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REGULATORS OF ANGIOGENESIS IN DIABETES AND TUMORS

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Stockholm 2005

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-628-6682-6

To my wonderful family

ABSTRACT

Angiogenesis is tightly regulated in order to provide adequate supply of nutrients and oxygen for normal cellular function. Tissue oxygenation represents a balance between the requirement for oxygen to maintain the cellular energy level and the potential risk of cellular oxidative damage. The master regulator of oxygen homeostasis is Hypoxia inducible factor-1 (HIF-1). Our basic hypothesis was therefore that HIF modulation might be relevant for diseases characterised by dysregulated angiogenesis, such as diabetes mellitus and tumors.

Chronic complications of diabetes are a major health problem, and a better understanding of their pathogenic mechanisms could offer a more rational therapy. Both hyperglycemia and hypoxia play important roles in the pathogenesis of diabetes complications and we propose that the interplay between these two factors is essential in determining the devellopement of chronic complications of diabetes. We demonstrated that high concentrations of glucose interfere with hypoxia-dependent stabilization of HIF-1α against proteasomal degradation in both primary human dermal fibroblasts and microvascular endothelial cells. The destabilizing effect of glucose was partially independent of prolyl hydroxylases (PHD). Hyperglycemia also inhibited transactivation and function of HIF-1 as demonstrated by a HRE (hypoxia responsive elements) reporter gene assay. In agreement with our *in vitro* observations we detect lower expression of HIF-1 α in diabetic wounds then in venous ulcers that share the same hypoxic environment but not the chronic exposure to hyperglycemia. Based on these findings we further investigated the potential therapeutic effect of HIF-1 α up-regulation in diabetic wounds by using DMOG, a chemical inducer of HIF-1 α which showed virtually no toxic effect on primary dermal fibroblasts *in vitro*. Local treatment with DMOG normalized the healing rate of experimental wounds in db/db mice suggesting that HIF-1 α modulation is a promising therapeutical approach for diabetic wounds.

Dysregulated angiogenesis is also a hallmark of tumor diseases. Kaposi's Sarcoma (KS) is a highly vascularised tumor, which depends strictly on angiogenetic stimuli. We have therefore investigated the HIF pathway in KS and its relation to the insulin like growth factor (IGF) system, known as a major factor in pathophysiology of different tumors. We demonstrated that KS cells express both HIF-1α and HIF-2 α even in normoxia and that IGF-I induced their expression, showing for the first time that a growth factor is able to induce accumulation of both HIFα congeners. HIF accumulation was followed by functional activation as demonstrated by a HRE reporter gene assay and by induction of HIF target genes (ex. VEGF). IGF-I induced accumulation of both HIF-1 α and HIF-2 α at a posttranscriptional level as demonstrated by qRT-PCR and cycloheximide chasing experiment and we suggest that IGF-I increased the translation of the α subunits. The clinical relevance of our findings was confirmed by the identification of high levels of both HIF isofoms in tumor cells in biopsies from patients with AIDS-related KS. It was further demonstrated that IGF-I and IGF-II are potent growth factors for KS cells and have an additive effect with VEGF. IGF-I receptor (IGF-IR) was identified as the receptor mediating the IGFs effect and we described an autocrine loop in KS cells in which IGF-II is the endogenous ligand for IGF-IR. Finally, it was demonstrated that blocking the IGF-IR with picropodophyllin, a recently described selective IGF-IR tyrosine phosphorylation inhibitor, induced apoptosis and decreased HIF accumulation and function in KS cells, suggesting a potential future therapeutic approach.

In conclusion, HIF is an important factor both in chronic complications of diabetes and in vascular tumor KS. We have highlighted that glucose and IGF-I regulate HIF by other mechanisms than the classic hypoxic regulation. Finally it was demonstrated that modulation of HIF can be a successful approach for treatment of both diabetic wounds and KS.

LIST OF PUBLICATIONS

This thesis is base on the following papers, which will be reffered to in the text by their Roman numerals:

I. **S.B. Catrina,** K.Okamoto, T. Pereira, K. Brismar, L. Poellinger. Hyperglycemia regulates HIF-1α (Hypoxia-inducible factor 1 alpha) Protein Stability and Function. *Diabetes, 2004, 53(12):3226-32*

II. **S.B. Catrina,** I.R. Botusan, J.Grunler, A. Rantanen, A. I. Catrina, K. Brismar. Inhibition of HIF prolylhydroxylases increases the wound healing rate in db/db mice. *Manuscript*

III. **S.B. Catrina**, M. Lewitt, C. Massambu, A. Dricu, J. Grunler, M. Axelson, P. Biberfeld, K. Brismar. Insulin-like growth factor-I receptor activity is essential for Kaposi's Sarcoma growth and survival. *Br J Cancer. 2005 Apr 25; 92(8):1467-74*

IV. **S.B. Catrina,** I. R. Botusan, A. Rantanen, A. I. Catrina, P. Pyakurel, M. Axelson, P. Biberfeld, L. Poellinger, K. Brismar. HIF-1α and HIF-2α are expressed in Kaposi Sarcoma and are modulated by IGF-I. *Submitted*

CONTENTS

LIST OF ABBREVIATIONS

AGE	Advanced glycozylation end product
Akt	Arrest defective protein-1
Alk	Activin receptor-like kinase
ARD-1	Protein kinase B
ARNT	Aryl hydrocarbon receptor nuclear translocator
bHLH	Basic helix-loop-helix
CHX	Cycloheximide
CCT	Cytosolic chaperonin containg TCP-1
CBP	CREB-binding protein
DFX	Deferoxamine
DMOG	Dimethyloxalylglycine
Erk	Extracellular signal- regulated kinase
FGF	Fibroblast growth factor
FIH-1	Factor inhibitin HIF-1
FLIP	FLICE inhibitory protein
Flt-1	Fms-like tyrosine kinase
Flk-1	Fetal liver kinase-1
HDMEC	Human dermal microvascular endothelial cells
HDF	Human dermal fibroblasts
HIF	Hypoxia inducible factor
HIV	Human immunodeficiency virus
HNF	Hepatocyte nuclear factor
HPLC	High performance liquid chromatography
HRE	Hypoxia responsive elements
IGF	Insulin growth factor
IGFBP	Insulin growth factor binding protein
IGF-IR	Insulin growth factor receptor
IR	Insulin receptor
KS	Kaposi's Sarcoma
KSIMM	Immortalized Kaposi's Sarcoma cell line
MDF	Mouse dermal fibroblasts
MDM2	Mouse double minute 2, human homologue
mTOR	Mammalian target of rapamycin
NLS	Nuclear localization signal
NOS	Nitric oxide synthase
MAPK	Mitogen activated protein kinase
ODD	Oxygen dependent domain
OG	Oxoglutarate
PARP	Poly (ADP-ribose) polymerase
PAS	Per-ARNT-sim protein
PDGF	Platelet derived growth factor
PHD	Prolyl hydorxylase domain-containing proteins
PI3K	Phosphatidylinositol-3OH-kinase
PKC	Protein kinase C
Ref-1	Redox factor-1
ROS	Reactive oxygen species
SCF	Skp-Cdc53-F-box
SRC	Steroid receptor coactivator
SUMO	Small ubiquitin-like modifiers
TAD	Transactivation domain
TCP-1	T complex protein-1
VEGF	Vascular endothelial growth factor
VEGF-R	VEGF receptor
VHL	Von Hippel-Lindau protein

Explanation separates us from astonishment,
which is the only gateway to the incomprehensible

Eugen Ionescu

1 RATIONALE

Angiogenesis represents the formation of new blood vessels and is controlled by a complex network of angiogenesis regulators. Abnormal angiogenesis is considered to be the hallmark of diseases such as diabetes and cancer which are among the most prevalent and costly health problems due to the high morbidity and mortality induced (1). Even though important progress has been made to develop therapeutic strategies for these diseases, there are still a lot of problems such as chronic complications of diabetes and metastasis which still are waiting for better management. A better understanding of the molecular pathways responsible for these clinical complications, might offer a better insight into disease pathogenesis resulting in new clues for future treatment. Moreover, specifically addressing these mechanisms in a laboratory setting might offer the premise for future drug development.

This thesis focuses on the investigation of angiogenesis regulators in diabetic foot ulcers and Kaposi's Sarcoma, two diseases which represent perfect clinical models of abnormal angiogenesis. By describing new pathogenic molecular pathways and suggesting new therapeutic approaches we hope to contribute to the future clinical research for the benefit of the patients.

2 BACKGROUND

2.1 Angiogenesis - a friend or a foe?

Angiogenesis represents the sprouting of new vessels from preexisting ones in contrast with vasculogenesis which represents the development of blood vessels from the *in situ* endothelial cells and arteriogenesis which refers to the stabilization of the sprouts by mural cells (2). Angiogenesis represents a complex process implying disruption of the endothelial growth constraints, activation of the cellular mechanisms against apoptosis, migration of the endothelial cells, specific interaction with the extracellular matrix and tube formation. In consequence for a normal and efficient angiogenesis several pathways need to be activated during a concerted action which maintains the fine balance between the growth promoting and growth inhibitory factors. The angiogenic switch depends on the induction of one ore several positive regulators of angiogenesis (3).

2.2 Regulators of angiogenesis

Several regulators of angiogenesis have been described to date. Among them, hypoxia and growth factors play a central role.

2.2.1 Hypoxia

Hypoxia is an essential stimulus for the vascular bed expansion. Primordially, cells are oxygenated by simple diffusion but as the organism growths a limit of oxygen diffusion is reached resulting in hypoxia that triggers the vessels growth. Angiogenesis, together with other concerted mechanisms such as acute modulation of respiration and circulation, glycolysis and erythropoiesis, represents a tissue adaptation to low oxygen tension. The molecular mechanism which lay behind the reaction of the organism to hypoxia is mediated by a transcriptional factor called hypoxia-inducible factor (HIF) which activates a myriad of target genes (see figure 1).

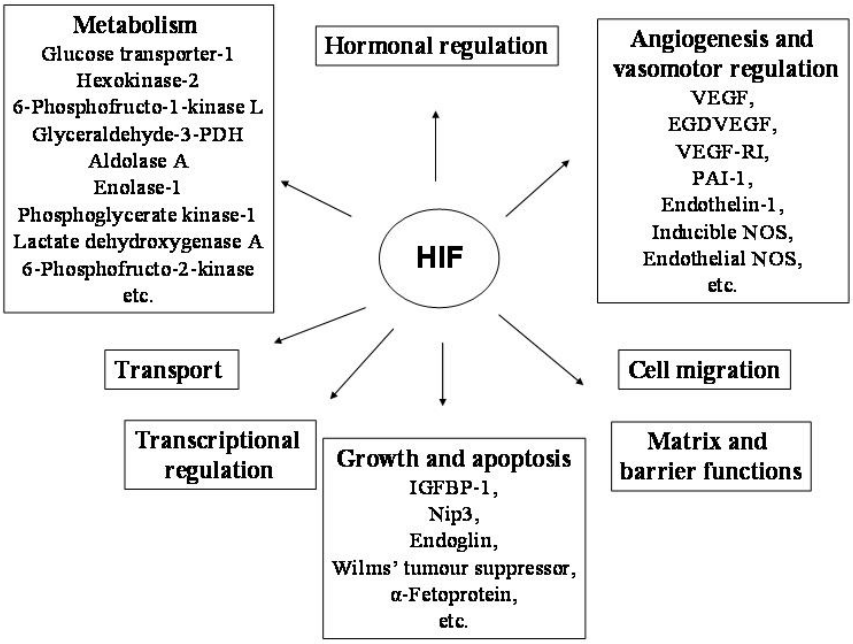


Figure 1. Trancriptional targets for HIF. Adapted from Schofield CJ and Rarcliffe RJ, Nature Reviews, 2004
Abbreviations: insulin growth factor binding proteins, IGFBP; endothelial-gland-derived vascular endothelial growth factor EGDVEGF; vascular endothelial growth factor receptor, VEGF-R; plasminogen-activator inhibitor-1, PAI-1; nitric oxide synthase, NOS.

2.2.1.1 Structural characterization of HIF

HIF was first discovered as a regulator of erythropoietin (4). It is a heterodimeric transcriptional factor that belongs to the basic helix-loop-helix (bHLH)-per ARNT sim (PAS) protein superfamily (5). It is composed of two subunits: α subunit and β subunit the late being also known as aryl receptor nuclear translocator (ARNT) (6). Only the

HIFα subunit is subjected to oxygen regulation. Structurally it has several common features with other members of the bHLH-PAS superfamilly. bHLH is located at the N-terminal part and it is composed of a basic rich residues domain, involved in DNA binding (7) followed by two α helices separated by a loop (8). The N-terminal part of the molecule also contains a relatively conserved region called PAS region. The C-terminal region of the molecule contains two domains involved in activation of transcription of the target genes, termed N- or C- TAD (transactivation domains). The bHLH motif induces dimerization and the PAS domain stabilizes it and determines the partner of choice. Two nuclear localization signals (NLS) have been identified, one in the bHLH region and the other in C-terminus (9).

Three α subunits have been described to date (figure 2). Their chromosomal localization are HIF-1α 14q21-q24, HIF-2α 2p21-p16 and HIF-3α 19q13-q32. HIF-1α contains 826 aminoacids. HIF-2α has 870 aminoacids with a 48% overall similarity with HIF-1α which is higher in the N-terminal region (10, 11). It also contains in the C-terminal region two transactivation domains with high similarity with HIF-1α (12). Even though HIF-3α structure is to some extent different, the N-terminal region is highly similar with the others' subunits contributing therefore to its capacity to dimerize with HIF-1β and to bind to hypoxia responsive elements (HRE). However, HIF-3α lacks the C-TAD region and in consequence can function as a repressor (13).

