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Supercritical carbon dioxide generated vascular endothelial growth factor encapsulated poly(DL-lactic acid) scaffolds induce angiogenesis *in vitro*

J.M. Kanczler [a], J. Barry [b], P. Ginty [c], S.M. Howdle [d], K.M. Shakesheff [c], R.O.C. Oreffo [a,*]

[a] *Bone and Joint Research Group, Developmental Origins of Health and Disease, University of Southampton, Southampton SO166YD, UK*
[b] *Baxter BioScience, Vienna, Austria*
[c] *School of Pharmacy, University of Nottingham, Nottingham NG72RD, UK*
[d] *School of Chemistry, University of Nottingham, Nottingham NG72RD, UK*

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Abstract

The ability to deliver, over time, biologically active vascular endothelial growth factor-165 (VEGF) through tailored designed scaffolds offers tremendous therapeutic opportunities to tissue-engineered therapies. Porous biodegradable poly(DL-lactic) acid (PLA) scaffolds encapsulating VEGF have been generated using supercritical CO_2 ($scCO_2$) and the kinetic release and angiogenic activity of these scaffolds examined *in vitro* and in an *ex vivo* chick chorioallantoic membrane (CAM) angiogenesis model. After processing through $scCO_2$, VEGF maintained its angiogenic activity as assessed by increased tubule formation of human umbilical vein endothelial cells (HUVEC) cultured on Matrigel (VEGF = 1937 ± 205 μm; $scCO_2$-VEGF = 2085 ± 234 μm; control = 1237 ± 179 μm). VEGF release kinetics from $scCO_2$-VEGF incorporated PLA monolith scaffolds showed a cumulative release of VEGF (2837 ± 761 ρg/ml) over a 21 day period in culture. In addition, VEGF encapsulated PLA scaffolds increased the blood vessel network in the CAM compared to controls; control, 24.8 ± 9.6; VEGF/PLA, 44.1 ± 12.1 (vessels/field). These studies demonstrate that the controlled release of growth factors encapsulated into three-dimensional PLA scaffolds can actively stimulate the rapid development of therapeutic neovascularisation to regenerate or engineer tissues.

Keywords: Angiogenesis; Tissue engineering; HUVEC; Scaffold; Polymer

Recent tissue engineering advances have led to the possibility of successful repair and restoration of function in damaged or diseased tissues [1–3]. The tissue engineering approach uses an appropriate cell source and a biocompatible/biodegradable scaffold to produce constructs that structurally and functionally mimic target tissue. To date, significant success has been achieved, particularly in areas of skin, bone and cartilage regeneration. The challenges that arise with these new constructs are that most tissue and organs are multiphasic in nature and contain multiple cell types. In addition, the regeneration of tissue is regulated by the interaction of various tissue inductive growth factors, the formation of a vascular supply to support the metabolic needs of the developing tissue mass, and a cell population capable of responding to the chemical cues to grow new tissue. Therefore, biomimetic scaffolds should be able to support multilineage cell types. However, a key issue surrounding the development and maintenance of newly engineered, three-dimensional tissue constructs remains the development of a vascular supply to provide oxygen and nutrients that facilitates growth, differentiation, and the functionality of the tissue once fully developed.

Strategies augmenting the development of new blood vessels are paramount for virtually any tissue undergoing

* Corresponding author. Fax: +44 2380796141.
E-mail address: roco@soton.ac.uk (R.O.C. Oreffo).
URL: WWW.mesenchymalstemcells.org (R.O.C. Oreffo).

