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Hypoxia-Regulated Pro- and Anti-Angiogenesis in the Heart

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1. Introduction

Maintenance of oxygen homeostasis is essential for the survival of all multicellular organisms. Specialized chemoreceptor cells can sense increased (hyperoxia) or decreased (hypoxia) oxygen levels and subsequently regulate cardiovascular and ventilatory rates. Additionally, all nucleated cells sense and respond to reduced O_2 availability acutely, through the activation of proteins, and chronically, through the regulation of gene transcription. Decreased oxygen levels stimulate the oxygen delivery system and provide mechanisms to activate cell death or survival pathways, depending on the context of the hypoxia signal. Several responses are developed by cells and tissues faced with a hypoxic challenge, particularly in the heart: 1) increased ventilation and cardiac output, 2) a switch from aerobic to anaerobic metabolism, 3) promotion of improved vascularization via angiogenesis, and 4) enhanced O_2 carrying capacity of the blood. Hypoxia occurs in physiological situations such as during embryonic development, as well as in pathological conditions such as ischemia, wound healing, and cancer.

Angiogenesis is the process by which blood microvessels are formed from existing ones, which is required for development and occurs in pathological conditions. It is also important for reducing myocardial hypoxia due to coronary and ischemic heart disease. In myocardial infarction or chronic ischemic heart disease, angiogenesis responds to tissue hypoxia by new vessel formation, which diminishes myocardial ischemia. Nevertheless, physiological angiogenesis is usually insufficient to re-establish an adequate blood supply to the myocardium, which decreases its proper functioning. Therapeutic angiogenesis in the heart aims at increasing new vessel formation in ischemic myocardium and thus improving myocardial function by increasing blood flow (oxygen and nutrient supply). This may contribute to preventing heart failure and sudden cardiac death. However, hypoxia also stimulates several angiogenic inhibitors in the heart while pro-angiogenic factors are increased. This spontaneous balance reaction is one of the causes for the failure of angiogenic therapy for ischemia heart diseases. Unfortunately, the area of anti-angiogenesis in the heart has remained unclear. This chapter reviews the effects of both angiogenic and anti-angiogenic reactions in the heart dependent on the endothelial response to hypoxic /ischemic stimulation.

2. Hypoxia-regulated angiogenesis in the myocardium

2.1 HIF1-α-mediated angiogenesis

Hypoxia-stimulated myocardial angiogenesis has well been studied in past decades. When the myocardium is deprived of blood, a process of ischemia, infarction, and myocardial remodeling is initiated. Hypoxia-inducible factor 1 (HIF-1) is a transcriptional activator of vascular endothelial growth factor (VEGF) and is critical for initiating early cellular responses to hypoxia in specimens of human heart tissue (Lee *et al.*, 2000). Upon initiation of the hypoxic signal, HIF1-α translocates to the nucleus, dimerizes with HIF1-β to form the HIF-1 complex and induces the expression of its transcriptional targets via binding to hypoxia-responsive elements (HREs) (Chilov *et al.*, 1999). HREs are present in many angiogenic genes, such as VEGF, angiopoietin-2, VEGF receptors (Flt1 and KDR), and neuropilin-1 (Hickey and Simon, 2006; Simons, 2005). Hypoxia can up-regulate these angiogenic molecules by several mechanisms, including direct transcriptional activation by HIFs or indirect up-regulation by HIF-induced molecules. In addition, previous studies suggested that hypoxia/reoxygenation promotes myocardial angiogenesis via an NF kappa B-dependent mechanism in a rat model of chronic myocardial infarction (Sasaki *et al.*, 2001).

Additional transcription factors induced by hypoxia, such as Related Transcription Enhancer Factor-1 (RTEF-1) and early growth response 1 (EGR-1), can both target VEGF to enhance angiogenesis (Shie *et al.*, 2004; Yan *et al.*, 2000). Additional angiogenic growth factors such as IGF are also induced by hypoxia, but can signal through a HIF-1-independent pathway (Slomiany and Rosenzweig, 2006).

