Progenitor Cells and Hypoxia in Angiogenesis

Verloop, R.E.

2011

Link to publication in VU Research Portal

citation for published version (APA)
Verloop, R. E. (2011). *Progenitor Cells and Hypoxia in Angiogenesis*.

Discussion

Introduction

Improving neovascularization (the formation of new blood vessels during adult life) is an important topic both in the field of tissue engineering as well as in the development of new cardiovascular disease therapies. A better understanding of the contribution of endothelial progenitor cells (EPC) and the specific adaptations of endothelial cells (EC) during chronic hypoxia to neovascularization is urgently required to initiate more successful (pre)clinical studies on restoring efficient perfusion. Because of the evolving insight in the nature and origin of EPC during the course of these studies, we studied both the 'classical' EPC as well as the later identified endothelial colony-forming cells (ECFC). These ECFC are identical to the blood-outgrowth endothelial cells (BOEC), as described in Chapter 5[1]. Initially we thought these cells might serve as an enforcement of the endothelial lining, either as cell replacement because of endothelial injury or apoptosis, or to keep up with the demand for endothelial cells, which are recruited by the angiogenic process. During the course of the studies it appeared that two subtypes of progenitor cells were present in the 'classical' EPC population, the early or myeloid EPC and the endothelial colony-forming cells (ECFC/BOEC). In addition, we examined the genomic response of endothelial cells exposed to a prolonged period of hypoxia. Mostly due to technical limitations, hypoxia is mainly studied in the acute phase of tissue repair and neovascularization. However, studying the prolonged effect of hypoxia on endothelial cells may give a better approximation of pathological conditions, such as seen in peripheral artery disease or chronic heart failure.

Various types of circulating angiogenesis-stimulating progenitor cells

When we initiated our first studies on the potential beneficial contribution of EPC to angiogenesis, crude cell populations such as unselected peripheral blood and bone marrow-derived mononuclear cells were generally used. To enhance neovascularization by postnatal vasculogenesis, we decided to study the effect of the hematopoietic stem-cell containing CD34^{+} cell population on various components of neovascularization (**Chapter 4**). At that time it was hypothesized that certain cells in this CD34^{+} cell fraction functioned as angioblasts, giving rise to endothelial progenitor cells and endothelial cells[2-4]. The CD34^{+} population was also chosen to avoid later risk of unwanted side-effects such as stimulation of atherosclerosis[5, 6].

Recruitment and retention of CD34^{+} progenitor cells to sites of tube formation were the two initial components of EPC biology we studied (**Chapter 3**). Whereas the CD34^{+} cells manifested an at random movement over the growth factor- and cytokine-stimulated endothelial monolayer, the progenitor cells remained associated

with a developing tube once they reached them. Related to inflammation, adhesion receptors might be responsible for the specific recruitment and retention. Furthermore, in agreement with other parallel studies[7-11] we documented a very low number of CD34^{+} cells that actually incorporated in the endothelial lining of developing tubes and induced a modest stimulation of tube formation. In contrast, addition of cultured EPC (in **Chapter 4** also indicated as circulating angiogenic cells) or CD34^{+} co-cultured with CD34^{-} cells dramatically enhanced tube formation. Despite the positive expression of a number of endothelial markers on these cultured EPC, such as endothelial nitric oxide synthase (eNOS), VE-cadherin (CD144), VEGF receptor 2 (VEGFR2, or kinase insert domain receptor (KDR)), CD146 and CD31, it is highly likely that the cells used in these experiments resemble the so-called early EPC or CFU-EC[12-16]. In contrast to the believe at that time, these colonies were later shown not to be composed of endothelial cells, but to consist of a core of round hematopoietic cells, including myeloid progenitor cells, monocytes and T lymphocytes, and spindle-shaped monocytes/macrophages at the periphery. Although these cells manifest very limited or no incorporation in the endothelial lining at all, it does not exclude a role for these cells in angiogenesis and arteriogenesis, as was also evident from our experiments. Their effect on angiogenesis resembles the potent stimulatory role of monocytes and macrophages, which has been described by many investigators[17-29].

