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Regeneration through autologous hypoxia preconditioned plasma

Ektoras Hadjipanayi[1,2] and Arndt F Schilling[1,3,*]

[1]Experimental Plastic Surgery; Clinic for Plastic and Hand Surgery; Klinikum Rechts der Isar, Technische Universität München; Munich, Germany; [2]Department of Plastic, Reconstructive, Hand and Burn Surgery; Bogenhausen Hospital; Munich, Germany; [3]Center for Applied New Technologies in Engineering for Regenerative Medicine (Canter); Munich, Germany

Cellular hypoxic preconditioning is being employed to obtain complex, yet physiological, secretomes rich is angiogenic factors. We previously proposed exposing peripheral blood cells (PBCs) to hypoxic stress stimulation, and demonstrated that controlled release of PBC-derived factor mixtures induces directional microvessel growth in vitro. Hypoxia therefore provides a useful tool for enhancing the angiogenic potential of blood plasma, by generating compositions based on PBCs' natural responses to a wound-like microenvironment. Here, we discuss various methods for preparing and delivering Hypoxia Preconditioned Plasma (HPP), i.e., plasma derived after extracorporeal conditioning of anticoagulated blood under physiological temperature and hypoxia. Special emphasis is given to those approaches that will likely facilitate the clinical translation of HPP-based therapies. We finally draw a comparison between HPP and other, currently available blood-based products, and present the case that its arrival paves the way for developing next-generation autologous therapies toward angiogenesis-supported tissue repair and regeneration.

Keywords: hypoxia, blood, plasma, preconditioning, factors, angiogenesis, wound, ischaemia, therapy, regeneration

Abbreviations: VEGF, vascular endothelial growth factor; MMPs, matrix metalloproteinases; PF-4, platelet factor 4; TSP-1, thrombospondin 1; FGF, fibroblast growth factor; IL-8, interleukin-8

Correspondence to: AF Schilling; Email: a.schilling@tum.de

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Introduction

Hypoxic preconditioning of cells has been proposed to be a promising strategy for generating complex, yet physiological, angiogenic factor protein mixtures which can be delivered to ischemic tissues (e.g., wounds, ulcers, burns) to aid re-perfusion, repair and regeneration.[1-4] The development of this concept has been motivated by the realization that the mechanisms underlying the inadequate induction of compensatory angiogenesis, seen in many chronic ischemic/hypoxic conditions, involve a blunting of the ability of cells to effectively upregulate angiogenic factor (e.g., VEGF, Angiopoietins) production, largely owed to dysfunctional hypoxia-inducible factor (HIF) programming.[5-8] This depressed signaling appears to be the result of cellular habituation to prolonged/repeated hypoxic episodes, such that cells no longer respond as they normally would after an acute ischemic challenge.[1,9] If it were then possible to provide an ischemic tissue with the complete set of protein factor signals that would normally be present, had it not become habituated to chronic hypoxic stress, this should restart angiogenesis and drive it to completion. Additionally, with regards to the vascularization of grafts (e.g., skin, fat, muscle, bone grafts) and implants, the delivery of hypoxia-induced secretomes could confer a head start (corresponding to the length of in vitro preconditioning) to successful take/integration, by establishing early angiogenic support.

Among the various canditate cell types that are suitable for hypoxic stress stimulation, peripheral blood cells (PBCs) represent an ideal autologous cell type, since their easy harvest and ample availability means that the need for lengthy cell population expansion cycles, required for example when using skin fibroblasts obtained through a biopsy, is circumvented. PBCs can therefore be cultured directly after being obtained from

the patient. Importantly, blood plasma (in patients with normal nutritional status) provides a physiological source of electrolytes, glucose and other nutrients for PBCs, which also removes the need to use additional culture medium, a standard requirement for in vitro cell culture.

