CHAPTER III

MATERIALS AND METHODS

Cell culture

Human bone cells were isolated from the fragments of alveolar process attach to the 3rd molar from patients undergoing surgery for impaction removal with informed consent. The protocol is approved by the Ethical Committee, Faculty of Dentistry, Chulalongkorn University. Alveolar process fragments were dissected under sterile conditions, cut into very small pieces and washed several times in calcium and magnesium-free phosphate-buffered saline (PBS), followed by a final wash in complete Dulbecco's modified Eagles medium (DMEM), containing 15% fetal bovine serum (FBS), Penicillin G (100 U/ml), Streptomycin sulfate(100 μg/ml), Amphotericin B (25 μg/ml) and L-glutamine (2 mM). The bone fragments were cultured in complete DMEM, at 37 C in 95% air/5% CO_2 for 4-5 days. During this period, cells were seen to migrate from the bone and formed seams around the bone fragments. The cells were maintained in culture until confluence. The cells were then seeded in 24 well plates at a density of 40,000 cells/well (for MTT assay) and in 35 mm tissue culture dishes at a density of 400,000 cells/dish (for RNA isolation) 4 h before irradiation. All of the experiments were performed using cells from passage 3rd- 5th.

Cell irradiation

Four hours after seeding cell, the culture vessels were irradiated with 0, 1, 2 doses using a dental x-ray machine generator (Gendex 1000, Gendex Corp., Illinois, USA). The radiographic parameters for a dose were 75 kVp, 15 mA, and 0.26 sec. The irradiated cells were then maintained in culture. Four hours after irradiation, total RNA was isolated to determine Bcl-2, Bax, Bad, Bcl-XL and Caspase-3 expression by RT-PCR assay and the toxicity of cell were evaluated by MTT assay after irradiation 24 h.

MTT assay

Cytotoxicity was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay which was based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermeable to cell membranes, thus, resulting in its accumulation within healthy cells. Solubilisation of the cells by the addition of the detergent solution containing 1:9 of DMSO and glycine buffer (0.1M glycine / 0.1M sodium chloride pH10) results in the liberation of the crystals which are solubilized. The number of surviving cells is directly proportional to the level of the formazan product created. The color can then be quantified using a simple colorimetric assay. The results can be measured spectrophotometrically using the absorbance at 570 nM (Genesys UV scanning, Thermospectronic, Roche, NY, USA). All measurements were done in triplicate.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from irradiated human bone cells using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to munufacturer's instruction. The concentration of RNA was determined using a spectrophotometer (Genesys UV scanning, Thermospectronic, Roche, NY, USA). One microgram of total RNA from each sample was used to generate cDNA by using Reverse transcription kit (Promega, Madison, WI, USA). Subsequently, polymerase chain reactions were performed using tag polymerase (Invitrogen, Brazil) by a thermocycler (Tpersonal, Whatman Biometra, Goettingen, Germany) to detect the expression level of Bcl-2, Bax, Bad, Bcl-XL, Caspase-3. The expression level of Glyceraldehyde-3-dehydrogenase (GAPDH) was used as an internal control. The PCR products were analyzed by electrophoresis in 2% agarose gel and visualized by Ethidium bromide fluorostaining. The band intensity was quantified using Scion Image software (Scion, Frederick, Maryland, USA). The reaction products were amplified using the following primer pairs (Table 3).

Table 3 Primer sequences used in PCR

Gene	Sense (5'-3')	Antisense (5'-3')	Expected product length (bp)	NCBI-BLAST accession
BCL-2	AGG AAG TGA ACA TTT CGG TGA C	GCT CAG TTC CAG GAC CAG GC	126	NM_000633.2
BAX	TGC TTC AGG GTT TCA TCC AG	GGC GGC AAT CAT CCT CTG	152	NM_138765.2
BAD	GAG TGA GCA GGA AGA CTC CAGC	TCC ACA AAC TCG TCA CTC ATC C	320	NM_032989.1
BCL-XL	TTA CCT GAA TGA CCA CCT A	ATT TCC GAC TGA AGA GTG A	166	NM_138578.1
CASPASE-3	CAA ACT TTT TCA GAG GGG ATC G	GCA TAC TGT TTC AGC ATG GCA C	240	NM_032991.2
GAPDH	TGA AGG TCG GAG TCA ACG GAT	TCA CAC CCA TGA CGA ACA TGG	375	NM_002046

Individual electrophoresis bands were analyzed by Scion Image analysis software (Scion Corporation, Frederick, Maryland) semiquantitatively, in which a particular band optical density was compared to the corresponding GAPDH (Y_{GAPDH}). The relative changes in expression (RE) for each band under investigation were calculated by dividing optical density values for a particular band by the optical density values for GAPDH: RE= (X/Y_{GAPDH}).

Statistical analysis

Results were expressed as mean ±SD. Statistical differences between control and irradiated group were determined using paired t-test. A p-value of less than 0.05 was considered statistically significant.