The peritubular positioning of the CD34^{+} progenitor cells in our studies has many similarities to that of myeloid progenitor cells, which undergo extravasation to become positioned around developing vessels in tumors where they stimulate tumor angiogenesis[30, 31]. Interestingly, when CD34^{+} and CD34^{-} cells were co-cultured the largest number of EPC colonies were formed, which were more successful in stimulating *in vitro* tubular sprouting than a pure population of CD34^{+} cells (**Chapter 4**). Virtually no cell clusters were formed when CD34^{+} and CD34^{-} cells were separated by a 0.4 µm pore trans-well system allowing diffusion of soluble factors without physical contact. These experiments suggested that direct cell-cell contact between CD34^{+}- and CD34^{-}-cells was necessary for endothelial differentiation of CD34^{+}-cells, but the involvement of platelet microparticles, which also cannot pass the filter and may contribute to the acquisition of endothelial marker proteins[32], cannot be excluded. Later on Van Beem *et al.* demonstrated that also the interaction between T lymphocytes and monocytes was essential for the extent of colony formation[15]. These results were in support of a myeloid nature of the CD34^{+} cell that we used in our initial studies. Furthermore, the modest purity of CD34^{+} cell preparations (90%) we used in these experiments may accommodate 'contaminating' cells with pro-angiogenic

potential or the capacity to differentiate into true endothelial cells. In support of this suggestion, we documented the appearance of clusters and confluent monolayers of cobblestone-shaped cells in long-term cultures of EPC (6 weeks), which phenotypically resembled mature endothelial cells. Also their *in vitro* growth kinetics showed a high similarity to the later characterized endothelial colony forming cells (ECFC).

As mentioned before, during the course of our studies, a novel type of EPC was identified and characterized. These so-called late-outgrowth endothelial cells, or endothelial colony-forming cells (ECFC, indicated as blood-outgrowth endothelial cell (BOEC) in **Chapter 5**), have the typical endothelial cobblestone morphology and display a wide range of endothelial markers, without the expression of hematopoietic or monocytic lineage markers CD45, CD14, CD11b or CD163[33-35]. Apart from the endothelial differentiation and high proliferative potential, these cells possess the capacity to form capillary-like tubes *in vivo* which connect to the resident vasculature, thereby becoming part of the systemic circulation[7, 8, 12, 36-40]. Both ECFC/BOEC and early EPC have been suggested as being suitable for therapeutic applications, to replace or support the host vasculature (Fig. 1)[12, 38, 41]. It is known for several years that cardiovascular risk factors and diseases, like hypertension and diabetes, negatively influence the number and functionality of the 'classical' EPC[42-48]. To circumvent this complication, genetic modification or incubation with pharmaceutical compounds have shown promising results in enhancing the potential of EPC in a preclinical setting[33, 49-52]. For ECFC, the number of reports on ECFC dysfunction is scarce[53, 54], but they also suggest a reduced presence in blood in diabetes and cardiovascular disease. Similar to the modification of 'classical' EPC, *ex vivo* modulation (e.g. priming) of ECFC is a valid option, for example by SDF-1, which enhanced binding to activated endothelium and stimulated FGF-2 and MMP-2 production by ECFC[55].

