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Cell-free carrier system for localized delivery of peripheral blood cell-derived engineered factor signaling: towards development of a one-step device for autologous angiogenic therapy

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ABSTRACT

Spatiotemporally-controlled delivery of hypoxia-induced angiogenic factor mixtures has been identified by this group as a promising strategy for overcoming the limited ability of chronically ischemic tissues to generate adaptive angiogenesis. We previously developed an implantable, as well as an injectable system for delivering fibroblast-produced factors *in vivo*. Here, we identify peripheral blood cells (PBCs) as the ideal factor-providing candidates, due to their autologous nature, ease of harvest and ample supply, and investigate wound-simulating biochemical and biophysical environmental parameters that can be controlled to optimize PBC angiogenic activity. It was found that hypoxia (3% O_2) significantly affected the expression of a range of angiogenesis-related factors including VEGF, angiogenin and thrombospondin-1, relative to the normoxic baseline. While all three factors underwent down-regulation over time under hypoxia, there was significant variation in the temporal profile of their expression. VEGF expression was also found to be dependent on cell-scaffold material composition, with fibrin stimulating production the most, followed by collagen and polystyrene. Cell-scaffold matrix stiffness was an additional important factor, as shown by higher VEGF protein levels when PBCs were cultured on stiff vs. compliant collagen hydrogel scaffolds. Engineered PBC-derived factor mixtures could be harvested within cell-free gel and microsphere carriers. The angiogenic effectiveness of factor-loaded carriers could be demonstrated by the ability of their releasates to induce endothelial cell tubule formation and directional migration in *in vitro* Matrigel assays, and microvessel sprouting in the aortic ring assay. To aid the clinical translation of this approach, we propose a device design that integrates this system, and enables one-step harvesting and delivering of angiogenic factor protein mixtures from autologous peripheral blood. This will facilitate the controlled release of these factors both at the bed-side, as an angiogenic therapy in wounds and peripheral ischemic tissue, as well as pre-, intra- and post-operatively as angiogenic support for central ischemic tissue, grafts, flaps and tissue engineered implants.

1. Introduction

While chronically ischemic tissues are constantly exposed to hypoxia, the primary angiogenic stimulus, they paradoxically appear to have a limited capacity to appropriately respond to hypoxic stress [1]. We, and other authors, have proposed that the mechanism underlying the inadequate generation of compensatory angiogenesis seen in many chronic ischemic/hypoxic conditions involves a blunting of the ability of cells to up-regulate angiogenic factors (e.g. VEGF, angiopoietins) in response to prolonged/repeated hypoxic episodes [1–4]. Exposing selected cell types to hypoxia *in vitro*, in order to stimulate production of angiogenic factor proteins, with subsequent spatiotemporally-controlled delivery of such complex, yet physiological, factor mixtures *in vivo* could therefore provide a solution to overcoming the limited ability of ischemic tissues to optimally up-regulate angiogenic signaling [5,6]. Effectively, this strategy then aims at overriding the habituated response of cells within an ischemic tissue to the constant oxygen micro-environment, thus restarting the angiogenic process and driving it to completion.

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Utilization of hypoxia as a tool for angiogenic induction harnesses the innate biological mechanism that naturally generates angiogenesis in the body, in physiological (e.g. embryogenesis), as well as pathological states (e.g. ischemia, wound healing, tumor formation) [7]. This approach has the advantage that it can be easily and successfully followed, even if the complex angiogenic factor cascades are mapped only incompletely. Exposing cells to hypoxia to promote production of angiogenic factors has, therefore, been widely investigated [8–10]. Indeed, conditioned media from hypoxic cell cultures have previously been described to induce angiogenesis *in vitro* and *in vivo* [11]. Furthermore, pre-conditioning peripheral blood mononuclear cells to hypoxia has been shown to increase their survival and angiogenic potency upon implantation into ischemic hindlimbs of mice [12], while grafting cord blood mesenchymal stem cells as spheroids in the same animal model improved therapeutic efficacy due to enhanced cell survival and paracrine activity, effects mediated by hypoxic cell preconditioning within spheroid cultures [13]. Consequently, there is an increasing focus on trying to decipher hypoxia-induced signaling spatial and temporal profiles [2,6,8], which will inform on the mechanisms mediating physiological and pathological angiogenic processes, as well as provide useful insights into the optimal modes of delivering hypoxia-based angiogenic therapies.

We have previously reported on two strategies for delivering hypoxia-induced signaling (HIS) *in vivo*, namely, implanting living [9] or non-viable [6] hypoxic cell-matrix depots that actively produce factors, or act as carriers of factors trapped within the matrix during *in vitro* pre-conditioning, respectively. In an effort to make this approach more clinically applicable, we recently developed an injectable system for localized and temporally-controlled delivery of cell-free matrix carriers loaded with fibroblast-generated, hypoxia-induced factors [2]. The central idea enabling this technology lies in the ability to engineer a composite matrix construct, in which a central cellular and a peripheral acellular compartment co-exist in culture, but remain spatially distinct through separation by a nano-porous filter. This filter prevents cell/pathogen movement into the acellular compartment. Angiogenic factor proteins, produced by cells residing in the central compartment in response to hypoxic exposure, can therefore be captured by the acellular matrix as they radially diffuse through it. Factor-loaded matrix fractions can be then locally delivered using an injectable sol–gel vehicle.

The aim of this study was to lay the foundation for translating this system's utility into a device-based tool that can be used at the bed-side. The primary pre-requisite for materializing this strategy is evidently the utilization of a cell type that can be directly seeded onto a matrix scaffold while being harvested, so that the need for conventional cell extraction from tissue and subsequent seeding is avoided altogether. With this in mind, we opted for using peripheral blood cells (PBCs), which can be conveniently obtained from a peripheral vein and directly delivered onto a scaffold by the same liquid medium in which they reside, i.e. plasma. The ample availability of peripheral blood cells also makes them the ideal autologous cell type for immediate/rapid cell culture, as the need for lengthy cell population expansion cycles is circumvented. Previous studies have shown that peripheral blood mononuclear cells (PBMCs) respond to stress (e.g. hypoxia/ischemia, inflammation, irradiation, ultrasound) by upregulating a wide range of angiogenic growth factors, such as VEGF [14–18], PDGF [19], bFGF [14,16], IL-8 [14], MMP-9 [14], and have the ability to induce angiogenesis *in vitro* [20,21], and *in vivo* upon implantation [12,22–26]. It has also been recently demonstrated that intravenous administration of the culture supernatant of irradiated apoptotic PBMCs confers cytoprotection to cardiomyocytes and inhibits tissue remodeling in rat and porcine acute myocardial infarction (AMI) models [14]. In various patient trials it was shown that transplantation of autologous mononuclear cells, from peripheral blood or bone marrow, increases leg perfusion in critical limb ischemia [27,28], improves cardiac function after AMI [29], and accelerates the healing of refractory skin ulcers [30,31]. These findings strongly provide evidence that peripheral blood is indeed a suitable source of angiogenic factor signaling cells.

Here we present a system for controlled delivery of PBC-generated, cell-free angiogenic factor mixtures. From an engineering standpoint, our previously described principle of combining the cell-scaffold and factor-carrier into one unit, yet keeping them spatially distinct by incorporating an intervening filter [2], provided the system's technological framework (Fig. 1). Within this context we investigated wound-simulating biochemical and biophysical environmental parameters that could act as useful controllers of PBC angiogenic activity, namely hypoxia and cell-scaffold composition/mechanical properties. A range of carriers that can be used to harvest these factors in the form of a wound-dressing or an injectable preparation was then tested, by quantifying VEGF retention-release, and assessing the ability of carrier releasates to induce endothelial cell tubule formation and directional migration *in vitro*.

2. Materials and methods

2.1. Rapid screening of angiogenic factors produced by PBCs

Peripheral blood (10 ml) was collected from a 23 yr old female healthy (BMI = 21 kg/m^2), non-smoker subject. The buffy coat was isolated by centrifuging the blood in EDTA-Vacutainer tubes (BD, Germany) at 3000 rpm/4 °C for 10 min, and mixed with 12 ml of serum-free (SF) media (AIM V, Invitrogen, Germany) (medium contained human serum albumin, a factor-stabilizer). PBC/SF-medium mixture (0.5 ml) was added to PET-, type I collagen-coated PET- or fibrin (Tissucol Duo S 1 ml Immuno, Baxter, Germany)-coated PET 1 μm pore membrane cell culture inserts (BD, Germany), before adding 2.5 ml SF medium per insert. Inserts were then placed in 6-well plates containing 3 ml SF medium per well. Cell culture was carried under hypoxia (3% O_2) or normoxia within a 37 °C/5% CO_2 incubator for 10 days. Media for each condition were then sampled from wells and analyzed with Angiogenesis Proteome Profiler array (R&D, USA), according to manufacturer's instructions. Utilization of the buffy coat, rather than whole blood, ensured precise control of oxygen levels in culture, as well as detection of only the factors generated by cultured PBCs, excluding any factors already present in plasma. Quantification of relative (test/control medium) factor levels was carried out by image analysis of array signals (quantification of mean spot pixel densities) from scanned x-ray film images (3 and 4 min exposures) using an imaging software (Image J, NIH, USA). Three samples were tested per condition.