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,[10-15] bFGF,[11,14] IL-8,[12,14] MMP-9,[14] and have the ability to induce angiogenesis in vitro,[16,17] and in vivo upon implantation.[18] Furthermore, preconditioning PBMCs under hypoxia has been shown to increase their survival and angiogenic potency upon implantation into ischemic hindlimbs of mice.[19] It has also been recently demonstrated that intravenous administration of culture supernatant from irradiated apoptotic PBMCs confers cytoprotection to cardiomyocytes and inhibits tissue remodeling in rat and porcine acute myocardial infarction (AMI) models,[14] while emulsions containing PBMC supernatant could enhance wound closure in a mouse model.[20] 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,[21,22] improves cardiac function after AMI,[23] and accelerates the healing of refractory skin ulcers.[24,25] These findings strongly provide evidence that peripheral blood is indeed a suitable source of angiogenic factor producing cells.

Hypoxia preconditioned plasma (HPP), i.e., plasma derived after extracorporeal conditioning of anticoagulated blood under physiological temperature (37 °C) and physiological hypoxia (1–5% O_2, i.e., below the O_2 tension of mixed venous blood), represents a special form of conditioned culture medium, in that its composition (concentrations and ratios of factors) is stoichiometrically (i.e., precisely) defined by the patient's natural cell population phenotype (i.e., blood cell type / count), in contrast to conditioned media typically obtained by ex vivo/in vitro reconstituted culture methods.[26] This confers an important advantage when considering the large inter-individual variation present in terms of gene expression and growth factor-induced cellular responses, and forms the very basis for its utilization as an autologous therapy. Indeed, we could previously show that there is statistically significant variation in the ability of PBCs to upregulate VEGF expression in response to hypoxia between healthy individuals (19 BMI-matched, non-smoker subjects tested),[27] an effect that may be driven by differences residing within the HIF system itself.[28] Importantly, this inter-individual heterogeneity could be indicative of possible differences in the physiological requirements for generating compensatory angiogenesis and achieving an adequate wound healing response. For example, patients with no coronary artery collateral circulation (which may be protective against AMI) have a significantly lower hypoxic induction of VEGF than patients with collaterals.[29] The idea of administering a personalized HPP-based therapy, to stimulate angiogenesis-supported tissue regeneration, thus appears to be self-evident.

Dependence of PBC Angiogenic Signaling on Ambient Temperature

The correlation of cellular metabolic and protein synthetic activity is well established.[30] Angiogenic factor expression by cultured PBCs will, therefore, predictably depend on the ambient temperature, a direct controller of cellular metabolism. To confirm this, we measured VEGF levels in isolated PBC cultures carried under normoxia (exposure to hypoxia was avoided in order to reduce basal VEGF expression, i.e., background noise), and physiological temperature (37 °C) or room temperature (20 °C, RT). We found that new VEGF production (distinguished here from passive VEGF release through activated platelets, by culturing PBCs on collagen-coated supports, thus inducing an early release of VEGF stored within platelets at the time of blood collection[31]) was closely dependent on ambient temperature; day 2 VEGF supernatant concentration was ~3-fold higher in 37 °C cultures (91 ± 18pg/ml) than in RT cultures (34 ± 2pg/ml) ($P < 0.01$). Cumulative VEGF concentration appeared to follow a similar temporal profile under both temperature settings, peaking at day 4 and then falling back toward day 2 levels, from day 8 onwards. At all time points, however, VEGF concentration in 37 °C supernatants was significantly higher than in RT supernatants ($P < 0.05$) (new data, supplementary to Hadjipanayi et al., 2013[27]).

PBC viability was examined over this time course. While there was a steady increase in cell death over time, almost doubling from day 2 to day 4 ($P < 0.05$), and from day 8 to day 12 ($P < 0.05$), no significant difference was seen between the two temperatures tested, at any time point (new data, supplementary to Hadjipanayi et al., 2013[27]). Thus, the observed differences in VEGF level between 37 °C and RT cultures were likely, primarily, the result of true differences in gene expression induced by greater cellular metabolic/protein synthetic activity under the higher temperature. These findings demonstrate the importance of carrying out extracorporeal conditioning of blood under physiological temperature, as this will produce HPP of higher angiogenic potency.

