Secreted Endothelial Cell Factors Immobilized on Collagen Scaffolds Enhance the Recipient Endothelial Cell Environment

Secreted endothelial cell factors immobilised on collagen scaffolds enhance the recipient endothelial cell environment

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ABSTRACT

Strategies to design novel vascular scaffolds are a continuing aim in tissue engineering and often such designs encompass the use of recombinant factors in order to enhance the performance of the scaffold. The established use of cell secretion utilised in feeder systems and conditioned media offer a source of paracrine factors which has potential to be used in tissue engineered (TE) scaffolds. Here we utilise this principle from endothelial cells, to create a novel TE scaffold by harnessing secreted factors and immobilising these to collagen scaffolds. This research revealed increased cellular attachment and positive angiogenic gene upregulation responses in recipient endothelial cells grown on these conditioned scaffolds. Also the conditioning method did not affect the mechanical structural integrity of the scaffolds. These results may advocate the potential use of this system to improve vascular scaffolds *in vivo* performance. Additionally this process may be a future method utilised to improve other tissue engineering scaffold therapies.

Keywords: Endothelial, secreted factors, paracrine, collagen scaffolds, autologous cells.
Abbreviations: EC (endothelial cell), ECSF (endothelial cell secreted factor), BM (basal media), ABM (adjusted basal media), E/N (1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide and NHS-hydroxysulfosuccinimide). NAVC (number of attached viable cells).

1. INTRODUCTION

A continuing challenge faced in vascular tissue engineering is how to improve treatments for cardiovascular disease and other such arterial conditions. Current strategies include a number of scaffold materials and/or therapies that mimic the native vessel wall, restore *in situ* endothelialisation, promote extracellular matrix (ECM) production, inhibit thrombogenicity, reduce inflammation and help stimulate neovascularisation and angiogenesis.[1,2] Tissue engineering has utilised a range of biomaterials including, decellularised ECM[3,4], synthetic biopolymers[5] and biodegradable polymers to create TE vascular grafts.[6–10]

Some of the most common types of scaffold are collagen based[11–13] and have been shown to promote cell attachment, migration, proliferation, differentiation and ECM production during remodelling and regeneration.[14] More recently they have incorporated growth factors and proteins such as vascular endothelial growth factor (VEGF)[15,16] and angiopoietin-1 (Ang1).[17,18] The use of growth factors and proteins has predominately focused on the concentration and release kinetics of these factors, whether they are designed to be retained within the scaffold,[19,20] or released.[21–23] Mainly there purpose is to enhance both the cell functionality, contact and to interact with the *in vivo* tissue.[24]

Additionally, cell secretion can also be a direct or indirect source of paracrine growth factors and proteins. Consequently this may be one way to partially recapitulate the intrinsic cell environment by using the cell secretion. The principle offered from cell secretion has been utilised directly in the cell culture as cell feeder layer systems to provide paracrine factors to recipient cells.[25] Cell feeder layers have been widely used to maintain pluripotency of human induced pluripotent stem cells (hIPSCs)[26,27] and human embryonic stem cells

(hESCs).[28,29] They have also shown promise in tissue regeneration[30,31] including secretion from mesenchymal stem cells (MSCs)[32–34] and endothelial progenitor cells (EPCs).[35,36]

In this *in vitro* study we use these principles of the feeder layer cell secretion technique to generate cell conditioned media and incorporate this into a novel TE scaffold. We achieve this by utilising endothelial cell-secreted factors (ECSFs) and immobilise these to collagen scaffolds and test for improved functionality by the attachment of recipient endothelial cells (ECs).

