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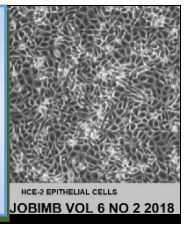

Expression of Beta Defensin Genes in Frozen Thawed and Cultured Immortalised Human Corneal Epithelial Cell Line

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ABSTRACT

Human beta defensins (hBD) are important host defence molecules at the ocular surface. In addition to their antimicrobial activities, hBD may also act as regulatory factors in recruiting and activating immune cells. Only hBD-1 – hBD-4 have been well characterised. To date, a complete profile of the beta defensin genes (*DEFB*) expression in immortalised human corneal epithelial cell line (HCE-2) has not been established. Therefore, this study is aimed to explore a spectrum of *DEFB* expression in HCE-2. Total RNAs were extracted from frozen thawed HCE-2 and cultured HCE-2 to ensure the gene expression were identical. The RNAs were reverse transcribed into cDNAs. The expression of 10 *DEFB* (*DEFB1*, *DEFB4A*, *DEFB103*, *DEFB104*, *DEFB105*, *DEFB106*, *DEFB109*, *DEFB123*, *DEFB126* and *DEFB127*) were analysed using polymerase chain reaction (PCR) and gel electrophoresis. *DEFB1* and *DEFB103* were the only hBD mRNAs found constitutively expressed in frozen thawed HCE-2 and cultured HCE-2. It is also interesting to note that PCR enhancer was needed to amplify the genes in cultured HCE-2. Our findings suggest that corneal epithelium constantly produce hBD-1 and hBD-3, which presumably provide the baseline defence against infection. Further investigation on the expression of these genes when HCE-2 stimulated with proinflammatory cytokines would help in better understanding of the ocular surface defence mechanism.

INTRODUCTION

Cornea is an important part of the eyes because it is located at the outermost layer of the eye. The cornea together with the lens provide the refractive power of the eyes. Corneal epithelium is a vital tissue that acts as microorganism barrier as our eyes are constantly under the attack of environmental pathogens [1].

The innate immune response is the body's first line of defence against invading pathogens. Studies over the past years uncovered that small peptides are also used as innate immune system to remove undesirable invaders. Our eyes are under constant pathogenic attack; thus, they release defensins to protect the eyes from this attack. Defensins are one of the antimicrobial peptides that involve in innate immune system that are secreted constitutively or induced upon infection by corneal epithelia [2]. Mammalian defensins are small in size with a molecular mass between three and six kDA and comprise of six conserved cysteine residues that form three intra-molecular disulphide bonds. In addition, classic mammalian defensins are 29 to 45 amino acids in length and most defensin genes constitute of two exon genes [3].

Defensins are believed to be among the earliest developed molecular effectors of innate immunity. Defensins are highly conserved molecules and consist of three classes which are α, β, and Θ defensins. However, only α, and β defensins are present in human whereas Θ defensins found in primates [4]. The human beta defensins (hBD) are primarily localised in epithelial surfaces [2]. More than 50 hBD including pseudogenes have been identified at the genomic and transcriptional level [5]. Beta defensin genes (*DEFB*) 1, 4A, 103, 104 and 109 were reported to express in corneal epithelial cells [6, 7].

The antimicrobial spectrum of defensins includes gram-positive and gram-negative bacteria, fungi, and viruses [8]. Defensins are cationic thus they will interact with the negative charge of the microorganism's membrane and cause pores formation. Formation of pores will lead to cell lysis [9]. Defensins may promote a rapid cellular immune response to infection through a chemotactic effect on monocytes. Defensins may also speed up wound healing via their mitogenic effect on epithelial cells and fibroblasts [10].

Defensin peptides have potentials to be developed as anti-microbial and anti-inflammatory drugs because they are produced innately and have a broad range of activities. To understand the activity of hBD, first the expression of the hBD genes need to be investigated *in vitro*. In our study, we screened a spectrum of hBD genes for their expression in SV40-transformed human corneal epithelial cells. To our knowledge, expression of a broad spectrum of hBD genes in immortalised human corneal epithelial cells was not reported yet.

