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Enhancing Angiogenesis Alleviates Hypoxia and Improves Engraftment of Enteric Cells in Polycaprolactone Scaffolds

Shivani Singh, Ph.D.¹, Benjamin M. Wu, D.D.S., Ph.D.^{1,2}, and James C.Y. Dunn, M.D., Ph.D.^{1,3}

¹Department of Bioengineering, University of California, Los Angeles, CA, 90095, USA

²Department of Material Science & Engineering, University of California, Los Angeles, CA, 90095, USA

³Department of Surgery, University of California, Los Angeles, CA, 90095, USA

Abstract

We examined whether expediting angiogenesis in porous polycaprolactone (PCL) scaffolds could reduce hypoxia and consequently improve the survival of transplanted enteric cells. To accelerate angiogenesis, we delivered vascular endothelial growth factor (VEGF) using PCL scaffolds with surface cross-linked heparin. The scaffold fabrication and characterization has been reported in our previous study. Enteric cells, isolated from intestinal tissue of neonatal mice and expanded *in vitro* for 10 days, exhibited high expression levels for contractile protein alpha-smooth muscle actin and desmin. The cultured enteric cells were seeded in scaffolds and were implanted subcutaneously in immunodeficient mice for 7 and 14 days. At day 7, the heparin-modified PCL scaffolds with VEGF exhibited significantly increased angiogenesis and engraftment of enteric cells, with a simultaneous reduction in hypoxia. At day 14, the blood vessels grew across the entire thickness of the scaffold and resulted in significantly diminished hypoxic environment; however, the transplanted cell density did not increase further. In conclusion, the enhancement of angiogenesis reduced cellular hypoxia and improved engraftment of enteric cells.

1. INTRODUCTION

In the absence of blood vessels, the diffusion of nutrients and cell proliferation is restricted to a depth of few hundred micrometers along the edges in tissue-engineered scaffolds (Volkmer *et al.*, 2008; Brown *et al.*, 2007; Dunn *et al.*, 2006). Rapid formation of blood vessels is essential to transport nutrients, alleviate hypoxia and achieve uniform cell engraftment in the scaffold. The in-growth of host vessels can be improved by the sustained delivery of angiogenic growth factors (Richardson *et al.*, 2001; Zisch *et al.*, 2003; Freeman and Cohen, 2009; Chiu and Radisic, 2010; Phelps *et al.*, 2010; Sun *et al.*, 2011).

Scaffolds with immobilized heparin have been used for sustained delivery of growth factors to enhance angiogenesis and tissue regeneration (Steffens *et al.* 2004; Nillesen *et al.*, 2007; Jeon *et al.* 2007; Jiang *et al.* 2010; Im and Lee, 2010). Heparin is a widely distributed polysaccharide in the extracellular matrix that electrostatically binds several growth factors while protecting them against enzymatic and thermal degradation (Klagsbrun, 1992; Brandner *et al.* 2006). The incorporation of heparin in the scaffolds has demonstrated ability

Full Address of the Corresponding Author: James C.Y. Dunn, Box 709818, 10833 Le Conte Avenue, Los Angeles, CA 90095, USA. Tel.: 310-267-2746, fax: 310-206-1120, jdunn@mednet.ucla.edu.

Disclosure Statement

No competing financial interests exist.

to maintain culture of various cell types. Hepatocytes cultured in heparin-derivatized polystyrene (Gutsche *et al.*, 1996), polyethylene glycol (PEG) (Kim *et al.* 2010) and alginate-chitosan (Seo *et al.* 2006) scaffolds exhibits enhanced proliferation and function *in vitro*. Heparin-PEG hydrogel supported the differentiation of the transplanted fibrochondrocytes *in vivo* (Lee *et al.*, 2011).

Investigators have shown that accelerating angiogenesis contributes to improvement in the engraftment of cells. The delivery of angiogenic growth factors increased vascularization and survival of the transplanted hepatocytes (Smith *et al.* 2004; Kedem *et al.* 2005; Lee *et al.* 2002) and myoblasts (Bouchentouf *et al.* 2008). Scaffolds coated with heparin-peptide amphiphile, delivering vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), improved angiogenesis and islet transplantation simultaneously (Rajangam *et al.* 2006). Similarly, delivery of bFGF using heparin- alginate hydrogel promoted nerve regeneration and angiogenesis (Ohta *et al.* 2004).

