JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE
J Tissue Eng Regen Med 2011; **5**: 69–84.
Published online 17 August 2010 in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/term.292

RESEARCH ARTICLE

Defining conditions for covalent immobilization of angiogenic growth factors onto scaffolds for tissue engineering

Loraine L.Y. Chiu[1], Richard D. Weisel[2], Ren-Ke Li*[2] and Milica Radisic*[1,3,4]
[1]*Department of Chemical Engineering and Applied Chemistry, University of Toronto, Canada*
[2]*Division of Cardiac Surgery, University of Toronto, Toronto General Research Institute, Canada*
[3]*Institute of Biomaterials and Biomedical Engineering, University of Toronto, Canada*
[4]*Heart and Stroke/Richard Lewar Centre of Excellence, University of Toronto, Canada*

Abstract

Rapid vascularization of engineered tissues *in vitro* and *in vivo* remains one of the key limitations in tissue engineering. We propose that angiogenic growth factors covalently immobilized on scaffolds for tissue engineering can be used to accomplish this goal. The main objectives of this work were: (a) to derive desirable experimental conditions for the covalent immobilization of vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang1) on porous collagen scaffolds; and (b) to determine whether primary endothelial cells respond to these scaffolds with covalently immobilized angiogenic factors. VEGF and Ang1 were covalently immobilized onto porous collagen scaffolds, using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) chemistry. To improve covalent immobilization conditions: (a) different reaction buffers [phosphate-buffered saline (PBS), distilled water, or 2-(*N*-morpholino)ethanesulphonic acid (MES)] were used; and (b) step immobilization was compared to bulk immobilization. In step immobilization, growth factors are applied after EDC activation of the scaffold, while in bulk immobilization, reagents are simultaneously applied to the scaffold. PBS as the reaction buffer resulted in higher amounts of VEGF and Ang1 immobilized (ELISA), higher cell proliferation rates (XTT) and increased lactate metabolism compared to water and MES as the reaction buffers. Step immobilization in PBS buffer was also more effective than bulk immobilization. Immobilized growth factors resulted in higher cell proliferation and lactate metabolism compared to soluble growth factors used at comparable concentrations. Tube formation by CD31-positive cells was also observed in collagen scaffolds with immobilized VEGF or Ang1 using H5V and primary rat aortic endothelial cells but not on control scaffolds.

Received 25 June 2009; Accepted 25 February 2010

Supporting information may be found in the online version of this article.

Keywords **covalent immobilization; vascular endothelial growth factor; angiopoietin-1; endothelial cell; tissue engineering; scaffold; collagen**

1. Introduction

The main goal of tissue engineering is to support cell growth and the assembly of functional tissues via integrated use of bioactive scaffolds and bioreactors. In particular, one of the main challenges in tissue engineering is to induce functional vascularization. Engineered tissues should have high thickness (~1 cm) and cell density

**Correspondence to: Milica Radisic, Institute of Biomaterials and Biomedical Engineering, Department of Chemical Engineering and Applied Chemistry, Heart and Stroke/Richard Lewar Centre of Excellence, University of Toronto, 164 College Street, Room 407, Toronto, Ontario M5S 3G9, Canada.*
E-mail: m.radisic@utoronto.ca;
Ren-Ke Li, MaRS Centre, Toronto Medical Discovery Tower 101 College Street, Room 3-702, Toronto, Ontario, Canada.
E-mail: renkeli@uhnres.utoronto.ca

($\sim 10^8$ cells/cm^3) to match the physiological properties of native tissues. To achieve this goal, we postulate that angiogenic growth factors can be incorporated within tissue engineering scaffolds to enable endothelial cell proliferation and tube formation.

Vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang1) are important growth factors that regulate the formation and maturation of blood vessels (Koblizek *et al*., 1998; Kim and Kiick, 2007). VEGF induces the growth of new blood vessels by promoting the dissolution of the basement membrane of the existing blood vessels, the migration and proliferation of endothelial cells, and the formation of tubes by endothelial cells (Kutryk and Stewart, 2003). Ang1 signals for the recruitment of pericytes to deposit new basement membrane, in order to stabilize the newly formed blood vessels (Kutryk and Stewart, 2003).

The angiogenic effects of VEGF and Ang1 have been extensively explored, using gene therapy (DeBusk *et al*., 2004; Chen *et al*., 2005; Zhou *et al*., 2005; Zacchigna *et al*., 2007; Arsic *et al*., 2003; Shyu *et al*., 2003), soluble growth factors (Cheng *et al*., 2007) and controlled release (Lee *et al*., 2004; Steffens *et al*., 2004; Hosack *et al*., 2008). Arsic *et al*. (2003) showed a synergistic effect of VEGF and Ang1 on blood vessel formation when using adeno-associated virus vectors for combined VEGF and Ang1 gene transfer. Benest *et al*. (2006) showed that combined use of VEGF and Ang1 led to more functionally perfused and mature microvessels compared to the use of individual growth factors. This benefit was due to a balance between sprouting and vessel formation. Controlled delivery of VEGF and Ang1 from hydrogels also elicited vascular maturity in mouse ear pinna (Hosack *et al*., 2008).

Soluble growth factors have also been used *in vitro* to improve the properties of engineered tissues. Cheng *et al*. (2007) cultivated neonatal rat heart cells within collagen scaffolds in culture media containing regulatory factors, such as insulin-like growth factor-1 (IGF1), platelet-derived growth factor-BB (PDGF) and Ang1. Soluble Ang1 improved neonatal rat heart cell viability *in vitro* (Cheng *et al*., 2007).

Covalent immobilization is another promising approach for providing growth factors (Chiu and Radisic, 2010), reviewed in Silva *et al*. (2009). Previously, VEGF has been immobilized onto surfaces using various methods. Taguchi *et al*. (2000) immobilized VEGF on poly(acrylic acid) surfaces, using carbodiimide chemistry. The co-immobilization of fibronectin and VEGF improved the growth of human umbilical vein endothelial cells (HUVECs). Koch *et al*. (2006) immobilized VEGF in collagen matrices using a homobifunctional crosslinker, disuccinimidyldisuccinatepolyethyleneglycol (SS-PEG-SS). The angiogenic effect in the collagen matrices was increased with covalently immobilized VEGF compared to admixtures of VEGF, as demonstrated by both *in vitro* endothelial cell growth and *in vivo* vessel growth in the chorioallantois membrane (Koch *et al*., 2006). Ito *et al*. (2005) mixed VEGF with photoreactive gelatin in water and photoimmobilized VEGF in a micropatterning fashion. This enhanced the growth and surface coverage of endothelial cells. Backer *et al*. (2006) incorporated a cysteine tag into VEGF, and then conjugated VEGF to a fibronectin-coated surface via the free sulphydryl group. The conjugated VEGF stimulated the growth of cells that expressed VEGFR-2.

By immobilizing growth factors onto biomaterials, they become protected against cellular inactivation and digestion. (Ito, 2008). As a result, the immobilized growth factors have sustained activity. Growth factor immobilization may also overcome the diffusional limitations of soluble factors, which must be transported to the centre of a three-dimensional scaffold (Shen *et al*., 2008). While controlled delivery of growth factors using hydrogels elicits responses in the surrounding environment, covalently immobilized growth factors may have an additional advantage of inducing local effects within the scaffold. Ito *et al*. (2005) immobilized VEGF on two-dimensional substrates to increase the growth of endothelial cells. Growth factors were also immobilized in patterns to achieve micropatterned HUVEC cultures and aid in the formation of blood vessel networks *in vitro* (Ito *et al*., 2005). More recently, VEGF was also immobilized into three-dimensional scaffolds (Shen *et al*., 2008; Koch *et al*., 2006).

We previously immobilized VEGF onto collagen scaffolds using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) chemistry to promote infiltration, survival and proliferation of D4T endothelial cells (Shen *et al*., 2008). Covalent co-immobilization of VEGF and Ang1 was required to promote tube formation (Chiu and Radisic, 2010). However, our previous work did not systematically vary the conditions for covalent immobilization and only the endothelial cell lines (D4T or H5V) were used. The main objectives of this work were: (a) to compare the effect of the immobilization buffers and timing of growth factor (VEGF and Ang1) application in the immobilization procedure on endothelial cell proliferation and tube formation; and (b) to demonstrate that primary endothelial cells were responsive to growth factors covalently immobilized on porous collagen scaffolds.

2. Materials and methods

2.1. Cell types

The cell types used in the study were D4T endothelial cells, H5V endothelial cells, and primary aortic rat endothelial cells. D4T endothelial cells were from an embryoid body-derived mouse endothelial cell line and were propagated in culture medium (D4T medium) consisting of Iscove's modified Dulbecco's medium (IMDM; Gibco, cat. no. 12 440-053) with 5% fetal bovine serum (FBS; Gibco, cat. no. 16 000-044), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco/Invitrogen, cat. no. 15 140 122). D4T cells were used for studies comparing PBS, distilled water and MES buffer, as well as comparing step and

DOI: 10.1002/term

bulk immobilization. H5V endothelial cells were from murine embryonic heart endothelium, and were grown in culture medium (H5V medium) consisting of Dulbecco's modified Eagle's medium (DMEM; Sigma, cat. no. D5796) with 4.5 g/l glucose, 4 mM L-glutamine, 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. H5V cells were used for studies comparing soluble and immobilized growth factors, as well as evaluating the effect of VEGF or Ang1 on tube formation. Primary rat endothelial cells were isolated from the aorta and cultured as described previously (Mizuno *et al*., 2005).

