How cancer cells hold their breath:
Does the treatment of cancer cells with EGF or VEGF reduce apoptosis under hypoxic stress?

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Abstract

Solid tumors are frequently characterized by regions of low oxygenation (hypoxia). Despite the large physiological stress it places on cells, hypoxia is often indicative of a poor prognosis and resistance to traditional anti-cancer therapies. Overactive epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) signaling has been shown to promote cell survival; thus, it was hypothesized that this may also account for resistance to hypoxia-induced apoptosis. This study examined the effects of treating hypoxic U251 cancer cells and HEK 293 transformed cells with EGF or VEGF. Dosage response trials determined that 0.1μg/ml of EGF and VEGF provided the most protection to hypoxic cells over 72 hours. Subsequently, both cell lines were treated with 0.1μg/ml EGF or VEGF and sampled at 24 hour intervals over a 72 hour time course in hypoxia. Hypoxic cancer cells underwent apoptosis less frequently when treated with EGF or VEGF in comparison with untreated cells. Additionally, the protective effects of EGF and VEGF increased with time in hypoxia. Characterizing the roles of growth factor signaling in the survival of hypoxic cancer cells is important, as it may allow for the design of specific anticancer therapies capable of sensitizing hypoxic cancer cells to apoptosis.

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List of Abbreviations

Akt… serine/threonine kinase activated by PI3K

Bcl-2… an anti-apoptotic protein, for which an entire family of pro-apoptotic and anti-apoptotic was named

BCS… bovine calf serum

BH… Bcl-2 homology

BNIP3… hypoxia-responsive pro-apoptotic Bcl-2 family member

c-Cbl… a ubiquitin ligase responsible for directing EGF receptor degradation

DMEM… Dulbecco's Modified Eagle Medium

EGF … epidermal growth factor

EGFR… epidermal growth factor receptor

ErbB 1, 2, 3, 4 … epidermal growth factor receptors

FBS … fetal bovine serum

HEK293 cells… human embryonic kidney cells given cancerous phenotype via viral transfection

HIF1-α … hypoxia inducible factor 1 transcription factor

MAPK… mitogen-activated protein kinase

Mcl-1… anti-apoptotic Bcl-2 family member

PI3K… phosphatidylinositol-3 kinase

TRAIL…tumor necrosis factor-related apoptosis inducing ligand

U251 cells… glioblastoma multiforme brain cancer cells

VEGF… vascular endothelial growth factor

Introduction

Apoptosis:

In healthy tissues, homeostasis is maintained via a delicate balance that exists between cell survival and cell death. This ensures that cells survive when their environment is accommodating, and undergo programmed cell death (apoptosis) if it is no longer supportive (Kabore et *al*., 2004). Apoptosis is an orderly cell death characterized by loss of adhesion to neighboring cells, mitochondrial depolarization, fragmentation of chromosomal DNA, and blebbing of the plasma membrane (Kabore et *al*., 2004). A family of cysteine proteases, known as caspases, carries out apoptosis. Caspases are synthesized as inactive precursors (pro-caspases), and are subsequently converted to active enzymes through proteolytic cleavage of characteristic aspartate residues. Caspases function as either initiators or effectors of proteolytic intracellular cascades, which result in the aforementioned morphological apoptotic alterations (Nomura et *al*., 1999). Research has indicated that two distinct, but parallel, apoptotic pathways result in the activation of effector caspases. These pathways are known as the extrinsic pathway, which is activated by the tumor necrosis factor death receptor family, and the intrinsic (mitochondria-dependent) apoptotic pathway, which is stimulated by endogenous molecules (Schuler et *al*., 2000). Both of the apoptotic pathways rely on several common initiator caspases (caspase-8 and caspase-9), which consequently activate the downstream effector caspases (caspase-3, caspase-6, and caspase-9). Thus, the regulation of caspase activation is important in governing apoptosis**.**

A cell's decision whether to survive and proliferate, or commit suicide, is largely dependent upon signals received from the extracellular environment. These signals, which range from changes in cell shape to ligation of surface receptors, or the presence of toxins,

either activate or suppress intracellular signaling molecules and alter cellular function in favor of survival or apoptosis. Diseases arise when the response of cells to their environment is diminished. In particular, cancerous cells have modifications in the regulatory mechanisms that control apoptosis (Kabore et *al.,* 2004). As a result, cancer cells may thrive in conditions wherein they would normally undergo apoptosis, such as hypoxia.

Hypoxia:

Rapid tumor growth requires an increasingly large supply of oxygen and nutrients. Although unregulated proliferation may be supported for a short time, malignant tumors quickly outgrow the carrying capacity of the surrounding vasculature (Wouters et *al*., 2003). This establishes a tumor microenvironment characterized by pockets of low oxygenation (hypoxia), acidity and low nutrient levels within the interior of the tumor (Figure 1; Wouters et *al*., 2003). Hypoxia is generally defined as regions with <2% oxygen, whereas normoxia is characterized by oxygen concentrations of approximately 21%. There are a variety of mechanisms whereby hypoxia may be established within a solid tumor, including an increased diffusion distance from normal vessels to cancer cells, and anemia, which is often a result of chemotherapy. Furthermore, hypoxia induces a tumor-associated vasculature, although it is incomplete, excessively permeable, and unable to penetrate in to the interior regions of the tumor. Thus, it is only able to supply the periphery of the tumor with oxygen, while the innermost region of the tumor becomes hypoxic (Vaupel et *al.*, 2001).

The largely diminished oxygen concentration associated with hypoxia places an enormous physiological stress on cells. In low oxygen conditions, the growth of both normal and cancerous cells is quickly inhibited, with necrotic cell death ensuing shortly thereafter. Necrosis is differentiated from apoptosis, as it refers to a swelling and lysing of cells, which is

not genetically programmed (Vaupel et *al.*, 2001). Hypoxic tumors are characterized by a reduced apoptotic response in comparison with normal cells. This is best explained by the fact that cancer is characterized by significant genomic instability that results from an increased mutation rate (Hanahan and Weinberg, 2000). Genome instability, which ranges from point mutations to gross chromosomal aberrations, allows for the clonal selection of cells with modifications permitting a tolerance of hypoxia (Semenza, 2000). Consequently, this results in a population of cancer cells that are largely resistant to hypoxia-induced apoptosis. Alterations that promote cancer cell survival in hypoxia usually relate to the expression of the hypoxia-dependent transcription factor hypoxia-inducible factor 1 α (HIF1-α), which regulates a collection of hypoxia-induced genes. The degradation of HIF1-α is oxygen dependent, thus it is degraded where oxygen is plentiful, but not in low oxygen conditions (Semenza, 2000). Among the HIF1-α regulated genetic modifications that permit cancer cells to survive hypoxic stress are an increased production of vascular endothelial growth factor (VEGF), which induces angiogenesis and apoptosis resistance, and decreased energy consumption via the activation of glycolysis (Semenza, 2003).

Hypoxic tumors are difficult to treat, as they often develop resistance to traditional therapies, such as radiation and chemotherapy, and they retain their resistance even after oxygenation is restored (Kinoshita et *al*., 2001). It was noticed early on that low oxygen reduces the efficiency of radiation therapy (Gray et *al*., 1953). Radiotherapy functions by producing reactive oxygen species, which subsequently damage and kill cells in the irradiated area. The low levels of oxygen associated with hypoxic tumors modify the reactions that irradiation causes in DNA, thereby decreasing the lethal lesions produced in comparison with well-oxygenated cells (Wouters et *al*., 2003). Thus, in order to achieve the same effect as in

oxygenated cells, much larger dosages of radiation, which may be harmful to healthy cells, are required for hypoxic tissues (Wouters et *al.*, 2003). Similarly, the efficacy of chemotherapeutic drugs relies on their delivery to the affected tissues. As previously mentioned, the blood vessels that supply solid tumors are often immature and excessively permeable; this results in poor transport of drugs to the tumor. Consequently, the drug dosages that actually reach the hypoxic cancer cells are not large enough to kill all of the cells, although they are sufficient for the development of treatment resistance (Vaupel et *al.*, 2001). Furthermore, many chemotherapeutic drugs are aimed at rapidly dividing cells (Shannon et *al*., 2003). Given that hypoxic cells show a reduced rate of proliferation, these treatments prove somewhat futile (Shannon et *al*., 2003).

Thus, somewhat paradoxically, hypoxia produces cell death in the tumor, yet it increases the net survival of cancer cells, as evidenced by treatment resistance. Given that the tumor-associated vasculature cannot form instantaneously, solid tumors undergo hypoxic stress before they can form microvessels (Pidgeon et *al*., 2001). Consequently, tumor cells must have a means of surviving in conditions wherein normal cells would undergo apoptosis. This suggests that adaptive responses to hypoxia and insufficient nutrients are crucial steps in the progression of a tumor (Pidgeon et *al*., 2001). If hypoxia were not beneficial to tumor growth, angiogenic signals should adequately vascularize entire tumors once they reach a certain age. Still, evidence indicates that hypoxia is a widespread attribute of solid tumors throughout their development. Clinical trials have demonstrated that tumors characterized by heterogeneity in oxygen levels are indicators of poor prognosis, as hypoxia induces malignant progression, metastasis, and angiogenesis, while reducing apoptotic potential (Nordsmark et

al., 2001). These characteristics are thought to be due in large part to excessive survival signaling mediated by growth factors, such as epidermal growth factor (EGF) and VEGF.