Our group has recently shown that VEGF delivered by porous polycaprolactone (PCL) scaffolds with surface cross-linked heparin exhibits sustained VEGF release *in vitro* and induces rapid angiogenesis *in vivo* (Singh *et al.* 2011). However, these studies utilized acellular scaffolds to deliver VEGF. In this study, our goal was to explore potential of the heparin-modified PCL scaffolds as delivery vehicle for VEGF and enteric cells. The scaffold fabrication and characterization has been reported in our previous study (Singh *et al.* 2011). We hypothesized that delivery of VEGF from the heparin-immobilized scaffold will accelerate angiogenesis, alleviate hypoxia and improve survival of the transplanted enteric cells.

2. MATERIALS AND METHODS

2.1. Animals

To track the *in vivo* survival of the transplanted enteric cells, transgenic mice strain FVB.Cg-Tg (GFPU) 5 Nagy/J (Jackson Laboratory, Bar Harbor, Maine), ubiquitously expressing green fluorescent protein (GFP) gene, were used as donors. The animals were maintained as a breeding colony and 1-day-old neonatal mice were used to harvest the enteric tissue. 8-week-old non-obese diabetic/severe combined immunodeficient mice (NOD-SCID, Charles River Laboratories, Wilmington, MA) were used as recipients. Autoclaved cages with irradiated diet and acidified water were used to house NOD-SCID mice. The animals were maintained and handled in compliance with the institutional regulations established and approved by the Animal Research Committee at the University of California, Los Angeles.

2.2. Isolation and expansion of enteric cells

1-day-old transgenic mice pups were used for the isolation of GFP-positive primary enteric cells. The entire intestine, from duodenum to colon, was excised and placed in cold Hanks' balanced salt solution (HBSS without calcium and magnesium; Invitrogen, Carlsbad, CA). The tissue was minced, centrifuged at 1 000 rpm for 15 min and resuspended in the digestion solution at 37 °C for 20 min. The digestion solution consisted of 1 ml of HBSS containing 1 mg/ml collagenase IV (Sigma, St. Louis, MO) and 0.25 mg/ml trypsin (Invitrogen). After dispersing the cells by vigorous shaking, the enzymatic activity was neutralized by addition of 4ml of 5% fetal bovine serum (FBS; Invitrogen). The dissociated cells were centrifuged at 1 000 rpm for 5 min, resuspended in HBSS and filtered through 70 µm nylon strainer (BD Bioscience, Bedford, MA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM), 10% FBS and 1% Antibiotic-Antimycotic (Invitrogen)

at 37 °C, 10% carbon dioxide, in humidified incubator for 10 days. The culture medium was changed every 2–3 days.

2.3. Preparation of scaffold

Porous polycaprolactone (PCL; B6003-2, Lactel Absorbable Polymers, Pelham, AL) scaffolds were prepared by solvent casting and particulate leaching technique. Briefly, PCL (10% w/w) was dissolved in a mixed solution of chloroform and methanol (65 chloroform : 35 methanol w/w). Sieved sucrose particles of 212–300 µm diameter were added to the PCL solution. The ratio of sucrose:PCL was 96:4 (w/w). The blend was mixed briefly to form homogeneous slurry and was cast into Teflon molds with inner diameter of 6 mm and height of 2 mm. Scaffolds were freeze-dried, and the sucrose was leached out in deionized water. The scaffolds were sterilized in 70% ethanol for 30 minutes and washed several times with phosphate buffered saline (PBS).