2.2. Effect of VEGF and Ang1 on different endothelial cell lines

Three independent experiments were conducted to study the effect of soluble VEGF and Ang1 on different endothelial cell lines, including: (a) low passage H5V (P5 and P6); (b) low passage D4T (P14, P15, P18); and (c) high passage D4T (P28, P30, P31). Cells, as a 10 μl suspension of 5000 cells, were seeded in each well of a 24-well plate ($n = 9$ for each of control, VEGF and Ang1 groups of each cell type). Culture medium (1 ml), which was specific for each cell line and supplemented with 50 ng/ml mouse recombinant VEGF-165 (Cell Sciences, cat. no. CRV014B) or 100 ng/ml human recombinant Ang1 (R&D Systems, cat. no. 923-AN-025) was added to each well. Culture medium that was not supplemented with growth factors was used for control samples. Culture medium was changed every other day. Brightfield images ($n = 3$–5) of each well were taken at ×200 magnification every day for 3 days. The number of cells in each image was counted to obtain cell density. The cell density was plotted against time, and the slope showed the cellular growth rate.

After 3 days of cultivation, XTT {sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulphonic acid hydrate} assay was performed on the samples, using cell proliferation kit II (Roche Diagnostics, cat. no. 1 465 015), according to the manufacturer's instructions.

2.3. Scaffold preparation

A metal borer was used to cut circular collagen scaffolds (diameter 7 mm, thickness 2 mm) from a sheet of porous collagen sponge (Ultrafoam collagen sponge, Davol, cat. no. 1 050 050). Both recombinant VEGF-165 and recombinant Ang1 were immobilized onto the scaffold, using EDC chemistry.

2.3.1. Step immobilization using PBS, water or MES as reaction buffers

EDC chemistry was performed in two ways. The first way was by step immobilization, in which the collagen scaffolds were immersed in 150 μL sterile filtered solution of EDC (Sigma, cat. no. E7750) and sulpho-NHS (*N*-hydroxysulphosuccinimide; Pierce Chemicals, cat. no. 24 510) in a 96-well plate. EDC/sulpho-NHS concentrations were 24 mg/60 mg, respectively, per 1 ml of 1 M phosphate-buffered saline (PBS; Gibco, cat. no. 10 010-023) or distilled water or 0.05M 2-(*N*-morpholino)ethanesulphonic acid (MES; Sigma, cat. no. M1317). The pHs of PBS and MES were measured to be 7.1 and 4.2, respectively (pH meter, InoLab pH 720). The ionic strength was 60 mM for PBS (Otto *et al*., 1999) and 35–70 mM for MES (Delomenie *et al*., 1997). PBS was used for the VEGF + PBS and Ang1 + PBS groups. Distilled water was used for the VEGF + DW and Ang1 + DW groups. MES buffer was used for the VEGF + MES and Ang1 + MES groups. EDC/sulpho-NHS solution in PBS was used for the PBS + EDC control sponges. PBS control sponges were immersed in 150 μL PBS. The activation of the collagen scaffold (i.e. EDC activation step) was allowed to proceed for 20 min at room temperature. The scaffold was then removed from the solution, dabbed onto a Petri dish surface to remove excess liquid, and immersed into 100 μL solution of VEGF or Ang1 in PBS. Both PBS and PBS + EDC control sponges were immersed in 100 μL PBS. This reaction (i.e. the immobilization step) was allowed to proceed for 1 h at room temperature.

2.3.2. Comparison of step vs. bulk immobilization using PBS and MES buffers

The second way to perform EDC chemistry was by bulk immobilization (Steffens *et al*., 2004), in which 100 μl solution of 1 μg/ml VEGF or Ang1 and EDC/sulpho-NHS (in the ratio of 24 mg:60 mg/1 ml PBS or MES) in PBS or MES was added to collagen scaffolds (i.e. VEGF + PBS bulk and VEGF + MES bulk groups). The reaction was allowed to proceed for 4 h at 37 °C. The immobilization buffers and growth factor concentrations used for various experimental groups are summarized in Table 1.

At the end of the reaction, the scaffolds were immersed in fresh PBS eight times for at least 5 min each time, incubated in culture medium for 1 h and dried on autoclaved Kim-wipes prior to cell seeding. In the figures and the Results section, the condition is step immobilization unless specifically indicated as bulk immobilization.

2.4. Cell seeding

After scaffold preparation, the collagen scaffolds were transferred to a clean 24-well plate. Before cell seeding, the desired cell number was centrifuged into a pellet and resuspended in the volume of cell type specific culture medium corresponding to 10 μl/collagen scaffold. D4T or H5V cells (50 000) were seeded onto the scaffolds in 10 μl culture medium for cell proliferation and metabolism studies. H5V cells (173 333) were seeded for evaluating tube formation. The scaffolds were incubated for 40 min at 37 °C for the cells to attach. After incubation, 1 ml fresh culture medium was added to each well. The samples were

DOI: 10.1002/term

Table 1. Solvents and concentrations of growth factors used

Name of experimental group	EDC activation step (first step)	Immobilization step (second step)	
	Solvent	VEGF in PBS (μg/ml)	Ang1 in PBS (μg/ml)
PBS	PBS*	–	–
PBS + EDC	PBS	–	–
VEGF + PBS (step, bulk)	PBS	1	–
VEGF + DW	Distilled water	1	–
VEGF + MES (step, bulk)	MES buffer	1	–
Ang1 + PBS	PBS	–	1
Ang1 + DW	Distilled water	–	1
Ang1 + MES	MES buffer	–	1

*No EDC or sulpho-NHS was added for the PBS group.

cultured for 7 days with 100% change and collection of medium on days 2, 4 and 7. The culture medium samples were stored at −20 °C for further lactate assay. For comparison between soluble and immobilized growth factors, the samples were cultured for 3 days with the collection of culture medium at the end of the cultivation period.

2.5. Soluble controls

For soluble control groups (i.e. S-VEGF and S-Ang1 groups), collagen scaffolds were not modified with growth factors, similar to the PBS group. Rather, soluble growth factors (50 ng/ml VEGF for the S-VEGF group or 50 ng/ml Ang1 for the S-Ang1 group) were added directly into the 1 ml culture medium after 50 000 H5V cells had been seeded onto the unmodified sponges.

2.6. XTT cell proliferation assay

The number of cells on the constructs after 7 days of cultivation was determined by performing XTT assay, using cell proliferation kit II (Roche Diagnostics, cat. no. 1 465 015), according to the manufacturer's instructions. Briefly, collagen samples were incubated with 100 μl culture medium and 50 μl of XTT labelling solution at 37 °C with 5% CO_2 for 4 h. The absorbance of the supernatant (100 μl) was measured at 450 nm using a plate reader (Apollo LB911, Berthold Technologies). Calibration curves were created by seeding known number of cells on collagen sponges (i.e. 0, 25 000, 50 000, 300 000 and 500 000 cells) and performing XTT assay on these standards. The amount of formazan dye formed, as indicated by the absorbance, is directly proportional to the number of viable and metabolically active cells in the scaffold.

2.7. Lactate production

Culture medium was collected from each well and stored at −20 °C on days 2, 4 and 7 of the cultivation period. A lactate assay kit (Biomedical Research Service Centre, cat. no. A-108L) was used to measure lactate concentrations in the culture medium samples (diluted 1 : 50 in distilled water), according to the manufacturer's instructions. Lactate assay works by the principle that the tetrazolium salt INT is reduced in a NADH-coupled enzymatic reaction to form a water-soluble formazan colour product. The intensity of the red-coloured product formed is directly proportional to the lactate concentration in the culture medium samples. The absorbance measurements were converted to lactate concentrations, using calibration curves from the series of lactate standards.

2.8. Quantification of immobilized VEGF and Ang1

VEGF and Ang1 ELISAs were performed by first preparing collagen scaffolds, as previously described. The immobilization solution (containing the remaining non-immobilized VEGF or Ang1) and washing solutions (eight washes containing the physically adsorbed VEGF or Ang1 washed off from the scaffold) were collected and stored at −20 °C overnight. ELISA was performed on these solutions (diluted 1 : 100 in PBS) to find the amount of VEGF or Ang1 that was not immobilized. The amount of immobilized VEGF or Ang1 was determined by subtracting the amount of VEGF or Ang1 remaining in the immobilization and washing solutions from the original amount of VEGF or Ang1 used in the immobilization solution (Chen *et al.*, 2005; Leclerc *et al.*, 2008; von Walter *et al.*, 2008). The amounts of VEGF and Ang1 were quantified using sandwich ELISA according to the manufacturer's instructions (Murine VEGF Enzyme-Linked ImmunoSorbent Assay Kit, Biovision, cat. no. K4364-1000 and Quantikine Human Angiopoietin-1 Immunoassay, R&D Systems, cat. no. DANG10).

2.9. Confocal microscopy on live cells

Staining was performed by incubating constructs with 200 μl solution of 1.5 μl carboxyfluorescein diacetate (CFDA; staining live cells green) per 1 ml PBS for 40 min

DOI: 10.1002/term

at 37 °C. Images were taken at ×200 magnification, using a confocal microscope (Zeiss LSM 510).