Growth Factor Signaling:

It has been proposed that the loss of regulation associated with tumor formation is largely the result of uncontrolled activation of survival signaling and decreased apoptotic signaling. Alterations in the expression and function of various growth factors and their corresponding receptors may be of great importance in aberrant signaling responses. Growth factors are signaling molecules that transmit cell proliferation, differentiation, migration and survival signals between cells by autocrine and/or paracrine mechanisms (Kabore et *al*., 2004).

Epidermal Growth Factor

Among the best-studied growth factors is epidermal growth factor (EGF), a 6-kilodalton peptide growth factor distinguished by the presence of six conserved cysteine motifs, which form three intra-chain disulfide bonds (George-Nascimento et. *al*, 1988). EGF is synthesized as a glycosylated transmembrane precursor, and is proteolytically cleaved from the cell surface as a mature growth factor. EGF is involved in several physiological processes, such as wound healing, upregulating VEGF transcription and stimulating the proliferation of epithelial cells, including glial and breast cells (Clarke et *al*., 2001; Maity et *al*., 2000). EGF interacts with a family of four related glycoprotein transmembrane tyrosine kinase receptors, including: ErbB1 (EGFR), ErbB2, for which no ligand has been identified, ErbB3, which has an impaired kinase function, and ErbB4 (Yarden and Sliwkowski, 2001). These receptors, which dock proteins at the plasma membrane, are characterized by an extracellular ligand binding domain, a transmembrane domain, and an intracellular tyrosine

kinase domain responsible for propagating signal transduction (Hackel et *al*., 1999). Ligand binding initiates the homo- or hetero-dimerization of ErbB receptors. Dimerization subsequently elicits the activation of internal tyrosine kinase activity, tyrosine autophosphorylation by ATP and the ensuing recruitment of intracellular substrates, resulting in mitogenic signaling. ErbB2 is the preferred signaling partner of the EGFR family as it enhances the signaling potency of its dimerization partner on multiple levels. For instance, association with ErbB2 yields decreased ligand-dissociation from the EGFR. Also, activated and internalized EGFR-ErbB2 heterodimers are preferentially recycled from the early endosome to the cell surface, rather than shuttled to the lysosome for degradation as would be the fate of activated EGFR-EGFR homodimers (Yarden and Sliwkowski, 2001). As a result, increased numbers of activated receptors are available on the surface of ErbB2 over-expressing cells, causing prolonged signaling.

Hyperactivity of the EGF signaling system, which is accomplished by over-expression of EGF, heterodimerization with the ErbB2 receptor, or mutations in the ErbB receptors, leads to increased survival signals received by cells (Levitzki, 2003). Studies indicate that EGF signaling is important in the growth and survival of several types of solid tumors, especially breast tumors and brain tumors of glial origin. Furthermore, constitutive expression or over-expression of EGFR is related to a poor prognosis for survival, disease progression and resistance to traditional therapies (Popov et *al*., 2004). For instance, treatment of epithelial cells with EGF has reduced apoptosis induced by the death receptors Fas and tumor necrosis factor-related apoptosis inducing ligand (TRAIL) (Gibson et *al*., 1999; Gibson et *al*., 2002). Moreover, research shows that EGF signaling protects cancer cells against hypoxia-induced apoptosis. In one case, ErbB2 over-expression was linked with overall increased tumor

viability and a significant increase in the population of viable hypoxic cells (Dragowska et *al*., 2004). EGF was also shown to effectively protect hypoxic MCF-7 breast cancer cells from apoptosis mediated by the pro-apoptotic protein BNIP3 (Kothari et *al*., 2003). Thus, hyperactivity of the EGF signaling pathway, either by over-production of ligand and receptors, or mutation in the receptors, results in an increase of survival signals received by the cell. Many of the proliferative and mitogenic functions of EGF are a result of intracellular signaling cascades transduced by its tyrosine kinase receptors (Figure 2). The phosphatidylinositol 3-kinase (PI3K) pathway, along with its downstream serine/threonine kinase Akt, and the mitogen-activated protein kinase (MAPK) pathway are two intracellular pathways activated by EGFR signaling (Yarden and Sliwkowski, 2001). Upon activation of tyrosine kinase receptors, these pathways are activated, leading to intracellular phosphorylation cascades. As a result, multiple downstream effector molecules, which favor cell survival via protein phosphorylation or modulation of gene transcription, are activated.

One of the main targets of regulation is the Bcl-2 family. Bcl-2 proteins constitute a related group of pro-apoptotic and anti-apoptotic proteins that form homo- or heterodimers through four conserved motifs known as Bcl-2 homology (BH) domains (Kabore et *al*., 2004). These proteins play a key role in mitochondria mediated apoptosis, as they release proteins such as cytochrome c from mitochondria, which subsequently leads to the activation of downstream pro-apoptotic proteins, including caspases (Nomura et *al*., 1999). EGF signaling through the PI3-AKT pathway has been implicated in shifting the balance between pro-apoptotic and anti-apoptotic Bcl-2 proteins in favor of survival. For instance, EGF upregulates expression of several anti-apoptotic Bcl-2 members, including Mcl-1, Bcl-2, and Bcl-xl (Henson et *al*., 2003; Janmaat et *al*., 2003; Jost et *al*., 2001). Along with activating

anti-apoptotic Bcl-2 proteins, AKT deactivates pro-apoptotic Bcl-2 proteins. Research has shown that AKT is responsible for the phosphorylation and consequent inactivation of the pro-apoptotic Bcl-2 protein BAD (Bacus et al., 2002). The MAP kinase pathway also reduces apoptosis upon stimulation by EGF, as it enhances the production of anti-apoptotic Bcl-2 transcription factors. Thus, regulation of Bcl-2 proteins via the PI3-AKT and MAPK pathways is an important mechanism by which EGF confers resistance to apoptosis upon cancer cells.

Vascular Endothelial Growth Factor

As previously stated, one of the downstream signaling targets of EGFR activation is VEGF upregulation (Clarke et al., 2001; Maity et al., 2000). VEGF, which was first described as vascular permeability factor, is a homodimeric glycoprotein expressed at elevated levels in a wide variety of human and animal cancers (Leung et al., 1989). The five isoforms of VEGF, which are produced by means of alternative splicing, can interact with homodimers or heterodimers of three tyrosine-kinase receptors named VEGF-1, VEGF-2, and VEGF-3 (Bergsland, 2004). These receptors have seven extracellular immunoglobulin homology domains and two intracellular tyrosine kinase domains ending with a C-terminal tail within the cytoplasm (Xie et al., 2004). Primarily, VEGF is responsible for the physiological and pathological growth and survival of blood vessels (angiogenesis). This plays a crucial role in tumor development and progression, as tumor-secreted VEGF activates endothelial cells and thereby enables the transport of blood and oxygen to the tumor (Plate et al., 1992). However, as previously mentioned, the tumor-associated vasculature is leaky and immature; this ensures that oxygen cannot reach certain regions of the tumor, which consequently sustains the hypoxic microenvironment (Mukhopadhyay et al., 1995).

Aside from a tumor's role in promoting angiogenesis by VEGF secretion, many tumor cells possess receptors for VEGF as well. Expression of VEGF receptors on tumors can provide resistance to apoptosis through autocrine signaling loops, which activate the PI3-Akt and MAPK pathways, and therefore favor cell survival (Pidgeon et al., 2001). Consequently, upregulation of VEGF expression in tumors generally predicts for treatment resistance, disease recurrence and reduced survival (Bergsland, 2004). One study showed that treating metastatic mammary carcinoma cells with VEGF reduced tumor cell apoptosis (Pidgeon et al., 2001). It was also found that VEGF enables leukaemic CMK86 cells to evade apoptosis induced by the chemotherapeutic drugs doxorubicin and etoposide by upregulating the anti-apoptotic Bcl-2 member MCl-1 (Katoh et al., 1998). Studies of cancer cells exposed to hypoxic stress have also demonstrated the importance of VEGF in protecting against apoptosis. Specifically, VEGF upregulation in hypoxia reduces the amount of hypoxia-induced apoptosis in cells deprived of nutrients (Baek et al., 2000). VEGF activates the MAP kinase and PI3-AKT pathways, and subsequently increases the proportion of the anti-apoptotic Bcl-2 protein relative to the pro-apoptotic protein BAX (Baek et al., 2000). This suggests that VEGF may be an important factor in the aggressive phenotype of tumors cells with heterogeneous oxygenation.

