Sunitinib deregulates tumor adaptation to hypoxia by inhibiting HIF-1α synthesis in HT-29 colon cancer cells

Hyun-Woo Shin, Chung-Hyun Cho, Tae-You Kim, Jong-Wan Park

Department of Pharmacology, Seoul National University College of Medicine, 28 Yeongeon-dong, Jongno-gu, Seoul 110-799, Republic of Korea

Department of Biomedical sciences, Seoul National University College of Medicine, 28 Yeongeon-dong, Jongno-gu, Seoul 110-799, Republic of Korea

Department of Internal Medicine, Seoul National University College of Medicine, 28 Yeongeon-dong, Jongno-gu, Seoul 110-799, Republic of Korea

Abstract

Sunitinib (SU11248, Sutent) is a class III/V receptor tyrosine kinase (RTK) inhibitor that exhibits potent anti-angiogenic and anticancer activities. Preclinical studies demonstrated that the sunitinib effects are attributed to inhibition of VEGFR and PDGFR phosphorylation. However, even in colon cancer cells lacking sunitinib-targeted RTKs, sunitinib effectively inhibits tumor growth in a xenograft model, and this raises a question about the mechanism underlying the in vivo anticancer action of sunitinib. Since hypoxia is a critical microenvironment that tumors face, we addressed the possibility that sunitinib deregulates tumor adaptation to hypoxia. First we found that sunitinib limits the colony growth of HT-29, which is a colon adenocarcinoma cell line lacking the RTKs, and that HIF-1α in the colonies is decreased by sunitinib. In cultured HT-29 cells, sunitinib suppressed HIF-1α under hypoxic conditions. Moreover, sunitinib repressed the activity of HIF-1α and subsequently decreased the expressions of HIF-1 downstream genes. Mechanistically, sunitinib blocked the 5′-UTR-dependent translation of HIF-1α. The HIF-1α suppression by sunitinib was also reproduced in a VHL-null renal cell carcinoma cell line, where HIF-1α is not degradable. In conclusion, the sunitinib inhibition of HIF-1 signaling could restrain tumor progression in hypoxic regions, which may contribute to anticancer effect of sunitinib.

1. Introduction

The class III/V receptor tyrosine kinases (RTKs) are expressed on various cancer cells and participate in autocrine loops that promote cell proliferation and survival, e.g. vascular endothelial growth factor receptor (VEGFR) in melanoma, platelet-derived growth factor receptor (PDGFR) in gliomas, stem cell factor receptor (KIT) in small cell lung cancer, and fetal liver kinase-3 (FLT3) in acute myelogenous leukemia [1]. On this basis, multi-targeted tyrosine kinase inhibitors have been developed as anticancer agents. Indeed, sunitinib has anti-angiogenic and anti-proliferative activities, and thus has been clinically used to treat advanced renal cell carcinoma (RCC) or gastrointestinal stromal tumor [2,3].

Although sunitinib was devised for blocking the class III/V RTKs, the precise mechanism of its anticancer action has not been fully understood. Compared to other cancer cell lines, the HT-29 colon cancer cell line seldom expressed the RTKs, and thus was less sensitive to sunitinib in culture conditions [4]. However, the growth of HT-29 xenografts was effectively inhibited by sunitinib, which was comparable with xenografts of other cancer cell lines expressing at least one of the RTKs [4,5]. Likewise, sunitinib inhibited in vivo growth of urothelial tumors, which also poorly responded to sunitinib in culture conditions [6]. Collectively, these reports suggest that sunitinib has anticancer activity through some other mechanism(s) in addition to its inhibition of the class III/V RTKs.

Hypoxia develops commonly in solid tumors and acts as a critical microenvironmental factor [7]. To survive in hypoxia, tumor cells actively run the adaptation processes including switch of energy metabolism and neovascularization, which are mainly provided by hypoxia-inducible factor-1 (HIF-1). HIF-1 is a heterodimeric transcription factor composed of HIF-1α and HIF-1β (alternatively named ARNT). In aerobic conditions, HIF-1α is prolyl-hydroxylated by PHD1–3, ubiquitinated by von Hippel-Lindau protein (pVHL), and finally degraded by 26S proteasome [8]. Moreover, HIF-1α is asparaginyl-hydroxylated by FIH-1, dissociates from CBP/p300 coactivators, and subsequently loses its transcriptional activity [9]. However, in oxygen-deficient conditions, HIF-1α becomes stable and active because both hydroxylation processes are blocked, and then, HIF-1α in association with HIF-1β transactivates numerous genes required for hypoxic adaptation [10]. In many human tumors, HIF-1α is highly expressed and its tissue level is positively related to tumor aggressiveness and poor outcomes of patients [11]. Thus, HIF-1α is viewed as the key factor that determines tumor cell fate in hypoxia and as a compelling molecule to target tumor microenvironment.