2.10. Preparation of frozen sections and CD31 immunostaining

After cultivation, the constructs were fixed in 10% neutral buffered formalin for 1 h at room temperature and kept in PBS at 4 °C for storage. The constructs were immersed in a solution of 30% sucrose in PBS overnight before cryosectioning. They were then each placed facing down in a separate cryomould (Cryomold Biopsy Disposable Vinyl Specimen Mold, 10 × 10 × 5 mm; Tissue-Tek, 4565) that had a thin layer of OCT compound (Embedding Medium for Frozen Tissue Specimens to ensure Optimal Cutting Temperature, Tissue-Tek, cat. no. 4583) at the bottom. The mould was filled up with OCT to cover the construct. The mould containing the sample was then submerged in liquid nitrogen to be snap-frozen. The snap-frozen samples were stored at −80 °C before being cryosectioned at −22 °C using a cryostat (Leica, cat. no. CM3050S), at a thickness of 10 μm and at intervals of 200 μm, starting from the top of the construct until 1400 μm below the top surface. Cut sections were put onto microscope slides (VWR, Microslides Superfrost Plus, 25 × 75 × 1 mm, cat. no. 48 311-703). The slides were air-dried at room temperature for 30 min. Then, they were fixed in 200 μl 100% acetone (ACS grade) for 20 min, until the acetone evaporated. The slides were blocked in 10% normal horse serum (NHS; Vector Laboratories, cat. no. S-2000) in PBS for 10 min at room temperature in a humidified chamber. The samples were then incubated in 100 μl primary antibody (rabbit polyclonal anti-CD31; AbCam, cat. no. AB28364-100) at a dilution factor of 1 : 50 at 4 °C overnight. On the next day they were incubated in 100 μl secondary antibody (Fluorescein goat anti-rabbit IgG; Vector Laboratories, cat. no. FI-1000) with Hoechst dye (Sigma, cat. no. B2883) at a dilution factor of 1 : 100 (for both antibody and Hoechst) for 1 h at room temperature. The slides were mounted with Fluoromount aqueous mounting medium (Sigma-Aldrich, cat. no. F4680), covered and imaged using fluorescence microscopy (Olympus IX2-UCB, In Vitro software). Five images/sample were taken and three samples were prepared for each experimental condition.

2.11. Haematoxylin and eosin (H&E) staining

After cultivation, the samples were fixed with 10% neutral buffered formalin (Sigma, cat. no. HT501129) for at least 24 h at room temperature. Fixed samples were sent to the Pathology Research Program (PRP) histology laboratory at University Health Network for paraffin embedding and sectioning, as well as H&E staining. Images were taken at ×400 magnification using optical microscopy (Leica Microsystems DM IL, type 090–153.001).

2.12. Statistical analysis and data representation

Statistical analysis was performed using SigmaStat 3.0 and GraphPad Prism 5.0. Differences between experimental groups were analysed by using *t*-tests, one-way ANOVA with *post hoc* Tukey tests or two-way ANOVA with Bonferroni post-tests. $p < 0.05$ was considered significant for all statistical tests. Results were plotted with GraphPad Prism 5.0 as means, with error bars representing standard errors.

3. Results

3.1. Effect of VEGF and Ang1 on different endothelial cell lines

The concentrations of VEGF (50 ng/ml) and Ang1 (100 ng/ml) were selected based on past studies on monolayers of endothelial cells (Satchell *et al*., 2004). It was found that the growth rate of H5V cells was 1.2- and 1.7-fold increased when the culture medium was supplemented with 50 ng/ml VEGF and 100 ng/ml Ang1, respectively, as compared to control (Figure 1A; see also Supporting information, Figure S1A). The growth rate of D4T cells at low passage numbers was 1.2- and 1.4-fold increased with VEGF and Ang1, respectively, compared to control (Figure 1B; see also Supporting information, Figure S1B). On the other hand, the growth rate of D4T cells at high passage numbers was slightly decreased, with Ang1 (0.92-fold of control) and 1.4-fold increased when supplemented with VEGF (Figure 1C; see also Supporting information, Figure S1C). In this study, passage numbers <20 are considered low.

After 3 days of cultivation, the final cell number within the sample wells was determined by XTT assay (Figure 1D). D4T cells at high passage numbers surprisingly showed slightly lower final cell numbers in VEGF- and Ang1-supplemented wells compared to control. Although both D4T cells at low passage numbers and H5V cells resulted in higher final cell numbers in VEGF- and Ang1-supplemented wells compared to the controls, the VEGF- and Ang1-supplemented samples of H5V cells showed a more significant difference than their control (1.7- and 1.2-fold control for VEGF- and Ang1-supplemented H5V cells, respectively, compared to 1.2-fold control for both VEGF- and Ang1-supplemented D4T cells). Moreover, only VEGF-supplemented H5V cells showed a statistically significant increase ($p < 0.001$) in the final cell number compared to the control. The results from cell growth rates (Figure 1A–C) and the final cell number at day 3 by XTT assay (Figure 1D) cannot be directly compared. While Figure 1A–C shows the growth rate in terms of total cell density at different time points, as obtained from the brightfield images, the XTT measurements in Figure 1D only account for live cells that are actively metabolizing.

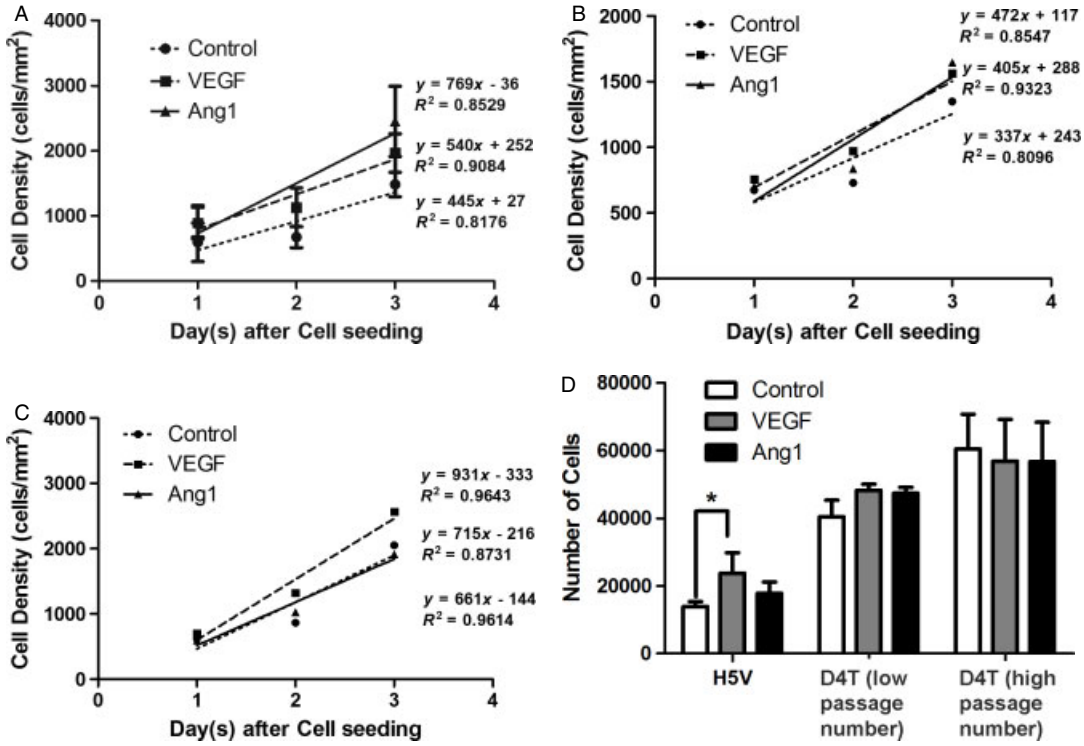

Figure 1. Growth of different endothelial cell lines as 2D monolayers in VEGF- and Ang1-supplemented culture medium. (A) Growth rate of H5V endothelial cells with no growth factors (control), 50 ng/ml VEGF or 100 ng/ml Ang1. (B) Growth rate of D4T endothelial cells at low passage numbers (error bars removed to avoid clustering). (C) Growth rate of D4T endothelial cells at high passage numbers. (D) XTT assay, indicating the total number of cells in the well after 3 days of cultivation. Low-passage D4Ts are P14, P15 and P18. High passage D4Ts are P28, P30 and P31. *$p < 0.05$ (two-way ANOVA with Bonferroni post-tests) was considered statistically significant

As a result, the H5V endothelial cell line at low passage number was considered more responsive to the growth factor treatment and these cells were chosen for further experiments involving immobilized VEGF and Ang1 on collagen scaffolds.

3.2. Effect of immobilization buffer on step immobilization of growth factors

The immobilization buffer for EDC chemistry was varied using PBS (1×), distilled water and 0.05 M MES buffer. Step immobilization involves first activating collagen scaffolds with EDC dissolved in PBS, distilled water or MES buffer, and then applying VEGF or Ang1. Since the desired end-effect of choosing the preferred buffer for the immobilization process is to attain higher proliferation and metabolic rates for the endothelial cells on the collagen scaffold, the conditions were compared by the final cell number and lactate production rate.

XTT assay provides an indication of cell proliferation during the cultivation period by measuring the final cell number within the scaffolds, seeded with the same initial cell number. The final cell number within the collagen scaffold (Figure 2A) was significantly higher in the VEGF + PBS group, as compared to the controls, PBS and PBS + EDC ($p = 0.016$, one-way ANOVA; $p = 0.016$, in comparison with PBS; $p = 0.007$, in comparison to PBS + EDC). Although the cell number in the VEGF + DW group was significantly higher than in the PBS + EDC control ($p = 0.035$), it was not significantly higher than in the PBS control ($p = 0.078$). The VEGF + MES group was not significantly different from both PBS and PBS + EDC controls ($p = 0.933$ in comparison to PBS; $p = 0.739$ in comparison to PBS + EDC). There was no significant difference between PBS and PBS + EDC controls ($p = 0.664$).

