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Human activated macrophages and hypoxia: a comprehensive review of the literature

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ABSTRACT

Macrophages accumulate in poorly vascularised and hypoxic sites including solid tumours, wounds and sites of infection and inflammation where they can be exposed to low levels of oxygen for long periods. Up to date, different studies have shown that a number of transcription factors are activated by hypoxia which in turn activate a broad array of mitogenic, pro-invasive, pro-angiogenic, and pro-metastatic genes. On the other hand, macrophages respond to hypoxia by up-regulating several genes which are chief factors in angiogenesis and tumorigenesis. Therefore, in this review article we focus mainly on the role of macrophages during inflammation and discuss their response to hypoxia by regulating a diverse array of transcription factors. We also review the existing literatures on hypoxia and its cellular and molecular mechanism which mediates macrophages activation.

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Introduction

Large phagocytic mononuclear leukocytes represent a population of bone marrow-derived (myeloid) cells which are known as monocytes (1). Monocytes constitute ~5-10% of leukocytes in the peripheral blood, where they circulate for several days before populating tissues as macrophages, in the steady state or during inflammation (2, 3). At sites of injury or microbial invasion, monocytes express chemokine receptors such as CCR2 and chemoattractants such as MCP-1 (Monocyte Chemoattractant Protein 1, also called CCL2, which is a ligand for CCR2) which elicits increased recruitment of monocytes to peripheral sites where they differentiate into macrophages and contribute to host defence, tissue remodelling and repair (4-7). Macrophages are "professional" phagocytic cells which act as an early line of defence in the immune system by recognising and engulfing pathogens such as bacteria and viruses (8). Phagocytosis is believed to be involved in macrophage activation and it results in the release of cytokines such as IL-1 (Interleukin-1), IL-6 and TNF (Tumour Necrosis Factor) which promote inflammation (9-12).

The presence of areas of low oxygen tension (hypoxia) is a hallmark of many pathological tissues such as solid tumours (13, 14), wounds (15) and site of infection and inflammation (16). Cells of the monocyte/macrophage lineage are involved in all of the above pathologies (17-19). It has been known for some time that macrophages accumulate in poorly vascularised and hypoxic sites and respond rapidly to hypoxia by altering the expression of a wide range of their genes (20, 21).

The inflammatory macrophages

Inflammation is a response of a tissue to injury which could be a simple wound or a complex autoimmune inflammation such as rheumatoid arthritis (22). It has been shown that macrophages are major players in the inflammatory response and secrete pro-inflammatory and antimicrobial mediators (23, 24). For example, it has long been known that macrophages activated *in vitro* by interferon- γ (IFN- γ) followed by a microbial trigger, can increase production of pro-inflammatory cytokines such as TNF and interleukins including IL-1 and IL-6 (25). Also, innate activation of macrophages by ligation of TLRs such as TLR-4

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with LPS (Lipopolysaccharide) is associated with microbicidal activity and production of other pro-inflammatory cytokines such as IFN- α and IFN- β (26, 27). Evidence to date suggests that macrophage-derived cytokines such as transforming growth factor- β (TGF- β), basic fibroblast growth factor and platelet-derived growth factor are important in tissue repair and remodelling (28, 29). In addition, it has also been shown that deactivation of macrophages, which is induced by presence of cytokines such as IL-10 or TGF- β , is associated with increased production of IL-4 which is an anti-inflammatory cytokine (25, 30).

Several studies have suggested that macrophages can be classified into two major groups, M1 and M2 (31-33). M1 macrophages are activated by IFN- γ , TNF (Tumour Necrosis Factor) or pathogen-associated molecular patterns such as LPS and can effectively destroy invading pathogens, tumour cells and foreign materials (25, 34). They act as antigen presenting cells and release pro-inflammatory cytokines such as TNF, IL-6, IL-1 and IL-12 and participate as inducer and effector cells in T helper 1 (Th1) responses (25, 34, 35). Accumulating evidence suggests that M2 macrophages, which result from culture in presence of IL-4, IL-13, IL-10 or TGF- β , can release anti-inflammatory cytokines, growth factors and mediators which are involved in wound repair and tissue remodelling and contribute as inducers in T helper 2 (Th2) responses (25, 34, 36, 37).

Overall, there are many stimuli which can push macrophages toward the activation phenotype. Hypoxia which often occurs in tumours and sites of infection can therefore activate macrophage expression of a broad range of genes including pro-inflammatory cytokines such as TNF- α , IL-1 and IL-6 (38-40).