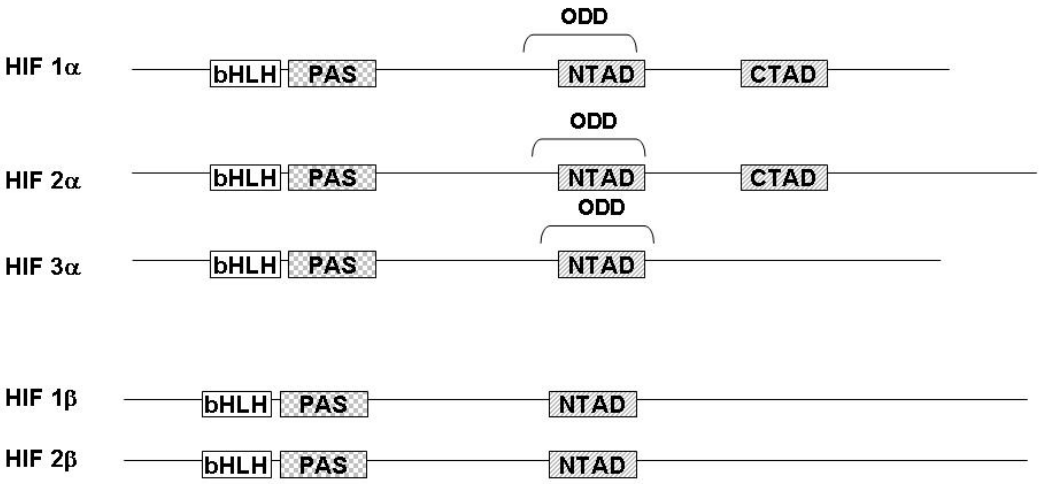


Figure 2. Schematic representation of the HIF α and β subunits.

Abbreviations: HIF, hypoxia inducible factor; PAS, Per-ARNT-sim; bHLH, basic helix-loop-helix; ODD, oxygen dependent degradation domain; NTAD, N-terminal transactivation domain, CTAD, C-terminal transactivation domain

There are several alternative splicing variants of HIF-1α able to interfere with the selection of the co-partner, to modify the specific binding to HRE or to act as negative factors in the translocation (14, 15). HIF-3α also exhibits multiple splicing variants (16). One of these variants exhibits an important inhibitory effect on the expression of hypoxia inducible genes being suggested to be responsible for the avascular phenotype of the cornea (17, 18).

HIF-1α is widely expressed in the tissues, while HIF-2α has a more restricted expression in the highly vascularized tissues (10) being completely absent in some tissue such as leukocytes (11). HIF-3α is expressed just in kidney, brain, heart and thymus (19).

2.2.1.2 Oxygen dependent regulation of HIF

Oxygen is the main regulator of HIF acting mainly at a postranslational level. A scheme of the oxygen dependent regulation of HIF is presented in figure 3. In normal oxygen levels the α subunit is polyubiquitinlated and directed to 26S proteasome. Ubiquitinilation requires the action of the E1 ubiquitin activating enzyme (only one identified to date) and an E2 conjugation enzyme (at least 25 identified). Finally the E3 ligase (100 members) catalyses the covalent binding of ubiquitin to its substrate (20). The E3 ligase for α subunits of HIF has been identified in the von Hippel Lindau (pVHL) protein complex (21, 22). The VHL complex functions as a tumor suppressor gene. It was first described in von Hippel Lindau disease, an inherited human cancer syndrome, in which patients carry a germline mutation in one allele and acquire a mutation or loss of function in the other allele and develop retinal, spinal, cerebral hemangioblastoma, clear-cell renal carcinoma and pheochromocytoma (23). The VHL ubiquitin ligase complex contains additional proteins called elongin B, elongin C, cullin 2 and Rbx1 and resembles SCF-like uibiquitin ligase complex from the yeast. The binding of VHL to elongin B and elongin C is facilitated by CCT (cytosolic chaperonin containing TCP-1) and protects VHL from autoubiquitinilation (24-26). The culin components need neddylation in order to recruit the E2 ubiquitin conjugated enzyme (27). VHL binds directly to the α subunits of HIF through its β subunit while the VHL α subunit binds to the elongins. It is still unclear in which subcellular compartment HIF-1α is ubiquitinilated (28, 29).

The key modification, allowing identification and direct binding of the HIF α subunit to VHL, resides in the hydroxylation of two critically proline residues in the presence of oxygen (30, 31). The reaction is catalyzed by a novel family of Fe^{2+} and 2-oxoglutarate (also known as α-ketoglutarate) dependent dioxigenases, designated as prolyl hydroxylase domain-containing proteins (PHD) (32-34). The two proline residues are located in the oxygen degradation domains (ODD), one at the N-terminal part (P402) and one at the C-terminal part (P564) (35). The proline residues are conserved in HIF-2α and in HIF-3 α. The PHD enzymes defined as "oxygen sensing" system are conserved from nematode worms and have three humans orthologues PHD 1-3, alternatively designated as HIF prolylhydroxylases (33) or EGLN (32). The name of ELGN comes from the original description of the gene in an abnormal egg laying phenoptype in Caernobitis elegans. All three enzymes have the capacity to hydroxylate HIF α subunits but PHD2 appears to be the key limiting enzyme for controlling the HIF α stability (36). Different PHDs members have a specific tissue (37) and cell distribution (38, 39) which together with different oxygen regulations (40) suggest potential distinct roles in the hypoxic pathway.

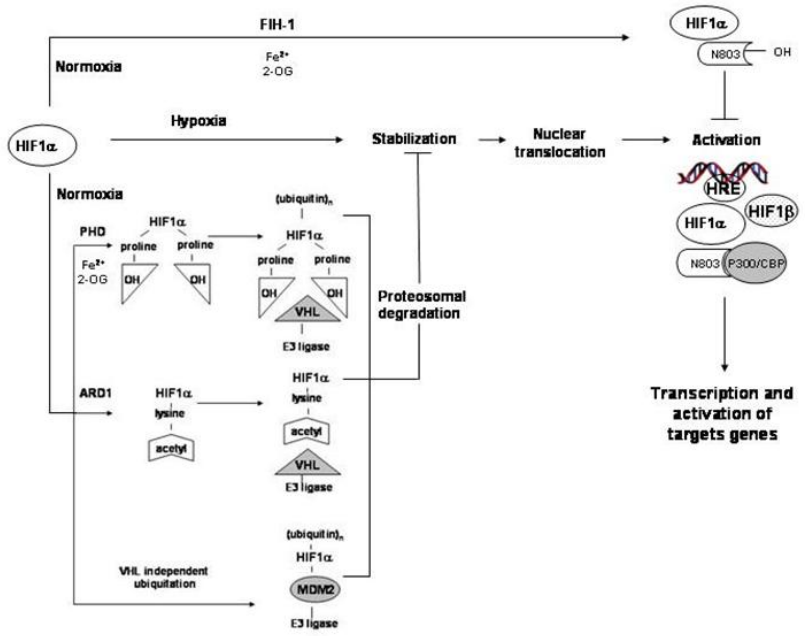


Figure 3. Oxygen dependent regulation of HIF1α

Abbreviations: HIF, Hypoxia inducible factor; FIH-1, factor inhibiting HIF-1; 2-OG, 2-oxoglutarate; N803, aspargine803; PHD, prolyl hydroxylase domain proteteins; ARD1, arest-defective-1; VHL, von Hippel-Lindau; MDM, murine double minute 2; HRE, hypoxia responsive elements, CBP, CREB-binding protein)

The biochemical characteristics of the PHDs are similar with collagen prolyl-4-hydroxylases which also needs O_2, Fe^{2+}, 2-oxoglutarate (2-OG) and abscorbate for their activity. However, these enzymes are unable to hydroxylate the HIF prolyl residues

(31). The PHDs incorporate two oxygen atoms, one in the critical HIF prolyl residues and one in the succinate residue. The reaction involves the decarboxylation of 2-OG and liberation of CO_2. The K_m of PHDs for oxygen is so close to the normal oxygen concentration (230-250μM), that even a small drop in oxygen level will affect the reaction, in opposition with collagen hydroxylases having a K_m for oxygen of 40μM (41). The Fe^{2+} requirement is confirmed by the intense stabilization of HIFα subunits induced by iron chelators, such as deferoxamine (DFX) or by its displacement with cobalt ions. Cobalt was suggested to also act by direct interfering with the interaction between HIFα subunit and pVHL (42). The importance of 2-OG is stressed by the HIFα stabilization induced by different small molecules able to displace 2-OG from the reaction (34). Ascorbic acid is also able to increase the PHD activity at least in cancer cells (43).

Acetylation of lysine 532 has been reported to be another posttranslational mechanism which favours interaction of the HIF-1α subunit with pVHL. The reaction is catalysed by an acetyltransferase termed Arest Defective Protein-1 (ARD-1) which also is itself modulated by oxygen, being down regulated in hypoxia (44). In accordance with the role of acetylation for HIF-1α stability and function is the observation that inhibition of histone deacetylases down regulates HIF-dependent angiogenesis (45).

In addition of PHD mediated VHL dependent degradation several other mechanisms which contribute to the stability of the HIF-α subunits have been described.

- A pVHL independent ubiquitination of HIF-1α has been described involving MDM2 as E3 ligase which is p53 dependent (46).
- The molecular chaperone heat shock protein 90 (hsp 90) has been shown to protect HIF-1α from degradation in a pVHL independent way (47) action which seems to require the PI3K/Akt pathway activation (48).
- Both the HIF-1α protein stability and function are increased by Jab 1 (jun activation domain-binding protein 1) (49).

The mechanisms of regulation discussed above interfere with the stability of the HIF-α subunit. However, once stabilized the HIF-α subunit is not necessarily active as a transcriptional factor. It needs interaction of the two TAD regions with transcriptional co-activators, such as CBP (CREB-binding protein), p300, steroid receptor coactivator-1 (SRC-1) and transcriptional intermediary factor-2 (9, 50, 51). The interaction is dependent as well on oxygen and involves another posttranslational modification, which resides in hydroxylation of asparagine 803 (N803) located in the C-TAD. The

enzyme catalyzing this reaction is asparagynyl hydroxylase and was first identified as an inhibitor of HIF transactivation and termed therefore Factor Inhibiting HIF-1 (FIH-1)(52). It was later shown that FIH-1 (53) is responsible for the oxygen dependent asparagyl hydroxylation which in turns inhibits the binding of CBP and p300 (54). FIH-1 belongs to the dyoxygenase family of proteins. As PHD the FIH-1 activity is dependent on Fe^{2+} and 2–OG but the K_m for oxygen is lower (90μM), suggesting that its activity is not as sensitive to small changes in oxygen concentrations. The FIH-1 K_m for 2-OG is half and for abscorbate is twice that for PHDs K_m, but in the same level as collagen hydroxylase's K_m (41). The crystal structure for FIH-1 suggests a putative binding region for VHL (55). FIH-1 transcription is not regulated by O_2 (56) and it is located in the cytoplasm (38).

Some other factors have been reported to increase the transcriptional activity of HIF-1α, such as the coactivator SRC-1 and redox factor-1 (Ref-1), which potentiate SRC-1 (57) as well as the VHL-associated KRAB-A domain containing protein (58) which acts as a transcriptional inhibitor.

2.2.1.3 Oxygen independent regulation of HIF

Even though there is no room to discuss that oxygen is the main regulation of the HIF stability and function, data has gathered about the contribution of different other players on HIF regulation. HIF-1α has been reported to be stimulated by growth factors, cytokines, hormones and other active substances (see table 1).

Table 1. Oxygen independent regulations of HIF

Growth factors	Insulin (59, 60) IGF-I (61) EGF (60, 62) Heregulin (63) HNF (64) PDGF (65), TGFβ (65),
Cytokines	IL-1 β (66) TNF- α (67)
Hormones	Androgens (68)
Other active substances	Trombin (65)
Posttranslational modifications	Phosphorylation* SUMOylation*

*Implicated in both oxygen dependent and independent regulation of HIF

Abbreviations:; insulin growth factor, IGF; epidermal growth factor, EGF, hepatocyte nuclear factor, HRN; platelet derived growth factor PDGF; transforming growth factor TGF; interleukin, IL; tumor necrosis factor, TNF; signal transducer and activator of transcription, SUMO.

Oxygen independent posttranslational modifications interfering with HIF activity have also been described. Phosphorylation, which is a well established mechanism to regulate intracellular signal proteins, is able modulate HIF activity. It has been suggested that HIFα phosphorylation is the consequence of the MAPK (69) and p38 activation (70) and does not modulate the protein stability but increases the transcriptional activity both in hypoxia and normoxia (71). Alternative mechanism to explain the increase transcriptional activity of HIF-1α after phosphorylation was provided by the higher affinity of HIF-1β for the phosphorylated HIF-1α form (72) and by activation of C300 through phosphorylation (73). SUMOylation is a process similar with ubiquitinilation able to modulate HIF-1α (74) by increasing both the stability and the transcription efficiency (75).

Different intracellular pathways contribute to both HIF-1α stabilization and activation by other stimuli than hypoxia. For example PI3-K (Phosphatidylinositol-3OH-kinase) activation is followed by accumulation and transactivation of HIF-1 α in several cell systems as demonstrated by experiments using PI3K inhibitors (60, 61) or dominant negative mutants of PI3-K (76). The downstream targets are AKT (76) and mTOR (mammalian target of rapamycin) (77-79). The involvement of GSK3β a downstream target for Akt in HIF stabilization is still unclear (80-82). The activation of PI3-K is followed by increased protein synthesis and does not seem to interfere with HIF-1α degradation (60, 61, 63). However, there are also reports questioning the contribution of PI3-K for HIF-1 α function (83, 84). A second pathway potential implicated in HIF activation is the MAPK pathway which enhances HIF-1 α transcriptional activity via ERK1/2 (69, 85). Although both HIF transactivation domains are sensitive to MAPK inhibition, they do not contain MAPK phosphorylation sites. It has been suggested that the effect on HIF-1 α activation is mediated via transactivation of p300 (73). As a third mechanism, the p38 pathway has been implied in the induction of HIF-1 α by chromium (86). It is interesting to note that contradictory reports suggesting lack of relevance for these pathways in HIF activation currently exist. These discrepancies might reside in differences in HIF regulation depending on the cell system. Moreover these pathways are not mutually exclusive and represent a fine tuning system difficult to dissect experimentally.

