Abstract

Analysis of the cells obtained from epithelial cell rests of Malassez through

single cell limiting dilution

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Summary

Introduction:

The maintenance of periodontal ligament (PDL) space between the alveolar bone and cementum is crucial for periodontal tissue homeostasis to prevent alveo-dental ankylosis, which is regulated by the molecular secretion of the PDL cell population. Loss of PDL tissue due to periodontitis or trauma may cause ankylosis to occur. This non-resilient support leads to a loss of function and eventually to resorption of the tooth root.

The epithelial cell rests of Malassez (ERM) are located in the PDL space. ERM are quiescent epithelial remnants of the Hertwig's epithelial root sheath (HERS) involved in forming tooth roots. Some studies have reported that ERM plays a pivotal role in the maintenance of the periodontal ligament space. It is observed that ERM is always found in the vital periodontal ligament areas of replanted teeth. Alternatively, ERM cells can differentiate to form bone, fat, cartilage, and neural cells *in vitro*, and they form bone and cementum-like structures *in vivo*. ERM functions have been reported, but the mechanism for the prevention of alveo-dental ankylosis has remained unclear.

In this study, we established clone cells derived from ERM by single-cell limiting dilution to analyze the characteristics of individual ERM cloned cells and clarify the role of ERM secreted proteins in preventing alveo-dental ankylosis or alveolar bone growth.

Materials and Methods:

Cloning: ERM cells were isolated from porcine periodontal ligament by the outgrowth method, from which a group of cloned ERM cells were obtained through the single-cell limiting dilution method.

Cell morphology: Isolated clone cells were observed for proliferation ratio and pattern of growth at the dish surface, and images were taken using a digital camera.

Proliferation assay: Growth rate was assessed by a CyQUANT Cell Proliferation assay kit at 10^4 cells/ml for six days.

Immunofluorescence and Immunocytochemistry: Each cloned ERM cell was checked for the epithelial marker cytokeratin wide (ck-wide) and the odontogenic epithelial marker cytokeratin-19 (CK-19).

Reverse transcriptase PCR (RT-PCR): RT-PCR was used to identify the mRNA expression of the p75 gene; CRUDE and ERM-2, -3, and -9 cells were used for comparison.

Real-time RT-PCR: Quantification of mRNA was evaluated by real-time RT-PCR for amelogenin, ameloblastin, sfrp5, and cytokeratin-14 (ck-14) genes.

Western blot analysis: Quantification of amelogenin protein was performed by western blotting.

Mineralization assay: Gingival epithelial (G.E), CRUDE ERM, and cloned ERM cells were co-cultured with human periodontal ligament fibroblasts (HPDLF) for 30 days, and the formation of hydroxyapatite crystals was quantified by Osteoimage™. Whether amelogenin is responsible for inhibition was tested by adding amelogenin, ameloblastin, and enamelin antibodies to the culture medium.

Alkaline phosphatase activity: ALP activity was measured from precipitated alizarin stains by a plate reader at 405 nm with LabAssay™.

In vivo transplantation: *In vivo* extracted maxillary first molars from 4-week-old Wistar male rats were transplanted into recipient rats' subcutaneous pockets after culturing with conditioned media of G.E, CRUDE ERM, and cell clones of ERM-2, -3, and -9.

The statistical significance of the difference was analyzed using one-way ANOVA and Scheffe's test.

Results and Discussion:

A total of 18 clones from epithelial-like cells were successfully obtained by single-cell limiting dilution, named ERM 1-18. Three cells (ERM-2, -3, and -9) were selected for further experiments based on their growth pattern and proliferation ratio. The cells from which all clones were obtained were named CRUDE ERM. All the cells were stained positive for ck-wide and CK-19, confirming that selected cells were ERM cells. ERM-2, which expressed high levels of ameloblast marker p75, also expressed high levels of amelogenin and less ameloblastin. ERM-3, which expressed the least p75, expressed significantly less amelogenin according to both real-time RT-PCR and western blotting, and had high expression of ameloblastin. According to real-time RT-PCR, ERM-2 and ERM-3 expressed significantly more sfrp5, a marker for inner enamel epithelial cells, and ck-14, a marker for outer enamel epithelial cells, respectively. Alizarin red staining in co-culture experiments showed intense

staining when HPDLF was cultured with no cells or with G.E cells. In contrast, HPDLF with CRUDE ERM or other ERM clones showed no staining, though ERM-3, the least amelogenin-expressing cell, showed a little. Only anti-amelogenin antibody was able to recover the inhibition of HPDLF mineralization. *In vivo*, teeth cultured in ERM-2-conditioned medium formed comparatively small alveolar bones. ERM-3 resulted in formation of larger bone, but the control group GE formed even larger bones along with alveo-dental ankylosis.

To our knowledge, this study is the first to isolate clone cells from CRUDE ERM and demonstrate morphological, phenotypic, and functional discrepancies within isolated clones. We also showed that ERM-secreted amelogenin might be related to the inhibition of alveolar bone regeneration, which may help prevent alveo-dental ankylosis.