In general, all groups showed an increasing lactate concentration from day 2 to day 7 (Figures 2, 3). Since lactate is accumulated during glycolysis in anaerobic metabolism, increasing lactate concentration is an indication of a trend from aerobic metabolism towards anaerobic metabolism. This is typical of a proliferating population of cells.

Lactate production rates give an indication of both cell number and the metabolic rate. Thus, lactate production provides different information about the cell population than final cell numbers, as evaluated by XTT assay. The lactate production rate (Figure 2B) increased from day 2 to day 7 for all groups ($p < 0.0001$). More importantly, the lactate production rates of VEGF + PBS and VEGF + MES groups were significantly higher than those in the controls, PBS and PBS + EDC, on day 7 ($p = 0.016$ two-way ANOVA; $p < 0.05$ Bonferroni post-test for VEGF + PBS compared to both PBS and PBS + EDC; $p < 0.001$ Bonferroni post-test for VEGF + MES compared to both

DOI: 10.1002/term

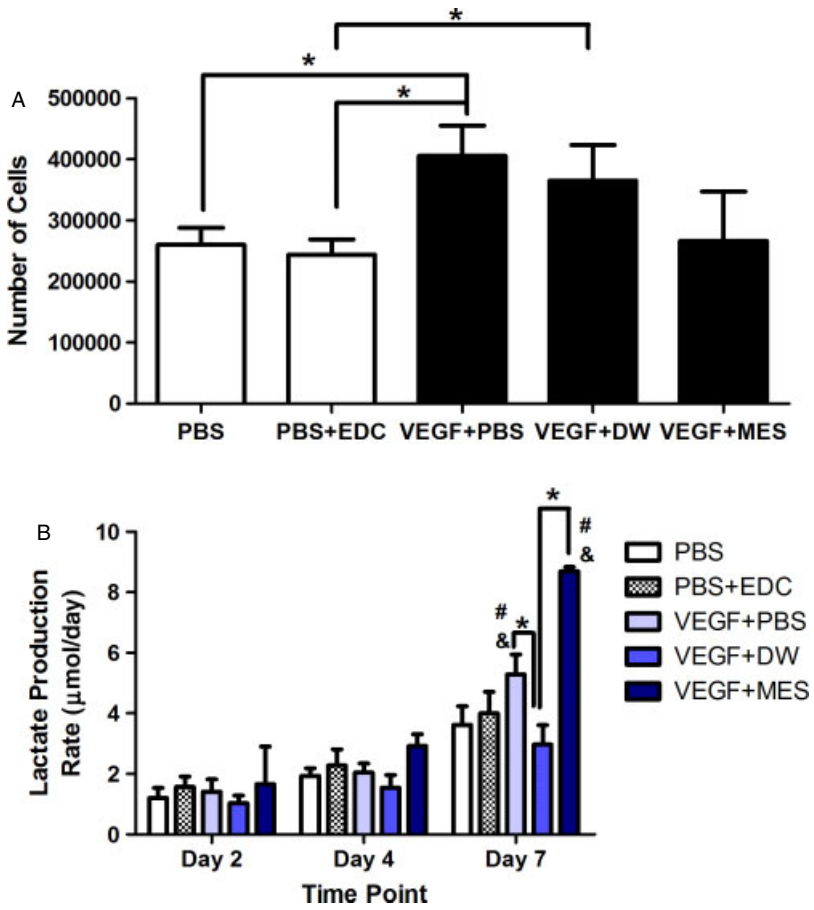

Figure 2. Effect of immobilization buffer on cell number in step immobilization of VEGF. (A) XTT assay indicating final cell numbers in collagen scaffolds at day 7. (B) Lactate production rate at various time points. *Statistically significant difference; # statistically significant difference compared to PBS control; &statistically significant difference compared to PBS + EDC control ($p < 0.05$; one-way ANOVA with *post hoc* Tukey test for cell numbers and two-way ANOVA with Bonferroni post tests for lactate production rates). PBS, control scaffold; PBS + EDC, scaffold treated with EDC crosslinker in PBS reaction buffer; VEGF + PBS, scaffold with immobilized VEGF, where PBS was used as a reaction buffer; VEGF + DW, scaffold with immobilized VEGF, where distilled water was used as a reaction buffer; VEGF + MES, scaffold with immobilized VEGF where MES buffer was used

PBS and PBS + EDC). The lactate production rate of VEGF + DW group was not significantly different from that in the controls ($p = 0.686$ in comparison to the PBS group; $p = 0.455$ in comparison to the PBS + EDC group). Rather, VEGF + DW had a significantly lower lactate production rate compared to the VEGF + PBS and VEGF + MES groups ($p < 0.01$ Bonferroni post-test for VEGF + PBS; $p < 0.001$ for VEGF + MES). Therefore, distilled water is the least preferred method for dissolving EDC and sulpho-NHS for the immobilization process, according to lactate production rates in the VEGF groups. Although VEGF + DW appeared to be a better condition than VEGF + MES according to XTT assay, cells in the VEGF + DW group are likely metabolizing at a slower rate by the end of day 7, according to the lactate production rate. This supports the results from XTT assay that PBS is the preferred buffer in EDC chemistry for the step immobilization of VEGF.

Similarly, PBS was found to be the preferred buffer in EDC chemistry for the step immobilization of Ang1. The final cell number within the collagen scaffold (Figure 3A) was significantly higher in the Ang1 + PBS group, as compared to the controls, PBS and PBS + EDC ($p = 0.005$, one-way ANOVA; $p = 0.003$, compared to the PBS control; $p = 0.001$, compared to the PBS + EDC control). The cell number for the Ang1 + DW group was significantly higher than in the PBS + EDC control ($p = 0.040$) but not in the PBS control ($p = 0.082$). The final cell number in the Ang1 + MES group was not significantly different from that in PBS or the PBS + EDC control ($p = 0.424$ in comparison to PBS; $p = 0.262$ in comparison to PBS + EDC).

The lactate production rate (Figure 3B) increased from day 2 to day 7 for all immobilized Ang1 groups ($p = 0.0013$). There was no significant difference amongst Ang1 groups at specific time points ($p = 0.115$). However, the Ang1 + PBS, Ang1 + DW and Ang1 + MES groups, respectively, had 3-fold, 2.7-fold and 3.8-fold increase in lactate production rate from day 2 to day 7, compared to 2.1 and 1.7 for the PBS and PBS + EDC controls. Although it appears that the Ang1 + MES group had the most drastic increase in lactate production rate, both the Ang1 + PBS and the Ang1 + DW groups showed much higher lactate production rate at day 7 compared

DOI: 10.1002/term

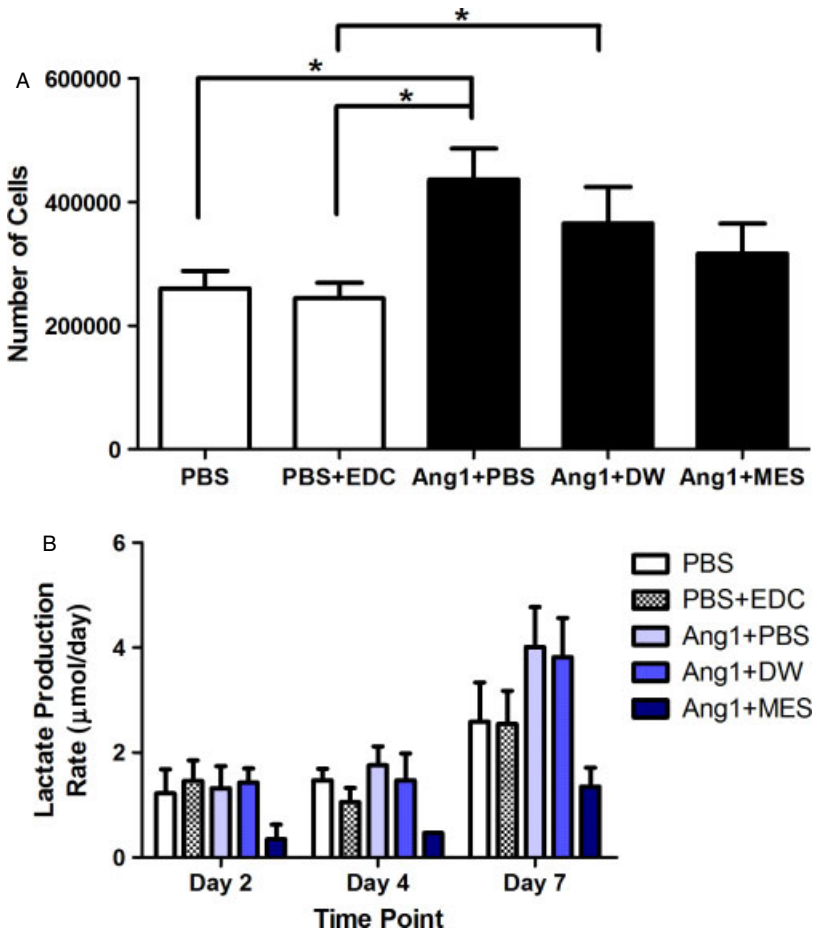

Figure 3. Effect of immobilization buffer on cell number in step immobilization of Ang1. (A) XTT assay indicating final cell numbers in collagen scaffolds at day 7. (B) Lactate production rate at various time points. *Statistically significant difference ($p < 0.05$; one way ANOVA with *post hoc* Tukey test for cell numbers and two-way ANOVA with Bonferroni post-tests for lactate production rates). PBS, control scaffold; PBS + EDC, scaffold treated with EDC crosslinker in PBS reaction buffer; Ang1 + PBS, scaffold with immobilized Ang1, where PBS was used as a reaction buffer; Ang1 + DW, scaffold with immobilized Ang1, where distilled water was used as a reaction buffer; Ang1 + MES, scaffold with immobilized Ang1, where MES buffer was used

to the Ang1 + MES group (4.01 µmol/day for Ang1 + PBS, and 3.82 µmol/day for Ang1 + DW were 3-fold and 2.8-fold of 1.35 µmol/day for Ang1 + MES, respectively).