Choice of progenitor cells in tissue repair
In support of the preferred use of ECFC/BOEC over the early EPC for vascular regenerating cell therapies, the biological activities of the ECFC/BOEC more closely resemble those of mature differentiated endothelial cells, as we and others have clearly demonstrated[56] (Chapter 5). As both EPC types are present in only very low numbers in the circulation (0.1-0.01% of the mononuclear cells), the superior *ex vivo* expansion is a useful characteristic of ECFC/BOEC to obtain sufficient cell numbers for therapy. Furthermore, ECFC/BOEC are able to incorporate into an existing endothelial monolayer, thereby forming VE-cadherin positive cell-cell junctions[56]. The formation of tubular structures *in vitro,* as well as their integration into a functionally perfused capillary plexus *in vivo* are reasons for further preclinical testing[38, 57,

[58]. Since ECFC/BOEC can be derived from different sources, like peripheral blood, cord blood and bone marrow, we made an analysis of potential differences in the pro-angiogenic properties of peripheral and cord blood. Although a slightly more angiogenic phenotype favors cord blood-derived ECFC/BOEC, the difference could be matched by administering VEGF to peripheral blood ECFC/BOEC. Because autologous cord blood so far is usually not available for the often aged cardiovascular patients, this latter fact is of great value. Until banking of autologous cord blood has come into effect, our results suggest that PB-BOEC might be equally eligible as CB-BOEC, since equal proliferation and tube formation could be reached by the addition of VEGF.
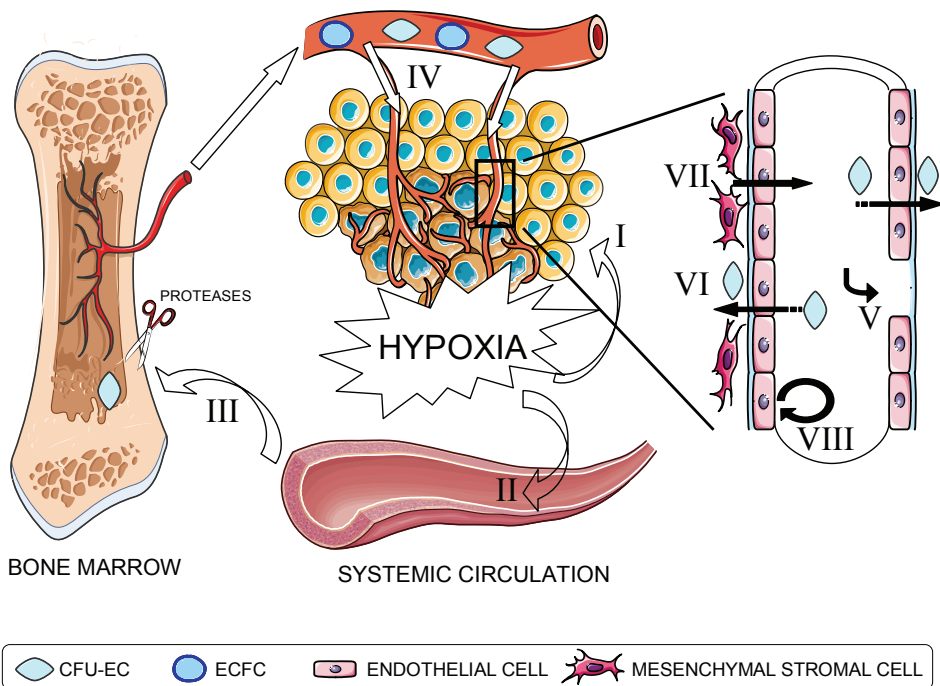
Fig 1. Contribution of endothelial progenitor cells to adult neovascularization
Hypoxia in a tissue induces HIF expression which controls the production of pro-angiogenic growth factors and cytokines in stromal cells (I). Proteins like VEGF and SDF-1 enter the circulation (II) and stimulate proteases (e.g. MMP-9) in the bone marrow (III), whereby resident progenitor cells are released in the systemic circulation. The progenitor cells of both CFU-EC and ECFC migrate towards areas of neovascularization (IV), where they position themselves either in the endothelial lining of developing blood vessels (V) or peri-vascular (VI). The ECFC support neovascularization by the supply of true endothelial cells which proliferate, migrate and form new blood vessels (VIII). Peri-vascular CFU-EC contribute to this process by the hypoxia-driven secretion of pro-angiogenic growth factors (VII).