MATERIALS AND METHODS

Cell line, reagents and consumables

Human corneal epithelial cell line (HCE-2), which is a primary culture of normal corneal epithelium immortalised with Ad12-SV40 hybrid virus by overnight incubation with virus at 37ºC was purchased from American Type Cell Culture (ATCC), USA. Keratinocytes serum free culture medium, bovine pituitary extract, epidermal growth factor and antibiotic/antimycotic, TrypLE Express, and phosphate-buffered saline (PBS) were purchased from Gibco (Invitrogen, USA). The additive supplement for HCE-2 such as fibronectin, collagen type 1, insulin and hydrocortisone were purchased from Nacalai Tesque INC, Japan.

Polymerase chain reaction (PCR) was carried out using GoTaq® PCR Core System I (Promega, USA) and peqGOLD *Taq* 'all inclusive' (VWR, Belgium). *DEFB* and *β-actin* primers were purchased from Integrated DNA Technologies, USA. RNA was extracted using Quick-RNA Mini Prep (Zymo Research, USA) and reverse transcribed using RT^2 First Strand Kit (Qiagen, Germany). The chemicals and reagents used in electrophoresis analysis were agarose gel (Vivantis, Inc. USA), TAE buffer (Vivantis, Malaysia), DNA loading dye (Norgen Biotek, Canada), 50 bp and 100 bp DNA ladders and RiboRuler High Range RNA ladder (Thermo Scientific, USA). Cell culture and molecular analysis consumables were purchased from Star Lab, Germany.

Thawing of cryopreserved HCE-2 for RNA extraction

Thawing procedure was carried out according to Kent [11] with some modifications. Cryopreserved vials containing HCE-2 were brought to room temperature and thawed by warming up the vial in between palms before immersing half of the vial into a 37°C water bath for about 1 min. One millilitre of the vial content was pipetted into a 2 mL microcentrifuge tube containing 1 mL of complete keratinocytes serum free medium and resuspended before centrifuging for 5 min at 12000 rpm. The supernatant was discarded. One millilitre of PBS was added to the cell pellet and centrifuged for 5 min at 12000 rpm to wash the cells. The washing steps were repeated three times. The pellet was subjected to RNA extraction after the final discardment of the supernatant.

HCE-2 cell culture

HCE-2 were grown in keratinocytes serum free medium that was prepared according to ATCC recommended procedure by supplementing with 0.05 mg/mL bovine pituitary extract, 5 ng/mL epidermal growth factor, 500 ng/mL hydrocortisone, 0.005 mg/mL insulin and 1% antibiotic/antimycotic. The cells were seeded in a T25 flask pre-coated with a mixture of 0.01 mg/mL fibronectin, 0.03 mg/mL bovine collagen type I and 0.01 mg/mL bovine serum albumin and incubated overnight in a CO_2 incubator (Eppendorf, USA) at 37°C.

The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 incubator. After reaching 80% of confluency, the cells were harvested using TrypLE Express dissociation enzyme and subjected to RNA extraction.

RNA extraction and cDNA synthesis

Total RNAs were extracted from frozen thawed and fresh cultured HCE-2 cells using Quick-RNA Mini Prep, according to the manufacturer's instructions. The concentration and purity of RNA samples were quantified using a nano-spectrophotometer (PcrMax, UK). The purity represented by OD 260/280 and concentration (ng/ µL) were recorded. RNA integrity was tested by running the RNA on 1% agarose gel. RNAs with purity between 1.9-2.0 with good integrity were subjected to reverse transcription polymerase chain reaction (RT-PCR) analysis using RT^2 First Strand Kit according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed into cDNA.

Primers

The primers for hBD and reference genes were designed using NCBI Primer-BLAST (**Table 1**). The hBD genes that were included in this study were *DEFB1*, *DEFB4A*, *DEFB103*, *DEFB104*, *DEFB105*, *DEFB106*, *DEFB109*, *DEFB123*, *DEFB126* and *DEFB127*. *β-actin* was used as the reference gene.

