CHAPTER III

MATERIALS AND METHODS

3.1 Medium

Keratinocyte growth medium (KGM, Clonetics, San Diego, USA) supplemented with penicillin-streptomycin, amphotericin B,and 0.15 mM $CaCl_2$ were used for HGEC culture throughout the study. RPMI 1640 medium supplemented with penicillin G (50 U/ml), streptomycin (50 μg/ml) and 10% heat inactivated fetal calf serum (FCS) (Gibco Laboratory, Grand Island, NY,USA) were used for PBMC culture.

3.2 Reagents

We used specific ultrapure TLR ligands for TLRs 2, 3, 4, 5, 7, 8 and 9 obtained from InvivoGen (San Diego, CA, USA) as shown in table 2.

Table 2: Toll-like receptor-specific ligands used in stimulating HGECs

TLR ligand	Ligand	Dose
TLR2	Ultrapure LPS from *Porphyromonas gingivalis*	50 μg/ml
TLR3	Polyinosine-polycytidylic acid (poly I:C)(Synthetic analog of double stranded RNA)	100 μg/ml
TLR4	Purified LPS from *Escherichia coli* K12 strain	10 μg/ml
TLR5	Purified flagellin from *Salmonella typhimurium*	5 μg/ml
TLR7	Loxoribine (Guanosine analog)	100 μM
TLR8	Single-stranded polyU oligonucleotide complexed with LyoVec™	5 μg/ml
TLR9	CpG oligonucleotide (CpG ODN) 2006	10 μg/ml

TNF-α was purchased form R&D Systems, Inc. (Minneapolis, MN,USA). (-)-Nicotine hydrogen tartrate salt was purchased form Sigma (St. Louis, MO,USA). Histopaque 1.077 and MTT were obtained from Sigma (St. Louis, MO,USA).

3.3 Cell isolation and culture

3.3.1 HGEC culture

Gingival tissue samples were collected from subjects who had clinically healthy periodontium with probing depth less than 4 mm. The biopsies of gingiva were obtained at the time of crown lengthening procedure for prosthetic reasons from Periodontal Clinic, Faculty of Dentistry, Chulalongkorn University. Informed consent was obtained prior to inclusion in the study. The protocol was approved by the ethics committee of Faculty of Medicine, Chulalongkorn University. The excised tissues were immediately placed in

sterile tube containing culture medium, keep on ice and transferred to the laboratory within a few hours for explantation.

The methods to obtain epithelium from the gingival tissues were that described by Boyce and Ham (1985) and Oda and Watson (1990). Briefly, The biopsies were washed once with DMEM medium to remove blood clots and adherent erythrocytes. The biopsies were minced with scissors into fragment of 1-3 mm^2, placed in 35 mm. tissue culture dishes and digested in 0.2% dispase (B-M Biochemicals, Indianapolis, IND., USA) for 24 h at 4°C, so that the epithelium were separated from the connective tissue. The digested tissues were washed and separated epithelium were cut into small pieces and trypsinzed (0.05% trypsin-EDTA, 37°C for 10 min). The epithelial cells were washed twice and cultured in KGM supplemented with penicillin-streptomycin, amphotericin B, and 0.15 mM $CaCl_2$ at 37°C, 5%CO_2. At confluence, the cells were trypsinized and maintained at approximately $2.5x10^4$ cells per well. The gingival epithelial cell culture at passage 2-4 were used for all the experimental assays.

3.3.2 Preparation of PBMC

Heparinized peripheral blood (20 ml) were obtained from healthy adult volunteers. PBMC were prepared as previously described by Boyum (1968). Briefly, heparinized peripheral blood were layered on Ficoll-Hypaque (Histopaque), and centrifuged for 30 minutes at 700 x g at 25 °C. PBMC were collected and washed twice with PBS and then resuspended in RPMI 1640 medium with 10% heat inactivated FCS. Cells were counted in haemocytometer and cell viability were assessed by trypan blue exclusion.

3.4 mRNA expression of TLR in HGECs

HGECs 1 x10^6 cells were trypsinized and washed twice. Total RNA were separated by using RNaeasy Mini kit from Qiagen (Chatsworth, CA, USA), according to the manufacturer's instructions.The RNA samples were further purified by successive treatment with DNase I (Qiagen, Chatworth, CA, USA). 1 μg of total RNA were reverse transcribed using ImProm-II Reverse Transcription System for RT-PCR, according to the manufacturer's instructions (Promega, Madison, WI, USA). TLR primer sequences shown in table 3 were used. PCR amplification were performed using Taq DNA polymerase (Qiagen, Chatworth, CA, USA) by the Mastercycler gradient (Eppendorf, Germany) for 35 cycles of 94°C for 30 seconds, 55°C (TLRs1-2, 4-8, 10) or 50°C (TLR3) or 65°C (TLR9) for 30 seconds, 72°C for 1 min, and then a final extension of 72°C for 2 min. Forward and reverse primer pairs selected for human TLRs 1-10 were purchased from Proligo (Singapore). The PCR products were separated on a 1.2 % agarose gel containing ethidium bromide and visualized by UV illumination. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as an internal standard. As a negative control, a PCR reaction were performed without an RT sample. PBMC were used as a positive control. The methods of RT-PCR for detecting mRNA expression of TLR on PBMC were the same as those of HGECs.

