 cycles of 15 minutes each, at a high level and at level 4. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide, and nonspecific protein was blocked with a universal blocking reagent (HK085-5K; Biogenex). All sections were washed with a phosphate-buffered saline solution and incubated with the primary antibody for 1 hour. The following primary antibodies were used: anti–Ki-67 (MIB-1) at a 1:100

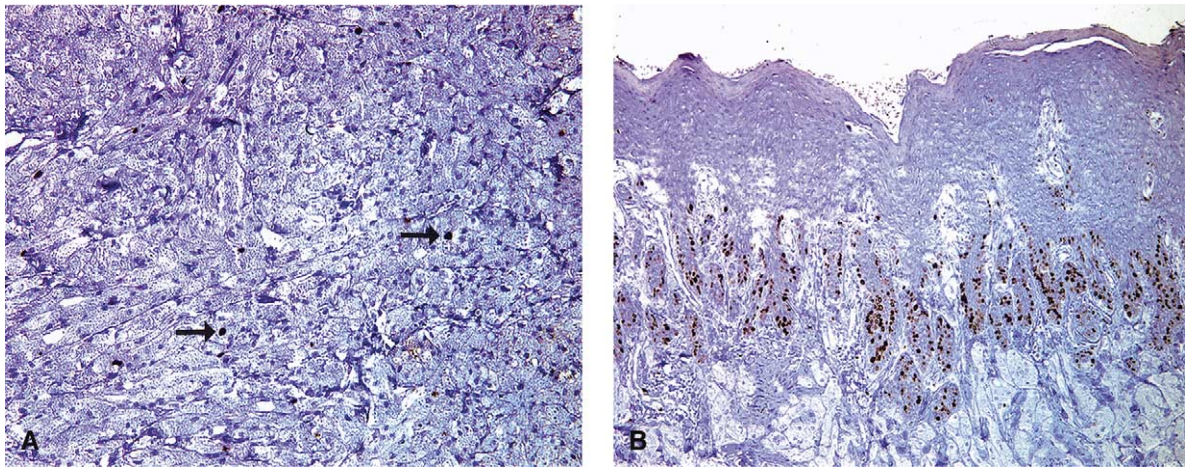

Fig 2. Expression of proliferating marker Ki-67 in oral granular cell tumors. **A,** Only isolated granular cells (*arrows*) are positive for Ki-67 (Ki-67, original magnification ×200). **B,** Strong Ki-67 immunostaining in the basal and parabasilar cells of overlying epithelium exhibiting marked pseudoepitheliomatous hyperplasia (Ki-67, original magnification ×100).

concentration (Biocare Medical, Walnut Creek, Calif); anti–Bcl-2 at a 1:50 concentration (Biocare Medical); and anti–caspase-3 (CPP32) at a 1:50 concentration (Novocastra, Newcastle-upon-Tyne, England). Sections were incubated with a secondary antibody (HK268-UK, 1:100, Biogenex) for 30 minutes. A StrepABComplex/HRP reagent (K0377; Dako, Carpinteria, Calif) was applied, and the known positive control section for each antibody was stained in each run to confirm the presence of appropriate immunostain activity. Negative controls were also included in each run.

To evaluate and compare the results of immunohistochemistry, both the positivity (ie, the percentage of GCT cells yielding a positive stain) and the intensity of staining were assessed. Positivity was classified in terms of the following: *negative*; ±, <5% positive cells; +, >5% to 20% positive cells; ++, >20% to 50% positive cells; and +++, >50% to 100% positive cells. Ki-67 positivity was assessed semiquantitatively by counting the number of positive nuclei in 100 successive GCT cells. The intensity of staining in the positive cases was graded as weak, moderate, or strong.

The study design was reviewed by the institutional review board of the University of Maryland, which granted an exemption from institutional review board approval (Exemption No. JJS-120101).