2.2.1.4 HIF function

When complete after dimerization, HIF translocates in the nucleus and binds to a cored sequence termed HRE, located in the promoter of more then 60 genes (87) which are up-regulated with few exceptions (88). As a consequence, HIF is involved in different processes such as angiogenesis, tumor development, cell survival, apoptosis, inflammation, erythropoiesis, vascular tone, metabolism, epithelial homeostasis, etc (see figure 1).

The role played by HIF in angiogenesis is essential as it is also stressed by the knockout mice models which lack different components of the system. Defect of HIF-1β (89, 90) is followed by embryo lethality at day 9.5-10.5 with a phenotype characterized by defective blood formation similar to mice lacking VEGF (91, 92). A defect in HIF-1α (93-95) also induces embryonic lethality with severe vascular, cardiac and neural abnormalities. The vascular defect is not a consequence of VEGF deficiency as far as VEGF expression in these mice is even up-regulated probably through a glucose modulation (96). The absence of redundancy of the HIF α subunits is stressed by the phenotype of the HIF-2 α knockout mice. There are four homozygous HIF-2 α mice models with very different phenotypes (97-100). In two of these models, mice die in utero with either catecholamine deficiency (97) or a defect in vessel remodeling but normal vasculogenesis (98). In other two models, the mice with postnatal survival have either respiratory distress syndrome due to decreased production of surfactant (99) or multiorgan pathology possibly due to impaired reactive oxygen species (ROS) homeostasis (100).

The relationship between HIF and angiogenesis in tumors is controversial. Even though most of the experimental and human pathology studies describe a correlation between HIF and vascular density, this relation was not always found (101). The role of the HIF in tumor development is also debated but most of the reports point out to a pro-tumoral function of HIF. Several approaches have been used to investigate the function of HIF in tumor development. Most of the tumors induced by embryonic stem cells deficient in HIF-1 α showed a decreased growth rate (95, 102, 103) but not all (93). Disruption of the link between HIF and its transcription coactivators (p300 and CREB) diminishes the tumor growth (104). Induction of HIF by disruption of both VHL gene alleles is embryonic lethal (105) while heterozygous VHL mice develop cavernous haemangioma of the liver (106). However, the model of VHL inactivation gave discordant results on tumorigenesis suggesting dependency on other

genes as well (107, 108). HIF-2 α enhancing role in tumor development seems to be sustained by different approaches: HIF-2 α knock-in instead of HIF-1 α increases tumorigenesis (109), stable overexpression of HIF-2 α but not HIF-1 α overrides the tumor suppressor activity of VHL (110, 111) or enhances the tumor growth in VHL defective tumors (112), while inhibition of HIF-2 α suppresses tumor growth (113, 114). However, a tumor suppressor role of HIF-2 α has been also reported (107, 115, 116). Recently, two familial cancers due to mutations in fumarate hydratase and succinate dehydrogenaze have been linked with up-regulation of HIF through inhibition of PHD by excess succinate and fumarate (117, 118). HIF-1α and HIF-2α overexpression was found in a large panel of solid tumors (119) and correlated in most of the studies with a poor prognosis (120).

2.2.2 Growth factors

2.2.2.1 Insulin growth factor

The insulin growth factor system is complex and dynamic and includes three ligands (IGF-I, IGF-II and insulin), three receptors (IGF-I receptor [IGF-IR], insulin receptor-[IR] and IGF-II receptor [IGF-IIR]) as well as six members of the IGF Binding Protein (IGFBP) family which can be modified in functions by specific proteases (121). The system has critical roles in normal physiology but it is also involved in different pathological processes such as cancer, diabetes and vascular diseases. The central player in the system is IGF-IR which is a tyrosin kinase receptor composed of two α and two β subunits, which have similar affinity for both IGF-I and IGF-II but much lower for insulin (122). The receptor has high similarity with the IR sharing 84% homology at the tyrosin kinase region (123) making it difficult to design specific inhibitors for the IGF-IR (124-126). The complexity of the signals is amplified by hybrid-receptors which can be formed between IR and IGF-IR in both normal and tumoral tissues (127-131).

IGF-I has different biological effects, which makes this protein a good candidate for an angiogenetic factor. It has high affinity binding sites on the endothelial cells of all the vascular beds (131-133), exhibits antiapoptotic properties (134) partially through activation of FLIP (135) and has antiinflamatory properties. IGF-I contributes to endothelial migration, proliferation, survival and progenitor cell mobilization (133, 136-138) and induces vasodilation, through activation of the constitutive NOS via Akt,

in both endothelial and vascular smooth muscle cells (139, 140). The essential role of IGF-I in normal vascular development was highlighted by the impaired retinal development in oxygen induced retinopathy, as a model for retinopathy of prematurity (ROP), in the IGF-I knock-out mouse despite the presence of VEGF (141). Moreover, a similar decrease in neo-vascularization, found in a vascular endothelial cell-specific knockout model of either the IR or IGF-IR, emphasizes the involvement of these receptors in endothelial function and in the cross talk with VEGF (142). Furthermore, implantation of a IGF-I pellet in the cornea of rabbits causes neovascularization (143). IGF-I potentiates the effect of other growth factors such as PDGF-BB, VEGF, FGF-2 on vascular biology (138)

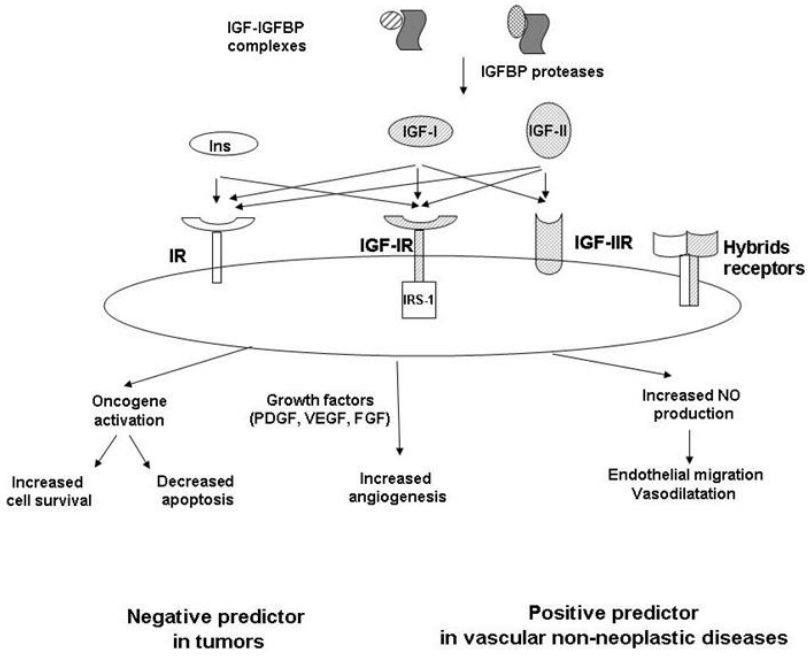


Figure 4. IGF system in tumoral and vascular diseases
Abbreviations: IGF, insulin growth factor; IGFBP, IGF binding proteins; IGF-IR, IGF-I receptor; IGF-IIR, IGF-II receptor; Ins, insulin; IR, insulin receptor; IRS, insulin receptor substrates; PDGF, platlet derived growth factor; VEGF, vascular endothelial growth factor; FGF, fibroblst grwoth factor; NO nitric oxide)

The relevance of IGF-I in vascular biology was also shown in diabetes by the demonstration that IGF-I was able to protect human retinal endothelial cells against glucose induced apoptosis (144). Patients with diabetes have low total and free IGF-I levels (145-147) that might represent a negative predictor of ischemic events (148).

The IGF system plays a critical role in cancer biology. The IGF-IR is present in most cancer cells (122) and it is required for malignant transformation induced by a variety of oncogenes (149, 150). Its contribution to cancer development is further supported by epidemiological studies demonstrating an increased relative risk for cancer in individuals with circulating IGF-I in the upper quartile (151) together with

interventional studies in animals where low IGF-I significantly reduced the growth, development and metastasis (152). Moreover, there is an up-regulation of IGF-II expression in different tumors (153) and clear evidence of loss of imprinting of IGF-II in a variety of human tumors (154).

Several methods to block the IGF-I system in tumors have been tested. The recent development of small molecules able to inhibit the IGF-IR tyrosine kinase has the advantage of high specificity and easy delivery (155).

2.2.2.2 Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF-A) belongs to a multipotent family of cytokines together with VEGF-B, -C, -D, -E and placenta growth factor (156). Alternative splicing of a single gene results in at least 5 different isoforms, with VEGF165 together with VEGF 121 and VEGF 189 being the most abundant forms and having the highest mitogenic activity (157). The VEGF receptors, previously designated as fms-like tyrosine kinase (Flt-1) and fetal liver kinase 1 (Flk-1/KDR), are currently known as VEGF-R1 and respectively VEGF-R2. Others receptors for the VEGF family have been described such as VEGF-R3/Flt-4 which is restricted to the lymphatic vessels (158) and neuropilins (neuropilin 1 and 2) that act as specific co-receptors for VEGF (159). Besides the complexity of the VEGF receptor family other factors modulate the VEGF activity. NO synthase for example is induced by VEGF in endothelial cells and functions as a negative regulator of VEGF activity through a negative feed-back mechanism (160, 161). In addition, angiopoietins that are a family of endothelial growth factors which bind to tyrosine kinase receptors Tie 1 and Tie2 play coordinated and complementary roles with VEGF in maintaining vascular homeostasis (162). VEGF binding to the receptors results in increased PI3K activity and phospholipase-Cγ activation with consecutive activation of PKC isoforms α and β (163). VEGF expression is relatively high during embryogenesis and decreases postnatal being up-regulated only at the site of active angiogenesis such as the proliferative phase of wound healing (164). The essential role of VEGF for embryonic vasculogenesis and angiogenesis is highlighted by the VEGF knock-out mice phenotype, where inactivation of a single allele resulted in embryonic lethality at day 11 and 12 and exhibited developmental abnormalities associated with defective vascularization and haematopoiesis and reduced angiogenic sprouting (91, 92).

Based on its central role in angiogenesis VEGF have been postulated to play an important role in the vascular abnormalities associated with diabetes (165). Interestingly both increased (166, 167) and unaltered (168, 169) serum VEGF were reported in patients with type 1 diabetes versus control. This can be due to either methodological differences or lack of relevance of the systemic levels of VEGF for the local changes. Reports investigating the local effect of VEGF at the site of the diabetic lesions further emphasized a role for VEGF in the disease. One of the most prominent actions of VEGF on the vascular beds, in addition to its antiapoptotic and proliferating effect, is the permeabilizing effect (170). The permeabilizing effect is partially explained by the ability to induce endothelial fenestration (171). The angiogenesis in wounds is regulated by microvascular permeability suggesting an important role for VEGF in this setting (172). Indeed VEGF increases the wounds healing rate in diabetic mice (173, 174) being important for optimal wound angiogenesis. However, other factors besides VEGF are essential for diabetic wound healing as far as VEGF blocking induces impairment of angiogenesis without delaying wound closure (175). Even though the VEGF system is extensively expressed in the kidney, neither VEGF nor VEGF blocking with specific aptamers influences the renal function in normal individuals (176, 177). However, expression of VEGF and its receptors appear to be up-regulated in the diabetes associated renal disease and VEGF inhibition results in beneficial effect of this clinical condition (178).

VEGF is an essential positive regulator of angiogenesis in cancer. Most tumors produce VEGF (157) and significantly higher levels of serum VEGF are found in patients with cancer (179). VEGF transfection induces a more aggressive phenotype of tumors in xenograft (180, 181). VEGF promotes cancer not only by stimulating angiogenesis but also by interfering with the function of the immune system through inhibition of dendritic cells maturation and modulation of natural killer cells adhesion tumoral microvessels (182). Successful clinical experience by blocking VEGF with the humanized anti-VEGF monoclonal antibody (Bevacizumab) in therapy of different malignancies confirm the importance of VEGF in tumoral angiogenesis and tumor biology (183). A variety of small-molecule receptor tyrosine kinase inhibitors that target VEGF receptors are currently developed for cancer therapy (184). Several other approaches to block VEGF have been proposed such as chimeric VEGF receptor proteins (185) and VEGF antisense oligonucleotides (186).

2.2.2.3 Other growth factors

Several other growth factors have been implicated to different extents as angiogenesis inducers.

Fibroblast growth factors (FGF) are a family of heparin-binding growth factors that mediate angiogenesis and tumor growth. FGFs exert their pro-angiogenic activity by interacting with various endothelial cell surface receptors, including tyrosine kinase receptors, heparan-sulfate proteoglycans, and integrins (187).

Platelet derived growth factors (PDGFs) and their tyrosine kinase receptors are involved in autocrine growth stimulation of tumor cells, stimulation of tumor angiogenesis and recruitment and regulation of tumor fibroblasts (188).

Transforming growth factor (TGF) has been described as both a stimulator and an inhibitor of the angiogenetic process both *in vivo* and *in vitro*. Recent reports unify these findings suggesting that specific activation of one of two different TGF receptors (endothelial cell-restricted ALK-1 versus broadly expressed ALK-5 receptor) is responsible for the switch between TGF-beta-induced biological responses (189).

2.2.3 Other regulators of angiogenesis

Beside hypoxia and growth factors, a large number of stimulatory and inhibitory angiogenesis regulators have been described as exemplified in table 2. The list however is more extensive and currently research in this area aims at identifying further members of the two categories.