Global Incubator Hypoxia vs. Local Cell-Induced Hypoxia

While exposing PBCs to hypoxic stress is an effective method for inducing angiogenic factor upregulation, it currently faces the common obstacle hindering the wider application of hypoxia-based strategies toward therapeutic angiogenesis, namely the limited availability of O_2-controlled incubators/chambers in clinical settings. A promising approach to overcome this limitation would be the employment of an engineered O_2 micro-environment, where O_2 tension is locally defined and continuously regulated by cellular O_2 consumption. By allowing PBCs to regulate their O_2 microenvironment, instead of exposing them to an artificial one, i.e., fixed/global hypoxia produced within an incubator, it

could be even possible to better simulate the conditions encountered in an in vivo wound setting. The direct correlation of pericellular O_2 tension with hypoxia-regulated gene expression, which also changes over time,[27] further suggests that a more physiological angiogenic response could indeed be achieved through cell-regulated hypoxia.

We previously showed that controlled, cell-mediated hypoxia can be achieved in the core of 3D collagen matrix depots, seeded at high density with dermal fibroblasts or vascular smooth muscle cells, by adjusting the total cell number and cell distribution within the depot.[32,33] Here, a VEGF response was elicited by cells exposed to low levels of O_2 (~3% O_2), primarily within the construct core.[32] Using a rabbit model, we demonstrated that subcutaneous implantation of living[2] or non-viable (frozen)[34] hypoxic cell-matrix depots, that actively produced factors or acted as carriers of factors trapped within the matrix during in vitro preconditioning, respectively, could induce directional infiltration of host endothelial cells into the depots within 1 wk. Importantly, this vascularization response was functional, as indicated by improvement of deep implant oxygenation. Our findings were validated by later work, in which grafting cord blood mesenchymal stem cells as spheroids in ischemic hindlimbs of mice was shown to improve therapeutic efficacy due to enhanced cell survival and paracrine activity, effects mediated by hypoxic cell preconditioning within spheroid cultures.[3] In this study, culturing cells as monolayer, where cells were not exposed to hypoxia, abrogated these effects. The findings of the work cited above, therefore indicate that cell-mediated hypoxia is a rational alternative to externally-controlled (i.e., incubator) hypoxia, in terms of its effectiveness as an angiogenic stressor.

PBC pericellular O_2 tension, being a function of cellular O_2 consumption, will predictably depend on the level of net aerobic metabolism, as well as the population cell number/viability. Both these parameters are directly related to the PBC seeding density, which is initially determined by the blood volume per unit area (BVUA) (**Fig. 1**). Since most cells, including PBCs, adapt to decreasing O_2 tension by reducing O_2 consumption,[35-39] with a compensatory shift to anaerobic (predominantly glycolytic[36,39]) metabolism, the development of persistent pericellular hypoxia in blood culture will have to rely on the presence of a minimum number of PBCs that remain viable and aerobically active (note; while hypoxia does not significantly reduce PBC viability compared with normoxia, a gradual increase in cell death is observed over time[27]). It is likely that, as a share of the total PBC population, this represents a statistically defined, rather than a fixed value, possibly highly varying between individuals (i.e., depending on blood cell count, age, pathology, genetic influence etc.). According to our preliminary data, a cell seeding density of ~$5x10^6$ WBCs/cm^2, corresponding to a BVUA = 1ml/cm^2, is sufficient for maintenance of severe pericellular hypoxia (< 1% O_2) over 7 d in cultures of blood obtained from healthy young subjects (new data, supplementary to Hadjipanayi et al., 2013[27]). Further studies, using larger samples, are admittedly required before a reproducible threshold PBC seeding density/BVUA range can be defined, which could then provide a reliable therapeutic guideline. Nonetheless, since the preparation of blood cultures with a defined BVUA can easily and cost-effectively be employed at the bedside, this approach will undeniably facilitate the clinical translation of HPP-based therapies.

