Effect of FGF-2 and PDGF-BB on a Co-Culture of Human Gingival Fibroblasts and Umbilical Vein Endothelial Cells
(Kesan FGF-2 dan PDGF-BB ke atas Ko-Kultur Sel Fibroblas Gingiva dan Sel Endotelial Vena Umbilikal Manusia)

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ABSTRACT
Gingival recession can be treated by root coverage procedure with tissue graft. The ideal gingiva graft should mimic the properties of the native gingiva. Gingival fibroblasts are main cells that reside in human gingiva, while the endothelial cells are the basis for blood vessel formation. The co-culture of these cells, will help in better understanding of gingival tissue regeneration. This study was aimed to determine the effects of fibroblast growth factor-2 (FGF-2) and platelet-derived growth factor-BB (PDGF-BB) on a co-culture of human gingival fibroblasts (HGFs) and human umbilical vein endothelial cells (HUVECs). In this in vitro experimental study, the medium for the establishment of monolayer and co-culture of these cells were first optimised. Then, the optimal concentrations of these growth factors were determined by assessing the cell viability using MTT assay. Next, to study the stimulatory effect of these growth factors, both HGF and HUVECs were co-cultured and gene expression analysis for fibroblast and angiogenic biomarkers was assessed using Real-Time RT-PCR. Cell viability assay showed that the effect of FGF-2 on HGF was dose-dependent and was optimum at a concentration of 5 ng mL\(^{-1}\), while that of PDGF-BB on HUVEC was optimum at a concentration of 20 ng mL\(^{-1}\). The stimulatory effect of FGF-2 and PDGF-BB was further supported by the Real-Time PCR results which showed that there is a significant expression of VIM, COL1A1, FN, CD31, VE-Cadherin, and VWF in the treatment group of both cells after three days of co-culture experiment, compared to control group. This study indicates a possible synergistic effect of FGF-2 and PDGF-BB growth factors in a co-culture of HGF and HUVEC leading to proangiogenic activity.

Keywords: Co-culture; FGF-2; HUVEC; PDGF-BB; tissue engineering

INTRODUCTION
Gingival recession is a complex soft tissue pathology, defined as an apical shift of the gingival margin, causing exposure of the root surface of a tooth (Jati et al. 2016). A wide range of surgical techniques have been proposed for the treatment of gingival recessions over the decades to ensure full root coverage and satisfactory aesthetic results (Shkreta et al. 2018). Among these, the soft connective
tissue grafts are most widely used and considered as a ‘gold-standard’ due to its high predictability (Gallagher & Matthews 2017). Soft connective tissue grafts are usually harvested from the palate and transplanted at the recession area to replace the receding tissue. Although the root covering is achieved, these grafts are not fully sufficient for the periodontal tissue to regain the physiological functions and it is coupled with certain limitations. These limitations include lack of adequate vascularisation, limited amount of available donor tissue and demand of a second surgical site, resulting in an additional trauma to the patient and associated risks such as pain, infection, donor-site morbidity and risks of rejection by the patient’s immune system (Moraschini & Barboza Edos 2016; Zuur et al. 2014). Pertaining to these limitations, tissue-engineered constructs are currently being explored in the field of biomedical engineering, however, desirable biocompatibility and bio-functionality still need to be explored.

Mass transfer limitation is a questionable challenge in tissue engineering strategies dealing with vascular research. As the tissue becomes thicker, cells existing at 200 µm and greater from the nearest capillaries would undergo hypoxia, followed by cell death. This diffusion limitation becomes increasingly crucial as the volume and cell population of engineered tissue increases. Blood vessels facilitate the transport of nutrients and oxygen via the vascular structure, which promotes cell migration, proliferation, differentiation and extracellular matrix (ECM) production; as well as the delivery of inflammatory cells to defend the host against pathogens (Traore & George 2017). In most tissue-engineered constructs, vascularisation is achieved by using endothelial cells (ECs). Moreover, apart from ECs, different cells population have been used within the same culture environment depending upon the tissue of interest.

Gingival tissue consists of collagen and blood vessels. Fibroblast and ECs are the common cells in this tissue. ECs are the building block of the vascular system and expected to form functional capillary networks in the tissue construct (Song et al. 2018). On the other hand, fibroblasts play an essential role in the angiogenic process through their production of ECM molecules (Um Min Allah et al. 2017). Previous study has been done using dermal fibroblast and human umbilical vein endothelial cells (HUVECs) in a co-culture system for promoting vascularization (Costa-Almeida et al. 2015). However, there is limited knowledge on the interaction of the cells in a co-culture system especially between human gingival fibroblasts (HGF) and HUVEC, which is very important to understand angiogenesis in gingival tissue.