Cellular cooperation in generating stable vessels

Despite the promise of EPC delivery in treating diseases associated with blood vessel disorders, (pre)clinical results on single EPC cell type infusions have been disappointing[59-61]. Given the different origins and functions of early EPC and ECFC/BOEC to neovascularization, Yoon *et al.* was among the first to investigate the possible synergism between these different cell types[40]. Injection of a mixture of the two types of cells resulted in superior neovascularization *in vivo* to any single-cell type transplantation. These results were supported by a more recent publication of Chade *et al.* who studied the effect of simultaneous delivery of early EPC and late-outgrowth EPC[62]. The pro-angiogenic role of growth factors secreted by the early EPC which coincided with the delivery of endothelial cells by the late-outgrowth EPC for neovascularization was suggested as a plausible explanation (Fig. 1). This described cooperation may have contributed to the significant stimulation of tube outgrowth by the relatively large number of early/classical EPC and the modest incorporation of supposed late-outgrowth EPC into the endothelial lining that we observed in our initial studies (Chapter 3). This type of combination therapy now awaits more thorough examination for possible therapeutic application.

Next to the induction of angiogenesis by growth factors, and the supply of endothelial cells (either by the resident blood vessel wall population, or by circulating progenitor cells) for capillaries, the developing blood vessels need to be stabilized by mural or perivascular cells. Mesenchymal stromal cells have been suggested as a promising source of angiogenesis-promoting and blood vessel-supporting cells[63-73]. Because of their multi-potency as well as immune suppressive properties, these cells make an interesting candidate for cell-based therapies[71, 74-82]. In fact, a positive role of paracrine factors produced by MSC on neovascularization has been described by a number of groups [83-103]. In agreement with other publications, we report the production and secretion of considerable amounts of pro-angiogenic factors, such as CXCL1, FGF-2, HGF, IL-6, MDK, u-PA, PGE_2, TGF-ß1, and VEGF by MSC (**Chapter 6**). Proliferation, one of the key components of angiogenesis was significantly increased when incubating human endothelial cells with conditioned medium from human fetal lung mesenchymal stromal cells (FL-MSC-CM). Furthermore, incubation of endothelial cells with FL-MSC-CM supplemented with the cytokine TNF-α significantly stimulated tube formation in a 3D fibrin matrix. VEGF and HGF were largely responsible for this stimulatory effect on angiogenesis, which is supported by a large number of reports[83, 87, 88, 91-100, 104-109]. Next to the production of growth factors and cytokines, proteases are also produced by MSC. MSC-derived uPA as well as MMP have been reported to have a stimulatory effect on endothelial tube formation

in 3D fibrin gels due, in part, to degradation of the matrix[105, 110].

In this context it is of interest to note that Au *et al.* used human bone marrow-derived mesenchymal stromal cells (hMSC) as a source of stabilizing perivascular cells[57]. MSC efficiently stabilized nascent blood vessels *in vivo* by functioning as perivascular precursor cells. The engineered blood vessels derived from human umbilical cord vein endothelial cells and hMSC remained stable and functional for more than 130 days *in vivo*. In contrast, single injection of endothelial cells resulted in the formation of tube-like structures, but these capillaries were non-functional and regressed within 30 days[57]. Co-implantation of progenitor cells for both endothelial cells as well as perivascular cells may have therapeutic potential above the use of terminally differentiated cells (Fig. 1). *In vivo*, the engineered vascular networks formed by coculture of human endothelial and mesenchymal progenitor cells isolated from blood and bone marrow remained patent at 4 weeks[58]. The formation of these long-lasting microvascular networks by postnatal progenitor cells obtained from less invasive sources constitutes an important step forward in the development of clinical strategies for tissue vascularization.