2. MATERIALS AND METHODS

2.1. Preparation and Experimental Setup

2.1.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) from an infant male Caucasian donor were obtained cryopreserved (500,000 cells) at passage 1 (PromoCell GmbH) and cultured and expanded to passage 5 (P5) in a humidified atmosphere of 5% CO_2/37°C in T-75 vented flasks (Corning®) and grown to 80% confluency. HUVECs were cultured according to a previously used endothelial cell culture protocol,[37–39] in brief, MCDB 131 medium (Life Technologies™) was supplemented with: 2% FBS (ThermoFisher Scientific); 1% L-Glutamine; 1% penicillin/streptomycin (Life Technologies™); 1 mg/L hydrocortisone; 50 mg/L of ascorbic acid (Sigma); 2 mg/L fibroblast growth factor (FGF); 10 mg/L epidermal growth factor (EGF); 2 mg/L insulin like growth factor (IGF); 1 mg/L vascular endothelial growth factor (VEGF) (PeproTech).

2.1.1.1. Basal media

Basal media (BM) consisted of MCDB 131 medium with 2% FBS omitted and all supplements (listed above) added for serum free cell culture conditions**.** For experimental conditions, 5 ml of the BM was incubated in a humidified atmosphere of 5% CO_2/37°C in T-75 vented flasks.

2.1.1.2. Cell conditioned basal media

HUVECs at P5 were washed three times using D-PBS/$CaCl_2$ and $MgCl_2$ free (Sigma). HUVECs were then cultured in 5 ml BM for 48 h and incubated in a humidified atmosphere of 5% CO_2/37°C in T-75 vented flasks

and grown to no more than 70-75% confluency to obtain cell conditioned BM. This media is generally known as cell conditioned media, for simplicity it is further referred to as adjusted basal media (ABM). The ABM was filter sterilised using 0.22 µM filter (Millex® GS Millipore) before use.

2.1.2. Collagen scaffolds

Scaffolds discs (10 mm diameter x 2 mm thick) were punched from sheets of commercially available Ultrafoam™ collagen (Davol Inc.) using a 10 mm disposable biopsy punch (Acuderm Inc.) on to the surface of a sterile 1.2 mm thick glass slide (ThermoFisher Scientific). According to the manufacturer's specifications, Ultrafoam™ is a water-insoluble, partial HCl salt of purified bovine dermal (corium) collagen formed as a sponge with interconnected pores. Collagen scaffolds soaked in D-PBS/$CaCl_2$ and $MgCl_2$ free (Sigma) served as the control groups for all experiments.

2.1.2.1. Conditioned collagen scaffolds via absorption

Scaffolds were soaked in either: PBS, BM or ABM and incubated 24 h at 37°C and mildly shaken in an orbital shaker (IKA KS 400 i) at 100 rpm in 100 ml Duran flasks during the conditioning process. Refer to Figure 1. for schematic overview of scaffold preparation.

2.1.2.2. Conditioned collagen scaffolds via immobilisation

Scaffolds were soaked for 40 min at room temperature with mild agitation in a D-PBS/$CaCl_2$ and $MgCl_2$ free (Sigma) solution of 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide - EDC (Sigma) and N-hydroxysulfosuccinimide - sulfo-NHS (Sigma) (E/N) at a concentration ratio of 16mg/24 mg ml^{-1} respectively and filter sterilised using 0.22 µM filter. The concentrations used had been used previously used concentrations for scaffolds.[40] The scaffolds were then subjected to three successive soaks in fresh D-PBS/$CaCl_2$ and $MgCl_2$ free (Sigma) for 10 min each at room temperature with mild agitation to remove any excess E/N. Scaffolds were soaked in either PBS, BM or ABM and incubated 24 h at 37°C and mildly shaken at 100 rpm in 100 ml Duran flasks during the conditioning process.

2.1.3. Cell seeding

P5 cells were used throughout this study at a seeding density of 5 x 10^5 cells per scaffold in 100 µl media with *n* = 4 for each condition. For cell seeding, cells were washed three times using D-PBS/$CaCl_2$ and $MgCl_2$ free (Sigma) and media changed into BM for 24 h and incubated in a humidified atmosphere of 5% CO_2/37°C in T-

75 vented flasks. Cells were seeded on to the collagen scaffolds in serum-free basal media within 12-well non-adherent culture plates (Greiner) and incubated for 1 h at 5% CO_2/37°C. Un-seeded scaffolds in each respective group served as the control. Serum-free basal media (1 ml) was then added to cover scaffolds and incubated 24 h and 48 h at 5% CO_2/37°C.

