

ABSTRACT

Title of thesis: The Role of Erythropoietin Signaling in Human Cancer

Ahmed Mohyeldin, Ph.D., 2004

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Hypoxia in solid tumors emanates from a structural and functionally disturbed vascular supply. Intratumoral oxygen levels are associated with poor prognosis, treatment resistance and cancer metastases, yet mechanisms for such phenomenon remain poorly understood. The **major objective** of this dissertation was to test whether or not erythropoietin (Epo), a hypoxia inducible cytokine, plays a role in astrocytoma treatment resistance and progression. The **specific aims** of this dissertation were to: **1) Determine whether or not hypoxia regulates the expression of Epo and EpoR (erythropoietin receptor) in astrocytomas. 2) Examine if Epo treatment results in treatment resistance in astrocytomas against chemotherapy. 3) Evaluate if Epo signaling promotes invasiveness in human astrocytomas.** We examined the expression of erythropoietin and its receptor using immunohistochemistry in human glioma and head and neck tumor biopsies. We also established several *in vitro* cell death and cell invasion assays to examine the effects of Epo signaling on human malignant astrocytoma and head and neck cancer cell lines. In

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addition, we developed primers to measure baseline and hypoxia-inducible Epo and EpoR mRNA expression in cancer cells with quantitative *RT-PCR*.

Collectively, this work answers key questions that provide insight into how hypoxia promotes cancer malignancy. Human cancers express Epo as well as functional EpoR. Expression of these proteins is most pronounced in hypoxic tumor regions and in invasive tumor margins. This work demonstrates that recombinant human Epoetin- α can directly stimulate the invasiveness of human cancer cells through Matrigel®. Epo also promotes tyrosine phosphorylation in human glioma cell lines. Hypoxia upregulates the expression of both Epo and EpoR in cancer cell lines and also promotes invasiveness. Moreover, hypoxia-induced invasiveness is blunted in stably transfected cells expressing a truncated form of the Epo receptor and diminished by Epo neutralizing antibodies. Together these findings suggest that autocrine or paracrine Epo signaling may play a significant role in cancer cell invasiveness. Furthermore, the use of Epo to treat anemia in cancer patients may have the deleterious side effect of promoting local cancer spread. Our work may also have profound implications for the treatment and management of cancer patients since Epo is used to treat anemia associated with cancer therapy.

**THE ROLE OF ERYHTROPOIETIN
SIGNALING IN HUMAN CANCER**

By

Ahmed Mohyeldin

Thesis submitted to the Faculty of the
Neuroscience Graduate Program
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DEDICATION

I dedicate this work to Cinda Helke(*b.1953-d.2004*) and John Sarvey(*b.1946-d.2003*), two amazing mentors who fought valiantly in their struggle against cancer and who enriched the lives of so many students they came in contact with. I am proud to have been a student of theirs during my years of graduate study at USUHS. In addition I dedicate this body of work in large measure to all scientific inquiry that advances the progress of medicine.

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INTRODUCTION

Hypoxic Adaptation and Erythropoietin

Aerobic life forms require oxygen (O₂) for the complete oxidation of fuels to efficiently generate high energy compounds that support survival. As the terminal electron acceptor in oxidative phosphorylation, molecular oxygen is maintained within a narrow range to avoid the risks associated with O₂ excess or deficiency¹. To achieve this, organisms have developed elaborate and highly specialized mechanisms to maintain oxygen homeostasis. In its excess (hyperoxia), organisms rely on a special repertoire of proteins that function to neutralize reactive species which threaten cellular life. During oxygen deficiency (hypoxia) organisms deploy rapid and long term strategies on systemic and cellular levels to adapt. On a systemic level, hypoxic adaptation includes reflex hyperventilation, increased erythropoiesis (generation of red blood cells) and neovascularization to increase oxygen delivery to tissues. On a cellular level, adaptive mechanisms increase glycolytic rates, enhance glucose uptake and upregulate cell survival proteins to compensate for the low energy yield and protect the cell from death related events.

For centuries physiologists and biologists have been fascinated with the molecular substrates underlying such adaptations. The understanding of how changes in oxygen pressure are sensed and transduced into altered organ and cell function is a subject of scientific and clinical importance. Insights into these mechanisms began as early as the 19th century when Paul Bert, known as the

father of aviation medicine, recognized a notable increase in red blood cell number in high altitude dwelling animals². The intimate relationship between hypoxic adaptation and erythrocytosis would foster a field of observations by several physiologists of the time (Viault, Carnot, Deflandre, Bonsdorff, Jalavisto, Reismann, and Erslev), ultimately converging to support a “humoral theory” for the regulation of red blood cell production³. The humoral hypothesis gained experimental validation with the identification⁴, biochemical purification⁵ and molecular cloning^{6, 7} of the hematopoietic factor erythropoietin (Epo). It is now appreciated that oxygen sensing mechanisms in the kidney respond to O₂ deficiency by increasing production of Epo. Transported via the blood stream, Epo acts on erythrocytic progenitors in the bone marrow to maintain appropriate hematocrit supplying tissues with the necessary oxygen requirement⁸. Since its discovery, the hypoxia-inducible expression of Epo has become a model system for studying the broader spectrum of oxygen-regulated gene expression. In fact, a deeper molecular investigation of this system over the past decade has culminated in the identification of the master transcriptional regulator of many of the responses associated with systemic and cellular hypoxic adaptation exhibited by metazoan life forms⁹.

The Pleiotrophic Functions of Erythropoietin

Today, the administration of recombinant human erythropoietin (rhEpo) has become an indispensable therapeutic adjunct to the treatment of patients with chronic anemia¹⁰. In fact, Epo pharmaceutical manufacturing and sales

represent a multi-billion dollar industry catapulting Epo into the top 10 list of revenue generating prescription drugs in the United States¹¹. However, research on erythropoietin has implicated this molecule in a broader spectrum of biological function than that initially anticipated. The methylation of the Epo gene restricts tissue expression to specific organs and thus for longest time Epo was thought to be solely expressed in the liver and kidney¹². Epo and its receptor (EpoR) however are expressed by several non-hematopoietic tissues such as the central nervous system¹³⁻¹⁵, retina¹⁶, heart^{17, 18}, lung¹⁹, breast²⁰, uterus²¹ and testis²². Several investigators have demonstrated that Epo and EpoR are expressed by several cell types including neurons^{23,24}, astrocytes²⁵, endothelial cells²⁶, vascular smooth muscle cells²⁷, placental trophoblasts²⁸, renal²⁹ and mammary epithelial cells³⁰. Epo signaling in these tissues mediates several biological roles including neuroprotection³¹, angiogenesis³², vasculogenesis³³, as well as cell proliferation³⁴ and cell migration²⁶. Uterine Epo production mediates angiogenesis during the estrus cycle²¹. In the lung, Epo regulates alveolar growth by limiting excessive proliferation and apoptosis¹⁹. Identification of the receptor on endothelial cells affirmed that Epo signaling plays a prominent role in vascular remodeling and in certain settings is involved in the development of the gut by promoting vili formation and endothelial cell migration^{20, 26}. It is not surprising that Epo is emerging as a multifunctional cytokine³⁵. Data from gene cloning has categorically placed Epo and EpoR as members of the cytokine superfamily type I, making them part of a class of growth factors largely known for their pleiotrophic functions. It is well known that the Epo receptor lacks any intrinsic

kinase activity. Gene expression is mediated via the recruitment of the tyrosine kinase JAK2 to mediate phosphorylation of various proteins and transcription factors³⁶.

Erythropoietin in the Central Nervous System

Cells from the nervous system were among the first non-hematopoietic tissues examined for Epo and EpoR expression. Studies on two rodent cell lines of neural origin, PC12 (clonal pheochromocytoma) and SN6 (septal cholinergic) cells, affirmed the presence of a functional erythropoietin receptor²⁴. Further investigation supported these findings when primary neuronal cultures from cerebral and hippocampal cells not only were positive for EpoR expression but demonstrated neuroprotection upon treatment with exogenous Epo^{31, 37}. The putative neuroprotective actions of Epo have been successful over a broad range of injury models both *in vivo* and *in vitro*. Epo ameliorates neuronal apoptosis associated with excitotoxicity³⁷, oxidative stress³⁸ and chemical neurotoxicity³¹. *In vivo*, Epo is protective against traumatic injury of the brain and spinal cord^{39, 40}. It prevents hypoxia/ischemia-induced DNA fragmentation in an experimental model of perinatal asphyxia⁴¹. Epo reduces infarct volume after middle cerebral artery occlusion⁴², improves functional recovery after brain injury⁴³ and is appreciated as an endogenous trophic factor that protects against retinal degeneration⁴⁴. Broader roles for Epo suggest that it could be an anti-inflammatory protective cytokine in an experimental model for autoimmune encephalomyelitis⁴⁵, and

recently decreasing expression of Epo in the brain may be associated with age-related neurodegenerative diseases⁴⁶.

Much like renal Epo, brain Epo is hypoxia inducible with limited expression in the normal adult brain when compared to the higher more ubiquitous expression of its receptor^{23, 25}. Cellular characterizations of the Epo producing cells in the nervous system reveal that astrocytes are the major source of Epo in the brain^{14, 25}. The trophic effects of Epo have been described on neurons³¹, oligodendrocytes³⁴, microglia⁴⁷, as well as astrocytes themselves^{34, 48, 49}. Epo is now recognized as a paracrine mediator of ischemic tolerance in the brain¹⁴. This hypothesis is evidenced by data from *in vitro* and *in vivo* studies which show that the neutralization of Epo signaling, either via Epo neutralizing antibodies or via soluble EpoR, exacerbates the ischemic injury. In addition to the brain, the heart and the retina appear to also rely on Epo signaling which suggests that the cytoprotective effects of Epo might be an endogenous mechanism exploited by several other tissues in times of metabolic stress.

Abnormal brain development exhibited by embryonically lethal EpoR gene knock-out animals supports a biological role of Epo signaling that extends beyond neuroprotection. Reintroduction of the Epo receptor partially restores normal brain development⁵⁰ and the administration of Epo *in vivo* was found to increase proliferation of neuronal progenitors⁵¹. In line with these observations, the Epo receptor was found to be expressed in the embryonic germinal zone during neurogenesis and in the adult subventricular zone where blockade of autocrine Epo signaling decreased the number of neuronal progenitors

produced⁵¹. Collectively these observations implicate a prominent role for Epo signaling in neurogenesis that extends beyond neuroprotection. Although several promising clinical trials are on their way, the clinical use of Epo for neuroprotection has yet to be incorporated as a standard of care.

Erythropoietin, Erythropoietin Receptor and Cancer

It is not surprising, those cancers which originate from tissues that utilize Epo signaling test positive for the expression of EpoR. In fact, our lab was the first to identify that Epo and EpoR were expressed by a broad range of human cancers that exploit Epo signaling⁵². Since then, Epo or EpoR expression has been associated with diverse human cancers and cancer cell lines screened by several investigators. To date, Epo receptor expression has been identified on numerous cancer cell lines or on neoplasms originating from the liver⁵³, kidney⁵⁴, ovary⁵⁵, breast⁵⁶, cervix⁵⁷, prostate⁵⁸, lung⁵⁹, and brain⁵².

The link between Epo and cancer is not a recent finding. In the 1960's, several clinical studies documented patients with renal carcinomas who presented with an elevated Epo plasma concentration and an abnormally high hematocrit^{60, 61}. The initial hypothesis was that the tumor cells ectopically expressed erythropoietin which accounted for the exhibited polycythemia. This hypothesis was supported by the fact that there was complete remission of the erythrocytosis following removal of the tumor and extracts from tumor tissues contained erythropoietic activity⁶¹. However, data that directly implicated Epo production to the cancer cells was lacking until 1991. Da Silva and colleagues⁵⁴

demonstrated for the first time using northern blot analysis, *in situ* hybridization and immunohistochemical analysis that Epo localized to the tumor cells themselves in three patients who had renal carcinomas and who were also polycythemic. Since then numerous studies have associated polycythemia with nearly 5% of all renal cancers⁶² as well as cancers originating from the liver^{63, 64}, adrenal glands⁶⁵, ovaries⁶⁶, mammary glands⁶⁷, uterus⁶⁸, testes⁶⁹ and even brain⁷⁰; all organs which are now known to utilize Epo signaling. Interestingly, in almost all these cases removal of the tumor resulted in a return to normal plasma Epo levels and remission of the erythrocytosis. The discovery of the ectopic expression of Epo in many of these neoplasms offers speculation on the biological relevance behind the production of this cytokine in the progression and growth of cancer especially because of its biological backdrop in the hematopoietic system and its anti-apoptotic/proliferative effects on erythroid progenitors.

The introduction of two human liver tumor cell lines in 1987 offered a practical and convenient approach to study Epo expression *in vitro*^{53, 71}, HepG2 and Hep3B cells. The two cell lines identified upregulate Epo production as pericellular PO₂ drops. This biological property made them an ideal model to study oxygen regulated gene expression thus focusing attention on the novel regulatory mechanisms behind cellular oxygen sensing which ultimately diverted investigations on the biological relevance of Epo in cancer. The study on these hepatoma cell lines primarily focused on the putative hypoxia-activated regulatory DNA sequences of the Epo gene. This work ultimately led to the

identification⁹ and biochemical purification⁷² of hypoxia-inducible factor-1 (HIF-1). HIF is a heterodimeric transcription factor that is now celebrated as a master regulator of oxygen homeostasis by transcribing a spectrum of genes essential for hypoxic adaptation¹.

The first series of investigations that aimed to test the biological relevance of Epo signaling in cancer emerged in the 1990's. Several of these studies demonstrated that Epo promoted proliferation and activated secondary messengers which implicated a functional and active Epo receptor on several hematopoietic and non-hematopoietic cancer cell lines. The most active research was on malignant lymphohematopoietic cells where several erythroleukemia cell lines were shown to possess receptors for Epo which upon activation inhibited apoptosis and promoted proliferation^{73, 74}. In fact several erythroleukemia cell lines require erythropoietin as a supplement in their media to maintain survival and other studies have speculated that Epo may even function as an autocrine growth factor in these cell lines⁷³. At the time recombinant human erythropoietin (rhEpo) was attracting increasing interest as an effective therapeutic adjunct for cancer-related anemia, so the aim of most of these early studies was to evaluate any potential cancer promoting effects that rhEpo may have on malignant non-hematopoietic cells. In 2000 Westenfelder and Baranowski⁶² were the first to discover that erythropoietin stimulated the proliferation of non-hematopoietic carcinoma cells. Using resected specimens from human renal carcinomas and two established renal tumor cell lines, they demonstrated the existence of authentic EpoR transcripts and protein expression. Tumors showed low-level

Epo expression and Epo treatment stimulated cell proliferation dose dependently in both cell lines.