Hypoxia

Molecular oxygen is essential for aerobic metabolism to maintain intracellular bioenergetics and to serve as an electron acceptor in many reactions (41). Ambient air is 21% O₂ (150 mm Hg) at sea level; however, most mammalian tissues have O₂ levels of 24-66 mm Hg (2%-9% O₂) (42). The term 'Hypoxia' describes low oxygen concentrations (43), which can affect and regulate many physiological and pathophysiological processes, including embryonic development (44) and wound healing (15). In biological systems, hypoxia usually occurs in pathological tissues including tumours, ischemic tissues, chronic obstructive pulmonary disease, atherosclerotic plaques (45, 46) and arthritic joints (47). It is known that a major obstacle to cell survival is reduction in oxygen availability, which is often confronted by cancer cells (48, 49). In general, rapid growth and abnormal angiogenesis at the site of the tumour, leads to insufficient blood

supply and consequent depletion of oxygen. This eventually results in the formation of necrotic and hypoxic regions in the inner parts of the tumour (49, 50). Vaupel and Meyer in 2007 showed that O₂ concentrations within cancerous tissues are reduced compared to surrounding normal tissue, with severe hypoxia correlating with invasion, metastasis and patient death (51). The oxygen concentration in these pathological tissues ranged from 0 to 15mmHg (52). Hypoxia is also found in healthy tissues such as the spleen (oxygen levels as low as 0.5% or 3 mmHg) (53) and it is also a condition seen in embryogenesis in which hypoxia signalling is considered necessary for normal development (46).

The role of hypoxic microenvironment in the pathogenesis and progression of human cancer was first proposed by Gray *et al* when intratumoral hypoxia was correlated with reduced efficiency of radiation therapy (54), and later on was discussed by other groups (55, 56). Hypoxia has also been shown to be linked to increased mutation rates (57), tumour invasion (58) and metastasis (59).

Genomic tools, including DNA microarrays, have enabled study of the global gene expression of many different cells and tissues under hypoxic stress (21, 60, 61) and more than 100 genes have been shown to be up-regulated by hypoxia. For example, hypoxia induces erythropoietin (EPO) (62), angiogenic cytokines such as vascular endothelial growth factor (VEGF) (63) and basic fibroblast growth factor (bFGF) which are required for adaption of the whole organism to general hypoxia by enhancing blood oxygen-carrying capacity and oxygen delivery (64). Also, hypoxic up-regulation of glucose transporter-1 (GLUT-1) which facilitates the transport of glucose across the plasma membranes of mammalian cells, has been detected in a variety of malignant tissues (65, 66).

It is well known that a variety of signalling pathways are activated by hypoxia (67, 68). Among these, the activation of the transcription factor hypoxia-inducible factor 1 (HIF-1) is a key element responsible for embryogenesis and up-regulation of numerous hypoxia inducible genes (69, 70). HIF-1-mediated gene expression allows an organism to respond to hypoxia by increasing oxygen delivery or adapting to decreased oxygen availability (71). Such targets for HIF-1, play critical roles in glycolysis, oxygen homeostasis, tissue remodelling, fat metabolism, angiogenesis, erythropoiesis and proliferation (72, 73).

Macrophages in hypoxia

It has been known for some time that macrophages are recruited and retained in poorly vascularised, hypoxic and necrotic sites including breast (74, 75) and ovarian carcinomas (76), wounds (77), atherosclerotic plaques (78) and arthritic joints

(79). In addition, It has been reported that chemoattractants such as colony stimulating factor 1 (CSF-1), MCP-1, VEGF and endothelin 1 recruit peripheral monocytes to tumour regions which are characterised by extremely low levels of oxygen and trigger differentiation into tumour associated macrophages (TAMs) (80). Several studies have shown that TAMs release a variety of enzymes and cytokines which promote tumour invasion, angiogenesis and metastasis, such as epidermal growth factor (EGF) and VEGF (19, 32, 81-83).

