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The IGFR1 inhibitor NVP-AEW541 disrupts a pro-survival and pro-angiogenic IGF-STAT3-HIF1 pathway in human glioblastoma cells

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ABSTRACT

Inappropriate activation of the IGF (insulin-like growth factor) system has been implicated in the growth and progression of a number of tumor types. Recent evidence indicates a possible role for the IGF system in modulating/mediating tumor cell response to hypoxia, a common occurrence in solid tumors, and particularly in malignant gliomas, causing tumor cells either to die, or to mount a pleiotropic adaptive response that is mainly orchestrated through activation of the hypoxia-inducible transcription factor HIF1. Experimental evidence suggests possible links between IGF- and HIF1-dependent signaling pathways, as well as a role for activated STAT3 in mediating their activities. Interestingly, *igf2* is among the target genes transactivated by HIF1, thereby providing the missing link in a hypothetical autocrine self-amplifying circuit.

The present study investigates the presence of the IGF-HIF1-VEGF axis in the human glioma cell line U-87 MG, and characterizes its molecular effectors. Our results show that exogenous IGF-I causes IGF1R and STAT3 activation, and increases HIF1α protein levels and HIF1 trascriptional activity, inducing VEGF release; a similar response, mediated by IGF-II release, is observed following HIF1α stabilization. The existence of an autocrine loop is confirmed by its down-regulation following inactivation of IGF1R (using the IGF1R-specific tyrosine kinase inhibitor NVP-AEW541), STAT3 (transfecting the cells with an expression vector encoding a dominant negative form of STAT3), or HIF1 (using the small molecule inhibitor YC-1). The ability of NVP-AEW541 to block this circuit could be beneficial in suppressing the growth and angiogenic potential of hypoxic glial tumors.

1. Introduction

The existence of an autocrine loop involving the IGF (insulin-like growth factor) system and HIF1 (hypoxia-inducible factor 1) is emerging as a survival strategy common to different tumor types [1,2]. The IGF signaling pathway is activated by binding of one of two soluble polypeptide ligands (IGF-I and IGF-II) to the tyrosine kinase IGF1R, a tetrameric receptor consisting of two extracellular α chains and two transmembrane β chains linked by a disulfide bond, bearing a high degree of homology with the insulin receptor (IR) [3]. Although IGF1R overexpression has been demonstrated in tumors, its activation is strictly dependent on ligand availability, which in turn can be regulated by the expression levels of the decoy receptor IGF2R (also known as the mannose-6-phosphate receptor) and of six IGF-binding proteins (IGFBPs). IGF1R activation has been implicated in several key features in malignancy, including loss of anchorage-dependent growth, evasion of apoptotic signals, proteolytic degradation of the extracellular matrix and tumor angiogenesis [4].

Interestingly, the IGF signaling pathway appears to be intimately connected with HIF1 activation, which indeed seems to mediate at least part of IGF-induced downstream effects. HIF1 is a heterodimeric transcription factor, the activity of which depends on the intracellular levels of its inducible α subunit. In the presence of oxygen, HIF1α is hydroxylated on two critical proline residues (Pro_{402} and Pro_{564}) in the so-called oxygen-dependent degradation domain (ODDD). This modification targets the protein for ubiquitylation by an E3 ligase complex (including the Von Hippel-Lindau tumor suppressor protein) and subsequent proteasomal degradation [5]. Growth factor tyrosine kinase receptor activation has been reported to increase HIF1α levels, as well as HIF1 activity, under both normoxic and hypoxic conditions, mostly through up-regulation of the PI3K/Akt and Ras/Raf/MAPK pathways [5,6]. HIF1 orchestrates a pleiotropic adaptive response to hypoxia by inducing the expression of more than 100 genes encoding glycolytic enzymes and glucose transporters (thereby facilitating the glycolytic switch in energy metabolism typically observed under hypoxic conditions), matrix metalloproteinases, and angiogenic as well as mitogenic and survival factors. Most notably, IGF-II is among the growth factors

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up-regulated by HIF1, thereby potentially participating in a tumor growth-promoting autocrine loop [7].

Members of the STAT (*signal transducers and activators of transcription*) family might also be involved in mediating signaling along an IGF-HIF1 axis. In unstimulated cells, STAT proteins are present in a latent, inactive form; upon engagement of cytokine and growth factor receptors by their respective ligands, STAT proteins are recruited to activated receptor complexes and phosphorylated on tyrosine residues, whereby they dimerize and translocate to the nucleus, to modulate target gene expression [8]. Constitutive activation of STAT proteins, and particularly of STAT3, has been demonstrated in a number of cancer cell lines and tumors, including gliomas [9,10], presumably due to dysregulation of growth factor receptor expression and/or activation. However, the role played by STAT3 in IGF signaling has only been investigated superficially [11,12].

Conditions required to activate a putative regulatory IGF-HIF1 loop at different points are common in solid tumors. Regions of intermittent or chronic hypoxia frequently develop within the tumor mass, due to the fact that proliferating cells rapidly outgrow existing vessels and to the aberrant function of newly formed tumor vessel; hypoxia-induced IGF-II release might then sustain the growth of IGF1R expressing tumors. Similarly, when the circuit is activated by increased availability of IGFs, the downstream effects of IGF1R might be greatly amplified through HIF1 activation.