RESULTS

Ki-67

Immunohistochemical analysis for Ki-67 revealed that only isolated GCs were positive, with strong nuclear staining (Fig 2, *A*). Positive cells accounted for 1% to 2% (±) of the total GC population in 7 patients and for less than 1% (±) in 5 patients. Immunoreactivity for Ki-67 was also evident in the basal and parabasal cells of the overlying epithelia of all patients (Fig 2, *B*). However, the pattern of Ki-67 staining in epithelia varied depending on the presence of PEH. More specifically, specimens characterized by the presence of PEH with proximity between GCs and oral surface epithelia exhibited Ki-67 staining in 10% to 30% (+ or ++) of the basal cells and in a similar percentage (+ or ++) of parabasal cells (Fig 2, *B*). In contrast, specimens lacking both PEH and close GC approximation to surface epithelium displayed only occasional Ki-67 positivity in basal cells (<5%, ±), whereas the percentage of parabasal cells with Ki-67–positive staining exceeded 50% (+++). The latter pattern of Ki-67 immunostaining was shared by oral surface epithelia located at the peripheral margins of the specimens, situated some distance from the GCT.

Bcl-2

Moderate to strong cytoplasmic immunostaining for Bcl-2 was seen in the GCs of all patients (Fig 3, *A* and *B*). In 10 patients, more than 90% of cells (+++) were positive. In the remaining 2 patients, the positive reaction was limited to a smaller number of cells; however, at least 50% of tumor cells in these GCTs exhibited staining (+++).

Oral surface epithelia immediately above and lateral to GCTs were consistently negative (Fig 3, *A* and *B*). Intervening striated muscle fibers were focally positive in 7 patients, diffusely positive in 2 patients, and neg-

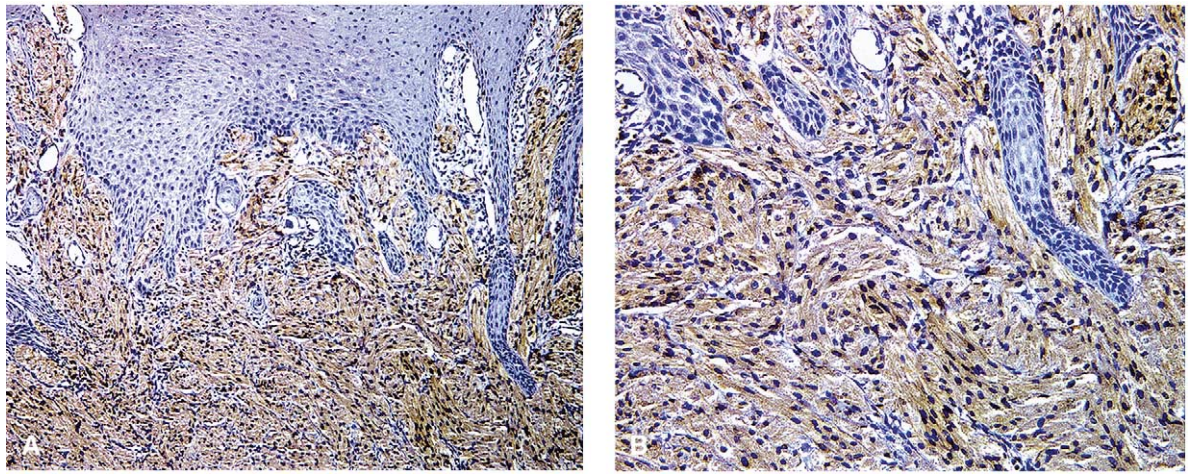

Fig 3. Bcl-2 protein expression in oral granular cell tumors. **A,** Granular cells display diffuse immunoreactivity for Bcl-2, whereas the overlying epithelial cells are consistently negative (Bcl-2, original magnification ×100). **B,** Higher magnification depicting strong cytoplasmic Bcl-2 staining in tumor cells (Bcl-2, original magnification ×200).

ative in 3 patients, the latter despite positive staining of adjacent tumor cells.