Moreover, hypoxia is associated with virtually all forms of vascular disorders, such as coronary and peripheral arterial diseases, including stroke, myocardial and limb ischemia; lung disorders; and diabetes (Fong, 2008). The post-ischemia neovascular response is largely driven by hypoxia-induced up-regulation and stabilization of VEGF, initiated in part by HIF1. In concert with enhanced expression of VEGF and its receptors, other growth factors and cellular receptors, including placental growth factor (PlGF), basic fibroblast growth factor (bFGF), IL-8 and ETS-1 are also differentially regulated in highly specific spatio-temporal expression patterns, orchestrated to acutely minimize parenchymal damage and to optimize subsequent healing and recovery. Physiological stresses such as hypoxia are regulated by a complex balance of both stimulatory and inhibitory signals that promote or inhibit angiogenesis. Specifically, understanding the role and regulation of genes during angiogenesis is becoming increasingly important in elucidating the compensatory hypoxic response.

2.2 Hypoxia upregulated microRNA-mediated angiogenesis

Recent studies have revealed important roles for microRNAs (miRNAs, miRs) in regulating endothelial cell function, especially in angiogenesis. miRNAs are a class of conserved non-coding small RNAs, with the ability to repress gene expression post-transcriptionally by targeting 3′-untranslated regions (3′UTRs) of mRNAs. Almost 700 miRNAs have been identified in humans, and the estimated number of miRNA genes is as high as 1000 in the human genome (Berezikov *et al.*, 2005). Current evidence demonstrates that miRNAs are important regulators of angiogenesis as well as cardiovascular development and disease.

Pro-angiogenic miRs (angiomiRs) regulate angiogenesis, for example, by targeting negative regulators in angiogenic signaling pathways, while anti-angiomiRs inhibit angiogenesis by targeting positive regulators of angiogenesis. Let7-f, miR-27b, mir-130a, miR-424, miR-296, and miR-210 have been shown to be pro-angiomiRs (Fish *et al.*, 2008; Ghosh *et al.*, 2010; Urbich *et al.*, 2008; Wurdinger *et al.*, 2008).

Let-7f and miR-27b exert pro-angiogenic effects as evidenced by the blockade of i*n vitro* angiogenesis with 2'-O-methyl oligonucleotide inhibitors (Kuehbacher *et al.*, 2008), while miR-210 is induced by hypoxia in endothelial cells and overexpression enhanced the formation of capillary-like structures. Additionally, overexpression of miR-210 enhanced VEGF-driven migration of normoxic endothelial cells, whereas inhibition of miR-210 decreased tube formation and migration (Fasanaro *et al.*, 2008).

Endothelial overexpression of miR-424 increased proliferation and migration, as a result of autocrine stimulation by HIF-1α–driven VEGF secretion. In addition to the *in vitro* changes in angiogenesis, miR-424 levels were significantly increased during vascular remodeling *in vivo*. Changes in miR-424 were observed in models of experimental myocardial infarction and PAD. Interestingly, the rodent homologue of miR-424, miR-322, was upregulated in peri-infarct cardiac tissues in rats following LAD ligation. Left ventricular tissues harvested away from the infarcted area also showed significantly higher levels of mu-miR-322. Ischemia seems to activate mu-miR-322 expression in the tissues surrounding the site of hypoxic stress as a survival mechanism to initiate collateral vessel growth (Ghosh *et al.* 2010).

Neoangiogenesis is essential for cardiac repair following myocardial infaction, when collateral vessels form at the site of the infarct and can maintain blood flow to ischemic tissue. The endothelial-specific miR-126 plays a role in neoangiogenesis following myocardial infarction and in the maintenance of vascular integrity. miR-126 potentiates MAP kinase signaling downstream of VEGF and FGF via Spred-1, an intracellular inhibitor of the Ras/MAP kinase pathway. miR-126 over-expression relieves the repressive influence of Spred-1 on the signaling pathways activated by VEGF and FGF, inducing angiogenesis. Elevation of miR-126 expression in the ischemic myocardium could enhance cardiac repair (Wang *et al.*, 2008).