Apart from using heterotypic cell population in a co-culture, exogenous molecules such as growth factors are used to achieve stable and mature vasculature within a construct. Numerous growth factors are known for their ability to actively regulate various functions of cells in tissue regeneration and in-vitro culture. FGF-2 and PDGF-BB are known to play important roles in fibroblast and EC activity, however, there is a dearth of information in the literature that assesses the effect of these two angiogenic growth factors on an in-vitro co-culture of HGF and HUVEC. Using the tissue engineering principle, i.e. the interaction between heterotypic cells population (HGF and HUVEC) in a co-culture and addition of exogenous growth factors (FGF-2 and PDGF-BB), this study will provide further understanding and aid in developing functional tissue graft for gingival regeneration.

**MATERIALS AND METHODS**

**CELLS AND MATERIALS**

Commercially available HGFs and HUVECs were used in this study. HGFs were purchased from ScienCell, USA, and HUVECs from Lonza, USA. For cell viability assay, both cells were seeded in a tissue culture 96-well flat-bottom plates purchased from Greiner Bio-One, Germany. For co-culture experiments, ThinCert™ (Greiner Bio-One, Germany) cell culture inserts for 6 well plates were used. Two polypeptide growth factors, fibroblast growth factor-2 (FGF-2) and platelet-derived growth factor-BB (PDGF-BB) were used in this study. FGF-2 as well as PDGF-BB were purchased from Sigma Aldrich, Germany.

**MONOLAYER CULTURE OF HGF AND HUVEC**

HGFs were cultured in Alpha Minimum Essential Medium (α-MEM, Gibco, USA) supplemented with 10% Fetal Bovine Serum (Gibco, South America), and 1% Penicillin Streptomycin (Pen/Strep) containing 10,000 units of penicillin (base) and 10,000 µg of streptomycin (base)/mL (Gibco, USA). HUVECs were cultured routinely in Endothelial cell growth medium-2 Bullet Kit (EGM™-2). Endothelial Basal Medium-2 (EBM™-2 Medium) purchased from Lonza, USA supplemented with EGM™-2 SingleQuots™Kit consisting of 0.1% human epidermal growth factor, 0.1% vascular endothelial growth factor, 0.1% R3- insulin-like growth factor-1, 0.1% ascorbic acid, 0.04% hydrocortisone, 0.4% human fibroblast growth factor-beta, 0.1% heparin, 2% Fetal bovine serum, 0.1% gentamicin/ amphotericin-B were used to formulate EBM™-2 to EGM™-2. The cultured cells were maintained at 37 °C, 5% CO₂, and 95% humidity. The cells from the 5th to 6th passage in culture were used for subsequent experiments.

**OPTIMISATION OF CO-CULTURE MEDIUM FOR HGF AND HUVECs**

To study the effect of growth factors in a co-culture of HGFs and HUVECs, it was mandatory to determine the
growth medium in which both cells can proliferate well. For monolayer culture, HGF, and HUVEC were grown in their complete media, α-MEM and EGM™-2, respectively. For co-culture experiments, two types of culture media were compared i.e. either both cells were grown in a 1:1 mix (by volume) of α-MEM and EGM™-2 or in α-MEM only. Then, cell morphology and proliferation were evaluated using inverted microscope (Carl Zeiss, Germany) to observe the growth of cells in both media.

**CELL VIABILITY ASSAY**

The 3,4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) assay was performed as previously described (Plumb et al. 1989) for evaluating the effect of growth factors on the viability of cells. Two separate culture conditions i.e. Group-A consisting of experiments performed with HGF in its complete growth medium (α-MEM) and in 1:1 medium containing α-MEM: EGM™-2 while Group-B was performed with HUVEC in its complete growth medium (EGM™-2) and in 1:1 medium containing α-MEM: EGM™-2, were tested. Briefly, HGFs and HUVECs were plated at a density of 5 × 10⁴ cells in 96-well plates in complete medium as well as in co-culture medium (previously optimized) and allowed to adhere overnight. After 24 h, media were replaced by fresh media and cultures were exposed to different concentrations of FGF-2 and PDGF-BB (5, 10, 15, 20, 25, and 30 ng each). Cells treated with only basic growth medium served as a negative control. After 3 days, MTT assay (5 mg mL⁻¹ in PBS) was performed as per standard procedures. Optical density (OD) was measured at a wavelength of 560~750 nm.