Many of these early studies collectively advocated some degree of caution with the use of Epo in correcting for anemia associated with cancer. Skeptics of this notion argued that until Epo demonstrated similar trophic effects *in vivo* the practice was safe and beneficial for patient survival. However, recent experimental data from *in vitro* and *in vivo* studies appears to weaken this contention. Investigations on the trophic role of Epo *in vivo* demonstrate that inhibition of erythropoietin signaling destroys xenografts of ovarian and uterine cancers in nude mice⁵⁵. Injections of EpoR antagonists in xenografts of stomach choriocarcinoma and melanoma cells inhibited angiogenesis, resulted in destruction of tumor masses and decreases in the phosphorylation levels of downstream Epo signaling targets⁷⁵. Treatment with exogenous rhEpo or an Epo mimetic peptide increased production of angiogenic growth factors and promoted endothelial cell proliferation and chemotaxis⁷⁶. Perhaps even more compelling two recent clinical trials designed to evaluate the therapeutic benefit of Epo in breast cancer and head and neck cancer were terminated prematurely due to adverse outcomes in the Epo treated group, including increased local regional spread and increased mortality^{77, 78}. Collectively these findings have drawn concern for the use of the Epo in the management of cancer-associated anemia⁷⁹ and have increased the urgency for a better understanding of Epo signaling in cancer promotion.

Currently, there is limited data on the prognostic significance of increased Epo expression or its receptor in cancer patients. Work from our lab has recently demonstrated that Epo and EpoR protein expression positively correlate with tumor grade in cervical⁵⁷, breast⁵⁶ and endometrial⁸⁰ carcinomas. In human endometrial carcinomas, EpoR expression in tumors was associated with advanced-stage disease, lymphovascular invasion and lymph node metastasis⁸⁰. Kaplan-Meier disease-related survival curves of endometrial carcinoma patients revealed that higher Epo expression is associated with adverse clinical outcome⁸⁰. Furthermore, our lab has identified that Epo can contribute to treatment resistance by blocking apoptosis associated with chemotherapeutic toxicity⁵⁷. In fact, it is a well known clinical observation that renal toxicity that develops in cancer patients who receive chemotherapy is reversed upon administration of Epo suggesting that the cytoprotective effects of Epo may also mediate tumor resistance⁸¹. While there have been proposals that Epo may promote angiogenesis^{32, 75, 76} and tumor cell survival via proliferation^{62, 76}, a defined biological role for Epo signaling in cancer treatment resistance and metastases is lacking. Thus the ambition of this proposal is to better define the biological activities that Epo can promote to enhance tumor growth in context of these findings.

Hypoxia and Cancer

The rapid proliferative rate of cancer cells culminates into a tumor size that eventually outstrips the vascular supply that is appropriately required for the

tissue mass resulting in intra-tumoral hypoxia⁸². Inadequate oxygen supply to growing tumors is further exacerbated by a faulty vasculature which is often times comprised of distended capillaries with leaky walls and compromised blood flow⁸². Accumulating evidence from experimental and clinical studies suggest a fundamental role for hypoxia in the malignant progression of a neoplasm⁸²⁻⁸⁴. Hypoxia is known to influence a series of biological parameters that provide tumor cells with intrinsic resistance to radiation⁸⁵ and chemotherapy^{86, 87} and drive malignant progression for increased potential for invasive growth^{88, 89}.

Regions of low oxygen are a common feature of most solid tumors including brain⁹⁰, prostate⁹¹, colon⁹², breast⁹³, cervical⁹⁴ and head and neck cancers⁹⁵. The degree of hypoxia is positively correlated with treatment resistance and death⁹⁶⁻⁹⁸. Hypoxia increases angiogenesis, glycolytic metabolism, cancer cell survival, and the probability of invasion and metastasis^{82, 98-101}. Although hypoxia can activate transcription of many genes with cancer promoting potential, the regulation of specific genes by hypoxia depends upon the given cell type and its stage of differentiation¹⁰². Thus, the relevant mediators of hypoxia-inducible malignant progression are not immediately obvious for all cancers. The recent identification of Epo and EpoR expressing cancers and the fact that Epo is an oxygen sensitive gene with cancer promoting potential makes hypoxia inducible Epo signaling an attractive candidate for mediating the aggressive phenotype that is often coupled to hypoxic cancer cells.

HIF-1, Cancer and Epo signaling

Analysis of the DNA sequences required for the hypoxic dependent transcription of the Epo gene led to the identification⁹, biochemical purification⁷² and molecular cloning¹⁰³ of hypoxia inducible factor-1 (HIF-1). HIF is an α,β -heterodimeric transcription factor that is a central mediator of cellular responses to low oxygen concentration¹⁰³. The transcriptional complex initiates the activation of a repertoire of genes that are involved in hypoxic adaptation¹⁰⁴. HIF has become an attractive therapeutic target in cancer biology because it appears to be the major mediator in the link between hypoxia and the malignant progression of neoplasms¹⁰². The oxygen-regulated HIF-1 α subunit of HIF-1 is highly expressed in cancer cells and its expression level in solid tumors is strongly correlated with angiogenesis, invasion, and poor clinical outcome^{105, 106}. Interfering with the HIF pathway leads to diminished xenograft tumor growth¹⁰⁷ and over-expression of HIF accelerates tumor development¹⁰⁸, increases treatment resistance⁸⁵ and metastatic potential^{88, 109}.

HIF-1 α expression is frequently seen in the hypoxic zones of tumor specimens¹⁰⁹. One of the pathological hallmarks of tumor hypoxia is the presence of necrotic foci that are surrounded by hypercellular zones referred to as pseudopalisades. Pseudopalisading cells have been shown to be hypoxic in nature and show nuclear expression of HIF-1 α ¹¹⁰. HIF-1 expression in this area is a major adverse prognostic factor and has been linked to the failure of current therapeutic modalities¹¹⁰⁻¹¹². Our lab has recently demonstrated that the expression of Epo and EpoR in cancer is highest in these hypoxic areas (Paper#1). It is plausible that tumor cells that are capable of expressing

erythropoietin rely on the biological properties of Epo signaling to escape the hypoxic stress. Hypoxic adaptation is known to upregulate trophic factors that protect against metabolic stress making Epo a likely candidate in such settings. In addition to trophic support cancer cells deploy mechanisms that initiate the escape from the hypoxic microenvironment via migration towards areas that are better oxygenated. Epo directly stimulates endothelial cell migration²⁶ which suggests that the activation of autocrine/paracrine Epo signaling by hypoxic tumor cells may act in a manner that mediates metastases and invasion. The fundamental role that hypoxia plays in tumor progression and the recent observation that several human cancers express Epo and EpoR make a compelling case for the evaluation of a hypoxia–inducible erythropoietin signaling loop in cancer promotion.

Does hypoxia inducible erythropoietin signaling promote cancer progression?

Recent preliminary data from our lab has identified that gliomas, the most common brain tumor often derived from astrocyte cells, highly express erythropoietin and erythropoietin receptor. Astrocytes are known to produce Epo under hypoxic stress thus providing a paracrine mediator of ischemic tolerance in the brain^{14, 25}. More recently, Epo has been found to have trophic effects on glial development. Treatment of astrocytes with Epo enhances their proliferation³⁴ and independently promotes stem cell initiation to a morphologically and

phenotypically similar astrocyte cell type⁴⁹. These observations are provocative for several reasons: If Epo signaling promotes proliferation or blocks apoptosis or enhances invasion in tumors as it does in some normal tissues, then EpoR activation may underlie many of the deleterious actions of hypoxia in cancer including resistance to therapy and pro-invasive behavior. Epo is also routinely given to patients undergoing chemo- and radiation-therapy to counter therapy-associated anemia¹⁰. This practice may produce deleterious effects by promoting cancer cell survival and resistance to therapy. Due to the trophic properties that Epo has on astrocytes we hypothesize that gliomas exploit an autocrine/paracrine Epo signaling loop that is enhanced under hypoxia and promotes glioma progression.

This body of work aims to answer key questions in support of this hypothesis. It is still not clear whether Epo and EpoR are regulated by hypoxia in cancer cells. Using several glioma cell lines we test whether hypoxia can regulate the expression of Epo and EpoR *in vitro*. Immunohistochemical staining of glioma specimens will reveal if the highest level of Epo and EpoR expression localizes to the hypoxic zones in tumors. Furthermore, Epo is known to be anti-apoptotic and neuroprotective over a variety of injury models. Exogenous erythropoietin protects cervical cancer cells from apoptosis induced by chemotherapeutic drugs⁵⁷. We tested if exogenous Epo can protect glioma cells in a similar manner. Finally, tumor hypoxia is intimately linked to the probability of invasion, metastasis and death. Epo promotes endothelial cell migration and upregulates proteases that degrade extracellular matrix²⁶. Hypoxia enhances

glioma invasion via mechanisms that remain largely unknown. We tested whether Epo could promote invasion in glioma cells and whether blockade of Epo signaling attenuated hypoxia enhanced invasion. All in all, the fundamental aim of this proposal explored whether hypoxia's role in tumor progression may be directly linked to autocrine/paracrine Epo signaling in cancer cells.

ERYTHROPOIETIN PROMOTES SURVIVAL AND INVASIVENESS OF ASTROCYTOMAS.

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Abstract:

Perinecrotic hypoxia within glioblastoma multiforme tumors induces the expression of genes, which may enhance the survival and invasiveness of cancer cells. The hypoxia-inducible pleiotropic hormone erythropoietin has recently been found to promote the development and survival of neurons and astrocytes. Since erythropoietin signaling has also been implicated in the malignant progression of some human cancers, we determined whether erythropoietin and its receptor were expressed in biopsies and cultured cells from human astrocytomas. We also determined whether erythropoietin signaling influenced malignant properties of human astrocytoma cells. Expression of both proteins was prominently observed in the hypoxic regions and invasive margins of glioma biopsies and erythropoietin receptor expression correlated with tumor stage. Erythropoietin receptor was also functionally upregulated by hypoxia in cultured glioblastoma cells. Both hypoxia and erythropoietin protected cultured glioblastoma cells from cisplatin cytotoxicity and also promoted the invasiveness of glioblastoma cells through Matrigel by potentiating metalloprotease activity. Hypoxia-enhanced cell invasion was attenuated in cells over-expressing a non-functional erythropoietin receptor. We conclude that hypoxia-inducible autocrine and paracrine erythropoietin signaling participates in the malignant progression of glioblastomas. Our findings suggest novel therapeutic targets for brain cancer treatment and warrant a closer evaluation of the use of erythropoietin in anemic cancer patients.

Introduction

Widespread tissue invasion and the development of therapeutic resistance by glioblastoma multiforme invariably results in death. Regions of low oxygen are a common feature of solid tumors and are highly characteristic of high-grade gliomas [1-3], in which the degree of hypoxia is positively correlated with treatment resistance and death [4-6]. Tumor hypoxia increases tumor angiogenesis, glycolytic metabolism, cancer cell survival, and the probability of invasion and metastasis [6-9]. These responses involve gene expression mediated via the hypoxia inducible transcription factor HIF-1 [10, 11]. The oxygen-regulated HIF-1 α subunit of HIF-1 is highly expressed in cancer cells and its expression level in human glioma biopsies is strongly correlated with angiogenesis, invasion, and poor clinical outcome [12-13]. Although HIF-1 can activate transcription of many genes with cancer promoting potential, the regulation of specific genes by HIF-1 depends upon the given cell type and its stage of differentiation [11]. Thus, the relevant mediators of hypoxia-inducible malignant progression are not immediately obvious for all cancers. Moreover, certain HIF-1 regulated gene products have multiple activities relevant to cancer promotion. These mediators may be particularly useful targets for the development of new therapeutic approaches.

One such HIF-1 regulated gene product is the glycoprotein hormone erythropoietin (Epo). Classically known for its role in erythropoiesis, Epo acts on erythrocytic progenitors in the bone marrow to maintain appropriate hematocrit [14]. However, erythropoietin has reemerged in the past decade as a pleiotropic

hormone with a wide range of biological roles in non-hematopoietic tissues such as the central nervous system [15-17], retina [18], heart [19,20], lung [21], breast [22], uterus [23] and testis [24]. Epo and its receptor EpoR, are expressed by several cell types including neurons [25, 26], astrocytes [27], endothelial cells [28], vascular smooth muscle cells [29], placental trophoblasts [30], and renal [31] and mammary epithelial cells [32]. Epo signaling in these cells and tissues mediates several biological roles including neuroprotection [33], angiogenesis [34], vasculogenesis [35], cell proliferation [36] and cell migration [28]. Activation of EpoR initiates several signaling pathways involving protein phosphorylation. Epo induced EpoR dimerization recruits the tyrosine kinase JAK2 (Janus Kinase 2) which then phosphorylates itself as well EpoR and the STAT-5 (signal transducer and activator of transcription-5) proteins [37]. EpoR also promotes Akt phosphorylation via the PI3 kinase pathway [38] as well as protein kinase C activation [39].

Epo signaling plays a pathogenic role in some forms of erythroleukemia [40], and recently several solid human cancers deriving mainly from organs that utilize Epo signaling, have also been shown to express both Epo and EpoR [41-46]. While there have been proposals that Epo may promote angiogenesis [34, 45, 46] and enhance cancer cell survival and proliferation in these tumors [41-46], a defined biological role for Epo signaling in cancer treatment resistance and metastases is lacking. Data from our lab suggests that expression of Epo signaling components correlates with poor prognosis and contributes to the progression and chemotherapeutic resistance of breast and cervical cancer [43,

44]. Local intra-tumoral blockade of Epo signaling can delay progression of ovarian, uterine, melanoma and stomach choriocarcinoma xenografts in nude mice [45, 47]. Moreover, two recent clinical trials designed to evaluate the therapeutic benefit of Epo in breast cancer and head and neck cancer were terminated prematurely due to adverse outcomes in the Epo treated group, to include increased local regional spread of cancer and increased mortality [48, 49]. Collectively these findings have drawn concern for the indiscriminate use of Epo in the management of cancer-associated anemia [50] and have increased the urgency for a better understanding of Epo signaling in cancer.

The actions of Epo as a paracrine mediator of ischemic and hypoxic tolerance [16, 51] in the nervous system have also sparked enthusiasm for its use as a neuroprotective agent. EpoR expression and the trophic effects of exogenous recombinant erythropoietin (rhEpo) have been described in neurons, oligodendrocytes, microglia, as well as astrocytes themselves [33, 36, 52, 53]. However, the main endogenous source of hypoxia inducible Epo expression in the nervous system appears to be astrocytes [16, 27]. Since hypoxic preconditioning and Epo can both stimulate angiogenesis and cell survival, we hypothesized that a hypoxia inducible autocrine/paracrine Epo signaling mechanism contributes to the malignant promotion of glioblastoma multiforme (GBM), which are primarily derived from astrocytes. In this study we report prominent expression of erythropoietin and its receptor in glioma biopsies and cell lines. We show that treatment of human glioma cells with rhEpo provides protection from cisplatin cytotoxicity and increases invasiveness through

Matrigel. We also implicate endogenous Epo signaling as a significant component of hypoxia-inducible invasive behavior in gliomas.