3. Therapeutic angiogenesis in the myocardium

In coronary artery disease (CAD), progressive occlusion of arteries can lead to collateral vessel development to supply the ischemic tissue. In spite of this, angiogenesis via neovascularization is not always sufficient as evidenced by the large number of revascularization procedures performed annually. The lack of a sufficient angiogenic response in part may be related to both the decreased production of angiogenic factors as well as a natural negative feedback by the upregulation of anti-angiogenic molecules. Therapeutic angiogenesis involves improving blood flow to ischemic tissue by the induction of neovascularization by angiogenic agents administered as recombinant protein or by gene transfer. To circumvent insufficient collateral vessel development, administration of recombinant protein or the genes that encode these proteins have been used as techniques of angiogenic therapy in preclinical and clinical trials. Yet, results of these trials have only been slightly beneficial for patients.

4. Anti-angiogenic genes in response to hypoxia

While hypoxia-induced VEGF initiates angiogenesis, it also up-regulates expression of molecules within the endothelium that act as negative feedback regulators to modulate excessive vascular sprouting and endothelial cell proliferation (Messmer-Blust *et al.*, 2009). Additionally, a previous report by An et al. demonstrated that Response Gene to Complement (RGC)-32 is another important VEGF-inducible gene that serves as a negative regulator of hypoxia-induced angiogenesis. RGC-32 expression was significantly increased under hypoxic conditions. Hypoxic induction of RGC-32 was also mediated by HIF-1α at both the transcriptional and posttranscriptional levels (An *et al.*, 2009). While HIF-1-induced RGS5 was not VEGF-mediated (Jin *et al.*, 2009), RGC-32 expression was significantly increased by VEGF but not by factors including TNF-α, FGF2 and IL-1β (An *et al.*, 2009). Unlike genes such as COX-2 (Wu *et al.*, 2003), RGC-32 did not follow the canonical VEGF-induced angiogenic pathway. Overexpression of RGC-32 destabilized vascular structure formation *in vitro* by down-regulating FGF-2 and cyclin E, which caused negative feedback to VEGF activation. Additionally, when angiogenesis was examined *in vivo* in a mouse model, RGC-32 drastically inhibited VEGF-induced angiogenesis in matrigel, attenuated the recovery rate in hindlimb ischemia and reduced tumor size (An *et al.*, 2009).

VEGF also induces Delta-like ligand 4 (Dll4), a part of the Notch signaling pathway expressed in the vascular endothelium, yet has anti-angiogenic properties (Lobov *et al.*, 2007). Lobov, et al. demonstrated that Dll4 is an antagonistic regulator of angiogenesis by injecting a soluble version of Dll4 (Dll4-Fc) that blocks Dll4/Notch interactions into the vitreous membrane of oxygen-induced ischemic retinopathic (OIR) mice. Blocking Dll4 significantly enhanced angiogenic sprouting while suppressing ectopic pathological neo-vascularization in the retinal vasculature (Lobov *et al.*, 2007). Therefore, Dll4 plays a role as a negative regulator of sprouting angiogenesis in response to the release of hypoxia-induced factors such as VEGF (Lobov *et al.*, 2007).

In addition to VEGF-induced anti-angiogenic genes, the hypoxia-induced VEGF itself is also subjected to negative regulation, such as by transcription factor E2F1 (Qin *et al.*, 2006). Under hypoxic conditions, VEGF is induced by HIF-1; however, E2F1 down-regulates expression of VEGF by associating with p53 and specifically down-regulating VEGF expression but not other hypoxia-inducible genes. Studies of E2F1-/- mice under hypoxic conditions revealed enhanced angiogenesis dependent on de-regulated VEGF, which illustrates a novel negative regulation to VEGF.