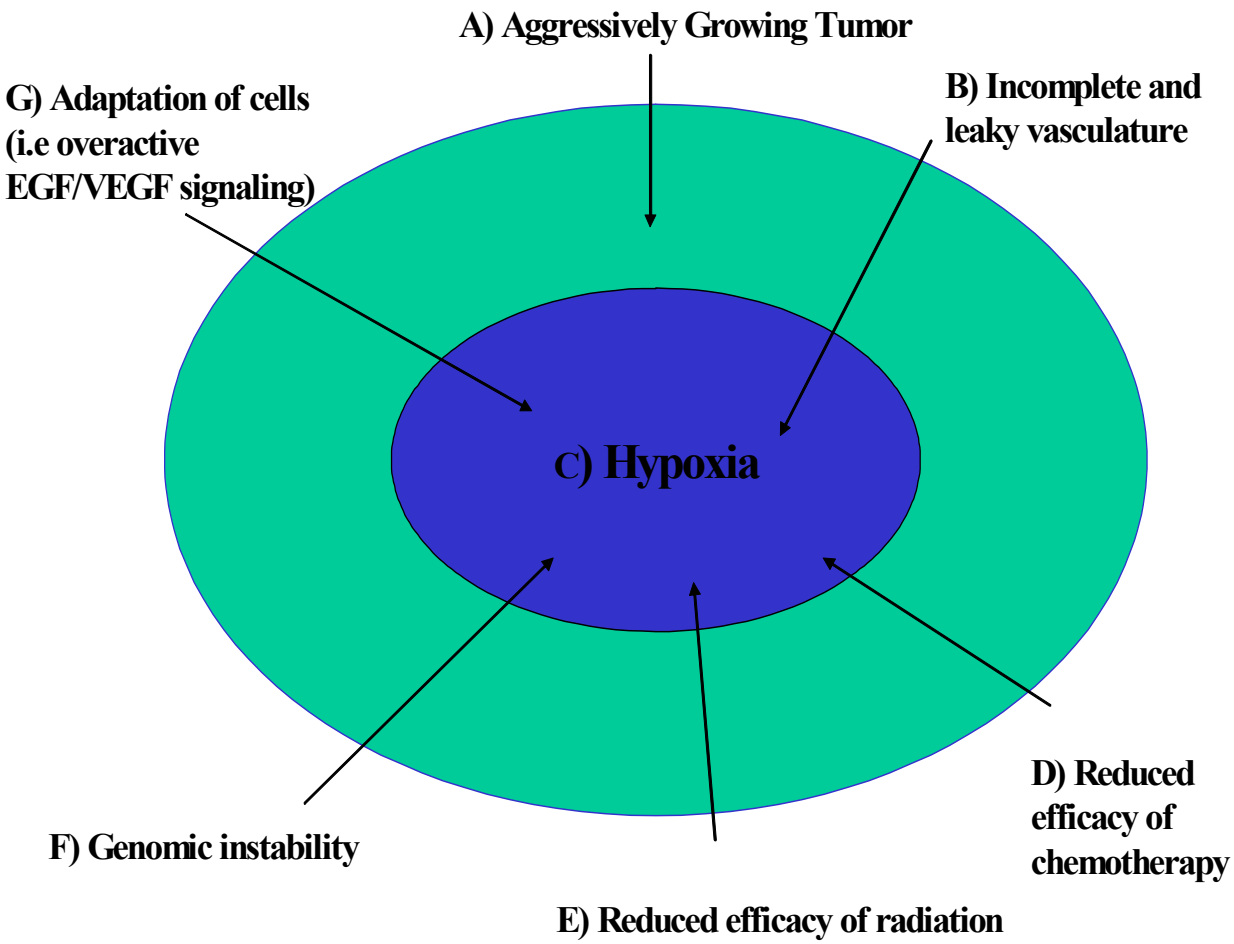


Figure 1: Biology of a hypoxic tumor. In a clockwise direction from the top: A) Rapidly growing tumors develop an B) incomplete and leaky vasculature, which C) results in hypoxia. Hypoxia reduces the efficacy of traditional anti-cancer therapies such as D) chemotherapy and E) radiation therapy, which results in a poor prognosis of survival. The ability of hypoxic cancer cells to evade apoptosis is thought to be due to F) genomic instability, which ensures the presence of cancer cells with G) adaptations that enable them to survive in low oxygen, such as overactive growth factor signaling.

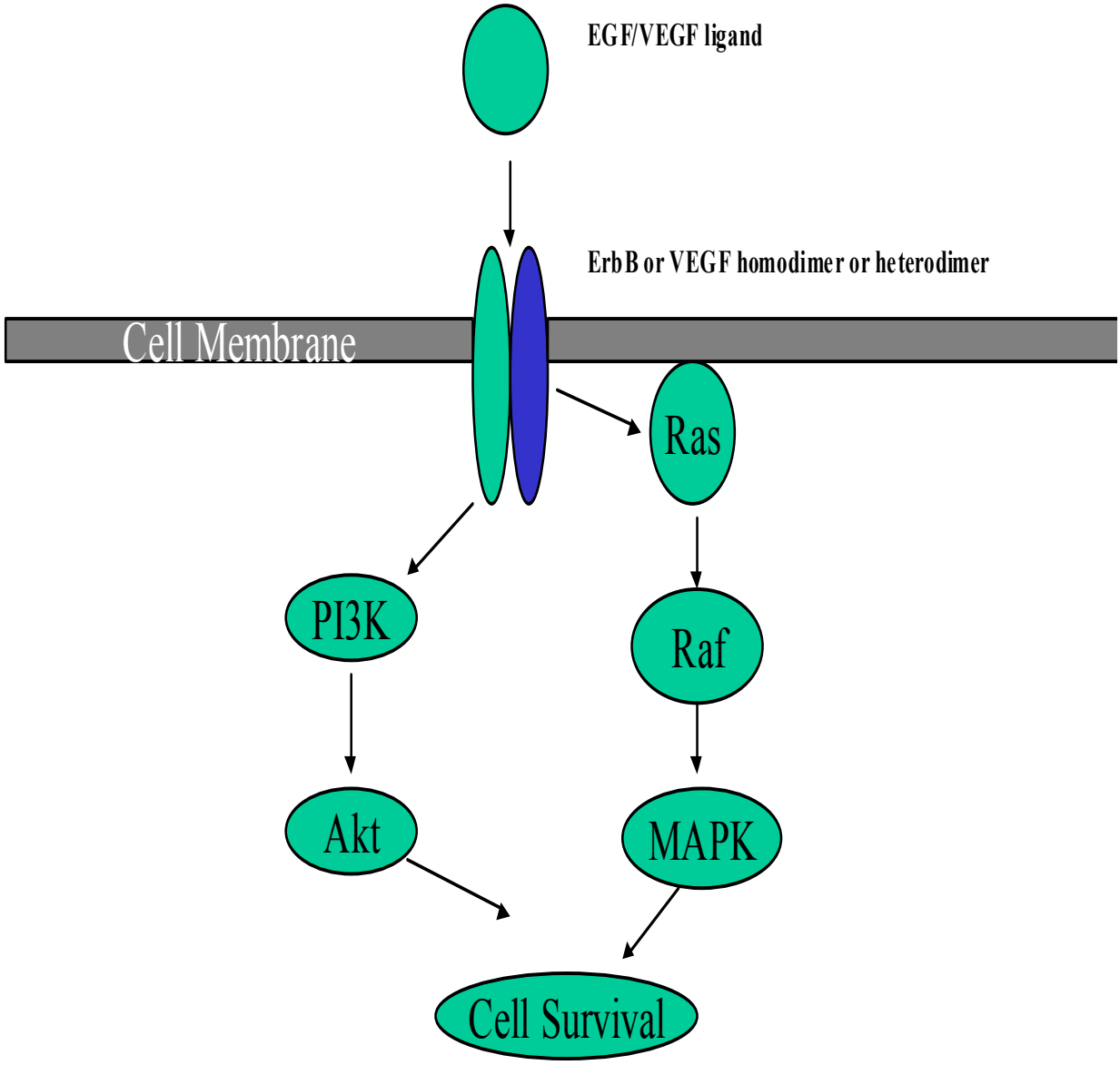


Figure 2: Simplified version of survival signaling pathways activated by EGF and VEGF. Transmembrane tyrosine kinase receptors of EGF and VEGF may be induced to dimerize by the binding of EGF or VEGF, or be constitutively active. Stimulation of the receptors causes phosphorylation of downstream proteins of the PI3-Akt and MAPK pathways, which ultimately results in increased survival (i.e. by affecting Bcl-2 proteins).

Treatment Targets:

Due to the fact that traditional cytotoxic treatments do not distinguish between tumor and host cells (Baselga, 2002), and are often ineffective against rapidly dividing tumors (Hanahan and Weinberg, 2000), the development of treatments specific for malignant cells has garnered much attention. Because the EGFR has been implicated in resistance to apoptosis, disease progression and a poor prognosis, targeting the EGFR, along with the other ErbB members, has become a sensible and attractive option. Additionally, anti-EGF treatments are not limited to cancers wherein the EGFR pathway is activated. This is because cross talk can occur with other intracellular pathways involving G protein coupled receptors (Leserer et *al*., 2000), or receptors for platelet-derived growth factor (He et *al*., 2001).

Among the more promising classes of potential drug treatments are tyrosine-kinase inhibitors and anti-receptor monoclonal antibodies. The intracellular catalytic portion of the ErbB receptors is required for signaling to take place. Thus, tyrosine-kinase inhibitor drugs have been designed to recognize characteristic residues and either transiently or permanently knock out the signaling of specific ErbB members by competing with ATP for receptor binding sites (Janmaat and Giaccone, 2003). The tyrosine kinase inhibitor Iressa, which is directed against the EGFR, has recently been approved for clinical trials. Iressa has been shown to elicit apoptosis and inhibit tumor proliferation and angiogenesis in animal and *in vitro* studies of brain cancer by activating the pro-apoptotic Bcl-2 protein BAD (Gilmore et *al*., 2002). Anti-angiogenic and anti-metastatic effects are likely due to the ability of Iressa to prevent the growth and cell-cell interactions of endothelial cells (Janmaat and Giaccone, 2003). When used in conjunction with chemotherapeutic drugs or radiation therapy, the pro-apoptotic effects of Iressa are synergistically enhanced (Magne et *al*., 2002; Williams et *al*.,

2002), thus leading to tumor regression.