Caspase-3

The GCs of all patients exhibited cytoplasmic staining for caspase-3 (Fig. 4, *A* and *B*). The percentage of positive GCs in GCTs ranged from 50% to 100% (+++), whereas the intensity of the immunostaining was moderate to strong in 5 patients and weak in 7 patients. Strong caspase-3 reactivity in the epithelia overlying and adjacent to GCTs was apparent in all patients (Fig 4, *A*). Positive staining was limited almost exclusively to the upper spinous and parakeratin layers. Striated muscle fibers traversing GCTs exhibited strong positivity in 7 patients.

DISCUSSION

The growth of a tumor reflects an imbalance between cell proliferation and cell death. Accordingly, alterations in cell proliferation– and apoptosis-related pathways and molecules have been identified in many benign and malignant neoplasms.[9-13] GCT is a well-recognized lesion that in the vast majority of cases behaves as a benign neoplasm; however, the molecular basis for its development and progression, which may be linked to disturbances in cell proliferation and apoptosis, has not been thoroughly investigated.

In this study of oral GCT, we first attempted to evaluate the proliferation state of the GCs by assessing the Ki-67 index. Increased levels of Ki-67 nuclear staining have been associated with oral epithelial dysplastic and malignant change, in certain cases carrying prognostic significance.[13] The very low number of GCT cells that exhibit a positive nuclear reaction for Ki-67 is indicative of the limited growth potential of these cells. Similarly, Lassaletta et al[20] reported that cells in GCTs of the larynx were only occasionally Ki-67–positive. In the study by Kaiserling et al,[21] none of the GCTs examined had reactivity for the cell proliferation markers proliferating cell nuclear antigen and Ki-67. In congenital epulis, a benign tumor with histopathologic features similar to GCT, few of the GCs (<5%) demonstrated immunoreactivity for both antigens proliferating cell nuclear antigen and Ki-67.[21] Our findings can be interpreted as consistent with the benign nature of the GCTs analyzed and in contrast to the high Ki-67 expression levels displayed by neoplastic cells of malignant GCTs.[8,19,22] Interestingly, in their study of malignant, atypical, and multicentric GCTs of soft tissue, Fanburg-Smith et al[19] disclosed that Ki-67 values of greater than 10% were an adverse prognostic factor with regard to survival.

We next investigated whether alterations in the immunohistochemical expression of apoptosis-related proteins could be linked to the pathogenesis of these tumors. Interestingly, GCT cells exhibited diffuse immunoreactivity for the antiapoptotic protein Bcl-2. This finding contradicts those in the study by Lassaletta et al,[20] where weak or negative Bcl-2 staining in laryngeal GCTs was interpreted as suggestive of the benign nature of this tumor. However, in the context of soft tissue tumors, Bcl-2 detection should not be considered a broadly useful marker of malignancy, because malignant mesenchymal neoplasms at times exhibit weaker Bcl-2 expression than their benign counterparts.[23] Indeed, benign soft tissue tumors with diffuse Bcl-2 ex-