5. Anti-angiogenesis in myocardial ischemia

Hypoxia, as a result of impaired blood flow, has a hazardous effect on organ structure and function, especially in the heart, i.e. myocardial ischemia. Acute coronary syndromes resulting from occlusion of one of the coronaries exposes the heart to ischemic conditions. Short periods of ischemia (~20 minutes) are reversible if followed by reperfusion. If coronary occlusion is prolonged, necrosis can propagate from subendocardium to subepicardium. Additionally, reperfusion beyond a few hours does not reduce myocardial infarct size.

Functional recovery of the ischemic tissues and organs is dependent on re-establishing collateral networks that sufficiently supply hyper-oxygenated blood to specialized cell

populations. In response to ischemic insults, most tissues in the body have the capacity to compensate for low levels of oxygen by mechanisms of vasodilation, angiogenesis, arteriogenesis, vascular remodeling, and hematopoiesis (Boutin *et al.*, 2008; Makino *et al.*, 2001). Coronary collateral vessels can increase blood flow to regions of the heart supplied by arteries with high-grade stenosis, thus protecting the myocardium from ischemia (Heinle *et al.*, 1974). Collateral formation is highly variable between patients and only partially attributable to differences in the degree of the coronary artery occlusive disease. For therapeutic angiogenesis, it is essential to understand the molecular mechanisms of angiogenesis and arteriogenesis in relation to tissue hypoxia.

Angiopoietin-2 (Ang2) is important in the inhibition of angiogenesis induced by hypoxia; previous reports determined that hypoxia enhances Ang2 expression both *in vivo* and *in vitro* (Oh *et al.*, 1999). The angiopoietin ligands/Tie receptors belong to a class of ligand/receptor families that play a critical role in angiogenesis, cell migration, proliferation, and survival. Ang1 and Ang2 are both ligands with a similar binding affinity for the Tie2 receptor, which is a member of the receptor tyrosine kinases (RTKs) and is predominantly expressed by vascular endothelial cells (Maisonpierre *et al.*, 1997). Ang1 induces angiogenesis via autophosphorylation of Tie2, while Ang2 competitively inhibits this effect (Maisonpierre *et al.*, 1997). Ang2 is an antagonist for the Ang1/Tie2 pathway, as supported by data from over-expressing Ang2 mice that showed a very similar phenotype to the Ang1 and Tie2 single knockouts (Yuan *et al.*, 2009). Interestingly, Ang2 possesses both partial agonistic as well as antagonistic action on Tie2 in the endothelium; alone, Ang2 is a weak activator of Tie2, whereas in the presence of Ang1, Ang2 inhibits Tie2 signaling. When the endothelium is stimulated with Ang1 and Ang2, Ang2 dose-dependently inhibits Ang1-induced Tie2 phosphorylation, Akt activation, as well as EC survival.

Though *in vitro* experimental studies have shown that VEGF, Ang-2, and Tie-2 but not Ang-1 expression is upregulated in ischemic myocardium, (Hashimoto *et al.*, 1994; Matsunaga *et al.*, 2003), clinical studies in patients with acute myocardial infarction have only shown increased peripheral blood VEGF (Kranz *et al.*, 2000). However, a more recent study showed that in addition to increased VEGF and Tie-2 levels, Ang-2 levels were also increased. Additionally, patients with evidence of myocardial damage (ie, AMI) had the highest levels of Ang-2, VEGF, and Tie-2 compared with other groups (Lee *et al.*, 2004).

6. Feedback loops in angiogenic inhibition in response to hypoxia in the heart

As a hypoxia-induced transcription factor, HIF-1 both stimulates and represses a multitude of genes important for adaptation to the low oxygen environment. Regulator of G protein Signaling 5 (RGS5) is a HIF-1-dependent, hypoxia-induced angiogenic inhibitor (Jin *et al.*, 2009) that functions as a negative regulator of G protein-mediated signaling (Adams *et al.*, 2000; Bell *et al.*, 2001). Previous reports showed that hypoxia specifically increased RGS5 expression in endothelial cells; RGS5 mRNA expression was induced by hypoxia while two other family members, RGS2 and RGS4, were not impacted (Jin *et al.*, 2009). In addition to changes in oxygen levels, HIF-1α played a key role in hypoxia-induced RGS5 expression by stimulating RGS5 promoter activity in endothelial cells. RGS5 slowed endothelial cell growth and significantly enhanced the apoptotic protein Bax, which led to increased apoptosis due to the change in the Bcl-2/Bax ratio (Jin *et al.*, 2009; Yang and Korsmeyer,