PCR

PCR was performed according to Razali [12] with some modifications. PCR was carried out to amplify the selected hBD genes and reference gene, *β-actin*. Optimisation of the PCR reaction was done using the frozen thawed sample. Hot Start PCR was performed to avoid non-specific amplification of DNA. The finalised amplification reactions were set up in a reaction volume of 20 µL containing 1 µL of cDNA and PCR reagents from Promega, USA, i.e. 200 µM dNTPs, 0.5 µM of forward and reverse primers, 1.5 mM $MgCl_2$ for all genes except for *DEFB103* was 2.5 mM, 1X *Taq* polymerase buffer and 2.5 U *Taq* polymerase.

Thermal cycling was initiated with denaturation step at 95ºC (10 min), followed by 40 cycles of denaturation at 95°C (30 sec), annealing at 57°C (30 sec) for all genes except for *DEFB103* was at 54°C (45 sec) and primer extension at 72°C (30 sec), then a final extension step at 72ºC (10 min) using a thermal cycler (peqSTAR 2X, Germany). The products of each PCR were analysed on ethidium bromide–stained agarose gels.

peqGold *Taq* 'all inclusive' PCR reagents were used according to manufacturer's protocol when *DEFB* genes could

not be amplified using GoTaq® PCR. The thermal profiles for all genes were as above.

Table 1. Primer sequences for RT-PCR and expected product lengths.

β-defensin gene (Product of the gene)	Primer sequence (5'- 3')	Product length (bp)
DEFB1 (hBD-1)	Forward: CTGGAAGCCTCTGTCAGCTC Reverse: TTACCACCTGAGGCCATCTC	151
DEFB4A (hBD-2)	Forward: GACCTTTATAAGGTGGAAGGC Reverse: ATGGCTCCACTCTTAAGGCAG	181
DEFB103 (hBD-3)	Forward: GGAGCTCTGCCTTACCATTG Reverse: CACGCTGAGACTGGATGAAA	189
DEFB104 (hBD-4)	Forward: CCCCAGCATTATGCAGAGAC Reverse: CTGTATTCTTGGCTGCGACA	150
DEFB105 (hBD-5)	Forward: TTTGGTTCAACTGCCATCAG Reverse: GTTCAGCCTGCAATTTCCAT	160
DEFB106 (hBD-6)	Forward: TTTCTCTTTGCCGTGCTCTT Reverse: GGTCCGACAGCATTTCAGAG	153
DEFB109 (hBD-9)	Forward: TGAGACTCCATTTGCTTCTCC Reverse: ACAGCACTTCATCCTTCTTCG	179
DEFB123 (hBD-23)	Forward: TGCTCTTATCCCAGCTGACTC Reverse: GGAGCATCTGTAACGGCATTT	77
DEFB126 (hBD-26)	Forward: CCATGGGGCTCTTCATGAT Reverse: CTGCAGATTTTCCTGCAATGTC	153
DEFB127 (hBD-27)	Forward: TTGCAATTCTGCTGTTCCAG Reverse: GGACGAGGTGGCTTTGTAAT	196
β-actin (β-actin)	Forward: GCAAAGACCTGTACGCCAAC Reverse: CGATCCACACGGAGTACTTG	156

Gel electrophoresis

Gel electrophoresis was carried out according to Razali [12] with slight modifications. One percent of agarose gel was prepared to determine the RNA integrity. RNAs were ran in the gel according to the RiboRuler High Range RNA Ladder manufacturer's protocol. PCR products were ran in 2% of agarose gel. Fifty or 100 bp DNA ladders were used as DNA molecular weight standards. Briefly, 1 µL of DNA loading dye was mixed with 5 µL of PCR product, and 1 µL of DNA ladder, respectively. Then, 6 µL of each sample mixture and 2 µL of ladder mixture were loaded into the wells of the gel. After completion of the run for 60 min at 80 volts, the gel was viewed and photographed using a gel documentation system (G:BOX, SynGene, USA).

RESULTS

The HCE-2 are moderately large epithelial cells that exhibit a pleomorphic appearance, have no definable borders, and show areas of multi-layered growth (**Fig. 1**).

