Effects of recombinant human amelogenin on the proliferation and differentiation of ameloblast cell and osteoblast cell

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［Objective］

Amelogenin has a hydrophilic C-terminal domain and a hydrophobic N- terminal domain, that exhibits strong polarity. Amelogenin is the major secretory product of ameloblasts in the developing teeth and is cleaved by serine proteases during the enamel maturation. It has been known that amelogenin plays crucial roles on the enamel mineralization. Recent studies suggest biological functions of amelogenin as a signaling molecule for the cell proliferation and differentiation. Extracted amelogenin proteins, which have been used in many studies, are the mixture of many different molecules, including cleaved fragments of amelogenin and growth factors. Therefore, purified full-length protein is required for clarifying the role of amelogenin.

The purpose of this study is to obtain recombinant human amelogenin from mammalian cell lines (EXPI cells), to examine the effects of recombinant human amelogenin on the differentiation and cell adhesion in rat ameloblast cells (HAT-7 cells) or mouse osteoblast cells (MC3T3-E1 cells).

［Methods］

(1) Construction of amelogenin and purified recombinant amelogenin

pAMELXv3-Myc-DDK-Histag plasmid was constructed and transfected into EXPI cells. Recombinant human amelogenin, which was secreted into the culture media, was purified with DDK agarose beads. Characteristics of the recombinant amelogenin were examined with silver staining, MEM-CODE staining, and Western blotting using antibodies against DDK-tag, c-Myc-tag, and amelogenin.

(2) Cell proliferation assay

HAT-7 cells and MC3T3E-1 cells were plated and were cultured in the presence or absence of recombinant human amelogenin or Emdogain® (EMD). Final DNA concentration was measured by cell proliferation assay kit.

(3) Alizarin red S staining and ALP assay

HAT-7 cells and MC3T3-E1 cells were cultured in osteogenic media in the presence or absence of recombinant human amelogenin or EMD. Cells were fixed and were stained with Alizarin red S for visualizing calcified nodules. Alkaline phosphatase activity was

measured spectrophotometrically using p-nitrophenol as a colorimetric substrate.

(4-1) Cell spreading assay

MC3T3-E1 cells were cultured in glass chambers coated with fibronectin, recombinant human amelogenin, or EMD. These cells were stained with $DIOC_6$ and DAPI.

(4-2) Effect of inhibitors on cell adhesion

The glass plate was coated with fibronectin or recombinant human amelogenin, and MC3T3-E1 cells were cultured in the presence of RGD peptide or fibronectin inhibitor.

(4-3) Immunostaining

Immunohistochemical analysis performed using an anti-paxillin antibody, and the fluorescence images were captured with confocal laser scanning microscopy.

[Results]

Western blot analysis with anti-DDK, anti-c-Myc, and anti-amelogenin antibodies showed multiple protein bands with the molecular mass of 30 kDa, 33 kDa, and 65 kDa in the culture media sample of human amelogenin expressing EXPI cells. Western blot analysis of the affinity purified sample with these antibodies showed two bands with the molecular mass of 27 kDa and 54 kDa. Essentially identical bands were detected with silver staining and MEM-CODE stain, indicating that the method with purified by DDK-tag provide the high-purity full-length recombinant human amelogenin. This method allowed us to purify 450 μg of recombinant human amelogenin from 50 mL culture media of EXPI cells.

Recombinant human amelogenin and EMD reduced the proliferation of the HAT-7 cells in a concentration dependent manner. A significant reduction was observed in the presence of 4 μg/mL recombinant human amelogenin or 100 μg/mL EMD. The calcium nodules and ALP activity in the HAT-7 cells were increased significantly by recombinant human amelogenin at a concentration of 4 μg/mL, but not by EMD.

Unlike HAT-7 cells, the proliferation of the MC3T3-E1 cells was accelerated by 100 μg/mL EMD, but not by recombinant human amelogenin. The formation of calcium nodules and increased ALP activities in MC3T3-E1 cells were increased with relatively low concentrations of recombinant human amelogenin (0.001~0.01 μg/mL) and 1 μg/mL

EMD. However, high concentrations of recombinant human amelogenin (4 μg/mL) and 100 μg/mL EMD decreased the formation of calcium nodules and ALP activities.

The effect of recombinant human amelogenin or EMD on the cell adhesion and spreading was examined using a confocal laser scanning microscopy. The area of the cell adhesion in the MC3T3-E1 cells was approximately 1.98-times on recombinant human amelogenin-coated glass than that on control (BSA-coated) glass. The RGD peptide and fibronectin inhibitor blocked recombinant human amelogenin or EMD mediated cell adhesion. In addition, accumulations of paxillin with the cell adhesion were observed by immunohistochemical analysis.

[Discussion]

In the present study, the purification of full-length recombinant human amelogenin was obtained using EXPI cells and DDK-tag. The 27 kDa and 54 kDa bands of the purified samples are thought to be the monomer and dimer of human amelogenin, respectively. These data suggest that secreted amelogenin molecules form hetero-oligomer in the purified solutions. EMD constituted for the most part by the cleaved form of amelogenin lacking the C-terminus region. The C-terminus region in amelogenin has affected the difference of the results in biological activities between EMD and full-length recombinant human amelogenin. For example, the mineralizations and ALP activity in HAT-7 cells were increased by recombinant human amelogenin, but not by EMD. These results suggest important roles of the C-terminus of amelogenin for promoting ameloblast differentiations. Interestingly, the mineralizations of MC3T3-E1 cells was increased by low concentrations of recombinant human amelogenin, it was decreased at the high concentrations.

Recombinant human amelogenin in this experiment had cell adhesive and spreading activity. The spreading of MC3T3-E1 cells on the dish coated with recombinant human amelogenin was associated with the accumulation of paxillin, a signal transduction adaptor protein in the integrin-mediated cell adhesion, and was blocked in the presence of RGD peptides or fibronectin inhibitors in the medium. These results strongly suggest that the recombinant human amelogenin promoted the cell adhesion through the

binding with integrin.

［Conclusion］

The method to purify the full-length recombinant human amelogenin in large scale was established. Effects of recombinant human amelogenin on the cell differentiation and proliferation were the difference between HAT-7 cells and MC3T3-E1 cells. Furthermore, recombinant human amelogenin had cell adhesive and spreading activity.