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repair or reconstruction. A number of groups have shown that administering regulators of angiogenesis such as VEGF or bFGF can stimulate resident endothelial progenitor cells to differentiate into mature endothelial cells with the potential to form new blood vessels [4,5]. VEGF, a 34–46 kDa homodimeric glycoprotein, which acts through its high infinity receptor VEGF-R2 (Flk-1/KDR), is one of the most important signalling molecules involved in early blood vessel development. Although the predominant action of VEGF has been demonstrated to reside on endothelial cells, it also binds to VEGF receptors expressed on monocytes, neurons, and osteoblasts [6,7]. Apart from the angiogenic effect of VEGF, it has been shown to induce haematopoietic stem cells recruitment from bone marrow, monocyte chemoattraction, neuronal protection, and bone formation [6–9]. The multiple roles of VEGF in bone remodelling is evidenced by studies implicating VEGF in fracture healing, blood vessel formation in endochondral bone formation, chondrocyte apoptosis, cartilage remodelling and endochondral growth plate ossification [10,11].

The ability to deliver, over time, biologically active VEGF through tailored designed scaffolds to sites of tissue regeneration offers tremendous therapeutic opportunities in a variety of tissue engineered scaffolds. The $scCO_2$ fluid mixing technology is a process that is performed at or below physiological temperatures without any harsh solvents, allowing bioactive factors to be encapsulated into biodegradable polymer scaffolds. The advantages of this process allow growth factors to be retained within scaffold constructs without compromising the activity [12]. Previously, we have shown that bioactive factors incorporated into biodegradable poly(DL-lactic acid) scaffolds by $scCO_2$ fluid mixing technology retain their activity when released from the scaffold process [13]. Such constructs were capable of releasing active BMP-2 and, significantly, promoting human bone marrow stromal cell differentiation and bone formation *in vitro* and *in vivo* [14].

In this study, we have set out to generate porous biodegradable scaffolds encapsulating VEGF using $scCO_2$ mixing technology and to examine the kinetic release and angiogenic activity of VEGF from these scaffolds *in vitro* and in an *ex vivo* chick chorioallantoic membrane angiogenesis model as a first step in the creation of constructs with angiogenic potential for application in tissue regeneration strategies.

Materials and methods

Recombinant human vascular endothelial growth factor-165 (VEGF) was purchased from Tebu-bio, Peterborough, UK. Endothelial cell culture growth medium and human vein endothelial cells (HUVECs) were from Promocell, Heidelberg, Germany. Human $VEGF_{165}$ Elisa Kit was from Chemicon, Hampshire, UK. Growth factor-reduced Matrigel® was purchased from BD Biosciences, Oxford, UK. Cell tracker green, ethidium homodimer-1, and DAPI were purchased from Invitrogen Ltd., Paisley, UK. All other materials were obtained form Sigma–Aldrich, Poole, UK unless stated.

VEGF incorporation into poly(DL-lactic acid) (PLA) scaffolds using supercritical CO_2 mixing technology. Ten micrograms of VEGF was resuspended in 120 μl of dH_2O. Twenty microliters of the reconstituted VEGF was pipetted onto 0.13 g of poly(DL-lactic acid) (MW 65,000) and mixed in Teflon moulds (10 mm deep and 10 mm in diameter). The VEGF/PLA mix was then lyophilised overnight in a freeze drier. The PLA was then foamed using $scCO_2$ to create the encapsulated growth factor PLA scaffolds as previously described by Quirk et al. and Howdle et al. [12,13]. For this, the polymer was plasticised at 35 °C under a pressure of 17.32 MPa. Upon release of the pressure, the pores are formed and fixed in the polymer structure thus creating a porous monolith composite with the encapsulate VEGF.

Cell culture of HUVEC on PLA scaffolds. HUVECs were expanded in endothelial cell growth medium, supplemented with 10% fetal calf serum (FCS). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2/balanced air and culture medium was changed every 48 h. HUVECs were identified by means of fluorescence microscopy using antibodies against von Willebrand factor (vWF). Cells were fixed in methanol/acetone (1:1), washed and incubated with a polyclonal rabbit anti-human vWF, 1:150 (Dako, Denmark). Cells were washed and incubated with donkey anti-rabbit IgG-FITC, 1:200 (Santa Cruz, USA) and then incubated with a nuclear stain, DAPI. Fluorescence images were taken using an Axiovert-200 fluorescent microscope (Carl Zeiss, UK). The PLA scaffolds were seeded with HUVEC (5×10^5 cells) and cultured for 28 days. To test for cell viability, scaffolds seeded with HUVEC (24 h and 28 days) were washed in 1× PBS and incubated with 10 μg/ml cell tracker green and 5 μg/ml ethidium homodimer-1 (CTG/EH-1) in tissue culture medium (TCM) for 1.5 h at 37 °C to label viable and necrotic cells, respectively. Scaffolds and cells were then incubated in TCM without CTG/EH-1 for 45 min before fixing in 95% ethanol. Cells were washed with 1× PBS and images of fluorescently labelled cells were captured using the Carl Zeiss Axiovision-3.1 software package.