2.2. Experimental Quantification

2.2.1. Protein quantitation

Media samples were then taken from the 6 condition groups after 24h of incubation with scaffolds and prior to cell seeding. Samples (*n* = 4 scaffolds) were analysed in quadruplicate using a Protein Quantitation Kit (BioVision®) according to manufacturer's protocol and the absorbance was measured in clear assay microplates (Greiner®) using a 595 nm filter in a Modulus™ II microplate multimode reader.

2.2.2. Mechanical testing of collagen scaffolds

Compressional mechanics of collagen scaffolds were accessed to determine the mechanical integrity post-modification due to cross-linking, soaking and shaking conditions. The compression testing and data interpolation are based on previously used methods for tissue engineered scaffolds.[41,42] Scaffolds *n* = 3 were measured in unconfined uniaxial compression testing using an Instron Model 5540 testing machine equipped with a 50-N load cell. The collagen scaffolds were compressed to 60% strain at a strain rate of 0.06mm/s. Incremental Young's modulus (i.e. the ratio of stress to strain) was calculated by measuring the slope of the stress-strain plot at incremental strain increases: (0-10%, 10-20%, 20-30%, 30-40%, 40-50% and 50-60%) as previously described.[43]

2.2.3. Scanning Electron Microscopy (SEM) of collagen scaffolds

SEM characterised the porous architecture of the collagen scaffolds post-modification due to cross-linking and/or soaking, then shaking conditions. The scaffolds tested, PBS unshaken, PBS shaken, PBE E/N unshaken and PBS E/N shaken. Scaffolds were snap-frozen then freeze dried using a FreeZone® 4.5 freeze-drier (Labconco®). The samples were then mounted on to metal stubs with double-sided carbon tape. Thin layers of a gold and palladium alloy were applied to each sample with an automated sputter coater (Polaron

SputterCoater). The samples were then examined at x 60 low magnification at 5kV (Hitach S-4700 SEM) as previously shown.[15]

2.2.4. CellTiter-Blue® Cell viability assay

The assay was performed according to the manufacturer's instructions (Promega). For the 6 condition groups, *n* = 4 scaffolds in duplicate readings to give SD of each group. A range of cell densities were also plated ($5x10^4$; $10x10^4$; $25 x10^4$; $5x10^5$ $7.5x10^5$ and $1 x10^6$ cells/ ml^{-1}) and counted to give a proportional ratio of cell number: fluorescence emitted within this standard curve. Samples were analysed in a Modulus™ II microplate multimode reader using filter of 525 nm Ex/580-640nm Em.

2.2.5. Live/Dead® Viability/Cytotoxicity assay

This assay was performed according to the manufacturer's protocol (Molecular Probes™ Life Technologies) for fluorescence microscopy on the seeded scaffolds. The working concentration of the calcein AM and EthD-1 dyes were diluted to 0.2 µM and 0.4 µM respectively from the suggested working concentrations of 2 µM and 4 µM, respectively. Scaffolds were washed 3 times to remove excess dye in D-PBS /$CaCl_2$ and $MgCl_2$ free (Sigma) and placed on a well slide with 25mm coverslip (Scientific Laboratory Solutions). Microscopy was performed using a Zeiss Axio Imager fluorescent microscope using a 40x objective.

2.2.6. DNA quantitation

Cell seeded scaffolds after the 24 and 48h growth periods were snap-frozen and stored at -20°C. Scaffolds were then freeze-dried overnight using a FreeZone® 4.5 freeze-drier (Labconco®) to remove any residual water content before DNA extraction. The scaffolds were then digested in a solution of D-PBS/ $CaCl_2$ and $MgCl_2$ free (Sigma), containing 2.5 U/ml papain extract (Sigma) 5 mM cysteine-HCl (Sigma) and 5 mM EDTA (Sigma) and samples were incubated overnight at 60°C. Cell extracts (n =4) of $5 x 10^5$ cells frozen at -20°C when scaffolds were seeded, served as the control. Samples (*n* =4 scaffolds) were mixed thoroughly before assay. A Quant-IT™ Picogreen® dsDNA assay kit (Life Technologies™) was used and performed according to the manufacturer's protocol based on 200 µl volume for microplate reader analysis. Samples were analysed in a Modulus™ II microplate multimode reader using filter of 490 nm Ex/510-570nm Em.

