Abstract: Bone resorption in the jaws is one of the most severe complications of keratocystic odontogenic tumors (KCOTs), and can be treated by either enucleation or marsupialization. However, the effects of marsupialization on bone regeneration adjacent to KCOTs, and the mechanisms involved, are still unclear. In this study, samples of 27 KCOTs were collected (20 before marsupialization and 7 after marsupialization) to detect the expression of bone regeneration-related molecules adjacent to KCOTs by immunohistochemistry and real-time quantitative polymerase chain reaction (qPCR). The results showed that bone formation was significantly enhanced in the KCOT capsule wall adjacent to bone after marsupialization, as demonstrated by alkaline phosphatase activity assay and immunostaining for bone morphogenetic protein. Moreover, immunohistochemistry revealed that osteoprotegerin (OPG) was up-regulated in the KCOT capsule wall adjacent to bone after marsupialization, while receptor activator of nuclear factor-κB ligand (RANKL) was down-regulated. Real-time qPCR also demonstrated increased expression of OPG after marsupialization, accompanied by a decrease in the expression of osteoclastogenesis-related molecules such as cathepsin K, matrix metalloproteinase-9, NFATc1, RANK and RANKL. This study provides further evidence that marsupialization may promote bone regeneration adjacent to KCOTs partially through regulation of the OPG/RANK/RANKL signaling pathway.

Keywords: marsupialization; KCOT; bone regeneration; ALP; OPG.

Introduction

Keratocystic odontogenic tumor (KCOT), also known as odontogenic keratocyst (OKC), is a benign intraosseous tumor of odontogenic origin with a characteristic lining of parakeratinized squamous epithelium and locally aggressive behavior (1). Bone resorption in the jaws is one of the most severe complications of KCOT and can easily give rise to large jaw defects or even pathologic fractures, especially when the KCOTs are large (2). Since it was first described in 1956, KCOT has been one of the most controversial pathological entities in the maxillofacial region.

Generally, KCOT can be treated by either enucleation or marsupialization, depending on its location, size, and...
proximity to vital structures such as the teeth, maxillary sinus, and the mandibular canal (3). Enucleation is the treatment of choice for small KCOTs and can be performed without damage to adjacent tissue, whereas marsupialization is usually performed for large ones, in order to minimize the cyst size and limit the extent of surgery (4). This approach is more conservative and creates a window in the wall of the cyst to relieve the intracystic positive pressure that might contribute to bone resorption due to marked compressive force (5), leading to gradual reduction of the cyst cavity size. Complete enucleation can then be subsequently performed when sufficient new bone has formed and the adjacent vital structures have been saved from damage. Our previous study has indicated that marsupialization could enhance bone density and reduce the cyst volume, as determined by X-ray measurement (6,7). Although the effects of marsupialization on the epithelium of KCOTs have been discussed (8), the effects on bone regeneration adjacent to KCOTs, and the mechanisms involved, remain to be further clarified.

Clarification of the receptor activator nuclear factor kappa B (RANK), receptor activator nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG) molecular system in osteoclastogenesis has been a major advance in bone biology (9). RANK, which belongs to the tumor necrosis factor (TNF) receptor superfamily, is a central activator of nuclear factor κB (NF-κB) and the signaling receptor for RANKL (10). RANKL can bind to RANK on the surface of pre-osteoclasts, leading to activation of osteoclasts (11). OPG, secreted by many cell types including osteoblasts, is a decoy receptor for RANKL that interrupts the interaction between RANKL and RANK, thereby inhibiting bone resorption (12). Therefore, the OPG/RANK/RANKL signaling pathway regulates the development and activation of osteoclasts, and consequently affects bone resorption. Importantly, several recent studies of odontogenic tumors have suggested the involvement of the OPG/RANK/RANKL signaling pathway in bone resorption due to KCOTs (13-16).

In the present study, we assessed the effect of marsupialization on bone regeneration adjacent to KCOTs, and investigated the involvement of the OPG/RANK/RANKL pathway in marsupialization-induced bone regeneration.

Materials and Methods

Reagents

Unless otherwise noted, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies used in this study were obtained from Santa Cruz (CA, USA).

Tissue samples

Samples of 27 KCOTs were collected at the Department of Oral and Maxillofacial Surgery, Hospital of Stomatology, Wuhan University. Twenty had been primary cases treated by marsupialization, among which seven had then been treated by enucleation after marsupialization. The clinical features of these KCOTs are listed in Table 1 and 2. Patients with nevoid basal cell carcinoma syndrome (NBCCS) were excluded from the study. All of the KCOT samples were diagnosed according to the latest World Health Organization classification of tumors, and the samples after marsupialization were subjected to double biopsy. All procedures were performed according to the National Institutes of Health guidelines regarding the use of human tissues, and this study was approved by the review board of the Ethics Committee of the Hospital of Stomatology, Wuhan University (034-2012).