© 2010 Elsevier Inc. All rights reserved.
Given that sunitinib inhibits the growth of tumor mass more efficiently than the proliferation of isolated tumor cells, we considered the possibility that sunitinib attenuates tumor adjustment to surrounding microenvironment, especially hypoxia. In the present study, we examined if sunitinib deregulates cellular adaptation to hypoxia in HT-29, and if so, investigated the mechanism by which sunitinib inhibits HIF-1 signaling.

2. Materials and methods

2.1. Reagents and antibodies

Sunitinib were purchased from Selleck biochemicals LLC (Housto, TX), and other chemicals from Sigma–Aldrich (St Louis, MO). Anti–HIF-1α antiserum for immunoblotting was generated as previously described [12]. Anti-HIF-1α monoclonal antibody (abcam, UK) and anti-Hypoxyprobe antibody (HPI, Inc., Burlington, MA) were used in immunofluorescence staining. DyLight™488 conjugated anti-mouse and Cy3 conjugated anti-rabbit secondary antibodies were from Vector laboratories (Burlingame, CA) and Jackson Immunoresearch (West Grove, PA), respectively. Other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture and treatments

HT-29 human colon cancer cell line was obtained from Korean Cell Line Bank (Seoul, Korea), human embryonic kidney (HEK) 293 cell line from the American Type Culture Collection (Manassas, VA), and VHL-null RCC4 cell line from the European Collection of Cell Cultures (London, UK). These cells were cultured in RPMI-1640 or Dulbecco’s modified Eagle’s medium, supplemented with 10% heat-inactivated FBS. All cells were grown in 5% CO2/20% O2. Colonies embedded in agar were punched out and incubated with pimonidazole hydrochloride (50 μg/ml of RNA was reverse-transcribed, and the cDNA obtained was amplified over 15 to 22 PCR cycles with 5 μl [1–32PIdCTP. The RT-PCR products (5 μl) were electrophoresed on a 4% polyacrylamide gel, dried, and then autoradiographed. Primers for human HIF-1α, VEGF, PGK1, PKD1, CAIX and β-actin were constructed as described previously [13].

2.6. Reporter gene construction and luciferase assay

HEK293 cells were transfected with the EPO reporter gene using calcium phosphate method [14]. HT-29 cells were transfected with the 5′-UTR reporter gene using Amaxa™ Cell line Nucleofector™ Kit R (Lonza Cologne AG, Germany). To evaluate the cap-dependent translation of HIF-1α mRNA, we cloned HIF-1α 5′-UTR (1–284) segment using RT-PCR, and then inserted it between thymidine kinase promoter and luciferase in the GL3 plasmid [15]. To evaluate the IRES-mediated translation of HIF-1α, we inserted the 5′-UTR segment between the GFP gene (5′ side) and the luciferase gene (3′ side) in the pcDNA plasmid. The β-gal expression plasmid was cotransfected to normalize gene expression. After stabilized for 16 h, transfected cells were split into 4 aliquots, and further cultured for 24 h. The cells were incubated in hypoxia with sunitinib for 16 h, and luciferase activities were analyzed using a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany).

2.7. Immunoblotting

Total cell lysates were separated on 8% sodium dodecylsulfate (SDS)/polyacrylamide gels, and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat milk in Tris/saline containing 0.1% Tween–20 for 1 h and then incubated overnight at 4 °C with a primary antibody diluted 1:1000. Membranes were incubated with a horseradish peroxidase (HRP)–conjugated secondary antibody (1:5000) for 2 h, and stained using an ECL Plus kit (Amersham Biosciences, Piscataway, NJ).

