Optimization of Endothelial Cell (HUVEC)-Gingival Fibroblasts Co-cultures in Perfused Degradable/Polar/Hydrophobic/Ionic Polyurethane (D-PHI) Scaffolds

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Statement of Purpose: Periodontal diseases affect nearly 50% of Americans 30 years and older[1]. Gingival tissue engineering is a potentially advantageous approach for reconstructing lost gingival tissue. Human gingival fibroblasts (HGFs) have been used previously to reconstruct the gingival lamina propria and mediate epithelial cell morphogenesis but gingival tissues need to be well-perfused for metabolic exchange and cell viability. Accordingly, perfusion systems and methods to promote angiogenesis should be considered for tissue-engineered constructs. Polyurethane hydrogels (D-PHI) have been shown to elicit less inflammatory response post-implantation[2] and perfused D-PHI scaffolds enhance the proliferation of HGF and collagen production[3]. Co-cultures of endothelial cells with fibroblasts in synthetic materials can support capillary formation[4] however the culture medium and cell seeding ratio must be optimized for each unique cell source and culture environment. The objective of the current study was to investigate the effect of culture medium and cell seeding ratio in the co-culture of HGF with endothelial cells (HUVECs) in a perfused scaffold with respect to promoting growth, capillary formation, and angiogenic factor production.

Methods: D-PHI scaffolds were fabricated via a free radical polymerization as outlined previously[3,5]. The cylindrical scaffolds were 2mm thick and 6mm in diameter and with a porosity of 80%. HGF-1 cells and HUVECs (from ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM, with 10% FBS and 1% antibiotics) and in F-12 K Medium (with 10% FBS, 1% antibiotics, 0.1 mg/mL heparin, and 0.05 mg/mL ECGS) respectively. For investigating the effect of culture medium, a mixture of both cell types (1:1) was seeded into D-PHI scaffolds for 24 hrs, which were then cultured in a custom-designed perfusion bioreactor (flow rate: 500 μL/min) for dynamic culture for 28 days. The samples were either cultured in a 50/50 mix of DMEM and F-12 K medium (by vol.), or in DMEM only. For investigating the effect of seeding ratio, a mixture of both cell types at ratios 2:1, 1:1, or 1:2 (HUVEC:HGF) was seeded and cultured under flow for 28 days. DNA content was quantified using Hoechst 33258. The metabolic activity and the production of angiogenic factors (VEGF, TGF-β1, FGF-2) were measured by a WST-1 assay and ELISAs respectively. The presence of capillary formation was assessed using immunofluorescence.

Results: The HUVEC-HGF co-culture was first optimized based on culture medium type. DNA content in the co-culture with the 50/50 mix medium showed a significant 3.1-fold increase in cell population over 28 days of culture ($p<0.01$) while the DMEM condition showed a 1.6-fold increase (**Fig. 1a**). Interestingly, the metabolic activity in both media types decreased significantly over 28 days (**Fig. 1b**), which may be attributed to contact inhibition mediated by HUVECs forming lumenal structures[6]. Immunofluorescence indicated that networks of HUVECs were observed only in the 50/50 condition (images not shown). In addition, VEGF and FGF-2 levels increased significantly (relative to D0) only in the 50/50 condition (data not shown). The HUVEC-HGF co-culture was also optimized based on cell seeding ratio (2:1, 1:1, and 1:2 (HUVEC:HGF)). DNA (**Fig. 1c**) and WST-1 data (**Fig. 1d**) suggest that the number of HGFs should be greater than that of HUVECs during initial seeding to achieve greater cell proliferation. A ratio of 1:2 (HUVEC: HGF) resulted in a drop in the metabolic activity after D1 and remained constant to D28 of culture. This level was greater than that in the other two ratios. TGF-β1 production was significantly greater for the 1:2 ratio (data not shown), suggesting that the increased number of HGFs may have upregulated TGF-β1, which would possibly lead to more ECM production and promote angiogenesis[7].

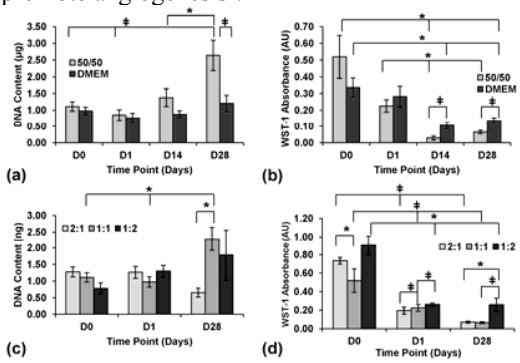

Figure 1. (a) DNA content and (b) metabolic activity of HUVEC-HGF dynamic co-cultures (1:1 HUVEC:HGF, total 80000 cells) on D-PHI scaffolds. Cells were cultured in either 50/50 mix medium or DMEM only. (c) DNA content and (d) metabolic activity of the co-culture at ratios of 2:1, 1:1, and 2:1 (HUVEC:HGF) on D-PHI scaffolds in 50/50 mix medium. n = 9, ±SE. Significantly different from each other (*$p<0.05$, ‡ $p<0.01$).

Conclusions: HUVEC-HGF co-culture in perfused D-PHI scaffolds with a 50/50 mix of media (by vol.) and greater fibroblast proportion exhibit enhanced growth, early signs of angiogenesis, and increased angiogenic factor production compared to co-culture in DMEM, which supports the potential of these conditions to produce a tissue-engineered construct for regenerating the gingival connective tissues in highly perfused conditions.

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Acknowledgements: NSERC Discovery Grant (360520), NSERC CGS D3, OGS, CellSignals Training Fellowship (STP-53877), Himsley H&A Memorial Prize.