Endothelial transcriptional profile of short-term and prolonged hypoxia
One of the aspects that received still little attention is the behavior of endothelial progenitor cells and mature (differentiated) endothelial cells present in a hypoxic area. In particular, the different effects of short-term and prolonged hypoxia on vascular endothelial cells received little attention in regenerative medicine. This is rather contradictory since a number of diseases, like cancer, heart ischemia and chronic infection, are usually accompanied by prolonged hypoxia[111-121]. Because of the lack of data on chronic hypoxic exposure we started a detailed genomic analysis of human endothelial cells subjected to short and prolonged periods of hypoxia. Comparison with five previously published microarray studies on short-term hypoxia-exposed endothelial cells[122-126] showed a substantial overlap in identity of the genes, more than originally anticipated. The stringency of microarray analysis, which was responsible for a considerable underestimation of the number of hypoxia-induced genes, resulted in the identification of highly significant 'common' hypoxia-regulated genes. Genes with lower statistical significance may therefore appear at random in the various studies. More importantly, when we compared the effects of acute versus chronic hypoxia, it became clear that the differences were limited and reflected, if any, a quantitative rather than a qualitative difference. Indeed, only eight genes responded qualitatively different to the duration of hypoxia, based on statistical analysis. Concerning HIF protein levels, HIF-1α was transiently induced by hypoxia, but it decreased rapidly

again after 12 hours, while the transient activation of HIF-2α lasted for more than 24 hours, but finally also decreased. These data apparently imply that other factors co-regulate the activity of the HIF-1 circuit during chronic hypoxia.

In cardiovascular (patho)physiology, chronic hypoxia and subsequent chronic activation of the HIF pathway can be both adaptive as well as deleterious. The shift from aerobic to glycolytic metabolism as enforced by HIF-1α regulation is a beneficial short-term effect, whereas long-term protective effects included HIF-1α-/HIF-2α-mediated induction of angiogenesis[127-130]. Conversely, in advanced ischemic heart disease the adaptive regulation of HIF appeared to be imbalanced. Chronic activation of the HIF pathway *in vivo* resulted in severe progressive heart failure, formation of malignant cardiac tumors with the capacity to metastasize, and premature death[131]. A study on human chronic heart failure supported these findings, suggesting the detrimental effect of a chronically activated HIF system[132]. Furthermore, prolonged HIF signaling potentially contributed to the pathogenesis of endothelial dysfunction, characterized by decreased vasorelaxation, increased thrombosis and inflammation, as well as an altered angiogenic potential; all factors intimately associated with progression and severity of cardiovascular disease[133]. Under our experimental conditions prolonged hypoxia dramatically reduced HIF protein levels (**Chapter 7**). Increased expression of PHD-2, PHD-3 and HIF-3α4, an alternatively spliced variant of HIF-3α with a HRE-inhibiting domain structure[134, 135], were most likely responsible for this time-dependent repression of HIF. The adverse effects of a fully active HIF system during chronic hypoxia can thereby be prevented[119, 131, 136-142]. In fact, when we conducted our study on the time-dependency of hypoxia, it was demonstrated that chronic hypoxia induced HIF-1α and HIF-2α 'desensitization', caused by augmented PHD expression and activity[143].In addition, it was demonstrated that the negative Ets transcription factor (Net) may play a role in the time-dependent regulation of hypoxia-induced gene transcription, as was evident by the requirement for both HIF-1α and Net in a large number of hypoxia-regulated genes. Net regulated the expression of several genes known to control HIF-1α stability, including PHD-2, PHD-3 and Siah2[144]. Recently, Serchov *et al.* demonstrated that during short-term hypoxia PHD-1 and PHD-3 interacted with Net resulting in its stabilization which coincided with HIF-1α stabilization[145]. In mouse endothelial cells, Net repressed PHD-2 and -3 expression, partially prolonging HIF-1α-mediated regulation. However, in prolonged hypoxia, the induction of PHD-2 and PHD-3 gradually predominated, resulting in degradation of both Net and HIF-1α[145].