Lactate production rate depends on the cell number as well as the number of endothelial cells that have entered the stationary phase of the growth curve (Hashimoto *et al.*, 1992); thus, discrepancies between cell number, as determined by the XTT assay and the lactate output, may be expected. Lactate production rate will be higher for the same number of cells in the growth phase compared to the stationary phase. It is possible that the cells on the VEGF + DW and Ang1 + MES scaffolds, which showed high cell numbers by day 7, had low lactate production rates because the cells had already entered the stationary phase at day 7, while cells in other groups were still in the growth phase.

Results from the XTT assay and lactate production rate both showed that the Ang1 step immobilization with PBS as a buffer promoted greater cell proliferation and metabolism, as compared to distilled water and MES buffer. Since PBS was found to be the preferred buffer for immobilization of both VEGF and Ang1, this finding may be advantageous in co-immobilization strategies.

3.3. Comparisons of step and bulk immobilization

Besides comparing the use of different buffers for the immobilization process, the EDC chemistry was also performed using two different methods: (a) step immobilization; and (b) bulk immobilization. Step immobilization involves: (a) immersing scaffolds into a solution of EDC/sulpho-NHS in PBS or MES buffer; and then (b) into a solution of VEGF or Ang1. The VEGF and Ang1 solutions were always prepared in PBS in order to maintain protein conformation. Bulk immobilization involves immersing the scaffolds into a solution containing all reagents (EDC/sulpho-NHS, VEGF or Ang1) prepared in either PBS or MES buffer.

XTT assay showed that VEGF + PBS step had the highest final cell number (Figure 4A) compared to other groups ($p = 0.0078$, one-way ANOVA), with statistically significant differences when compared to PBS

DOI: 10.1002/term

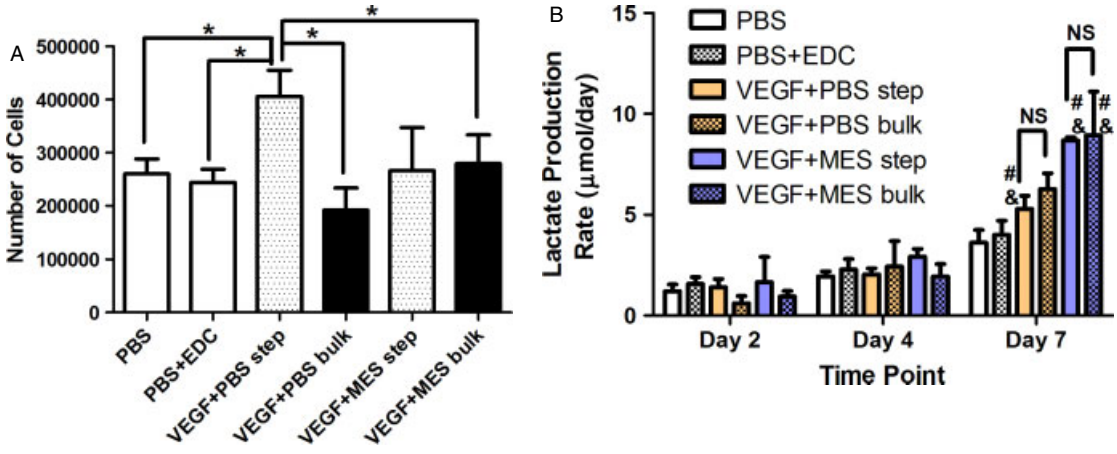

Figure 4. Comparison of step and bulk immobilization of VEGF. (A) XTT assay indicating final cell numbers in collagen scaffolds after 7 days of cultivation. (B) Lactate production rate. NS, no significant difference. *Statistically significant difference; # statistically significant difference compared to PBS control; &statistically significant difference compared to PBS + EDC control ($p < 0.05$; one-way ANOVA with *post hoc* Tukey test for cell numbers and two-way ANOVA with Bonferroni post-tests for lactate production rates). PBS, control scaffold; PBS + EDC, scaffold treated with EDC crosslinker in PBS reaction buffer; VEGF + PBS step, scaffold with immobilized VEGF, where PBS was used as a reaction buffer in step immobilization; VEGF + PBS bulk, scaffold with immobilized VEGF, where PBS was used as a reaction buffer in bulk immobilization; VEGF + MES step, scaffold with immobilized VEGF, where MES buffer was used in step immobilization; VEGF + MES bulk, scaffold with immobilized VEGF, where MES buffer was used in bulk immobilization

($p = 0.016$), PBS + EDC ($p = 0.007$) and VEGF + PBS bulk ($p = 0.005$). There was no significant difference between the VEGF + MES step and the VEGF + MES bulk groups ($p = 0.906$).

The lactate production rate (Figure 4B) significantly increased from day 2 to day 7 for both step and bulk immobilized groups ($p < 0.0001$). The VEGF + PBS step, VEGF + MES step and VEGF + MES bulk groups all had significantly higher lactate production rates at day 7 compared to PBS and PBS + EDC controls ($p < 0.001$ compared to PBS control; $p < 0.01$ compared to PBS + EDC control). There was no significant difference between the lactate production rates of the VEGF + PBS step and VEGF + PBS bulk, and between the VEGF + MES step and VEGF + MES bulk at day 7 ($p = 0.933$ for PBS groups; $p = 0.891$ for MES groups).

Since the VEGF + PBS step had significantly higher cell number according to the XTT assay compared to both controls and its corresponding bulk group (VEGF + PBS bulk), it was chosen as the preferred covalent growth factor immobilization method.

3.4. Amounts of immobilized growth factors by ELISA

The amounts of VEGF or Ang1 immobilized onto collagen scaffolds for this study are listed in Table 2. Here, the VEGF + PBS group had significantly higher amounts of immobilized VEGF compared to both VEGF + DW ($p = 0.0002$) and VEGF + MES groups ($p = 0.022$). Both Ang1 + PBS and Ang1 + DW groups had two-fold increase in the amount of immobilized Ang1 compared to Ang1 + MES group. Thus, PBS buffer improved the amounts of growth factors immobilized for both VEGF and Ang1.

Table 2. Amount of immobilized VEGF or Ang1 as quantified by ELISA

	Amount of immobilized growth factor	
Name of experimental group	VEGF (ng)	Ang1 (ng)
PBS	–	–
PBS + EDC	–	–
VEGF + PBS step	60.68 ± 2.90[a]	–
VEGF + PBS bulk	43.67 ± 6.32	–
VEGF + DW step	36.13 ± 4.67	–
VEGF + MES step	48.45 ± 3.90	–
VEGF + MES bulk	50.26 ± 3.25	–
Ang1 + PBS step	–	47.90 ± 4.67
Ang1 + DW step	–	44.47 ± 5.42
Ang1 + MES step	–	21.92 ± 9.24

[a]Significantly higher VEGF amount compared to VEGF + PBS bulk, VEGF + DW step and VEGF + MES step groups ($p < 0.05$).

3.5. Comparison of soluble and immobilized growth factors

The effects of soluble and immobilized growth factors were compared using a 3-day cultivation of H5V endothelial cells (Figure 5). Based on previously presented results, step immobilization with PBS as the buffer was used for immobilized groups (VEGF + PBS step and Ang1 + PBS step groups). Groups with soluble growth factors (S-VEGF and S-Ang1 groups) involve seeding cells onto unmodified collagen scaffolds and adding growth factors into the culture medium. The amounts of soluble growth factors (50 ng/ml VEGF or Ang1) that were added to the culture medium were similar to the amount of immobilized VEGF or Ang1, as quantified by ELISA (Table 2).

Both S-VEGF and S-Ang1 groups had no significant difference in final cell number (Figure 5A, B) as compared to PBS and PBS + EDC controls (one-way ANOVA,