2.2. Quantification of angiogenic factor protein levels under normoxic and hypoxic culture

Peripheral blood (5 ml) was collected into EDTA-Vacutainer tubes from 19 non-smoker healthy subjects (9 males; age = 38.3 ± 6 yrs, BMI = 26.3 ± 1.3Kg/m^2, and 10 females; age = 30.7 ± 2.2 yrs, BMI = 22.5 ± 1Kg/m^2). All subjects provided consent as approved by the ethics committee of the Heinrich Heine University, Düsseldorf, Germany. Blood was mixed with 5 ml SF-medium and aliquoted into 6-well plates (2.5 ml/well). Plates were placed in a normoxic or a hypoxic incubator (3% O_2), and cultured for 7 days, after which media were sampled from wells. ELISAs for vascular endothelial growth factor (VEGF), angiogenin (ANG), and thrombospondin-1 (TSP-1) (all from R&D, USA) were carried out on samples obtained from culture supernatants, according to manufacturer's instructions. Two samples from each subject were tested per factor for each condition (normoxia/hypoxia). For analysis of the temporal profile of factor expression, the buffy coat was isolated from 10 ml peripheral blood collected from a 23 year old female healthy (BMI = 21 kg/m^2), non-smoker subject and mixed with 12 ml of serum-free (SF) media. PBC/SF-medium mixture (0.5 ml) was added to cell culture inserts (type I collagen-coated PET 1 μm pore membrane), before

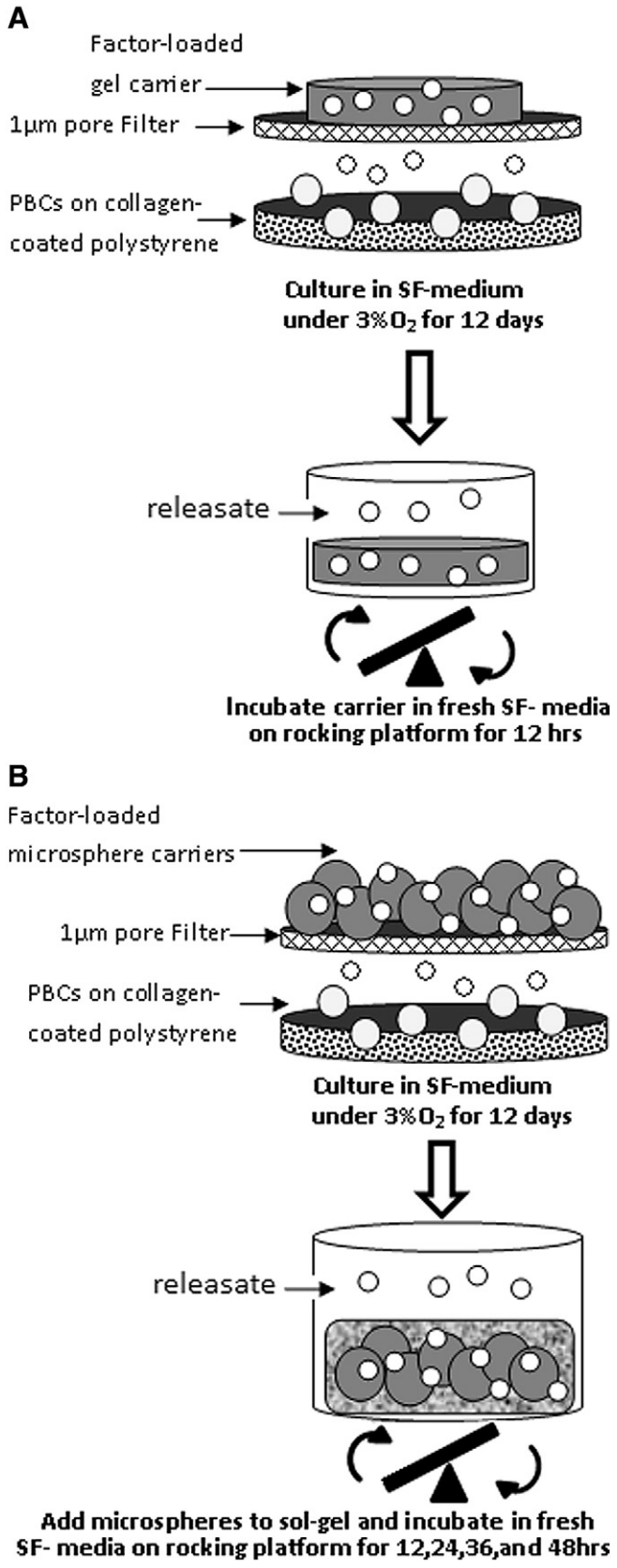


Fig. 1. Schematic showing system for loading PBC-produced, hypoxia-induced factor mixtures onto cell-free gel (A) and microsphere carriers (B), and method from obtaining fresh serum-free (SF) media containing factors released from carriers (releasates).

adding 4.5 ml SF medium per insert. Inserts were then placed in 6-well plates containing 5 ml SF medium per well. Cell culture was carried under hypoxia (3% O_2) or normoxia within a 37 °C/5% CO_2 incubator for 16 days. Media from wells were sampled at 4, 8, 12, 16 days and replaced by fresh media allowing culture to continue. ELISAs for VEGF, interleukin-8 (IL-8), TSP-1 (all from R&D, USA) were carried out on samples obtained from well media, according to manufacturer's instructions. Four samples were tested for each factor, per time point ($n = 4$).

2.3. Assessment of the effect of oxygen tension and scaffold material composition on PBC viability

One milliliter of PBC/SF-medium mixture, prepared as described above, was added to PET 1 μm pore membrane culture inserts, before adding 1 ml SF medium per insert, and placing inserts in 6-well plates containing 2 ml SF medium per well. Cell culture was carried out at 37 °C under hypoxia (3% O_2) or normoxia, for 2, 4, 8 and 12 days. In another setup, 0.5 ml of PBC/SF-medium mixture, was added to PET-, type I collagen-coated PET-, or fibrin-coated PET 1 μm pore membrane culture inserts, before adding 1.5 ml SF medium per insert. Inserts were then placed in 6-well plates containing 2 ml SF medium per well. Cell culture was carried under hypoxia (3% O_2) for 10 days. At the end of the culture period, media (cell-free) were harvested from wells and centrifuged for 5 min at $300 \times g$. Triton-X-100 was added to media in inserts to a final concentration of 1% and allowed to react for 2 h at 37 °C for complete cell lysis (max LDH release). Lactate dehydrogenase assay reaction mixture was prepared according to manufacturer's (Roche, Germany) instructions and mixed with 100 μl of media from wells or inserts in 96-well plates. Plates were incubated at 25 °C for 30 min in the dark, before measuring absorbance at a wavelength of 492 nm. SF-medium was tested as background control, and values were subtracted from all data points. Percentage cytotoxicity was calculated from the ratio of well/insert values. Three samples were tested for each condition.

2.4. Characterization of cell-scaffold material properties

2.4.1. Effect of scaffold material composition on factor expression

PBC/SF-medium mixture (0.5 ml) was added to PET-, type I collagen-coated PET-, or fibrin-coated PET 1 μm pore membrane culture inserts, before adding 1.5 ml SF medium per insert. Inserts were then placed in 6-well plates containing 2 ml SF medium per well. Cell culture was carried out under hypoxia (3% O_2) for 10 days. Media for each condition were then sampled from wells and analyzed by ELISA for VEGF, ANG and TSP-1 (R&D, USA) according to manufacturer's instructions. Three samples were tested per factor per condition.

2.4.2. Effect of scaffold matrix stiffness on factor expression

One milliliter of PBC/SF-medium mixture, prepared as described above, was added to 6-well plates as follows: Group I: wells with a polystyrene surface, Group II: wells containing 5 ml type I bovine collagen gels (*c*: 4 mg/ml, composition of 80 % collagen (Biochrom, Germany), 10% 0.5 M NaOH and 10% 10×MEM with phenol red as pH-indicator; prepared by drop-wise neutralization of acid-soluble collagen solution with alkali), Group III: wells layered by a sheet of plastic compressed type I bovine collagen. Compression of collagen gels was carried out on a blotting paper support under fixed mechanical loading for 5 min, and resulted in an increase in gel Young's modulus from ~50 kPa to ~2000 kPa [32]. After adding cells, 4 ml SF medium was added per well. Cell culture was carried out under normoxia within a 37 °C/5% CO_2 incubator for 10 days, after which media were sampled from wells and tested for VEGF by ELISA. Three samples were tested per condition.

