Hypoxia-Inducible Factor-1α and Hypoxia-Inducible Factor-2α Are Expressed in Kaposi Sarcoma and Modulated by Insulin-like Growth Factor-I

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Abstract

Purpose: Neoangiogenesis is essential for tumor development. Hypoxia-inducible factor (HIF), a transcriptional factor composed of two subunits (α and β), plays a key role in this process, activating proangiogenic factors such as vascular endothelial growth factor (VEGF). The HIF α subunits are critically regulated by oxygen and are also modulated by growth factors. Kaposi sarcoma (KS) is a highly vascular tumor that releases large amounts of VEGF and for which we have recently described an essential role for the insulin-like growth factor (IGF) system. We therefore investigated the expression of HIF α subunits in biopsies from KS tumors and their modulation by IGF-I in KSIMM, a KS cell line.

Results: Both HIF-1α and HIF-2α were expressed in KS biopsies in all tumoral stages. HIF-1α immunopositivity increased through the tumor development with highest expression in the late nodular stages. In KSIMM cells, IGF-I induced accumulation of both HIF α subunits. The induction suggests a translation mechanism as documented by cycloheximide chase experiment coupled with constant RNA levels as evaluated by quantitative real-time PCR. IGF-I – induced HIF α accumulation was followed by an increase in HIF function as assessed both by reporter gene assay and by induction of endogenous target gene expression (VEGF-A). Specific blockade of IGF-I receptor with αIR3 antibody or with picropodophyllin, a specific IGF-IR tyrosine kinase inhibitor, diminishes the basal and IGF-I – dependent induction of both HIF α congeners.

Conclusion: These novel findings show the coupling between the IGF and HIF signaling in KS and suggest a coordinated contribution by these pathways to the characteristic vascular phenotype of this tumor.

Neovascularization represents an essential pathogenic step in the development and evolution of tumors. Hypoxia plays an important role for expansion of the vascular bed both in normal and in tumor tissue. The hypoxia-inducible factor (HIF) is the most prominent transcriptional factor that activates the hypoxic expression of target genes involved not only in angiogenesis [e.g., *vascular endothelial growth factor* (*VEGF*)] but also in glycolysis, cell proliferation, and apoptosis, and adapt the cell to low oxygen concentrations. HIF is a heterodimer consisting of α and β subunits, which are constitutively produced in the cells. Only the α subunit is subjected to oxygen regulation. An oxygen degradation domain has been identified in the α subunit (1), which has been found to require hydroxylation of two critical proline residues in the presence of oxygen (2, 3) by a family of iron (II) – dependent prolylhydroxylases (4, 5). In the hydroxylated form, it binds to the von-Hippel Lindau protein, which functions as an E3 ubiquitin ligase and targets HIF-1α for rapid degradation via the proteosome pathway. Under hypoxic conditions, prolylhydroxylation of HIF-1α is blocked, allowing stabilization of the subunit that becomes free to bind the HIF-1β subunit and form the HIF-1 transcription complex. HIF-1 binds to the hypoxic responses element (HRE) – containing promoters of the regulated genes (60 described to date), which adapt the cell to the hypoxic environment. The transcriptional activation of HIF is subjected to an independent regulation that is also oxygen dependent (6).

A number of isoforms of the α subunit have been identified. Whereas HIF-3α lacks structures for transactivation and can act as a negative regulator (7 – 9), HIF-2α has a high degree of similarity in structure and function with HIF-1α but has a restricted tissue distribution (10).

HIF-1α and HIF-2α are expressed in many tumors and their expression has been linked with a poor prognosis for a variety of tumors (11). Their expression in tumor tissue but not in

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most of the normal tissues is mainly considered to reflect the relative hypoxia in a rapidly growing tissue (12). However, new data suggest that HIF-1α or HIF-2α can be activated by different oncogenes, cytokines, and growth factors. In consequence, up-regulation of HIF in tumors is not just a response of the tissue to hypoxia but represents a step in the oncogenic transformation process and it is a driving force in tumoral neovascularization, at least partially via stimulation of VEGF expression.

Kaposi sarcoma (KS), the most frequent tumor in acquired immune deficiency syndrome (AIDS) patients, is a highly vascularized tumor characterized by the presence of proliferating spindle cells admixed with mononuclear inflammatory cells (lymphocytes, macrophages, and plasma cells). The tumor-associated angiogenesis is a hallmark for KS, and the tumor biology strictly depends on angiogenetic stimuli, including VEGF (13). Moreover, the characteristically spindle-shaped KS cells produce a large amount of VEGF (14). Although HIF-1 is one of the most potent activators of VEGF and a key regulator of angiogenesis, there is no information available on HIF function in KS.

We therefore investigated the expression of HIF-1α and HIF-2α in biopsies from patients with AIDS/KS. Both HIF congeners were highly expressed in tumoral tissue through all stages of disease development (15). The highly vascularized phenotype of KS tumors suggests that HIF up-regulation is unlikely to be induced by local hypoxia. Taking into account that we have recently shown that the insulin-like growth factor (IGF) system is essential for KS biology (16) and that IGF-I has been reported to modulate HIF-1α (17–19), we have investigated the influence of IGF-I on HIF-1α and HIF-2α function in KSIMM, an established KS cell line. We were able to show that the KSIMM cells expressed both HIF-1α and HIF-2α and that IGF-I stimulated their accumulation and HIF function. In contrast to substances mimicking hypoxia, such as $CoCl_2$, which protect HIF α subunits against degradation, IGF-I induced both α subunits at the translation level. Blocking the IGF-IR diminished the accumulation of HIF-1α and HIF-2α, which is followed by a decrease of VEGF expression. These findings suggest that interference with the IGF system could contribute to a decrease in the vascularization of the tumor, which, in turn, is the hallmark of KS.