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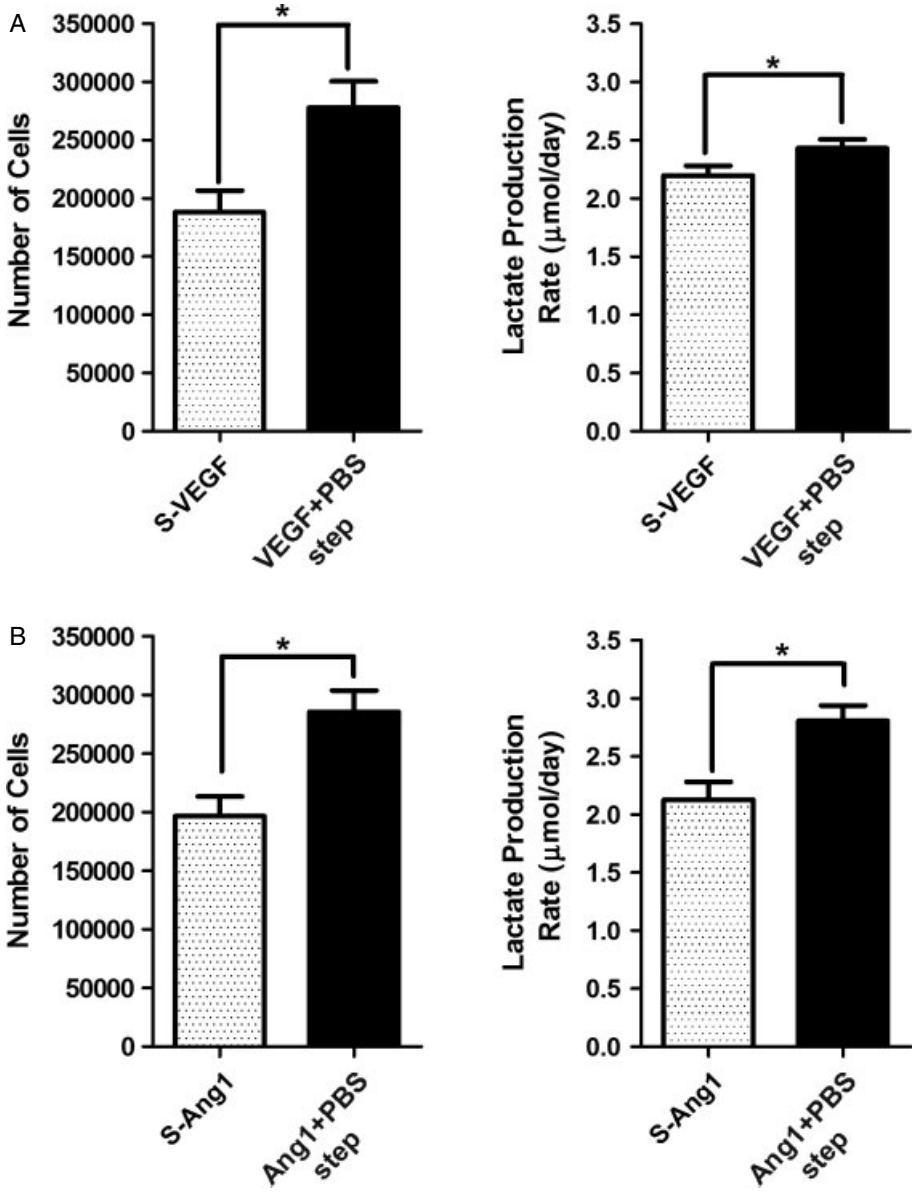

Figure 5. Comparison of soluble and immobilized growth factors using H5V endothelial cell line. Final cell number and lactate production rate for (A) VEGF + PBS step vs. S-VEGF. (B) Ang1 + PBS step vs. S-Ang1. *Statistically significant difference ($p < 0.05$; *t*-test). S-VEGF, control scaffold placed in culture medium with 50 ng/ml soluble VEGF; VEGF + PBS step, scaffold with immobilized VEGF, where PBS was used as a reaction buffer in step immobilization; S-Ang1, control scaffold placed in culture medium with 50 ng/ml soluble Ang1; Ang1 + PBS step, scaffold with immobilized Ang1, where PBS was used as a reaction buffer in step immobilization

$p = 0.0794$). However, both VEGF + PBS step and Ang1 + PBS step groups had significantly higher cell number as compared to PBS and PBS + EDC controls (one-way ANOVA, $p = 0.0078$). Moreover, immobilized groups had significantly higher cell number compared to their corresponding soluble groups (*t*-tests, $p = 0.0417$ for S-VEGF vs. VEGF + PBS step, $p = 0.0016$ for S-Ang1 vs. Ang1 + PBS step). Similarly, immobilized groups showed significantly higher lactate production rates (Figure 5A, B) as compared to their corresponding soluble groups (*t*-tests, $p = 0.0450$ for S-VEGF vs. VEGF + PBS step, $p = 0.0013$ for S-Ang1 vs. Ang1 + PBS step).

3.6. Effect of immobilized VEGF and Ang1 on tube formation by H5V endothelial cells

Besides cell proliferation and metabolism, immobilized VEGF and Ang1 (step immobilization using PBS as a buffer) also promoted tube formation when a high density of 173 333 cells was initially seeded onto the collagen scaffolds (compared to a low density of 50 000 cells for cell proliferation and metabolism studies). Confocal microscopic images of CFDA-stained samples (Figure 6A) showed elongated cells lining tube structures for VEGF + PBS and Ang1 + PBS, but not the PBS group. Similar results were observed with

DOI: 10.1002/term

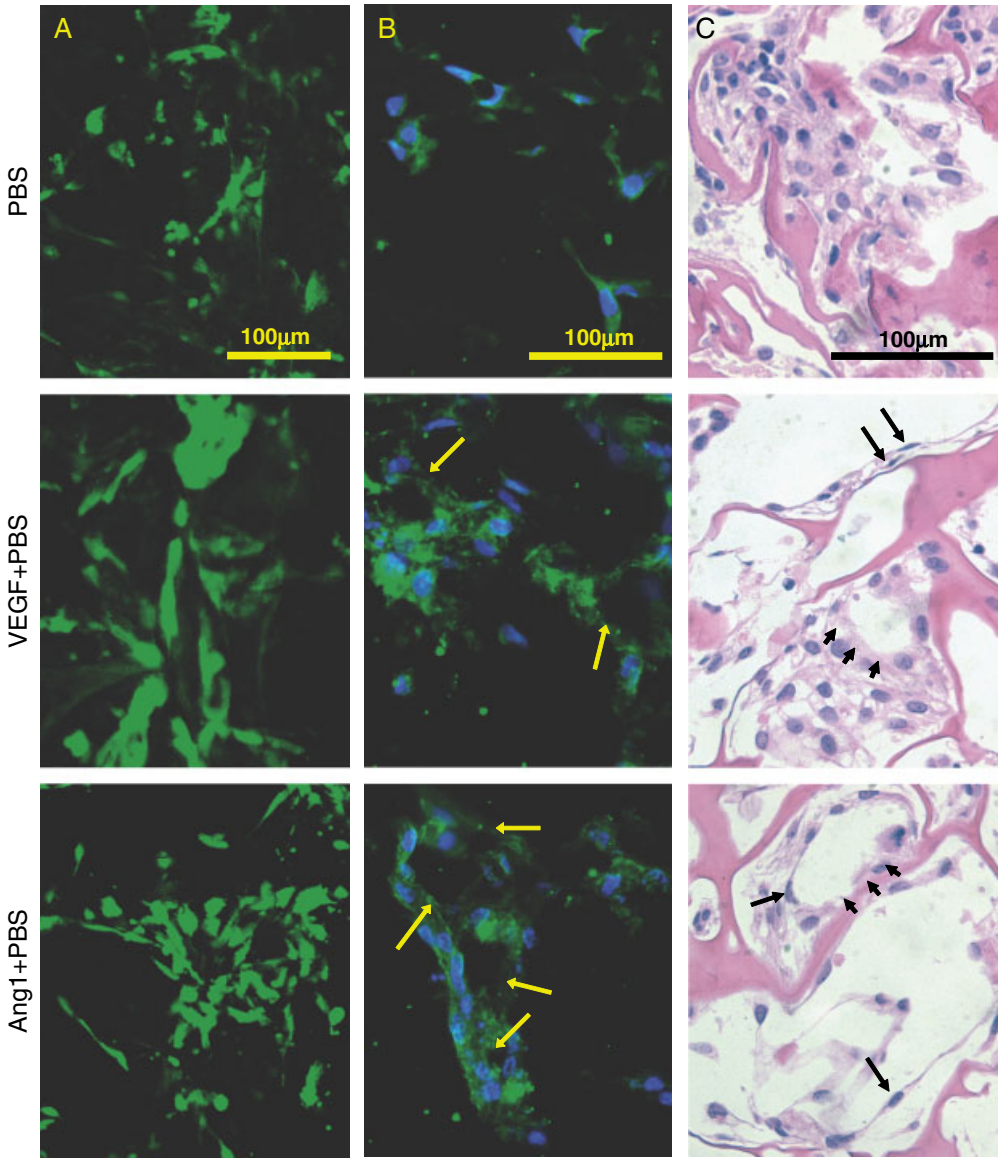

Figure 6. Cell morphology and tube formation in 7 days of *in vitro* cultivation of H5V endothelial cells. A high density of 173 333 cells was initially seeded. (A) Confocal microscopy images showing (CFDA staining of live cells). (B) CD31 immunofluorescence staining of frozen sections (circular structures formed by CD31 positive cells are indicated by arrows; cell nuclei stained with Hoechst dye). (C) H&E staining (black arrowheads, circular structures; arrow, elongated cells). PBS, control scaffold; VEGF + PBS, scaffold with immobilized VEGF, where PBS was used as a reaction buffer; Ang1 + PBS, scaffold with immobilized Ang1, where PBS was used as a reaction buffer

cluster of differentiation molecule-31 (CD31) staining (Figure 6B), a common endothelial cell marker. Circular structures formed by cell–cell contact of the endothelial cells were shown for the groups with immobilized VEGF or Ang1. In contrast, the PBS group showed sparsely distributed endothelial cells. H&E-stained images (Figure 6C) showed elongated cells with nuclei in a peripheral position, suggestive of endothelial cells in capillaries. These cells also connected to form circular structures, consistent with the CD31 staining. The PBS group showed rounded cells in random clusters. The immobilization of VEGF and Ang1 described here can be used to promote vascularization of engineered tissues in future studies.