Materials and Methods

Cell Culture and Treatments

Human U251, U87 and U373 glioma cells were cultured in Eagle's Minimum Essential Medium (MEM) and supplemented with 10% fetal bovine serum (FBS). Normal human astrocytes (Clonetics, lot 1F1475), were cultured in Dulbecco's Modified Eagle's Medium (GIBCO) containing 5.5mM glucose with 10% FBS and were switched to Eagle's MEM for some experiments as indicated. C6 and normal rat astrocytes were cultured using DMEM. Rat astrocytes were cultured from (E-16-E18) Wistar rats according to modified methods by McCarthy and de Vellis [54]. Hep3B cells were maintained in high glucose DMEM with 10% FBS and DU145 cells were cultures 1640 RPMI (Sigma) supplemented with 10% FBS. All media were supplemented with 1% (v/v) penicillin/ streptomycin. Cell lines were maintained in 21% O₂, 5% CO₂ and 74% N₂ in a humidified cell incubator at 37°C. For hypoxia treatments, culture dishes were sealed in a humidified chamber and continuously flushed with a gas mixture of 1% O₂, 5% CO₂ and 94% N₂ and incubated at 37°C.

Immunohistochemistry

Paraffin sections (5µm) of 25 human GBMs were obtained from the Armed Forces Institute of Pathology and 16 Grade II and Grade IV gliomas were selected from the Surgical Pathology files of the University of Pennsylvania

Medical Center were processed for immunohistochemistry. Sections, deparaffinized in xylene and rehydrated in graded alcohol, were steamed in 0.01 M sodium citrate buffer (pH 6.0) for 20 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS/10% NGS or NHS. Slides were incubated with primary antibodies against Epo (rabbit polyclonal, H-162; Santa Cruz Biotechnology, 1:200 dilution), EpoR (rabbit polyclonal, C-20; Santa Cruz Biotechnology; 1:200 dilution) and HIF-1 α (Mouse monoclonal, cat#H72320; Transduction Laboratories, 1:200 dilution) overnight at 4°C in 0.3% Triton X/PBS with 1.5% NGS or NHS. Slides were then washed three times with 0.2% Triton X/PBS and incubated for 60 min with biotinylated goat antirabbit IgG or anti-mouse IgG secondary antibody (1:200 dilution; Vector Laboratories, Inc.). After incubation with horseradish peroxidase-conjugated streptavidin (Streptavidin HP detection system; Research Genetics, Huntsville, AL) for 40 min, slides were developed with diaminobenzidine chromogen (Research Genetics) for 10 min and counterstained with hematoxylin. Negative controls included the omission of the primary antibody. The specificity of the Epo and EpoR antibodies was confirmed previously by our lab and others [42, 43, 45]. In addition, the specificity of the Epo and EpoR immunoreactivity was also evaluated by antibody absorption test: the primary antibody was pre-incubated with blocking peptide for Epo (rhEpo, R&D Systems, Minneapolis, MN) (10:1 peptide:antibody ratio) or EpoR (Santa Cruz Biotechnologies Inc.), which completely abolished immunohistochemical staining.

Interpretation of immunohistochemical stains

Immunohistochemical stains for Epo and EpoR were interpreted semiquantitatively by assessing the intensity and extent of staining on the entire tissue sections present on the slides according to a four-tiered (values ranging from 0 to 3) scale [43]. For Epo cytoplasmic, and for EpoR cytoplasmic and/or membrane immunoreactivity was considered positive. The percentage of weakly (value of 1), moderately (value of 2) and strongly (value of 3) staining cells was determined, so that the sum of these categories equated with the overall percentage of positivity. A staining score was then calculated as follows: Score (out of maximum of 300)= sum of 1 x percentage of weak, 2 x percentage of moderate and 3 x percentage of strong staining. For statistical analysis, the Wilcoxon signed rank test was used for the comparison of median EpoR expression levels in Grade II vs. Grade IV gliomas. Median EpoR immunohistochemical expression levels were compared using the Kruskal-Wallis one-way analysis of variance by ranks followed by Dunn's multiple comparison test, when appropriate. Statistical significance was determined if the two-sided p value of a test was less than 0.05.

Preparation of Nuclear Extracts and Whole Cell Extracts

For nuclear extracts, cells were washed with cold PBS, harvested on ice and centrifuged at $1000 \times g$ for 3 minutes. Cells were washed once with Buffer A (10mM Tris-HCl pH 7.5, 1.5 mM MgCl₂, 10 mM KCl, freshly supplemented with 2mM DTT and protease inhibitors) and each treatment sample was resuspended

in 200 μ l of Buffer A and cell suspension was homogenized with 25 strokes in a glass douncer with type b pestle. Homogenized cells were pelleted by centrifugation (4°C) for 10 minutes at 12,000 x g and the pellet was suspended in 50 μ l of Buffer C (420 mM KCl, 20mM Tris-HCl pH 7.5, 20% Glycerol, 1.5 mM MgCl₂, freshly supplemented with 2mM DTT and protease inhibitors(Roche)) and rotated for 30 minutes in the cold room. The suspension was then centrifuged (4°C) for 30 minutes at 20,000 x g and the supernatant was collected for western blot analysis or gel shift assay. Whole cell extracts were prepared by lysing cell pellet in RIPA buffer(0.1% SDS, 1% NP-40, 5mM EDTA, 0.5% Sodium Deoxycholate, 150mM NaCl, 50mM Tris-HCl, freshly supplemented with 2mM DTT and protease inhibitors) for 30-60 min on ice. Lysates were centrifuged (4°C) at 16,000 x g for 10 min and supernatant was collected for western blot analysis.

Immunoprecipitation and Western Blot Analysis

HIF 1 α western blots were performed by running 50 μ g of nuclear protein lysate on 4-12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes by standard procedures. Using a mouse monoclonal anti-HIF 1 α antibody (Transduction Laboratories), membranes were blocked with TBS/Tween with 5% nonfat milk for one hour and then incubated overnight with HIF antibody (1:400). Membranes were visualized by secondary antibody (anti-mouse, Amersham Biosciences) and enhanced chemiluminescence reagent (Pierce, Rockford, IL). EPOR western blots were performed by running 35 μ g of whole cell protein

extract on 4-12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes by standard procedures. Membranes were processed as previously described and treated with EPOR antibody 1:1500 (Santa Cruz, C-20) and visualized by secondary anti-rabbit antibody, 1:5000 (Pierce, Rockford, IL). p-STAT5 immunoprecipitation was performed by lysing cells with Extraction Buffer (400mM NaCl, 10mM Hepes pH 7.5, 1.5mM MgCl₂, .1 mM EGTA, 5% glycerol, freshly supplemented with 1mM DTT, protease inhibitors, 1mM NaO₂V₄). 400µg of cell lysate from each treatment sample were incubated at (4°C) with .5µg of STAT5 antibody (rabbit polyclonal, C-17; Santa Cruz Biotechnology) for 2hrs. Protein A-agarose beads were added, and samples were incubated for 1hr and then centrifuged. 50 µl of loading buffer were added to each sample and boiled for 5 minutes. Lysates were electrophoresed on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were processed as previously described and probed with p-STAT5 antibody (1:1000). EPOR(C-20, Santa Cruz) or p-EPOR (sc188, Santa Cruz) immunoprecipitation experiments were performed according to published methods by [55].

Electrophoretic Mobility Gel Shift Assay

HIF-1 electrophoretic mobility shift assay was performed as described previously[10]. The oligonucleotide probe from the erythropoietin enhancer region contained the HIF-1 binding site (5-GCCCTACGTGCTGTCTCA-3).

RNA Extraction, RT-PCR and quantitative PCR

Total RNA from the cultured cells was extracted using RNeasy kit (Qiagen). For each RT-PCR reaction, 1µg of total RNA was used with SUPERScript One-Step RT-PCR system (Invitrogen). The following sense and antisense primers were used: EPO mRNA (GI 31229, nucleotides 498–517 and 732–713). The following forward/reverse primers were used: for human HPRT1 (GenBank: NM_000194): 5'-TGACACTGGCAAAACAATGCA-3' / 5'-GGTCCTTTTCACCAGCAAGCT-3'; MMP2 : 5'- GTGGATGCCGCCTTTAACT-3' / 5'-GGAAAGCCAGGATCCATTTT-3' ; uPAR: 5'-AGCTATCGGACTGGCTTGAA-3' / 5'-CTTCGGAATAGGTGACAGC-3'. HotStarTaq DNA Polymerase (Qiagen) was used for RT-PCR amplifications. For quantitative real-time PCR analysis, the SYBR Green PCR Master Mix (Perkin Elmer) and ABI Prism 7700 Detection System was used. Single band amplification was verified through multicomponent analysis.

Cell Viability Assays

Cell Counting Assay. U251 cells were cultured with MEM in 6 well plates at ~40-60% confluence. 24hours after seeding, serum media was removed and fresh serum free media with or without cisplatin (10µg/ml) was added to the cells for 48 hours. Each treatment condition was done in triplicate and after 48 hours cells were analyzed for cell morphology and counted under phase contrast microscopy. Cells attached to the surface of the well were counted from 5 predetermined fields from each treatment condition.

MTT Assay. U251 cells were cultured in 48 well plates and using the same cisplatin cell killing paradigm as previously mentioned, after 48 hours of treatment 20µl of MTT solution (10mg/ml) was added to each well (500µl of media). After 4 hours of incubation in 37°C, media was removed and the blue formazan salt was dissolved with isopropyl alcohol. Spectrophotometric analysis was performed on the dissolved product and plate readings were taken at a 570nm wavelength as an index of cell viability. The difference in viability between treatment groups was statistically analyzed using a two-tailed student's *t* test.

Cell Invasion Assay

Cell invasion experiments were performed using 24-well Biocoat Matrigel™ Invasion Chambers with an 8-µm pore polycarbonate filter according to manufacturer's instructions (Beckton Dickinson Labware, Bedford, MA). For all cell lines, regular growth factor matrigel invasion chambers were used (cat#35-4480, BD Biosciences). For experiments with DU145 cells, reduced growth factor invasion chambers were used (cat#35-4483). Prior to experimentation all invasion chamber inserts were hydrated according to manufacturer's protocol. Briefly, cells in the growing phase were trypsinized and suspended at a concentration of (4×10^5 cells/ml). The lower compartment of the plates received 750µl of serum free media and/or the treatment condition designated in each experiment. 2×10^5 cells were plated in each insert and allowed to invade for 48 hours at 37°C in a humidified incubator with 21% O₂. For hypoxia experiments, cells were added to inserts and plates were incubated in a humidified hypoxia

(1% O₂) chamber, sealed and allowed to invade for 48 hours. All drug treatments were performed prior to hypoxia exposure. Cells that remained inside the insert after 48 hours were thoroughly wiped with a cotton swab and invading cells were fixed and stained using Diff-Quick® Stain Solution (Dade Bering, Newark, DE). Invading cells were quantified by counting the number of stained cells in five predetermined fields. All treatments groups were performed with an *n* of 6 inserts. The difference in invasion between treatment groups was statistically analyzed using a two-tailed student's *t* test unless otherwise noted.

Transfections

Plasmids encoding the full-length genomic DNA for the erythropoietin receptor (EpoR-f) and a truncated form (EpoR-t) were kindly donated by Dr Yukio Nakamura (RIKEN Bioresource Center, Ibaraki, Japan). The DNA was purified for transfection using the QIAfilter Maxi Plasmid Kit (Qiagen Sciences, MD) and 5 µg was introduced into the cells using Lipofectamine 2000 (Invitrogen, CA) as described by the manufacturer. A mock transfected cell line was also prepared using the pEGFP-C1 vector (Clontech, CA). Cells were placed under selective pressure 24h post-transfection, using 750 µg/ml G418. Western analysis and flow cytometry was used to confirm expression of foreign EpoR expression.

Analysis of EpoR surface expression by flow cytometry

Washed cells (1×10^5) were resuspended in 0.5% (w/v) bovine serum albumin (BSA) in Dulbecco's phosphate-buffered saline (PBS) containing 1 µg/ml of

human IgG (R&D Systems, MN) and incubated for 15 min. Cells were then incubated on ice for 20 min with either 0.5 µg anti-EpoR conjugated to phycoerythrin (R&D Systems) or an isotype control antibody. The cells were washed twice in PBS, resuspended in 0.5% BSA-PBS followed by analysis on an Epic XL-MCI flow cytometer (Coulter Corporation, FL). Dead cells were gated out using 7-amino actinomycin D (Sigma-Aldrich, MI).

Materials

Erythropoietin was purchased from (AMGEN Pharmaceuticals), Epo neutralizing antibody (cat#AB286NA, R&D Systems, Minneapolis, MN), MMP Inhibitor (cat# 444264, Calbiochem, San Diego , CA). Cisplatin (P4394, Sigma, St.Louis, MO).

Epo Elisa

Epo Elisa kit (cat# DEPOO, R&D Systems, Minneapolis, MN) was used according to manufacturer's instructions to measure erythropoietin protein released in media from Hep3B cells. Experiments were done in 10cm dishes and media from Hep3B cells exposed to normoxia (21% O₂) or hypoxia (1% O₂) for 24 hrs was collected and assayed directly for Epo protein expression.

Results

Expression of Epo and EpoR in human astrocytomas.

To determine whether Epo signaling was present in astrocytomas we analyzed the immunohistochemical expression of Epo and EpoR in human tumor biopsies. Analysis of 25 GBM specimens showed glioma cell-specific Epo staining in 23 samples and EpoR immunoreactivity in all 25 samples (figure 1A). Little or no staining for Epo or EpoR was found in the normal adult brain elements. GBMs are high grade tumors characterized by neovascularization, regions of necrosis and prominent invasion into normal brain tissue. The most prominent staining we observed in the GBM specimens was in the pseudopalisading cells bordering pockets of necrosis within the tumor (figure 1B). These characteristic perinecrotic areas of GBM are known to be markedly hypoxic (56) with strong expression of hypoxia-inducible genes [57-59]. Expression of HIF-1 α protein is markedly elevated in these areas and was observed to co-localize with Epo and EpoR staining (figure 1B). Cancer cells in the invasive border between tumor and normal brain also showed markedly increased expression of Epo and EpoR as did infiltrating cells found in remote white matter and grey matter (figure 1A). Prominent staining for Epo and EpoR was also seen in perivascular glioma cells and within hypertrophied endothelium of tumoral vasculature (figure 1A). In a separate analysis of 7 Grade II astrocytomas vs. 9 Grade IV (GBM) adult astrocytomas a significantly greater expression of EpoR was seen in the GBM specimens (figure 2A, B). Two thirds on the GBM samples also stained prominently for matrix metalloprotease 2

(MMP2), a marker for invasive phenotype, while MMP2 staining was absent from all grade II tumors (figure 2A).