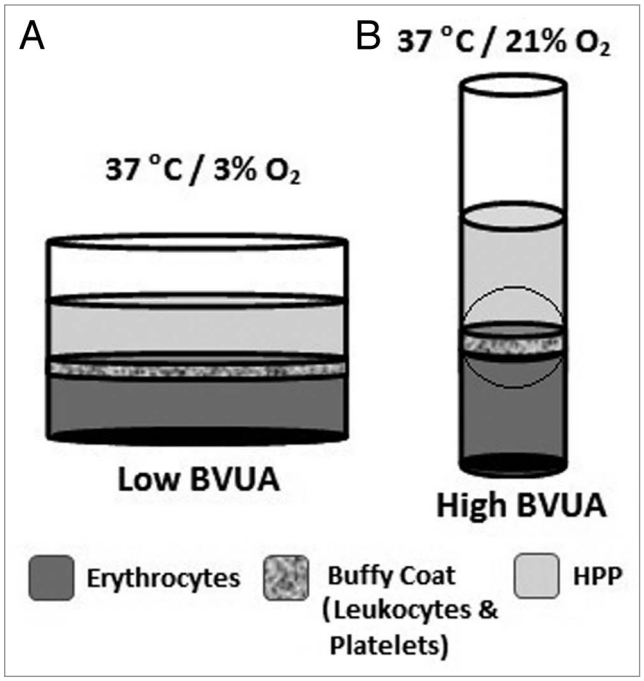

Figure 1. Schematic comparing the two approaches for culturing PBCs under hypoxic stress-stimulation, to obtain hypoxia preconditioned plasma (HPP). (**A**) Blood can be cultured at a low blood volume per unit area (BVUA; area refers to the well cross-sectional area), under global hypoxia, within an O_2-controlled chamber. Here, the large surface area ensures the uniform exposure of PBCs to the chosen O_2 tension. (**B**) Blood can be cultured within a normoxic chamber at a high BVUA, so that O_2 consumption by PBCs gradually generates a pericellular hypoxic micro-environment (shown by the circle). Here, the profile of O_2 tension adjacent to the buffy coat layer will be determined by the proportion of PBCs that remain viable and aerobically active. Typical values for a low and high BVUA are < 0,25ml/cm^2 and > 1ml/cm^2, respectively (based on preliminary data).

Controlling HPP Composition

Since hypoxia-induced PBC factor expression varies over time,[27] the length of hypoxic conditioning provides a key controller of HPP composition, in terms of the concentrations and ratios of factors present. For example, we showed that in contrast to VEGF, that is upregulated in hypoxia-exposed PBCs, the expression of the anti-angiogenic factor TSP-1 is initially upregulated, but then downregulated

under prolonged hypoxia.[27] We and other authors have previously discussed the inhibiting effect of hypoxia on anti-angiogenic signaling (e.g., TSP-1), as a possible natural mechanism for optimizing the long-term effectiveness of angiogenic factor cascades.[27,40,41] These findings, once again, highlight the dependence of physiological angiogenesis on the delicate balance of pro- and anti-angiogenic mediators, whose levels and ratios are dynamically regulated in both the temporal, as well as the spatial dimension.

With respect to determining the optimal length of the conditioning phase it is evidently important to consider the complete (to the extent that this is characterized) proteomic profile (i.e., pro- and anti-angiogenic factors), and not just pro-angiogenic factor expression, as this will ultimately determine the mixture's angiogenic effectiveness. Understanding the temporal profiles of factor expression is especially important in acute settings, where a compromise has to be met between urgency of application and potency of the preparation. For example, it could be envisaged that a composition derived after a short conditioning period (e.g., 24–48hrs) could be initially administered for providing early angiogenic support to the wound, while a more potent composition is being prepared. However, in addition to VEGF and TSP-1, studied in our previous study as exemplar, the temporal expression of other major factors has to be analyzed before a therapeutic timeline can be defined, and will be the focus of future work.