Regarding endothelial cells, hypoxia and its principal transcription factor HIF are considered the major driving forces for neovascularization[126, 146-151]. Apart from the formation of new capillaries, supplying arterioles need to enlarge, since short-term effects, like vasodilatation, are no longer sufficient to adequately regulate the perfusion of the expanded vascular bed[124, 152]. In agreement with others, we showed that the HIF pathway was markedly induced in endothelial cells during short-term hypoxia, with increased protein levels of HIF-1 and -2α[153-155]. These transcription factors migrate to the nucleus, where they form an active transcriptional complex with HIF-1β[156-159]. By binding to hypoxia-responsive elements inside the promoter regions of genes, a large number of angiogenic growth factors, receptors and proteases are synthesized, like vascular endothelial growth factor (VEGF)[160], placental growth factor (PlGF)[161], platelet-derived growth factor B (PDGF-B)[162], basic fibroblast growth factor (bFGF)[162], and stromal-cell derived factor 1 (SDF-1)[163]. Collectively, these factors promote endothelial cell survival, proliferation, and migration, whereby they try to restore tissue perfusion[164-170]. Although the current believe is that the majority of angiogenic molecules is synthesized and secreted by perivascular and stromal cells inside the hypoxic area[24, 163, 171-173], we found that endothelial cells also can contribute to this process by increased expression of the growth factors VEGF-A, VEGF-B, and PlGF as well as the proteases MMP2 and MMP10 (**Chapter 7**). Apparently some autocrine pathway is utilized to promote survival during prolonged periods of hypoxia, and perhaps even activates proliferation, migration, and capillary tube formation[166-169, 174, 175]. It is suggested that this response is triggered by hypoxia-induced activation of the MEK/ERK2-pathway, and, as documented in some studies, independent from VEGF, although this remains debatable[176-178].

Our studies have provided a first step to better understand the short- and long-term responses of human endothelial cells to hypoxia. Future studies have to verify whether there is a specific response of the endothelial genome to hypoxia. In this respect the considerable contribution of HIF-2α, which is more tissue-specific than HIF-1α may play a role[179-181].

Next to the important role of HIF, KLF-2 was identified as a potential hypoxia-regulated transcription factor, given its reduction during hypoxia which coincided with a number of direct and indirect KLF-2 target genes (**Chapter 7**). It appears that hypoxia partially mimics a pro-inflammatory transcription profile with increases in a number of proinflammatory and profibrotic genes that were repressed by KLF-2, such as MCP1, PAI-1, E-selectin and endothelin[182, 183]. Furthermore, a recent publication demonstrated the potent inhibitory role of KLF-2 on angiogenesis[184]. Mechanistically, KLF-2 promoted HIF-1α degradation in a von Hippel-Lindau protein-independent

but proteasome-dependent manner. The down-regulation of KLF-2 thus may also contribute to limit this way of HIF-1α degradation. It is too early to estimate whether KLF-2 might be a suitable target for modulating the angiogenic response in disease states.

The information on gene induction by acute and prolonged hypoxia in human umbilical vein and microvascular endothelial cells may act as a reference for future studies on the effects of various degrees and durations of hypoxia on early EPC and ECFC. Precursors of EPC typically reside in the hypoxic osteoblastic niche of the bone marrow. However, once these cells enter the blood, they become exposed to much higher oxygen tension. The chemo-attraction of these cells in the ischemic tissue, as driven by factors like VEGF and SDF-1, results in a completely new hypoxic environment with different properties being demanded from these cells. Understanding of their behavior at those sites is needed for optimal use of such cells in tissue repair.

Finally, although this thesis mainly focuses on the role of hypoxia on angiogenesis and adult vasculogenesis in neovascularization, it goes without saying that growth and development of larger supplying blood vessels is equally important in improving tissue perfusion[185]. During the acute phase of hypoxia, small capillaries are rapidly formed in, and around, the border of the hypoxic area. However, their survival is completely dependent on the proper connection to supplying arterioles. In a large number of types of tumors this process is imbalanced resulting in malfunctioning vasculature[186, 187]. In developing granulation tissue and wound repair more organized vascular structures are formed. The resident collateral vessels need to grow and mature in order to bypass the blockage and find connections to the newly formed capillary plexus. Without this process, the small blood vessels as formed in hypoxia-induced angiogenesis rapidly become obsolete and are swiftly degraded[188-191].