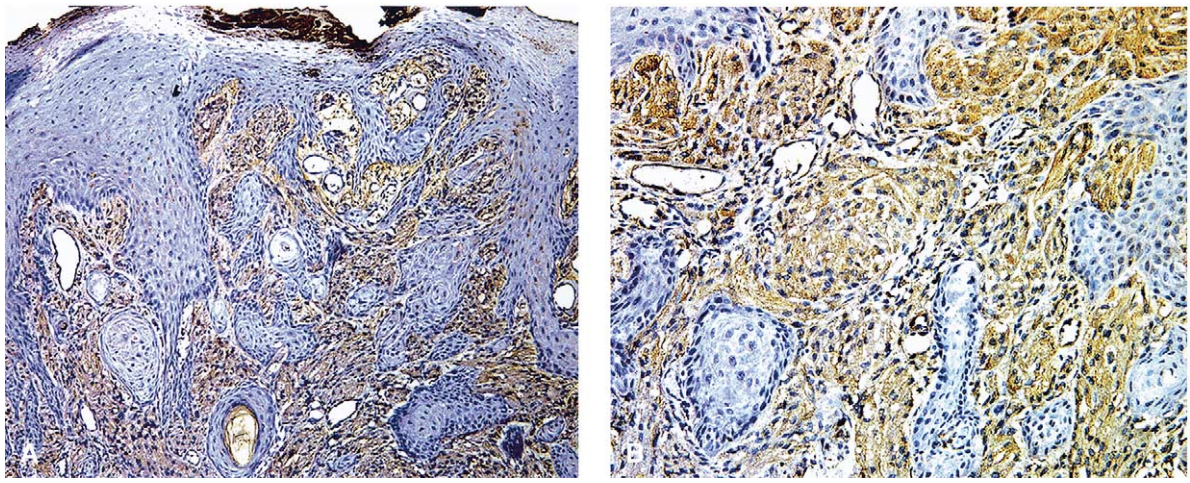

Fig 4. Caspase-3 protein expression in oral granular cell tumors. **A,** Diffuse caspase-3 immunoreactivity of tumor cells is depicted. Positive epithelial cells, mainly in the parakeratin layer, are also observed (caspase-3, original magnification ×100). **B,** In this field, tumor cells had a strong cytoplasmic reaction for caspase-3, whereas the epithelial cells are negative (caspase-3, original magnification ×200).

pression such as solitary fibrous tumor, hemangiopericytoma, and schwannoma, a condition which may favor the growth of neoplastic cells, have been reported.[23] Likewise, Bcl-2 overexpression may also be related to the pathogenesis of oral GCT, because the inhibition of apoptosis could lead to prolonged life for GCs in GCTs. In contrast, it should be recalled that Bcl-2 expression has been identified in healthy tissues, including those of smooth muscle and neural origin. It has been proposed that the pattern of Bcl-2 expression in soft tissue tumors often parallels that in their healthy tissue counterparts.[23] Consistent with the findings of several studies supporting the origin of GCTs from Schwann cells,[2,4,5,7] one reasonable hypothesis is that GCT tumor cells retain the Bcl-2 positive immunohistochemical profile of their progenitor cells. This theory is in agreement with the results of previous studies showing Bcl-2 expression in both normal neural cells and their related neoplasms.[23] In contrast, most of our patients had only focal Bcl-2 staining of striated muscle fibers adjacent to tumor cells. Similarly, Miettinen et al[23] noticed that skeletal muscle generally failed to exhibit Bcl-2 staining, with the exception of atrophic or regenerative striated muscle fibers adjacent to tumors, where Bcl-2 immunohistochemical reactivity was seen. Absence of BcL-2 staining in the epithelia overlying the tumors was comparable with that observed in healthy epithelium.[10]

Caspase-3 represents another important molecule in apoptosis. It belongs to the caspase family of cysteine proteases, whose enzymatic action leads to the cleavage of cellular substrates during apoptotic cell death.[17,18,24] Of the caspases, caspase-3 has been characterized as the most directly linked to apoptosis.[17,24] It has been suggested that caspase-3 expression may signify increased vulnerability to apoptosis or activation of the apoptotic cascade, or both.[18] Caspase-3 has been identified in a number of benign and malignant neoplasms, including ameloblastoma,[25] squamous cell carcinoma,[11] breast carcinoma,[12] neuroblastoma,[26] and lymphoma.[9] Certain healthy cells and tissues, but no Schwann cells, also express caspase-3.[27] Immunohistochemical detection of caspase-3 does not necessarily confirm function, but the reactivity seen in GCT cells in our patients may be an indication of the occurrence of apoptotic death in the GC populations. However, it has been demonstrated that Bcl-2 can prevent the processing and activation of caspase-3 without affecting its expression levels.[17,28] Thus, Bcl-2 up-regulation, as demonstrated in our patients, may counteract the proapoptotic function of caspase-3.[28] Nonetheless, the localization of caspase-3 expression in the upper layers of the epithelium may signal the participation of this molecule in the terminal differentiation process of keratinocytes. Another finding that was compatible with our results was that striated muscle fibers have been shown to display variable immunoreactivity for caspase-3, ranging from a negative to a strongly positive reaction.[27]