A number of strategies have been devised to disrupt the IGF signaling axis, ranging from forced expression of M6P/IGF2R or IGFBPs to the development of small interfering RNAs, antisense oligonucleotides, monoclonal antibodies and catalytic inhibitors targeting IGF1R [13–15]. Similarly, HIF1 has also been considered as a therapeutic target [16,17]: several small molecule inhibitors have been described [17]; an antisense oligonucleotide targeting HIF1α mRNA, as well as a small molecule inhibitor (PX-478) are currently undergoing phase I clinical trials [18,19]. Merging these two approaches might lead to mutual potentiation of their effects in tumors expressing an overactive IGF-HIF1 circuit.

Based on these premises, the present study investigates the role played by the IGF-HIF1-VEGF axis in a human glioma cell line, and its possible regulation through an autocrine circuit. Gliomas are the most common neoplasms in the adult human brain and they are frequently characterized by coexistence of intense neoangiogenesis and hypoxic and necrotic regions; in addition, the IGF system has been implicated in tumorigenesis in the CNS, and particularly in the pathogenesis of glial tumors [13,20]. Our results indicate that: (a) an autocrine loop, involving IGF1R, STAT3, HIF1 and IGF-II, exists in U-87 MG cells, regulating tumor cell survival and VEGF production; and (b) the circuit can be effectively disrupted by inhibiting IGF1R activation with the selective pyrrolo[2,3-*d*]pyrimidine derivative NVP-AEW541 [21], which reduces both cell viability and VEGF release. These observations suggest that pharmacological targeting of the IGF1R may provide a significant therapeutic benefit, especially in hypoxic tumors featuring autocrine regulation of the IGF-HIF1 axis.

2. Materials and methods

2.1. Reagents

Standard chemicals and cell culture reagents were purchased from Sigma–Aldrich srl. (Milan, Italy), unless otherwise indicated; NVP-AEW541 was generously provided by Novartis (Basel, Switzerland).

2.2. Cell culture, cytotoxicity studies and HIF1α stabilization

The human glioblastoma cell line U-87 MG (ATCC® HTB-14™) was obtained from the American Type Culture Collection (Manassas, VA, USA), and authenticated by morphological inspection, growth curve analysis and short tandem repeat profiling, using the Promega PowerPlex® 1.2 system (Promega, Madison, WI, USA) and the Applied Biosystems Genotyper® 2.0 software (Applied Biosystems, Foster City, CA, USA) for amplicon analysis. Cells were maintained in DMEM supplemented with 10% fetal bovine serum (Euroclone, Italy), 1% glutamine, 1% antibiotic mixture, 1% sodium pyruvate, 1% non-essential aminoacids, at 37 °C in a humidified 5% CO_2 atmosphere and were routinely checked for *Mycoplasma* infection, using the Mycoplasma Plus™ PCR Primer Set (Stratagene, Agilent Technologies, La Jolla, CA).

To assess cell survival following NVP-AEW541 exposure, 4×10^4 U-87 MG cells/well were seeded onto 24-well plates and allowed to grow for 24 h before treatment with different NVP-AEW541 concentrations (0.25, 0.5, 1.0 and 2.5 μM). After 24 h (and subsequently at 48 and 72 h for growth curves), cells from 3 replicate wells per treatment were detached by trypsinization and resuspended in PBS containing 0.2% Trypan blue; viable cells were counted using a Bürker hemocytometer following 10 min incubation.

To achieve HIF1α stabilization, cells were exposed to 50 μM $CoCl_2$ for 24 h, a condition that mimics hypoxia by inhibiting prolyl hydroxylation of the ODDD and its subsequent interaction with the Von Hippel-Lindau protein [22].

2.3. Vectors and transfections

U-87 MG cells were transiently transfected with an expression plasmid (pEF-HA-STAT3F) containing a full-length cDNA encoding hemagglutinin peptide (HA)-tagged STAT3F, a dominant negative form of STAT3 in which Tyr_{705} is replaced by phenylalanine, subcloned into a pEF-BOS expression vector (originally provided by Prof. S. Nagata, University of Osaka Medical School, Japan [23]); control cells were transfected with the pEF-BOS plasmid. pEF-HA-STAT3F was a generous gift from Prof. T. Hirano (Department of Molecular Oncology, University of Osaka Medical School, Japan [24]). Log-phase growing cells (5×10^6) were harvested and transfected with 30 μg of DNA, using an EasyJet electroporator system (Equibio, Ashford, UK), under conditions optimized according to the manufacturer's instructions (200 V, 1500 μF). All experiments were performed starting 48 h after transfection.

The pBabe-STAT3C expression vector, containing a cDNA encoding a constitutively activated form of STAT3, was a generous gift from J. Bromberg et al. [25] and was transfected into U-87 MG cells by lipofection, using Lipofectamine 2000® (Invitrogen, Carlsbad, CA, USA). Briefly, 1×10^6 cells were seeded onto 100 mm Petri dishes and allowed to grow for 24 h before lipofection with the pBabe-STAT3C plasmid; control cells were transfected with the "empty" pBabe plasmid. STAT3C expression in transfected cells persisted for 6–8 days; all the experiments were performed within 5 days after transfection.

U-87/HRP-EGFP cells were obtained from U-87 MG cells by transient transfection with a plasmid containing the EGFP (enhanced green fluorescent protein) cDNA under the control of an artificial hypoxia-responsive promoter (HRP), consisting of five copies of a 35-bp fragment from the HRE of the human VEGF gene and a human cytomegalovirus (CMV) minimal promoter (kindly provided by Dr. Y. Cao) [26]. Cells were seeded onto 6-well plates (3.5×10^5 cells/well) and allowed to attach for 24 h before lipofection using Lipofectamine 2000®. EGFP expression in transfected cells (following HIF1α stabilization with $CoCl_2$ at 50 μM for 24 h) persisted for 6–8 days; all the experiments were performed within 5 days after transfection.