Table 2. Regulators of angiogenesis

Stimulators of angiogenesis*	Inhibitors of angiogenesis*
Angiogenin	Angiostatin
Angiopoietin-1	Canstatin
Cytokines (TNFα, IL-8)	Endostatin
Chemokines	Heparinases
Integrins	TSP-1
Nitric oxide synthase	Cytokines (IL-2, Il-4, IL-18, IFN)
Matrix metallproteinases	Hormones (HCG, PRL 16kDa)

*This is a selective presentation, adapted from (190).

Abbreviations: tumor necrosis factor (TNF), interleukin (IL), interferon (IFN), human chorionic gonadotropin (HCG), prolactin (PRL), thrombospondin (TSP)

2.3 Angiogenesis and disease

2.3.1 Diabetes

A number of abnormalities associated with angiogenesis have been reported in diabetes. We face a paradox with complications associated with either stimulated or inhibited angiogenesis. An increased angiogenesis in the retina leads to proliferative retinopathy and an aberrant angiogenesis in the vessel wall leading to plaque instability. On the other hand there is a deficient angiogenesis which contributes to both chronic foot ulcers and neuropathy. Defective arteriogenesis has also been reported (191, 192) together with impaired release of the endothelial progenitor from the bone marrow with defective function (193, 194). The angiogenesis paradox in diabetes and its chronic complications is still not fully explained. We focus our following discussion on diabetic chronic foot ulcers, a model of deficient angiogenesis.

2.3.1.1 Diabetic foot ulcers – general considerations

Diabetic foot ulcers are major contributors to health care costs and hospitalization. Fifteen percent of the patients with diabetes will develop foot ulcers during their life and 85% of the non traumatic amputations are preceded by foot ulcers, half of these interventions being performed in diabetic patients (195).

A diabetic foot ulcer is defined as a full-thickness wound below the ankle in a diabetic patient (196). Until now more than 10 different systems have been developed to classify diabetic foot ulcers for clinical practice with a final international consensus (196) followed by a new "research classification"(197) which facilitates the communication in the field. It should be pointed out that the late consensus takes in account other important characteristics that influence the clinical management and prediction to heal then just the extension of the lesion as the classical systems. The PEDIS system (perfusion, extent/size, depth/tissue loss, infection and sensation) is based on these major characteristics and its validation is awaited (197). The advantage of a system which takes in account at least some of these characteristics (infection and perfusion) in prediction of the amputation has been shown (198).

2.3.1.2 Diabetic foot ulcers – pathogenesis

The development of foot ulcers is the consequence of the combination of several factors that leads to ulceration. The main causes for foot ulcers are peripheral diabetic neuropathy and micro- and macro- angiopathy. In a vast majority of patients the presence of neuropathy is the *first major* component causing pain insensitivity (199, 200). In conjunction with sensory neuropathy most of the diabetic patients which are prone to foot ulcers have also peripheral autonomic neuropathy that keeps arteriovenous shunts followed by local edema and low tissue oxygenation (201). Autonomic neuropathy is followed by a decreased activity of the sweats gland which causes dry skin prone to fissuring. The *second component* is trauma which is usual the consequence of high pressure during walking. In normal conditions the foot has the ability to distribute the high forces that apply to the plantar surface and therefore to avoid high pressures. In the presence of motor neuropathy, the weakness and wasting of small intrinsic muscles lead to muscular imbalance, with characteristic clawing of the toes and plantar flexion of the metatarsal heads leading to high pressures areas. Limited joint mobility also contributes to the appearance of high pressure regions. The *third component* is the impaired wound healing related to reduced blood supply (discussed below), abnormal expression of growth factors and local edema. The *fourth component* is the effect of high glucose per se.

The normal inflammatory phase in wound healing is essential for bringing healing messages through cytokine and growth factors secretion and angiogenetic stimuli. In diabetic foot ulcers the healing process is blocked in the inflammatory phase inducing in consequence a delay in granulation and epithelisation (202, 203). Even when healed the quality of the scar is low because collagen synthesis is impaired in the diabetic state (204) which partially explain the increased risk of recurrence.

Different growth factors which play an essential role in wound healing have been reported to have an abnormal expression in diabetic wounds (205, 206). Moreover "trapping" of the growth factors by macromolecules, such as alpha 2-macroglobulin, that leak in the dermis, may hinder growth factors to act on the target cells (207). The excessive amount of metalloproteinases, characteristic for chronic wounds (208), together with the leukocyte-derived proteases in infected wounds can also contribute to a lower bioavailability of the growth factors. The last but not the least a direct influence of diabetes i.e. hyperglycemia on the cell sensitivity is to be taken in account as it has

been observed in fibroblasts derived from the diabetic wounds that develop a phenotype resistant to growth factors (209-212).

2.3.1.3 Diabetic foot ulcers – angiogenesis

Several mechanisms are involved in the regulation of angiogenesis in diabetes, in general and in diabetic foot ulcers, in particular. Genetic mechanisms for example play certainly a role taking in account that endothelial dysfunction was reported even in patients with impaired glucose tolerance and in relatives of type 2 diabetes (213, 214). However, it has become clear from the interventional studies that chronic hyperglycemia is the essential player (215, 216). The endothelial cells are vulnerable to high glucose concentrations because they are not able to decrease the entrance of glucose in the cell in case of high outer concentration (217). Several mechanisms have been proposed to explain the deleterious effect of high glucose concentrations on the biology of the cells. Historically the first pathway proposed was the *polyol pathway*, which suggested that a chronic high intracellular glucose concentration is reduced to sorbitol and further oxidized to fructose by aldose reductase (which is a detoxifying enzyme that reduces aldehydes in corresponding alcohols). The reaction consumes NADPH which is needed for regenerating reduced glutathione, an important antioxidant. In this way activation of the aldose reductase decreases the antioxidant capacity of the cells (218). The second mechanism suggested to mediate the deleterious effect of high glucose on endothelial cells is the *production of AGE (advanced gylcozylated end products) precursors* inside the cells. AGE precursors alter the activity of different intracellular proteins (219). By diffusing out from the cells they modify matrix proteins (220) or circulating proteins which in turn bind to AGE receptors and stimulate production of inflammatory cytokines and growth factors and activate the pleiotropic transcription factor NF-κB (221-224). The third pathway activated by high glucose and considered to be involved in diabetic endothelial dysfunction is the *activation of PKC* α, β, δ by diacylglycerol formed from excessive glucose (225). PKC activation is followed by several intermediate mechanisms that contribute to the vascular pathology in diabetes, such as activating of TGF-β, PAI-1, endotelin, VEGF, NF-κB, decreasing eNOS, etc. The fourth mechanism assumed to mediate the deleterious effect of hyperglycemia on endothelial cells is represented by an increased flux on the *hexosamine pathway* where fructose-6 phosphate is diverted from glycolysis to glucosamine 6 phosphate (by GFAT glutamine: fructose 6 phosphate

amidotransferase) and finally to UDP N-acetylglucosamine (226). N-acetylglucosamine modifies posttranslational different proteins such as transcription factors (like SP1) and gene expression (i.e. increase of PAI and TGF-β1) with deleterious effect on diabetic blood vessels (227).

Recently an unifying mechanism which stands for all the deleterious effect of glucose has been proposed which resides on the increase of ROS production in mitochondria and which is alone able to activate all the four mechanisms mentioned before (228). It has been proposed that the high levels of ROS produced as a consequence of high intracellular glucose levels cause strand breaks in nuclear DNA which activates poly (ADP-ribose) polymerase (PARP) that inhibits the GAPDH and by this the precursors of the glycolytic pathway accumulates and turn on all the fourth pathways already described (229).

Peripheral vascular disease has been identified in 30% of patients with diabetic foot ulcers (199). Peripheral vascular disease is represented in patients with diabetes by macroangiopathy which is identical with the arteriosclerotic changes seen in patients without diabetes (though more distal) and by microangiopathy which is unique to the diabetic patient. Although there is no occlusive lesion in the diabetic microcirculation, as traditionally thought (230), there are functional lesions, which encompasse endothelial as well as smooth muscle cells, including deficiency of the nerve axon reflex which controls the vasodilatation after injury (231, 232). The impairment of the diabetic foot at the level of microcirculation can be considered as functional ischemia due to the vessels inability to dilate under conditions of stress. Beside this functional lesion, morphological changes are also present such as thickening of the basal membrane with reduction of the capillary size but with normal capillary density (233-235). Thickening of the basal membrane impairs the diffusion of nutrients, migration of inflammatory cells and decreases the elastic properties of the vessels wall limiting their capacity for vasodilatation.

2.3.2 Kaposi's Sarcoma

KS is a highly vascular tumor being the most common malignant tumor associated with AIDS.

2.3.2.1 Kaposi's Sarcoma – general considerations

Kaposi Sarcoma first described in 1872 (236) and traditionally encountered in a minority of patients has attracted more attention in the last period of time from the recognition of the AIDS-associated Kaposi Sarcoma (237). The disease is now classified in four clinical forms:

- Classic KS which typically occurs in the extremities of elderly non-HIV infected men of Mediteranean or Jewish ancestry.
- African KS which was largely endemic before the appearance of HIV, affecting also women and children and having a rapid disease progression. However, KS in Africa has now reached epidemic proportions because the explosive spread of AIDS.
- AIDS-related KS which, before introduction of highly active anti-retrovirally therapy (HAART), affected one third of the patients with AIDS and was often the presenting sign and had a high aggressive behavior.
- Transplant-related KS seen in immunosuppressed, transplanted patients which may be chronic or rapidly progressing.

All the forms share a common histological appearance that has been divided into different progressive stages (238). Early "patch-plaque"-stage KS lesions are characterized by the proliferation of small, irregular endothelial lined spaces surrounding new blood vessels together with infiltration of mononuclear inflammatory cells. The more advanced "nodular"-stages of KS consist of the proliferation of spindle-shaped cells which probably represent the tumor cells. The origin of the KS spindle cells is unclear being suggested to have endothelial, lymphatic or mesenchymal progenitor cell origin (239).

Supported by compelling epidemiological studies, an infectious agent was sought and subsequently discovered in AIDS-KS lesions by representational difference analysis (240). Kaposi's Sarcoma-associated herpesvirus, KSHV, the eighth human herpesvirus (HHV8), is a member of the γ2-herpesviridae subfamily. The closest related HHV is the oncogenic herpesvirus, Epstein–Barr Virus (EBV or HHV-4). KSHV infection is also associated with two rare lymphoproliferative disorders, primary effusion lymphoma (PEL) (241) and multicentric Castleman's disease (MCD) (242). HHV8 is associated with all the forms of KS (243).

2.3.2.2 Kaposi's Sarcoma – pathogenesis

The pathogenesis of KS is still unclear and involves mechanisms dependent on HHV8 but also on the local microenvironment involving inflammatory cytokines and angiogenetic factors.

The contribution of KSHV in the pathogenesis of KS is supported both by epidemiological and pathogenic data. Epidemiological surveys correlate the prevalence of the virus in a certain population with the risk to develop KS (244). HHV8, in advanced KS lesions, is present in nearly all the spindle cells (245) where it can induce expression of different proteins which have the ability to induce cell growth, block apoptosis and to promote angiogenesis and down modulate the host immune responsiveness (viral-cyclin, viral-FLIP, kaposin, G-protein-coupled receptor, etc) (246).

The tumor microenviroment is an important aspect of tumor cell progression. Signal changes between tumor cells and the surrounding tissue have been shown to stimulate proliferation, survival and migration of the cancer cells (247). Spindle cells together with the surrounding inflammatory cells express high levels of IL-6, FGF, TNF-α, VEGF, oncostatin-M and interferon-γ which have stimulating effects on the growth of the tumor cells but also are able to induce endothelial cells to aquire characteristics similar to the KS spindle cells (248). Several matrix metalloproteinases (MMP) secreted either by the KS spindle cells or by inflammatory cells from the lesions contribute, as well to the expansion of the tumor (MMP-1, MMP-3 MMP-9, MMP-12, MMP-19) (249, 250).
The male preponderance of adults with KS is still unexplained but there were suggestions on hormonal influence on KS development (251).

2.3.2.3 Kaposi's Sarcoma – angiogenesis

Both KS spindle cells and host cells stimulated by tumor cells are able to produce angiogenic factors that contribute to the characteristically highly vascular phenotype of the tumor. These molecules are produced at high levels in all the lesion stages and they usually manifest growth promoting effects on KS spindle cells, as well.

Vascular endothelial growth factor (VEGF) is suggested to have a central paracrine/autocrine role for tumor growth and tumoral angiogenesis (252). A strong expression of the VEGF receptors is found both on the KS spindle cells and on the stromal vessels around the tumors (253) and contribute to the tumor angiogenic behaviour. To support VEGF-R significance in KS biology, VEGF chimeric toxins are highly effective on experimental KS tumors (254). It has been reported also that HHV-8 is able to up-regulate Flk-1 when infecting endothelial cells contributing in this way to the KS transformation (255). Moreover HIV-1 transactivating gene protein (Tat) which is released by the infected T cells and which is highly angiogenic acts also on Flk-1 (256). VEGF contributes as well to the tumor associated edema as far as the increased vascular permeability in KS can be blocked by a neutralizing anti VEGF antibody (257).

Moreover, VEGF-C has been reported to be a growth factor for KS spindle cells (258).

bFGF is produced and released by the KS cells and it contributes highly to the tumoral angiogenesis (259). bFGF is also produced and released by the KS cells and it contributes highly to the tumoral angiogenesis (259). bFGF has autocrine and paracrine growth and chemotactic activities, and stimulates angiogenesis (260). It contributes to the development of the KS lesion (261) and it even produces KS-like lesions when the recombinant protein is injected in nude mice (262, 263). Its agiogenic action is synergized by Tat (262). It has been reported that the experimental KS development and related angiogenesis can be blocked by antisense against FGF (264).