In this study, all GCTs behaved in a benign, nonaggressive fashion and did not recur after conservative surgical excision. However, the potential for aggressive clinical behavior in a rare subset of GCTs is supported in the literature. In a study by Lack et al,[29] five of 24

incompletely excised GCTs recurred. The multicentric occurrence of GCT has been also reported,[8,19,30] and malignant behavior of GCT with metastatic spread—albeit exceedingly rare—has been described.[1,8,19] Despite efforts to establish histopathologic criteria for discrimination among benign, atypical, and malignant GCTs, the presence of metastases remains the only reliable criterion for malignancy, given that even cytologically bland GCTs may metastasize.[19,22] Future research endeavors should focus on comparing the expression of cell proliferation and apoptotic markers encountered in typical, recurrent, and malignant GCTs, with the goal of identifying patterns of expression predictive of tumor biological behavior. In this regard, the detection of higher degrees of tumor cell proliferation, angiogenesis, and p53 expression in a case of cutaneous malignant GCT relative to its benign counterpart necessitates further investigation.[22]

An interesting histopathologic finding in GCT is the frequent occurrence of PEH in the epithelium overlying the tumor, a pattern mimicking—and occasionally mistaken for—the invasion of squamous cell carcinoma into connective tissue.[1] The cause of PEH is largely unknown. In our study, GCTs with PEH exhibited a noteworthy increase in Ki-67 staining of basal cells of the overlying epithelia. Proximity of the epithelium to the underlying GCs appears to be a prerequisite for up-regulation of basal cell proliferation. This relationship between enhanced basal cell proliferative activity and the development of PEH implies that the stimulation of basal cell proliferation occurs through interaction between GCs and neighboring epithelial cells, providing a plausible explanation for the association of PEH with GCT. Future investigations should address the molecular basis for the epithelial and mesenchymal cell interactions that result in PEH. This alteration in epithelial cell behavior may be the result of either direct apposition of epithelial cell and GC populations or caused by the influence of soluble proteins produced by tumor cells, such as epidermal growth factor, transforming growth factor–α, nerve growth factor, and calretinin.[31-34]

It would be interesting to evaluate whether similar changes in the proliferative capacity of epithelial cells are observed in PEH typically encountered in other disorders, such as median rhomboid glossitis, blastomycosis and other deep fungal infections, keratoacanthoma, Wegener's granulomatosis, and necrotizing sialometaplasia.[1] Aside from providing insight into the pathogenetic mechanisms of reactive epithelial growth, the assessment of the differential expression of Ki-67 and other proliferative markers in PEH and a comparison with frank cancerous lesions may prove diagnostically useful by allowing discrimination in equivocal cases. For example, Phillips and Helm[35] showed that differences in the distribution of proliferating cell nuclear antigen among PEH in keratoacanthoma and squamous cell carcinoma of the skin may facilitate their distinction.

Low cell proliferation activity in GCT is in accordance with its benign clinical behavior. Caspase-3 expression may suggest an activation of the apoptotic cascade in GCs, but the persistence of cells in the tissues could be attributed to the expression of Bcl-2 protein, functioning as a survival factor through the inhibition of apoptosis. Increased proliferation activity of the basal epithelial cells in GCTs with PEH may represent an induction phenomenon mediated by hitherto unidentified molecules produced by the GCs. Future studies should elucidate the molecular basis for the observed protein changes and seek potential diagnostic applications. A comparison of the immunohistochemical profile of GCT with those of other benign and malignant soft tissue tumors of the oral cavity, including the rare malignant variant of GCT, will enhance our understanding of the roles that cell proliferation and apoptosis play in the development and progression of this tumor and raise the possibility of providing potentially useful markers for the prediction of the clinical behavior and prognosis of a given neoplasm.

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