2.2.7. RNA isolation

Cell seeded scaffolds after the 24 and 48h growth periods were snap-frozen in 350µl Trizol® (Sigma) and stored at -80°C until preparation. Upon thawing, the scaffolds were homogenised using a TissueRuptor™ device (Qiagen) and centrifuged at 12000 rpm to obtain an aqueous layer and this was subjected to a chloroform extraction and 70% ethanol precipitation. The RNA was then prepared using an RNeasy® kit (Qiagen) according to the manufacturer's protocol. The RNA (100ng/µl) was used to prepare cDNA using ImProm-II™ Reverse Transcription System (Promega) according to the manufacturer's Instructions.

2.2.8. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

The qRT-PCR was performed in triplicate using three independent cDNA samples with additional respective RT-samples to investigate gene expression after seeding on scaffolds. Sensifast™ SYBR® High-ROX (Bioline) was used in the reaction and the reaction was performed using a lightcycler® 480 Instrument II (Roche Life Science) for standard program of 45 cycles. Relative quantification of the RT-PCR results was carried out using the $2^{-\Delta\Delta ct}$ method.[44,37] Forward and reverse primer sequences (Sigma) were as follows: Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) forward primer '5-GTCTCCTCTGACTTCAACAG-3', reverse primer, '5-GTTGTCATACCAGGAAATGAG-3'; vascular endothelial growth factor A (*VEGFA*) forward primer '5-AGACCAAAGAAAGATAGAGCAAGACAAG-3', reverse primer '5-GGCAGCGTGGTTTCTGTATCG-3'; matrix metalloproteinase 1 (*MMP1*) forward primer '5-AGCTAGCTCAGGATGACATTGATG-3' reverse primer 5'-GCCGATGGGCTGGACAG-3'; von Willebrand factor (*vWF*) forward primer 5'-GCAGTGGAGAACAGTGGTG-3', reverse primer 5'-GTGGCAGCGGGCAAAC-3'; Angiopoietin-1 (*Ang1*) forward primer 5'-ATTCTGAATGGTGGGGAGCA-3', reverse primer 5'- TGTGCTGGGATGGGAAAGAT-3'; platelet/endothelial cell adhesion molecule (*PECAM/CD31*) forward primer 5'- ATTGCAGTGGTTATCATCGGAGTG-3', reverse primer 5'-CTCGTTGTTGGAGTTCAGAAGTGG-3'; Tissue inhibitor of matrix metalloproteinase-2 (*TIMP2*) forward primer 5'-AATGCAGATGTAGTGATCAGG-3', reverse primer 5'-TCTATATCCTTCTCAGGCCC-3'.

2.3 . Statistical analysis

Data are presented as average ± standard error mean. Statistical significance was determined by performing one-way ANOVA with $n = 4$ for protein quantitation assay, CellTiter-Blue® cell viability assay and the DNA quantitation assay. For qRT-PCR $n = 3$ and for compression testing $n = 3$. All data presented with significance accepted p-value < 0.05.