3.7. Effect of covalently immobilized VEGF and Ang1 on primary rat aortic endothelial cells

Results obtained using primary rat aortic endothelial cells (Figure 7, 8) are consistent with those obtained using H5V endothelial cells. Using primary cells and step immobilization of VEGF and Ang1 in PBS buffer, significantly higher cell numbers and lactate production rates were obtained compared to the corresponding soluble groups (S-VEGF and S-Ang1) after 3 days of cultivation (Figure 7; *t*-tests, $p < 0.0001$ for cell number of both VEGF and Ang1 vs. their corresponding soluble groups; $p = 0.0352$ and $p = 0.0121$ for lactate production rates of VEGF vs. S-VEGF and Ang1 vs.

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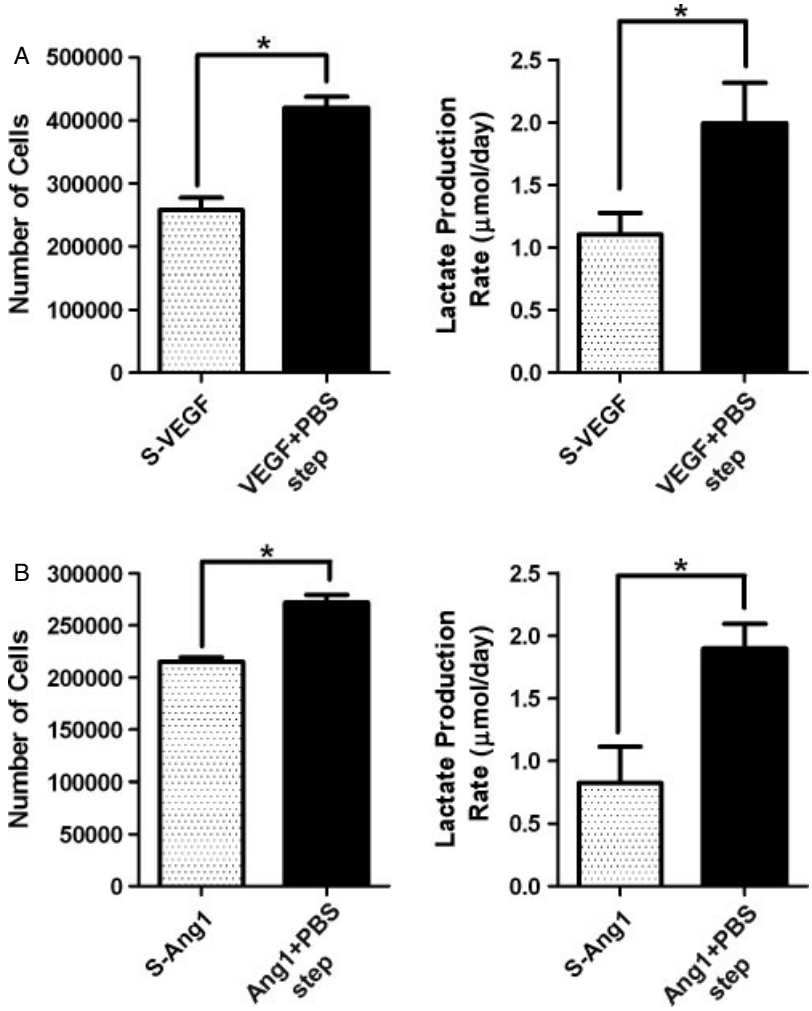

Figure 7. Comparison of soluble and immobilized growth factors using primary endothelial cells. Final cell number and lactate production rate for (A) VEGF + PBS step vs. S-VEGF. (B) Ang1 + PBS step vs. S-Ang1. *Statistically significant difference ($p < 0.05$; *t*-test). S-VEGF, control scaffold placed in culture medium with 50 ng/ml soluble VEGF; VEGF + PBS step, scaffold with immobilized VEGF, where PBS was used as a reaction buffer in step immobilization; S-Ang1, control scaffold placed in culture medium with 50 ng/ml soluble Ang1; Ang1 + PBS step, scaffold with immobilized Ang1, where PBS was used as a reaction buffer in step immobilization

S-Ang1, respectively). Live/dead staining demonstrated tube formation by the primary endothelial cells in the immobilized groups compared to random distribution of cells in the soluble groups (Figure 8). This suggests that primary endothelial cells are also responsive to the covalently immobilized growth factors.

3.8. Probability of interference with receptor binding domains

In this work, EDC chemistry was used to immobilized VEGF and Ang1 onto collagen scaffolds. EDC reacts with carboxyl groups of the first molecule to form an amine-reactive *O*-acylisourea derivative, which is converted into amine-reactive sulpho-NHS esters in the presence of sulpho-NHS (Grabarek and Gergely, 1990). The reactive esters then react with amine groups on the second molecule to form stable amide bonds between proteins.

Immobilization of growth factors can interfere with the receptor binding region of the growth factors if the amino acids from the receptor binding region are involved in the immobilization reaction. To determine the probability of the immobilization reaction interfering with receptor binding, the number of free COOH and/or NH_2 groups in the side chains of amino acids within the receptor binding domain was divided by the total number of amino acids in the receptor binding domain (see Supporting information, Figure S2). This analysis was performed for both VEGF (Table 3) and Ang1 (Table 4).

In step immobilization, when EDC is added first to the collagen scaffold, the carboxyl groups of collagen scaffold are activated and the growth factors most likely provide amine groups. Thus, the interference is due to the probability of amine groups of the receptor binding domain being bound to collagen. When EDC is added to VEGF or Ang1 first, the carboxyl groups of the growth factor are activated and the interference is due to the probability of carboxyl groups of the receptor binding domain being used in binding to collagen.

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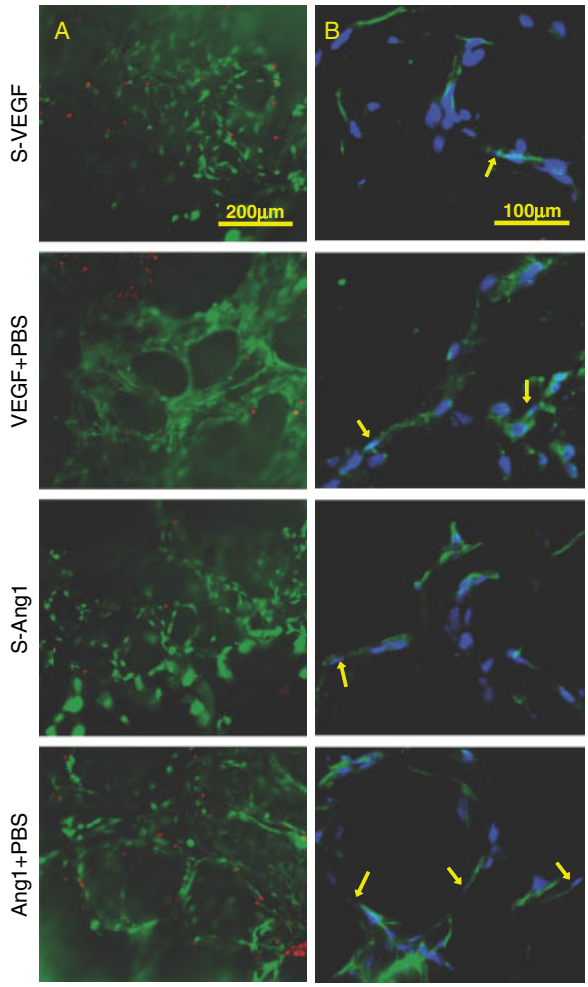

Figure 8. Formation of tube-like structures after 7 days of *in vitro* cultivation of primary rat endothelial cells. A high density of 173 333 cells was initially seeded. (A) Live/dead staining at day 7 (CFDA staining of live cells; PI staining of dead cells). (B) CD31 immunofluorescence staining of frozen sections (elongated phenotype of CD31 positive cells indicated by arrows; cell nuclei stained with Hoechst dye)

Bulk immobilization, which involves adding all reagents together simultaneously, allows growth factors to act as both carboxyl and amine molecules. In this case, interference is the total effect from both probabilities of carboxyl and amine groups in the receptor binding domain being bound to collagen.

Although the cell culture results clearly demonstrate the bioactivity of the immobilized growth factors (Figures 2–5), the probability of interference in the range from 23% for step immobilization to 32–38% for bulk immobilization (Tables 3 and 4) motivates the need for more specific chemistries. For example, copper-free click chemistry suitable for *in vivo* applications has been recently described and may be investigated in future studies (Chang *et al*., 2010).

Besides showing less interference, step immobilization also allows more growth factors to bind to collagen, whereas collagen has more chance of binding to itself in bulk immobilization. In bulk immobilization, the collagen scaffolds are immersed in a solution with both the crosslinkers and the growth factors for a long period (i.e. 4 h), allowing collagen to crosslink to itself. On the other hand, in step immobilization, the scaffolds are first activated with the crosslinker EDC for a short period of time before reacting the activated scaffolds with VEGF or Ang1 without the presence of excess crosslinkers. Although this explains the higher immobilized amount of VEGF in the VEGF + PBS step group compared to its corresponding bulk group (Table 2), the step and bulk groups of VEGF + MES did not show a significant difference in the immobilized VEGF amount. One possible explanation is the change of VEGF conformation under the low pH of MES buffer and, in turn. the lowered ability of VEGF to bind to collagen in both the step and bulk groups.

Table 3. Probability of interference for VEGF immobilization*

VEGF	Carboxyl	Amine	Total
Receptor-binding domain	2	3	5
Entire sequence	25	42	67
Number of amino acids in receptor-binding domain			13
Interference (%)	15.4	23.1	38.5

*Calculated by dividing the number of carboxyl or amine residues in the receptor binding domain by the total number of amino acids in the receptor binding domain, as represented in the Supporting information, Figure S2.