Determining the levels and bioactivity of $scCO_2$ treated VEGF. Lyophilised VEGF (10 μg) was placed through the $scCO_2$ process and was analysed to determine loss factors during the process compared to that of untreated VEGF. Quantification of the levels of VEGF *in vitro* was determined by an ELISA kit according to the manufacturer's instructions. Both VEGF lyophilised powder and the $scCO_2$-VEGF was resuspended in 1× PBS. Ten and one nanograms per milliliter of control VEGF and $scCO_2$-VEGF were assayed for levels of VEGF. Activity of the $scCO_2$ treated VEGF compared to that of untreated VEGF was assessed by the Matrigel tube formation assay [15]. *In vitro* formation of tubular structures was observed on growth factor-depleted Matrigel diluted 1:1 with MEM-199 in 48-well plates. 5×10^4 cells were seeded onto the Matrigel-coated wells in culture medium supplemented with 10% FCS. Following cell adhesion to the Matrigel (2 h at 37 °C), the FCS containing media was removed and the cells were washed with 1× PBS before the addition of the VEGF (10 ng/ml), $scCO_2$-VEGF (10 ng/ml) or the vehicle alone in media containing 0.2% bovine serum albumin. After 16 h of incubation the cells were observed with a Zeiss inverted microscope and experimental results (tubule length formation and number of networks) were recorded using an image analyser software package, Axiovision 3.1.

Determining the kinetic release and bioactivity of $scCO_2$-VEGF incorporated PLA scaffolds. VEGF release kinetics from the monolith $scCO_2$-PLA/VEGF scaffolds was determined using an ELISA kit as detailed above. PLA (mean weight $= 30.0 \pm 0.8$ mg) and $scCO_2$-PLA/VEGF (mean weight $= 29.6 \pm 3$ mg) scaffolds were placed in 24-well plates and incubated with 1× PBS (pH 7.4). The supernatants were collected on days 4, 7, 14, and 21 and stored at −80 °C until assayed by the VEGF-ELISA.

The CAM assay was used to determine the activity of VEGF incorporated into the PLA scaffold. Fertile chick eggs were obtained from PD Hook Hatcheries, Oxfordshire, UK and incubated for 10 days in a multihatch incubator (Brinsea Products, Sandford, UK) at 37 °C in a humidified atmosphere. At day 10 a square window was created and the control PLA scaffold and VEGF encapsulated scaffolds were placed on top of the chorioallantoic membrane of the chick egg. The shell window was resealed and the egg was incubated without turning for a further 4 days. The CAM and scaffold were removed and photographed with a stereomicroscope with a digital camera (Canon Powershot G2) attached

and the number of blood vessels around the explants were calculated to determine the increase in angiogenesis. The chorioallantoic membranes and scaffold explants were then fixed in 95% ethanol, dehydrated in a graded series of alcohols and embedded in low-melting-point paraffin wax. 6 μm sections were prepared for histology and stained for Alcian Blue and Sirius Red.

Statistical analysis. The statistical analyses were performed using GraphPad Prism software. Differences among groups were determined either by Student's *t* tests or one-way ANOVA with a post-Dunnett's test according to experimental design and were considered to be significantly different if $P < 0.05$.