Hypoxia regulates Epo and EpoR and activates Epo signaling in glioma cells.

In order to investigate whether human glioblastomas were able to respond to Epo signaling we examined the expression of EpoR in cell lines by western blot analysis and RT-PCR. The U251, U87, and U373 human glioblastoma cell lines were all found to express EpoR protein at a much higher level than normal human astrocytes (figure 3A). U251 cells treated with the clinically utilized Epoetin alpha (rhEpo) displayed prominent tyrosine phosphorylation of several cellular proteins as well as enhancement of JAK2, STAT5, and Akt phosphorylation (figure 3B). U251 cells cultured 6h in hypoxia (1%O₂) showed a marked accumulation of HIF-1 α protein and HIF-1 DNA binding activity in nuclear extracts vs. cells cultured under normoxia (21%O₂) (figure 3C). This was paralleled by an increase in Epo and EpoR mRNA by RT-PCR analysis. Longer treatment with hypoxia (24h) induced EpoR protein as observed by western blotting and also enhanced endogenous phosphorylation of EpoR (figure 3C) suggesting the presence of a hypoxia-inducible autocrine/paracrine Epo pathway.

Epo protects glioma cells against cisplatin toxicity

Treatment of U251 cells with rhEpo did not alter cell proliferation or the cell cycle distribution profile (data not shown) but did provide dose-dependent protection against cisplatin cytotoxicity (figure 4A, B). Even treatment of cells for

as little as 5 min with rhEpo afforded sustained protection 24 h later (figure 4C). Significant protective effects of Epo were not seen at doses below 10 U/ml, however pretreatment of cells with 24h hypoxia allowed significant protection to be observed at 1 U/ml (figure 4D,E). Moreover, the hypoxia induced increase in Epo sensitivity was found to correlate with time dependent hypoxic induction of EPOR mRNA by quantitative real time PCR (figure 4F).

Epo and hypoxia promote invasion of glioma cells through Matrigel.

Given the significant expression of Epo and EpoR on invasive glioma cells seen in tumor biopsies, we examined the effects of rhEpo on invasion of astrocytomas across Matrigel in modified Boyden chambers. We found that rhEpo dose dependently promoted Matrigel invasion by U251 cells (figure 5A). The invasion of C6 rat astrocytoma cells was also dose dependently stimulated by rhEpo while rat primary astrocyte appeared to display a bell-shaped dose response curve (figure 5B). The response of rat cells to rhEpo was less robust overall and was statistically significant only when analyzed via a one-tailed t- test. Both hypoxia and rhEpo stimulated invasiveness of U251 and U373 glioma cells as well as DU145 human prostate carcinoma and Hep3B human hepatoma cells (figure 5C). The latter are known classically for their pronounced Epo production under hypoxia.

When directly compared, both U251 and Hep3B cells showed nuclear HIF-1 α accumulation under hypoxia (figure 6A). However, Hep3B cells displayed much higher basal and hypoxia-inducible Epo mRNA than U251 cells (figure 6A-C). An Epo neutralizing antibody reduced hypoxia stimulated, but not serum

stimulated invasion in U251 cells (figure 6D). This approach also reduced the U251 invasion promoting effect of hypoxia-conditioned medium from Hep3B cells (figure 6E). Using the same Epo neutralizing antibody in the glioma experiments, Epo protein from hypoxic treated Hep3B conditioned media (24h treatment) was removed via immunoprecipitation. Conditioned media was treated with 10 µg/ml of Epo neutralizing antibody for two hours and antibody bound Epo was removed with protein A-agarose beads. Samples from this media were analyzed for Epo protein using an Epo Elisa assay to demonstrate loss of Epo protein (figure 6E) and corresponding media from the various treatment groups as indicated were used to promote glioma invasion. Hypoxic Hep3B conditioned media promoted the most glioma invasion, which was blunted when Epo was removed from the media via immunoprecipitation (figure 6F).

Epo mediates invasion via an increase in MMP-2 activity.

Immunohistochemical analysis revealed strong correlation between protein expression levels of HIF-1 α , EpoR and MMP-2 in pseudopalisading cells of GBM specimens (figure 2). Recent evidence demonstrates that pseudopalisades in glioblastomas are hypoxic, express extracellular matrix and are formed by an actively migrating cell population [56]. We found that inhibition of matrix metalloprotease (MMP) activity lowered the invasion promoting effects of Epo in glioma cells (figure 7A) and both hypoxia and Epo treatments resulted in increased MMP-2 activity in the media (figure 7B). However, while hypoxia clearly induced mRNA expression of MMP2 as well as uPAR, Epo failed to demonstrate a reproducible increase in these same genes (figure 7C).

Overexpression of truncated EpoR reduces hypoxia-inducible invasiveness.

In order to directly implicate a role for the Epo signaling in hypoxia-inducible Matrigel invasiveness, we generated U251 cells overexpressing either a full length EpoR or a truncated EpoR, which lacked the cytoplasmic signal transducing portion. Successful transfection of these constructs was verified by western blotting with antibodies generated against the cytoplasmic portion of the EpoR (figure 8A) or via cell sorting of cells stained with a fluorescence tagged antibody generated against the extracellular portion of the EpoR (figure 8B). Based on the flow cytometry data, approximately 30% of all cells were successfully transfected with the full length EpoR and about 50% were successfully transfected with the truncated EpoR. Although no clear differences were seen between the two transfected cell types in cell viability (figure 8C) or basal rate of invasiveness (data not shown), a clear reduction of hypoxia-inducible invasiveness was observed in cells transfected with the truncated EpoR (figure 8D). Taken together our data support a collaborative role for hypoxia and Epo signaling in the survival and invasiveness of human gliomas and other cancers.

Discussion

The major finding of this current investigation is that hypoxia-inducible Epo signaling in human astrocytomas promotes invasiveness and cytoprotection against a chemotherapeutic agent. Epo and EpoR immunoreactivity is markedly increased in glioma cells as compared to normal adult brain elements and

appears to correlate with tumor grade. These observations are consistent with previous findings of upregulated Epo and EpoR expression in other human cancers [41-46]. In light of the recently identified potent angiogenic properties of Epo [28, 34], the immunoreactivity for Epo and EpoR that we observed associated with tumor vasculature supports an important role for Epo signaling in tumor angiogenesis. Epo and EpoR were also specifically enriched in the pseudopalisading glioma cells surrounding necrotic foci. These regions are known to be hypoxic [56] and to strongly express HIF-1 α protein [56-59, figure 1A]. GBM cells in culture also express functional EpoR, which is upregulated by hypoxia. The EpoR gene is not known to have a Hypoxia Response Element (HRE) HIF-1 binding site and hypoxia may regulate EpoR indirectly by increasing Epo levels. Epo is known to upregulate the expression of its own receptor in certain cell lines and endothelial cells [61-63]. Enhancement of the EpoR signaling pathway (figure 3C) by hypoxia suggests that glioma cells retain an autocrine Epo/EpoR signaling mechanism similar to that described for normal astrocytes.

Tumor hypoxia correlates with poor clinical outcome [4-6]. Our observation that hypoxia and rhEpo both protect glioma cells in culture from cisplatin toxicity suggests a role for hypoxia-inducible Epo signaling in chemotherapeutic resistance. The ability of hypoxia to potentiate Epo reversal of cisplatin toxicity may result from the hypoxic upregulation of erythropoietin signaling elements described above. Although a clear signaling pathway has yet to be implicated in the cytoprotective effects of Epo on cancers cells, the JAK-STAT pathway is

emerging as a strong candidate. STAT5 regulates bcl-2 and bcl-X_L expression, two anti-apoptotic proteins that are activated by Epo in erythroblasts and neurons.

Tumor hypoxia is also a strong driving force behind cancer metastasis and invasion [8, 9, 65]. Although Epo has been shown to promote the motility of endothelial cells [28] and enterocytes [22], our study is the first to report that Epo can enhance invasion of human glioma and other cancer cells through connective tissue (figure 5). Epo also enhanced normal rat astrocyte invasion suggesting a broader biological role for Epo signaling in cell migration through tissues. This function may underlie the ability of Epo signaling to promote migration of erythroblasts through the bone marrow matrix and in to blood. Invasion of brain tissue by endothelial cells during angiogenesis and astrocytes during reactive astrocytosis may be similarly promoted by Epo. Epo neutralizing antibodies as well as the overexpression of a truncated EpoR also attenuated hypoxia induced glioma invasion. These findings implicate Epo signaling as a component of hypoxia-inducible invasion of glioma cells.

Pseudopalisading glioma cells in GBM tumors express enhanced gelatinase activity characteristic of an invasive phenotype [56, figure 2] and the Epo enhanced invasion in glioma cells was blocked by an MMP inhibitor (figure 6A). Moreover, conditioned media from hypoxia and Epo treated glioma cells demonstrated higher levels of active MMP-2 (gelatinase A). HIF-1 regulates the hypoxia-inducible expression of several invasion promoting genes including MMP2 and the urokinase plasminogen-activating enzyme receptor (uPAR).

Although hypoxia induced these genes in glioma cells Epo could not do the same despite increasing active MMP-2 levels. These findings imply the existence of novel interactions between hypoxia and Epo signaling pathways in promoting cancer cell invasiveness. Combined Epo/EpoR expression may allow tumor cells to survive hypoxic conditions in regions of tumor overgrowth and escape regions of poor oxygenation by invading surrounding, more vascularized tissue.

Biological actions of Epo signaling in cancer cells are just beginning to be appreciated. The recent termination of two clinical trials due to adverse outcome in rhEpo treated patients has heightened the importance of understanding Epo actions on cancer cells [48, 49]. Our data that Epo signaling enhances cancer invasion and promotes resistance to therapy strongly advocate a re-examination of the indiscriminate treatment of cancer patients with rhEpo.

Figure 1. Expression of Epo and EpoR in human glioma biopsies (A)

Immunohistochemistry. Expression of Epo and EpoR in human glioma biopsies (n=25), (i, ii) Epo and EpoR positive cells invading through cortex, (iii, iv) Gemistocytic astrocytoma cells expressing Epo/EpoR, (v, vi) Epo and EpoR staining in glioma cells and tumoral vascular elements, (vii, viii) Prominent Epo and EpoR staining in perivascular glioblastoma cells. (B) Immunohistochemistry. HIF-1 α , Epo and EpoR expression is most intense in the hypoxic zones of glioma biopsies.

Figure 2. Expression of HIF-1 , EPOR and MMP-2 in glioma biopsies. (A)

Immunohistochemistry. Grade IV glioma biopsies display higher levels of HIF-1 α , EpoR, and MMP-2 protein expression than Grade II samples (n=9). Furthermore, there was strong overlap in the intensity of expression between HIF-1 α , EpoR and MMP-2 which often localized to the hypoxic zones and the leading edge in tumor margins. (B) Immunohistochemistry. Grade IV glioma biopsies displayed higher levels of EpoR expression than Grade II gliomas, $P < 0.05$; n=9.

Figure 3. Hypoxia regulates Epo and EpoR expression and promotes Epo signaling in U251 cells. (A) Western blot analysis. Human glioma cell lines display higher EpoR protein expression than normal human astrocytes. (B)

Western blot analysis. U251 glioma cells display functional EpoR. Stimulation with Epo (200 U/ml) for 15 minutes increased general tyrosine phosphorylation, p-JAK2, p-STAT5 and p-AKT protein levels. (C) Western blot analysis, gel shift,

PCR analysis. Treatment of U251 cells with hypoxia (1% O₂) for 4 hours increased HIF-1 α protein levels and DNA binding. 8 hour treatments with hypoxia increased Epo and EpoR mRNA levels and 24 hours of hypoxia (1% O₂) increased EpoR protein expression. In addition, EpoR phosphorylation was enhanced with hypoxia (24hrs) U251cells when compared to their normoxic controls.

Figure 4. Epo promotes glioma cell survival. (A) Cell morphology. Cells treated with Epo demonstrated normal cell morphology when compared to cisplatin treated controls. More cells in the Epo treated group were attached to surface of the plate and healthy adherent cells from each treatment group were counted for quantification. (*P<.05). (B) Cell counting assay. Epo treatment protected U251 cells from cisplatin induced cytotoxicity. (C) MTT assay. Pre-treatment with Epo for various time points protects U251 cells against cisplatin induced cytotoxicity. (*P<.05). (D) (E) Hypoxia (1% O₂) and Epo synergize to enhance cell survival in U251 glioma cells against cisplatin cytotoxicity. Cells were treated with low and high doses of Epo under normoxic (21% O₂) and hypoxic (1% O₂) conditions during cisplatin treatment for 48 hours. (*P<.05). (F) Quantitative-PCR. EpoR mRNA levels in U251 cells demonstrated significant fold inductions after 8 and 24 hour treatments with hypoxia (1% O₂). (*P<.05).

Figure 5. Hypoxia and Epo promote matrigel invasion in several human cancer cell lines. (A) Cell invasion assay. Treatment of U251 cells with Epo

(48hrs) promotes the invasion of glioma cells through matrigel. (B) Cell invasion assay. Epo treatment (48hrs) enhances C6 glioma cells and normal rat astrocytes invasion through matrigel, however hypoxia (1% O₂) promoted C6 glioma invasion only. (one-tailed t-test, *P<.05). (C) Cell invasion assay. U251 and U373 glioma cell lines demonstrate Epo (200 U/ml) and hypoxia (1% O₂) enhanced invasion. (*P<.05). (D) Cell invasion assay. Epo (200 U/ml) and hypoxia (1% O₂) enhanced invasion in several other cell lines including prostate (DU145) and hepatoma (Hep3B) cells. (*P<.05).

Figure 6. Epo signaling is a component of hypoxia mediated invasion. (A) Western blotting, PCR. Hypoxia (1% O₂) for 4 hours increased HIF-1 α protein expression in U251 and Hep3B cells. Hypoxia treatment for 24 hours increased Epo mRNA in both cell lines as measured by RT-PCR and quantitative-RT-PCR analysis. (B) and (C). (D) Cell invasion assay. Hypoxia (1% O₂) induced invasion (48hrs) in U251 cells is attenuated with Epo neutralizing antibody (9 μ g/ml). Epo neutralizing antibody (9 μ g/ml) does not attenuate serum induced invasion in U251 cells. (*P<.05). (E) Epo Elisa assay. Cell invasion assay. Epo from hypoxic conditioned Hep3B media was immunoprecipitated using (10 μ g/ml) of Epo neutralizing antibody and media was collected for Epo Elisa for protein expression. (*P<.05). (F) 24 hour Hypoxic (1% O₂) Hep3B conditioned media promotes invasiveness in U251 cells when compared to normoxic (21% O₂) conditioned media and this effect is attenuated with the Epo neutralizing antibody. (*P<.05).