1996). Furthermore, RGS5 inhibited VEGF-induced angiogenesis through the p38 MAPK-dependent pathway *in vitro* and *in vivo.*

Additionally, a more recent report demonstrated that insulin growth factor binding protein-6 (IGFBP-6) was upregulated in vascular endothelial cells in response to prolonged hypoxia and can inhibit angiogenesis *in vitro* and *in vivo* (Zhang *et al.* 2011). Sequence analysis of the human IGFBP-6 promoter suggested that hypoxia induces the expression of IGFBP-6 in VECs, likely via a HIF-1-mediated mechanism, as there are 8 canonical HREs located in the promoter region of human IGFBP-6 gene. Interestingly, hypoxia has been shown to rapidly induce IGFBP-1 (Kajimura *et al.*, 2005; Tazuke *et al.*, 1998), whereas the up-regulation of the IGFBP-6 gene is not observed until 48 h of hypoxia, implying that IGFBP-6 induction by hypoxia may act as a negative feedback mechanism in hypoxia-induced angiogenesis.

Furthermore, previous studies suggest that HIF1α function is regulated by mitogen activated protein (MAP) kinases (Berra *et al.*, 2000; Minet *et al.*, 2000; Richard *et al.*, 1999). Specifically, the closely related MAP kinase, BMK1/ERK5, is necessary for vasculature remodeling and maintenance. Embryos deficient for the BMK1 MAP kinase die between E10.5 and E11.5 due to angiogenic failure and cardiovascular defects. BMK1 deficiency leads to increased VEGF dysregulation, which impedes angiogenic remodeling and vascular stabilization. Additionally, BMK1 negatively regulates transcription from VEGF during hypoxia. Further research confirmed BMK negative regulation using a conditional BMK1 knockout mouse, which revealed that vascular integrity was compromised and EC apoptosis was increased (Hayashi *et al.*, 2004; Pi *et al.*, 2004; Sohn *et al.*, 2002). Upregulation of BMK1 negatively regulates HIF1α and angiogenesis by increasing HIF1α ubiquitination and degradation in endothelial cells (Pi *et al.*, 2005).

7. Hypoxia-induced apoptosis

During angiogenesis, endothelial cells undergo proliferation, reorganization, and stabilization to establish a mature vascular network. Apoptosis occurring in endothelial cells causes an inhibitory effect on cell proliferation, which has a similar impact to that of anti-angiogenic factors. Hypoxia promotes apoptosis in endothelial cells, as demonstrated by changes in p53 protein levels (Hammond and Giaccia, 2005; Stempien-Otero *et al.*, 1999). Several studies suggest that HIF-1α stabilizes p53 and contributes to hypoxia-induced p53-dependent apoptosis (Xenaki *et al.*, 2008). A recent report proposed that P300/CBP-associated factor (PCAF), an HIF-1α cofactor, regulates the balance between cell cycle arrest and apoptosis in hypoxia by modulating the activity and protein stability of both p53 and HIF-1α (Xenaki *et al.*, 2008). Previous reports demonstrated that p53 can also inhibit angiogenesis by the following mechanisms: elevating thrombospondin-1 levels, repressing both VEGF and FGF-2, and by inducing degradation of HIF-1 (Folkman, 2006).