Results

Adhesion viability of HUVEC on PLA scaffolds

HUVECs, were characterised by their typical cobblestone morphology and positive expression for vWF (Fig. 1A and B). Following seeding onto PLA scaffolds, extensive cell adhesion could be observed within 24 h and good cell viability as evidenced by strong expression of the cell viability marker, cell tracker green and negligible ethidium homodimer-1 expression, a marker for cell necrosis (Fig. 1C). In extended culture, over a period of 28 days, viable HUVECs were still observed adhered and proliferating on the PLA scaffolds. Some cell death was noted with the positive expression of ethidium homodimer-1 (EH-1) in some of the HUVEC adhered to the PLA scaffolds (Fig. 1D).

Concentration levels and bioactivity of VEGF after $scCO_2$ processing

A VEGF specific ELISA was used to quantitate VEGF levels after processing through $scCO_2$ compared to unprocessed VEGF controls. Following $scCO_2$ processing of VEGF the reconstituted form of $scCO_2$ treated VEGF (10 and 1 ng/ml) showed no significant decrease in VEGF levels compared to the control form of VEGF (10 and 1 ng/ml) (Fig. 2A). Critical in any release design strategy for a given growth factor will be an ability to demonstrate cumulative temporal release. VEGF release kinetics from $scCO_2$ VEGF incorporated PLA monolith scaffolds showed a cumulative release of 2837 ± 761 ρg/ml amount of VEGF (0.78% ratio release of encapsulated VEGF) over a 21 day period in culture (Fig. 2B).

Following confirmation of negligible loss of VEGF content as a consequence of the $scCO_2$ process, we determined the level of activity of the $scCO_2$-VEGF by their ability to stimulate HUVEC cultured on three-dimensional reduced growth factor Matrigel matrix. Reconstituted $scCO_2$-VEGF retained its bioactivity, as evidenced by the ability to induce endothelial cell tube formation in HUVEC on Matrigel compared to control and untreated VEGF (Fig. 3A–C). Untreated VEGF (10 ng/ml) as well as the $scCO_2$ treated VEGF (10 ng/ml) significantly increased the tubule length (μm/field). Thus we observed a significant