2.5. Test of VEGF release from cell-free carriers

One milliliter of PBC/SF-medium mixture was prepared as above, mixed with 1 ml SF-medium and added to type I collagen-coated 6-well plates (Fig. 1). Carriers were added to culture inserts (1 μm pore PET membrane) as follows: gel carriers (3 ml) tested were polihexanid gel (Ringer solution, polyhexanid concentrate 0.04%, glycerol 8,5%, hydroxyl-ethyl cellulose 2.4%), type I collagen gel (4 mg/ml) and hydrosorb gel (Ringers solution, glycerol, hydroxy-ethyl cellulose, carboxy-methyl cellulose) (Paul Hartmann AG, Germany). One milliliter of SF-medium was then added per insert. Microsphere carriers tested were polystyrene beads (125–212 μm) coated with denatured porcine-skin collagen or pronectin F (Sigma, Germany). Microspheres were added to inserts in excess (1 g per insert) and mixed with 2 ml SF-medium. Culture was carried under hypoxia (3% O_2) for 12 days, after which gel carriers were removed from inserts, added to 1.5 ml fresh SF-media, and incubated for 12 h on a rocking platform to allow diffusion of factors from gels into media (Fig. 1A). Media containing released factors (releasates) were then sampled and tested for VEGF by ELISA. Microsphere carriers were removed from inserts, added to 2 ml type I collagen gel or 2 ml fibrin gel (Fig. 1B). Gels containing microspheres were incubated in 2 ml SF-media on a rocking platform, and media (200 μl) containing released factors were sampled at 12, 24, 36 and 48 h and tested for VEGF by ELISA. Three samples were tested per carrier type ($n = 3$).

2.6. Test of microsphere release from gel vehicles

Fluorescent-labeled type I-coated polystyrene microspheres (FluoSpheres, Invitrogen, Germany) (15 μl) were mixed with 5 ml of type I neutralized collagen gel or hydrosorb gel and added to 6-well plates. Collagen gels were allowed to set at 37 °C for 30 min. One milliliter of PBS was added per well and gels were incubated on a rocking platform for 12 h, after which they were centrifuged at 1200 rpm for 5 min. Fifteen microliters of PBS was sampled from each well after mechanical agitation and after centrifugation, and observed with fluorescence microscopy for quantification of released microspheres. Three samples were tested per gel type ($n = 3$).

2.7. Test of the angiogenic effect of carrier releasates

Factor-loaded gel and microsphere carriers were stored at −20 °C for 1 month, before obtaining their releasates as described above. The angiogenic potential of releasates was then tested in an *in vitro* angiogenesis assay, by assessing their ability to induce tubule formation in human umbilical vein endothelial cells (HUVECs, CellSystems, Germany) seeded on factor-reduced Matrigel (BD, Germany). HUVECS were seeded at a density of 10×10^3/well, with 50 μl of test or control media added per well (μ-Slide Angiogenesis, Ibidi, Germany), and cultured in 5% CO_2/37 °C for 12 h. Cells were then stained with Calcein AM (PromoKine, Germany) and tubule formation was observed with phase contrast microscopy. Assessment of the extent of capillary-like network formation was carried out by counting the number of tubules and nodes (a node was defined as the point of intersection of two or more tubules), and quantification of tubule length. Collagen gel releasates were also tested in the aortic ring model to assess their ability to induce microvessel sprouting. Aortic rings were dissected from

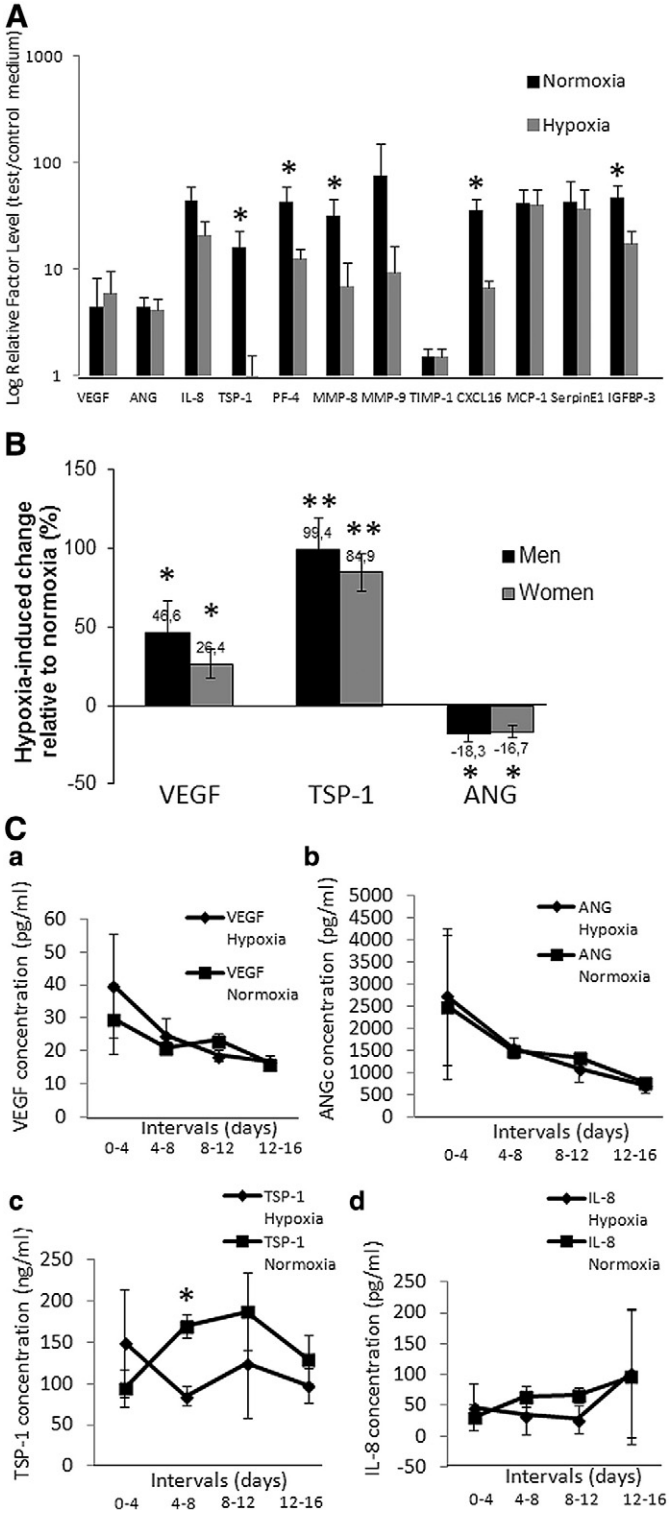
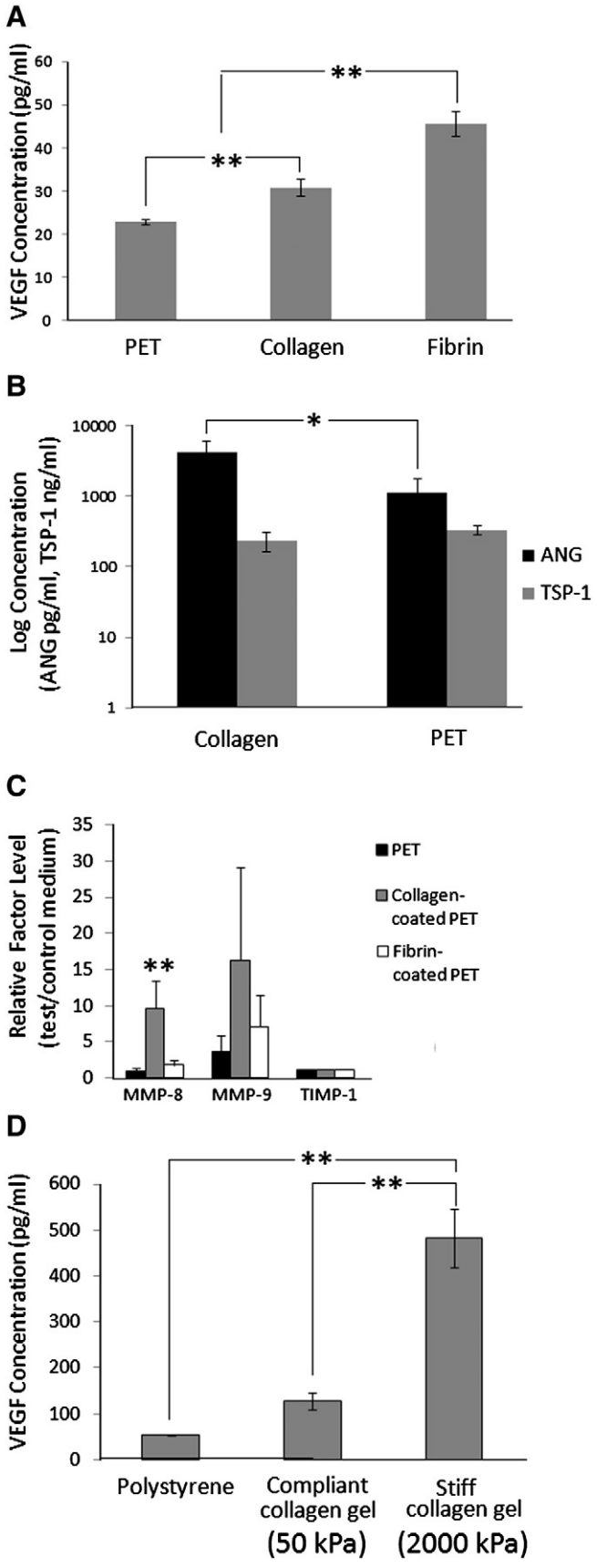