**CO-CULTURE OF HGF AND HUVEC IN TRANSWELL SYSTEM**

Non-contacting co-culture system (Renaud & Martinoli 2016) was used to study the gene expression levels of fibroblast biomarkers (Vimentin (VIM), Collagen type I, alpha 1 (COL1A1), and Fibronectin (FN)) and angiogenic biomarkers (Cluster of differentiation-31 (CD-31), Vascular endothelial cadherin (VE-CAD), and Von Willebrand factor (v-WF)) in the presence of growth factors. Briefly, prior to cell seeding, to promote cell attachment in a multiple well-plate and in ThinCert™ cell culture transwell inserts, recommended amount of culture medium was added and placed into the incubator at 37 °C for 1 h. Next, HGFs (1 × 10⁶ cells) were seeded in the multiple well-plate (6 well-plate; lower compartment) while HUVEC (1 × 10⁶ cells) seeded on membrane transwell insert (upper compartment) and allowed to grow overnight. This is for initial equilibrium period for cell attachment. After 24 h of overnight incubation, the upper and the lower compartments were combined and growth factors (5 ng FGF-2 and 20 ng PDGF-BB) were added to initiate the experiment. Co-cultures were maintained for 72 h at 37 °C, 5% CO₂, and 95% humidity. Following that, the culture media were aspirated from both the compartments and cells from each group were harvested to quantify and isolate RNA for Real-Time PCR. Control group consisting of HGF only, HUVEC only, and HGF-HUVEC only, whereas HGF-HUVEC with growth factors were the treatment group.

**RNA EXTRACTION AND REAL-TIME RT-PCR ANALYSIS**

Total RNA was isolated from cells of all the groups and quantified by measuring the absorbance at 260 and 280 nm using Eppendorf BioPhotometer plus machine. One-Step real-time RT-PCR was performed to study the gene expression of fibroblast and angiogenic biomarkers using SensiFAST™ SYBR® Hi-ROX One-Step Kit exactly to the manufacturer’s instructions. The mRNA expression level of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to normalize the data and the non-template control (NTC) was included in each experiment. The genes primer sequences were designed using NCBI Primer-Blast as shown in Table 1. The reactions were performed on the StepOnePlus™ Real Time PCR System (Applied Biosystems, USA) in MicroAmp™ Fast 8-Well Strip, 0.1 mL (Applied Biosystems, USA) covered with MicroAmp® Optical 8-Cap Strips (Applied Biosystems, USA) manually set up in triplicates. PCR conditions were as follows: 45 °C for 10 min, 95 °C for 2 min followed by 40 cycles of 95 °C for 5 s (denaturation step), 60 °C for 10 s (annealing step) and 72 °C for 5 s (extension step) during which fluorescence was measured. mRNA expression levels were recorded as threshold cycles (Cₜ). Data was acquired using the StepOnePlus™ Real Time PCR Software (Applied Biosystems, USA). After the real-time RT-PCR run, relative quantification was performed using the ΔΔCT method (Pfaffl 2001).

**STATISTICAL ANALYSIS**

The data are expressed as mean ± standard error of the mean (S.E.M). All experiments were done in triplicate. Statistical analysis for the cell viability assay was performed using one-way analysis of variance (ANOVA) for comparison between two means followed by Bonferroni post-hoc test, whereas, real-time RT-PCR data were processed by Kruskal Wallis test followed by Mann-Whitney U test for comparisons between groups using IBM SPSS software v 22.0 (SPSS, Chicago, IL, USA). All statistical analyses were performed at the significance level of p < 0.05.

**RESULTS AND DISCUSSION**

**OPTIMISATION OF HGF-HUVEC CO-CULTURE**

The typical morphology of HGF showed spindle-shape and HUVEC showed a cobblestone-like shape as shown Figure 1. The co-culture medium was optimised prior to co-culture of HGF-HUVEC. For this, two sets of experiments were performed. In the first experiment, both cells were grown in a 1:1 mix (by volume) of α-MEM
EFFECT OF FGF-2 AND PDGF-BB ON THE PROLIFERATION OF HGF AND HUVEC

It has been shown that for Group A (HGF in α-MEM and HGF in α-MEM: EGM™-2) (Figure 3(A), 3(B)), the effect of FGF-2 was dose-dependent and the highest number of viable cells were seen at an optimum concentration of 5 ng mL⁻¹, whereas, for Group B (HUVEC in EGM™-2 and HUVEC in α-MEM: EGM™-2) (Figure 4(A), 4(B)), the effect of PDGF-BB was measured, and the most viable cells were seen at an optimum concentration of 20 ng mL⁻¹.