Figure 7. Epo signaling promotes invasion in glioma cells via increased

MMP activity. (A) Cell invasion assay. Epo induced invasion in U251 cells is blocked with a non-specific MMP inhibitor (50 μ M). (*P<.05). (B) MMP-2 Fluorescent enzyme assay. Hypoxia and Epo increase MMP-2 activity. Conditioned media from hypoxic (1% O₂) and Epo (200 U/ml) treated U251 cells (24 hours) showed higher MMP-2 activity. The amount of active MMP-2 present in the media was assayed via the level of fluorescence present of a cleaved peptide product. (*P<.05). (C) Quantitative PCR. Hypoxia (1% O₂) treated U251 cells exhibited elevated MMP-2 and uPAR mRNA when compared to normoxic controls. Epo (200 U/ml) treated cells did not demonstrate any increase in MMP-2 or uPAR mRNA. (*P<.05).

Figure 8. Truncated EPOR attenuates hypoxia enhanced invasion in U251

cells. (A) Western blot analysis. Stably transfected U251 cells expressing full-EPOR (EPOR-f) and truncated-EPOR (EPOR-t), antibody used for western only recognizes cytoplasmic portion of the receptor. (B) Flow cytometry. EPOR surface levels of non-transfected cells, EPOR-f and EPOR-t U251 cells as expressed by relative mean fluorescence units assayed via a directly conjugated phycoerythrin EPOR antibody. (C) MTT assay. MTT reduction capabilities of U251 cells expressing (EPOR-f) and (EPOR-t) demonstrate no significant difference in MTT reduction activity. (D) Invasion assay. U251 EPOR-t demonstrated diminished hypoxia enhanced invasion when compared to U251

EPOR-f cells. Hypoxia enhanced invasion EPOR-f cells when compared to normoxic EPOR-f control cells(*P<.05).

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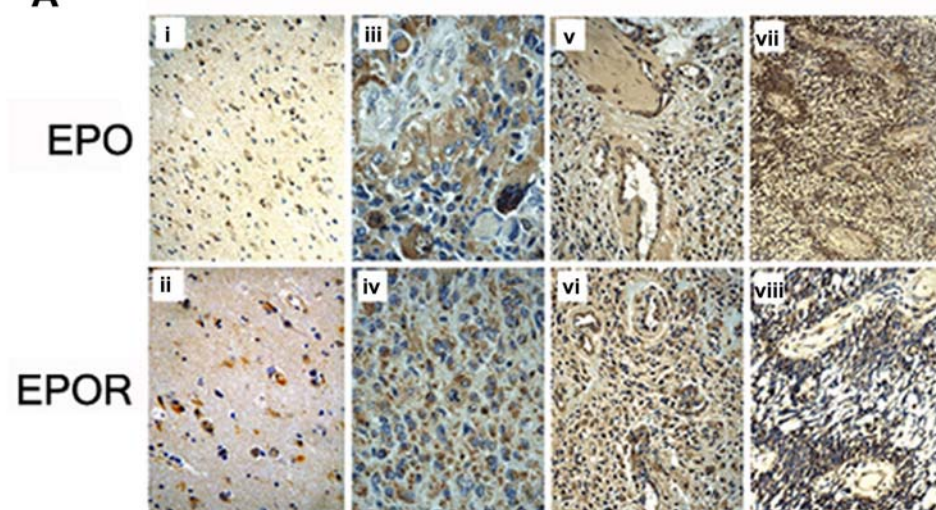
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Figure 1

A



B

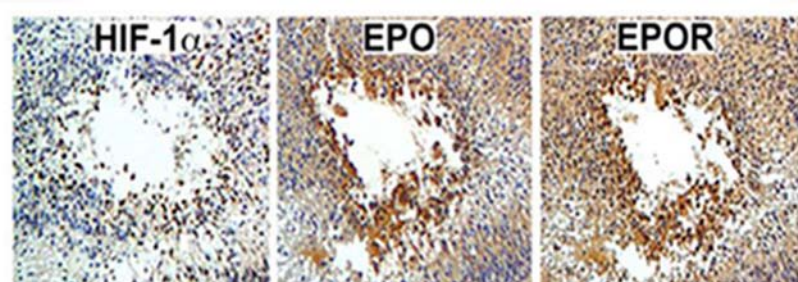


Figure 2

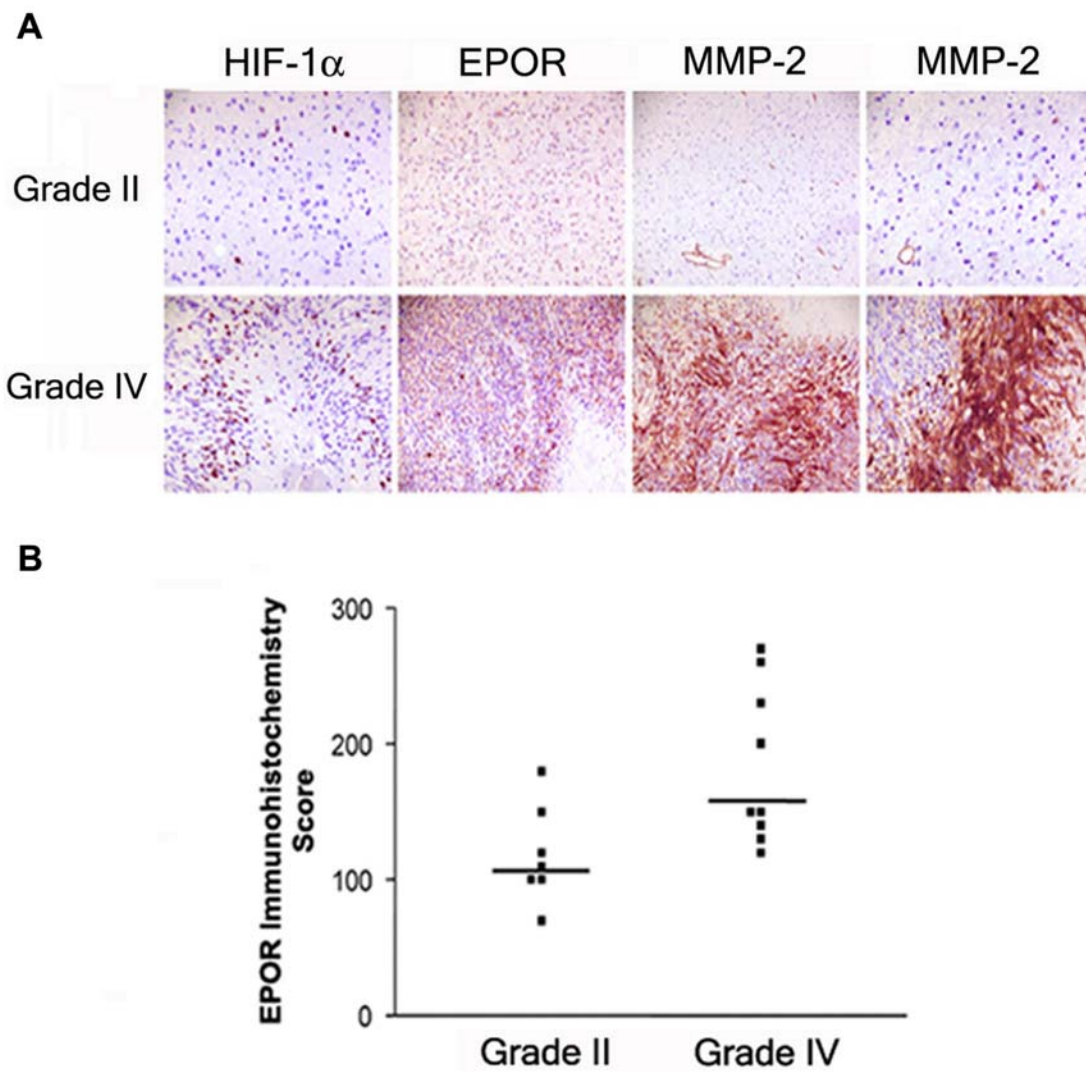
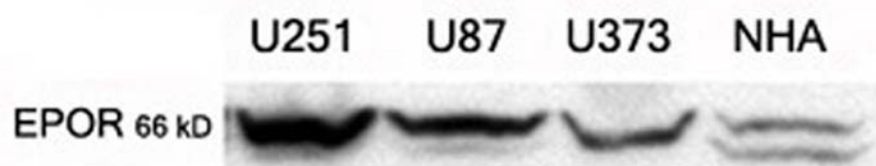
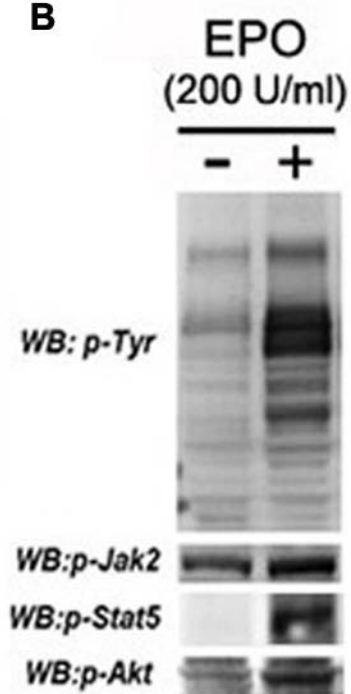