Monoclonal antibodies (mAbs) have been developed which target several members of the EGFR superfamily, including EGFR and ErbB2. These antibodies have high specificity for their cognate receptors, and have synergistic apoptotic effects when used in conjunction with chemotherapy and radiotherapy (Hinoda et *al*., 2004). Cetuximab is one example of an EGFR-targeted antibody. Cetuximab is a chimeric human/mouse mAb, which functions by binding to the extracellular portion of the EGFR, thereby causing internalization of the receptor. Studies show that binding of Cetuximab inhibits tyrosine kinase activity, cell proliferation, and angiogenesis (Perrotte et *al*., 1999), especially when used with chemotherapeutic drugs (Inoue et *al*., 2000). Conversely, Herceptin is a monoclonal antibody specifically targeted against the ErbB2 receptor (Levitzki, 2003). Women suffering from metastatic breast cancer expressing high levels of ErbB2 have benefited from Herceptin treatment. This is reflected by statistically significant increases in overall response rate and survival time (Slamon et *al*., 2001). Herceptin causes the internalization and degradation of ErbB2 by recruiting the ubiquitin c-CBL ligase to ErbB2, and initiating lysosomal degradation (Prenzel et *al*., 2001). Research has shown that Herceptin may be an effective anti-cancer therapy under hypoxic conditions, as it sensitizes MCF-7 breast cancer cells to hypoxia-induced apoptosis (Kothari et *al*., 2003). Thus, several antibodies and tyrosine kinase inhibitors targeting EGF and the EGFR superfamily are currently in clinical trials, and their effectiveness *in vivo* appears promising.

Since VEGF signaling is of great importance in angiogenesis and apoptosis resistance in solid tumors, anti-VEGF therapies have become the subject of intensive research. Inhibition of either the VEGF ligand or its receptor may cause regression of tumor-associated

blood vessels, reduce anti-apoptotic effects, and normalize the vasculature, thereby allowing chemotherapeutic drugs to reach the tumor (Rosen, 2002). Like most drugs targeted to the EGF receptor, many anti-VEGF ligand or receptor therapies rely heavily on mAbs. For instance, bevacizumab is a humanized mAb targeted against the VEGF ligand (Ignoffo, 2004). Bevacizumab binds all five isoforms of VEGF with high affinity (Ignoffo, 2004). *In vivo* studies involving mice with human cancers have demonstrated that bevacizumab significantly reduces the size and number of xenografted liver tumors, especially when used in combination with traditional chemotherapeutic or radiation therapies (Borgstrom et *al*., 1999; Lee et *al*. 2000). Since VEGF receptors on tumor cells provide resistance to apoptosis, disrupting the function of the receptors can sensitize cancer cells to apoptosis induced by drugs or radiation (Rosen, 2002). A novel perspective taken on anti-VEGF treatment is that it also allows for the normalization of the tumor-associated vascular tissue (Jain, 2005). Less permeable microvessels allow for more effective delivery of chemotherapeutic drugs to the tumor, and a higher intra-tumor oxygen concentration augments the efficacy of radiation therapy (Jain, 2005). Thus, anti-VEGF therapies have multiple anti-tumor effects, including inhibition of angiogenesis, increased oxygenation, and the blockage of anti-apoptotic stimuli. Furthermore, since angiogenesis is not very prevalent in adult physiology (being involved in only blood clotting and the menstrual cycle), its inhibition is generally accompanied by relatively few side-effects (Jain, 2005). Anti-VEGF therapy also has several potential advantages over chemotherapy and radiation therapy *in vivo* relating to the development of resistance (Harmey and Bouchier-Hayes, 2002). Since cancer cells are genetically unstable, they can quickly become resistant to treatment, as they acquire mutations that allow them to circumvent apoptosis. However, the cells responsible for angiogenesis are predominantly

non-malignant endothelial cells. These cells do not exhibit the same genetic instability, thus they are not as likely to contain mutations that enable treatment resistance to develop (Harmey and Bouchier-Hayes, 2002).

Rationale for Experiment:

EGF and VEGF favor cancerous progression through increased survival signaling and decreased apoptotic signaling. The roles of EGF and VEGF in resistance to various apoptotic stimuli and malignant progression are well studied. As a result, both EGF and VEGF, along with their receptors, have become extremely attractive targets in anti-cancer therapies. However, their abilities to reduce hypoxia-induced cancer cell death are not well characterized. Thus, it would be valuable to determine to what extent EGF and VEGF protect cancer cells from hypoxia-induced death. This study examined the ability EGF and VEGF to protect U251 glioblastoma multiforme cancer cells, and HEK 293 transformed cells (embryonic kidney cells given a cancerous phenotype through viral transfection) from apoptosis under hypoxic stress. A comprehension of the specific effects of VEGF and EGF signaling on anti-apoptotic pathways may have potential ramifications in the design of future anti-cancer therapies.

Hypothesis:

It was hypothesized that treating hypoxic HEK 293 and U251 cells with EGF or VEGF would reduce the amount of hypoxia-induced apoptosis over 72 hours.

Materials and Methods

Cell Culture:

U251 cancer cells and HEK 293 transformed cells were obtained from existing samples. Cells were maintained in a humidified 5.0% CO_2, 37°C incubator in Dulbecco's Modified Eagle Medium (DMEM). The medium for HEK 293 cells was supplemented with 5% bovine calf serum (BCS) and 1% Penicillin-Streptomycin. Conversely, medium for U251 cells was supplemented with 5% fetal bovine serum (FBS), 1% Penicillin-Streptomycin, 1% 100mM sodium pyruvate and 1% 200mM L-glutamate. The constitution of these media had previously been determined to be conducive to the growth of their respective cell lines.

Hypoxic chamber:

A Forma Scientific Anaerobic Chamber was utilized for all hypoxic treatments. An O_2 concentration of less than 1% was established within the chamber via the perfusion of a high purity grade gas containing 5% CO_2, 10% H_2 and 85% N_2. Hypoxia was maintained within the chamber by regularly inundating the chamber with the gas, which was drawn through a three-step air filter system of charcoal, palladium, and a dessicant. All media used in the hypoxic chamber were stored inside the chamber without a lid for at least four days prior to use. This ensured that the oxygen would be removed from the medium, therefore it could not be utilized by the cells. In order to ensure the low oxygen content of the chamber, BBL Gas Pak anaerobic test strips (which turn blue in color at an O_2 concentration of 0.01%) were used.

Hypoxic/Normoxic Treatment:

Cells from each line were incubated in the normoxic and hypoxic chambers in the absence of growth factors for 0, 24, 48 and 72 hour intervals. Prior to incubation in the

hypoxic chamber, the medium was aspirated from each plate, thereby ensuring that the cells were not able to obtain oxygen from the medium. At time 0, media that had been found to produce the desired cell death responses for their respective cell lines, were added to the cells. The HEK 293 transformed cells were grown in a DMEM medium with 2% BCS and 1% Penicillin-Streptomycin. A DMEM medium containing 2% FBS, 1% Penicillin-Streptomycin, 1% 100mM sodium pyruvate and 1% 200mM l-glutamatine was utilized for U251 cells. As a control for the oxygenating effect of adding growth factors in subsequent experiments, a volume of water corresponding to the largest volume of growth factor used was added.

EGF Dosage Response:

Prior to treatment with growth factors, both cell lines were tested in order to determine what time frame would produce approximately 50% cell death under hypoxic stress (it is difficult to draw conclusions about growth factor treatment from more confluent cells, as they are more resistant to apoptosis). It was determined that a 72 hour period of incubation in hypoxia for each cell line yielded the desired apoptotic response. HEK 293 and U251 cell lines were therefore treated with recombinant human EGF (Sigma) for a 72 hour period in 6-well plates with 1ml of medium. The cells were incubated in hypoxia and normoxia at EGF concentrations of 0.01µg/ml, 0.1µg/ml, 1µg/ml and 10µg/ml. The 1µg/ml and 10µg/ml treatments were prepared by directly adding an appropriate amount of the 1µg/µl EGF stock solution to the cells. A 1µg/ml solution was also prepared, in which 1µl of the stock solution was added to 1 ml of sterile water. This was required as a large dilution was necessary for the 0.01µg/ml and 0.1µg/ml treatments. As with the non-treated hypoxic and normoxic time-trials, the tissue culture medium was aspirated and replaced with appropriate hypoxic and normoxic media for all growth factor experiments. The EGF concentration exhibiting the

greatest anti-apoptotic effect was used for the time response trials.

EGF Time Response:

An EGF concentration of 0.1μg/ml was found to yield the greatest protective effect for both U251 and HEK293 cells after 72 hours of hypoxic stress. Correspondingly, a 0.1μg/ml solution of EGF was applied over time intervals of 0, 24, 48, and 72 hours to each cell line. This set of experiments allowed the percentage of death observed in cells grown in the presence of EGF to be compared to the amount of death in cells lacking EGF at 0, 24, 48 and 72 hours in hypoxic and normoxic conditions.

VEGF Dosage Response:

HEK 293 and U251 cell lines were treated with recombinant human VEGF (Sigma) for a 72 hour period in 6-well plates with 1ml of medium. Cells were treated with VEGF concentrations of 0.001μg/ml, 0.01μg/ml and 0.1μg/ml, which were achieved by adding the appropriate amount of the 5 μg/ml VEGF stock solution directly to the cells. The VEGF concentration resulting in the largest reduction of apoptosis was then applied over a 72 hour time course. VEGF dosage responses were performed only once due to time limitations.

VEGF Time Response:

A VEGF concentration of 0.1μg/ml was found to have the largest protective effect during the dosage response trials. Therefore, the HEK293 and U251 cells were treated with 0.1μg/ml VEGF and sampled every 24 hours over a 72 hour time course. The stock solution concentration of VEGF was 5μg/ml, therefore 20μl were added to the 1ml of medium in each well in order to obtain the desired dilution. The proportion of apoptosis in hypoxic and normoxic cells after treatment with VEGF was then compared against the amount of apoptosis in non-treated hypoxic and normoxic cells.