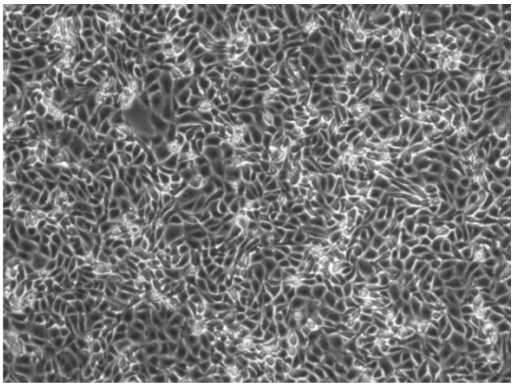

Fig. 1. Morphology of HCE-2 viewed under an inverted microscope at 40x magnification.

After extracting the total RNAs from frozen thawed and cultured HCE-2, the quantity and purity of the RNA samples were measured. RNA samples with purity between 1.9-2.0 were subjected to gel electrophoresis to ensure the extracted RNAs have good integrity prior to gene expression analysis. **Fig. 2** (a) and (b) clearly show two bands on each lane indicating intact 28s and 18s RNA. There is no sign of RNA degradation, thus the RNA samples were suitable for the gene expression analysis.

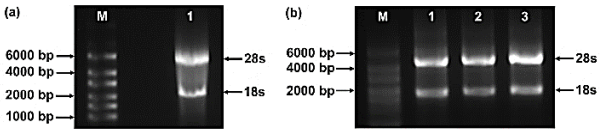

Fig. 2. RNA integrity of (a) frozen thawed HCE-2 and (b) cultured HCE-2 validated via gel electrophoresis. M: RNA size marker; Lane 1, 2, and 3: total RNA extracted from HCE-2.

Optimisation of PCR amplification was done using frozen thawed HCE-2 to determine the best annealing temperature and time, and $MgCl_2$ for all the selected genes. PCR reactions were carried out using GoTaq® PCR. However, interestingly we found that there were no PCR products of hBD genes observed in the gel when cDNA of cultured HCE-2 was amplified using GoTaq® PCR. Hence, we used peqGOLD *Taq* 'all inclusive' that contains enhancer for hard to amplify templates. We found that *DEFB1* and *DEFB103* expressed in both frozen thawed (**Fig. 3a**) and fresh cultured HCE-2 (**Fig. 3b**) but not the other investigated hBD genes. Gel images of some non-expressing hBD genes were not shown.

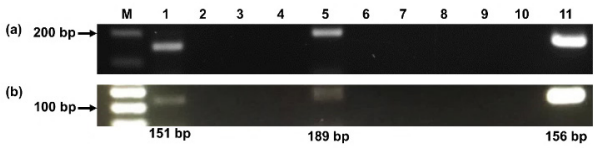

Fig. 3. Gel-based RT-PCR analysis to determine the expression profile of hBD genes. (a) Expression of hBD genes and *β-actin* from frozen thawed HCE-2. (b) Expression of hBD genes and *β-actin* from fresh cultured HCE-2 amplified using peqGOLD *Taq* 'all inclusive'. Lane M: DNA ladder; Lane 1: *DEFB1* expression evaluated as positive; Lane 3: *DEFB4a* expression evaluated as negative; Lane 5: *DEFB103* expression evaluated as positive; Lane 7: *DEFB104* expression evaluated as negative; Lane 9: *DEFB105* expression evaluated as negative; Lane 11: *β-actin*; Lane 2, 4, 6, 8, 10: non-template control for *DEFB1, DEFB4a, DEFB103, DEFB104, DEFB105*.

We also subjected the cDNA of the fresh cultured sample to PCR without adding the enhancer into the PCR reaction mixtures to confirm that the *DEFB1* and *DEB103* were only able to be amplified because of the enhancer. We found that amplification of *DEFB1* and *DEB103* failed without the enhancer (**Fig. 4**).

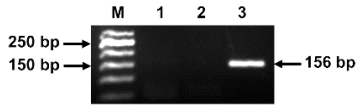

Fig. 4. Expression of *DEFB1*, *DEB103* and *β-actin* from fresh cultured HCE-2 amplified using peqGOLD *Taq* 'all inclusive' without addition of enhancer. Lane M: DNA ladder; Lane 1: *DEFB1* expression evaluated as negative; Lane 2: *DEFB103* expression evaluated as negative; Lane 3: *β-actin*.