Materials and Methods

Materials. Recombinant human IGF-I was provided by Pharmacia (Stockholm, Sweden). Cell culture reagents were from Life Technologies (Stockholm, Sweden). Complete Protease Inhibitor tablets were bought from Roche Diagnostics Corp. (Indianapolis, IN). Picropodophyllin was prepared as previously described (20). The mouse monoclonal anti-HIF-1α and anti-HIF-2α antibodies were obtained from GeneTex, Inc. (San Antonio, TX), mouse monoclonal-anti-CD34 (endothelial antigen) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), secondary peroxidase-linked antimouse IgG was from Amersham Biosciences (Uppsala, Sweden), and mouse monoclonal anti-IGF-IR antibody (αIR3), used for cell culture, was obtained from Oncogene Research Products (Boston, MA). All other reagents used for immunohistochemistry were from DAKO (Glostrup, Denmark).

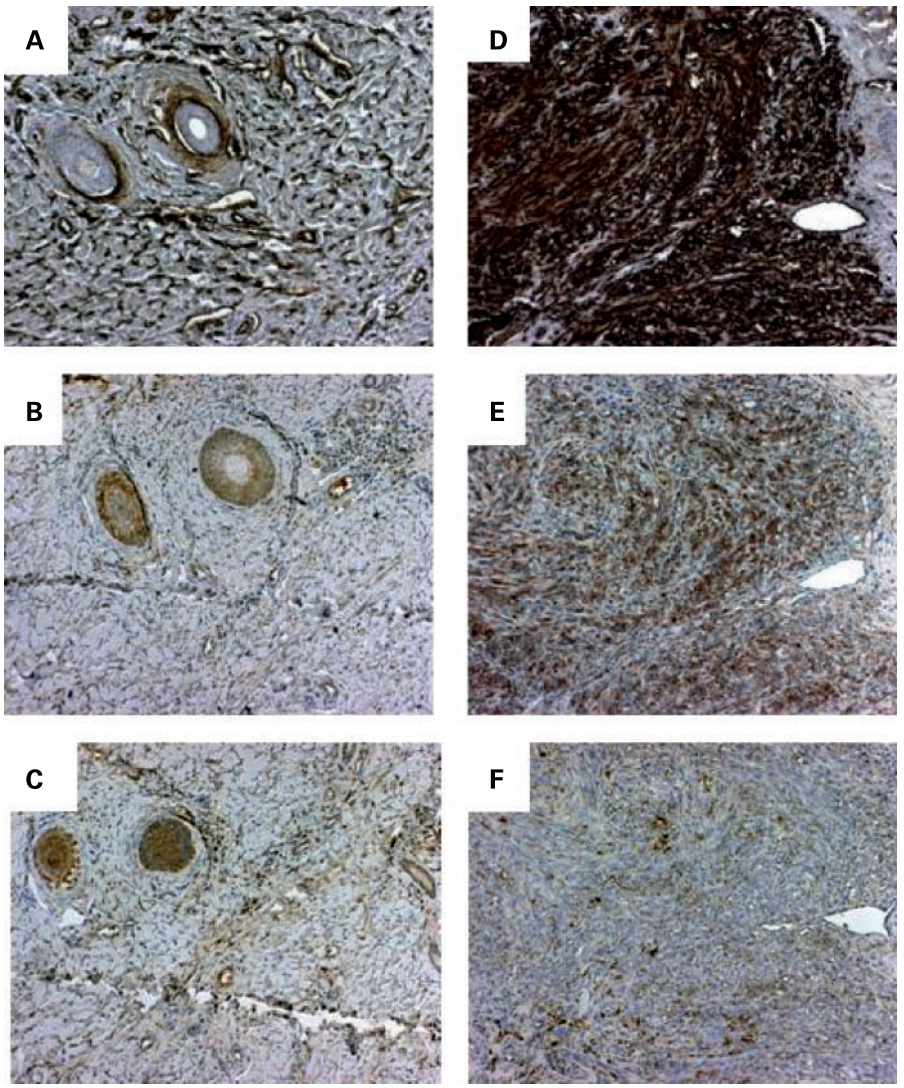
Tissue preparation and immunohistochemistry analysis. Diagnostic, surgical biopsies of AIDS-related KS lesions from patients in Tanzania (Department of Pathology, Muhimbili Medical School, Dar El. Salaam, Tanzania; $n = 15$, nine nodular and six patch lesions) were formalin-fixed, embedded in paraffin, and sectioned for histopathologic and immunohistochemical evaluation. The patients' HIV status was evaluated by serology using the Wellcozyme Recombinant anti-HIV-1 ELISA VK56 (Murex Diagnostics, Toronto, Ontario, Canada). 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-2α using a catalyzed signal amplification system (K1500, DakoCytomation, Glostrup, Denmark) according to indications from the manufacturer. CD34 staining was done as previously reported (16) to define the borders of the KS lesions. Matched IgG isotype controls were included for each marker. All biopsies were evaluated semiquantitatively using a four-point scale (0, <5% positive tumoral cells; 1, between 5% and 15% positive tumoral cells; 2, between 15% and 30% positive tumoral cells; and 3, >30% positive tumoral cells) in a random order by an observer who was unaware of the patient's identity and disease stage.