Fig. 2. Oxygen tension influences the expression profile of PBC-produced angiogenesis-related proteins. A) Plot showing the profile of protein factor expression, as analyzed by proteome profiler assay, when PBCs were cultured on collagen-coated PET membranes and exposed to either normoxia or hypoxia (3% O2) for 10 days. *Y*-axis represents the ratio of factor level in culture supernatant over control serum-free medium. Three samples were tested for each condition. B) Plot showing the percentage change in the level of VEGF, TSP-1 and ANG expression when PBCs were cultured under hypoxia (3% O2) compared to when PBCs were cultured under normoxia for 7 days. Whole blood samples, obtained from 19 non-smoking healthy adults, were cultured under hypoxia and normoxia on a polystyrene surface for 7 days. Quantitative analysis of factor protein levels in cell culture supernatants was carried out by ELISA, with 2 samples tested for each subject, per factor. C) Plots showing the temporal profile of VEGF (a), ANG (b), TSP-1 (c) and IL-8 (d) expression by PBCs cultured on collagen-coated PET membranes under hypoxia (3% O_2) and normoxia for 16 days. Media supernatants were sampled every 4 days, and factor levels were quantified by ELISA. Three samples were tested per factor per condition. Error bars represent s.d. (A,C) or s.e.m. (B), $^*p < 0.05$ and $^{**}p < 0.001$ refers to hypoxic vs. normoxic levels.

female adult mice as previously described [33], underwent overnight serum starvation in opti-MEM Reduced Serum medium (Life Technologies, Germany) and embedded into Matrigel bilayer matrix (50 μl/layer in 96-well plates). Releasates, control serum-free medium (AIM V, Invitrogen, Germany) or VEGF-containing medium (90 ng/ml) was added (150 μl/well) to the rings, before culturing them in 5% CO_2/37 °C. Medium change was carried out every 3 days, while rings were observed with phase contrast microscopy at 0, 3, 5 and 8 days and photographed, with all 4 quarters per ring analyzed for sprouting (formation of structures of connected cells that are attached, at their base, to the ring). Three rings were tested per condition. The ability of releasates to induce directional endothelial cell invasion though a Matrigel-coated PET membrane (BD Falcon, FluoroBlok), over 24 h at 5% CO_2/37 °C was tested using the BD BioCoat Angiogenesis system (BD, USA) which allows exclusive visualization of invasive cells labeled with $DiIC_{12}$ Fluorescent Dye (BD, USA), according to manufacturer's instructions. An inverted fluorescence microscope was used to view each well and quantification of percentage cell invasion, relative to control medium, was carried out by image analysis (Image J, NIH, USA). A minimum of 5 fields were analyzed per sample, with 3 samples tested for each condition.



2.8. Statistical analysis

For each experimental condition $n \geq 3$ was used. Data are expressed as mean ± standard deviation or mean ± standard error, as noted. Statistical analysis was carried out using Student's independent *t*-test where a maximum of 2 groups was compared or one-way ANOVA accompanied with multiple comparison tests for analysis of more than 2 groups, using SPSS 14 software. The probability of a type one error was set to 5% ($\alpha = 0.05$), unless noted otherwise.

3. Results

3.1. Regulation of PBC factor expression by oxygen tension

Oxygen tension was the first environmental factor investigated as a means of controlling angiogenic factor production by PBCs. Proteome profiler assay was first used to rapidly screen the expression profile of angiogenesis-related proteins released in culture supernatants when PBCs were seeded on type I collagen-coated PET membranes and cultured under normoxia or hypoxia (3% O_2) for 10 days. As shown in Fig. 2A a range of factor proteins was present in the media of both normoxic and hypoxic cultures, as compared with control serum-free media, including pro-angiogenic (VEGF, ANG, IL-8), anti-angiogenic (TSP-1, PF-4) and matrix remodeling (MMP-8, MMP-9, TIMP-1) factors.

VEGF, TSP-1 and ANG were the three factors selected for quantitative analysis by ELISA. Peripheral blood was collected from 19 healthy, non-smoking subjects and cultured for 7 days under normoxia or hypoxia. Fig. 2B shows that VEGF and TSP-1 expression were significantly up-regulated in hypoxic, relative to normoxic cultures (hypoxic vs. normoxic population mean supernatant concentration values for VEGF and TSP-1 were 50.5 ± 5.2 pg/ml vs. 41.9 ± 6.2 pg/ml ($p < 0.05$) and 242.6 ± 11.4 ng/ml vs. 135.5 ± 11.1 ng/ml ($p < 0.001$), respectively), while ANG expression was down-regulated under hypoxia (hypoxic

Fig. 3. Effect of cell-scaffold material composition and matrix stiffness on PBC angiogenic factor expression. A) Plot showing VEGF concentration in culture supernatants when PBCs were cultured on PET-, collagen-coated PET or fibrin-coated PET membranes under hypoxia (3% O2) for 10 days. B) Plot showing ANG and TSP-1 concentration in culture supernatants when PBCs were cultured on collagen-coated PET- or naked PET-membranes under hypoxia (3% O2) for 8 days. C) Plot showing the expression profile of matrix-remodeling proteins when PBCs were cultured on PET-, collagen-coated PET or fibrin-coated PET membranes under hypoxia (3% O2) for 10 days. *Y*-axis represents the ratio of factor level in culture supernatant over control serum-free medium. D) Plot showing VEGF concentration in culture supernatants when PBCs were cultured on a polystyrene surface, compliant (~50 kPa) collagen gel matrix or stiff (~2000 kPa) collagen gel matrix under normoxia for 10 days. Three samples were tested for each condition. Error bars represent s.d., $^{*}p < 0.05$, $^{**}p < 0.01$.

vs. normoxic ANG supernatant concentration was 3192.5 ± 108 pg/ml vs. 3938 ± 160.8 pg/ml ($p < 0.001$)). For all factors tested, no significant difference was found in factor expression between male and female subjects.

Analysis of the temporal profile of factor expression revealed that the rate of factor production was not constant (Fig. 2C). VEGF and ANG showed a gradual down-regulation over time, under both normoxia and hypoxia (Fig. 2Ca,b). Expression of TSP-1 was interesting in that while PBCs under hypoxia produced ~50% more of the factor compared to PBCs under normoxia in the first 4 days, this pattern was reversed over time (Fig. 2Cc). Indeed, cumulative TSP-1 levels by 12 days culture were 359.3 ± 34.4 ng/ml vs. 452 ± 45.4 ng/ml ($p < 0.05$) for hypoxic vs. normoxic PBCs, respectively. Thus, TSP-1 expression under hypoxia appeared to undergo gradual down-regulation, following an initial upregulation, as also suggested by the proteome profiler data (Fig. 2A). In contrast to VEGF, ANG and TSP-1, the expression of IL-8 was relatively stable over time, under both hypoxia and normoxia (Fig. 2Cd).

3.2. Effect of cell-scaffold material properties on PBC factor expression

The two parameters tested with regards to the cell-scaffold were material composition and mechanical properties, in this case matrix stiffness. We first investigated the effect of scaffold material on factor expression, by culturing PBCs on three different scaffolds under hypoxia; naked PET membrane, type I collagen-coated PET membrane and fibrin-coated PET membrane. In this setup, utilization of the same underlying substrate (i.e. PET membrane), ensured that the matrix stiffness felt by cells was comparable in all three scaffolds tested [34]. As shown in Fig. 3A, the highest VEGF level was produced by PBCs cultured on fibrin-coated PET membrane, followed by collagen-coated and naked PET membrane ($p < 0.01$). Similarly, ANG expression was significantly higher when PBCs were cultured on collagen-coated than on naked PET membrane ($p < 0.05$) (Fig. 3B). In contrast, there was no significant difference in TSP-1 levels between the two scaffold supports (Fig. 3B). Analysis of matrix-remodeling proteins was carried out by proteome assay, and revealed that PBCs cultured on collagen-coated PET membrane produced the highest level of MMP-8 and MMP-9 (Fig. 3C). TIMP-1 expression was comparable in all three scaffolds tested.

