CHAPTER III

MATERIALS AND METHODS

3.1 Samples and cell culture

Bone samples were collected from patients undergone surgery of alveolar bone. Informed consent was obtained prior to inclusion in the study. The protocol was approved by the Ethics Committee of Faculty of Dentistry, Chulalongkorn University. The explant was immediately placed in a sterile tube containing Dulbecco's modified Eagle's medium supplemented with penicillin G, streptomycin (50 μg/ml) and amphotericin B (2.5 μg/ml) and 10% heat-inactivated fetal bovine serum (FBS) (Gibco Laboratory, Grand Island, NY). The sample was washed twice with phosphate buffer solution (PBS) to remove blood clots and adherent erythrocytes. The surrounding soft tissues was removed by scraping with a sterile scalpel. Then it was cut into small pieces and transferred to a 35-mm tissue culture dish containing 2 ml of Dulbecco Minimal Essential Medium with F12 nutrient mixture (1:1) (DMEM/F12) supplemented with penicillin G (50 U/ml), streptomycin (50 μg/ml) and amphotericin B (2.5 μg/ml) and 10% heat-inactivated fetal bovine serum (FBS) (Gibco Laboratory, Grand Island, NY), incubated at 37°C in a humidified atmoshphere of 95% air and 5% CO_2. Culture medium was changed twice weekly. When the cells migrating from the explants were confluent, they were subcultured into a 75 cm^2culture flasks (Corning Incoperation, Acton, USA). To determine ALP expression, cells in passage 1 were plated in one well of 24-well plate and were cultured in inducing agents [DMEM/F12 supplemented with penicillin G (50 U/ml), streptomycin (50 μg/ml) and amphotericin B (2.5

μg/ml),10% heat-inactivated fetal bovine serum (FBS) (Gibco Laboratory, Grand Island, NY), 50 μg/ml of AA and 10 mM of β-GP , medium with 50 μg/ml of ascorbic acid (AA) and 10 mM of β-glycerophosphate (β-GP)] for 14 days. Then, cells were wash ed three times with PBS and fixed with 70% alcohol for 10 min. ALP activity were tested using 500 μl of the substrate for ALP [5-bromo-4-chloro-3-indolylphosphate/nitroblue-tetrazolium salt, BCIP/NBT 228.7mg (Sigma *FAST*) in 10 ml of distilled water]. Positive stained cells were included in this study. Cells in passage 2 were either frozen in fetal bovine serum/DMSO (9:1) in liquid nitrogen for further use, or subcultured for experimental purposes. Cells in the 3rd or 4th passage were used in the following experiments.

3.2 Osteogenic marker gene expression by RT-PCR analysis

Cells were seeded into 60-mm culture dishes (1.8-2.5x10^5 cells/dish). They were incubated overnight. Then, the culture medium were replaced with the inducing medium, except the negative control group which was cultured in the medium without AA and β-GP. Culture medium was changed twice weekly. At days 3, 7, 14, 21, 28, cells from one 60-mm culture dish was trysinized. RNA was extracted using RNeasy Mini Kit (Qiagen, Chatworth, CA, USA), according to the manufacturer's instructions. The RNA samples were further purified by successive treatment with DNase I (Qiagen, Chatworth, CA, USA). These RNA extracts were stored at -80°C. One microgram of total RNA was used for reverse transcription with random hexamer and Improm-II reverse transcriptase (Improm-II™, Promega Corp,USA.), following the manufacturer's instructions. The cDNA was used for detection of bone markers; COLIA2, ALP, BSP2, OPN, OCN, by polymerase chain reaction

(PCR). PCR amplifications were performed using Taq DNA polymerase (Qiagen, Chatworth ,CA, USA) by the Mastercycler gradient (Eppendorf, Germany) for 35 cycles of 95°C for 30 seconds (ALP, COLIA2, BSP2, OPN, GAPDH) or 1 min (OCN), 55°C for 1 min (ALP, COLIA2, BSP2, OPN) or 60°C for 1 min (OCN, GAPDH), 72°C for 1 min, and then a final extension of 72°C for 2 min. Specific primer sequences for these genes are as follows:

Table 1. Primer sequences used for PCR

Gene	Primer sequence	Product size (bp)	Reference
COLIA2	5' GGACACAATGGATTGCAAGG 3' 5' TAACCACTGCTCCACTCTGG 3'	461	(Noth et al., 2002)
ALP	5' ACGTGGCTAAGAATGTCATC 3' 5' CTGGTAGGCGATGTCCTTA 3'	475	(Ling et al., 2005)
BSP2	5' TTAGCTGCAATCCAGCTTCC 3' 5' CTCCCCCTCGTATTCAACG 3'	408	(Lallier et al., 2005)
OPN	5' GCATCACCTGTGCCATACC 3' 5' CATTCAACTCCTCGCTTTCC 3'	522	(Lallier et al., 2005)
OCN	5'ATGAGAGCCCTCACACTCCTC 3' 5' GCCGTAGAAGCGCCGATAGGC 3'	297	(Noth et al., 2002)
Human GAPDH	5'TCATCTCTGCCCCCTCTGCTG 3' 5'GCCTGCTTCACCACCTTCTTG 3'	438	(Nukaga et al., 2004)

PCR products were separated by electrophoresis in a 1.2% agarose gel containing ethidium bromide. The target bands were visualized with a UV illuminator and analyzed with the image analysis software (Gel Imaging Analysis, Bio Imaging system, Inc., Gene Genius, UK). The band intensities for each gene were measured. The data were presented in relative value to the band intensities of endogenous GAPDH. The experiments were repeated using cells derived from human alveolar bones from three different individuals.

The human osteoblastic cell line, SaOS2, were used as a positive control for all primers. The cDNA of peripheral blood mononuclear cells (PBMC) were used as a negative control for bone marker primers.

3.3 Alkaline phosphatase activity assay

To determine the ALP activity, cells were seeded into 24-well tissue culture plates (Coster, U.S.A.) ($1.5x10^4$ cells/well). They were incubated overnight. Then, the culture medium was replaced with the inducing medium, except the negative control group which was cultured in medium without AA and β-GP. Culture medium was changed twice weekly. At days 3, 7, 14, 21, 28, cells were washed three times with PBS and fixed with 70% alcohol for 10 min. 500 μl of the substrate for ALP was added to the fixed cells for 10-15 min, then washed three times with PBS. The experiment was performed in triplicate for each time point.

3.4 Alizarin red S staining of mineralized nodules

Cells were cultured and fixed as described in the method for ALP activity assay. Fixed cells were stained with 500 μl of Alizarin red S for 1 hour and then washed by 70% alcohol for 3 times. The wells were air-dried after washing. Each staining was done in triplicate.

3.5 Budget

1. Cell culture flask and media	20,000 Baht
2. RNeasy Mini Kit	20,000 Baht

3. RT-PCR kit	40,000 Baht
4. Agarose gel	4,000 Baht
5. Reagent	10,000 Baht
: ascorbic acid, β-glycerophosphate, BCIP/NBT, Alizarin red S	
Total	*94,000 Baht*