Table 4. Probability of interference for Ang1 immobilization*

Ang1	Carboxyl	Amine	Total
Receptor-binding domain	20	49	69
Entire sequence	60	128	188
Number of amino acids in receptor-binding domain			216
Interference (%)	9.3	22.7	31.9

*Calculated by dividing the number of carboxyl or amine residues in the receptor binding domain by the total number of amino acids in the receptor binding domain, as represented in the Supporting information, Figure S2.

4. Discussion

Covalent attachment of angiogenic growth factors to biomaterials is a useful strategy to develop a matrix with enhanced angiogenic capabilities (Koch *et al*., 2006). This in turn has significant potential applications in the field of tissue engineering. The use of covalently immobilized growth factors in the context of cardiovascular tissue engineering is just emerging. Previously, we have used covalent immobilization of VEGF alone to promote proliferation of D4T endothelial cells (Shen *et al*., 2008). We have also improved proliferation and tube formation by H5V endothelial cells, using co-immobilization of VEGF and Ang1 (Chiu and Radisic, 2010). However, we have not systematically compared the conditions for growth factor immobilization, a topic of the current study. In our previous work, we only used endothelial cells lines (D4T and H5V) whereas primary endothelial cell were

DOI: 10.1002/term

used here. In this study, we chose the EDC chemistry (Shen *et al*., 2008) because it is a simple and water-based technique. Washing after the immobilization procedure leaves the collagen scaffold free of any excess crosslinking reagents and byproducts.

EDC is active in the pH range 4.5–7.5. Different reaction buffers were previously used for EDC chemistry. For example, Vepari and Kaplan used 0.1 M MES solution with 0.9% sodium chloride, pH 5.6, as the reaction buffer to minimize horseradish peroxidase (HRP) non-specific adsorption on the protein scaffold (Vepari and Kaplan, 2006). Wissink *et al*. (2000) carried out the EDC chemistry in 0.05 M MES buffer. MES buffer was used to minimize hydrolysis of EDC (Wissink *et al*., 2000). Lee *et al*. (2009) used 200 mM phosphate buffer at pH 8 for surface activation and aptamer immobilization on carboxylated Si nanowires by EDC chemistry.

In this study, VEGF and Ang1 immobilized in PBS buffer led to increased cell proliferation and metabolism within collagen scaffolds compared to the use of distilled water and MES buffer (Figures 2, 3). Changes in pH and salt concentration can alter the electrostatic interactions between amino acids within the protein, thus affecting the conformation and activity of the protein. Different conformations allow different amino acids in the protein to be accessible to reactions and receptor binding. In this particular study, conformational change can affect (a) the ability of VEGF or Ang1 to bind to collagen and (b) the bioactivity of immobilized VEGF or Ang1.

Varying the pH of the EDC reaction was previously shown to result in different immobilization yields (Olde Damink *et al*., 1996). The VEGF + PBS group had a significantly higher amount of immobilized VEGF compared to both the VEGF + DW and VEGF + MES groups. PBS and distilled water have neutral pH values, while MES buffer is acidic. PBS contains 144 mg/l potassium phosphate monobasic, 9000 mg/l sodium chloride and 795 mg/l sodium phosphate dibasic. These salts do not change the pH of the solution, but have a dehydrating action and affect the ionic strength of the solution. Salts can affect hydrogen bonds between amino acids within the protein. Distilled water has no inorganic salt content. It may be due to the inorganic salts present in PBS that VEGF has a more desired conformation for EDC chemistry in order to bind to collagen.

Moreover, the function of a protein is determined by its conformation. Previously, it was found that VEGF-165 did not phosphorylate Erk1/2 at low pH (pH 5.5), while it stimulated Erk1/2 activation with peak activation time of 5 min at pH 7.5 and 10 min at pH 6.5 (Goerges and Nugent, 2003). Increased pH also improved Erk1/2 activation for VEGF-121. VEGF-induced Erk1/2 activation is involved in proliferation and migration of endothelial cells (Kim *et al*., 2002). VEGF was thought to be at its stored state at the lower pH of 5.5 (Goerges and Nugent, 2003). Interestingly, the lowered activity of VEGF was not permanent, and the activity was recovered after washing at pH 7.5 (Goerges and Nugent, 2003). In the current study, VEGF was immobilized onto the collagen scaffold at a lower pH for the VEGF + MES group compared to the VEGF + PBS group. The covalently immobilized VEGF was likely hindered in its ability to recover activity once immobilized to the scaffold at its stored state.

The purpose of comparing two cell lines at different passage numbers was to demonstrate the limitations of using cell lines, especially at high passage numbers, for research exploring the effect of growth factors. Although cell lines are a convenient source, due to their availability and high proliferation rates, confirmation of the effect of immobilized growth factors on the primary cells was required, since primary cells are involved in ultimate tissue engineering applications, particularly in the promotion of *in vivo* angiogenesis. In this study, primary rat aortic endothelial cells demonstrated an increase in the final cell number and the lactate production rate when cultivated on scaffolds with immobilized growth factors compared to the cultivation with soluble growth factors (Figure 7). These cells were also able to form tube-like structures when cultivated on scaffolds with covalently immobilized VEGF and Ang1 (Figure 8).

The Ang1 + PBS group showed increased cell growth compared to the Ang1 + DW group (Figure 3A), although similar amounts of Ang1 were immobilized (Table 2), as determined by ELISA. For example, the Ang1 + DW group did not show significantly higher cell number compared to PBS control, while the Ang1 + PBS group did. There may be discrepancies between the parts of Ang1 molecules recognized by the ELISA antibody and the cell receptors that are responsible for the observed results. The effect of using different immobilization buffers on cell growth is probably due to a combination of both the amount of immobilized growth factors and the protein conformation that the growth factors show when immobilized.

Step immobilization was the preferred method of immobilization compared to bulk immobilization, according to cell proliferation results (Figure 4). This may be due to two phenomena. First, there is a lower probability of interference for the receptor binding domain in step immobilization (Tables 3, 4). This means there is less chance of the receptor-binding domain being involved in the immobilization reaction, thus allowing the domain to remain bioactive to cultivated cells. Second, scaffolds for bulk immobilization were immersed in a solution with EDC for a longer time than scaffolds for step immobilization. There is a greater chance that the collagen molecules in these scaffolds start to bind to themselves, which is a reaction competing with the immobilization reaction. The result is a lower amount of growth factors immobilized onto the scaffolds (Table 2). There was an increased amount of immobilized VEGF for the VEGF + PBS step group compared to the VEGF + PBS bulk group ($p = 0.020$) (Table 2). However, there was no difference for the VEGF + MES step and bulk groups ($p = 0.7239$), likely since VEGF changed conformation under lower pH (as discussed above) and had decreased ability to bind to collagen in both step and bulk immobilization.

Collagen scaffolds with immobilized growth factors mimic the *in vivo* microenvironment by promoting a local

DOI: 10.1002/term

regulation and guidance of cell activity (Boontheekul and Mooney, 2003). Multiple growth factors can be co-localized to elicit the appropriate sequence of events. Specifically, immobilized VEGF and Ang1 provide local cues to better guide cell proliferation and tube formation within the scaffold, as compared to soluble growth factors. The improved cell proliferation and metabolism by immobilized growth factors compared to soluble growth factors is consistent with the expected improvement in stability and extended signalling (Fan *et al*., 2007; Ito, 2008). In addition, covalent immobilization may increase the local concentration of the growth factors at the scaffold pore walls, which are the attachment sites for the cells. More importantly, growth factor immobilization can render bioactivity to three-dimensional biomaterials, natural or synthetic, and promote desired cell–material interactions. This is especially important for tissue engineering.

The main novelty of the current study is the demonstration that primary cells, specifically rat aortic endothelial cells, respond to immobilized growth factors by enhanced proliferation and tube formation. As such, this approach may be a useful platform for engineering vascularized tissues *in vitro* and *in vivo*. Another important outcome of this work is confirmation that step immobilization and utilization of PBS as a buffer for both reaction steps improve the bio-availability of the growth factors, as demonstrated by the cell proliferation rates. In our future work, the long-term *in vivo* effects of scaffolds with immobilized angiogenic factors will be explored.

5. Conclusion

Different conditions for the immobilization of VEGF and Ang1, using EDC chemistry on collagen scaffolds, were tested, and the outcomes were evaluated by the ability of the scaffolds to promote endothelial cell proliferation and metabolism, as well as the amount of immobilized growth factors determined by ELISA. Our results indicate that: (a) PBS is the preferred buffer for the immobilization process, as compared to distilled water and MES buffer; (b) step immobilization is a preferred method of immobilization, compared to bulk immobilization; (c) low passage H5V endothelial cells are more useful in studying the effect of immobilized growth factors, since they were more responsive to VEGF and Ang1, as compared to D4T endothelial cells; and (d) primary rat aortic endothelial cells responded to the immobilized growth factors by proliferation and tube formation. The covalent immobilization of VEGF and Ang1 to three-dimensional scaffolds may represent a novel method for the vascularization of engineered tissues.

Acknowledgements

This study was supported by a Canadian Institutes of Health Research (CIHR) grant for the Cardiac Regeneration Project (CARE project), a Heart and Stroke Foundation Grant-in-Aid (No. NA6077), and a NSERC Alexander Graham Bell Canada Graduate Scholarship (to L.L.Y.C.).

Supporting information on the internet

The following supporting information may be found in the online version of this article:

Figure S1. Effect of VEGF and Ang1 on different endothelial cell lines as 2D monolayers in a 24-well plate

Figure S2. Amino acid sequences of VEGF and Ang1. (A) Mouse VEGF. (B) Human Ang1

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