In order to test the effect of matrix stiffness on factor expression, we seeded PBCs on compliant and stiff collagen hydrogel matrices (Young's moduli of ~50 kPa and ~2000 kPa, respectively). Importantly, the increase in matrix stiffness was achieved here by irreversible removal of interstitial fluid from the matrix through plastic compression, rather than by chemical modification, thus ensuring that material composition remained the same in both conditions [32]. Fig. 3D shows that VEGF protein expression was significantly higher (~4-fold increase, $p < 0.01$) in stiff versus compliant matrices. According to our previous results comparing collagen-coated vs. naked PET membranes (Fig. 3A), seeding PBCs on collagen gel matrices yielded higher VEGF levels than seeding them on a polystyrene surface, although this difference was only significant for stiff matrices ($p < 0.01$) (Fig. 3D). This confirms the dependence of factor expression on both matrix stiffness and material composition, parameters which are inevitably interlinked [35].

3.3. Effect of oxygen tension and cell-scaffold on PBC viability

In order to identify whether the previous findings of changes in factor expression were partly mediated by changes in PBC viability, we measured the cytotoxic effect of hypoxia and scaffold material composition, using the LDH assay. While there was an increase in total cell death over time (reaching ~50% by 12 days, $p < 0.05$), no significant difference in cytotoxicity was observed between normoxia and hypoxia, at any given time point, when PBCs were cultured on PET membrane supports (Fig. 4A). Furthermore, collagen scaffolds

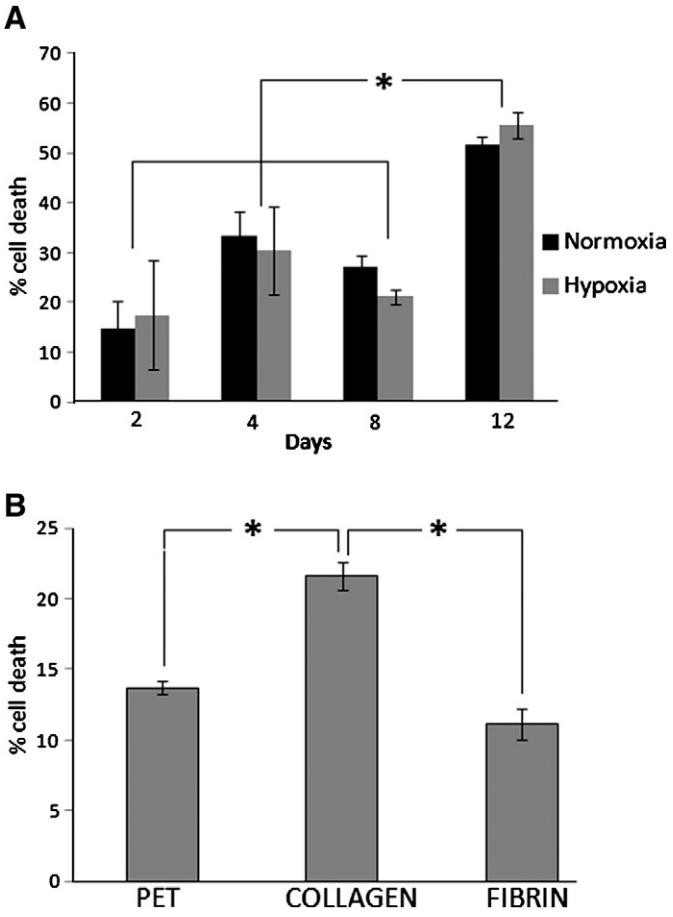


Fig. 4. Effect of oxygen tension and cell-scaffold material composition on total PBC viability. A) Plot showing percentage cell death relative to total cell number, as measured by LDH cytotoxicity assay, in PBCs cultured on PET-membranes under normoxia or hypoxia (3% O_2) at 37 °C for 2, 4, 8 and 12 days. B) Plot showing percentage cell death relative to total cell number in PBCs cultured on PET-, collagen-coated PET or fibrin-coated PET membranes under hypoxia (3% O2) for 10 days. Three samples were tested for each condition. Error bars represent s.d., $^*p < 0.05$.

appeared to induce a small, but significant, increase in cell death (~10%, $p < 0.05$) compared to PET or fibrin scaffolds, over 10 days culture (Fig. 4B). Therefore, the increase in factor levels measured when PBCs were seeded on collagen scaffolds occurred despite a reduction in cell number.

3.4. Cell-free factor carrier system

Based on our previous findings, we cultured PBCs on collagen-coated polystyrene (i.e. biomimetic and stiff) supports, under hypoxia, and collected the factors produced over 12 days within gel or microsphere carriers. Analysis of VEGF concentration in gel carrier releasates, obtained after 12 h incubation of gels in factor-free media, showed that collagen gel retained 4–5 fold more VEGF than hydrosorb gel and polyhexanid gel ($p < 0.05$) (Fig. 5A). At the end of the culture period, collagen gels had a VEGF retention ratio (ratio of VEGF concentration in gel to that in culture medium) of 26 ± 10%. Analysis of VEGF release from microsphere carriers, loaded with PBC-derived factors, showed that both collagen-coated and pronectin-coated microspheres were able to retain VEGF (Fig. 5B). When microspheres (collagen-coated or pronectin-coated) were embedded into collagen gels, there appeared to be an initial burst-release of VEGF into media within the first 12 h (Fig. 5B), after which VEGF concentration in media remained stable, suggesting that a delayed diffusive equilibrium was established between the collagen gel and medium. In contrast, embedding collagen-

coated microspheres into fibrin gels resulted in a more gradual VEGF release, with the 12 h medium VEGF concentration being significantly lower (~50% less, $p < 0.05$) than that from collagen gels containing pronectin-coated microspheres, and showing a ~30% increase from 12 h to 24 h (Fig. 5B). Furthermore, there was no difference in the initial (12 h) or equilibrium (48 h) concentrations of VEGF released from collagen gels containing collagen-coated or pronectin-coated microspheres, indicating that the observed difference in VEGF release profiles was primarily effected through the gel type used as delivery vehicle, and not the microspheres used as factor carriers, in this system.