Table 1. Nucleotide sequence of the primers used for amplification of HIF-1α, HIF-2α, VEGF, and β-actin by qRT-PCR

Primer	Orientation	Sequence
HIF-1α	Forward	5′-TGCTCATCAGTTGCCACTTCC-3′
	Reverse	5′-CCAAATCACCAGCATCCAGAAGT-3′
HIF-2α	Forward	5′-AGTGCATCATGTGTGTCAACTACG-3′
	Reverse	5′-GGGCTTGAACAGGGATTCAGTC-3′
VEGF	Forward	5′-GCTGCTCTACCTCCACCATGC-3′
	Reverse	5′-CCATGAACTTCACCACTTCGTG-3′
β-actin	Forward	5′-CTCTTCCAGCCTTCCTTCCTG-3′
	Reverse	5′-CAGCACTGTGTTGGCGTACAG-3′

Cell culture. KS IMM cells, kindly provided by Dr. A. Albini (Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy), were cultured in DMEM supplemented with 2 mmol/L L-glutamine, 100 IU/mL penicillin and streptomycin, and 10% heat-inactivated bovine serum in a humidified atmosphere with 5% CO_2 at 37°C. Experiments to detect HIF α subunit protein expression and RNA levels were carried out in 100-mm Petri dishes in singleton, whereas luciferase reporter gene experiments were done in six-wells plates in triplicate. The experiments were repeated at least thrice. Twenty-four hours following plating, the cells were rinsed once with PBS, serum-starved for 24 hours, and incubated with testing substances for the times indicated, with volume <0.01%.

Western blot analysis. After treatment with the indicated compounds, KSIMM cells were chilled on ice and collected in iced PBS. The cell pellet obtained after centrifugation (5,000 × *g*, 5 minutes, 4°C) was frozen in liquid nitrogen. After thawing, the cells were lysed in 100 μL radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 8.8), 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% Igepal, 0.1% SDS supplemented with protease inhibitors cocktail plus freshly added 0.5 mol/L Na_3VO_4, 1 mmol/L DTT, and 1 mmol/L phenylmethylsulfonyl fluoride] followed by centrifugation at 4°C for 20 minutes at 20,000 × *g*. Protein concentrations of the supernatant were measured using the Bradford assay (Bio-Rad, Sundbyberg, Sweden). An equal amount of proteins (70 or 100 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-C, Amersham Biosciences) and blocked overnight with 5% nonfat milk in 0.1% Tween PBS at 4°C. The membrane was incubated with the primary antibody diluted in blocking buffer 2 hours at room temperature. After several washes, the membrane was incubated with the secondary peroxidase conjugate antibody (1:3,000) diluted in blocking buffer for 1.5 hours. After extensive washing with PBS, the complexes were visualized using enhanced chemiluminescence (Amersham Biosciences) according to the instructions from the manufacturer. Radiographs were analyzed using Image J 1.32 (NIH, Bethesda, MD) and the net intensity



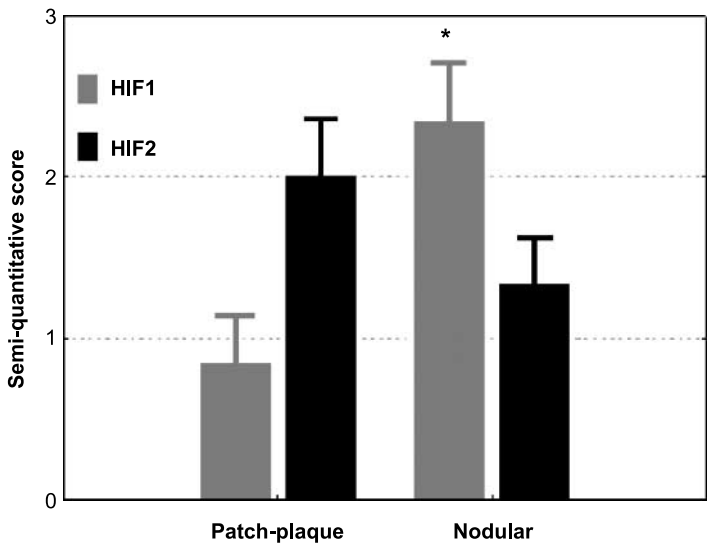


Fig. 1. AIDS-KS biopsies show immunoreactivity for HIF-1α and HIF-2α. Serial sections, paraffin-embedded, from a case with patch KS (*A-C*) and from a case with nodular KS (*D-F*) stained for CD34 (*A* and *D*), HIF-1α (*B* and *E*), and HIF-2α (*C* and *F*). Original magnification, ×100. The graph shows the results of the quantification of the staining. *Columns,* mean; *bars,* SE; *, $P < 0.05$.