2.8. Statistical methods

Statistical analysis was performed using the statistical software package SigmaStat for Windows SPSS 12.0 (SPSS, Chicago, IL). Values were described as mean ± SD. The Mann–Whitney U test was used to compare reporter activities. Differences were considered statistically significant at the P < 0.05 level. All statistical tests were two-sided.

3. Results

3.1. Sunitinib limits the colonial growth of HT-29 by downregulating HIF-1α

We checked whether sunitinib affects the mass formation of cancer cells. HT-29 cells formed colonies in semisolid medium without adherence to a solid substratum. Interestingly, sunitinib inhibited the growth of colony, whereas it did not block the formation of colony. The number of large colonies (>50 μm diameter) was noticeably reduced in the presence of sunitinib, while the number of small colonies (<50 μm diameter) did not differ (Fig. 1A). Under confocal microscopy, hypoxic areas were detected within colonies and HIF-1α was prominently expressed in there (Fig. 1B, top), whereas small colonies were not hypoxic and scarcely expressed HIF-1α (Fig. 1B, middle). We next tried to examine whether sunitinib inhibits HIF-1α in the HT-29 colonies. However, as no large colonies were built in the presence of 5 or 10 μM sun-
itinib, we chose the colonies treated with 1 μM sunitinib, which showed a partial inhibition of colony growth. Consequently, HIF-1α expression was substantially reduced, but still remained, in the colonies positively stained with hypoxyprobe (Fig. 1B, bottom).

3.2. Sunitinib deregulates HIF-1 signaling in HT-29 colon cancer cell line

We next investigated the effect of sunitinib on hypoxic induction of HIF-1α in cultured HT-29 cells. HIF-1α was markedly induced after 8 h hypoxia, which was attenuated by sunitinib (Supplementary Fig. 1A). When the incubation time was prolonged to 24 h, the hypoxic expression of HIF-1α was more sensitively reduced by sunitinib (Fig. 2A). HT-29 cell viability was not altered 8 h after sunitinib treatment, and slightly decreased 24 h after treatment with sunitinib at more than 10 μM (Supplementary Fig. 2). Since the HIF-1 inhibition versus cytotoxicity occurs at lower concentrations, the sunitinib inhibition of HIF-1α might not be a secondary event due to its cytotoxicity.

3.3. Sunitinib represses HIF-1 activity

HT-29 cells were treated with sunitinib in hypoxia for 16 h, and mRNAs of HIF-1 target genes were analyzed using a semiquantita-
tive RT-PCR. Consequently, sunitinib suppressed the hypoxic induction of HIF-1 target genes, such as PGK1, PDK1 and CAIX (Fig. 2B). Exceptionally, the mRNA level of VEGF, a HIF-1 targeted angiogenic factor, was not reduced by sunitinib. Also, we found that HIF-1α mRNA level was not altered by sunitinib, suggesting that sunitinib down-regulates HIF-1α at the post-transcriptional level. Next, we tried to evaluate the HIF-1 activity using the EPO-enhancer reporter system, but HT-29 cell line was not suitable for this analysis because of its poor uptake of the EPO-Luc plasmid. Instead, we used HEK293 cells to perform this experiment. Supplementary Fig. 1B showed that sunitinib suppressed HIF-1α also in HEK293. After 16 h hypoxia, EPO-enhancer activities were markedly enhanced, and sunitinib significantly inhibited this hypoxic activation (Fig. 2C). These findings further support the HIF-1 inhibitory effect of sunitinib.

3.4. Sunitinib inhibits synthesis, not stabilization, of HIF-1α protein

To understand how sunitinib suppresses HIF-1α, we first investigated the possibility that sunitinib stimulates the degradation of HIF-1α. To analyze the oxygen-dependent degradation of HIF-1α, we induced HIF-1α in normoxia using desferrioxamine (a PHD inhibitor), and stopped

---

**Fig. 2.** Sunitinib downregulates HIF-1α. (A) Dose-dependent repression of HIF-1α protein level by sunitinib in HT-29. HT-29 cells were incubated under normoxic (N) or hypoxic (H) conditions in the presence of sunitinib for 24 h. HIF-1α and ARNT proteins in total cell lysates were analyzed by Western blotting. (B) Sunitinib attenuates the hypoxic induction of HIF-1 target genes. RNAs were isolated from HT-29 cells subjected to normoxia (N) or hypoxia (H) in the presence of sunitinib for 16 h. The mRNAs of HIF-1α and its target genes were analyzed by RT-PCR and autoradiography. PGK1 indicates phosphoglycerate kinase 1; PDK1, pyruvate dehydrogenase kinase 1; CAIX, carbonic anhydrase IX. (C) Sunitinib-induced HIF-1 inhibition. Epo-enhancer and β-galactosidase reporter plasmids were co-transfected into HEK293 cells. After 16 h incubation, luciferase and β-galactosidase activities were measured. *P < .05 versus the hypoxic control.