A study by Burke *et al* (2003) showed that certain genes are up-regulated by macrophages under hypoxic conditions. They used cDNA array hybridization to determine the effect of hypoxia on mRNA of 1185 genes in primary human monocyte-derived macrophages (HMDM). This study showed hypoxia induced mRNA up-regulation of the enzyme matrix metalloproteinase-7 (MMP-7), neuromedin B receptor and DNA-binding protein inhibitor (Id2) as well as known hypoxia inducible genes such as VEGF and GLUT-1 (21). Another cDNA array research by White *et al* (2004) also revealed more than 30 mRNA pro-angiogenic genes which were up-regulated by hypoxia in primary macrophages. Among these genes, apart from VEGF, the best characterized ones were fibroblast growth factor 2 (FGF2), IL-8, macrophage migration inhibitory factor (MIF) and cyclooxygenase- 2 (COX-2) (84). In addition, it has been demonstrated that hypoxic macrophages up-regulate a number of transcription factors, such as HIF-1, which in turn up-regulate a broad array of genes including VEGF and GLUT-1 whose products promote tumour growth and angiogenesis (21, 85- 87).

Hypoxia-responsive transcription factors

Hypoxia activates a diverse array of transcription factors such as activator protein-1 (AP-1) (88, 89), cAMP-response element binding protein (CREB) (90, 91), specific protein 1 (SP-1) (92-94) and most importantly HIF-1 (95), which in turn activates a broad array of mitogenic, pro-invasive, pro-angiogenic, and pro-metastatic genes (96, 97). Since the discovery of HIF-1 by the Semenza lab in the early 1990s, it has been recognised as the central importance and described as the “master regulator” of the transcriptional response to hypoxia (96).

Hypoxia-Inducible Factors (HIFs)

There are two main types of HIF, HIF-1 and HIF-2 (98, 99) which are the predominant transcription factors mediating the effects of hypoxia on gene expression (100, 101). HIF-1, the most ubiquitously expressed and best characterised member of the family is recognised as a master regulator of hypoxic signalling whose activation has been shown to regulate the expression of over 70 genes at the transcriptional level (102).

Both HIF-1 and 2 are heterodimeric molecules consisting of α and β subunits which belong to a family of basic helix-loop-helix proteins (103). The HIF- β subunit, known as ARNT (Aryl hydrocarbon Receptor Nuclear Translocator) is found in the nucleus in both normoxia and hypoxia (98), whereas the α subunit is constitutively produced but is subjected to rapid degradation in the presence of oxygen (with a half-life of less than five minutes), only being stable in the absence of oxygen (i.e. hypoxia) (102). The HIF-1 α subunit contains an oxygen-dependent degradation domain (ODD) and two transactivation domains (TAD) which are required for transcriptional activation activity of HIF-1, being capable of binding to two transcriptional co-activators, CREB binding protein (CBP) and p300 (104-107). In normoxia, specific proline residues at positions 402 and/or 564 in the ODD of the HIF- α subunit are hydroxylated by prolyl hydroxylase enzymes (PHD) (108, 109). PHD is a family composed of prolyl 4-hydroxylases (PHD1-4) which require iron (Fe (II)), 2-oxoglutarate, O₂ and ascorbate as substrates; their activity is reduced in hypoxia due to the limitation of O₂ concentration (110, 111). Hydroxylation of HIF-1 α acts as a signal for recognition by the tumour suppressor VHL (von Hippel-Lindau protein), leading to ubiquitination and proteasomal degradation (Figure 1) (112-114).

In addition to prolyl hydroxylation by PHDs, another oxygen-dependent modification occurs in the transactivation domains of HIF- α subunit. It is dependent on the presence of an asparagine hydroxylase enzyme known as factor inhibiting HIF (FIH) (115, 116). In this oxygen-dependent regulatory mechanism, FIH blocks the interaction between HIF- α with p300 and CBP by hydroxylating an asparagine residue at position 803, thus inhibiting the activity of the HIF-1 α transactivation domain (Figure 1) (117, 118).

In hypoxia, PHD activity decreases and enables rapid accumulation of HIF- α in the nucleus where it dimerises with the HIF- β subunit and binds to hypoxia response elements (HREs) in the promoters of various genes (70, 119). The decrease in oxygen availability also impairs FIH which results in a decrease in HIF- α subunit asparagine hydroxylation, allowing increased recruitment of transcriptional co-activators (p300/CBP) which eventually leads to enhanced transcriptional activation of HIF target genes (Figure 1) (16, 115, 120) which are implicated in many different aspects of oxygen delivery and metabolism including vasodilatation (nitric oxide synthases), iron metabolism (transferrin) (121), glucose transporters (GLUT-1), angiogenesis (VEGF), enhanced blood oxygenation (erythropoietin) (122) and glycolysis (phosphoglycerate kinase) (95).