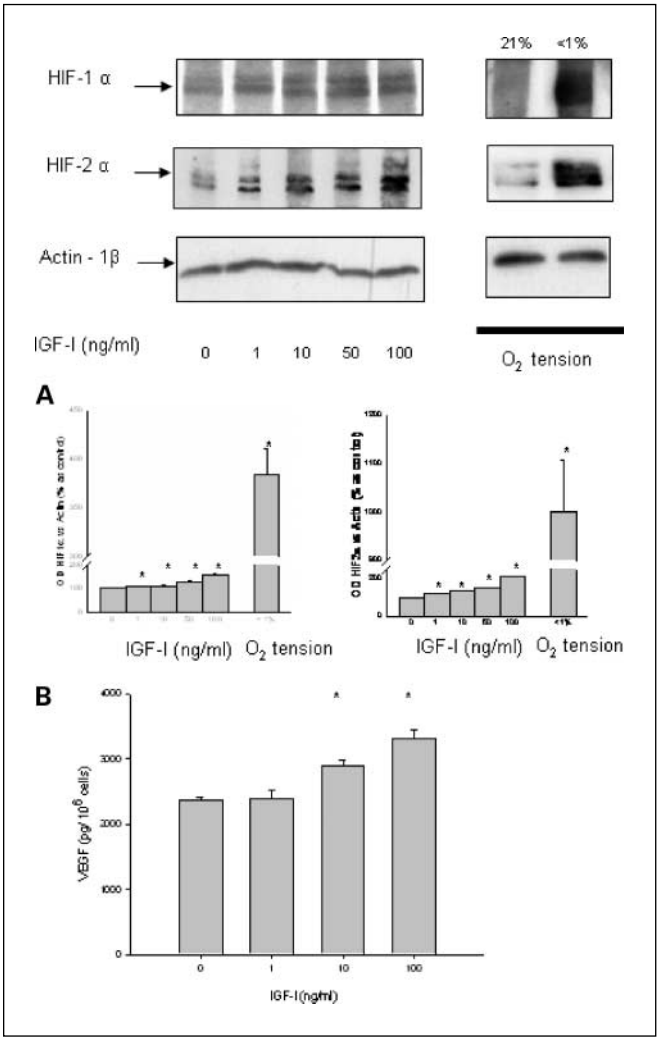


Fig. 2. IGF-I induces, in a dose-dependent manner, both HIF-1α and HIF-2α accumulation in KSIMM cells and VEGF production in cell culture medium. *A,* KSIMM cells, starved for 24 hours, were exposed to either IGF-I or vehicle in normoxia or to vehicle in normoxia (21% O_2) or hypoxia (1% O_2) for 6 hours. Whole-cell extracts were prepared and analyzed by Western blotting using anti-HIF-1α or anti-HIF-2α antibodies. *Columns,* mean from five experiments; *bars,* SE. *, $P < 0.05$, significant difference. *B,* KSIMM cells, starved for 24 hours, were exposed to either IGF-I or vehicle for 24 hours when the VEGF presence in the conditioned medium was quantified by ELISA. *Columns,* mean from three experiments; *bars,* SE. *, $P < 0.05$, significant difference.

of the specific bands (HIF-1α and HIF-2α) corrected for β-actin expression were expressed as percentage of the control-treated cells.

Reporter gene assay. To assay the effect of IGF-I on the transcriptional activity of HIF-1α, we have used a plasmid (pT81/HRE-luc) that contains three tandem copies of the erythropoietin HRE in front of the herpes simplex thymidine kinase promoter and the luciferase gene (21). KSIMM cells were plated at a density of 1.6×10^5 per well in six-well plates and transfected on the second day (~70-80% confluency) with the reporter plasmid (1 μg/well), in fresh medium without antibiotics, using the FuGENE 6 transfection reagent (Roche Diagnostics) according to instructions from the manufacturer. After 24 hours, the medium was changed with DMEM without FCS and further incubated for another 24 hours when the indicated compounds were added or hypoxia was started. After 24 hours, the luciferase activity was determined as described by the manufacturer (Promega, Inc., Madison, WI), and the reporter gene activity was standardized to the β-galactosidase activity in the cell lysate measured as described by the manufacture (Promega). The results were expressed as fold induction relative to the activity of the control sample.

RNA isolation and cDNA synthesis. Total RNA from cells was extracted from KSIMM cells, using Trizol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from 1 μg total RNA with Superscript III reverse transcriptase with UDG transacetylase (Invitrogen) according to the manufacturer-recommended protocol. The cDNAs were stored at −20°C until use in quantitative real-time PCR (qRT-PCR).

Quantitative real-time PCR. Oligonucleotide primers specific for HIF-1α, HIF-2α, VEGF-A, GLUT1, and glyceraldehyde-3-phosphate dehydrogenase (Table 1) were designed using OligoPerfect or Lux programs.[6] Real-time PCR was done in ABI Prism 7700 light thermocycler using the SYBRgreen Platinum kit and ROX reference dye (Invitrogen). A three-step (15 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C) 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 prism Sequence Detection System 1.9.1. A pilot experiment with a five-point standard curve for the target genes (*HIF-1α*, *HIF-2α*, and *VEGF-A*) and the reference gene (*β-actin*) 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 (22). All experiments were repeated at three separate occasions and the samples were analyzed in duplicate for each gene.

VEGF measurement. VEGF protein released into the conditioned medium of KS IMM cells was measured using a commercial ELISA kit (R&D Systems, Abingdon, United Kingdom) 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 medium was collected following 24 hours of incubation with different concentrations of IGF-I and assayed in duplicates following the instructions from the manufacturer. Viable cells (trypan blue exclusion) were counted and the values were expressed as picograms VEGF per 10^6 living cells.

Statistical analysis. All values are presented as mean ± SE. Data were analyzed by one-way ANOVA with a Tukey *post hoc* test or Kruskall-Wallis one-way ANOVA with Dunn's method, as appropriate, using SigmaStat v2.03 SPSS, Inc.

Results

HIF-1α and HIF-2α are expressed in KS biopsies. To characterize the expression of both HIF-1α and HIF-2α, we investigated KS biopsies obtained from 17 HIV-positive patients. In all the cases studied, both HIF α subunits were expressed throughout the tumor area but not in the normal dermal tissue that surrounded them. HIF-1α staining had both a nuclear and cytoplasmic pattern and increased significantly from the early patch biopsies (0.8 ± 0.3; Fig. 1B) to the late nodular KS biopsies (2.3 ± 0.4; $P < 0.05$; Fig. 1E). HIF-1α was expressed in spindle tumoral cells, endothelial cells, infiltrating leukocytes, and the basal layer of the epidermis.

No difference in HIF-2α expressions was observed between nodular (1.3 ± 0.3; Fig. 1F) and patch cases (2.0 ± 0.4; Fig. 1C). HIF-2α immunostaining had a similar distribution pattern as HIF-1α.

[6] http://www.invitrogen.com.

HIF-1α and HIF-2α are stimulated by IGF-I in KSIMM cells. For further studies concerning the regulation of HIF-1α and HIF-2α in KS, we have used KSIMM, an established KS cell line that has the characteristics of the KS spindle cells and produce large highly vascularized tumors when injected s.c. in nude mice (23). We have recently reported for these cells the importance of IGF-IR for cell proliferation and survival (16). Therefore, we extended our investigation on the modulation of the HIF α subunits by IGF-I.