Immunohistochemistry

All specimens were fixed in buffered 4% paraformaldehyde and embedded in paraffin. The paraffin-embedded tumor tissues were then serially sectioned (4 µm in thickness), and selected slides were routinely stained with hematoxylin-eosin (HE). All of the tumor sections were reviewed in a blinded manner by two pathologists. Immunohistochemical analyses were then conducted in accordance with our previous procedures (17,18). The staining intensities for the tested markers in the KCOT capsule wall adjacent to bone were estimated semi-quantitatively as follows: –, no staining; +, mild; ++, moderate; or ++++, intense.

Alkaline phosphatase activity assay

Alkaline phosphatase (ALP) activity was measured in human samples using an ALP assay kit (Sigma-Aldrich) with p-nitrophenyl phosphate as substrate. Briefly, fresh frozen sections were fixed in ice-cold acetone for 5 min and then air-dried for about 5 min. Subsequently, the slides were placed into ALP substrate working solution at 37°C for 60 min. After being checked microscopically and washed in distilled water, all the sections were counterstained with hematoxylin for 1 min and finally mounted directly with aqueous medium.

Real-time quantitative PCR

Isolation of total RNA, synthesis of cDNA, and real-time qPCR were carried out in accordance with our previous description (17). GAPDH was selected as an internal
control for each experiment. The primer nucleotide sequences for PCR were designed as follows: CatK: 5'-ACCGGGGTATTGACTCTGAA-3' and 5'-GAG-GTCAGGCTTGCATCAAT-3'; MMP9: 5'-CCTGGAGACCTGAGAACCAATC-3' and 5'-CCACCGAGTGTAACCATAGC-3'; NFATc1: 5'-GCATCACAGGGAAGACCGTGTC-3' and 5'-GAAGTTCAATGTCGGAGTTTCTGAG-3'; OPG: 5'-CGCTACCTTGAGATAGAGTT-3' and 5'-CCAAGACACTAAGCCAGTTA-3'; RANK: 5'-CAGAACTAAGCTCAGTATGA-3' and 5'-GAATGCCAAGCTGCAGCAAC-3'; RANKL: 5'-TCGTTGGATCACAGCACATCA-3' and 5'-TATGGGAACCAGATGGGATGTC-3'; GAPDH: 5'-CCATGTTCGTCATGGGTGTGAACCA-3' and 5'-GCCAGTAGAGGCAGGGATGATGTTTC-3'.

Statistical analysis

Fisher’s exact test and Student’s t-test were used for statistical analysis. Differences at $P < 0.05$ were considered to be significant.

Results

Effects of marsupialization on the epithelial tissue of KCOTs

The results of HE staining showed that marsupialization led to substantial histological changes in the epithelial tissue of KCOTs. In most of the samples before marsupialization, the lining of epithelial cells was well defined with uniform thickness, and parakeratosis was evident (Fig. 1, upper panel). On the other hand, samples after marsupialization mostly showed hyperplastic, stratified, non-keratinizing squamous epithelium as well as thick connective tissue, being no longer compatible with the microscopic appearance of KCOT (Fig. 1, lower panel).

Effects of marsupialization on bone formation adjacent to KCOTs

We then explored the effects of marsupialization on bone formation adjacent to KCOTs using bone ALP activity assays (19). As shown in Fig. 2, enhanced ALP activity was widely detected in pre-osteoblasts, osteoblasts,
lining cells on the surface of trabeculae, and some newly embedded osteocytes. In areas of new bone formation in samples subjected to marsupialization, stronger bone ALP activity was observed in the osteoid. However, no ALP staining was detectable in deeply embedded osteocytes and the calcified bone matrix. In the KCOT capsule wall adjacent to bone in samples before marsupialization, the activity of bone ALP was much lower than that of samples after marsupialization.