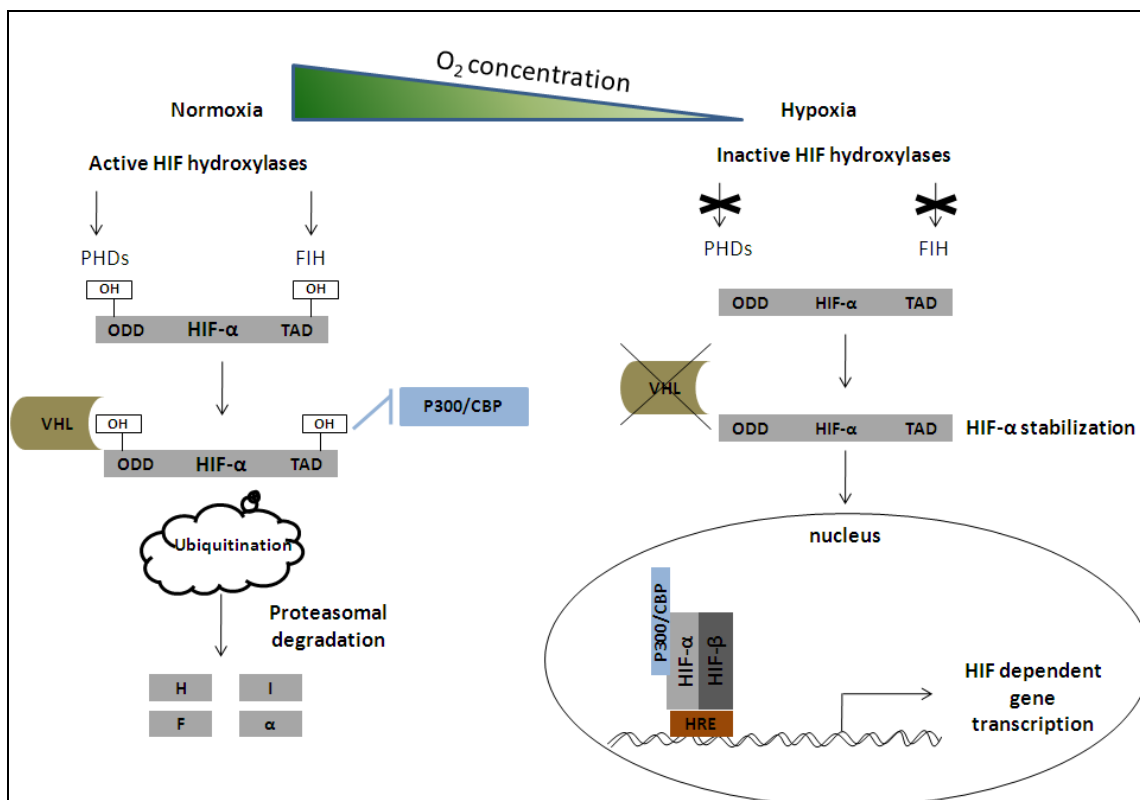


Figure 1. Regulation of HIF-1 in normoxia and hypoxia

In normoxia, the HIF- α subunit is first hydroxylated by PHD at two specific proline residues at positions 402 and/or 564 in the ODD region and then by FIH which blocks the interaction between HIF- α and p300 and CBP by hydroxylating an asparagine residue at position 803, thus inhibiting the activity of the HIF-1 α transactivation domain. Both mechanisms then act as a signal for recognition by the tumour suppressor VHL leading to ubiquitination and proteasomal degradation. In hypoxia, reduced activity of PHD causes rapid accumulation of HIF- α in the nucleus where it dimerises with the HIF- β . This complex then binds to HREs in the promoters of various genes. Also, the activity of FIH enzyme will be impaired by the decrease in O_2 availability, leading to reduction of hydroxylation of HIF- α subunit asparagine. This mechanism allows increased recruitment of transcriptional co-activators (p300/CBP) which eventually leads to enhanced transcriptional activation of HIF target genes.

Hypoxia responsive elements (HREs)

Previous studies showed that HIF-1 binds to hypoxia responsive elements, a consensus sequence in the promoter of about 200 HIF target genes (among which around 100 genes have been confirmed) and initiates transcription by recruiting transcriptional co-activators such as p300/CBP (16, 97).

The minimal cis-regulatory element (CGTG) required for hypoxic induction of gene transcription was first identified by Semenza who also determined that this core HRE consensus sequence is required but is not sufficient for effective gene activation in response to hypoxia (95, 123). Analysis of 107 HIF-1 responsive genes showed that neighbouring nucleotides occur with non-random frequency, especially in the 5' flanking bases, demonstrating that a fully functional HRE requires neighbouring DNA binding sites for additional transcription factors or co-activators, which may act to amplify the hypoxia response (16).