Other molecules with angiogenic proprieties have been also described to be produced in KS tumors as: PDGF-B (265), HGF (266, 267), inflammatory cytokines (268).

3 AIMS

3.1 General aim

The general aim of the present thesis was to investigate pathogenic mechanisms involving angiogenesis regulators in two disease models (diabetes and Kaposi's Sarcoma) and to identify new therapeutic targets.

3.2 Specific aims

- To characterize the interaction between high glucose levels and HIF
- To characterize the mechanism of glucose-dependent HIF destabilization
- To describe the therapeutic effect of HIF induction in diabetic wounds
- To characterize the interaction between IGF and HIF in Kaposi's Sarcoma cells
- To characterize the mechanisms by which IGF induces HIF accumulation
- To describe the importance of IGF system for Kaposi's Sarcoma cell biology
- To describe the effect of IGF-IR blocking on Kaposi's Sarcoma cells function

4 MATERIAL AND METHODS

Cell culture (Paper I-IV)

Human dermal fibroblasts (HDFs) and human dermal microvascular endothelial cells (HDMECs) were purchased from PromoCell (Heidelberg, Germany). Primary mouse skin fibroblasts (MSF) culture was established as below. Only cells between passages 4-9 were used. KS IMM cells were kindly provided by Dr. A. Albini (Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy).

HDFs and HDMECs were maintained in commercially supplied fibroblast and endothelial cell growth media while KSIMM, or HDFs and MSF during experiments were cultured in DMEM supplemented with 2 mM L-glutamine, 100 IU/ml penicillin and streptomycin and 10% heat-inactivated bovine serum (FBS) in a humidified atmosphere with 5% CO2 at 37^{o}C.

Establishing fibroblast primary cell culture (Paper II)

Primary mouse skin fibroblasts (MSF) culture was established using the skin explant technique. Skin samples from db/db or from control mice, cleaned from subcutis when needed, were cut in small pieces (2-3-mm) and placed in 6 wells plate under sterile coverslips. Two ml of complete DMEM were added to each well.

Hypoxia induction (Paper I, II, IV)

Hypoxia studies were run in a Modular Incubator Chamber (Billups-Rothenberg) flushed with a gas mixture of 95% N2 and 5% CO2 until the concentration of O2, measured with an oxygen meter (Dräger) decreased to 1%.

Western blot analysis (Paper I-IV)

The cells were collected in phosphate buffer saline (PBS), and after centrifugation the cell pellet was frozen in liquid nitrogen. After thawing, the cell pellet was resuspended in 70 µl of extraction buffer (10 mM HEPES pH 7.9, 400 mM NaCl, 0.1 mM EDTA, 5% (v/v), glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) (paper 1) or in RIPA buffer (50 mM Tris HCl pH 8.8, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Igepal, 0.1% SDS supplemented with protease inhibitors cocktail plus freshly added 0.5 M Na_3VO_4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) (paper 2, 4) followed by centrifugation at 4^0 C for 20 min at 20 000 g.

Between 50-100 µg of total cellular proteins (measured by Bradford's assay with BSA standards) were blotted after SDS–polyacrylamide gel electrophoresis to a nitrocellulose filter and blocked overnight with 5% non-fat milk in 0.1% Tween PBS at 4^0C. The membrane was then incubated with the primary antibody diluted in blocking buffer 2 h at room temperature. After several washes, the membrane was incubated with the secondary peroxidase conjugate antibody diluted in blocking buffer for 1.5 hours. After extensive washing with PBS, the complexes were visualized using enhanced chemiluminescence (Amersham Biosciences).

Reporter Gene Assay (Paper I, IV)

To assay the effect of glucose on the transcriptional activity of HIF-1α, we have used a plasmid (pT81/HRE-luc), which contains three tandem copies of the erythropoietin hypoxia response element (HRE) in front of the herpes simplex thymidine kinase promoter and the luciferase gene. The cells to be transfected were plated in 6 wells plates and transfected at 70-80% confluency with the reporter plasmid (0.5 µg/well), after changing the medium to Optimem (Life Science Technologies Inc), using the FuGENE 6 transfection reagent (Roche Diagnostics Corp. IN, USA) according to manufacturer's instructions. After different incubation protocols depending on the

experiments (generally after 48 hours of incubation) luciferase activity was determined as described by the manufacturer (Promega Inc., Madison, WI, USA)**,** and the reporter gene activity was standardized to protein content and expressed as –percent induction relative to the activity of the control conditions.

RT-PCR (Paper I)

The expression levels of HIF-1α RNA were evaluated by RT-PCR using β-actin as internal control. At the end of the incubation period, the cells were collected in phosphate buffer saline (PBS) and after centrifugation 50 µl RNA*later* (Ambion Inc. USA) was added to the pellet, which was kept at –70^{o}C until analysis. Total RNA was extracted using Micro-to-Midi Total RNA purification kit (Invitrogen, Paisley, UK). After spectrophotometric quantification, reverse transcription was carried out using random primers and SuperScript ^{TM} II RT (Invitrogen). First strand cDNA was amplified using HIF-1α specific primers 5'-CTGTGATGAGGCTTACCATCAGC-3' (left) and 5'-CTCGGCTAGTTAGGGTAC ACTTC-3' (right) or ß-actin specific primers using 5'-GACAGGATGCAGAAGGAGAT-3' (left) and 5'-TTGCTGATCCACATCTGCTG -3' (right). The amplification parameters were set for HIF-1α 94 ^{o}C for 2 min, 55 ^{o}C for 0.3 min, 68 ^{o}C for 1 min, 72 ^{o}C for 1 min (26 cycles total) and for β-actin 94.5 ^{o}C for 5 min, 55 ^{o}C for 0.3 min and 72 ^{o}C for 1 min (24 cycles total). Both reactions were in the linear range of amplification. The PCR products were electrophoresed on a 1.2% agarose gel containing ethidium bromide.

Quantitative real-time PCR (qRT-PCR) (Paper II, IV)

Total RNA from cells was extracted from cells or tissues, using Trizol reagent (Invitrogen). First strand cDNA was synthesized from 1 µg total RNA with Superscript

III reverse transcriptase (RT) with UDG-transacetylase (Invitrogen) according to the manufacturer's recommended protocol. The cDNAs were stored at -20 ºC until use in quantitative real-time PCR.

Oligonucleotide primers specific for HIF-1α, HIF-2α, VEGF-A, GLUT1, IGF-II, VEGFR1 and GAPDH were designed using OligoPerfect or Lux programs (Invitrogen.com). Real time PCR was performed in ABI Prism 7700 light thermo cycler using the SYBRgreen Platinum kit and ROX reference dye (Invitrogen). A three-step (15 s at 94 ºC, 30 s at 60 ºC and 30 s at 72 ºC) cycling protocol was used for 40 cycles. Fluorescence data was collected at two points to monitor both amplification and melting curves. The data was analyzed using ABI prisma SequenceDetectionSystem 1.9.1. A pilot experiment with a 5-point standard curve for the target genes and the reference gene (GAPDH) showed no major differences in the amplification efficiencies. Shortly, the threshold cycle (C_T) values of the targets were subtracted from the C_T values of the reference and the difference in C_T values was plotted against the logarithm of the template amount. The slope of the resulting straight line was <0.1, indicating that the PCR efficiencies were comparable.

In the following experiments we ran one standard curve for the reference gene, and calculated the relative expression levels in each sample by a comparative ($\Delta\Delta C_T$) method. All experiments were repeated at three separate occasions and the samples were analyzed in duplicate for each gene.

Immunohistochemistry for HIF-1α in frozen material (Paper I)

Punch biopsies were obtained from the center of diabetic or venous ulcers using local anaesthesia when needed (xylocain with epinephrine, Astra-Zeneca, Sweden) and immediately deep frozen in liquid nitrogen (-70^0C). The biopsies were sliced by

cryostat (8μ) and directly fixed in acetone at 4^0C for 10 min. After washing 3 times with PBS, endogenous peroxidase was blocked with H_2O_2 (0.3%) at room temperature for 15 min. The slides were incubated with the mouse monoclonal anti HIF-1α antibody (2.95μg/ml) diluted in PBS containing 1%BSA, 0.1% Triton X-100 at 4^oC overnight. On the second day a biotin-labelled horse anti mouse antibody was added for 30 min**,** and the sections were incubated with HRP ABC-complex (ABC Elite kit, Vector Laboratories, CA, Burlingame) at room temperature for 45 min. The reaction was finally developed with DAB (Vector Laboratories, CA, Burlingame), and the slides were stained with haematoxylin. For validation of the method, the same protocol was applied to HDFs grown on glass cover slips and incubated for 6 hours in the hypoxic chamber (1%O_2) or kept in a normal incubator (21%O_2)

Immunohistochemistry for HIF-1α and HIF-2α, IGF-IR, CD34 in formalin fixed, paraffin embedded material (Paper III, IV)

Diagnostic, surgical biopsies of AIDS-related KS lesions were formalin-fixed, embedded in paraffin and sectioned for histopathological and immunohistochemical evaluation. Paraffin sections were deparaffinized, rehydrated and pretreated by microwave heating in citrate buffer, pH 6. Serial sections were stained with monoclonal antibodies against HIF-1α and HIF-2 α using a catalyzed signal amplification system (K1500, DakoCytomation, Glostrup, Denmark) and with antibodies against IGF-IR and CD34 using ABC immunohistochemistry with an anti-avidin enhancement technique. After rinsing and blocking with normal horse serum, serial sections were incubated with anti IGF-IR or anti CD34 antibody for 2 h at 37^0C. This was followed by rinsing and incubation with biotinylated secondary antibody (horse anti-mouse) for 40 min and another rinsing and incubation with ABC peroxidase (30 min). To increase the

sensitivity of ABC, a biotinylated antibody against avidin (30 min) and a second ABC peroxidase treatment (30 min) was performed. Bound ABC was visualized by incubation with fresh DAB for 2-10 min. Matched IgG isotype controls were included for each marker. All biopsies were evaluated semi-quantitatively using a four point scale for HIF α subunits (0 - less than 5% positive tumor cells, 1 - between 5 and 15% positive tumor cells, 2 – between 15 and 30% positive tumor cells, and 3 – more than 30 % positive tumor cells) in a random order by an observer who was unaware of the patient's identity and disease stage.

IGF-IR immunostaining in KSIMM cells cultured on chamber slides and fixed in PFA, 4% in PBS pH 7.4, was performed using the monoclonal mouse IgG1 antibody at a concentration of 10 μg/ml.

Evaluation of cell proliferation by ^{3}H-Thymidine incorporation assay (Paper II, III)

After 44 h of incubation with testing substances, 1μCi ^{3}H-thymidine was added to each well. Four hours later the cells were washed twice with 0.9% ice-cold NaCl. The cell-associated radioactivity, precipitated with 5% TCA, was determined by liquid scintillation counting. Results are expressed as a percentage of the control, untreated cells. In the last paper the cell-associated radioactivity Wallac Trillux 1450 microbeta counter was used.

Evaluation of cell apoptosis

MTT assay (Paper III)

After 44 h of incubation with PPP or control (DMSO) the number of viable cells was evaluated using the MTT viability assay. Briefly, 20 μl MTT (3-[4, 5- dimethylthiazol-2- yl]- 2, 5- diphenyltetrazolium bromide; thyazolil blue) (5 mg/ml) in DMEM were

added to each well. After 4 hours of incubation, the formazan crystals, produced by viable cells, were dissolved with 100 µl 0.004 N HCl-isopropyl alcohol for 5 min. The cell survival rate was calculated from the optical density at 570 nm after substracting the optical density at 620nm. Results are expressed as a percentage of the control treated cells.

TUNEL assay and morphological evaluation (Paper III)

TdT- mediated dUTP nick end labelling (TUNEL reaction) was carried out using a fluorescein labelled *in situ* cell death detection kit (Roche, Bromma, Sweden). Briefly, the cells fixed in paraformaldehyde (PFA) were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate solution for 2 min on ice and 50 µl TUNEL reaction reagent/sample was applied over the cells. After 60 min incubation at 37^{o}C in the dark, the slides were examined by fluorescence microscopy. For light microscopy evaluation the slides were incubated for 30 min, 37 ^{o}C with 50 µl converter POD in PBS, 0.1% BSA and exposed to DAB (3,3'- Diamino benzidine) solution (0.5 mg/ml) (Sigma). Finally, the samples were counterstained with Meyer hematoxilin. The classical morphological apoptosis criteria of nuclear condensation, membrane blebbing and formation of apoptotic bodies combined with TUNEL positive reaction of the nuclei were used to evaluate, by light microscopy, the apoptotis, which was expressed as an apoptotic index (percentage of apoptotic cells from 500 counted cells).

FACS with annexin V/ propidium iodide (Paper III)

Following incubation with specified substances, cells were trypsinised and than stained with annexin V and propidium iodide as specified by the manufacturer (R&D Systems, Oxon, UK) and analysed by flow cytometry. In order to compare the effect of different substances on the survival rate we generated histograms for annexin V fluorescence

and positive cells were gated and expressed as percentages from total number of the acquired cells.

IGF-I and IGF-II assay in cell culture medium (Paper III)

IGF-I and IGF-II in conditioned cultured medium were assayed after HPLC separation from IGFBPs by radioimmunoassay (RIA) using high affinity antibodies. The IGF-I assay was previously described (Bang et al., 1991) and has detection limit 0.07 ng/ml with intra- and interassay coefficients of variation 4 and 11%. IGF-II assay was performed under the same conditions as IGF-I assay except that IGF-II labelled by the chloramine T method was used as a tracer and an anti IGF-II mouse monoclonal antibody (S1F2) was used as first antibody in a final concentration of 1:150,000 (Upstate Biotechnology, Lake Placid, NY) and the separation was performed using an anti-mouse IgG (Sac-Cel, IDS Ltd, Boldon, UK). The detection limit was 0.05 ng/ml with intraassay coefficient of variation of 3%.