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 (Fig. 2A). IGF-I was able to stimulate both HIF-1α and HIF-2α accumulation in a concentration-dependent manner (Fig. 2A).

We have also investigated the effect of IGF-I on VEGF secretion, known to be both an essential growth factor for KS and a target gene for HIF. IGF-I increased the production of VEGF in a dose-dependent manner in KSIMM cell culture medium (with a maximum of 3,321 ± 129 pg/10^6 cells; $P < 0.05$ for concentrations >10 ng/mL; Fig. 2B).

IGF-I and the hypoxia mimetic, $CoCl_2$, have different dynamics for inducing accumulation of HIF-1α and HIF-2α in KSIMM cells. To investigate the mechanisms behind the IGF-I–induced HIF-1α and HIF-2α accumulation in KSIMM cells, we have first compared the dynamics 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 (24, 25). Both HIF-1α and HIF-2α proteins were rapidly induced by IGF-I (30 minutes), whereas the stabilizing effect of $CoCl_2$ was obvious only after 3 hours with a weak induction at 2 hours (Fig. 3). The same "late" induction of α subunits was observed after treatment with deferoxamine, which also interferes with degradation of α subunits in a similar way as $CoCl_2$ (26) (data not shown). Taken together, these data suggest that IGF-I acts by a different mechanism than hypoxia mimetics to induce accumulation of HIF-1α and HIF-2α in KSIMM cells.

IGF-I induces HIF-1α and HIF-2α through a posttranscriptional mechanism. IGF-I 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 (Fig. 4A). This shows 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 did a cycloheximide chase experiment. After induction of HIF α subunits with either IGF-I or $CoCl_2$ (4 hours treatment), the cells where maintained in the presence of inducers and in the absence (Fig. 4B1) or presence (Fig. 4B2) of cycloheximide, which blocks the translation of the proteins (27). The levels of HIF-1α and HIF-2α were followed in dynamic by Western blot with specific antibodies. In the absence of cycloheximide, both stimuli ($CoCl_2$ and IGF-I) maintained constant levels of the HIF α subunits during the next hour (Fig. 4B1), as expected from the previous shown time point experiments (Fig. 3). After blocking the protein synthesis with cycloheximide, both HIF-1α and HIF-2α where minimally decreased during the next hour in the presence of $CoCl_2$, which is known to interfere with their degradation (24, 25). However, IGF-I–stimulated HIF α subunits started to decrease within 15 minutes and virtually disappeared during 1 hour after exposure to cycloheximide (Fig. 4B2), which clearly show that IGF-I does not protect the HIF α subunits against degradation. Together with the data that shows that IGF-I acts at the posttranscriptional level, the cycloheximide chase observation further supports that IGF-I induces translation of both HIF-1α and HIF-2α.

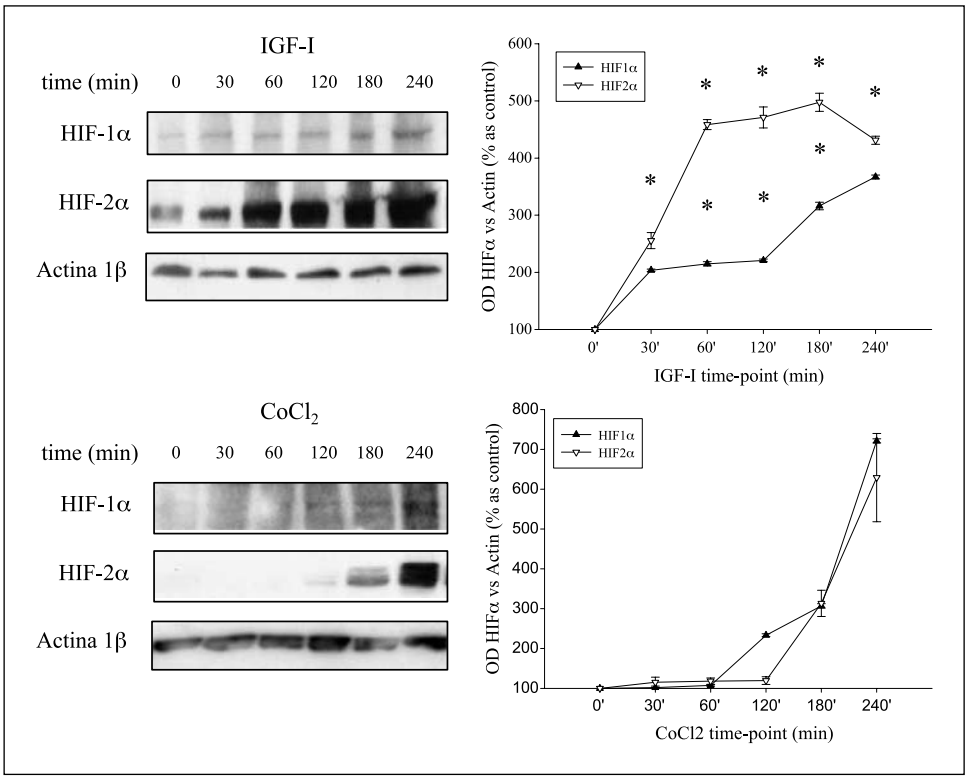


Fig. 3. IGF-I and $CoCl_2$ induce accumulation of HIF-1α and HIF-2α in KSIMM cells in a different dynamic. KSIMM cells, starved for 24 hours, were exposed to either IGF-I or $CoCl_2$ for the indicated period of time. Whole-cell extracts were prepared and analyzed by Western blotting using anti-HIF-1α or anti-HIF-2α antibodies. *Points,* mean from three experiments; *bars,* SE. *, $P < 0.05$, significant difference.