DISCUSSION

Defensins have many functions such as to promote epithelial healing, chemoattract, and maybe non-antigenic which can be suitable source that have broad spectrum of activity [13,14]. Therefore, defensins as therapeutic agents for eye inflammation would be interesting as they are naturally occurring effector molecules of the innate immune system. We investigated the expression of 10 hBD mRNAs, namely hBD-1, hBD-2, hBD-3, hBD-4, hBD-5, hBD-6, hBD-9, hBD-23, hBD-26 and hBD-27 in frozen thawed HCE-2 and cultured HCE-2 using RT-PCR technique.

Good quality RNAs were extracted from frozen thawed HCE-2 and cultured HCE-2 samples. A good quality human RNA has two ribosomal subunits bands in the gel which are 18s and 28s. Another characteristic of good quality RNA is that the 28s band is twice the intensity of 18s. The RNAs extracted from both types of samples fulfilled the condition in gel electrophoresis. The purity of frozen thawed HCE-2 and cultured HCE-2, that was measured using nano-spectrophotometer were in the range of 1.9 - 2.1 indicating high purity RNAs. High purity RNA can produce high quality cDNA by reverse transcription.

In most cases high amount of RNA is needed for gene product analysis. Therefore, cells are passaged several times to obtain adequate amount of material. Frequent passaging may cause alteration in gene expression profile between earlier and higher passages [15]. In this study, we extracted total RNAs directly from frozen thawed cells and cultured HCE-2 to determine whether the expression profile of the spectrum of the genes will be still alike. We found that the amplification products from cultured HCE-2 were identical to frozen thawed HCE-2, nonetheless only possible when enhancer was added in the PCR mixture. Gene amplification was successful without enhancer in the frozen thawed HCE-2 probably because the cells were cryopreserved in DMSO. DMSO is one of the enhancing agents utilised during PCR amplification to increase yield, specificity and reproducibility [16].

Our study showed that the frozen thawed HCE-2 and cultured HCE-2 expressed *DEFB1* (hBD-1 mRNA) and *DEFB103* (hBD-3 mRNA). Haynes *et al*. [10] reported that *DEFB1* expressed in the tissues of conjunctiva, cornea and lacrimal gland. McDermott *et al*. [17] and McIntosh *et al*. [7] found that *DEFB1* and *DEFB103* were constitutively expressed in primary cultures of human corneal epithelium cells. However, McDermott *et al*. [17] found *DEFB4A* (hBD-2 mRNA) only expressed when stimulated by proinflammatory cytokines such as IL-1β, acting through mitogen-activated protein (MAP) kinase and nuclear factor (NF)-κB pathway, which differed from McIntosh *et al*. [7] finding, where in their culture the gene was constitutively expressed.

In 2010, Garreis *et al*. [18] found expression of *DEFB104* (hBD-4 mRNA) in SV40-transformed human corneal epithelial cells. Our present finding is not in line with theirs. McIntosh *et al*. [7] and Huang *et al*. [19] also reported contradicting findings of *DEFB104* expression in primary cultures of human corneal epithelium cells. Similarly, *DEFB109* (hBD-9 mRNA) also found not expressed in the HCE-2 in this study, however Mohammed *et al*. [20] reported *DEFB109* constitutively expressed in SV40-transformed human corneal epithelial cells. Contradicting results obtained although same type of *in vitro* model used perhaps due to the variance in culture conditions and environment.

Alike *DEFB4A*, *DEFB4* and *DEFB109*, mRNAs of hBD-5, hBD-6, hBD-23, hBD-26 and hBD-27 also did not express, probably because of the expression of these hBDs are tissue specific or inducible by stimulant such as proinflammatory cytokines. McIntosh *et al*. [7] and Huang *et al*. [19] also did not detect mRNA expression of hBD-5, hBD-6, hBD-23, hBD-26, and hBD-27 in ocular epithelium samples.

In this preliminary study, the findings suggest that immortalised human corneal epithelium cell expresses *DEFB1* and *DEFB103* constitutively, presumably to provide baseline defence against infection. This study also suggests that PCR of cultured HCE-2 needs addition of enhancer to amplify gene products. Further investigation is required to determine the expression of the selected genes when HCE-2 stimulated with proinflammatory cytokines.

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