Perspective

The concept of progenitor cells that can differentiate into true endothelial cells with high proliferative potential or into perivascular-positioned, angiogenesis-promoting cells is attractive, but also highly debated. As mentioned before better characterization and identification of ECFC and its circulating precursor as well as the early outgrowth/circulating angiogenic cells in the blood by specific markers would help to improve the isolation, separation and amplification of these cells, and the possible use of these cells for autologous and possibly heterologous transplantation.

Hypoxia plays a key role in the overall biology of progenitor cells[24, 163, 173]. Upon impaired tissue perfusion, hypoxia induces the expression of various cytokines and growth factors in perivascular cells (vascular fibroblasts, smooth muscle cells). These factors initiate the mobilization of endothelial progenitor cells and circulating angiogenic cells from the bone marrow to the systemic circulation[192, 193]. Locally, hypoxia is responsible for a cytokine gradient across the blood vessel which, presumably, is responsible for progenitor cell homing and extravasation, whereby these cells positioned themselves in and around the developing vessels[194]. By secreting proangiogenic molecules, the perivascular myeloid cells support resident endothelial cells and endothelial progenitors to proliferate and thereby neovascularization. In all of the above-mentioned steps, proteases are indispensable. Manipulating mobilizing and/or entrapment signals may offer therapeutic opportunities to stimulate angiogenesis[30, 195-198].

In vivo, a number of diseases are most likely responsible for the failure to adequately compensate for reduced perfusion upon vascular occlusive events. The resulting hypoxia would normally activate the HIF system, thereby initiating a cascade of adaptive processes to restore perfusion. However, in type 2 diabetes, high glucose is responsible for a decrease in transactivation by HIF-1α during hypoxia[199]. Covalent modification of the co-factor p300 by the dicarbonyl metabolite methylglyoxal resulted in impaired binding to HIF-1α and a reduction of VEGF production, thereby hampering neovascularization. It is therefore not surprising than compounds are being designed to stimulate HIF signaling. The clinical use of PHD and FIH inhibitors has been suggested in ischemic disorders such as myocardial infarction, stroke, and peripheral artery disease to promote neovascularization and restore tissue oxygenation. These organic small molecules interfere with the utilization of iron and/or 2-oxoglutarate by the PHD resulting in inhibition of HIF prolyl hydroxylation and activation of HIF-dependent transcription[200-207]. Promising results have been documented in the preclinical treatment of stroke, anemia, myocardial infarction, and ischemic renal disease[208-215]. Although some of these agents have now entered human clinical trials, the safety of chronically administering HIF prolyl hydroxylase inhibitors to humans remains an important issue, especially given the potential links between HIF and cancer[212, 216, 217].

Adenoviral or peptide-based gene delivery of stabilized HIF-1α is another strategy, and has been shown to effectively enhance angiogenesis and vascular maturation in dermal wounds, reduce infarct size in a preclinical model of myocardial infarction, and improved outcome in a mouse model of diabetic peripheral artery disease[218-225].

In addition to this preclinical data, a clinical study had provided evidence that genetic variation at the HIF-1α locus contributes to variation in the arteriogenic response to ischemia. The relative risk for the absence of collaterals was increased five-fold in cardiovascular patients with the variant allele[226]. In these patients, a relative deficiency of HIF-1α may prevent arteriogenesis and thus provide a rational for HIF-1α gene therapy[227].

Altogether, this thesis provides a wide platform of fundamental research and results on two important determinants of neovascularization, being hypoxia and pro-angiogenic progenitor cells. The described mechanisms may help to initiate novel preclinical experiments to increase the knowledge on stimulating neovascularization, supporting the fields of cardiovascular research as well as tissue engineering.

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