Figure 3

A



B



C

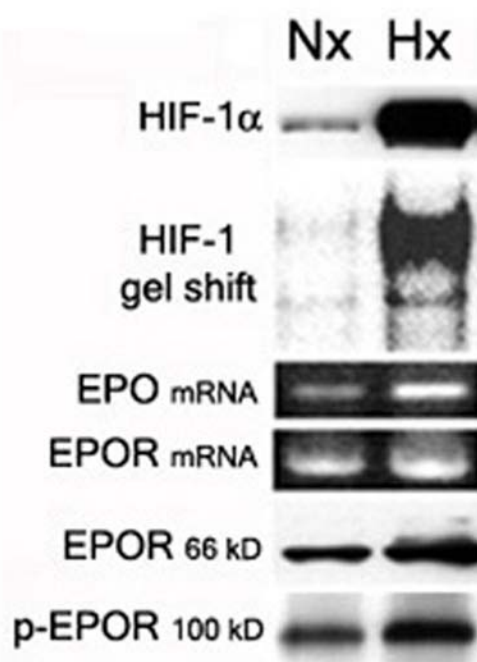


Figure 4

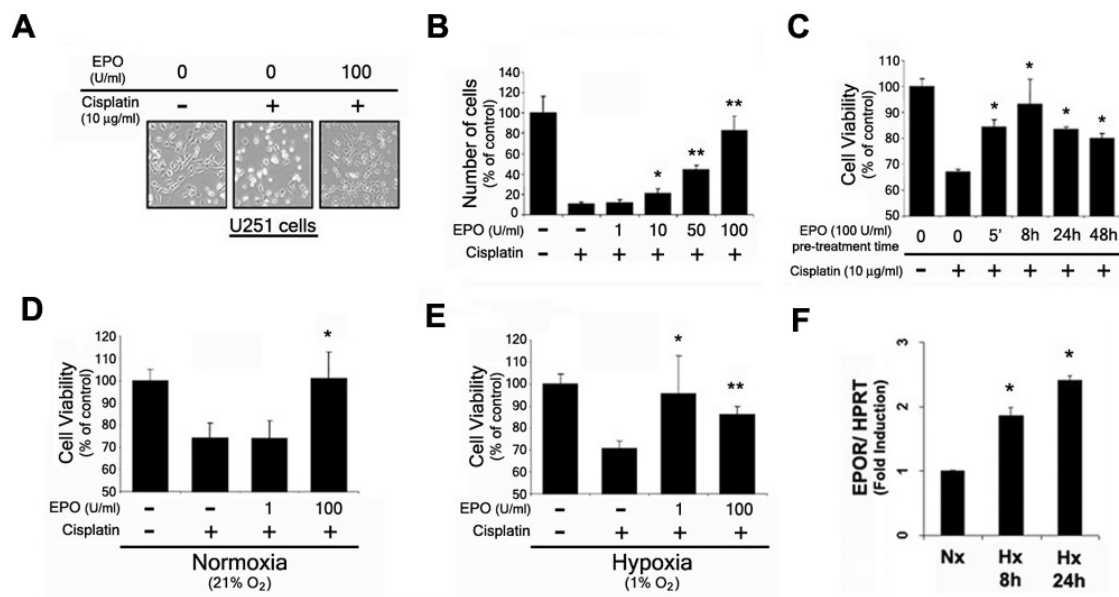


Figure 5

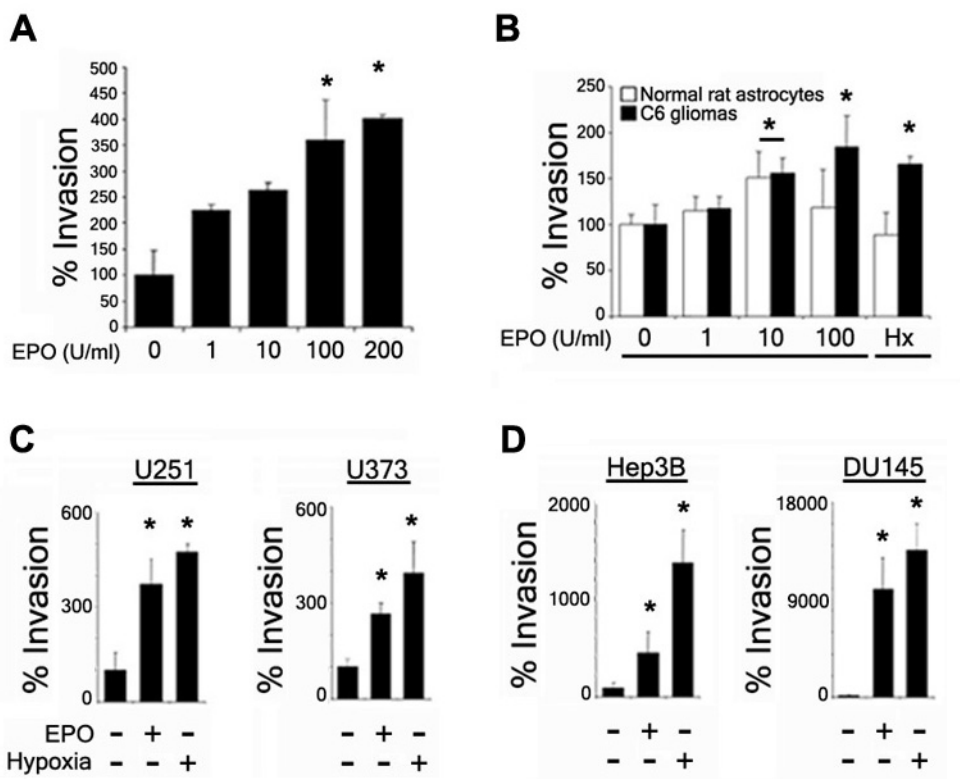


Figure 6

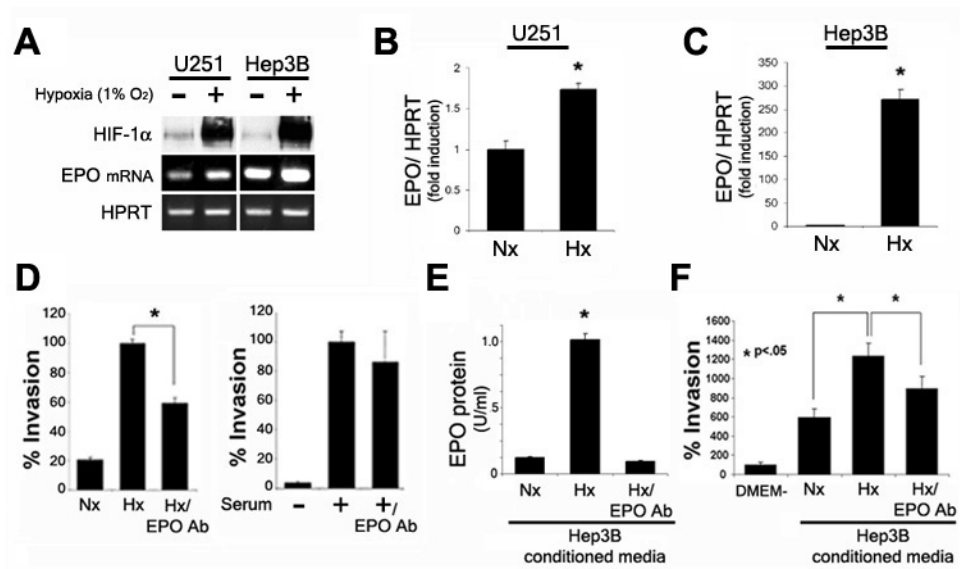


Figure 7

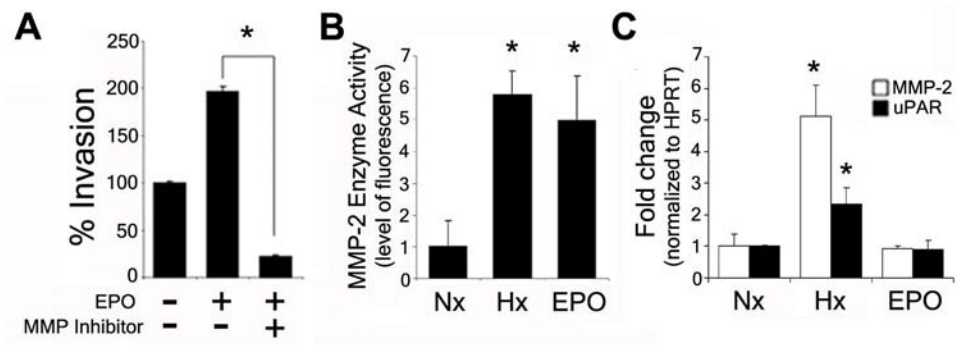
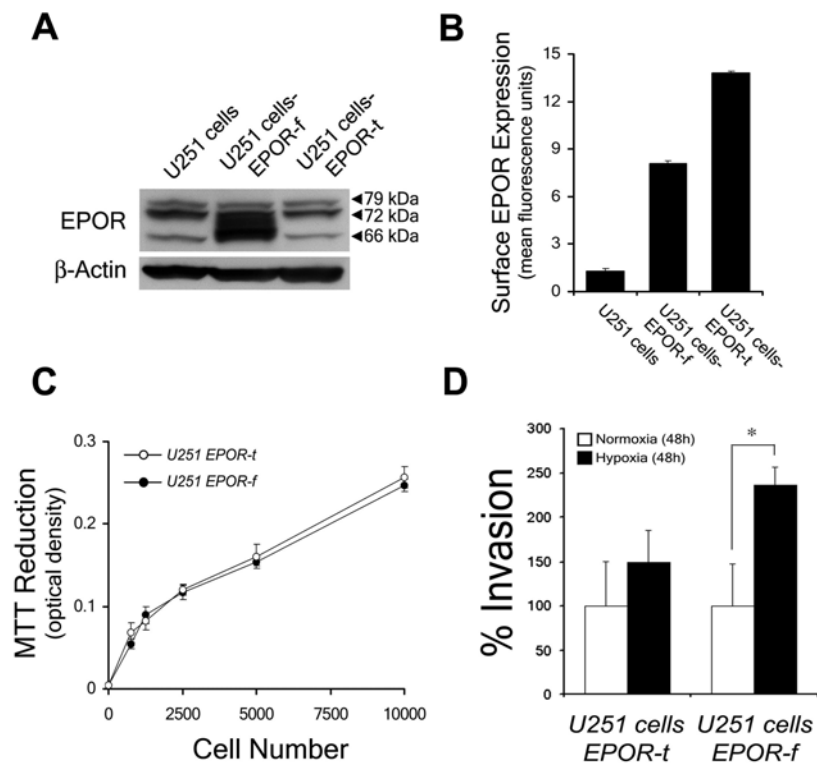


Figure 8



**ERYTHROPOIETIN SIGNALING PROMOTES INVASIVENESS OF HUMAN
HEAD AND NECK SQUAMOUS CELL CARCINOMA.**

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The opinions or assertions contained herein are the private ones of the author, and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

SUMMARY

Background: Erythropoietin (Epo) is used for managing anemia in cancer patients. However, recent studies have associated adverse outcomes with this practice. We investigated the expression and function of Epo signaling mechanisms in human head and neck tumor biopsies and cell lines.

Methods: Surgical biopsy specimens from patients with head and neck cancer were evaluated for the expression of erythropoietin and erythropoietin receptor (EpoR) using immunohistochemistry. Human head and neck cancer cell lines were evaluated for Epo and EpoR gene expression. Epo responsiveness of the cell lines was assessed by Epoetin- α induced tyrosine phosphorylation of the JAK2 protein kinase. Transmigration assays across Matrigel coated filters were used to examine effects of Epoetin- α on cell invasiveness.

Findings: In 32 biopsies we observed significant association between disease progression and expression of Epo and its receptor EpoR. Expression was highest in malignant cells, particularly within hypoxic and infiltrating tumor regions. Basal and hypoxia-inducible Epo and EpoR expression was observed in human head and neck cancer cell lines, which responded to Epoetin- α treatment with prominent JAK2 phosphorylation and enhanced invasiveness. Inhibition of JAK2 phosphorylation reduced both basal and Epo-induced invasiveness.

Interpretation: Our findings support a role for autocrine Epo signaling in the malignant progression and local invasiveness of head and neck cancer. Activation of this mechanism by recombinant Epo (rhEpo) treatment may

contribute to the increased locoregional spread recently reported in rhEpo treated cancer patients.

Introduction

Epo treatment increases hematocrit and improves fatigue in anemic cancer patients¹. However, recent studies have raised the possibility that rhEpo treatment may also exert direct biological actions on human cancer cells²⁻⁵. Two recent clinical trials were in fact stopped prematurely due to clinical worsening associated with rhEpo use^{6, 7}. One of these trials evaluated 351 head and neck cancer patients and found poorer locoregional progression-free survival in rhEpo treated patients vs. the placebo group⁷. The mechanisms underlying this observation remain unknown.

Expression of the Epo and EpoR genes in neoplastic lesions has recently been correlated with poor prognosis in several human cancers including breast⁸, cervical³, and endometrial carcinoma⁹. Newly appreciated, non-hematopoietic biological activities of Epo such as promotion of angiogenesis¹⁰ and inhibition of apoptosis¹¹ may contribute to disease progression in human cancers. We performed this study to determine whether Epo signaling mechanisms had biological effects in head and neck cancer. We investigated the expression of Epo and EpoR in human head and neck cancer specimens and explored biological effects of Epo on head and neck carcinoma cell lines.

Methods

Clinical samples and clinical data

Study protocols involving human material were approved by the University of Pennsylvania Institutional Review Board. Thirty two cases of head and neck squamous cell carcinoma biopsies or tumor resections (larynx - 9, aryepiglottic fold - 5, epiglottis - 4, tongue - 4, retromolar trigone - 4, cervical lymph node – 6) were selected from the Surgical Pathology files of the University of Pennsylvania Medical Center. Hematoxylin and eosin (H&E) stained slides of all cases were reviewed and the diagnoses confirmed. Invasive carcinomas as well carcinomas *in situ* were also evaluated. All specimens were primary resection or pretreatment biopsies, from patients with no prior treatment with Epo.

Immunohistochemistry and immunocytochemistry

Immunohistochemical assays were performed on formalin-fixed paraffin-embedded sections as described previously^{3, 8}. Five µm-thick sections were cut and deparaffinized in xylene and rehydrated in graded alcohols. All slides were steamed in 0.01mol/L sodium citrate buffer (pH 6.0) for 20 minutes. Endogenous peroxidase activity was blocked by .3% hydrogen peroxide in methanol for 20 min. Slides were incubated with the antibodies against erythropoietin (Epo, rabbit polyclonal, H-162, 1:200 dilution, Santa Cruz Biotechnologies), erythropoietin receptor (EpoR, rabbit polyclonal, C-20, 1:400 dilution, Santa Cruz Biotechnologies) overnight at 4°C. Slides were then washed five times with Tris Buffered Saline containing Tween 20 (TBST, pH 7.6, DAKO) and incubated for 30 minutes at room temperature with horseradish peroxidase labeled dextran polymer coupled to anti-rabbit antibody (DAKO EnVision + System HRP, DAKO),

developed with diaminobenzidine for 10 minutes and counterstained with hematoxylin. For Epo and EpoR immunohistochemistry, slides of fetal liver and placenta were used as positive controls. The specificity of the Epo and EpoR antibodies were confirmed previously². In addition, the specificity of the EpoR and Epo immunoreactivity was also evaluated by antibody absorption test: the primary antibody was preincubated with blocking peptide for EpoR (Santa Cruz Biotechnologies Inc.) or human recombinant Epo (rhEpo, R & D Systems, Minneapolis, MN) (10:1 peptide:antibody ratio), which resulted in complete abolishment of immunohistochemical staining. The specificity of the immunostaining reaction is further supported by other experiments using a mouse monoclonal anti-Epo (Clone 9C21D11, R&D Systems) and a rabbit polyclonal anti-EpoR antibody (Upstate Biotechnology, Lake Placid, NY)^{3, 8, 9}, which resulted in an immunostaining pattern similar to that obtained with the antibodies used in the current study. For cell line staining, cells were fixed with 10% formalin and stained for Epo as described above.

Interpretation of immunohistochemical stains

Immunohistochemical stains for Epo and EpoR were interpreted semiquantitatively by assessing the intensity and extent of staining on the entire tissue sections present on the slides according to a four-tiered (0-3) scale³. For Epo cytoplasmic, and for EpoR cytoplasmic and/or membrane immunoreactivity was considered positive. In the case of dysplasias or *in situ* carcinomas, first, the percentage of total epithelial thickness showing positive staining was determined

(e.g. 50% if the basal half or 75% if the basal three fourth of the squamous epithelium showed positive immunostaining, etc.). In the case of invasive tumors, first the total percentage of positively staining tumor cells was determined. Then the percentage of weakly (1), moderately (2) and strongly (3) staining cells was determined, so that the sum of these categories equated with the overall percentage of positivity. A staining score was then calculated as follows: Score (out of maximum of 300) = sum of 1 x percentage of weak, 2 x percentage of moderate and 3 x percentage of strong staining.

Statistical analysis

The Wilcoxon signed rank test was used for the comparison of median EpoR and Epo immunohistochemical expression levels in invasive squamous cell carcinoma, squamous cell dysplasia, and the adjacent benign squamous epithelium. Median EpoR, and Epo immunohistochemical expression levels in benign epithelia, dysplasia, and invasive carcinoma were compared using the Kruskal-Wallis one-way analysis of variance by ranks followed by Dunn's multiple comparison test, when appropriate. Statistical significance was established if the two-sided p value of a test was less than 0.05.

Cell Culture and Hypoxia Treatments

Human JHU-O22SCC (from here on referred to as 022) and UM-SCC-22B (from here on referred to as 22B) cancer cells were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (GIBCO) containing high glucose (25mM) with 10% FBS,

respectively. All media were supplemented with 1% (v/v) penicillin/ streptomycin. Cell lines were maintained in 21% O₂, 5% CO₂ and 74% N₂ in a humidified cell incubator at 37°C. For hypoxia treatments, culture dishes were sealed in a humidified chamber and flushed with a gas mixture of 1% O₂, 5% CO₂ and 94% N₂ and incubated at 37°C for the time indicated.

Western Blotting and Immunoprecipitations

For cell extract preparation, cell pellets from 100% confluent 10cm culture dishes were lysed in RIPA buffer (0.1% SDS, 1% NP-40, 5mM EDTA, 0.5% Sodium Deoxycholate, 150mM NaCl, 50mM Tris-HCl, with 2mM DTT and protease inhibitors cocktail) for 60 minutes on ice. Lysates were centrifuged (4°C) at 16,000 x g for 10 minutes and supernatant was collected for western blot analysis. For HIF-1 α and EpoR western blotting, whole cell lysates were resolved using 4-12% polyacrylamide-SDS gel (100 μ g for HIF-1 α and 50 μ g for EpoR). Proteins were transferred to nitrocellulose membrane, blocked with 5% nonfat dry milk in TBS-T (50mM Tris, pH 7.6, 150mM NaCl, 0.1% Tween-20) and probed with HIF-1 α monoclonal antibody 1:350 (Transduction Laboratories) and EpoR rabbit polyclonal antibodies (C20) 1:1500 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. Horseradish peroxidase conjugated secondary antibodies were used to probe membranes: sheep anti-mouse (Amersham Pharmacia Biotech, Piscataway, NJ) for HIF-1 α 1:2000 and goat anti-rabbit for EpoR (1:5000). Immunoreactive bands were visualized using chemiluminescence (SuperSignal WestPico Chemiluminescence kit; Pierce,

Rockford, IL). Phospho-Jak2 (p-JAK2) immunoprecipitations were performed as previously described¹². Briefly, cells were lysed and immunoprecipitations were performed with 5µg of JAK2 antibody (Upstate Biotechnology Inc, Lake Placid, NY) and protein A-Sepharose beads (Boehringer Mannheim, Indianapolis, IN). Immunoprecipitates were separated on 8.75% polyacrylamide-SDS gel, transferred to nitrocellulose membrane and probed with a monoclonal anti-phosphotyrosine 4G10 antibody 1:1000 (Upstate Biotechnology Inc.).