**Fig. 3.** Sunitinib inhibits HIF-1α synthesis, not its stability. (A) Oxygen-dependent degradation of HIF-1α. HT-29 cells were cultured to reach 60% confluence. After 2 h of pre-treatment in fresh media with sunitinib, cells were incubated under hypoxia for another 4 h. At the indicated period of re-oxygenation, HIF-1α levels were analyzed by Western blotting. (B) HIF-1α protein synthesis. HT-29 cells were treated with 10 μM MG132 to prevent the degradation of newly synthesized HIF-1α, and HIF-1α levels were determined at the indicated time by Western blotting.
the further synthesis of HIF-1α using cycloheximide. Since this experiment was done in normoxia, we can analyze HIF-1α degradation via pathways other than the PHD/pVHL/proteasome system. Even in this experiment, sunitinib did not affect HIF-1α degradation (Supplementary Fig. 3). Next, we checked the possibility that sunitinib inhibits the synthesis of HIF-1α protein. To analyze the synthesis rate of HIF-1α protein, we blocked the further degradation of HIF-1α using MG132 (a proteasome inhibitor), and examined the time course of HIF-1α accumulation. Fig. 3B showed that HIF-1α protein was substantially synthesized after 4 h and the de novo synthesis of HIF-1α was severely impaired by sunitinib. This indicates that sunitinib down-regulates HIF-1α by blocking its protein synthesis.

3.5. Sunitinib inhibits the 5′-UTR-driven translation of HIF-1α

With regard to the regulation of HIF-1α translation, two distinct mechanisms have been reported, namely, 5′ cap-dependent translation and IRES-dependent translation [16,17]. The HIF-1α 5′-UTR segment is the main target site for these translational pathways. To determine which translational pathway is regulated by sunitinib, we used two reporter plasmids, namely, Tk/5′-UTR-Luc reporter designed for cap-dependent translation and CMV/GFP-5′-UTR-Luc reporter for IRES-dependent translation [15]. Both reporter activity were found to be dose-dependently reduced by sunitinib (Fig. 4A and B). Since AKT signaling is known to regulate HIF-1α translation [18], we measured the cellular levels of phospho-AKTs as indices for AKT activation, and found that sunitinib reduced AKT phosphorylation at Thr308 and Ser473 residues (Fig. 4C). These results suggest that sunitinib reduces HIF-1α 5′-UTR-driven translations by inhibiting AKT or its upstreams. Based on our results, sunitinib could inhibit HIF-1α even in cancer cells lacking HIF-1α degradation pathway because it blocks HIF-1α synthesis. Actually, sunitinib noticeably suppressed HIF-1α expression in VHL-null RCC4 cell line that constitutively expresses HIF-1α even in normoxia (Fig. 4D). Sunitinib is expected to show anti-HIF activity in various types of cancers.

4. Discussion

This study originated from a question; how does sunitinib inhibit the growth of tumors lacking the class III/V RTKs. At the starting-point heading for a plausible mechanism, we focused tumor hypoxia inevitably developed during mass formation. In hypoxic surroundings, glycolysis is rapidly enhanced to compensate for ATP depletion. In this process, HIF-1 transactivates key genes involved in glucose transport and glycolysis, such as GLUT-1, GLUT-3, phosphofructokinase L, PGK1, and lactate dehydrogenase-A. Accordingly, the anti-tumor effect of HIF-1 inhibition can be explained by decreased level of these genes. In the present study, we demonstrated that sunitinib restrains the growth of HT-29 colony and down-regulates HIF-1α by inhibiting its translation. The sunitinib inhibition of HIF-1α signaling may impair tumor adaptation to hypoxia, which may be one of the principal mechanisms of the tumor shrinkage by sunitinib.