Table 3: Primer sequences of TLR, HBD-2 and GAPDH

Product	Forward primer	Reverse primer	Amplicon size (bp)
TLR1*	CGTAAAACTGGAAGCTTTGCAAGA	CCTTGGGCCATTCCAAATAAGTCC	885
TLR2*	GGCCAGCAAATTACCTGTGTG	CCAGGTAGGTCTTGGTGTTCA	614
TLR3*	ATTGGGTCTGGGAACATTTCTCTTC	GTGAGATTTAAACATTCCTCTTCGC	319
TLR4*	CTGCAATGGATCAAGGACCA	TCCCACTCCAGGTAAGTGTT	622
TLR5##	CCTCATGACCATCCTCACAGTCAC	GGCTTCAAGGCACCAGCCATCTC	355
TLR6$	ACTGACCTTCCTGGATGTGG	TGGCACACCATCCTGAGATA	404
TLR7$	ACAAGATGCCTTCCAGTTGC	ACATCTGTGGCCAGGTAAGG	207
TLR8*	CAGAATAGCAGGCGTAACACATCA	AATGTCACAGGTGCATTCAAAGGG	636
TLR9##	GCGAGATGAGGATGCCCTGCCCTACG	TTCGGCCGTGGGTCCCTGGCAGAAG	510
TLR10$	GGCCAGAAACTGTGGTCAAT	AACTTCCTGGCAGCTCTGAA	287
HBD-2**	CCAGCCATCAGCCATGAGGGT	GGAGCCCTTTCTGAATCCGCA	255
GAPDH#	TGATCTCTGCCCCCTCTGCTG	GCCTGCTTCACCACCTTCTTG	435

(* primer sequence by Saikh et al., 2003, ## primer sequence by Schaefer et al., 2004, $ nucleotide sequences were determined from PubMed (National Center for Biomedical Information), ** primer sequence by Krisanaprakornkit et al.,2000, # primer sequence by Nukaga et al., 2004 and the primers were custom designed using primer 3 software.)

3.5 HBD-2 expression after stimulation of HGECs by TLR ligands

HGECs (1.2 x10^5 cells/ml) in 48-well plates were treated with either a) various single TLR ligands: *P.gingivalis* LPS (50 μg/ml), poly(I:C) (100 μg/ml), *E. coli* LPS (10 μg/ml), *Salmonella typhimurium* flagellin (5 μg/ml), loxoribine (100 μM), ssPolyU (5 μg/ml), and CpG ODN 2006 (10 μg/ml); b) TNF-α (30 ng/ml); c) combination: *P.gingivalis* LPS (50 μg/ml) plus TNF-α 30 ng/ml.

After stimulation with TLR ligand(s), TNF-α, or the combination *P.gingivalis* LPS and TNF-α for 24 h, the cells were collected for measurement of HBD-2.

Total RNA were isolated and reverse transcribed as mentioned. PCR conditions for HBD-2 were described as in Krisanaprakornkit, et al. (2000). Briefly, PCR amplification were performed using Taq DNA polymerase on the Mastercycler gradient for 28 cycles of 95°C for 30 seconds, 65°C for 30 seconds, 72°C for 1 min, and then a final extension of 72°C for 7 min. HBD-2 specific primer (Table 3) was purchased from Proligo. The PCR products were separated on a 1.2 % agarose gel containing ethidium bromide and visualized by UV illumination.

3.6 Nicotine treatment of HGECs

We first determined the non-toxic doses of nicotine for HGEC culture. $3x10^4$ HGECs were cultured overnight in 96 well plate. Nicotine concentrations at 0.1, 0.3, 1 and 10 mM were added into HGEC cultures, incubated for 24 h and the cell viability was assessed by MTT assay. The viable HGECs were detected using MTT dye, which forms blue formazan crystals that were reduced by mitochondria dehydrogenase present in living cells. After 24 h with nicotine treatment, the culture supernatants of each HGEC culture were removed, replaced with 25 μg MTT in 100 μl volume. After 4 h incubation, 200 μl of dimethyl sulfoxide was added into each well to dissolve the formazan crystal. The survival rate of the cells was calculated from spectrophotometer measurement at 570 nm wavelength (anthos, zenyth 200rt). Data obtained from the MTT assay was shown as relative percent, by comparing with the control. The experiments were performed in duplicate using cells prepared from two different donors.

We then tested the effect of nicotine on stimulated HGEC culture. Nicotine at non-toxic doses was added to HGECs ($1.2 x10^5$ cells/ml), which were stimulated with the combination of *P.gingivalis* LPS and TNF-α, in 48-well plates. After 24 h incubation, the cells were collected for measurement of HBD-2.

3.7 Statistic analysis

Statistical comparisons among treatment conditions with respect to production of HBD-2 were analyzed using SPSS V11.5 software. The parametric paired sample t-test was used for normally distribution data, and

the nonparametric Wilcoxon signed rank test was used for non-normally distributed data. A value of $p< 0.05$ was considered statistically significant.

3.8 Budget

1.	KGM	30,000 Baht
2.	Trypsin-EDTA, Antibiotic	4,000 Baht
3.	RNeasy Mini kit	25,000 Baht
4.	RNase free DNase	6,000 Baht
5.	Taq Polymerase	10,000 Baht
6.	dNTP	7,000 Baht
7.	$CaCl_2$	2,000 Baht
8.	HBD-2 primer and TLR primer	23,000 Baht
9.	TLR ligands	100,000 Baht
10.	Nicotine	10,000 Baht
11.	Plastic ware	11,500 Baht
	Total	228,500 Baht