Fibroblast growth factor is a cellular growth factor that bind to heparin and heparin sulphate. MTT result of this study showed that the effect of FGF-2 on HGF is reported to be dose-dependent which is consistent with the previous studies (Nishimura & Terranova 1996; Palmon et al. 2000) in which local application of this growth factor on regeneration of periodontal tissues has been investigated and demonstrated that FGF-2 promotes the growth and proliferation of human PDL cells and exerts a dose-dependent effect on PDL and gingival fibroblast migration (p<0.01). Moreover, the effect of this growth factor has been studied on the proliferation and apoptosis of cultured HGF (Tanimoto et al. 2013) and the result has been in agreement with the stimulatory effects. In this study, the highest number of viable cells were seen at an optimum concentration of 5 ng mL⁻¹ which is different with Walters et al. (2005), who performed a study to test the role of growth factors (FGF-2, PDGF, and TGF-β) on HGF minocycline uptake. Their study showed that both FGF-2 and PDGF at a concentration of 10 ng mL⁻¹ significantly enhanced the minocycline uptake and the effect was reported to be dose-dependent.

PDGF-BB is a heparin-binding growth factor known to play a critical role in the maturation and remodelling of vessels during later stages of development and in angiogenesis (Minardi et al. 2017). Because endothelial proliferation is necessary for the formation of new vessels, angiogenic growth-regulatory molecules would be expected to induce mitogenesis in vascular ECs. In this study, it has been shown that the effect of PDGF-BB on HUVEC was not dose-dependent and that the most viable cells were seen at the optimum concentration of 20 ng mL⁻¹. Binding of PDGF to its receptors leads to activation of the receptor tyrosine kinase and to subsequent initiation of cytoplasmic signal transduction pathways, in turn leading to the migration, proliferation, and differentiation of PDGF-responsive cell types. Previously, growth response of PDGF-BB to HUVEC at the site of injury has been assessed (Zetter & Antoniades 1979). Their result showed that no significant increase in cell number or colony size was seen when HUVEC initially was seeded at a density of 1 × 10⁴ cells. However, with the addition of 0.5 ng mL⁻¹ PDGF-BB, a four-fold (4.8 × 10⁴) increase in cell number was observed along with the increase in the colony size after 8 days that suggest the mitogenic activity of purified platelet mitogen (Zetter & Antoniades 1979). On the contrary, a dose-dependent effect of both PDGF-AB and -BB was also observed in a conventional two-dimensional culture where cell numbers were increased more by PDGF-BB and proved to be a potent mitogen. However, no effect on capillary EC
proliferation was seen even with higher concentrations (20 ng mL\(^{-1}\)) of PDGF-AB which demonstrated that this isoform has no mitogenic effect (Marx et al. 1994).

Of interest, bovine aortic ECs that displayed an ‘angiogenic’ phenotype in culture have PDGF-β receptors and respond to PDGF-BB was shown to form cords and tubes. Moreover, it has been shown that PDGF-BB at 1 ng mL\(^{-1}\) (p<0.05) and at 3 ng mL\(^{-1}\) or higher (p<0.001) dose-dependently increased DNA synthesis (Battegay et al. 1994). Shimizu et al. (1999) studied the effect of PDGF-BB on cultured chick cardiac myocytes and the results of viability assay showed that the effect of PDGF-BB was dose dependent and that the maximal mitogenic effect and increased DNA synthesis was achieved at 5 ng mL\(^{-1}\). The results of these studies are not in agreement to our current findings on HUVEC where the effect was not dose-dependent. The possible explanation could be because of the phenotypically distinct cellular population (bovine aortic ECs, cardiac myocytes, and HUVEC) that are associated with differences in cell shape, spatial organisation, the nature of the substrate, and the ECM in the microenvironment. However, in general, the stimulatory effects of PDGF-BB, irrespective of the type of ECs and cellular activity are corroborated with present study and are thought to be suggestive of the angiogenic response with HUVEC in the culture.

EFFECT OF FGF-2 AND PDGF-BB ON GENE EXPRESSION OF FIBROBLAST AND ANGIOGENIC BIOMARKERS IN HGF AND HUVEC

The expression level of each gene was measured, and normalized to GAPDH expression. The Kruskal Wallis test for fibroblast and angiogenic gene biomarkers of the treatment group (with growth factors) showed significant changes in expression level (p < 0.05) after three days of co-culture experiment compared to control group(s) in which genes were slightly expressed but not significant (p > 0.05) (Figure 5(A)-5(F)). Results showed that the \(r^2\) and percentage (%) of VIM, COL1A1, FN, CD31, VE-Cadherin, and vWF are 0.989, 95.92%; 0.99, 99.36%; 0.996, 109.42%; 0.978, 97.29%; 0.988, 95.60%; 0.991, 95.02%, respectively.