RT-PCR and quantitative RT-PCR analysis for erythropoietin gene expression

Total RNA from cells was isolated using the RNeasy Mini Kit (Qiagen Inc.). cDNA was generated from 5 µg of total RNA using iScript™ cDNA Synthesis Kit (BIO RAD). PCR was conducted using MAXIscript SP6 (Ambion) with 1 µL of cDNA template and 0.3 µM of forward and reverse primers. The primers for Epo were : forward [5'-TCACTGTCCCAGACACCAAA - 3'] and reverse [5'-GGGAAGAGTTGACCAACAGG - 3'], which correspond to base pair 378 to 518. PCR cycling conditions were 40 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 60 seconds. PCR products were run on a 4% agarose gel along with a 50 bp ladder (Invitrogen Corp.) Primers for control gene HPRT were: forward [5'- TGACACTGGCAAAACAATGCA -3'] and reverse [5'-GGTCCTTTTCACCAGCAAGCT -3'].

For quantitative real-time PCR analysis, the SYBR Green PCR Master Mix (Perkin Elmer) and the Bio Rad Detection System was used. Single band amplification was verified through multicomponent analysis. Primers for GLUT3

were: forward [5'-TGACGATACCGGAGCCAATG -3'] and reverse [5'-TCAAAGGACTTGCCCAGTTT -3']. Primers for control gene GUS were: forward [5' -GAAAATATGTGGTTGGAGAGCTCATT -3'] and reverse [5'-CCGAGTGAAGATCCCCTTTTTA-3'].

Cell Invasion Assay

Cell invasion experiments were performed using 24-well Biocoat Matrigel™ Invasion Chambers with an 8-µm pore polycarbonate filter according to manufacturer's instructions (cat#35-4480, Beckton Dickinson Labware, Bedford, MA). Prior to experimentation all invasion chamber inserts were hydrated according to manufacturer's protocol. Briefly, cells in the growing phase were trypsinized and resuspended at a concentration of 2×10^5 cells/ml in media with 0.5% FBS. The lower compartment of the plates received 750µl of serum free media. All drug treatments were added to the lower compartment of the plate prior to cell plating. 1×10^5 cells were plated in each insert and allowed to invade for 48 hours at 37°C in a humidified incubator with 21% O₂. Cells that remained inside the insert after 48 hours were thoroughly wiped with a cotton swab and invading cells were fixed and stained using Diff-Quick Stain Solution (Dade Begring, Newark, DE). Images of invading cells were captured and quantified by counting the number of stained cells in five predetermined fields at 20x magnification (average number of cells per field for O22 cells and 22B cells under serum free conditions was 5 and 61) All treatments groups were performed with an *n* of 6 inserts. The difference in invasion between treatment groups was

statistically analyzed using a one-tailed student's *t* test. Recombinant erythropoietin was purchased from AMGEN (Thousand Oaks, CA), AG490 was from Sigma (St. Louis, MO).

Role of the Funding Source

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Results

To investigate whether Epo signaling might play a direct role in head and neck squamous cell carcinoma progression we examined the immunohistochemical expression of Epo and EpoR proteins in biopsy samples obtained from oral cavity, oropharyngeal, hypopharyngeal and laryngeal lesions of patients not previously treated with rhEpo. Our analysis revealed high levels of both Epo and EpoR expression in the carcinomas examined (25/32, and 32/32 respectively). In normal tissue, EpoR staining was low and confined to the basal epithelial layer (Figure 1a). Strong EpoR staining was seen throughout dysplastic epithelium, in invasive carcinoma cells, and in lymph node metastases. Tumoral vascular elements also showed prominent EpoR immunoreactivity. Epo staining of normal elements was undetectable in most samples (Fig. 1a). Within tumors however, Epo immunoreactivity was typically seen in the peri-necrotic rims, which are known to be severely hypoxic¹³. Discretely intense Epo staining was also seen in invasive carcinoma cells, but was not as uniformly expressed as

EpoR staining. A statistically significant correlation between tumor progression and immunohistochemical staining for EpoR (Fig 1b) and Epo (Fig. 1c) was demonstrated.

We next explored the biological regulation and actions of Epo signaling in cancer cells using human head and neck squamous cell carcinoma (HNSCC) cell lines. EpoR expression was observed by western blotting in several HNSCC cell lines including the 022, 22B (Fig. 2a), 22A, 37B, 37A, 15A and 1483 cell lines (Data not shown). Epo gene expression is regulated by the hypoxia-inducible transcription factor HIF-1¹⁴. The 022 and the 22B HNSCC cell lines are known to differ significantly in their basal expression level of the oxygen responsive HIF-1 α subunit of HIF-1¹⁵. In addition to displaying higher basal HIF-1 α levels, the 22B cells also displayed higher EpoR protein expression (figure 2a). Following 24h exposure to hypoxia (1%O₂), both cells lines accumulated HIF-1 α , and increased expression of EpoR protein (figure 2a). While both cell lines expressed Epo mRNA (figure 2b) and Epo protein (figure 2e), Epo expression did not increase after 24 hours of hypoxia treatment in either cell line when compared to other HIF-1 mediated hypoxia inducible genes such as the glucose transporter, GLUT3 (figure 2c and 2d).

The 22B cells also display greater invasiveness through Matrigel-coated Boyden chambers than the 022 cells¹⁵ (figure 2f and 2g). To find out whether Epo signaling could influence the invasive behavior of HNSCC cells we first examined whether EpoR could be activated by rhEpo in these cells. Upon binding Epo, dimerization of EpoR recruits and activates the JAK2 tyrosine kinase, which then

phosphorylates itself along with other signaling components¹⁶. As shown in figure 3a, rhEpo (in the form of Epoetin- α) promoted tyrosine phosphorylation of JAK2 in both O22 and 22B cells. Moreover, the Epoetin- α stimulated JAK2 phosphorylation was blocked by the specific JAK2 inhibitor AG490¹⁷. Remarkably, Epoetin- α also promoted invasiveness in both cell lines (figure 3b). The O22 cells displayed a greater response to Epo although this may have been due to the much higher basal invasiveness seen in the 22B cells. A bell-shaped dose-response relationship was observed for Epo induced invasiveness has been previously reported for other cellular actions of Epo¹⁸. The Epo-induced HNSCC cell invasion was blocked by AG490 thus implicating involvement of the EpoR-JAK2 signaling pathway (figure 3c). The high basal invasiveness of 22B cells was also blunted by AG490 (figure 3d) suggesting that an autocrine Epo signaling mechanism may play a role in the invasion of some HNSCC.

Discussion

We have shown that Epo signaling elements are prominently expressed in head and neck cancers. Other studies have recently identified biologically active Epo signaling in human breast and uterine cancers and have correlated the expression of Epo and EpoR with poor prognosis^{3, 8, 9}. The correlation we report here between Epo and EpoR expression and malignant progression in head and neck cancer is consistent with these previous observations. The mechanism underlying *Epo* and *EpoR* gene expression in cancer cells are not entirely clear. Expression of both genes can be stimulated by hypoxia^{14, 19} and the HNSCC cell

lines we examined displayed hypoxia-inducible upregulation of EpoR expression rather than Epo. The higher normoxic expression of EpoR in the 22B cell line correlates with the higher basal HIF-1 α expression and invasiveness in these cells¹⁵. HIF-1 α is the key regulatory subunit of HIF-1, a transcription factor that controls gene expression of Epo and other hypoxia responsive genes. High basal and hypoxia-inducible HIF-1 expression is observed in solid tumors²⁰ and has been linked to increased angiogenesis²¹, enhanced invasiveness^{15, 20, 22}, and poor clinical outcome²³. It is possible that some of the adverse effects correlated with HIF-1 expression in cancer are mediated via Epo signaling. Although hypoxia did not induce Epo mRNA expression in either cell line, RT-PCR experiments and immunocytochemical detection of Epo protein support an active constitutive production of the cytokine. Epo is known to exhibit tissue restricted expression with hypoxia and several known growth factors modulate its expression levels. The mechanisms regulating the normoxic expression of Epo in these cells remains an important question to be answered. Methylation of the CpG sites in the Epo promoter interfere with HIF-1 binding which ultimately restricts Epo gene expression under hypoxia. However, it is well known that organs such as the liver during development express a steady level of Epo expression until this signal is turned off and hypoxic renal production of Epo takes over. The regulators that control hepatic expression of Epo may be the same regulators that are controlling the normoxic expression in cancer cells. Candidate regulators include retinoic acid. Retinoic acid is known to bind to the Epo enhancer and drive the expression of Epo in embryonic carcinoma cells that

do not express O₂-regulated Epo expression³¹. Furthermore decreased retinoic acid binding to the Epo enhancer in the liver has been suggested be an important switch from retinoic acid control to hypoxic control of Epo expression. Another transcription factor that appears to be linked to the constitutive expression of Epo in the liver is GATA4. Most GATA transcription factors have been found to repress Epo expression under hypoxia when over-expressed, however, GATA4 is able to bind to the Epo promoter and increase Epo expression³².

Although the ability of Epo to promote angiogenesis and improve cell survival has been suggested to play a role in human cancer, our report is the first to demonstrate an effect of Epo on cancer cell invasiveness. Exogenous Epo activated JAK2 phosphorylation and stimulated cell invasion of both HNSCC cell lines while a JAK2 inhibitor blocked this effect. JAK2 can activate several intracellular signaling cascades including the phosphorylation of the STAT family of transcription factors¹⁶. STATs have been implicated in tumorigenesis²⁴ previously but have yet to be examined for a role in invasiveness. Activation of the JAK-STAT signaling pathway by Epo is well appreciated in erythroid precursors and in endothelial cells. Epo is known to induce an invasive, pro-angiogenic phenotype in endothelial cells as well as neovascularization *in vivo*²⁵. These processes correlate with Epo induced JAK2 phosphorylation and matrix metalloprotease -2 production in endothelial cells^{25, 26}. In addition, rhEpo can promote migration of enterocytes²⁷ in addition to stimulating the migration of burst-forming unit erythroids (BFU-E) from the bone marrow to the spleen²⁸. The ability of Epo signaling to increase the migratory or invasive behavior of cells may

thus be a widespread but underappreciated activity important for normal development and physiology^{29, 30}. Constitutive or hypoxia-inducible expression of this activity in cancer cells may contribute to malignant progression.

Biological actions of Epo signaling in cancer cells are just beginning to be appreciated. The recent termination of two clinical trials due to adverse outcome in rhEpo treated patients has heightened the importance of understanding Epo actions on cancer cells. Our data suggests that Epo signaling enhances cancer invasion and that indiscriminate treatment of cancer patients with rhEpo should be re-examined.

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Figure 1. EpoR and Epo immunohistochemistry in head and neck squamous cell carcinoma. **(a)** Top row: A prominent increase in EpoR staining (brown color) is seen in biopsies with dysplastic (left panel) and invasive carcinoma cells (middle panel) as well as in tumoral vasculature (right panel). Middle row: EpoR immunoreactivity in normal epithelium (left panel), dysplastic epithelium (middle panel) and invasive carcinoma (right panel). Bottom row: Epo immunoreactivity in normal epithelium (left panel), peri-necrotic tumor region (middle panel), and invasive carcinoma (right panel). **(b)** EpoR and Epo expression in lymph node metastasis. EpoR staining is seen in metastatic cancer cells (M) but not in normal lymphocytes (L). Epo staining is most prominent in the malignant cells bordering necrotic regions (N). **(c)** Correlation of EpoR and Epo immunoreactivity with malignant progression. P-values of EpoR staining were calculated for benign and dysplasia (** $p < 0.01$), benign and carcinoma (** $p < 0.001$), and dysplasia and carcinoma ($p > 0.05$). P-values of Epo staining were calculated for benign and dysplasia (** $p < 0.001$), benign and carcinoma (** $p < 0.001$), and dysplasia and carcinoma (* $p < 0.05$). Bars indicate median immunostaining score values. N.S.= not significant.

Figure 2 Differential invasiveness of HNSCC cell lines correlates with higher HIF, and EpoR expression. **(a)** Differential expression of HIF-1 α and EpoR expression in O22 and 22B HNSCC cells. For hypoxia treatment, cells were exposed to 1% O₂ for 24 hours. **(b)** PCR amplification of Epo from O22 and 22B cell cDNA with HPRT as control gene. **(c)** Quantitative real-time PCR analysis of Epo and

GLUT3 mRNA levels in O22 cells after 24 hours treatment with hypoxia. The amount of each mRNA in samples was normalized to the average of HPRT1 mRNA and GUS mRNA in the same sample. **(d)** Quantitative real-time PCR analysis of Epo and GLUT3 mRNA levels in 22B cells after 24 hours treatment with hypoxia. The amount of each mRNA in samples was normalized to the average of HPRT1 mRNA and GUS mRNA in the same sample. **(e)** Epo immunocytochemistry demonstrated protein expression in normoxic O22 cells and 22B cells, Epo antibody concentration 1:200, no primary control exhibited no staining (data not shown). **(f)** 22B cells display higher invasive potential as assayed with Matrigel coated Boyden chambers for a 48 hour period under serum free conditions, * $p < 0.05$. **(g)** Cells were allowed to invade in Boyden chambers with or without Matrigel under serum conditions to assess total motility, and under serum free conditions with Matrigel for 48 hours to determine differential invasive capacity of the two cell lines.

Figure 3 Epo signaling mediates invasion in HNSCC cell lines. **(a)** Exogenous rhEpo (10U/ml) treatment enhances phosphorylation of JAK2 and this activation is blocked with AG490 (20 μ M), * $p < 0.05$. **(b)** Epo promotes cell invasion through Matrigel coated Boyden chambers under serum free conditions (48hrs), * $p < 0.05$. **(c)** Epo (10 U/ml) induced invasion in O22 cells is blocked with AG490 (20 μ M) treatment, * $p < 0.05$. **(d)** Basal invasion of 22B cells is reduced with AG490 (20 μ M) treatment only under serum free conditions,* $p < 0.05$.

Figure 1.

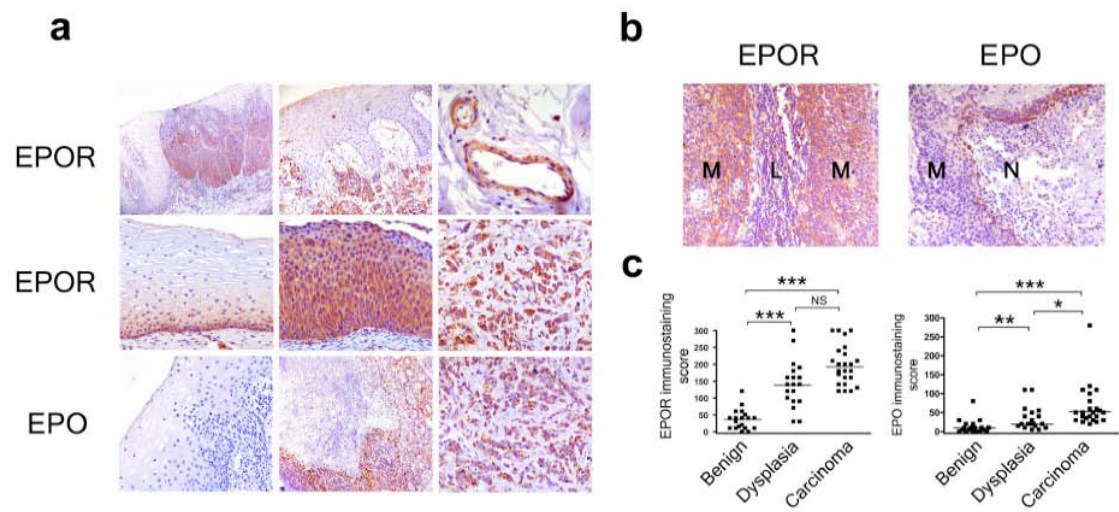


Figure 2.