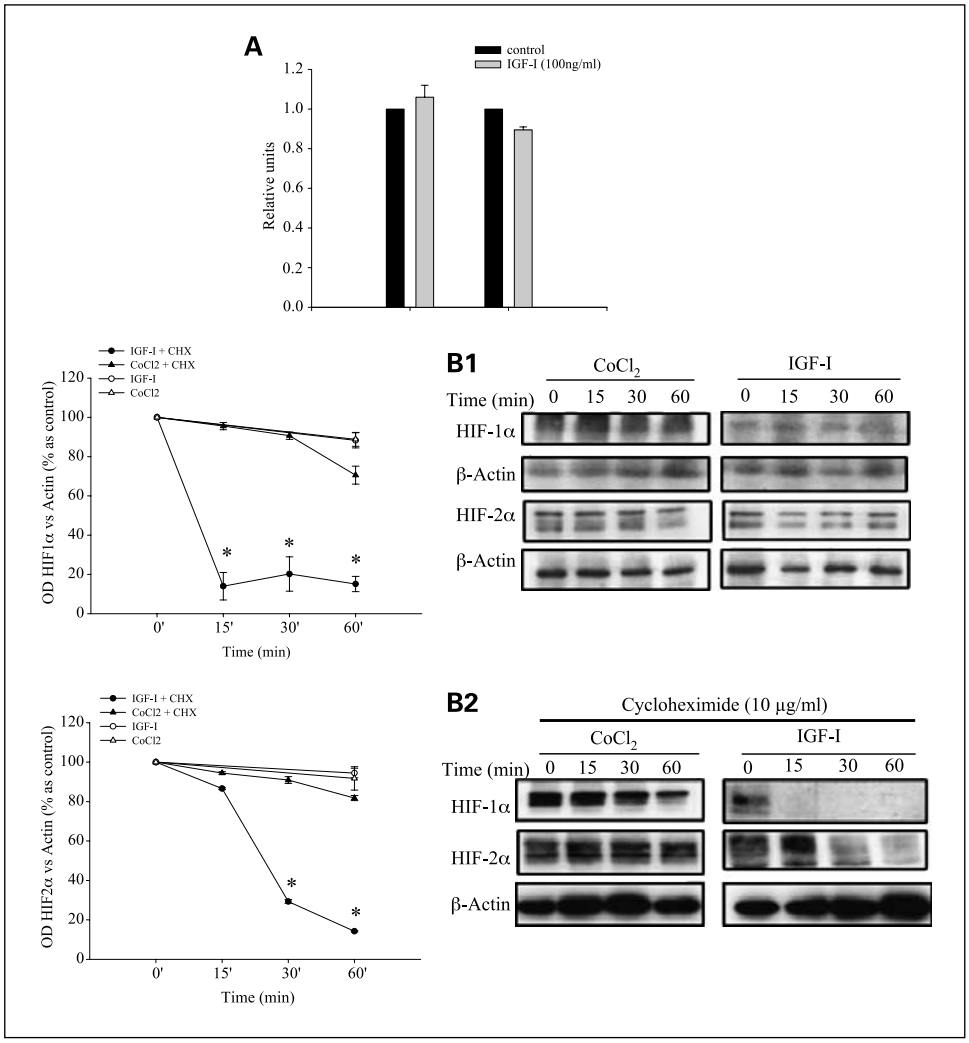


Fig. 4. IGF-I induces accumulation of HIF-1α and HIF-2α at posttranscriptional level depending on protein synthesis. *A,* KSIMM cells, starved for 24 hours, were exposed for 6 hours to either IGF-I or vehicle, after which the levels of HIF-1α and HIF-2α RNA were analyzed by qRT-PCR. *Columns,* mean from five experiments; *bars,* SE. *B,* KSIMM cells, starved for 24 hours, were exposed for 4 hours to either IGF-I or $CoCl_2$ to maximally induce both HIF-1α and HIF-2α. Cells were maintained in the presence of the above stimuli, in the absence (*B1*) or presence (*B2*) of cycloheximide. Whole-cell extracts were prepared from the time points indicated and analyzed by Western blotting using anti-HIF-1α, anti-HIF-2α antibodies, and β-actin antibodies. *Points,* mean from three experiments; *bars,* SE. *, $P < 0.05$, significant difference.

IGF-I induces HIF function in KSIMM cells. 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 a HRE-reporter construct and exposed the cells to either IGF-I or hypoxia (Fig. 5). Both hypoxia and IGF-I significantly induced a luciferase reporter gene after 24 hours of exposure (24.3 ± 4.2–fold and 3.78 ± 2.3–fold, respectively; $P < 0.005$).

Moreover, we evaluated by qRT-PCR the effect of IGF-I on the expression of *VEGF-A*, which is target gene for HIF. IGF-I induced in 6 hours a 1.47 ± 0.08–fold increase of the VEGF-A mRNA in KSIMM cells (Fig. 6).

Blocking IGF-IR decreases the HIF-1α and HIF-2α accumulation and the expression of their target genes. The profound effect of IGF-I on the HIF-1α and HIF-2α protein accumulation and function prompted us to investigate the effect of picropodophyllin, a new, specific IGF-IR tyrosine kinase inhibitor, which has the potential to be used as therapeutic agent (20) on both HIF α subunits accumulation and on VEGF expression. Treatment of KSIMM cells with picropodophyllin not only abolished the IGF-I–stimulated HIF-1α and HIF-2α accumulation and VEGF mRNA but even decreased VEGF expression to a lower level than in untreated cells (0.55 ± 0.07–fold; Fig. 6). The functional involvement of the IGF-IR in the induction of both HIF α subunits was also confirmed by complete abolishment of the IGF-I effects when the cells were coincubated with αIR 3 monoclonal blocking antibody (Fig. 6) (28).