Additionally, marsupialization led to a significant increase in the level of BMP-2/4 expression. Immunohistochemistry showed that the level of BMP-2/4 expression was very low in the KCOT capsule wall adjacent to bone in samples before marsupialization (Fig. 3, upper panel), whereas intense expression of BMP-2/4 was detected after marsupialization (Table 3), especially in areas close to the bone surface (Fig. 3, lower panel). All of the above findings strongly suggested that marsupialization was able to promote the formation of bone adjacent to KCOTs.
Effects of marsupialization on immunoreactivities of OPG and RANKL

To further clarify the mechanisms underlying the effects of marsupialization, we investigated the involvement of the OPG/RANK/RANKL signaling pathway (20). Immunoreactivity for both RANKL and OPG was detected in the cytoplasm and membrane of cellular components in the KCOT capsule wall adjacent to bone, whereas samples after marsupialization demonstrated a higher rate of OPG-positivity than samples before marsupialization (Fig. 4, Table 4). In contrast, the level of RANKL expression was obviously lower in the KCOT capsule wall adjacent to bone in samples after marsupialization relative to samples before marsupialization (Fig. 5, Table 5), suggesting that marsupialization affected the OPG/RANK/RANKL signaling pathway.

Effects of marsupialization on expression of mRNA for the tested molecules

To verify the effects of marsupialization on bone formation at the molecular level, we then measured the levels of expression of mRNA for several bone regeneration-related molecules in seven specimens before marsupialization and five samples after marsupialization using real-time qPCR analysis. The data revealed that OPG showed higher levels of expression in nearly all of the samples after marsupialization. Meanwhile, when compared with samples before marsupialization, the levels of mRNA expressions for CatK, MMP9, NFATc1, RANK, and RANKL were found to be significantly decreased after marsupialization (Fig. 6).

Discussion

Although the treatment of KCOT remains controversial, large lesions are often managed by marsupialization. This is a more conservative approach that avoids extensive surgery by reducing the high fluid pressure within the cavity and allowing newly formed bone to fill the jaw defect. Thus, marsupialization can save adjacent vital structures such as the teeth, maxillary sinus and inferior
alveolar canal (21). Recent studies have demonstrated that marsupialization is highly effective for decreasing the size of the cyst before enucleation (22). It has also been suggested that marsupialization may inhibit interleukin-1α expression and epithelial cell proliferation in KCOTs (23). A long-term follow-up analysis has shown that marsupialization might reduce the aggressive growth characteristic of KCOTs, although it does not reduce the tendency of KCOTs to recur (24). A number of studies have focused on the effects of marsupialization on KCOTs. Our present study showed that marsupialization was able to significantly affect the epithelial tissue of KCOTs, as demonstrated by the presence of hyperplastic stratified non-keratinizing squamous epithelium in most of the samples after marsupialization, mostly in accordance with previous studies (23,25). Interestingly, our study also demonstrated glass-like degeneration in several samples after marsupialization (data not shown). However, a larger number of samples will be needed to determine whether these changes are related to marsupialization. In addition, we found that marsupialization enhanced ALP activity at the junction between soft and hard tissue, especially in pre-osteoblasts and osteoblasts. We also demonstrated that marsupialization significantly up-regulated the expression level of BMP-2/4, the most important growth factor for bone formation. The above findings suggest that marsupialization can promote bone formation adjacent to KCOTs, being consistent with our previous study involving X-ray examinations.

Several recent studies have explored the role of the OPG/RANK/RANKL pathway in KCOTs, and showed that an imbalance in the expression of these factors may contribute to the bone/tooth resorption associated with KCOTs (13-16). Our immunohistochemical results showed that the level of OPG expression was significantly up-regulated in the KCOT capsule wall adjacent to bone in samples after marsupialization, whereas the expression of RANKL was down-regulated. Also, real-time qPCR demonstrated an increase of OPG expression in samples after marsupialization, accompanied by decreased expression of osteoclastogenesis-related molecules such as cathepsin K (CatK), matrix metalloproteinase-9 (MMP9), NFATc1, RANK and RANKL. These data indicate that marsupialization may affect the OPG/RANK/RANKL signaling pathway. Considering that the high pressure within the cyst cavity may induce RANKL expression and result in the formation of osteoclasts, we speculated that the effect of marsupialization on the OPG/RANK/RANKL signaling pathway may be due to the release of intracystic pressure (26,27). In addition, marsupialization might induce inflammation and eliminate the possibly hypoxic environment of the cavity (8,28), affecting activation of the OPG/RANK/RANKL signaling pathway. However, further studies are required to clarify the precise mechanisms involved.

In summary, our present study has provided further evidence that marsupialization can promote the regeneration of bone adjacent to KCOTs, possibly through regulation of the OPG/RANK/RANKL signaling pathway. Our results shed new light on the mechanisms underlying the effects of marsupialization, and might lead to the development of new therapeutic strategies for KCOTs.

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Conflict of interest
The authors have no conflict of interest to declare.

References