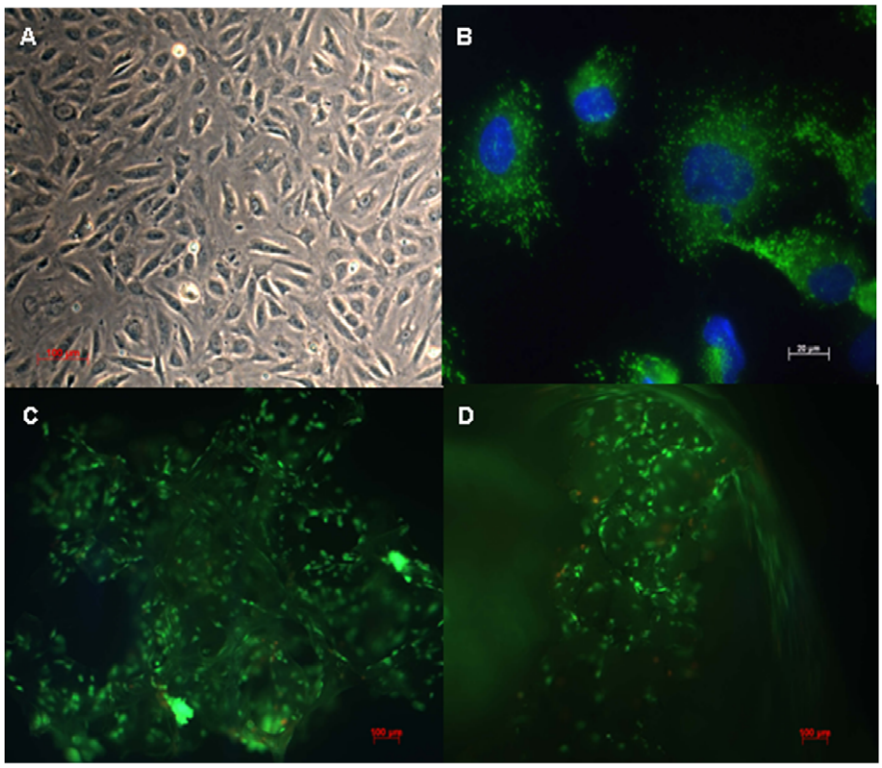

Fig. 1. HUVECs grown in culture with typical cobblestone morphology (A) and positive immunostaining for vWF with DAPI nuclear counterstain (B). Live (green)/dead (orange) cell tracker green/ethidium homodimer-1 staining of HUVEC cultured on PLA scaffold for 24 h (C) and 28 days (D), respectively.

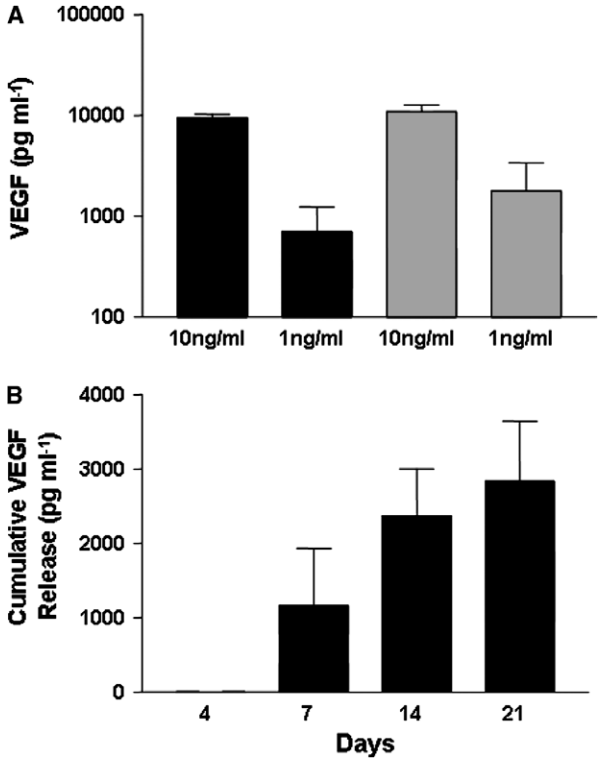

Fig. 2. Analysis of VEGF by ELISA, VEGF levels after treatment with $scCO_2$ quantified by ELISA (A), control VEGF (■); $scCO_2$ treated VEGF (■). *In vitro* release kinetics of VEGF from PLA encapsulated VEGF scaffolds as determined by ELISA against human VEGF (B). Data plots represent means ± SD, $n = 3$ experiments.

increase ($P < 0.001$) in tubule length in VEGF (1937 ± 205) and $scCO_2$ treated VEGF (2085 ± 234) in comparison to control (1237 ± 179) (Fig. 3D). Similarly, quantification of enclosed endothelial cell networks (networks/field) showed a significant increase ($P < 0.05$) in three independent experiments in VEGF (13.3 ± 1.8); $scCO_2$ treated VEGF (15.2 ± 3) compared to control (6.1 ± 1.1) (Fig. 3E).

Induction of angiogenesis

To validate the angiogenic inductive capacity of the $scCO_2$ VEGF/PLA scaffolds, samples were cultured on chorioallantoic membrane cultures. Macroscopic observations of the chorioallantoic membrane incubated with PLA scaffolds (Fig. 4A) or the $scCO_2$ VEGF/PLA scaffolds (Fig. 4B) showed that after 4 days incubation the scaffolds were enveloped in the allantoic membrane. As expected the VEGF/PLA scaffolds implanted into the CAM significantly increased the number of vessels surrounding the scaffolds compared to control PLA scaffold. Control = 24.8 ± 9.6; VEGF/PLA = 44.1 ± 12.1 (Fig. 4E). Histological analysis confirmed an extensive increase in blood vessel numbers surrounding the VEGF encapsulated PLA scaffolds compared to the control scaffolds (Fig. 4C and D).