The heparin was immobilized on the surface of the porous PCL scaffold by cross-linking heparin using carbodiimide reaction. The chemicals 2-(N-Morpholino)ethanesulfonic acid (MES; cat. No. M3671), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC; cat. no. E1769), N-hydroxysuccinimide (NHS; cat. no. 130672) and unfractionated heparin sodium salt derived from porcine intestinal mucosa (cat. no. H4784) were purchased from Sigma. The scaffolds were saturated with 0.05 M MES buffer (pH 5.5) for 15 minutes prior to the reaction. Subsequently, the scaffolds were immersed in freshly prepared solution of heparin (1% w/v), 0.5 M EDC and 0.5 M NHS in MES buffer and vortexed briefly. After 15 hours of reaction at room temperature, the scaffolds were extensively washed with distilled water to remove the byproducts.

The cross-linking reaction between heparin molecules forms a 500–800 nm thick layer over PCL struts as visualized under transmission electron microscope (Singh *et al.* 2011). Fourier transform infrared spectra of heparin-modified PCL show new bands corresponding to amide C=O and -OH groups (Singh *et al.* 2011). Scanning electron microscopy reveals sub-micron roughness on the surface of heparin-PCL scaffolds (Singh *et al.* 2011). There was 7.5 ± 1.6 mg of heparin per scaffold as determined by toluidine blue assay (Singh *et al.* 2011).

The human rhVEGF165 (R&D Systems, Minneapolis, MN) was loaded on the scaffold by applying a solution of 10 µg of growth factor in 60 µl PBS in three aliquots onto the scaffold. The VEGF loaded scaffold was allowed to dry in laminar hood for 40 minutes at room temperature and immersed in 1 ml of PBS at 4 °C for overnight to remove freely diffusible VEGF.

Our previous studies used radiolabeled ^{125}I -VEGF to show that heparin-PCL scaffold exhibit higher retention of VEGF than the unmodified PCL scaffold (Singh *et al.* 2011). Heparin-PCL scaffolds loaded with 10 µg of VEGF retains approximately 4 µg of VEGF as compared to 1 µg of VEGF retained by the unmodified PCL upon 24 hours of incubation in media containing serum. The release rate of VEGF from heparin-PCL scaffolds during the first two weeks is approximately 100 ng/day. The phosphorylation of VEGFR2 receptor in endothelial progenitor cells confirms the bioactivity of VEGF in scaffolds.

2.4. Cell seeding and scaffold implantation

After 10 days of *in vitro* culture, the confluent enteric cells were trypsinized immediately before seeding on the scaffold and counted with a hemocytometer. The heparin-modified PCL scaffolds were seeded with 100 000 cells in 50 µl media. The un-modified PCL scaffolds were seeded with 25 000 cells 50 µl media. The seeded scaffolds were incubated

for 3 hours at 37 °C in a humidified incubator prior to implantation to allow cell attachment. Mice were anesthetized with isoflurane and a 1-cm long dorsal midline incision was made to create two subcutaneous pockets on the either sides of the incision. Two scaffolds, of the same type, were implanted in each mouse for all the experiments; the skin was sutured with 3-0 Vicryl (Ethicon, Somerville, NJ). Three scaffolds were implanted in 3 separate mice for each group and utilized in immunohistochemical analyses. Five scaffolds implanted in 5 different animals were used to assess the genomic GFP DNA for each group. Analyses of the mRNA protein expression for each group utilized separate mice to implant 5 scaffolds for day 7 and 3 scaffolds for day 14 time-points.

2.5. Hypoxyprobe-1 injection and scaffold harvest

At day 7 and 14 post-implantation, the mice were infused with Hypoxyprobe-1 (HPI Inc., Burlington, MA) before euthanasia to enable detection of cellular hypoxia by immunohistochemistry. The Hypoxyprobe-1 (HP1) forms adduct with thiol groups of the proteins in the hypoxic cell and has been used to visualize hypoxia in tissues by other investigators (Bouchentouf *et al.* 2008; Kim *et al.* 2009). The animals were administered intraperitoneal injection of 3 mg/100 µl HP1 in saline two hours before sacrifice. At the time of harvest, mice were euthanized and one of the scaffolds from each mouse was fixed promptly in cold solution of 10% formalin in PBS for histological processing. The other harvested scaffold was frozen in liquid nitrogen and stored at -80 °C for RNA or DNA extraction.