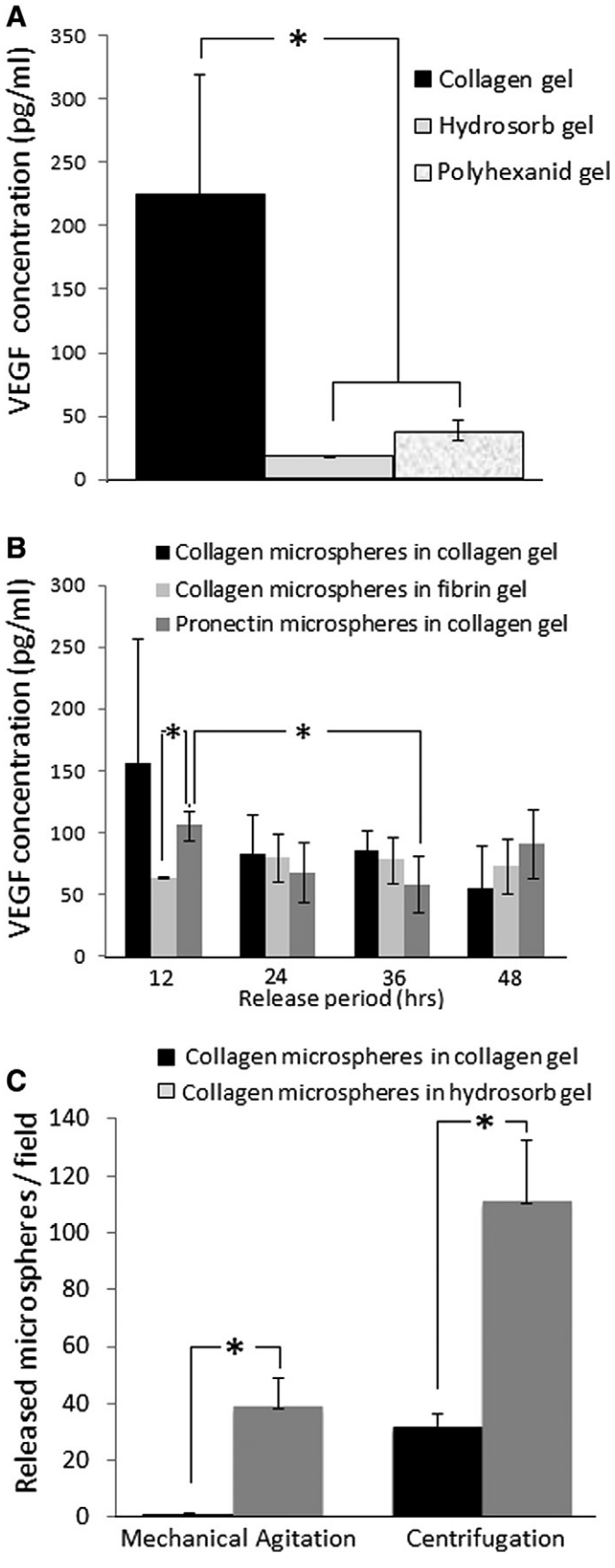


Fig. 5. Gel and microsphere carriers retain and release PBC-derived factors. A) Plot showing VEGF concentration in releasates from gel carriers, after 12 h incubation in fresh serum-free media. Gel carriers tested were type I collagen gel, hydrosorb gel and polyhexanid gel. B) Plot showing cumulative VEGF concentration in releasates from collagen- and pronectin-coated microsphere carriers that were embedded in collagen or fibrin sol–gel, over 12, 24, 36 and 48 h. Carriers were loaded with factors produced by PBCs that were cultured under hypoxia (3% O_2) for 12 days. Three samples were tested per carrier type. C) Plot showing the number of collagen-coated microsphere carriers released from type I collagen gels or hydrosorb gels after 12 h incubation on a rocking platform or centrifugation at 1200 rpm for 5 min. Three samples were tested per gel type. Error bars represent s.d., $^*p < 0.05$.

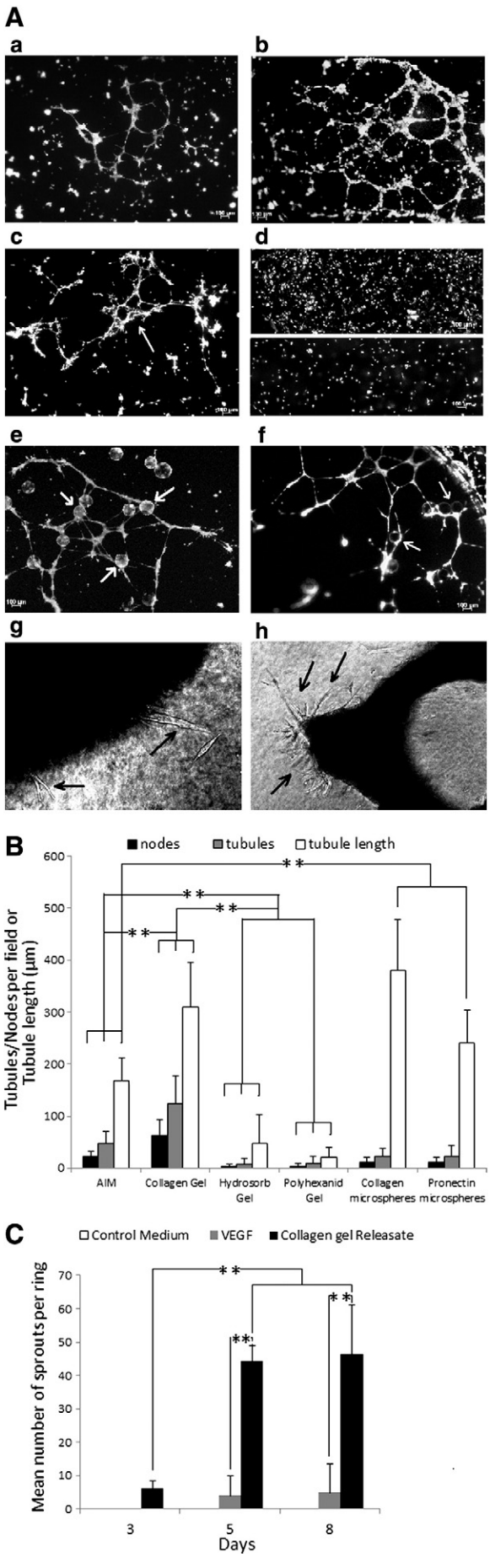
In order to test the effect of gel composition on the release of microsphere carriers, collagen-coated fluorescent microspheres were embedded into either collagen or hydrosorb gels. As shown in Fig. 5C, the number of microspheres released from collagen gels into media was significantly lower than that released from hydrosorb gels, both after 12 h mechanical agitation and 5 min centrifugation ($p < 0.05$). Therefore, incorporating microspheres into a gel of identical material composition resulted into greater microsphere retention, suggesting that this difference was, at least partly, likely mediated by improved integration of the microspheres into the gel, in addition to any differences in gel matrix structure/porosity.

3.5. Angiogenic potential of carrier releasates

Releasates from gel carriers were stored at −20 °C for 1 month, before being tested on HUVEC-seeded Matrigel for assessing their ability to induce tubule formation. It was found that collagen gel releasates significantly increased the number of tubules (~2 fold increase, $p < 0.01$) and nodes (~3 fold increase $p < 0.01$) formed, compared to control medium (Fig. 6A, B). The tubules induced by collagen gel releasates also had approximately double the length of those formed in control medium ($p < 0.01$). In contrast, hydrosorb gel and polyhexanid gel releasates appeared to perform significantly worse than control medium, in terms of all three parameters analyzed ($p < 0.01$) (Fig. 6A, B). Releasates from collagen- and pronectin-coated microspheres, embedded in collagen gel, were found to induce a significant increase in tubule length, which was 2 and 1.5 fold greater than that induced by control medium, respectively ($p < 0.01$) (Fig. 6A, B). In these cultures, the number of tubules formed was lower than control, indicating that formation of longer tubules was promoted through improved tubule connectivity.

Analysis of microvessel sprouting in the mouse aortic ring assay revealed that collagen gel releasates induced sprouting as early as 3 days of culture, at which time point, control and VEGF-containing medium did not appear to have any angiogenic effect (Fig. 6A, C). By 5 days, a significantly greater number of sprouts had formed in rings incubated with collagen gel releasates than VEGF-containing medium ($p < 0.01$). This difference persisted at 8 days, although no further increase in number of sprouts was observed.

The ability of carrier releasates to induce directional endothelial cell migration through a Matrigel membrane was tested using an endothelial cell invasion assay. In accordance to the above results, collagen gel releasates significantly induced ~2 fold higher cell invasion than control medium and hydrosorb gel ($p < 0.01$) (Fig. 7A, B). Polyhaxanid gel releasates appeared to inhibit cell invasion, which was 50% lower than control medium (Fig. 7A, B). From the microsphere carriers tested, releasates from collagen-coated microspheres, embedded in collagen gel, were found to induce ~1.5 fold higher cell invasion compared to



control medium (Fig. 7A, B). This effect, however, was weaker than that induced by collagen gel releasates ($p < 0.05$).

4. Discussion

We previously discussed the merits of delivering hypoxia-induced angiogenic factor mixtures in a spatiotemporally-controlled manner, as a means to overcome the limited ability of chronically ischemic tissues to generate adaptive angiogenesis [6,9]. This led us to develop an injectable, cell-free system for localized delivery of fibroblast-produced factors [2]. Here, we identify peripheral blood cells (PBCs) as the ideal factor-providing candidates, due to their autologous nature, ease of harvest and ample supply. Use of patient autologous cells provides the advantage that possible immunological and infectious adverse effects, associated with administration of blood products, can be prevented. It also paves the way for developing personalized angiogenic therapies.

The findings of this study indicate that angiogenic factor production by PBCs is closely regulated by their residing micro-environment. Hypoxia (3% O_2) was found to initially induce an upregulation of VEGF and TSP-1, while down-regulating ANG expression, compared to normoxia. Importantly, since cell viability was not found to be negatively impacted by hypoxia, it is likely that the measured differences in factor protein levels were the result of true changes in gene expression. The effect of hypoxia on PBC-driven angiogenic processes appears therefore to be a complex one, since it involves interplay of both pro-angiogenic (e.g. VEGF, ANG) and anti-angiogenic (e.g. TSP-1, PF-4) factors. Indeed, a previous investigation showed that angiogenic monocytes actively produce TSP-1, suggesting that their ability to mediate angiogenesis results from a complex interplay of positive and negative regulators [36], while another study found that the production of angiogenic stimulators or inhibitors by platelets depends on their mode of activation [37]. Additionally, as we previously showed for fibroblast-generated hypoxia-induced factors [2], it was also found here that PBC factor expression exemplifies significant temporal variation, as the cells adapt/habituate to a given oxygen tension. In particular, the down-regulation of the angiogenic inhibitors TSP-1 and PF-4 observed under hypoxia, over time, may be indicative of a gradual optimization of PBC angiogenic activity, as the cells respond to the hypoxic stress. Consideration of the temporal profile of factor expression will aid the precise definition of the length of hypoxic conditioning, and how this can be best correlated to the different phases of wound healing, which are themselves underlined by a timely sequence of angiogenic events [38].