Discussion

The development of intricate, three-dimensional engineered tissue will depend upon the ability to organise a multitude of signals into a coordinated, time-dependent regenerative vehicle. Thus, the ability to deliver specific growth factors, through controlled-release devices, to cells within the regenerating tissue/scaffold structure to develop vascular networks *in vivo*, offers significant hope for patients with various ischemic diseases [16–18]. In these studies we demonstrate that powdered PLA and VEGF can be combined using a $scCO_2$-mixing technology to produce encapsulated VEGF into a PLA scaffold. In this procedure, the efficient processing of the liquefied polymer in $scCO_2$ at near physiological temperatures results in a homogeneous distribution of the bioactive factor throughout the polymer matrix, creating a porous monolith scaffold with encapsulated active VEGF. The material system developed for our studies are based on poly(lactide)s [i.e. poly(lactic acid) (PLA)], a widely used biomedical polymer with FDA approval for use in humans that has been safely used as a biodegradable scaffold for the regeneration of many tissues [13,21–25].

Recently, a combined growth factor delivery system of VEGF and PDGF released in an orchestrated spatio-temporal manner from a polymeric structure were able to generate mature vessels [19]. Also, Huang et al. [20] were able to deliver a combination of VEGF and BMP-4 to increase blood vessel formation and enhance bone regeneration.

An area where growth factor release from biodegradable scaffolds has made significant progress is within the field of bone regeneration. Previously, Yang et al., demonstrated that human recombinant Bone Morphogenic Protein-2 (rhBMP-2) could be encapsulated into scaffolds and promote human bone marrow stromal cell adhesion, proliferation, and differentiation with extensive evidence of new bone formation. This release of a growth factor BMP-2 and TGF B1 was also shown to be released from CaP cement and Titanium-fiber mesh scaffolds compared with non-loaded implants [26]. Thus while originally, this technology was used to develop scaffolds for therapeutic intervention in a variety of muscoskeletal diseases by release of an osteogenic agonist, BMP-2 to promote osteogenesis, the development of a vascular supply is of paramount importance in maintaining the growth of the scaffold and regenerated tissue. These studies show that VEGF can withstand the supercritical CO_2 process and maintain its activity by stimulating the angiogenic response of HUVEC *in vitro* as well as promoting vessel formation by the release of VEGF from PLA scaffolds in an *ex vivo* chorioallantoic membrane assay. It has recently been shown that VEGF can be released from a porous poly(lactic-co-glycolide) scaffold [1]. The current studies demonstrate a similar result profile although it should be noted our release kinetics were slightly less than that stated by Murphy et al. Following implantation of the VEGF/PLA scaffold into the chorioallantoic membrane there was an increase in the number of