2.6. RNA extraction and qRT-PCR

The mRNA expression of contractile proteins was compared between the native intestinal tissue, the expanded enteric cells and the harvested scaffolds. To extract the mRNA from the enteric tissue, the freshly excised intestinal tissue was frozen in liquid nitrogen. The cells cultured *in vitro* for 10 days were trypsinized, centrifuged and the cell pellet was frozen immediately. The frozen samples were disrupted by using Kontes pellet pestle (Kimble Chase, Vineland, NJ) and were mixed with the lysis buffer supplied with the RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA isolation was performed following Qiagen RNeasy Mini Kit protocols. RNA samples were treated with DNase (Qiagen). The amount of total RNA was quantified by reading its absorbance at 260 nm. The real-time reverse transcription polymerase chain reaction (RT-PCR) was conducted by using the QuantiTect reverse transcriptase and master mix (Qiagen), 30 ng of extracted sample RNA and pre-made primers and probe mixes (Mm01546133_m1 for *Acta2*, Mm00802455_m1 for *Des*, Mm00443000_mH for *Myh11* and Mm99999915_g1 for *Gapdh*; Applied Biosystems, Foster City, CA). Real-time PCR was carried out on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). *Gapdh* was used as control for the total amount of RNA used in each reaction. The threshold cycle value for each sample was the cycle number at which the fluorescence exceeded the threshold limit. All the samples were processed in duplicates. Gene expression levels of the sample were normalized to that of the intact enteric tissue and were computed as $0.5^{(CT_{CA} - CT_{CB})}$. CT is the threshold cycle number for a specific gene in the sample and CG is the corresponding threshold cycle number for its *Gapdh*. CA is the threshold cycle number for the same gene in the enteric tissue, and CB is the corresponding threshold cycle number for its *Gapdh*.

2.7. DNA Extraction and qPCR

The engraftment of the transplanted enteric cells was evaluated by quantifying the genomic GFP DNA content of the scaffolds at day 0, 7 and 14. For day 0 time-point, the heparin-modified PCL scaffolds and unmodified scaffolds were seeded with 100 000 cells and 25 000 cells, respectively, in 50 µl media; the scaffolds were incubated for 3 hours at 37 °C to

allow cell attachment and frozen at -80°C . To generate a standard curve for each set of implantation experiment, 1 000 000 enteric cells cultured *in vitro* for 10 days were frozen and stored at -80°C . The DNA was isolated from the samples according to the manufacturer's manual for the Qiagen DNeasy Blood and Tissue Kit. The genomic DNA isolated from 1 000 000 enteric cells was serially diluted 10-fold to produce three values ranging from 1 000 000 to 10 000 cells for the GFP DNA standard curve. The real-time quantitative polymerase chain reaction (qPCR) was performed by using the QuantiTech master mix (Qiagen), 0.015 volume fraction of the total DNA and 90 nM of forward and reverse primers and 25 nM of probe. Probes were labeled with 6FAM, a fluorescent reporter dye with TAMRA at the 3' end. The sequence for the probe was 5'-CCGACAAGCAGAAGAACGGCATCA-3. Sequences for the forward and reverse GFP primer were sense, 5-ACTACAACAGCCACAACGTCTATATCA-3 and antisense, 5-GGCGGATCTTGAAGTTCACC-3. The threshold cycle value for each sample was the cycle number at which the fluorescence exceeded the threshold limit. Each sample was processed in duplicates. A standard curve with a linear relationship was obtained by plotting the threshold cycle number against the known number of GFP-positive cells on a logarithmic scale. The transplanted GFP cells in the scaffolds were computed from the threshold cycle values using the equation of the standard curve.

2.8. Immunohistochemistry and analysis

The *in vitro* cultured enteric cells were fixed with 10% formalin for 10 minutes, stained with rabbit anti-GFP antibody (Abcam, Cambridge, MA) and mouse anti- α SMA antibody (Sigma), combined with fluorescent secondary antibodies (Invitrogen).