HIF-1α mediates hypoxia-induced apoptosis through the up-regulation of genes as well as suppression of genes such as Bcl-2 (Carmeliet *et al.*, 1998; Jin *et al.*, 2009). One member of the Bcl-2 family, Bcl-2/E1B interacting protein (BNIP3) is up-regulated under hypoxic conditions (Chen *et al.*, 1997; Kothari *et al.*, 2003). Kothari and colleagues showed that blocking hypoxia-induced BNIP3 expression using siRNA or a mutant BNIP3 inhibits hypoxia-induced cell death. Additionally, hypoxia-mediated BNIP3 expression occurs by direct binding to an HRE in the human BNIP3 promoter, and mutation of this HRE site

eliminates the hypoxic responsiveness of the promoter. Furthermore, hypoxia-induced BNIP3 expression was detectable 24 h after initial up-regulation of HIF-1α, indicating that BNIP3 expression occurs much later in the hypoxic response than genes such as VEGF (Kothari *et al*., 2003).

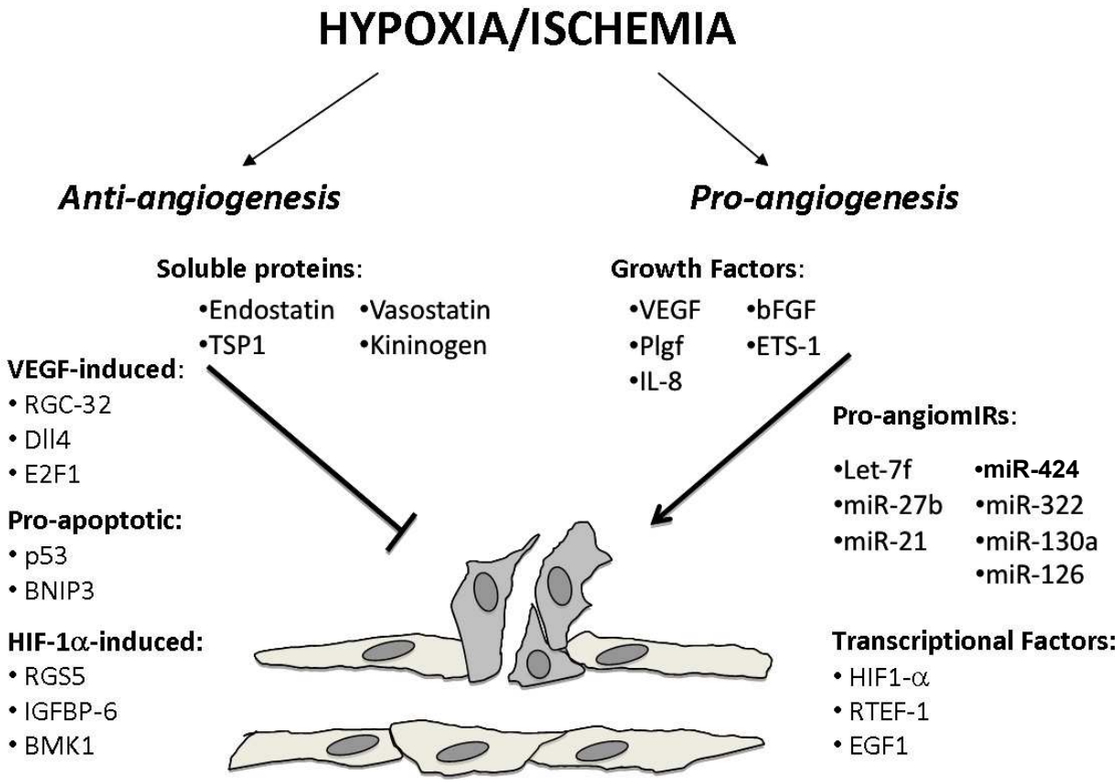

Fig. 1. Schematic illustration of hypoxia/ischemia-induced factors that can balance the decision between pro- and anti-angiogenesis in endothelial cells (EC). It is classified by direct indirect induction through molecules such as HIF-1, VEGF and transcription factors. This delicate equilibrium can easily be influenced by diverse factors, including angiogenic cytokines, local remodeling of the ECM, growth factors, inflammation, and most importantly, exposure to hypoxia.