VIM, COL1A1, and FN are fibrous proteins that are the major component of ECM secreted by fibroblasts. Usually, expression of these proteins is linked to support and facilitate cellular attachment and communication by activating signalling pathways (Albelda & Buck 1990; Manimegalai et al. 2016). On the other hand, CD-31, VE-Cad, and v-WF are commonly known as vascular endothelial cell specific markers which are majorly involved in vascular biology and angiogenesis (Goncharov et al. 2017). In this study, the expression of these biomarkers was significantly observed in a co-cultured group with the addition of optimised concentration of GFs (FGF-2 and PDGF-BB) when compared to control. Previously, studies have been done on the synergistic role of these two angiogenic growth factors in a combination to study the vascular stability and angiogenesis (Cao et al. 2003; Li et al. 2010; Nissen et al. 2007; Sufen et al. 2011). It has been confirmed that the establishment of the functional vascular network requires the addition of more than one angiogenic factor. The expression of these genes in the presence of FGF-2 and PDGF-BB in a co-culture of HGF and HUVEC could be because of the synergistic role of these two growth factors. The underlying mechanism may be complex, but it is believed that addition of FGF-2 upregulates the PDGFR-α and PDGFR-β which activates PDGF receptor-transduced signalling pathways leading to more pronounced cellular interactions in a co-culture environment (Cao et al. 2003).

| Gene  | Primer sequences 5' to 3' | Accession no. | Amplicon size (bp) |
|-------|--------------------------|---------------|-------------------|
| FN    | CGGAGAGACAGGAGAATAGCCCCT | NM_001306132  | 150               |
|       | TTGCTGCTTGCGGCGCTGTC     |               |                   |
| COL1A1| TACAGCGTCACTGTGATGGGC    | NM_000088     | 61                |
|       | TCAATCAGTCTCTGCCCCAG     |               |                   |
| VIM   | CTTTGAACGCAAAGTGGAATCT   | M14144        | 119               |
|       | CCACATCGATTGGACATGCT     |               |                   |
| v-WF  | GAATGGTGCTGTACGGCTGG     | NM_000552     | 57                |
|       | CACGCATCGCTCTGACAC       |               |                   |
| CD-31 | TCTAGAAGGAAAGGCCTCCCT    | NM_001101655  | 145               |
|       | TGGGAGCAGGCAGGTCCA       |               |                   |
| Primer | Forward | Reverse | Accession no | Amplicon size (bp) |
|--------|---------|---------|--------------|-------------------|
| VE-CAD | AGAAGAAGCCTCTGATTGG | TGTGACTCAGGAAGAAGCTG | NM_001795 | 113 |
| GAPDH  | CAACAGCGACACCCACTCTC | CACCCTGTTGCTGTAGCCAAA | NM_002046 | 115 |

FIGURE 1. Typical morphology of HGF and HUVEC in a monolayer culture. Arrows in red showing: A. Spindle-shaped morphology of HGF, and B. Cobblestone-like shape of HUVEC.

FIGURE 2. Images of HGF and HUVEC morphology in 1:1 mix (by volume) of α-MEM and EGM™-2 at day 1 until 5 using an inverted microscope. A-C. HGF proliferated and maintained its spindle shaped morphology, and D-F. HUVEC proliferated and maintained its cobblestone-like shape (magnification: × 100).
FIGURE 3. Effect of FGF-2 on the viability of HGFs based on MTT assay. A. Effect of various concentration of FGF-2 on HGF in growth medium containing α-MEM, and B. Effect of various concentration of FGF-2 on HGF in growth medium containing 1:1 mix (by volume) of α-MEM and EGM™-2. The values of OD of the different concentrations were normalised to the average OD value of the control group. Data are presented as mean ± S.E.M., # indicates a non-significant difference (p > 0.05) between the groups. Other pairs are significant
CONCLUSION

Together, data from our study conclude the importance of using co-culture approach for studying the cellular behaviour of heterotypic cell population. Co-culture of HGF and HUVEC plays a significant role in the expression of fibroblastic and angiogenic genes, however addition of FGF-2 and PDGF-BB in the co-culture further enhanced these effects. These findings supported the important role of these growth factor in angiogenic activity which need to be further explored in the co-culture system. As vascularisation in tissue engineered constructs for treating gingival recession is essential, further investigations could...
be carried out using this co-culture method with the present of these growth factors for better understanding the cellular interactions between HGF and HUVEC.

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