Abstract

Effect of extracellular matrix protein of Epithelial rests of Malassez supernatants during enamel crystallization of tooth germ

2022

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Introduction:

Enamel covers the surface of the tooth and is the hardest tissue in the body. It is composed of approximately 95–97% hydroxyapatite (HAP), 1% enamel protein, and 2% water. Tooth decay is caused by bacteria, and when enamel is lost, it cannot be fully regenerated. Although lost enamel can be restored with composite resin, glass ionomer cement, or porcelain, it is preferable to replace it with HAP, which has a regular structure like enamel. Enamel forms in the extracellular environment through the action of various complex proteins and proteases in three stages: secretory, transitional, and maturation. Amelogenin (Amelx) is essential for the formation of enamel HAP. In Amelx-deficient mice, enamel thickness was only 10–20%, and the resulting enamel was hypocalcified, had a disorganized crystalline structure, and was abnormally colored. Amelx and other EMPs are cleaved by the 45 amino acid tyrosine-rich Amelx peptide (TRAP), which contains the proteases enamelysin (MMP-20) and kallikrein 4 (KLK4). TRAP is involved in enamel formation, and point mutations in TRAP have been reported to lead to defective enamel formation in mice.

After root formation, degraded Hertwig's epithelial sheaths (HERS) migrate into the space of the periodontal ligament to form filamentous or reticular aggregates as remnant epithelial cells of Malassez (ERM). Although the function of ERMs remains to be elucidated, evidence suggests that they are composed of a clonogenic epithelial population that exhibits both epithelial and mesenchymal stem cell characteristics. They have also been reported to secrete enamel matrix proteins such as Amelx. Clonal ERM cells isolated from porcine dentate ligament (PDL) exhibit high Amelx expression.

This study aims to promote crystallization of immature enamel in dental embryos by using ERM-CM instead of recombinant full-length or spliced forms of Amelx, which are complex, expensive, and require safety considerations.

Methods:

Cloned ERM cell culture

For this study, ERM-2, a clone of CRUDE ERM, was provided by Dr. Islam. ERM-2 was selected based on the highest expression level of Amelx.

RT-PCR and qPCR analysis of Enamel matrix proteins

Total RNA was extracted from cells by the acid-guanidine-thiocyanate/phenol-chloroform method using TRizol. Aliquots of total cDNA were amplified using Amelx, Ambn, KLK4, MMP-20, and GAPDH primers.

Histological and immunohistochemical studies on extracted tooth germs

A histological and immunohistochemical study was performed to investigate the detailed distribution of ameloblast cells.

Collection of conditioned mediums

ERM-2 clonal cells and porcine gingival epithelial cells were cultured in KGM. When the confluence of cells reached 5×10^4 cells/ml, the medium was replaced with KBM. After four days of cell culture, the supernatant was collected and used as a conditional medium.

Organ culture with conditioned mediums and analysis by scanning electron microscopy

Mandibular molar tooth germs were dissected from C57BL/6J mice on postnatal day 3. Using previously stored ERM-2 supernatants, tooth germ organs were grown in 24-well dishes. Organ culture was continued for 1, 3, 7, and 14 days. The surface layers of the enamel structure were examined with a scanning electron microscope.

si-RNA transfection in ERM-2 cells and SEM analysis

ERM-2 cells were transfected with si-RNA targeting negative (scramble) and specific enamel matrix proteins such as amelogenin, ameloblastin, KLK4, and MMP20. After two days of incubation, the supernatants were collected from each group of cells. RT-PCR was performed to determine the expression of the target genes in the siRNA transfection experimental condition. SEM was performed to visualize the enamel structure's surface layer.

SPSS analysis

All values are expressed as mean ± SE for the respective groups. Statistical analysis was performed using IBM SPSS Statistical tool for iOS. The results were compared using the Mann–Whitney U test as statistically significant.

Results and Discussion:

Hematoxylin-eosin staining of mouse tooth germ sections at PN3 showed that they were in the late bell stage with a thin enamel layer. Immunohistochemical staining showed that the AmelX protein was specifically positive in ameloblasts with enamel formation. In ameloblasts with thin enamel, the expression of the AmelX protein was low. On day 14 of tooth germ organ culture with CM, ERM-2 formed clear hexagonal crystals on the enamel surface. No clear columnar structures were observed in the gingival epithelial cell. These results suggest that enamel growth can be induced by ERM-2 CM.

Our previous study found high levels of amelogenin expression in ERM-2 clonal cells, but the levels of other EMPs were not investigated. qRT-PCR was used in this study to evaluate the expression of the AMELX, AMBN, KLK4, and MMP-20 genes in ERM-2. ERM-2 exhibited significantly higher levels of EMPs than CRUDE ERM. To investigate the role of these EMPs in enamel mineralization, si-RNA-based knockdown experiments were carried out. qRT-PCR confirmed the knockdown of the Amelx, Ambn, KLK4, and MMP-20 genes in ERM-2 cells. All target genes were expressed when si-RNA was transfected into ERM-2. All si-EMP genes showed a significant decrease in expression when compared to the si-RNA negative control. However, si-EMPs ERM-2 significantly reduced the mRNA expression of other EMPs. SEM analysis of tooth germs cultured with si-negative transfected ERM-2 CM showed deposition, elongation, and compaction of columnar structures on days 3 and 7. On day 14, the columnar structures aggregated densely and showed hexagonal HAP-like crystal maturation. ERM-2 CM, which was transfected with si-EMPs and si-KLK4, and si-MMP-20, did not form columnar structures on the tooth germ surface. This study discovered that ERM CM contains EMPs, KLK4, and MMP-20, as well as other amelogenesis-related proteins, and demonstrated that ERM CM promotes the formation of enamel-like hexagonal hydroxyapatite columnar structures in organ- cultured tooth germs.

Conclusion:

ERM clones with high Amelx content in CM were able to promote the formation of enamel prisms in ex vivo tooth germ organ culture. However, the exact mechanism of interaction between Amelx, Ambn, KLK4 and MMP-20 molecules is unknown and needs further investigation.