The paraffin-embedded scaffolds were cut into 6- μm sections. The sections were stained with rat anti-mouse CD31 antibody (BD Biosciences, Franklin Lakes, NJ) or, mouse anti-hypoxypore-1 antibody (HPI Inc., Burlington, MA), followed by biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA). The staining was visualized with diaminobenzidine peroxidase (DAB) substrate (Vector Laboratories) at 200 $\times$ magnification. The blood vessel density, blood vessel area and hypoxic area within the scaffolds were measured using ImageScope software (Aperio Technologies, Vista, CA). The sections stained with rabbit anti-GFP antibody were detected fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (Invitrogen). The total percent of GFP-positive area in the 6- μm scaffold section was scanned and analyzed at 200 $\times$ magnification using Ariol SL-50 image analysis system (Genetix, San Jose, CA).

For multiple immunostaining, the agarose-embedded scaffolds were cut with a Vibratome (Ted Pella, Redding, CA) into 200- μm sections. The sections were stained with anti-mouse CD31 antibody, anti-GFP antibody, anti- α SMA antibody or, anti-hypoxypore-1 antibody, combined with fluorescent secondary antibodies (Invitrogen). The samples were visualized on a Zeiss Axioskop microscope and a Leica TCS-SP MP confocal microscope.

2.9. Statistics

The relative gene expression, genomic GFP DNA, anti-GFP positive area, anti-hypoxypore-1 positive area, anti-CD31 positive vessel area and vessel density at each time point were analyzed using ANOVA. To measure significant difference between groups, the post hoc testing of pairwise differences was performed using the Tukey's Studentized Range (HSD) test. The data were considered statistically significant when $p < 0.05$.

3. RESULTS AND DISCUSSION

The enteric tissue is used as a source of smooth muscle cells for intestinal tissue engineering. The contractile function of the intestine is carried out by smooth muscles that constitute the prevalent cell type in the enteric tissue. Majority of the enteric cells cultured *in vitro* for 10 days were anti- α SMA-positive (Figure 1A). The cultured enteric cells expressed high level of mRNA for contractile markers α SMA and desmin; the mRNA expression of the contractile protein MHC in the cultured cells was same as in native tissue (Figure 1B). Our results indicate that *in vitro* expansion of enteric cells enriched the smooth muscle cell population, while the population of other cells declined. Other researchers have reported high expression of smooth muscle specific α SMA, desmin, and smoothelin, with negligible presence of S100-positive glial cells and absence of c-kit positive interstitial cells of Cajal when the colonic smooth muscle tissue were cultured *in vitro* for 10 days (Stanzel *et al.* 2010).

The scaffolds implanted subcutaneously in NOD-SCID mice were retrieved at day 7 and day 14. Grossly, the heparin-PCL scaffold loaded with 10 μ g of VEGF exhibited increased redness at day 14, suggesting improved angiogenesis within the scaffold (Figure 2). The seeding density of the enteric cells was based on quantification of the GFP genomic DNA using qPCR. Equal density of adhered cells in the scaffolds was achieved when heparin-modified PCL scaffolds were seeded with 4-times the number of cells compared to those seeded in the PCL scaffolds. Therefore, 100 000 enteric cells were seeded on heparin-modified PCL scaffolds and 25 000 enteric cells were seeded on PCL scaffolds to achieve the same initial cell density in all scaffolds. The enteric cell distribution within scaffolds was confirmed with anti-GFP immunofluorescence after cell seeding (Figure 3A). The possible reason for lower attachment of cells in heparin-PCL scaffold could be the electrostatic repulsion between negatively charged glycocalyx of the enteric cells and negatively charged sulfate groups in heparin.

At day 7 after implantation, immunofluorescence demonstrated that most of the blood vessels were confined to the periphery of the heparin-PCL scaffolds loaded with 10 μ g of VEGF (Figure 3B). The successive section stained with anti-HP1 showed hypoxic GFP-positive cells away from the in-growing blood vessels. The GFP cell density was also less in the hypoxic regions. As anticipated, the presence of GFP-positive cells was more uniform in heparin-PCL scaffold with VEGF since it stimulated earlier angiogenesis. The confocal analysis showed that the GFP-positive cells were interdispersed between the network of CD31-positive vessels and were co-localized with the α SMA at day 7 (Figure 4).