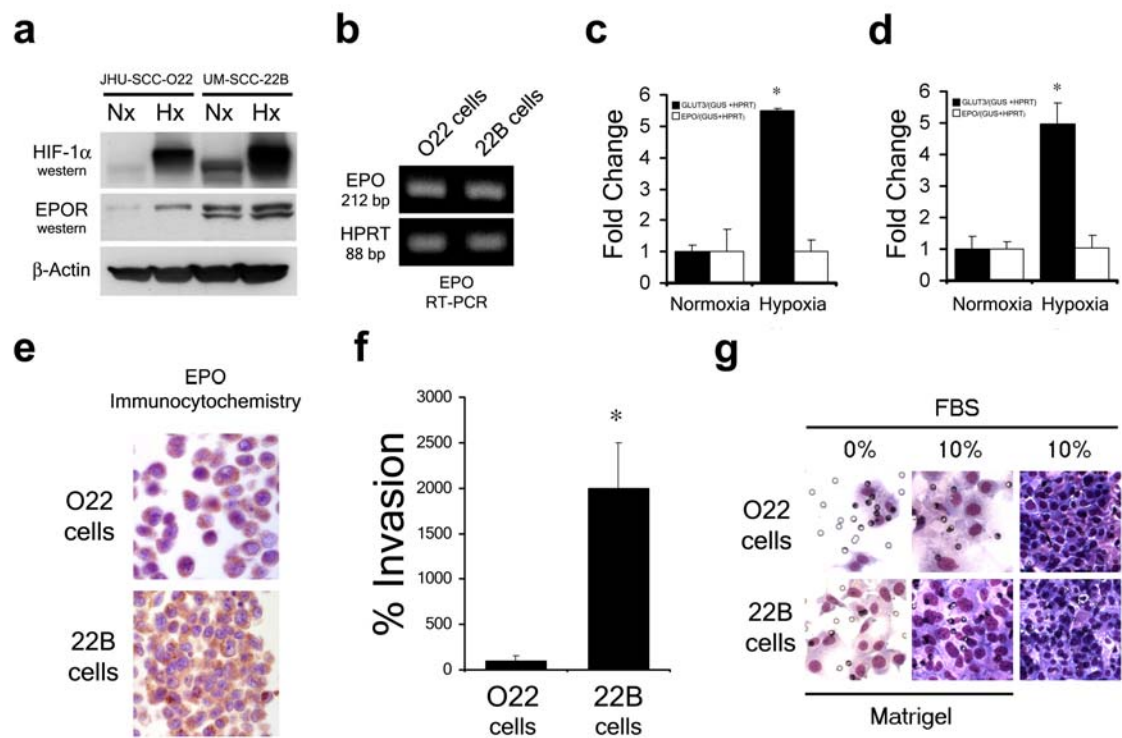
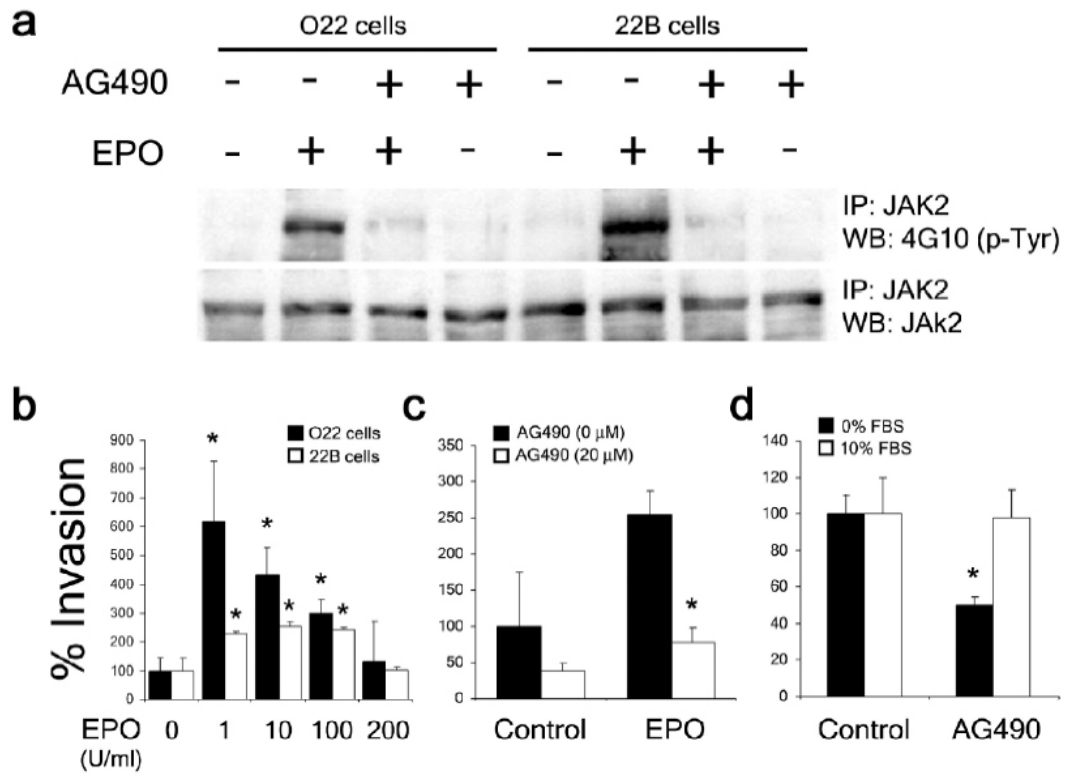


Figure 3.



DISCUSSION

Erythropoietin is a primary regulator of red blood cell production and thus an essential component of hypoxic adaptation in vascular organisms⁸. rhEpo has become indispensable in the treatment of chronic anemia. In the past decade Epo has emerged as a pleiotrophic cytokine with broad anatomical expression and biological functions that extends beyond hematopoiesis³⁵. In various settings Epo promotes angiogenesis, cytoprotection and cell migration. Interestingly, neoplasms which originate from tissues that utilize Epo signaling express high levels of functional erythropoietin receptor⁵². Interfering with autocrine Epo signaling in tumor xenografts disturbs tumor angiogenesis and cancer growth while exogenous Epo administration promotes proliferation in several cancer cell lines^{55, 62, 75, 76}. Tumor hypoxia is a pathophysiological consequence of disturbed microcirculation. Although it has been strongly associated with tumor propagation, resistance to therapy, tissue invasion and poor outcome, the mechanisms linking these relationships remain poorly understood and may be cell type specific. Since Epo is an oxygen sensitive gene with cancer promoting potential we proposed that hypoxic cancer cells exploit Epo signaling as a necessary mechanism for mediating the aggressive phenotype often coupled with hypoxic cancers. The major ambition of this proposal was to better define the biological activities that Epo can promote to enhance tumor growth. We chose to examine our hypothesis in the context of a common and aggressive form of brain cancer called Glioblastoma. Gliomas, are often derived from low

grade astrocytomas and astrocytes have been known to produce Epo and to respond to Epo trophic support.

Our access to glioma biopsies and glioma cell lines and our tools to study various parameters of Epo biology made our lab well equipped to investigate the role of erythropoietin signaling in glioma cells. Immunohistochemical analysis of GBMs for Epo and EpoR immunoreactivity revealed markedly enhanced expression levels compared to normal adult brain elements. EpoR expression levels in Grade IV glioma specimens when compared to Grade II gliomas were significantly higher, an observation that is consistent with previous findings in other cancers. In light of recent findings that increased Epo expression was significantly associated with adverse clinical outcome in human endometrial carcinomas, this strong correlation between EpoR expression and tumor grade suggests that it may be an adverse prognostic factor in gliomas and other cancers. The high expression of Epo and EpoR in hypoxic zones of glioma specimens suggested that their expression levels could be hypoxia regulated. To test this hypothesis we examined several glioma cell lines in context of other well established hepatic cancer cell lines that are known to express Epo in an oxygen sensitive manner. We found that exposure to hypoxia not only increased Epo expression but also increased EpoR transcript and protein levels as well. Although the EpoR gene is not known to have a HIF binding site molecularly identified by a Hypoxic Response Element (HRE) binding sequence, there have been reports that hypoxia may regulate EpoR indirectly via increasing Epo levels. Tumor hypoxia is primarily known for its contributions to current failures in

therapeutic modalities against cancer^{111, 112}. Our work shows that both hypoxia and Epo can protect glioma cells from chemotherapeutic toxicity. In addition, we provide data that demonstrated a synergistic relationship between the two. This observation could have profound implications on the treatment and management of anemic cancer patients who receive rhEpo. This practice may synergize with tumor hypoxia to produce deleterious effects by promoting cancer cell survival and resistance to therapy. Epo's ability to enhance endothelial cell migration prompted us to examine its effects on glioma cells. Both hypoxia and Epo enhanced invasion and increased levels of active proteases that degrade extracellular matrix. Blockade of Epo signaling under hypoxia enhanced invasion attenuated glioma cell invasion by 50% suggesting that hypoxic glioma cells exploit an active autocrine/paracrine Epo signaling loop to promote invasion. Exogenous Epo was found to induce a pro-invasive phenotype on various cancer cell lines originating from other organs besides the brain^{20, 26}. Although the mechanism by which Epo enhances invasion is not entirely clear, it appears that it is dependent on a class of proteases called matrix metalloproteinases that degrade extracellular matrix. All in all, these observations may explain the underlying mechanism behind the association between hypoxia and cancer invasion and metastases.

Hypoxia is a strong and adverse prognostic factor in tumors of the head and neck^{96, 97}. Moreover, a recent clinical trial aimed to test the beneficial effects of Epo treatment in head and neck cancer patients was terminated prematurely due to poorer locoregional progression-free survival in the Epo treated group⁷⁸.

To test whether Epo was a contributing factor in such clinical observations our second body of work focused on screening several head and neck cancer specimens for Epo and EpoR expression and examined if the biological invasion enhancing effects of Epo carried over in cell culture models. In line with our previous research, Epo and EpoR were highly expressed in head and neck specimens compared to normal tissue and both Epo and EpoR expression correlated with tumor grade. Exogenous Epo treatment enhanced invasion of head and neck cancer cell lines suggesting that the current therapeutic practice of administering Epo to cancer patients may account for the deleterious consequences observed in the recently terminated clinical trial. While hypoxia appeared to increase EpoR protein levels in head and neck cell lines, Epo expression was not hypoxia inducible. Previous reports have discovered that methylation of the CpG sites in the Epo promoter interfere with HIF-1 binding which ultimately restricts Epo gene expression under hypoxia¹². Despite a lack of induction with hypoxia, head and neck cancer lines expressed an active constitutive production of the cytokine as evidenced by immunocytochemistry. When autocrine Epo signaling is interrupted via the drug AG490, a JAK2 inhibitor, basal invasion is reduced by 50% suggesting that like glioma cells head and neck cells exploit endogenous Epo signaling to promote invasion. Since low tumor oxygenation is intimately associated with invasion of head and neck cancers, this observed mode of upregulation of EpoR under hypoxia may sensitize some head and neck cancer cells to peripherally derived Epo and thus contribute to invasion under hypoxic conditions. Even though the mechanisms

regulating the normoxic expression of Epo in these cells remain unknown, several transcription factors that are known to regulate the normoxic expression of Epo in the liver during development serve as interesting candidates for investigation (for more details see page 78 Paper #2). Moreover, the clinical cases we investigate represent polyclonal cancer cell populations that may have different Epo expression mechanisms than the monoclonal cell lines we investigated.

In order to directly implicate a role for the Epo signaling in hypoxia-inducible Matrigel invasiveness, we generated U251 cells overexpressing either a full length EpoR or a truncated EpoR, which lacked the cytoplasmic signal transducing portion. Successful transfection of these constructs was verified by western blotting with antibodies generated against the cytoplasmic portion of the EpoR or via cell sorting of cells stained with a fluorescence tagged antibody generated against the extracellular portion of the EpoR. Based on the flow cytometry data, approximately 30% of all cells were successfully transfected with the full length EpoR and about 50% were successfully transfected with the truncated EpoR. Although no clear differences were seen between the two transfected cell types in cell viability or basal rate of invasiveness (data not shown), a clear reduction of hypoxia-inducible invasiveness was observed in cells transfected with the truncated EpoR. Taken together our data support a collaborative role for hypoxia and Epo signaling in the survival and invasiveness of human gliomas and other cancers.

Collectively this body of work introduces two more human cancers, gliomas and head and neck carcinomas that appear to utilize Epo signaling for their malignant progression. In addition, this is the first report that documents Epo's invasion promoting effects on cancer cells. We propose that hypoxia inducible Epo signaling is major mechanism by which glioma cells enhance their invasion under hypoxic conditions and protect themselves from chemotherapeutic toxicity. With these observations and the recent termination of two clinical trials that evaluated Epo's efficacy in cancer patients, the management of anemic cancer patients who receive Epo as an adjuvant therapy should be reassessed. Hypoxia inducible autocrine/paracrine Epo signaling may be an additional mechanism behind the association between how hypoxia promotes cancer malignancy and in some cancers Epo signaling may be a major mechanism. Interruption of Epo signaling may serve as a potential therapeutic strategy in the treatment of cancer. In support of this hypothesis, data from *in vivo* and *in vitro* experiments indicate that blocked of Epo signaling destroys tumor xenografts and reduces invasion of cancer cells. In addition this work may provide insight into the mechanisms that link smoking to cancer. Heavy smokers are known to exhibit signs of hypoxia¹¹³ and erythrocytosis¹¹⁴ due to high levels of carbon monoxide exposure from tobacco smoke. Experimental data suggests that elevated carboxyhemoglobin levels increase tumor hypoxia¹¹⁵, elevate plasma erythropoietin levels¹¹⁶ and increase radiation resistance to therapy¹¹⁵. Furthermore, patients who continue to smoke during cancer therapy have poorer local tumor control and survival than abstainers of the same clinical stage¹¹⁷.

Elevated Epo levels in heavy smokers could promote tumor progression or increase malignancy.

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