3. RESULTS

3.1 Evaluation of scaffold properties

3.1.1. Retainment of ECSFs

The level of protein released into or extracted from different conditioning fluids was investigated. The protein concentration in the conditioning fluids were measured after agitation with the scaffolds to show the influence in processing methods. The protein released into the PBS group had an average concentration of 0.32 μg/μl (as the collagen scaffold itself is a source of protein), while in the BM group this was higher at 0.35 μg/μl, due to the additional components present within the media. Additionally the ABM group had the largest concentration at 0.4 μg/μl, with the presence of ECSFs in the media. In the functionalised groups, which showed significant difference between PBS E/N (0.3 μg/μl) and BM E/N (0.31 μg/μl), these displayed 25 % and 22.5 % less protein compared to ABM group, respectively. The surface functionalisation step demonstrated that the ECSFs were retained in the scaffolds, as shown by the reduction of free protein constituents found in the media (conditioning fluid) observed for these groups, with the ABM E/N group (0.35 μg/μl) having 12.5% less in the conditioning fluid compared to the (non-functionalised) ABM scaffolds, with a similar trend being shown in the other respective groups (Figure 2.).

3.1.2 . Scaffold integrity

In these experiments we assessed the effect of post-modification of the scaffolds, due to soaking and/or E/N surface functionalisation within agitation conditions and to determine if the mechanical integrity was modified. The compression properties of the collagen scaffolds were tested, as was the corresponding surface topography analysed by SEM. Large differences in Young's modulus were seen at low strain intervals (10-20%) with a maximum of 50% difference observed between PBS shaken (0.4 kPa) and unshaken (0.20 kPa). At high

strain interval of 50-60% the compression difference between PBS shaken (3.08 kPa) and PBS unshaken (2.78 kPa) was reduced to 9.7% difference. No statistical significant differences were shown between the groups throughout the intervals of the Young's modulus (Table 1.). The surface topography between the scaffold groups showed no vast difference in the macroporous or microporous structure of the collagen (Figure 3.) with pour sizes varying between 50-200 µm approximately across the surface.

3.2 Assessment of scaffold functionality

3.2.1. EC Attachment and viability

The ability of cells to attach to the scaffolds was assessed and also their viability once attached to the scaffolds (Figure 4A). The number of attached viable cells (NAVC) after 24 h displayed a significant progressive increase across the six scaffold conditions with ABM scaffolds (30 x 10^3 cells attached) compared to 33% and 10% less attached cells in BM and PBS scaffolds, respectively. The E/N treated scaffolds showed greater NAVC than untreated scaffolds across all groups. The NAVC was most profound with ABM E/N scaffolds (35 x 10^3 cells attached) compared to 71% less attached cells in PBS scaffolds (10 x 10^3 cells attached). However at 48 h there was an increase in the NAVC, which was significantly greater in all these scaffold groups, with the ABM E/N scaffolds (110 x 10^3 cells attached) compared to 45% less cells attached in PBS E/N scaffolds. Comparing the difference between 24 h and 48 h values of NAVC within the six conditions, the level increased exponentially. The amount of DNA retained on the scaffolds from cell attachment showed the same trend across the scaffold groups at 24 h. This was then further increased within these scaffold groups at 48 h, with ABM E/N showing the greatest concentration of DNA retained on the scaffold at 1500 ng/ml (Figure 4B). However the difference in DNA concentration between the groups at 24

h and 48 h is not exponential unlike the NAVC (described above). Visual microscopy using Live/Dead® Viability/Cytotoxicity assay showed NAVC on the scaffolds with the greatest amount on the ABM E/N scaffolds (Figure 5).

3.2.2. Gene expression of ECs on scaffolds

The analysis of gene expression accessed the functionality of the recipient ECs (seeded) and determined if the scaffold preparation method effected the EC response in terms of expression of key angiogenic and regulatory genes (Figure 6. and Figure 7.). The results indicate a progressive increase in notably *VEGFA* and *Ang1*, across all scaffold groups, with ABM scaffolds showing the greatest increase of gene expression, further enhanced by E/N surface functionalisation. This ABM E/N group showed this greatest level of expression compared to the lowest level with PBS scaffolds and this was significantly 6-fold and 3-fold higher in *VEGFA* and *Ang1* respectively at 24 h. These levels increased 6.5-fold higher and 3.5 fold higher at 48 h. The key functional gene *CD31* was also increased in all groups, with the greatest level in the ABM E/N scaffolds. For the same comparison with PBS scaffolds the levels were 3-fold higher at 24 h and significantly 2.5 fold higher at 48 h. The *vWF* expression showed marginal differences when compared across groups, with the largest increase (2-fold higher) in PBS scaffolds between the 24 h and 48 h time point. *MMP1* was significantly 2-fold lower in the E/N functionalised scaffolds at 24 h and 48 h when compared to untreated scaffolds. Conversely the expression of *TIMP2,* showed a significant increase (2-fold) in the E/N scaffolds at 24 h to 48 h.