Discussion

KS is characterized by the proliferation of characteristic spindle cells, neovascularization, and infiltration with inflammatory cells. The tumor, classically seen rarely and just in elderly subjects, has encountered a burst with HIV infection, being the most frequent tumor in AIDS patients (29). Although highly active retroviral therapy has hampered the tumor extent, KS still represents an important health problem, as many patients develop drug resistance and in most cases the duration of the response is limited (30). Notably, the majority of the patients are located in geographic regions with limited access to highly active retroviral therapy (31).

The highly vascular phenotype represents a hallmark for KS, and identification of pathways that contribute to its neovascularization is a priority for a potential efficient therapy. Here, we describe for the first time that both HIF-1α and HIF-2α, which are key players in angiogenesis, are expressed in

AIDS-related KS biopsies. Moreover, we were able to describe that IGF-I, recently described by us as an essential growth factor for KS (16), induces accumulation of both HIF-1α and HIF-2α and stimulates their function in KSIMM, a specific KS cell line (23). Furthermore, by showing that blocking of the IGF-IR causes decrease in both HIF as well as in VEGF expression, we suggest a new therapeutic option to decrease the angiogenetic potential of the tumor.

Overexpression of HIF is encountered in most of the solid tumors (32, 33) and there is suggestion to be associated with lack of response to therapy (34). The mechanism for the up-regulation of HIF in tumoral tissue can reside on the hypoxic environment that is common in neoplasms and correlates with a bad prognosis (35–37). It can also rely on the abnormal regulation of HIF in tumors by oncogenes or growth factors (38). Taking into account the highly vascular phenotype of the KS tumors and that the immunostaining for HIF α subunits is present all over the tumoral tissue and not just in "perinecrotic areas," it is unlikely that the overexpression of HIF α isoforms is a consequence of hypoxia. Although the HIF α isoforms have a high similarity in structure and function, there are data that suggest different functions for HIF α isoforms (39). HIF-2α seems to be more important for promoting tumor development (40–42) than HIF-1α, which has been recently reported to have a retarding effect on a von-Hippel Lindau associated renal cell carcinoma (41). KS is a multistage progressive disease that begins as early "patch-plaque stage" and evolves to late "nodular" stage (43). Our observation that HIF-2α is overexpressed starting from the early stages of the tumor development is in agreement with the tumor-enhancing role attributed to this HIF α isoform. Whether the predominance of HIF-1α in nodular forms of the tumor is a self-limiting reaction of the tumor or the consequence of hypoxic environment due to the tumor development warrant further investigation. The progressive infection of the tissue with HHV8 during the multistage evolution of KS (44) can offer another explanation of the full activation of HIF-1α only in the late stages, taking into account the recent finding that an HHV8 oncogene (KS-associated Herpes virus G protein–coupled receptor) is able to activate HIF-1α (45).

Our recent observation on the essential role played by IGF-I in KS tumor biology (16) coupled with previous reports on the interaction between IGF system and HIF-1α and VEGF (17, 19, 46) prompted us to further investigate the modulation of HIF α subunits in one of the three established KS cell lines, KSIMM (23). We showed that KSIMM cells express both HIF α subunits even in normoxia and were up-regulated by hypoxia and hypoxia mimetics. Moreover, IGF-I was able to increase the accumulation of both HIF-1α and HIF-2α in a dose-dependent manner over a broad range of concentrations. To our knowledge, this is the first article that 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 specific activation of certain genes by either HIF-1α or HIF-2α (39), the common up-regulation of both isoforms of the HIF α 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.

Previous reports have suggested a difference in the mechanism of regulation of HIF-1α by hypoxia and by growth factors or oncogenes (17, 19). However, no such investigation regarding HIF-2α regulation is currently available. Thus, we have investigated the mechanisms by which IGF-I regulates HIF-2α in comparison with HIF-1α. We have first made the observation that $CoCl_2$ up-regulates both HIF-1α and HIF-2α later than IGF-I, suggesting a different mechanism of action between the two stimuli. We have further shown that IGF-I does not interfere with HIF α subunit degradation, as the IGF-I–induced HIF α subunits quickly disappeared when the protein synthesis was blocked with cycloheximide. IGF-I induced accumulation of both HIF-1α and HIF-2α at a posttranscriptional level and we suggest that it acts by increasing the translation of the α subunits. 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 HIF α subunits by growth factors compared with hypoxia (38). It is not surprising 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 (47). In other cell systems, members of the IGF-insulin family induce HIF-1α accumulation by activating the phosphatidylinositol 3 kinase, mammalian target of rapamycin pathway (48), and/or mitogen-activated protein kinase (19), although, for the first one, conflicting results have been reported (49). Our preliminary observations suggest the involvement of both pathways in the IGF-I modulation of HIF-1α and HIF-2α in KSIMM cells (data not shown).

Accumulation of HIF α subunits is not always followed by an increase in its transcriptional activity (50). We therefore investigated HIF function either by transient transfection with HRE-reporter plasmids or by evaluation of endogenous target genes (*VEGF*). Both approaches showed that IGF-I activates HIF function in KSIMM cells. Because a recent report has suggested that the HIF α subunits have different effects on gene activation, the contribution of each HIF α subunit in IGF-I–induced HIF target genes warrants further investigation (39).