Apoptosis Assay:

Following incubation in the hypoxic/normoxic chambers, the extent of apoptosis in U251 and HEK 293 cells was determined. Initially, HEK 293 cells were detached from the culture dish by rapidly washing fresh medium over the plate. Conversely, U251 cells form peptide bonds with the culture dishes, thus they were treated with a pre-warmed trypsin-EDTA solution for approximately two minutes in order to detach them from the plate. Cells were then transferred to labeled 15ml screw-cap tubes and centrifuged at 1200 rpm for five minutes at 24˚C. After centrifugation, all but 100µl of medium was aspirated from each tube. The tubes were put on ice, thereby preventing light exposure or additional growth. Subsequently, each tube received 2µl of a solution containing acridine orange (100 µg/ml) and ethidium bromide (100 µg/ml) in PBS orange staining reagent. The cell solution was gently mixed, and 10µl was added to each microscope slide. For each sample, the percentage of apoptotic cells was quantified under a UV microscope using a fluorescein filter set for the detection of condensed DNA in apoptotic cells. Nuclei of non-apoptotic cells uniformly stain green, whereas early apoptotic nuclei appear green with orange spots, and nuclei in later stages of apoptosis are bright orange.

Statistical Analysis:

The mean percentages of apoptotic cells were compared via a one-way analysis of variance (ANOVA). The null hypothesis, which stated that the treatment of hypoxic cancer cells with EGF or VEGF does not reduce apoptosis, was tested. Comparisons were made between growth factor-treated and untreated hypoxic cancer cells of the same cell line and period of incubation. Treated normoxic cells were compared with untreated normoxic cells as

controls for all growth factor experiments, as growth factors were not expected to reduce apoptosis in these relatively unstressed cells.

Results

EGF Dosage Response:

A dose response was required in order to determine the concentrations of growth factor that could protect cells against hypoxia induced apoptosis. A 72 hour period was chosen for both U251 and HEK 293 cells. This time interval of hypoxia produced an ideal amount of apoptosis in untreated cells for examining the effects of growth factor treatment. In comparison with the 50.0$\pm$ 2.2% of apoptotic cells counted in untreated U251 cells under hypoxic stress, cells treated with 0.01μg/ml EGF exhibited a slight reduction to 41.6$\pm$1.6% apoptosis ($p = 0.064$; Figure 3a). However, the apoptotic response dropped sharply at an EGF concentration 0.1μg/ml where there was 30.0$\pm$ 2.1% apoptosis ($p = 0.001$) and subsequently leveled off at 1μg/ml ($p = 0.001$) and 10μg/ml ($p = 0.001$; Figure 3a). Under normoxic conditions, the U251 cells did not have a significant response to EGF treatment (Figure 3a).

Hypoxic HEK 293 cells experienced a non-significant reduction in apoptosis upon treatment with 0.01μg/ml EGF ($p = 0.096$), as untreated HEK 293 cells had 60.3$\pm$4.1% rate of apoptosis, while treated cells died in 50.7$\pm$1.8% of cases (Figure 3b). Like the U251 cells, the levels of apoptosis decreased abruptly at a concentration of 0.1μg/ml to 41.8$\pm$2.3% ($p = 0.017$), and remained essentially constant at 1μg/ml ($p = 0.017$) and 10μg/ml ($p = 0.016$; Figure 3b). Normoxic HEK 293 cells treated with EGF exhibited less apoptosis than cells exposed to hypoxic stress, but did not undergo less apoptosis than untreated normoxic cells (Figure 3b).

EGF Time Response:

A time course experiment was performed in order to compare the percentages of apoptotic cells in treated and untreated conditions over a 72 hour time course. It was

determined from the dosage response trials that an EGF concentration of 0.1μg/ml was the best suited for the EGF time trial, as the larger concentrations had no further reduction of apoptosis. A time course of 72 hours, with samples taken every 24 hours, was used for all time trials. U251 and HEK 293 cells were incubated in hypoxic and normoxic conditions as previously described. The percentage of apoptosis at each 24 hour interval was determined via acridine orange staining.

The mean percentage of apoptotic U251 glioblastoma multiforme cells at time 0 was 6.3±1.4%, whereas 14.2±0.7% (Figure 4a) of untreated U251 glioblastoma multiforme cells underwent apoptosis at 24 hours incubation in hypoxia. Hypoxic U251 cells treated with 0.1μg/ml EGF for 24 hours exhibited 12.2±0.7% cell death, which does not represent a significant reduction in apoptosis ($p = 0.129$; Figure 4a). However, treating U251 cells for 48 hours reduced cell death from 27.1±1.4% to 21±1.7% ($p = 0.045$; Figure 4a). Similarly the 72 hour treatment of hypoxic U251 cells produced significant results, as it decreased cell death from 50.0±2.2% to 33.8±4.3% ($p = 0.005$; Figure 4a). Thus, treating U251 glioblastoma cancer cells with 0.1μg/ml EGF effectively reduced apoptosis, with the effect size increasing over time. Contrary to expectation, the treated and untreated normoxic U251 cells showed significant differences in cell death at 24 and 48 hours (Figure 4a). However, significant differences in normoxic apoptosis did not appear to be correlated with EGF treatment, as treated cells exhibited more apoptosis than untreated cells at 48 hours. It is unclear why these results were obtained. Interestingly, there was an increase in apoptosis over the 72 hours in normoxic cells. Apoptosis increased from 2.5±0.7% to 9.3±0.7% in treated cells, and from 5.7±0.5% to 9.1±0.9% in untreated U251 cells over 72 hours in normoxia (Figure 4a). This may be explained by the fact that as cells grow together, there is a depletion of nutrients and

space, which leads to elevated levels of cell death.

At time 0, 7.3±0.4% of HEK293 cells were apoptotic (Figure 4b). After 24 hours in hypoxia, 20.2±1.0% of untreated HEK 293 cells had undergone apoptosis, whereas 17±1.2% of HEK293 cells died when treated with EGF for the same duration ($p = 0.134$; Figure 4b). Untreated HEK 293 cells incubated for 48 hours decreased from 32.2±1.4% apoptosis, to 22.7±0.6% apoptosis when treated with EGF in hypoxia ($p = 0.003$; Figure 4b). Similarly, apoptosis in hypoxic HEK293 incubated for 72 hours decreased from 60.3±4.1% to 42.0±1.8% when treated with EGF ($p = 0.015$; Figure 4b). This indicates that treatment of HEK293 cells with 0.1μg/ml EGF diminishes hypoxia-induced apoptosis. Conversely, normoxic HEK 293 cells treated with EGF did not show a decrease in cell death, although apoptosis increased over the 72 hour duration (Figure 4b).

VEGF Dosage Trial:

HEK 293 and U251 cancer cells were treated with VEGF concentrations of 0.001μg/ml, 0.01μg/ml and 0.1μg/ml over a 72 hour period in hypoxia and normoxia. The mean percent apoptosis in untreated U251 cells over 72 hours was 50.0±2.2%, while treatment of U251 cells with 0.001μg/ml VEGF decreased the levels of apoptosis to 44.0% ($p = 0.411$), 0.01μg/ml VEGF reduced apoptosis to 32.0% ($p = 0.032$), and treatment with 0.1μg/ml VEGF yielded 26.0% apoptosis ($p = 0.008$; Figure 5a). Conversely, the amount of cell death in normoxic conditions did not depend on the amount of VEGF used to treat U251 cells (Figure 5a).

The proportion of apoptosis in untreated HEK 293 cells was determined to be approximately 60.3±4.1%. Hypoxic HEK293 cells treated with 0.001μg/ml EGF exhibited 46.5% apoptosis ($p = 0.627$), cells treated with 0.01μg/ml VEGF underwent apoptosis 41.0%

of the time ($p = 0.230$), and a VEGF concentration of 0.1 μg/ml reduced the mean percentage of apoptotic cells to 28.5% ($p = 0.015$; Figure 5b). Apoptosis in treated and untreated cells under normoxia was, however, not dependent on the concentration of VEGF (Figure 5b).

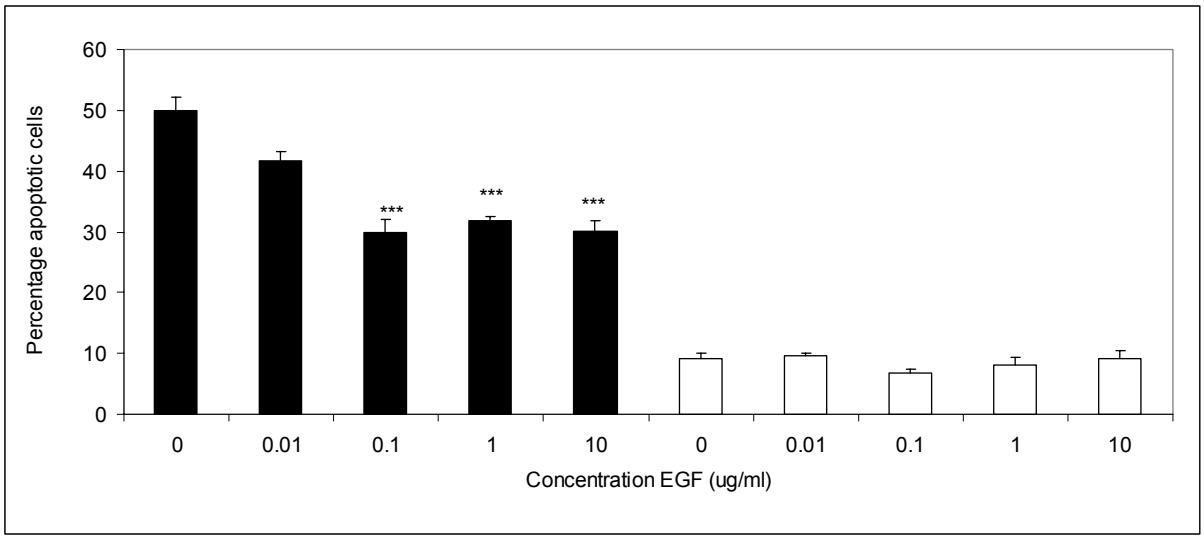
VEGF Time Response:

A VEGF concentration of 0.1μg/ml was found to reduce apoptosis more than the other concentrations tested in the dosage response trials. At time 0, 6.3±1.4% of U251 cells had undergone apoptosis (Figure 6a). Treatment of hypoxic U251 cells with VEGF did not reduce apoptosis significantly, as the percent of apoptotic cells decreased only slightly from 14.2±0.7% in untreated cells to 14±2.3% in cells treated with VEGF for 24 hours ($p = 0.817$; Figure 6a). Similarly, VEGF treatment of U251 cells incubated for 48 hours in hypoxia had no effect on apoptosis, as the mean percentage of apoptosis was 27.0±1.4%, essentially the same as 26.7±4.6% in untreated cells ($p = 0.913$; Figure 6a). However, there was a significant reduction in apoptosis at the 72 hour interval, as mean percentage of apoptosis decreased from 50.0±2.2% in untreated cells, to 37.2±2.2% in cells treated with VEGF ($p = 0.010$; Figure 6a). As a result, it appears that treatment of U251 cells with VEGF is capable of reducing hypoxia-induced apoptosis, however the effects are not significant until 72 hours of exposure to hypoxia. VEGF treatment did not reduce U251 cell death after 24 and 72 hours in normoxia, as VEGF-treated and untreated cells underwent very similar amounts of apoptosis (Figure 6a). Conversely, EGF-treated cells underwent a significantly higher proportion of apoptosis than untreated cells after 48 hours of incubation, although the reason for this is unclear (Figure 6a).

At time 0, 7.3±0.4% of HEK293 cells had undergone apoptosis (Figure 6b). When HEK 293 cells were treated with 0.1μg/ml VEGF, the 24 hour interval displayed a decrease in apoptosis from 20.2±1.0% in untreated cells to 15.6±0.44% in VEGF-treated cells ($p = 0.036$;

Figure 6b). Untreated HEK293 cells incubated in hypoxia for 48 hours exhibited 32.2±1.3% apoptosis, whereas treated cells died only 18.5±1.6% of the time, which is a very significant reduction ($p = 0.000$). 72 hour treatments also showed strong decreases in apoptosis, as VEGF treatment induced a drop in apoptosis from 60.3±4.1% to 37.2±3.6% ($p = 0.013$). It therefore appears that VEGF effectively reduces the proportion of hypoxia-induced apoptosis in HEK293 transformed cells. Interestingly, the HEK293 cells appeared to resist apoptosis to a greater extent than U251 cells when treated with VEGF. It is not immediately clear why this would occur, however the HEK 293 cells may have more VEGF receptors, or they may simply be more responsive to the VEGF ligand. The HEK 293 cells treated with VEGF did not differ from untreated cells in normoxia, although cell death did increase with time.

(a)



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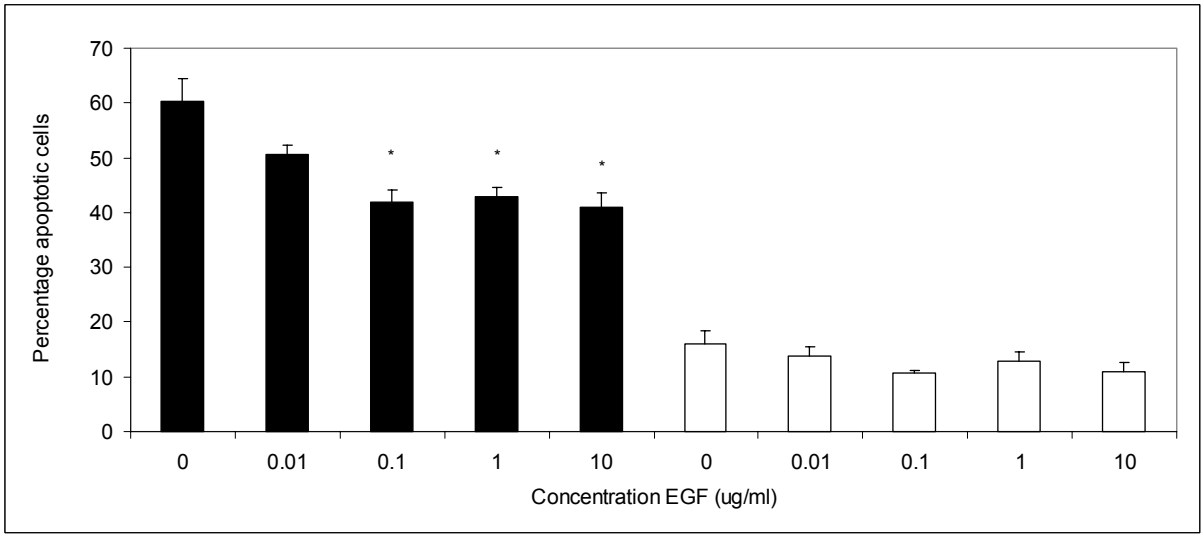
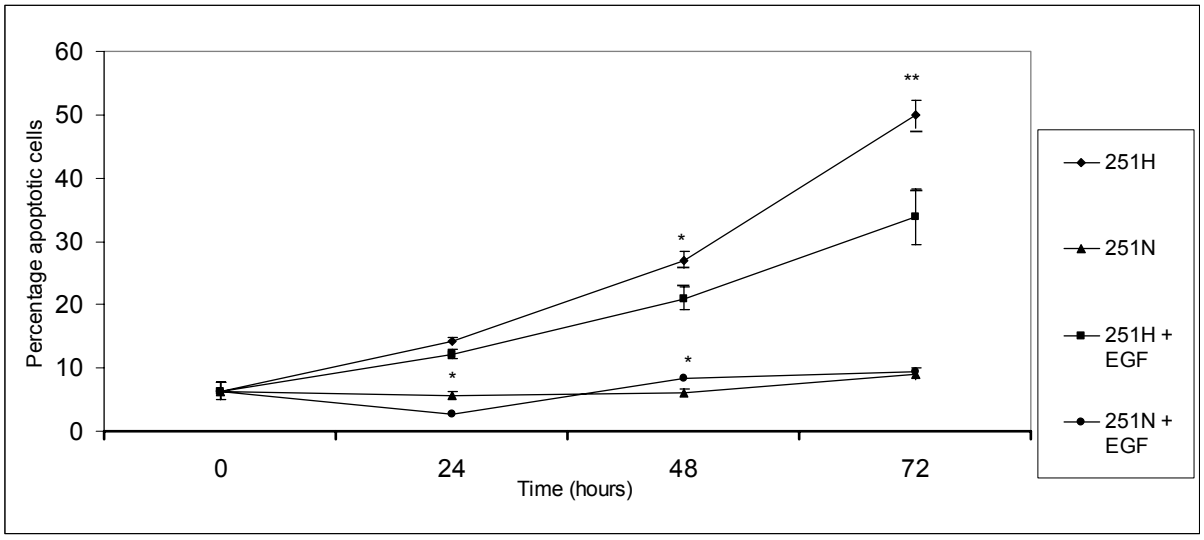


Figure 3: Dose response experiments with (a) U251 and (b) HEK293 cell lines treated with varying concentrations of EGF over 72 hours. Cells were incubated in hypoxia (black) and normoxia (white) in independent trials, and cell death was determined by acridine orange apoptosis assays. The mean percentage of apoptosis was plotted where the error bars represent the standard error of the mean. A one-way ANOVA was utilized in the comparison of the untreated sample to treated samples. * indicates that $p \leq 0.05$, ** indicates that $p \leq 0.01$, and *** indicates that $p \leq 0.005$.

(a)



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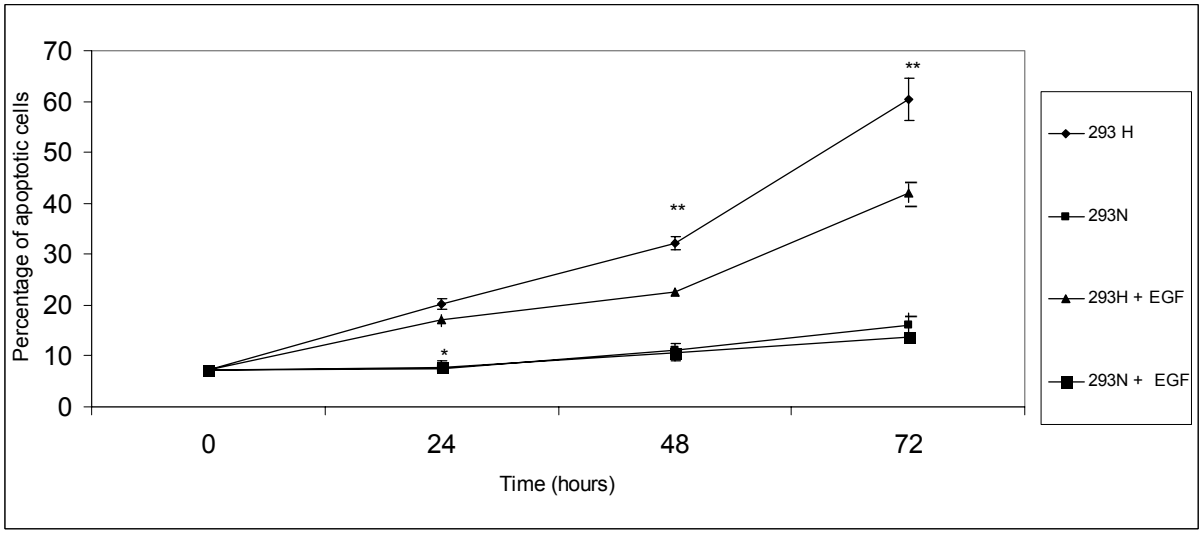

Figure 4: The percent cell death observed in independent trials of (a) U251 and (b) HEK293 cells incubated in hypoxia or normoxia over a time course of 0-72 hours in the absence or presence of 0.1μg/ml EGF. "H" refers to hypoxic conditions, and "N" refers to normoxia. The mean percentage of apoptosis was plotted where the error bars represent the standard error of the mean. A one-way ANOVA was utilized in the comparison of the untreated sample to treated samples. * indicates that $p \leq 0.05$, ** indicates that $p \leq 0.01$, and *** indicates that $p \leq 0.005$.