Cell-scaffold material properties were found to be an equally important environmental parameter determining factor expression by PBCs, and could thus provide an additional tool for engineering the factor-mixture composition. The scaffold material had a direct effect, as shown by the significantly higher level of VEGF released by PBCs seeded on a fibrin-coated or collagen-coated PET membrane, compared to naked PET membrane. It could be argued that both collagen and fibrin offer more biomimetic environments [32,39],

Fig. 6. Angiogenic potential of carrier releasates. A) Image panel showing the *in vitro* angiogenic response (tubule formation) seen when releasates from gel and microsphere carriers, loaded with PBC-derived factors, were added to endothelial cell-seeded Matrigel and cultured for 12 h (a–f) or to mouse aortic rings embedded in Matrigel (g–h). a: negative control medium, b,c: collagen gel releasate (complex capillary-like structures arrowed in c), d: hydrosorb gel (upper image) and polyhexanid gel (lower image) releasate, e: collagen microspheres embedded in collagen gel releasate, f: pronectin microspheres embedded in collagen gel releasate (arrows in e and f show microspheres in contact with tubules) g: VEGF-containing medium 5 days culture, h: collagen gel releasate 3 days culture (arrows show microvessel sprouts from aortic rings). Bars = 100 μm. B) Plot showing the mean number of tubules and nodes, and mean length of tubules formed on endothelial cell-seeded matrigel by the gel and microsphere carrier releasates after 12 h culture. C) Plot showing the mean number of sprouts per ring, induced by control medium, VEGF-containing medium (90 ng/ml) or collagen gel releasate over 8 days culture. Three samples were tested per carrier type. Error bars represent s.d., $^{*}p < 0.05$, $^{**}p < 0.01$.

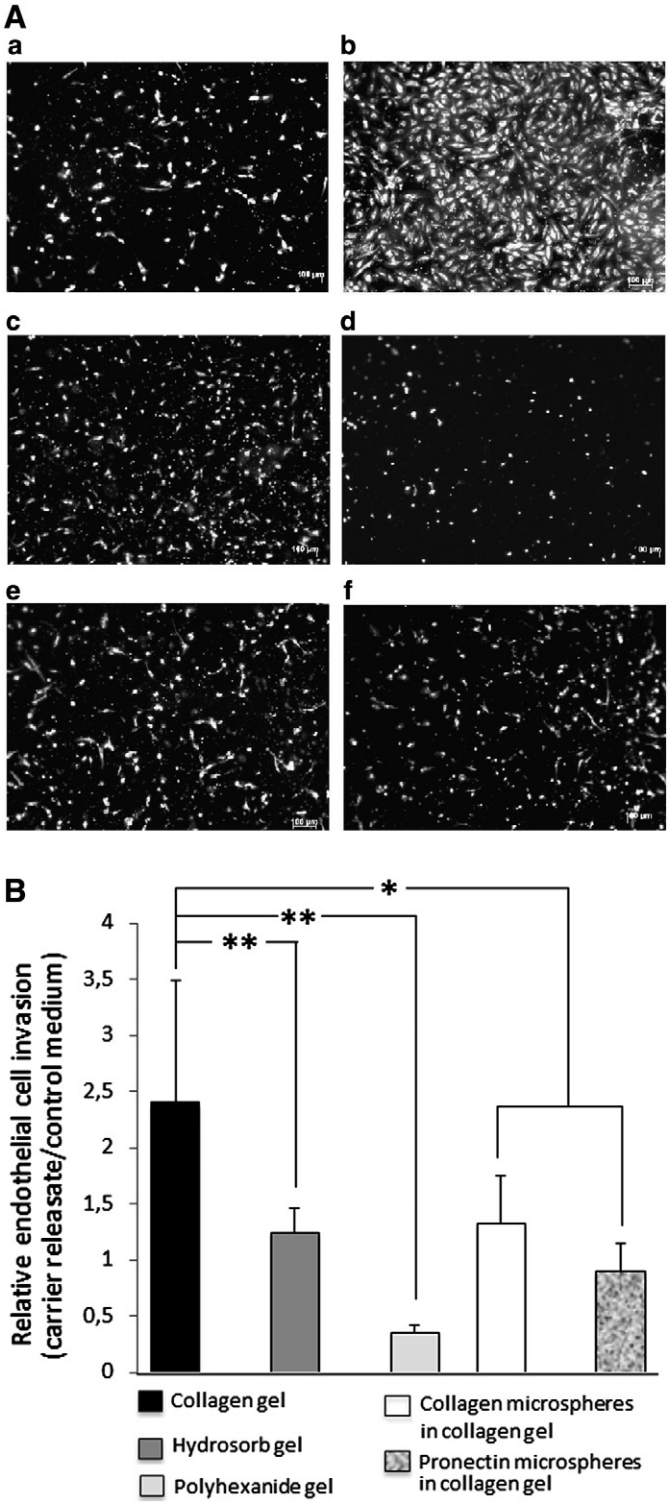


Fig. 7. Carrier releasates induce directional endothelial cell migration. A) Image panel showing the endothelial cell invasion through matrigel membrane induced in response to the releasates from gel and microsphere carriers loaded with PBC-derived factors. A: negative control medium, B: collagen gel releasate, C: hydrosorb gel releasate, D: polyhexanid gel releasate, E: collagen microspheres embedded in collagen gel releasate, F: pronectin microspheres embedded in collagen gel releasate. Bars = 100 μm. B) Plot showing the percentage relative invasion (carrier releasate/SF control medium) of endothelial cells through matrigel membrane, induced by gel and microsphere carrier releasates. Three samples were tested per carrier type. Error bars represent s.d., $^{*}p < 0.05$, $^{**}p < 0.01$.

reminiscent of those encountered by PBCs during extravasation and wound healing (for example, VEGF is released from activated platelets when they come in contact with collagen in injured vessels during blood coagulation [40]). What was perhaps more unexpected, was the finding that scaffold mechanical properties also had a significant effect on factor expression. Indeed, when PBCs were seeded onto the same collagen scaffold, but the stiffness of the matrix was increased by compression-mediated fluid removal, cells produced significantly more VEGF (~4-fold up-regulation) on scaffolds of higher stiffness. It is well documented that cells respond to matrix stiffness by modifying cell behavior, e.g. proliferation [32,41], migration [35], differentiation and gene expression [39,41,42]. Changes in the stiffness of the matrix to which PBCs are exposed occur in many pathophysiological processes such as hypertension [43], myocardial infarction [42], clotting and wound healing [44], and are also inevitably encountered during PBC migration through various tissues. To our knowledge, this is the first report of the regulating role of matrix stiffness in PBC angiogenic factor expression, in a controlled *in vitro* model. For the purpose of optimizing cell-scaffold characteristics, a biomimetic (e.g. fibrin and/or collagen) matrix of high stiffness would thus provide the strongest stimulus for factor production.

Here we show that PBC-generated engineered factor mixtures could be harvested within cell-free gel carriers. While loading of factors onto collagen gels was, at least partly, mediated through trapping of macromolecules by the nano-porous fibrillar matrix, some factors such as VEGF, MMPs could also exhibit specific binding to the gel [45,46]. It could be indeed shown that collagen gel was more effective in retaining VEGF compared to hydrosorb and polyhexanid gels, two commonly used wound-management gels. This difference was in turn translated into a stronger induction of *in vitro* endothelial cell tubule formation and directional migration by collagen gel releasates. Interestingly, we found that hydrosorb and polyhexanid gel releasates not only induced a weaker angiogenic response compared to collagen gel releasates, but also performed significantly worse than control medium. It is likely that this was due to the additional presence of angiogenesis-inhibiting factors in their releasates, factors absent in control medium. This unmasking of anti-angiogenic factor effect, seen here, highlights once again the complex balance of physiological angiogenic factor cascades, and the sensitivity of cellular physiology to changes in their composition. In this context, a biomimetic collagen matrix provides an ideal carrier for complex factor mixtures, capturing factors at the physiological concentrations and ratios that they would be presented to cells within the wound micro-environment.