Role of HIF-1 in macrophages

As previously mentioned, macrophages are associated with a number of inflammatory sites such as atherosclerotic plaques (124), myocardial infarcts (125), rheumatoid arthritis (126), healing wounds (127), sites of bacterial infection and malignant tumours (20, 128, 129) in which hypoxia is present. In hypoxia, macrophages rely heavily on HIFs for energy production and activity, express HIF-1 α protein abundantly and increase transcriptional activation of HIF target genes (21, 85). Unusually, macrophages are also significantly dependent on HIF-1 regulated genes for energy production in normoxia (130).

Some early studies using a rat alveolar macrophage-derived cell line and the human monocytic cell line (THP-1) reported that short term hypoxia did not increase HIF-1 α mRNA, suggesting that HIF-1 α is regulated by hypoxia by decreased protein stability (128, 131). However, our recent study showed increases in HIF-1 α mRNA levels after long term hypoxia (5 days) in human primary macrophages and also we observed that this up-regulation is mediated by increased transcription rather than increased mRNA stability. Similar increases in HIF-1 mRNA in hypoxia have been reported by other groups in non-macrophage cell types but the subject is still somewhat controversial (132).

An increased level of HIF-1 α protein in activated macrophages was first demonstrated by Hollander *et al* in 2001 (126) in inflamed joints of patients suffering from rheumatoid arthritis and later by Talks *et al* in 2000 (133) in tumour sections and Burke *et al* in 2002 (85) in isolated hypoxic human primary macrophages *in vitro*. Also, other studies showed increased levels of HIF-1 α in inflammatory cells of healing wounds and suggested that this could be due to a release of inflammatory cytokines such as TNF- α which can strongly increase HIF-1 α protein levels in cells after injury, leading to increased expression of HIF-1 responsive genes such as VEGF which regulate the process of tissue repair (134). Other groups have also investigated HIF-1 activity during differentiation of monocytes to macrophages (135). It was shown by Oda *et al* in 2006 that both HIF-1 α and HIF-1 β protein levels increase markedly during the differentiation of monocytes to macrophages in the monocytic cell line (THP-1) and in monocytes from human peripheral blood (136). They suggested that activation of protein kinase C (PKC) and mitogen-activated protein kinase (MAPK)-signalling pathways are responsible for this increase in HIF-1 gene expression (136).

Non-hypoxic up-regulation of HIF-1

Despite the name, numerous studies have now shown that HIF-1 α can be induced by a variety of stimuli in addition to hypoxia. The key studies in this field are reviewed below.

Lipopolysaccharide (LPS)

LPS is a component of the cell wall of Gram-negative bacteria (137). It binds to the CD14 and TLR4 cell surface receptors of monocyte/macrophages (138, 139) leading to activation of a number of genes that are often associated with hypoxia, many of which are believed to be up-regulated independently of HIF-1 (140-143). Several studies have shown that LPS treated macrophages up-regulate genes such as VEGF, GLUT-1 and iNOS (inducible nitric oxide synthase) which

are known to be regulated by HIF-1 (128, 131). In contrast to hypoxia, which is generally considered not to up-regulate HIF-1 α mRNA, LPS has been shown to stimulate HIF-1 α expression at transcriptional level under normoxia in alveolar-derived rat macrophages and human primary macrophages through a NF- κ B site in the promoter of the HIF-1 α gene (128, 131). It was shown that LPS increases HIF-1 α protein expression in a time and dose-dependent manner which in turn modulates hypoxic gene activation (128). Also, an induced HIF-1 α mRNA and protein expression in differentiated THP-1 cells treated with LPS under normoxia has been reported (131). This study, using RNAi against MAPK and also a specific inhibitor of this pathway, showed down-regulation of LPS-induced HIF-1 α mRNA and protein in THP-1 cells suggesting a role for the MAPK pathway in LPS-dependent HIF-1 α induction (131).