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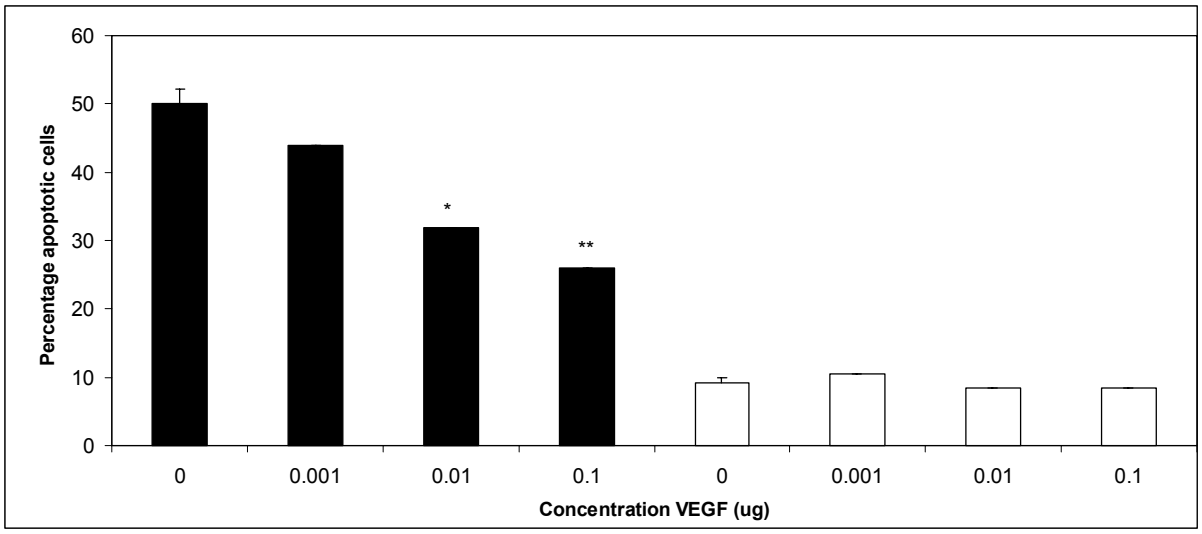

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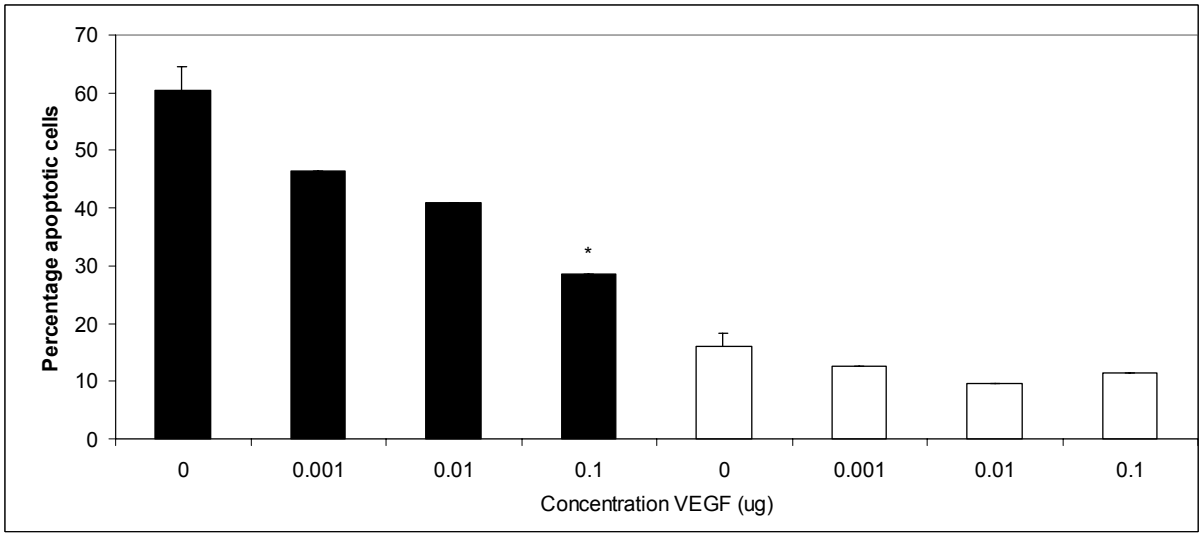
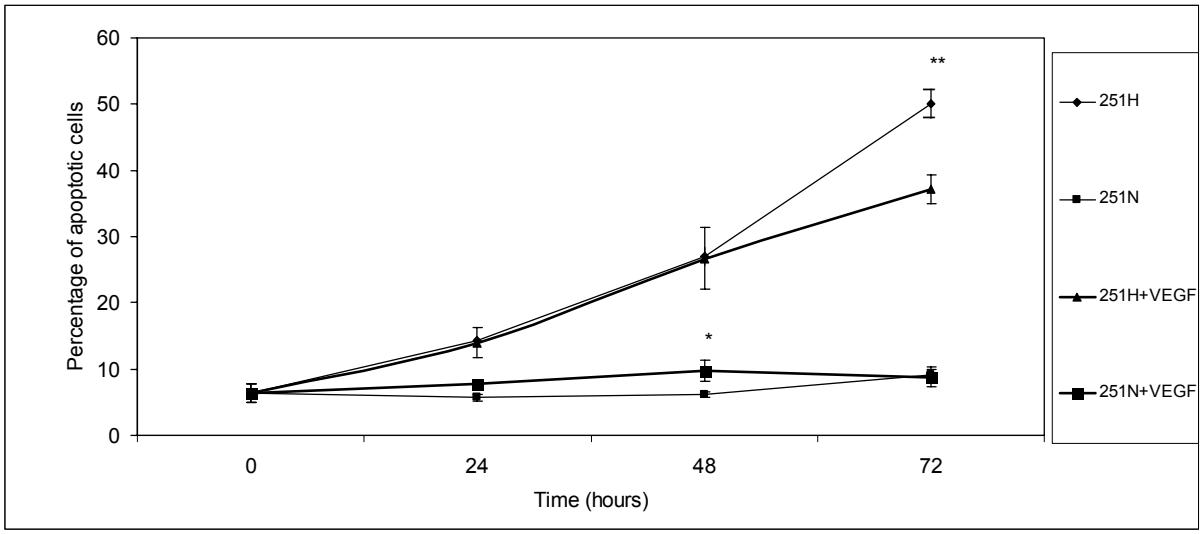


Figure 5: Dose response experiments with (a) U251 and (b) HEK293 cell lines treated with varying concentrations of VEGF over 72 hours. Cells were incubated in hypoxia (black) and normoxia (white) in independent trials, and cell death was determined by acridine orange apoptosis assays. The mean percentage of apoptosis was plotted where the error bars represent the standard error of the mean. A one-way ANOVA was utilized in the comparison of the untreated sample to treated samples. * indicates that $p \leq 0.05$, ** indicates that $p \leq 0.01$, and *** indicates that $p \leq 0.005$.

(a)



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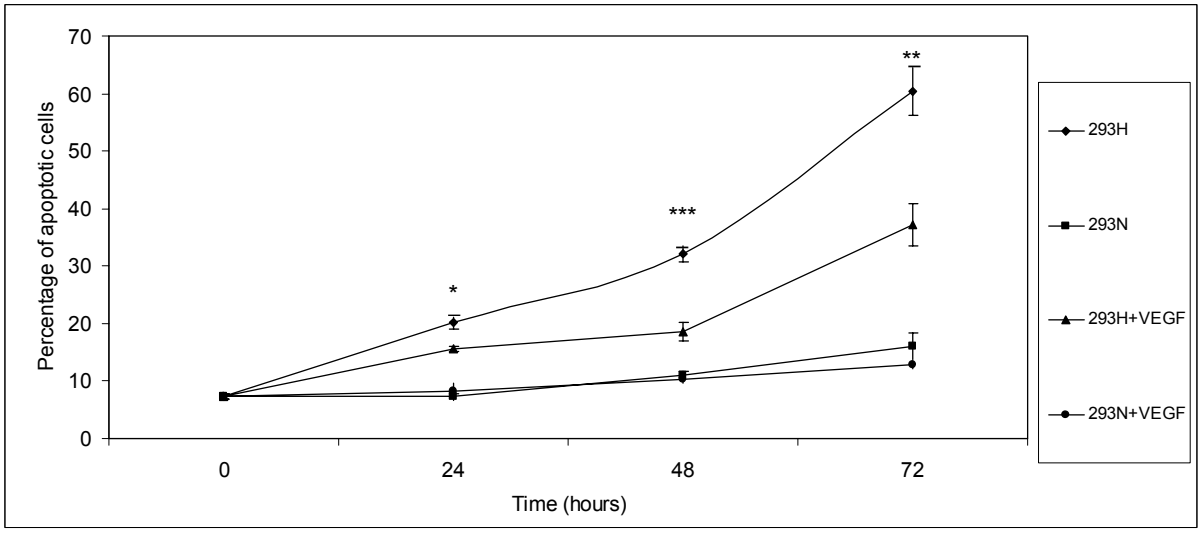

Figure 6: The percent cell death observed in independent trials of (a) U251 and (b) HEK293 cells incubated in hypoxia or normoxia over a time course of 0-72 hours in the absence or presence of 0.1μg/ml VEGF. "H" refers to hypoxic conditions, and "N" refers to normoxia. The mean percentage of apoptosis was plotted where the error bars represent the standard error of the mean. A one-way ANOVA was utilized in the comparison of the untreated sample to treated samples. * indicates that $p \leq 0.05$, ** indicates that $p \leq 0.01$, and *** indicates that $p \leq 0.005$.