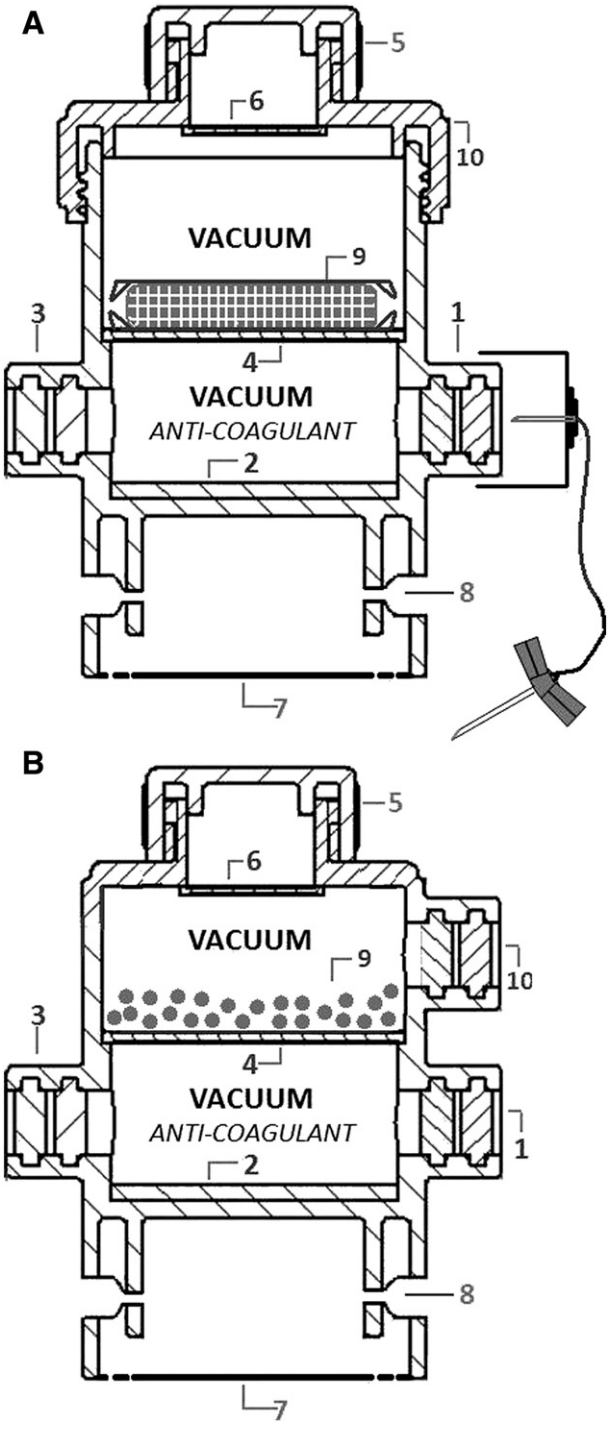
Collagen gel releasates were also shown to be effective in stimulating microvessel sprouting in the aortic ring assay, where they performed significantly better than VEGF. This finding again provides evidence to the idea that physiological angiogenesis can only be recapitulated by providing the complete set of cellular signals, rather than using a single/few (recombinant) factor(s). For example, the presence of MMPs (e.g. MMP-9) in collagen gel releasates may have contributed to the increased sprouting observed [47]. Successful angiogenic induction in this model also suggests that PBC-derived carrier releasates could have therapeutic utility in cardiac, as well as peripheral ischemic tissue.

Importantly, factor mixtures were tested after 1 month freeze storage of loaded carriers at −20 °C, suggesting that this process does not interfere with factor stability or bioactivity, as we previously showed for fibroblast-derived protein mixtures [2]. Mixtures were also shown to be effective when factors were derived from a relatively small amount of blood (1 ml of PBC suspension used per carrier). Using the same system, the concentration of collagen gel carrier-loaded VEGF corresponding to 10 ml of blood would predictably be in the nanogram range, which has previously been shown to be sufficient for angiogenic induction *in vivo* [48,49]. Furthermore, the retention capacity of collagen gel carriers could be improved through physical and/or chemical

modification of their matrix structure/porosity, e.g. by plastic compression fabrication [6] and cross-linking [50], respectively.

Releasates from collagen- and pronectin-coated microspheres were also effective in inducing tubule formation *in vitro*. Delivery of VEGF into media from microsphere carriers through fibrin sol–gel was slower, lacking the 12 h burst release seen with collagen sol–gel. Therefore, the presence of fibrin, a known VEGF-binding protein [51], in the delivery vehicle promoted a more controlled factor release. Controlled/sustained release of factor(s) *in vivo* is indeed important for overcoming their short half-life period and promoting stabilization of the formed vasculature [52–54]. Interestingly, we could observe direct contact between endothelial cells/tubules and the microspheres. This suggests that the role of microspheres could extend beyond that of delivering loaded factors, to that of contact guidance, as previously shown with fibrin microbeads [55]. Indeed, the ability to provide a combination of chemotactic and haptotactic cues could be useful in tissue sites of extensive vascular compromise, where a local angiogenic source is lacking. Furthermore, since we showed that it is possible to control the release of microsphere carriers, and their loaded factors, through the sol–gel used as delivery vehicle, it would be feasible to regulate both these type of signaling using this hybrid system.

The system described here for obtaining cell-free carriers loaded with PBC-derived engineered factor mixtures could be readily integrated into a simple, one-step device (Fig. 8). The ability of such a device to house all steps of the process (i.e. blood collection, cell culture and factor harvest/delivery) provides a significant improvement over currently available bed-side systems for delivering autologous blood-based preparations, such as platelet rich plasma gel (e.g. Autologel system) [56], as it is now possible to deliver hypoxia-induced (i.e. angiogenesis-targeting) factor mixtures of higher protein concentration and potency, rather than only the factors already stored within cells at the time of blood collection. Relevant to this is also the utilization of whole blood, instead of any given cellular fraction, thus circumventing the need for cell isolation, which would evidently add a pre-handling step. From a biological perspective, we consider using whole blood advantageous, as it is known that in addition to the peripheral blood mononuclear cells, both the platelet and granulocyte fractions are strong angiogenic factor providers [40,57–60]. Finally, the possibility to deliver factors both in the form of a wound dressing (gel carrier; Fig. 8A) and an injectable preparation (microsphere carriers mixed with sol–gel; Fig. 8B), makes this device applicable to both open (e.g. wounds, ulcers, burns) and closed (e.g. transplanted tissue) tissue sites requiring angiogenic support. Such factor-loaded biomimetic (i.e. biodegradable) matrices, could potentially also serve as scaffolds for promoting tissue regeneration, as well as repair.



5. Conclusions

In this study we investigated a range of environmental factors that could potentially influence the angiogenic activity of PBCs, and therefore provide a means to control/modify this behavior. Engineered factor protein mixtures could be harvested within cell-free carriers, and were shown to be effective in inducing an angiogenic response *in vitro*. The proposed device design integrates this system and enables

Fig. 8. Schematic showing a prototype device for one-step harvesting and delivering angiogenic factor mixtures from autologous peripheral blood. A) Prototype version for delivering factors in the form of a wound dressing. B) Prototype version for delivering factors as an injectable preparation. A desired volume of blood (e.g. 10 ml) is added to the lower compartment, initially under vacuum, by aspiration through a port (1) that is designed to be compatible with standard blood-drawing equipment (e.g. BD Vacutainer ® system). The lower compartment contains an anticoagulant (e.g. EDTA) so that added blood remains in liquid state, ensuring uniform distribution of blood cells onto the matrix scaffold support at the bottom (2). Serum-free medium is then added to the lower compartment through a second port (3), completely fills the lower compartment and slightly extends, though the nano-porous filter (4), into the upper compartment. The filter prevents movement of cells and pathogens into the upper compartment. The vented cap (5), communicating with a nano-porous hydrophobic membrane for gas-exchange (6), is then opened. The device is now ready for conditioning, in a 37 °C 5% CO_2 incubator, under hypoxia (1–10% O_2). For space-efficiency, multiple devices can be stacked on top of each other in an incubator, by removing the device base lid (7) and fitting it onto the vented cap of the device below. Air flow is maintained through the base apertures (8). During culture, blood cells up-regulate production of angiogenic factor proteins that diffuse into the upper compartment, through the nano-porous filter, and are trapped within a cell-free carrier (9). The carrier can either be a nano-porous matrix (e.g. collagen gel) protected within two porous meshes (A, 9), or comprise micro-particles (e.g. collagen-coated microspheres) (B, 9). At the end of conditioning, the wound-dressing matrix carrier is removed by unscrewing the lid of the upper compartment (A, 10). Factor-loaded microspheres are combined with a sol–gel delivery vehicle (e.g. collagen gel) that is added though a port in the upper compartment (B, 10), and mixed by agitation to obtain an injectable preparation.

controlled delivery of one-step harvested PBC-derived factors, in the form of a wound dressing or an injectable preparation. The simple design, cost-effectiveness and one-step functionality make this device a promising tool to be used both at the bed-side, as an angiogenic therapy in wounds and peripheral ischemic tissue, as well as pre-, intra- and post-operatively as angiogenic support for central ischemic tissue, grafts, flaps and tissue engineered implants.

Disclosure statement

In the past 5 years AFS has provided consulting services to IPB and has received institutional support by Biomet, Curasan, Eucro, Heraeus, and Johnson & Johnson. There are no royalties to disclose. The device described in this article is protected under a patent (PCT/EP2013/051910), first filed in Feb., 2012 by E. Hadjipanayi, H.G. Machens and A.F. Schilling. This study was carried out under the umbrella of the Emacure™ Project (for more info please visit www.emacure.org).

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