The HPLC separation of the IGFs from IGFBPs was performed using a size-exclusion chromatography after incubation for 30 min at 22 ^{0}C of 50 μl conditioned culture medium with 50 μl column buffer (acetic acid 0.2M, trimethylamine 0.1M and Triton X-100 0.5 g/l, pH 2.8 adjusted with sulphuric acid).

VEGF measurement (Paper IV)

VEGF protein released into the conditioned media of KSIMM cells was measured using a commercial ELISA kit (R&D Systems, Abingdon, UK) with a lowest detectable level of 5 pg/ml, and inter- and intra-assay coefficients of variation of 6.7% and 3.5%, respectively. Conditioned media was collected following 24-h of incubation

with different concentrations of IGF-I and assayed in duplicates following the manufacturer's instructions. Viable cells (trypan blue exclusion) were counted and the values were expressed as pg VEGF per 10^6 living cells.

Animals and experimental protocol and wound model (Paper II)

Male mice C57BL/KsJm _/_*Leptdb* mice (*db_/db_*) (Stock 000662) and their normoglycemic heterozygous littermates were obtained from Charles River (Belgium), housed five per cage in a 12h light/ 12h dark cycle at 22^0C and provided *ad libidum* with standard laboratory food and water. At 11 weeks of age the mice were caged individually for 1 week and then wounded as below. The experimental procedure was approved by the North Stockholm's Ethical Committee for Care and Use of Laboratory Animals.

Following blood glucose control, general anesthesia was performed with Medetomidin (1mg/kg) and Ketamine (75mg/kg). The hair of the back was shaved with an electric clipper followed by a depilatory cream. The skin was rinsed with alcohol and two full-thickness wounds extending through the panniculus carnosus were made on the dorsum on each side of midline, using a 6 mm biopsy punch. A transparent dressing (Tegaderm, 3M, Minneapolis, MN) was applied to cover the wounds after topical application of 100 μl of the DMOG (2 mM diluted in paraffin) or control (paraffin). Atipamezol 1 mg/kg was used for recovery after anesthesia. Following the surgical procedure the animals were individually housed. During the first two days after surgical procedure the animals received s.c. bupremorphine (0.03mg/kg) twice a day for relieve any possible distress caused by the procedure. The dressing was either replaced or glued if the wound was exposed before closure. At the end of the experiment which was defined as the moment when the wounds were 90% closed, the animals were

euthanized and the wounds were harvested (one frozen in liquid nitrogen and the other one fixed in paraformaldehyde 4% in PBS).

Treatment with DMOG or vehicle (100 μl) were applied thorough the dressing using a 30 Gauge needle every other day.

Digital photographs were recorded at the day of surgery and every other day after wounding. A circular reference was placed alongside to permit the correction for the distance between the camera and the animals. The wound area was calculated in pixels, using the Image J 1.32 (N.I.H., USA), corrected for the area of the reference circle and expressed as percent of the original area.

Tissue preparation and histological analysis (Paper II)

After fixation in formalin the samples were dehydrated and embedded in paraffin. Five-micron thick sections were mounted on SuperFrost slides and stored at room temperature. For histological evaluation sections were deparaffinized and rehydrated followed by hematoxylin and eosin staining. All slides were then evaluated by light microscopy by an independent observer unaware of the biopsy identity, using a semi-quantitative score to evaluate vascularity, granulation as well as dermal and epidermal regeneration. We used a four point scale to evaluate vascularity (1- severe altered angiogenesis with only 1-2 vessels per field and endothelial edema, thrombosis and/or hemorrhage, 2-moderately altered angiogenesis with 3-4 vessels per site, moderate edema and hemorrhage, but absence of thrombosis, 3-mild altered angiogenesis with 5-6 vessels per site, moderate edema but absence of thrombosis and hemorrhage and 4-normal angiogenesis with more than 7 vessels per site with only mild edema but absence of thrombosis and hemorrhage) and granulation tissue formation (1-thin granulation layer, 2-moderate granulation layer, 3-thick granulation layer, 4-very thick

granulation layer) and a 3 point scale to evaluate dermal and epidermal regeneration (1-little regeneration, 2-moderate regeneration and 3-complete regeneration).

5 RESULTS AND DISCUSSION

This work investigated the role of angiogenesis modulators in the pathogenesis of diabetes and Kaposi's Sarcoma, two disease states characterized by abnormal angiogenesis. We hope to have contributed to the current knowledge in this field by identifying new pathogenic pathways and proposing new therapeutic approaches.

5.1 High glucose impairs HIF-1α stabilization

Prolonged exposure of the tissues to hyperglycemia is currently considered as the primary pathogenic factor for development of complications in diabetes. During recent years hypoxia has also been suggested to be relevant in this context. However, when this thesis was designed at the beginning of year 2000 little was known about the interplay between glucose and hypoxia and its relevance for the clinical condition.

We first focused our investigations on the effect of glucose on HIF expression by two cell types essentially affected in the development of chronic complications of diabetes: primary human fibroblasts and endothelial cells (**Paper I)**. We did not detect HIF expression in normoxic cells and glucose did not change its level. This is in agreement with the observation made on normal retinal cells, where high glucose does not affect the normoxic levels of HIF-1α protein (269). However, the glucose effect seems to be cell type specific, as far as it has been reported to modulate HIF expression in tumoral cells where higher levels of HIF-1α where encountered in normal glucose concentration then in low glucose concentrations (270). In contrast, glucose impaired in a dose dependent manner hypoxia-dependent accumulation of HIF-1α protein in fibroblasts and endothelial cells, an effect that was mimicked by equivalent molar concentrations of mannitol. These findings suggest that osmolarity could play an essential role in HIF-1 α accumulation, which differs from the previously reported lack of influence that mannitol had on HIF-1α expression in ovarian tumor cells (271). This difference might be explained by differences of the HIF induction mechanisms used in these studies (hypoxia versus arsenite) as well as differences in the cell phenotype. The importance of the cell type for the glucose modulation of HIF-1α accumulation is also supported by our observation of an inducible effect of hyperglycemia on HIF-1α hypoxic stabilization in some cancer cell lines (SB Catrina et al, non published data, figure 5). The effect of hyperglycemia was stable and could be detected as early as 6h

after incubation. This points out that the interference of HIF-1α stabilitzation induced by hyperglycemia can stay behind both to a defect of the immediate cell reaction to hypoxia (relevant for acute ischemic events) but also to chronic adaptation of the tissues to low oxygen tensions (relevant for chronic complications).

In order to further characterize the effect of hyperglycemia on hypoxia-regulated stabilization of HIF-1α we next investigated the transcriptional activation function of HIF-1α following exposure to high glucose concentrations. To study this we used

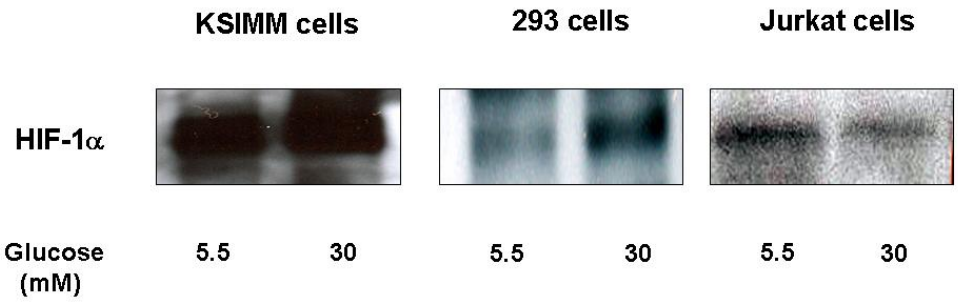


Figure 5. Hyperglycemia effect on HIF-1α stabilization is cell type specific.
Abbreviations: Kaposi sarcoma cells, KSIMM; hypoxia inducible factor, HIF.

HDFs transiently transfected with an HRE-driven luciferase reporter gene that generated a strong hypoxia-dependent response. Exposure of the cells, under hypoxic conditions, to glucose concentrations higher then 5.5 mmol/l, produced a dose-dependent inhibition of the transactivation response. Thus, in hypoxic cells the glucose-induced impaired HIF-1α protein stability correlates with a decrease in functional activity of HIF-1α. As for the protein stability experiments, mannitol mimicked the glucose effect in the reporter gene assay consistent with the suggestion that osmolarity may play an important effect. Glucose has been reported to interfere with hypoxia-dependent activation of the liver pyruvate kinase promoter (272). Because of the high homology between the HRE and glucose responsive elements (GRE) of this promoter, it has been proposed that the corresponding transcription factors HIF-1α and upstream stimulating factors could compete for binding to either response element, thereby inducing a decrease of the cognate activation pathway (272). In our

study, the glucose-induced decrease in functional activity of HIF-1α in hypoxic cells was followed by an impaired stabilization of HIF-1α protein, providing an alternative mechanism of regulation of HIF-1α function by glucose in hypoxia. In the case of cellular responses to hypoglycemia, a link between glucose concentrations and hypoxia signaling has also been observed (93-95). Embryonic cells deficient in HIF-1α are unable to upregulate several HIF-1 target genes at low glucose concentrations (93-95). However, the modulation by hypoglycemia of HIF-1α target genes seems to be dependent on the cell type varying from inhibition to stimulation (273). In our study, we show for the first time that high levels of glucose also influence HIF-1α protein levels and function in both primary fibroblasts and endothelial cells by negative interference with hypoxia-dependent stabilization of HIF-1α.

5.2 Mechanisms of glucose dependent HIF-1α destabilization

The next step was to investigate the mechanisms by which glucose interferes with the hypoxia-dependent HIF-1α stabilization (**Paper I)**.

To determine if glucose interferes with the transcription of HIF-1α we investigated by RT-PCR the RNA expression. Hyperglycemia did not modify HIF-1α RNA levels in either hypoxia or normoxia suggesting that high concentrations of glucose impair the hypoxic stabilization of HIF-1α at the posttranscriptional level. The same lack of influence of hyperglycemia on HIF-1α RNA could be confirmed later by quantitative RT-PCR in SaOs cells (an osteoblast-like cell line) (unpublished data). To confirm that the effect of hyperglycemia is at the posttranslational level we evaluated the HIF-1α proteasome-mediated degradation in normoxia which defines a central mechanism of regulation of HIF-1α. For this purpose we studied the interaction between hyperglycemia and HIF-1α stability in the presence or absence of MG132, a specific inhibitor of proteasome activity. In the presence of MG132, the effect of high concentrations of glucose or mannitol on HIF-1α disappeared, suggesting that hyperglycemia interferes with hypoxia-dependent stabilization of HIF-1α against proteasomal degradation. HIF-1α is targeted to proteasomal degradation after binding to von Hippel-Lindau, which functions as E3-ubiquitin-protein ligase. The interaction with von Hippel-Lindau requires the O_2-dependent hydroxylation of at least one of the two proline residues by specific PHD (32-34). We thus investigated whether the

inhibitory effect of hyperglycemia on HIF-1α stabilization is dependent on the proline hydroxylation using EDHB and DMOG, two different inhibitors of PHD (**Paper I, II)**. The PHD inhibitors induced HIF-1α stabilization in normoxia at the same level as hypoxia, confirming the efficiency of the chemical to block PHD in HDFs. It is not surprising to see that hypoxia increases the HIF-1α accumulation in the presence of PHD inhibitors, because hypoxia may activate different mechanisms involved in protection of HIF-1α against degradation. In addition to a decrease in proline hydroxylation, hypoxia modulates nuclear translocation, coactivator recruitment within the nucleus (9), HIF-1α acetylation (44), and so forth. Hyperglycemia was able to decrease the HIF-1α stabilization in the presence of two PHD inhibitors in both normoxia and hypoxia, suggesting that other mechanisms, in addition to proline hydroxylation, may be involved in regulation of HIF-1α protein turnover in the presence of high glucose.

Several reports pointed out that p53 is able to destabilize HIF-1α by a von Hippel-Lindau–independent mechanism [rev. in (274)]. Moreover, p53 is induced by hyperglycemia (275) which prompted us to verify whether the effect of hyperglycemia on HIF-1α stabilization is mediated by p53. The persistence of the destabilizing effect of hyperglycemia on HIF-1α in p53 deficient fibroblasts suggests that p53 is not involved in the hyperglycemic-induced degradation of HIF-1α.

Another von Hippel-Lindau–independent proteasomal degradation mechanism for HIF-1α that involves heat shock proteins (HSPs) 90 and/or 70 was recently reported (47, 48). Inhibition of these chaperones is followed by a decrease in HIF-1α protein stability and transcriptional activity in the same way as we observed after exposure of the cells to high glucose levels. It is therefore tempting to speculate that HSPs could be the mediators of the destabilizing effect of hyperglycemia on HIF-1α, especially if we take in account that HSP 70 is decreased in patients with diabetes or in experimental diabetes (rev. in (276).

5.3 Diabetic wounds express low levels of HIF-1 α

To assess the in vivo significance of hyperglycemia-induced inhibition of HIF-1α protein stability at hypoxia, we studied the expression of HIF-1α by immunohistochemistry in biopsy materials of diabetic chronic foot ulcers and chronic venous ulcers (**Paper I)**. We compared these two chronic types of lesions because

they had similar hypoxic environment but not the hyperglycemic condition. We observed both nuclear and cytoplasmic expression of HIF-1α in fibroblasts of venous ulcer biopsy material. The cytoplasmic presence of HIF-1α could be due to enhanced protein stabilization and accumulation before nuclear translocation (277). In contrast, low levels of HIF-1α protein characterized the biopsy material from diabetic ulcers, generating faint cytoplasmic staining in scattered fibroblasts. These data suggest that inhibition of hypoxia-induced stabilization of HIF-1 protein by hyperglycemia *in vitro* also occurs *in vivo*.