While HIF-1 mainly targets pro-angiogenic genes, there are feedback molecules to counteract this up-regulation, such as endostatin, which down-regulates HIF-1α (Abdollahi *et al*., 2004). Hypoxia-induced endostatin inhibits VEGF-induced angiogenesis by preventing proliferation and migration of endothelial cells (Abdollahi *et al*., 2004; Heljasvaara *et al*., 2005; Morbidelli *et al*., 2003) and simultaneously up-regulates anti-angiogenic genes, including thrombospondin (TSP1), vasostatin, and kininogen (Abdollahi *et al*., 2004). Reduced oxygen supply in FVB mice exposed to a hypobaric hypoxia chamber showed enhanced MMP production leading to a subsequent increase of endostatin generation in the lung and aorta (Paddenberg *et al*., 2006). These results were confirmed by Suhr et. al, who reported a significant increase in the endostatin and MMP-9 plasma concentration following hypoxic conditions in human cyclists (Suhr *et al*., 2007). Further studies done on TSP-1 by Morgan-Rowe et. al. showed that it is induced in hypoxia-conditioned media and blocks

proliferation of HMEC-1 cells. Increased TSP-1 levels were found following 24 hours of hypoxia in the HMEC-1 media. Additionally, caspase 3 levels were increased followed by inhibition of proliferation and subsequent apoptosis, despite the elevation of VEGF (Morgan-Rowe *et al.,* 2011).

8. Equilibrium between hypoxia-induced angiogenesis and anti-angiogenesis/apoptosis in the heart

Hypoxia promotes pro- and anti-angiogenesis as well as apoptosis in endothelial cells. Apoptosis occurring in endothelial cells causes an inhibitory effect on cell proliferation, which has a similar impact to that of anti-angiogenic factors, as demonstrated by changes in p53 levels in hypoxia. It is important for these three reactions to remain in equilibrium during myocardial ischemia. Additionally, several factors can be both pro- and anti-angiogenic in subsequent studies illustrating the intricacies innate to blood vessel growth.

9. Clinical relevance to coronary artery diseases

Current approaches available for patients with ischemic heart disease include medical therapy or coronary revascularization by percutaneous coronary angioplasty or coronary artery bypass grafting. Problems with these approaches include that many of the patients are not candidates for coronary revascularization procedures or achieve incomplete revascularization with these procedures. It is necessary to discover candidate molecules able to stimulate myocardial angiogenesis for therapeutic application. Preliminary clinical experiences suggest that therapeutic angiogenesis may provide additional blood flow to incompletely revascularized areas. Therapeutic angiogenesis with either HIF-1 or VEGF resulted in a marked increase in blood flow and improved cardiac function in animal studies without apparent toxicity (Simons, 2005). However, the results of clinical trials have been inconsistent and largely disappointing (Simons, 2005), presumably because the negative feedback of angiogenesis during hypoxia may explain the inadequate natural collateral circulation in hypoxic coronary diseases, in which upregulated VEGF inhibits other proangiogenic signaling or triggers angiogenic inhibitor signaling.

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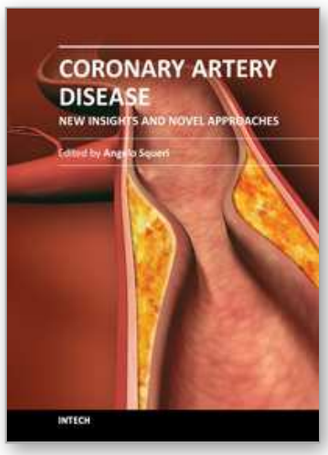

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Coronary Artery disease is one of the leading causes of death in industrialized countries and is responsible for one out of every six deaths in the United States. Remarkably, coronary artery disease is also largely preventable. The biggest challenge in the next years is to reduce the incidence of coronary artery disease worldwide. A complete knowledge of the mechanisms responsible for the development of ischaemic heart disease is an essential prerequisite to a better management of this pathology improving prevention and therapy. This book has been written with the intention of providing new concepts about coronary artery disease pathogenesis that may link various aspects of the disease, going beyond the traditional risk factors.

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