Phosphoinositide (PI) 3-kinase signalling

PI3-Kinase activities have been found in all types of eukaryotic cells and are linked to a diverse set of major functions of the cell, including cell growth, proliferation, motility, differentiation and survival (144-146). PI3-kinase phosphorylates the hydroxyl group at position 3 of the inositol ring of phosphatidylinositol (147). PI3-kinase has been the focus of intense study as increasing evidence suggests a key role for PI3-kinase pathway in many human diseases including allergy, inflammation, heart disease and cancer (148, 149). An interesting mechanism was proposed via which the normoxic activation of PI3-kinase could increase the rate of HIF-1 α translation in vascular smooth muscle cells (VSMC) (150). It has been previously reported that activation of PI3-kinase by growth factors and hormones leads to the recruitment and activation of a downstream effector of PI3-kinase, known as the mammalian target of rapamycin (mTOR) (151, 152). mTOR activation results in increased phosphorylation and inactivation of 4E-binding protein 1 (4E-BP), the eukaryotic translation initiation factor, and activation of p70-S6 kinase 1 which leads to increased protein synthesis (151, 153). Inactivation of 4E-BP and activation of p70S6K has been shown to increase translation of HIF-1 α mRNA through the 5' untranslated region (5'UTR) (150, 154). This is believed to be the main mechanism responsible for HIF-1 α induction through the PI3-kinase dependent pathway, resulting in increased VEGF expression in vascular smooth muscle cells and human tumour cell lines (155, 156).

Cobalt (CoCl₂) stabilisation of HIF-1 α

It has been demonstrated that CoCl₂ induces hypoxia-regulated genes by stabilizing HIF-1 α in

normoxia (112). As outlined before, hydroxylation of the proline residues, which reside in the oxygen-dependent degradation domain of HIF-1 α , by prolyl hydroxylase is one of the key mechanisms that mediate the binding of VHL with HIF-1 α which eventually leads to proteasomal degradation of HIF-1 α (109, 111). In a study, it has been suggested that iron is a critical factor for the activity of PHD as these enzymes have an iron-binding centre (110). In addition, this study suggested that CoCl₂ may act as a competitor for iron, inactivating PHD by binding and engaging an iron-binding site in the proline hydroxylase. Due to this enzymatic inhibition, HIF- α is not targeted for proteasomal degradation (110). Beside the inactivation of PHD by CoCl₂, another mechanism via which HIF- α could be stabilized by cobalt, has been proposed (157). In this process, cobalt stabilizes HIF-1 α protein by direct binding to the ODD in HIF-1 α , thereby preventing the interaction between HIF-1 α and VHL protein and subsequently inhibiting proteasomal degradation which results in HIF-1 α stabilization (157).

Desferrioxamine (DFO) stabilization of HIF-1 α

Since the introduction of DFO in the 1960s, it has been widely used as a chelating agent to bind free iron in the bloodstream and removing excess iron from the body (158). Several studies have demonstrated that normoxic cells treated with DFO induced HIF-1 target genes such as EPO (159), VEGF (160) and GLUT-1 (161) by inducing the accumulation of HIF-1 protein. An early study demonstrated that DFO disrupts pVHL-HIF- α complex formation which is required for ubiquitination and proteasomal degradation of HIF-1 α in normoxia (112). It has been demonstrated that DFO inhibits hydroxylation of HIF-1 α by chelating the iron required for the activity of PHD enzyme (157). Therefore, due to inhibition of HIF- α hydroxylation, the pVHL-HIF- α complex formation is inhibited causing HIF- α stabilization which results in induction of HIF-1 target genes (162-164).

Conclusion

In this review article, we provided evidence which show hypoxic activated human macrophages could regulate broad array of angiogenesis and tumorigenesis genes. In addition, further ground working experiments suggested that high-level transcription of such genes in hypoxia appears to occur via a HIF-1 dependent mechanism which can be activated by hypoxia and DFO in addition to CoCl₂. A better understanding of how hypoxic regulated genes are influenced by hypoxia in human macrophages will hopefully be helpful for the development of future therapies for a range of different diseases such as vascular disorders like atherosclerosis, where hypoxia-induced genes

accumulation plays a key role in disease development. As macrophages have been shown to accumulate in the areas with low oxygen tension where hypoxic regulated genes are up-regulated, the knowledge of how such genes promoter is induced by hypoxia by elucidation of the hypoxia responsive elements could be an additional advantage for future tumour gene therapy whereby a therapeutic gene could be engineered to be regulated by the hypoxia responsive promoter. Macrophages transfected with this construct could be used in the delivery of the therapeutic gene to radiotherapy and chemotherapy resistant hypoxic tumour sites where the gene would be locally induced. In addition, hypoxic up-regulation of such genes by human macrophages which are recruited and retained in hypoxic and necrotic sites could be a potential prognostic factor in patients with malignant tumours.

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