5.4 HIF-1α up-regulation normalizes the healing rate of experimental diabetic wounds

Based on our *in vitro* and *in vivo* findings we hypothesized that the defect of a diabetic cell to adapt to hypoxia might play a central role in determining the clinical development of chronic complications of diabetes. Previous investigations suggested that beside glucose levels, oxygen levels are important for devellopment of chronic diabetic foot ulcers (278, 279) and diabetic neuropathy (280, 281). We therefore choosed to investigate the relevance of glucose induced HIF-1α destabilization and HIF-1α therapeutic modulation for healing of experimental diabetic wounds using db/db mice (**Paper II)**, known to have a delayed wound healing similar to that in humans (282). Db/db mice have been widely used to study the diabetic wound pathophysiology and potential therapies (283, 284) and we have observed that in mouse fibroblasts glucose has the same destabilizing effect on HIF-1α accumulation as in human cells (figure 6).

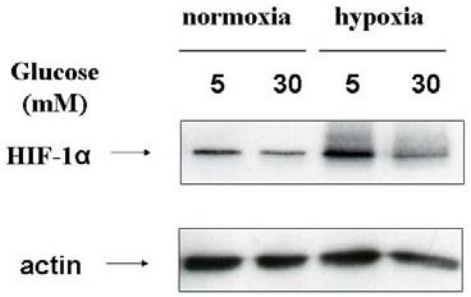


Figure 6. Hyperglycemia impairs HIF-1a stabilization in mouse dermal fibroblasts.
Abbreviations: hypoxia inducible factor, HIF.

The next step was to identify potential therapeutic candidates for *in vivo* modulation of the HIF-1α. We have therefore investigated the ability of two compounds which interfere with PHD activity, by either chelating Fe^{2+} (DFX) or by competing with 2-oxoglutarate (DMOG), to induce HIF-1α accumulation in skin fibroblasts during incubation with different glucose concentrations and oxygen tension. As expected both substances (DMOG 200 μM, DFX 100μM) were able to induce HIF-1α accumulation in normoxia in primary fibroblasts. The HIF-1α induction in normoxia and hyperglycemia was higher than the hypoxic stabilisation, suggesting that both substances are potential candidates for locally stabilizing HIF-1α in diabetic wounds.

To avoid a potential toxic effect, we have further investigated the effect of DMOG and DFX on the proliferation rate of dermal mouse fibroblasts (MSFs) in order to select the best candidate for *in vivo* studies (**Paper II)**. DMOG was practically not interfering with the MSFs proliferation rate up to a concentration of 200 μM. In contrast DFX inhibited the proliferation rate of MSFs at concentrations higher then 1 μM. Similar results were obtained on human dermal fibroblasts (HDFs) (figure 7). Even though potentially more toxic at high doses, further investigations of the effect of DFX on wound healing at low, non-toxic concentrations are warranted taking in account other properties of DFX, such as its antioxidant effects, which are foreseable positive for diabetic complications therapy (285). Moreover it has been reported that DFX has higher affinity for FIH than for PHD opening the possibility to use lower nontoxic, doses of DFX that could fully activate HIF-α transactivation even in the absence of an obvious stabilization (286).

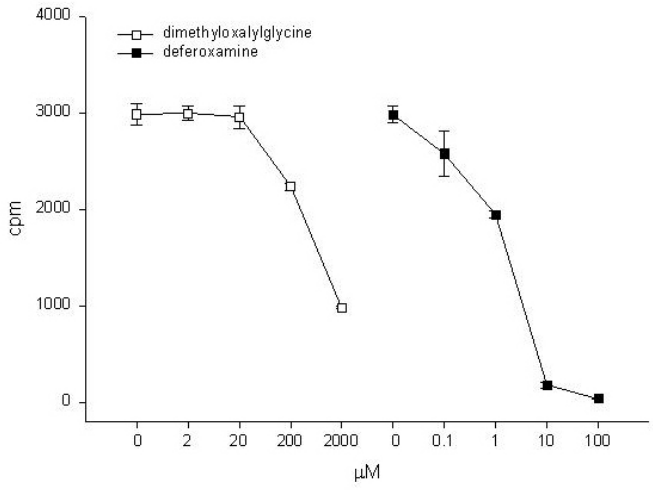


Figure 7. DMOG effects on human dermal fibroblasts.
Abbreviations: cpm, counts per minute; DMOG, dimethyloxalylglycine.

We continued our investigations by studying the effect of topical DMOG application on the healing rate of the diabetic wounds in db/db mice. Two groups were included for comparison: vehicle treated db/db mice and vehicle treated heterozygous normoglycemic littermates. We have applied a concentration of 2 mM taking in account the dilution due to wound fluid. A wound dressing was used to minimize the skin contraction and to allow a prolonged contact of the therapy with the wound surface. Analysis of the wound closure was performed through digital processing of pictures taken every other day until 90% closure (as evaluated by two independent observers). Wounds healed 90% after 14 days in db/db mice and after 12 days in their normoglycemic littermates. DMOG (2mM), applied every other day on db/db mice wounds, accelerated wound healing with a 90% closure after 12 days. The difference in the wound healing dynamic between untreated and DMOG treated db/db mice became statistically significant at day 8 and persisted at all the following time points. Moreover, no differences between db/db DMOG treated animals and their heterozygous normoglycemic littermates were observed with the exception of day 2, when the normoglycemic mice had smaller wound area. The difference in the apparent wound area observed in the first days between db/db mice (DMOG or vehicle treated) on one side and normoglycemic littermates on the other side could be explained by the fact that large parte of wound healing in rodents is due to contraction which is minimized by the splitting effect of the obesity in db/db animals (282). The treatment had no influence on the blood glucose levels which did not vary significantly in either group.

To better characterize the therapeutic effect of the topical application of DMOG we evaluated the histological characteristics of the wounds. Diabetic non-treated wounds were characterized by a thin to moderate granulation tissue formation with a low vascular count as well as reduced regeneration of the dermal and epidermal layers. Endothelial edema, thrombosis as well as an increase in the adipose tissues were present to different extent in all non-treated diabetic wounds. In contrast, a thick granulation tissue, moderate vascular count as well as good regeneration of the dermal and epidermal layers were present in DMOG treated diabetic wounds. Occasional endothelial edema but no signs of thrombosis and/or hemorrhage were present. The non-diabetic control mice showed almost full recovery with complete regeneration of the dermal and epidermal layers, moderate to high vascular count as well as a thick granulation tissue with low number of infiltrating inflammatory cells. Histological evaluation demonstrated a significant better wound healing in controls and DMOG treated diabetic mice as compared to diabetic non treated mice as seen by significant

higher scores for vascularity as well as for dermal and epidermal regeneration. Granulation tissue formation tended to be higher in controls and diabetic treated mice as compared to diabetic non-treated mice but the difference did not reach statistical significance.

To determine the functional consequence of DMOG-dependent PHD inactivation in the wounds we measured by qRT-PCR the mRNA for HIF-1α as well as several HIF-1α target genes. Treatment with DMOG was able to induce both VEGF-R1 and IGF-II which are target genes for HIF and which both are relevant for wound healing and tissue regeneration. As expected, DMOG did not modulate HIF-1α mRNA taking in account that the compound induce HIF-1α accumulation and function by acting at posttranscriptional levels. We did not detect changes in the VEGF mRNA levels. This is not surprising, in the light of the recent observation that VEGF is not induced by hypoxia in db/db mice (287). Wound healing is not only dependent on the angiogenesis. Normal wound healing in presence of impaired angiogenesis was reported following local treatment with soluble VEGF receptor (175). Thus, the positive effect of induction of HIF on wound healing may involve apart from angiogenesis other complex mechanisms.

5.5 IGF I induces HIF-1α and HIF-2 α in Kaposi's Sarcoma cells

Apart from hypoxia, growth factors have an essential role in the regulation of angiogenesis. IGF-I is potential relevant for disease states characterized by abnormal angiogenesis being involved in both vessel and tumour biology. Therefore we continued our investigation by studying the interaction between HIF and IGF in KS, a disease model in which both growth factors and angiogenesis are essential.

To study the *in vitro* effect of IGF-I on KS we used one of the three established KS cell lines, KSIMM. These cells produce highly angiogenic and hemorrhagic tumors when injected in nude mice, mimicking the human disease (288). We have described for the first time that HIF-2α and to a lesser extent HIF-1α were expressed even in normoxia in KSIMM cells and both congeners were highly up-regulated by hypoxia (**Paper IV)**. IGF-I was able to stimulate both HIF-1α and HIF-2α accumulation in KSIMM cells, in a concentration dependent manner. We further investigated whether the accumulation of HIF−1α and HIF−2α proteins induced by IGF-I was also followed by activation of their function. For this purpose we transiently transfected the KSIMM cells with an

HRE-reporter construct and exposed the cells to either IGF-I or hypoxia. Both hypoxia and IGF-I induced significantly the luciferase reporter gene after 24 hours exposure. Moreover, IGF-I induced a significant increase in the expression of both VEGF-A and GLUT1, two target-genes for HIF, as evaluated by quantitative RT-PCR. The functionally relevance of our findings was confirmed at the protein levels, IGF-I being able to significantly increase the VEGF secretion in the medium of KSIMM treated cells. To our knowledge this is the first report which shows that IGF-I is able to increase both HIF α isoforms in the same cell system. In the light of the recently reported observation on the specifically activation of certain genes by either HIF-1α or HIF-2α (289), the common up-regulation of both HIFα isoforms encountered in KSIMM cells, suggests that IGF-I generally activates the HIF target genes and by this, it largely contributes to the characteristically vascular phenotype of KS.

5.6 Mechanisms of IGF dependent HIF accumulation

In order to investigate the mechanisms behind the IGF-I induced HIF-1α and HIF-2α accumulation in KSIMM cells, we evaluated the IGF-I effect at the transcriptional level. IGF-I did not modify the levels of mRNA for either HIF-1α or HIF-2α as measured by qRT-PCR at the same time points where we observed an increase in both HIF-1α and HIF-2α protein accumulation (**Paper IV)**. This demonstrates that IGF-I acts at a posttranscriptional level to induce both HIF-1α and HIF-2α in KSIMM cells. To further dissect the mechanism by which IGF-I induces accumulation of HIF α subunits we compared the dynamic of the accumulation of the HIF α subunits after treatment with $CoCl_2$ and IGF-I. $CoCl_2$ functions as a hypoxia mimetic and stabilizes the α subunit by interfering with its degradation (51, 290). $CoCl_2$ induced both HIF-1α and HIF-2 α later than IGF-I, suggesting a different mechanism of action between the two stimuli. We have further demonstrated that IGF-I did not interfere with the HIFα degradation, as far as in contrast with $CoCl_2$ induced- HIF-α subunits, the IGF-I induced HIF-α quickly disappeared when the protein synthesis was blocked with cycloheximide (CHX). IGF-I induced accumulation of both HIF-1α and HIF-2α at a posttranscriptional level and we suggest that it acts by increasing their translation. Our observation on the IGF-I induced translational stimulation of both HIF-2α and HIF-1α is in agreement with previous reports that have suggested a different regulation of HIFα subunits by growth factors as compared with hypoxia (291). It is not surprinsing that

IGF-I stimulates the translation of HIF α subunits taking in account that all the members of the IGF-insulin family generally activate the translational machinery (292).

5.7 KS lesions express high levels of HIF-1 α and HIF-2 α

To study a potential clinical relevance of our results we investigated the expression of both HIF-1α and HIF-2α in KS biopsies obtained from 11 HIV positive patients (**Paper IV)**. In all the cases studied both HIF α subunits were expressed throughout the tumour area but not in the normal dermal tissue which surrounded them. HIF-1α staining had both a nuclear and cytoplasmatic pattern and increased significantly in the late "nodular" KS biopsies compared with the early "patch" biopsies. HIF-1α was expressed in spindle tumoral cells, endothelial cells, infiltrating leucocytes and the basal layer of the epidermis. Even though the distribution of the HIF-2α immunostaining had a similar cellular distribution pattern as HIF-1 α, there was no difference in HIF-2 α expressions between "nodular" and "patches" cases. Over-expression of HIF is encountered in most of the solid tumors (119, 293) and is suggested to be associated with lack of response to therapy (294). The mechanism for the up-regulation of HIF in tumoral tissue can be due to the hypoxic environment that is common in neoplasms and correlates with a poor prognosis (295-297). It can also be an consequence of the abnormal regulation of HIF in tumors by oncogenes or growth factors (291). Taken in account the highly vascular phenotype of the KS tumors and that the immunostaining for HIFα subunits are present all over the tumoral tissue and not just in "perinecrotic areas" it is unlikely that the over-expression of HIFα isoforms is a consequence of hypoxia. Even though the HIFα isoforms have a high similarity in structure and function there is data which suggests different functions for the HIFα isoforms (289). HIF-2α seems to be more important for promoting tumor development (112, 113, 298) than HIF-1α which recently has been reported to have even an antitumoral effect on a VHL- associated renal cell carcinoma (112). KS is a multistage progressive disease which begins as an early "patch-plaque stage" and evolves to a late "nodular" stage (299). Our observation that HIF-2α is over-expressed already in the early stages of the tumor development is in agreement with the tumor promoting role of this HIFα isoform. Whether, the predominance of HIF-1α in the nodular forms compared with the early stage of the tumor is a self limiting reaction of the tumor or the consequence of hypoxic environment due to the tumor development warrants further

investigation. The progressive infection of the tissue with HHV8 during the multistage evolution of KS (300) can offer another explanation of the fully activation of HIF-1α only in the late stages taking in account the recent finding that an HHV8 oncogene (Kaposi Sarcoma-associated Herpes virus G protein-coupled receptor) is able to activate HIF-1α (301).