Controlling HPP Delivery

In addition to controlling the timing of delivery, the ability to locally deliver HPP-based compositions is equally important, in order to generate spatial factor gradients that promote chemoattraction of endothelial cells toward the target site.[42] For stimulating directional angiogenesis, HPP-factors could be loaded onto suitable carriers (e.g., nano-porous polymeric matrices) that could be topically applied to the wound or be injected subcutaneously through sol-gel vehicles.[4,27] In addition to using an exogenous sol-gel, localized injectable delivery could be achieved by taking advantage of the innate sol-gel properties of HPP, which can be induced to form a fibrin gel-matrix through activation of the coagulation cascade by combining it with thrombin/calcium (note; this is feasible in HPP prepared with at least 1 wk preconditioning, unpublished data). In such applications, exogenous fibrinogen could be optionally added to HPP, depending on the desired volume of the resulting gel-matrix, as well as the length of the conditioning phase (note; fibrinogen has a biological half-life of about 100 h). The in vivo-formed fibrin matrix then sequesters the factors at the site of injection, by specific binding (e.g., VEGF[43]) and/or passive trapping, and ensures their controlled release.[4] This also helps to avoid unwanted side-effects such as vascular leakage and ectopic angiogenesis. Such factor-loaded biomimetic/biodegradable matrices could, furthermore, potentially serve as scaffolds for migrating host cells (e.g., fibroblasts, endothelial cells) at a defect site, hence promoting tissue repair and regeneration.

Device for One-Step HPP Preparation and Controlled Delivery

Hypoxic stress stimulation is evidently accompanied by a significant degree of cell death.[27,32] Administration of apoptotic cellular material could potentially induce immunological and/or inflammatory adverse reactions. Consequently, safe utilization of HPP and other hypoxia-induced secretomes requires the development of systems for purification of the released proteins, by filtering out cellular components.

We recently reported on a cell-free carrier system for controlled delivery of protein factors present in HPP.[27] In our system, PBCs were cultured under wound-simulating conditions, on collagen or fibrin scaffolds under controlled hypoxia (3% O_2 / 37 °C), in order to promote upregulation of PBC angiogenic signaling. PBC-derived factors could be collected within cell-free collagen gel carriers, simultaneously as they were being produced, by diffusion through a nano-porous filter membrane separating the blood and carrier compartments. The angiogenic capacity of factor-loaded gels was demonstrated by the ability of their releasates to stimulate tubule formation, directional endothelial cell migration and sprouting in in vitro Matrigel assays. This system could be integrated into a simple bioreactor device that enables one-step sterile harvesting of HPP, in the form of cell-free matrix carriers for localized factor delivery (**Fig. 2**). Such a device will allow clinicians to carry out blood conditioning at the bedside, thus simplifying the currently laborious methodology of safe tissue handling and culturing procedures.

Comparison of HPP with Platelet-Based Therapies

Platelet activation and release of factors stored in their granules has long been advocated as a useful strategy for obtaining angiogenic compositions based on platelet concentrates, such as platelet-rich plasma (PRP) and PRP gel / platelet-rich fibrin matrix (PRFM).[44] However, it should not be dismissed that, in addition to a host of pro-angiogenic mediators, certain factors released by platelets (e.g., PF-4, TSP-1) are strongly anti-angiogenic. Such factors, especially when present in excess (i.e., supraphysiological concentrations, such as those found in platelet concentrates), might negatively impact the angiogenic effectiveness of platelet-rich products, by competing with or negating the effect of pro-angiogenic factors. This could indeed explain the limited success of PRP therapies in treating chronic wounds to date.[44,45]