At day 14 after implantation, scaffolds with heparin and VEGF displayed CD31-positive vessels throughout the entire thickness of the scaffold; there was a concomitant decline in the hypoxic regions (Figure 5). The transplanted GFP-positive cells were more uniformly distributed across the thickness in scaffolds with increased angiogenesis. In contrast, the scaffold with insufficient angiogenesis had enteric cells confined near the scaffold-tissue interface and had sparse GFP cells in the interior. The hypoxic cells were located at a distance of approximately 300 μ m away from the blood vessels in the scaffolds (Figure 6).

The total vessel density and percentage area occupied by vessels were quantified from the 6- μ m sections of the scaffold stained with anti-CD31 (Figure 7). At day 7 and 14, heparin-PCL scaffolds loaded with VEGF resulted in a 3-fold increase in angiogenesis over scaffolds without heparin and VEGF (Figure 8A and B). The combination of heparin and VEGF had a 2-fold increase in vessel density and percentage area over the scaffolds with either VEGF or heparin alone at day 14. The percentage of hypoxic area also decreased significantly in heparin-PCL scaffold with VEGF at day 14 (Figure 8C).

The transplanted cell engraftment was quantified by the total GFP genomic DNA content and by evaluating the percentage of scaffold area stained with anti-GFP (Figure 8D and E). At day 7, both methods showed significantly more enteric cells in heparin-PCL scaffold with VEGF as compared to scaffolds without heparin and VEGF. At day 14, scaffolds with heparin and VEGF maintained the high enteric cell engraftment among all the groups. There was no significant difference in GFP cell density between day 7 and day 14 time-points in heparin-PCL scaffolds loaded with VEGF.

Compared to the native intestinal tissue, the mRNA expression of the crucial contractile marker smooth muscle MHC was significantly lower in all of the scaffolds at day 7 and day 14 (Figure 8F). The retrieved implants exhibited expression of other early contractile markers α SMA and desmin. Our results indicate that the enteric smooth muscle cells may not have fully differentiated to the contractile phenotype at day 14. The normal intestinal smooth muscle cells are contractile and non-proliferative. The expansion of smooth muscle cells in culture is known to cause the decline in contractile phenotype and acquisition of a synthetic or proliferative phenotype.

The enteric cell density did increase significantly in heparin-PCL scaffold with VEGF, from 300 cells/mm³ at day 0 to 2 000 cells/mm³ at day 7. In comparison, the cell density in unmodified PCL scaffold was only 550 cells/mm³ at day 7. Recently, Oh *et al.* reported high proliferation of muscle-derived stem cells and their differentiation into smooth muscle cells *in vitro* when cultured in PCL microspheres with immobilized heparin and bFGF (Oh *et al.* 2011). The high proliferation of enteric cells in scaffolds with immobilized heparin was contrary to the inhibitory effect of soluble heparin on the proliferation of vascular smooth muscle cells reported by investigators (Beamish *et al.* 2009; Blaukovitch *et al.* 2010).

Additional factors will be required to achieve high cell density and restore contractile function of the transplanted enteric cells. It has been shown that the circular smooth muscle cells derived from colon exhibited high proliferation in presence of platelet-derived growth factor (PDGF-BB) and insulin-like growth factor-1 *in vitro* (Stanzel *et al.* 2010). The expression of MHC in the vascular smooth muscle cells is up regulated upon mechanical stimulation (Niklason *et al.* 1999).

In conclusion, the enhancement of angiogenesis by delivery of VEGF in heparin-PCL scaffolds alleviated hypoxia and increased engraftment of the enteric cells. Besides improving nutrient transport, additional biochemical and mechanical cues may be necessary to support proliferation and differentiation of the transplanted enteric cells.

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Figure 1. Characterization of enteric cells cultured *in vitro* for 10 days. Cells were immunostained with anti-GFP and anti- α SMA antibodies (A). Scale bar = 200 μ m. mRNA expression of contractile markers relative to the enteric tissue (B). Values represent the mean and standard deviation of measurements from five experiments. (*) $p < 0.05$, (#) $p < 0.005$.