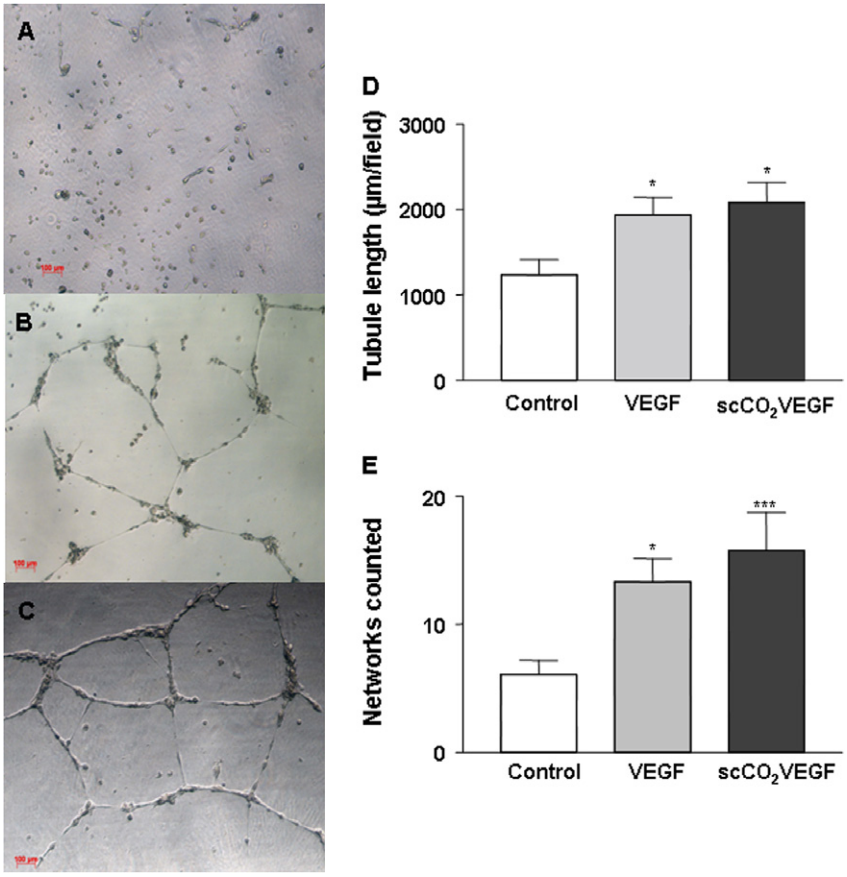

Fig. 3. Representative images demonstrating that $scCO_2$ treated VEGF can induce network tube formation of HUVEC on a growth factor reduced Matrigel basement membrane compared to that of untreated VEGF; control (A) 10 ng/ml VEGF (B) 10 ng/ml $scCO_2$/VEGF (C). $scCO_2$/VEGF showed elevated increases in HUVECs tubule length (D) and the number of tubule networks formed (E) showing no loss of VEGF activity. Results represent means ± SEM calculated from three independent studies ($^{***}P < 0.001$; $^{*}P < 0.05$ vs. unstimulated HUVEC).

blood vessels generated in close proximity to the scaffold. This is comparable to the report of Murphy et al. who demonstrated blood vessel growth in scaffolds implanted into the rat cranium defect model. Our preliminary work with VEGF/PLA scaffolds inserted into a critical femur defect model showed an increase in bone formation (unpublished observations).

Although, VEGF is a predominant factor in angiogenesis, it also plays a critical role in the skeletal growth and endochondral bone formation. The absence of vascularisation is a major determinant of failure of bone healing in cases of non-union fractures. In addition, Gerber et al. have shown that hypertrophic chondrocytes express VEGF mRNA in the epiphyseal growth plate, signifying that a VEGF gradient is needed for cartilage invasion by metaphyseal blood vessels and growth plate morphogenesis [11]. During endochondral bone formation VEGF coordinates blood vessel formation, chondrocyte apoptosis, cartilage remodelling, and the ossification of the endochondral growth plate [10,11]. Within the first 11 days of bone healing osteoprogenitor cells, chondrocytes, and osteoblasts express VEGF [27]. VEGF plays a key role in the cross talk between endothelial cells and osteoblasts during bone formation and fracture healing, where VEGF increases osteoblast differentiation [28]. A number of studies have also indicated that inhibition of VEGF decreases blood vessel invasion, impairs callus mineralization and trabecular bone healing [10,11,29]. Hence, the development of a vascular supply in fracture healing and osteogenesis maybe amplified by endogenous production of angiogenic and osteogenic mediators [30]. In support of such a strategy, a number of recent studies have shown that the combination of angiogenic and osteogenic factors can stimulate bone healing and regeneration [1,8,9].