We have recently shown that IGF-IR blocking diminishes the proliferation of and induces apoptosis in KSIMM cells, and we

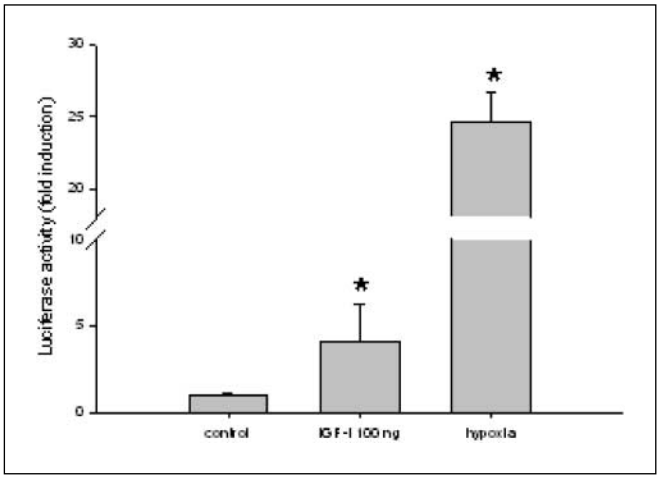


Fig. 5. IGF-I increases the functional activity of HIF. KSIMM cells were transiently transfected with an HRE-luciferase reporter construct and exposed to IGF-I (100 ng/mL) or hypoxia (1% O_2) for 24 hours. Luciferase activity was standardized to β-galactosidase activity and expressed as fold induction relative to the activity of the vehicle-treated cells in normoxia. *Columns,* mean from three experiments; *bars,* SE. *, $P < 0.05$, significant difference.

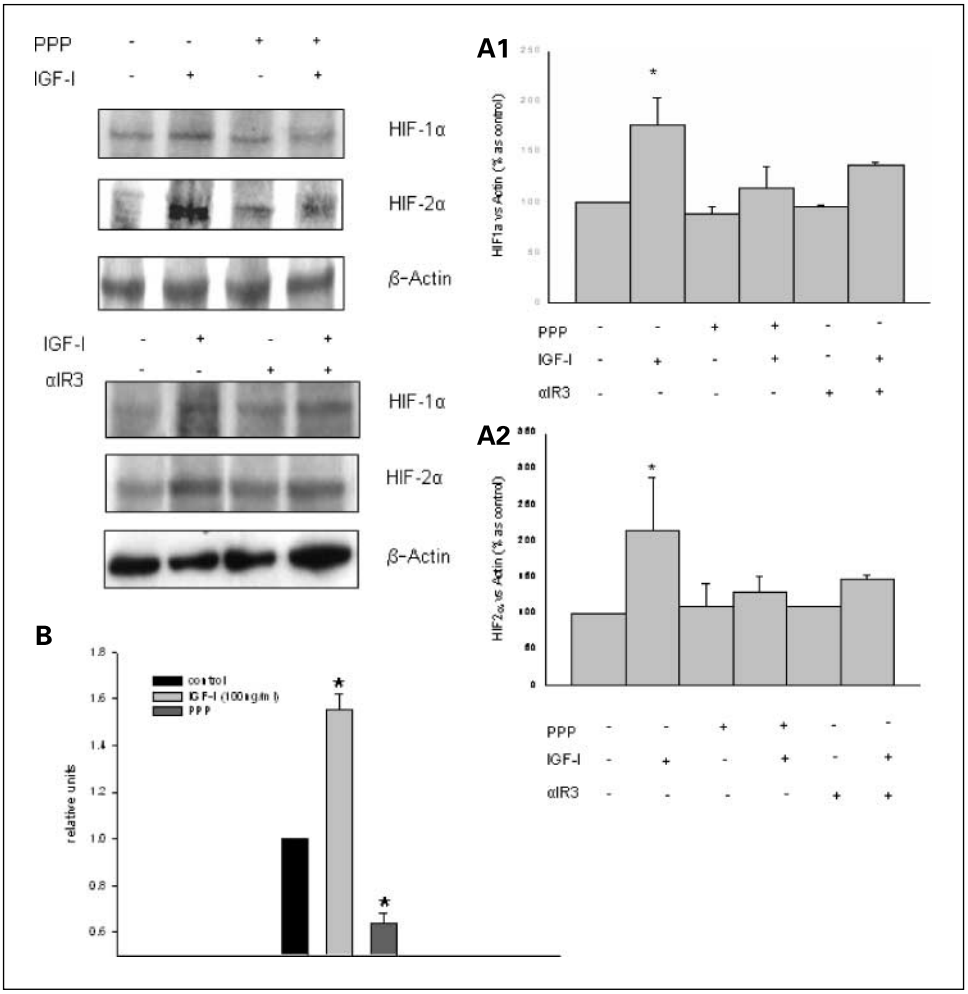


Fig. 6. The IGF-I effect on HIF-1α and HIF-2α accumulation and VEGF expression is abolished by blocking the IGF-IR. KSIMM cells, starved for 24 hours, were exposed for 6 hours to IGF-I (100 ng/mL) or vehicle in the presence or absence of 500 nmol/L picropodophyllin (*A1*) or αIR3 (*A2*), when the whole-cell extracts were prepared and analyzed by Western blotting using anti-HIF-1α or anti-HIF-2α antibodies (*A*) or VEGF mRNA were analyzed by qRT-PCR (*B*). *Columns,* mean from four experiments; *bars,* SE. *, $P < 0.05$, significant difference.

have proposed this approach as a potential therapeutic tool for KS (16). Neovascularization is an essential characteristic of tumors and in the case of KS is a defining phenotype. We have therefore tested the effect of IGF-IR blocking on HIF α accumulation and VEGF expression. We were able to show that treatment of KSIMM cells with picropodophyllin, a newly specific IGF-IR tyrosine kinase inhibitor, not only cancelled the effect of IGF-I on the accumulation of both HIF-1α and HIF-2α and induction of VEGF expression but it also decreases VEGF under the control levels. This effect is in agreement with our observation on the existence of an autocrine antiapoptotic loop in KSIMM cells (16), which is also relevant for the angiogenetic potential of these cells. Our observation is in agreement with previous reports that have suggested a combined antiangiogenic and antitumor effect for other IGF-IR–specific kinase inhibitor (51).

In conclusion, we have shown that IGF-I is able to activate the HIF-VEGF system in KS cells. We suggest that agents that interfere with IGF system can be of use to inhibit the defining angiogenetic stimuli in these tumors.

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