Figure 2.

Macroscopic evaluation of the scaffolds seeded with enteric cells and harvested at day 7 and day 14. PCL and heparin-modified PCL scaffolds were seeded with 25 000 and 100 000 enteric cells, respectively. V0 and V10 represent scaffold without VEGF and loaded with 10 μ g of VEGF, respectively. H0 and Hm represent scaffolds without heparin and with heparin cross-linked for 15 hours, respectively. Scale bar = 3 mm.

Figure 3.

Immunofluorescence evaluation of the scaffolds at day 0 (A) and day 7 (B). PCL and heparin-modified PCL scaffolds were seeded with 25 000 and 100 000 enteric cells, respectively. The enteric cells, blood vessels and hypoxia in 200- μ m thick successive sections of the scaffold were visualized using anti-GFP, anti-CD31 and anti-hypoxyprobe-1 (HP) antibodies, respectively (B). V0 and V10 represent scaffold without VEGF and with 10 μ g of VEGF, respectively. H0 and Hm represent scaffolds without heparin and with heparin cross-linked for 15 hours, respectively. Scale bar = 500 μ m.

Figure 4. Confocal image of immunostained 200- μ m section of scaffold retrieved at day 7. 100 000 enteric cells were seeded in scaffold cross-linked with heparin for 15 hours and loaded with 10 μ g of VEGF. Enteric cells and blood vessels were localized using anti-GFP and anti-CD31 antibodies, respectively (A). Alpha-smooth muscle actin-positive enteric cells were visualized using anti- α SMA and anti-GFP antibodies, respectively (B). Scale bar = 200 μ m.

Figure 5.

Immunofluorescence evaluation of scaffolds at day 14. The enteric cells, blood vessels and hypoxia in 200- μ m sections of the scaffold were visualized using anti-GFP, anti-CD31 and anti-hypoxypore (HP) antibodies, respectively. V0 and V10 represent scaffold without VEGF and with 10 μ g of VEGF, respectively. H0 and Hm represent scaffolds without heparin and with heparin cross-linked for 15 hours, respectively. Scale bar = 500 μ m.

Figure 6.

Spatial distribution of blood vessels and hypoxic cells in the heparin-PCL scaffold harvested at day 7 and day 14. 100 000 enteric cells were seeded in scaffold cross-linked with heparin for 15 hours and loaded with 10 μ g of VEGF. Hypoxic area (outlined blue) and vessel cross-sectional area (outlined red) in the scaffold was identified using anti-hypoxyprobe-1 and anti-CD31 antibodies, respectively. Scale bar = 400 μ m.

Figure 7.

Angiogenesis in scaffolds harvested at day 7 and day 14 evaluated by DAB staining with anti-CD31. V0 and V10 represent scaffold without VEGF and with 10 μg of VEGF, respectively. H0 and Hm represent scaffolds without heparin and with heparin cross-linked for 15 hours, respectively. Scale bar = 100 μm .

Figure 8.

Quantification of angiogenesis (A, B), hypoxia (C), enteric cell engraftment (D, E) and relative gene expression (F). CD31-positive blood vessels in scaffold sections were measured as average vessel density (A, n=3) and percentage of scaffold area occupied by vessels (B, n=3). Hypoxia was measured as percentage scaffold area occupied by hypoxyprobe-positive cells (C, n=3). Enteric cell engraftment was determined by quantitative PCR of GFP DNA (D, n=5) and by immunohistochemical staining of scaffold sections using anti-GFP antibody (E, n=3). mRNA expression levels of contractile markers in scaffolds relative to the native enteric tissue was determined (F, n=5 for day 7 and n=3 for day 14). V0 and V10 represent scaffold without VEGF and with 10 μ g of VEGF, respectively. H0 and Hm represent scaffolds without heparin and with heparin cross-linked for 15 hours, respectively. Values represent the mean and standard deviation of measurements of scaffold harvested from n number of experiments. (*, &, ψ , π , #, +) $p < 0.05$.