Similar to normal tissue, engineered tissue requires blood vessels to grow and to remain viable. A plethora of complex three-dimensional-engineered tissues have been developed over the last few years, however, the main obstacle in the success of these tissues is the need for adequate vascularisation. Vascularisation of tissue constructs *in vitro* may help in maintaining cell viability during tissue growth and induce the vascular structural organisation upon implantation. To address this problem, some success has been achieved in developing an artificial vasculature by isolating and growing endothelial cells on polymer scaffolds [31,32] or by using endothelial progenitor cells as a source for engineering blood vessels [33]. Furthermore, co-implantation of endothelial cells and mesenchymal cells form

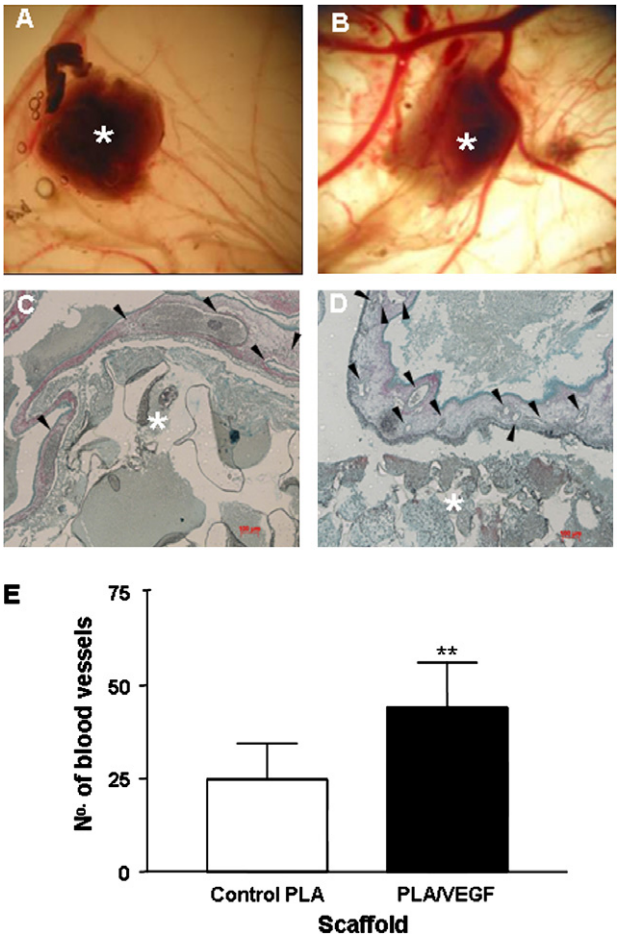

Fig. 4. Macroscopic evaluation of the angiogenic activity of PLA scaffolds (*) with and without encapsulated VEGF in the CAM evaluated as the number of blood vessels surrounding an implant, PLA scaffold (A), PLA encapsulated VEGF scaffold (B). Original magnification: (A and B) 40×. Histological analysis of the scaffolds incorporated into the CAM showed a highly vascularised tissue surrounding the scaffolds with more vessels (arrowheads) adjacent to the VEGF encapsulated PLA scaffold (D) than that of control PLA scaffold (C). Number of vessels surrounding the VEGF encapsulated capsule was significantly increased compared to the controls (E). Control = 24.8 ± 9.6; VEGF/PLA = 44.1 ± 12.1. $^{**}P < 0.01$ vs. PLA scaffold alone. Data represent mean number of vessels ±SD ($n = 9$ eggs/group).

stable networks of vessels has been reported [34], while a combination of myoblasts, embryogenic fibroblasts and endothelial cells grown on PLLA:PLGA scaffolds have improved the vascularisation in skeletal muscle tissue [3]. In the latter case, the addition of embryonic fibroblasts increased levels of VEGF and hence promoted endothelial cell formation [3].

In conclusion, the current studies demonstrate that growth factors encapsulated into PLA scaffolds can be released in an active form to stimulate angiogenesis. The development of biomimetic scaffolds which can release growth factors stimulating a resident population of stem and progenitor cells may mimic the repair and formation of new tissue structures such as bone. The ability to release factors such as VEGF in combination with osteogenic factors such as BMPs, is currently under investigation in our laboratories and should encourage the development of a vasculature into the new generation of scaffolds, to promote and maintain the neo tissue development *in situ* with exciting therapeutic potentials therein for tissue regeneration strategies.

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