5.8 IGF promotes survival of Kaposi's Sarcoma cells

As IGF is not only a pro angiogenetic factor, but also a tumor growth promoting factor, we went further to investigate the effect of IGF on KS proliferation. IGF-I stimulated the proliferation of KS IMM cells in a dose-dependent manner (**Paper III)**. IGF-I has been shown to be a growth factor for different tumours and prospective epidemiological data suggested that high circulating levels of IGF-I confer increased risk for different solid tumors (reviewed in (121)). For KSIMM cells, IGF-I had the same growth promoting potency as VEGF, which is postulated to be one of the most important growth factors for KS tumors (302). Furthermore, IGF-I and VEGF have an additive effect on the KSIMM proliferation rate, which suggests that these two growth factors may act at least in part independently to promote growth. VEGF and IGF-I cellular pathways, in normal endothelial cells, have been reported to either act independently (141) or to interact (303). The cooperation of the growth promoting effect between VEGF and IGF-I is even more interesting in the light of our observation that IGF-I is able to stimulate VEGF secretion in KSIMM cells, suggesting a complex interaction (**Paper IV)**.

5.9 KS cells express IGF-I receptor

Based on the previously presented data we concluded that the IGF is an essential growth factor for Kaposi's Sarcoma as well as a tempting therapeutic target. However, an essential step in designing specific therapeutic approaches is the characterization of the pathogenic pathway. Therefore we first investigated the receptor which mediates the growth-promoting effect of IGF-I (**Paper III)**. We have first characterized pharmacologically the receptor involved by comparing the growth promoting effect of insulin with des (1-3) IGF-I, which is an IGF-IR agonist that binds minimally to the IGFBPs. Des (1-3) IGF-I stimulated the growth rate of KSIMM cells in a dose dependent manner and was far more potent than insulin suggesting that IGF-IR is the

mediator of IGF-I effects on KSIMM cells. The presence of this receptor in KSIMM cells was confirmed by immunohistochemistry (**Paper III)**. The functional involvement of these receptors as mediators of the IGF-I growth promoting effect was further demonstrated by the complete abolishment of the IGF-I growth promoting effect when the cells were co-incubated with an anti IGF-IR specific monoclonal blocking antibody (αIR3). Moreover, we were able to demonstrate that the IGF-IR mediates antiapoptotic signals in KS cells, as far as blocking of the IGF-IR induced cell apoptosis. The profound effects which followed the specific blocking of the IGF-IR on basal non stimulated KSIMM cells suggested the presence of an autocrine loop essential for both proliferation and survival of these tumoral cells. We have in consequence analysed the secretion of IGF-I and IGF-II by KSIMM cells, as potentially equal potent ligands for IGF-IR. Both IGF-I and IGF-II mRNA expression have been documented previously in AIDS-KS cultured cells (304) but, to our knowledge, there is no previous report on their secretion by KS cells. To avoid the known interferences of IGFBPs with IGF-I and IGF-II assays, which could give false positive results we have first separated them by HPLC (**Paper III)**. We were able to detect IGF-II but not IGF-I in the conditioned medium. The presence of the IGF-II as member of the endogenous IGF system in KS cells is not surprising. There is clear evidence of IGF-II is up-regulated in different tumors (153).

The relevance of our findings was confirmed by the identification of IGF-IR expression in the spindle cells of AIDS-related KS biopsies. No clear difference in the IGF-IR expression between early and late KS stages was observed, suggesting a constant IGF-IR expression during the characteristic multistage development of KS tumours.

5.10 IGF receptor blocking decreases HIF accumulation and promotes apoptosis in Kaposi's Sarcoma cells

The presence of IGF-IR in the tumor specimens from patients with AIDS-KS in addition to the essential functional role of the IGF-IR in KSIMM biology prompted us to investigate the potential therapeutic effect of IGF-IR blocking. Numerous attempts have been made to block the IGF system for treatment of tumors [reviewed in (305)] but most of the methods have had different disadvantages such as lack of specificity, difficulties of drug delivery etc. One of the recent most promising approaches is to use specific small molecules that inhibit IGF-IR tyrosine kinase (121). In this light, we have tested the effect of PPP, which has been shown to be a potent and a specific

inhibitor of the IGF-IR tyrosine kinase (124). Treatment of the KSIMM cells with PPP induced a dose dependent apoptosis in the same dose range reported for other IGF-IR positive cells (**Paper III)**. We would like to point out that the compound is active even in the presence of foetal calf serum (FCS), which shows that blocking the IGF system overrides the survival signals from other growth factors. Moreover, we were able to demonstrate that treatment of KSIMM cells with PPP cancelled the effect of IGF-I on the accumulation of both HIF-1α and HIF-2α and on the induction of VEGF expression (**Paper IV)**. VEGF mRNA levels decreased after treatment with PPP even below the control basal levels which is in agreement with our suggestion on the existence of an autocrine antiapoptotic loop in KSIMM cells (306). This also becomes relevant for the angiogenetic potential of these cells. Our observation is in agreement with previous reports which have suggested a combined antiangiogenic and antitumoral effect for other IGF-IR specific kinase inhibitors (126).

6 POINTS OF PERSPECTIVES

This thesis has been designed with the aim to identify new pathogenic mechanisms, relevant for diseases characterized by dysregulated angiogenesis such as chronic complications of diabetes and tumors. We found new mechanisms which affect different regulators of angiogenesis and proposed new therapeutic approaches not applied at present.

The knowledge on the role played of hypoxia, in general, and of HIF, in particular, in diabetes was limited when this work started at the beginning of year 2000. We demonstrated that hyperglycemia interferes with hypoxia-dependent stabilization and function of HIF-1α. Based on this observation we proposed that the deficiency of the diabetic cell to adapt to hypoxia might play a central role in pathogenesis of chronic complications of diabetes. We have further confirmed our hypothesis by showing the positive effects of HIF induction in an experimental model of diabetic wounds. These results might offer the premises for conducting clinical studies on wound healing in patients with diabetes. A substance (deferoxamine), which we used efficiently *in vitro* to induce HIF-1α is already clinically approved for other indications. A successful result could extend the application to other diabetic complications in which angiogenesis is impaired (e.g. neuropathy, arteriosclerosis). Furthermore, identification of the critical residues for hyperglycemia-induced HIF-1α destabilization will allow us to tailor more specific future therapies.

As our main research focus was on regulators of angiogenesis we choose to extend our studies to the opposite end of dysregulated angiogenesis, namely excessive angiogenesis in tumors. KS is a highly vascularised tumor and its biology is strictly dependent on angiogenetic regulators. Here we were also able to demonstrate that HIF modulation influences disease pathogenesis. Moreover, we described the interaction between HIF and IGF in KS, and we noted the importance of the IGF-I receptor in KS proliferation and apoptosis. The specific IGF-IR inhibitor (PPP) used in our *in vitro* settings has proved to be efficient and non toxic in previous *in vivo* tests on tumors implanted in mice. Thus a direct continuation of the present work will be to test the compound in KS implanted in mice. After completing additional studies in different experimental models, clinical studies with PPP in KS might result in new treatment

strategy. Furthermore a deeper analysis of the pathways involved in mediating the IGF-I activating effect on HIF in KS will enable design new therapeutic approaches. Finally by using the same research approach, other tumors susceptible to such therapies could be identified.

Identification of the IGF-I stimulating effect on HIF-1α in KS cells could be also the starting point for evaluating IGF-I as a strategy for inducing angiogenesis and stimulating the wound healing

7 CONCLUDING REMARKS

1. HIF is an important factor and a potential therapeutic target in chronic complications of diabetes.

- High levels of glucose influence HIF-1α protein levels and function in both fibroblasts and endothelial cells
- Hyperglycemia interferes with hypoxia-dependent stabilization of HIF-1α against proteasomal degradation, partially independent of PHD.
- Diabetic wounds express lower levels of HIF-1α as compared to venous ulcers, confirming the clinical relevance of the *in vitro* findings.
- Both DMOG and DFX are potential candidates for inducting HIF-1α in diabetic wounds
- Topically applied DMOG normalized the healing rate of diabetic wound in an experimental animal model by increasing vascularization and epidermal regeneration suggesting that chemical inhibition of PHD is a promising therapeutic approach for treatment of diabetic foot ulcers.

2. IGF-I is an essential factor in determining the growth and the pro-angiogentic potential of KS tumoral cells, suggesting that interference with IGF-I system is a promising therapeutic target.

- IGF-I induces HIF-1α and HIF-2α accumulation in KS cells via a posttranscriptional mechanism independent of the classic hypoxic regulation.
- IGF-I induces HIF-1 function and HIF target genes in KS cells
- IGF-I is a growth and angiogenesis promoter in KS cells
- *In vivo* KS lesions express high levels of HIF-1 α and HIF-2 α in addition to IGF-IR
- Specific blocking of the IGF-IR decreases HIF accumulation function and induces apoptosis in KS cells, suggesting a new therapeutic approach to treat these tumors.

8 ACKNOWLEDGEMENTS

I am happy that I arrived at this point, to write my second thesis. I promise that it will not be followed by another PhD thesis, at least not in medicine. I am here today just because I was lucky enough to meet people who made it possible.

I would like to start by thanking my supervisor Prof. **Kerstin Brismar** for her continuous support, energy and enthusiasm. She has the power to make things happen in a pleasant and peaceful way. She shares her time with everyone who needs any kind of help, as one may wonder if she ever can find a bit of time for herself. She is an example of how a clinician can do basic research. I have to confess that I would have never started a second PhD if I hadn't met her.

To my co-supervisor Prof. **Lorenz Poellinger** for introducing me to the hypoxia field. I appreciate his gentle way modulating my "Latin-Balkanic way" to write rebuttal letters. I take the opportunity to apologize for literally hunting him to have his opinions on our papers.

To my co-supervisor Doc. **Moira Lewitt** for her rigorous approach in experimental design and for sharing with me her knowledge and experience on the IGF system.

Prof. **Mihai Coculescu** my first PhD supervisor, for his support during these years to maintain my professional relation with Bucharest. He is the person who fooled me into becoming an endocrinologist, so he is the person to blame.

I would like to pay tribute to the late Professor **Viktor Mutt.**

Prof. **Peter Biberfeld** for all the help during years, for pleasant collaboration and interesting discussions. .Prof. **Gustav Dallner** for his inexhaustible energy and enthusiasm for science and for life in general. Prof. **Magnus Axelson** for nice collaboration. Prof. **Kerstin Hall** for her critical and constructive remarks.

I wish to specially acknowledge **Ileana Botusan** who gave me the opportunity to conduct her first steps in science and who became in short time a valuable scientist and friend, playing an important role in completing the experimental work of this thesis. **Jacob Grünler** for his continuous support in everything related to the lab and not only that. I admire his patience and support, and his ability to tolerate my first stammers in Swedish. I am happy that I had the opportunity to know him outside the lab and to enjoy his and **Sonia**'s friendship. **Ken Okamoto** for his patience with my Latin (and definitely not my Japanese) way of solving and handling the laboratory mistakes I made throughout our collaboration. I think that we made a very good team with continuous night and day shifts. **Anja Ranatanen** for bringing an active atmosphere in the lab. **Jing Wang** for the late talks around the scientific and non scientific world. **Octavian Savu** for keeping me in touch with the lab work during my thesis writing. **Katrin Brandt** for carefully organizing the cell culture room, **Henrik von Horn** for

his never ending questioning of science. **Inga Lena Wivall** and **Elvi Sandberg** for their technical assistance and for making things easy in the lab.

Anica Dricu for interesting scientific collaboration and for introducing me to Kerstin**. Teresa Pereira** for her continuous help in HIF lab techniques. **Charles Massambu**, **Pawan Pakyarel** for amiable collaboration. **Alena** and people in the gastro Lab for making cohabitation on the same floor pleasant: **Kristina, Sofie, Cecilia, Therese, Mark and Vjiolé. Gerg Jambor** for linquistic advice.

It is time to mention the people who made my life pleasant outside the scientific world. They have not contributed directly to the results of this thesis but without them life would not be as fun as it is. To make the story short: life is not just in the lab.
Anki Popescu and **Constantiu (Puiu) Mara** the first Romanian friends who welcome us in Stockholm and for constantly being so close to us. **Maria** and **Iulian Necula** for being so kind, so hospitable and helpful, for making us feel home when we visited them. **Eni** and **Doru Dricu** for being all the time so energetic and making good things without a glance, for believing so deeply in the Romanian spirit. **Mihai Cotfas** for his invaluable support at the beginning of my Swedish period and more. **Ana Cotfas** for her way of making us very welcome in her house even when the boat arrived late in the evenings. **Florin Sirzen** for being such a good friend despite geographical distance.

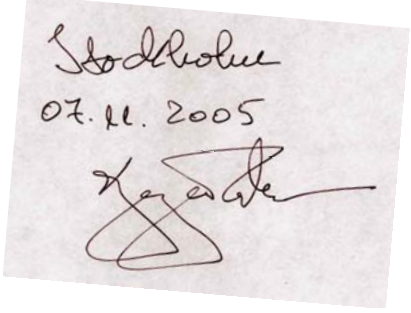
My dearest friends **Claudia** and **Dan Minulescu** (good that I wait a bit with the thesis to have the opportunity to write a common family name) for being the best friends and godparents anybody can have. To **Irina** and **Dan Ionescu** for their eternal friendship, and when I say this I believe that 27 years can define eternity. **Vali Leanca** for his deep friendship and for his way of being happy and making things happen in a very happy way.

To **Flori** and **Marinică** for the warm atmosphere you feel when you meet them, **Oto** and **Georgiana** for always have a special perspective on life. To **Dori** for having such a wonderful girl and for taking so much care of her, for showing her the same values I believe in.

To my special **Tanti** for being like my second mother.

To my parents **Veronica** and **Sergiu** for their love and support, for believing so much in me, and for giving me the feeling that I always can easily reach what I want.

To my wife **Anca** for being who she is, for her continuous love and support, for being my half. To our daughter **Ioana** the sunshine of our lives. Vă pupă tata pe amândouă.



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