4. DISCUSSION

The majority of recent studies have used the incorporation of recombinant factors to enhance the preformance of TE scaffolds for specific treatments. This can often be complex with varying success, especially when multi-factors are employed.[45] An alternative approach to produce the growth factors or proteins is by the use of cell secreted factors. This has been previously achieved by one of two methods: by the use of cell feeder layers; or obtaining conditioned media. One successful strategy used MSC-derived conditioned medium that promoted proliferation of cardiac progenitor cells (CPC), inhibited apoptosis induced by hypoxia and serum starvation and furthermore upregulated expression of a cardiomyocyte-related gene.[46] This strategy of paracrine cell secretion has been utilised in many regenerative medical applications, namely with the use of stem cells, whereby the paracrine secretion from these cells elicits a response in recruitment of host cells to the tissue environment.[47,48]

In this study we generated a conditioned media in a bovine-free serum containing the endogenous ECSFs and attached this to the scaffold using a number of techniques. This process demonstrated an increase in the level of protein present initially by conditioning scaffolds using BM but more so by using a cell conditioned media (ABM) scaffold. By using a surface cross-linking reaction, we were able to retain and further enhance the conditioned scaffolds and show a greater significant effect in viable cell attachment when these scaffolds have the presence of ECSFs. Nevertheless, the cross-linking reaction served to increase the retention of bound factors upon agitation of BM and ABM scaffold conditions and even enhanced the performance of PBS scaffolds. The further benefits of also using a cross-linking approach such as E/N, proved not only to un-affect the collagen structural and mechanical integrity but to also slightly enhance the stability of any collagen degradation at 48 h.[49] E/N cross-linking has been widely used in the immobilisation of recombinant growth

factors to collagen scaffolds[15-17] but here we were also able to demonstrate a stable, sustained effect when immobilising media.

The key finding was we determined an enhancement effect from this scaffold modification method. This was initially observed using BM and became more profound when ABM scaffolds were used and then further increased by functionalising with E/N, displaying significant differences between the groups tested. In order to represent the phenotype from the attached recipient cells, key gene expression was evaluated. There was also an unaltered endothelial phenotypic response from the attached autologous cells in serum-free conditions, however there was an enhanced effect in angiogenic genes. In addition we were able to show that the collagen integrity was stable between 24 h and 48 h, as *MMP1* representing collagen degradation was reduced when scaffolds were E/N treated. Likewise the inverse expression of *TIMP2* representing collagen integrity was increased when scaffolds were E/N treated.

While the preliminary findings of this study are promising there are important limitations and other parameters that exist which should be considered. An important limitation is in the scaffold type used, which dose not have potential as a vascular subsitute, that said there is potenital to use this novel conditioning process on other scaffolds types. Additionally there are limitations in the process used to produce the conditioned media which could be modified to adjust of the secreted factors produced. A number of mechanisms could be used to achieve this, such as modification to the serum-free culture[50] or by exploting hypoxic conditions to over produce secreted factors.[51] Furthermore the long term activity could be invesitgated to access the potential for an off-the-shelf scaffold approach using this processing technique. Nevertheless, these studies have shown the potential of a cell

secretion method for TE scaffold applications and also provide this method within a serum-free environment.

5. CONCLUSIONS

Here we have demonstrated a scaffold model utilising a novel cell secreted method for specifically ECs. Taken together our results and the core principle of this method highlight the potential that could be extended to other cell types, tissue environments and suitable scaffold materials in tissue engineering and regenerative medicine applications. This strengthens the case for its potential as a translatable clinical process for improvement in scaffold preformance.

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7. AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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