Hypoxic preconditioning, on the other hand, offers a means for optimizing the angiogenic potential of blood plasma through hypoxia-induced changes in PBC factor expression, without merely relying on the release of platelet-derived factors. While this does occur to a certain degree, platelet activation is reduced, since HPP is conditioned in an anticoagulated state. For example, PF-4 levels in 5 d-conditioned HPP were found to be > 30% lower than in blood serum ($P < 0.05$) (new data, supplementary to Hadjipanayi et al., 2013[27]). It is also of interest to note that during wound healing the onset

of angiogenesis is temporally distinct from the early inflammatory phase, in which platelets are primarily involved in hemostasis (angiogenesis is typically induced 3-4 d after wounding),[46] while different platelet stimuli appear to evoke distinct secretion of pro-angiogenic and anti-angiogenic factors.[47] This suggests that platelets might play a regulatory, rather than a directly stimulatory role in new vessel formation. HPP could represent an improved alternative to platelet-rich products, by providing temporally-defined compositions based on PBCs' natural responses to hypoxic stress, i.e., conditions normally encountered within a healing wound. Treatment with HPP could therefore replenish the missing signaling in the non-healing wound, restart physiological angiogenesis where it has stalled, or support it, so that the regenerative process can progress to completion.

Conclusion

Hypoxia preconditioned plasma provides an optimized autologous hypoxia-induced secretome, since it is easy to harvest, while its factor composition is defined by the physiological and patient-specific cellular responses that mediate effective wound healing. It therefore forms a robust platform for the future development of hypoxia-based therapies toward angiogenesis-supported tissue repair and regeneration.

Disclosure of Potential Conflicts of Interest

In the past 7 y A.F.Schilling has provided consulting services to IPB and has received institutional support by Biomet, Curasan, Eucro, Heraeus, and Johnson and Johnson. There are no royalties to disclose. The device described in this article is protected under a patent (WO/2013/113821), filed in Feb. 2012 by E. Hadjipanayi, H.G. Machens and A.F. Schilling.

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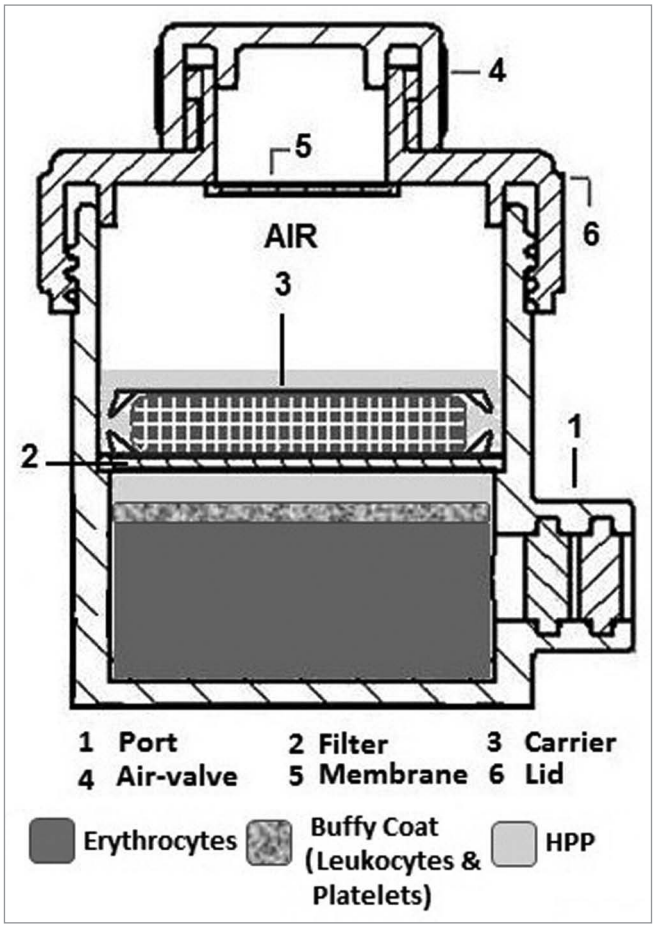

Figure 2. Schematic showing the bioreactor device for one-step harvesting and delivering protein factors present in hypoxia preconditioned plasma (HPP), through a cell-free matrix carrier. The device incorporates a nano-porous filter between the blood and carrier compartments, so that HPP sterilization and removal of cellular material occurs automatically during factor loading onto the matrix carrier. Note that since the plasma is passively separated from the blood cells, which sediment over time during conditioning, no centrifugation is required for HPP isolation (adapted from Hadjipanayi et al., 2013[27]).

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