In hypoxic tissue, HIF-1 induces the expression of VEGF, which is closely associated with the induction of the neovasculature in human cancer [19]. On contrary to the genes involved in glycolysis, we observed that the VEGF gene was not inactivated after sunitinib treatment in HT-29 cells. This can be explained in molecular and clinical aspects. Though HIF-1 is a potent activator to target the VEGF gene, VEGF expression is regulated through multiple pathways and different transcription factors. The 5′-flanking region of the VEGF gene contains a number of binding sites for transcription factors.
factors, such as HIF-1, signal transducer and activator of transcription-3, activator protein-1 and 2, specific protein-1, early growth response factor-1, nuclear factor-xB, Smads, estrogen, and prolactin [20]. Moreover, VEGF mRNA has an AU-rich element at its 3'-UTR, and thus is regulated at the post-transcriptional level [21]. Presumably, in spite of HIF-1 inhibition, VEGF expression may be compensated through multiple factors in the presence of sunitinib. Indeed, many clinical studies showed that the plasma level of VEGF significantly increased in cancer patients during sunitinib treatment [22,23]. These reports support our idea that sunitinib may activate the compensatory mechanism to increase VEGF expression.

Basically, with respect to its anticancer action, sunitinib blocks the RTKs located on stromal or endothelial cells, eventually suppressing the stromal proliferation and angiogenesis. In addition, sunitinib may block autocrine pathways via the inhibition of RTKs located on tumor cells, leading to inhibition of signal transduction pathways related with cell survival or proliferation [24]. In this study, we also found that the sunitinib inhibition of HIF-1α synthesis was accompanied with the inactivation of AKT, a potential downstream of sunitinib-targeted RTKs. Furthermore, reporter results revealed that sunitinib inhibits the 5'UTR-dependent translation of HIF-1α which is known to be regulated by the AKT pathway [25]. Therefore, it is speculated that sunitinib blocks some RTK-mediated autocrine loops involved in HIF-1α synthesis. However, it is also possible that sunitinib directly inhibits AKT or its upstream kinases, and the precise mechanism is an open question.

It should also be noted that sunitinib suppressed HIF-1α in VHL-null RCC cells. This means that sunitinib could be used as a HIF-1α inhibitor even in the situation that HIF-1α is not degradable. Actually, sunitinib is currently used as a first-line drug in treating advanced RCC which poorly responds to conventional chemotheraphy and radiation. In ~75% of RCC, the VHL gene is mutated or inactivated, and subsequently active HIF-1α and/or HIF-2α are constitutively expressed, which is associated with aggressive behaviors of RCC. Therefore, the HIF signaling is generally viewed as a very promising target for RCC therapy. Interestingly, Burkitt et al. found that disruption of HIF-1α, HIF-2α, or both genes led to improved tumor response to sunitinib [26]. This enhanced response was explained by two potential mechanisms. First, tumor angiogenesis and perfusion were inhibited synergistically by sunitinib and the HIF inhibition. Second, the HIF inhibition directly inhibited survival and proliferation of cancer cells. No matter what is sunitinib’s mechanism, the HIF inhibition in RCC cells may be associated with good outcomes of sunitinib therapy in RCC patients. The implication of HIF-1α expression was also mentioned in gastrointestinal stromal tumor (GIST), another major indication of sunitinib. Prognosis in GIST was significantly poorer in patients with tumors expressing HIF-1α than in patients with tumors lacking HIF-1α [27]. In addition, high expression of HIF-1α in GIST was significantly correlated with tumor recurrence/distant metastasis [28]. These clinical works suggest that HIF-1α is a valuable therapeutic target and an ancillary prognostic factor.

5. Conclusions

We demonstrated that sunitinib limits the growth of HT-29 colony and suppresses HIF-1 signaling in HT-29 cells. Our results imply that HIF-1 deregulation may be one of the principal mechanisms of the tumor shrinkage by sunitinib. In the same time, the correlation between HIF-1α level and clinical efficacy of sunitinib should be further investigated in the future, which will provide the rational therapeutic indication of sunitinib. Furthermore, sunitinib could be applied for treating many HIF-related diseases, rather than is restricted to its current approved indications.

Acknowledgment

This work was supported by the Korea Healthcare technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea. (A091081).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.06.060.

References