Discussion

The significance of hypoxia in epithelial-derived solid tumors is becoming more and more apparent. Not only is hypoxia able to confer resistance to traditional anti-cancer therapies, but it has also been shown to repopulate tumors subsequent to treatment (Kinsoshita et *al*., 2001). The paradox of hypoxia being a poor prognostic indicator, despite being a severe physiological stress, may be at least partially understood by the increased survival of cancerous cells with regulatory alterations that enable their continued existence in hypoxia. Herein, we have shown that growth factors, specifically EGF and VEGF, are important in reducing the apoptotic response in hypoxic HEK293 and U251 cells over a 72 hour time course.

Over-activation of the EGF signaling pathway occurs in a wide variety of cancers. This predicts for aggressive tumor growth and a reduced prognosis of survival, as EGF signaling activates anti-apoptotic responses (Popov et al., 2004). For instance, as mentioned above, treating cancer cells with EGF reduces apoptosis associated with the FAS and TRAIL death receptors (Gibson et *al*., 1999; Gibson et *al*., 2002), and over activation of the ErbB2 receptor counteracts the effects of the drug Taxol (Yu et *al*., 1998). Our study has shown that EGF can reduce the apoptotic response in U251 glioma cells and HEK 293 transformed cells under hypoxic stress. Thus, EGF appears to hinder apoptotic signaling in cancer cells under hypoxic stress. Interestingly, the dosage response trials found that treatments in excess of 0.1μg/ml did not further reduce apoptosis in either cell line. It is unclear why this should be the case, although it is possible that the EGF receptors are saturated at a concentration of 0.1μg/ml. Consistent with our findings, another study demonstrated that EGF effectively

protected hypoxic MCF-7 breast cancer cells from apoptosis induced by the pro-apoptotic Bcl-2 protein BNIP3 (Kothari et *al*., 2003). This suggests, as our study did, that the aberrant activation of EGF signaling is important in protecting cancer cells from hypoxia-induced apoptosis.

In spite of the ability of VEGF to induce vascularization, small regions of hypoxia remain long after the initiation of angiogenesis (Wouters et *al*., 2004). Accordingly, it is believed that hypoxia has a net positive effect on tumor progression. This may be due to the tumor promoting anti-apoptotic effects conferred upon cancer cells by autocrine VEGF signaling (Pidgeon et *al*., 2001). We have demonstrated that the treatment of cancer cells with VEGF inhibited apoptosis in hypoxic stress in comparison with untreated cells. Interestingly, VEGF appeared to exert a greater protective effect on HEK293 cells than on U251 cells. It is unclear why this may have occurred, however it is possible that the HEK293 cells possess more VEGF receptors, or are more sensitive to VEGF. Our findings correspond well with other research, which has shown that hypoxia reduces apoptosis in serum-deprived HepG2 cancer cells by upregulating the concentration of VEGF (Baek et *al*., 2000). The fact that VEGF and the VEGF-2 receptor were found to be upregulated in hypoxia indicates that VEGF may serve as a survival factor in a self-enhancing fashion (Baek et *al*., 2000). Thus, through VEGF upregulation, hypoxia provides survival signals to cancer cells prior to the establishment of microvessels. Further confirmation of VEGF's ability to enable the circumvention of apoptosis in cancer cells comes from treatment of the serum-deprived hypoxic cells with recombinant human VEGF. As in the present study, human VEGF was capable of reducing apoptosis resulting from hypoxia (Baek et *al*., 2000). These results are

taken together as strong evidence that hypoxia makes tumor cells less susceptible to apoptosis- inducing stimuli.

No attempts were made to characterize the mechanisms through which EGF and VEGF allowed cancer cells to resist apoptosis. However, on the basis of previous research, one may speculate that activation of the aforementioned survival pathways, such as PI3-Akt and MAPK, played a pivotal role. The importance of Akt in cancer survival under hypoxic stress is supported by the finding that hypoxia-resistant MCF-7 cells, which overexpress the ErbB2 receptor, are sensitized to apoptosis by wortmannin, a potent inhibitor of the PI3-Akt pathway (Bacus et *al*., 2002). Hypoxia has been shown to initiate the PI3K-dependent activation of Akt signaling; this subsequently induces downstream targets to favor survival (Chen et *al*., 2001). This directly implicates signaling via the PI3-Akt pathway in protection against hypoxia-induced apoptosis. Additionally, the MAPK pathway, which is also stimulated by EGF and VEGF, activates several downstream targets that alter expression of several Bcl-2 members (Johnson and Lapadat, 2002). The activation of the extracellular regulated kinase (ERK) induces cell survival through the upregulation of the transcription factor ELK1, which activates anti-apoptotic Bcl-2 members, and blocks mitochondrial apoptosis (Johnson and Lapadat, 2002). Thus, the activation of the MAPK pathway by growth factor signaling in hypoxia should also be the subject of future research. As discussed above, the PI3-Akt and MAPK pathways transduce survival signals, which cause downstream targets such as Bcl-2 proteins to favor cell survival. Similarly, aberrant survival of cancer cells in unaccommodating microenvironments, including hypoxia, may also be a result of defects in the apoptotic machinery itself. For example, transformed BMK cells subjected to hypoxia *in vivo* exhibited profound apoptotic death when the mitochondrial apoptotic pathway

was intact. However, apoptosis was prevented by overexpression of the anti-apoptotic Bcl-2 protein, or by the loss of the pro-apoptotic BAX and BAK proteins (Nelson et *al*., 2004).

EGF and VEGF are not the only growth factors implicated in providing cancer cells resistance to apoptotic stimuli. Several studies show that treating cancer cells with insulin-like growth factor (IGF), which is required for normal growth of cells, reduces the apoptotic response. IGF may also be capable of protecting against hypoxic stress, as it diminished the percentage of MCF-7 cells that underwent apoptosis when incubated in low oxygen (Kothari et *al*., 2003). Furthermore, over-expression of the IGF1 receptor (IGF1R) promotes increased survival in cells exposed to hypoxia, low pH and low glucose (Peretz et *al*., 2002). Immunoblot analyses showed increased receptor protein levels in cells exposed to hypoxia. This suggests that the hypoxic microenvironment induces expression of the IGF1R, and that the subsequent over-expression of the receptor may increase cell survival in such conditions (Peretz et *al*., 2002). Platelet-derived growth factor (PDGF) is a peptide growth factor involved in the stimulation of cell growth, reorganization of actin, inhibition of gap junction communication, and inhibition of apoptosis (Apte et *al*., 2004). Stimulation of autocrine PDGF signaling is thought to contribute to the early transformation, progression, and aggressive nature of glioblastomas, melanomas, pancreatic cancer, and prostate cancer (Apte et *al*., 2004). While the ability of PDGF to prevent apoptosis in hypoxic cancer cells is not well characterized, studies of healthy brain cells show that the expression of PDGF and its receptor correlate with tolerance to hypoxic stress (Simakajornboon et *al*., 2001). As with EGF and VEGF, Akt signaling and the subsequent phosphorylation of downstream targets, such as BAD, was involved (Simakajornboon et *al*., 2001). Thus, in addition to EGF and VEGF, several other growth factors are capable of diminishing apoptosis in cancer cells under

hypoxic stress. This further supports our findings that growth factor signaling makes cancer cells in hypoxia less responsive to apoptosis. Further research should be done to better characterize the action of these growth factors on reducing hypoxia-induced apoptosis, and how they may interact with EGF and VEGF signaling *in vivo*.

In light of their multiple anti-apoptotic effects, VEGF, EGF, and their receptors have become the focus of exhaustive research. Recent clinical trials of drugs that block the action of EGF and VEGF ligands and receptors have shown great promise in the treatment of solid tumors. For instance, Iressa, an orally active tyrosine kinase inhibitor that is selective for the EGFR blocks the signal transduction pathways implicated in cell survival (Magne et *al*., 2002). Iressa has been particularly beneficial in the treatment of head and neck cancers, wherein EGFR expression is amplified. Previous data have shown that Iressa has numerous effects on tumor cells including cell cycle arrest, increased apoptosis and reduction in cell proliferation (Magne et *al*., 2002). Iressa has also been shown to sensitize cells incubated in hypoxia to apoptosis, as it dramatically reduced the proportion of viable hypoxic cells in LCC6 and MCF-7 metastatic mammary tumors (Warburton et *al*., 2004).

Further, the monoclonal antibody Herceptin, which is targeted at breast cancers, inhibits the signaling of ErbB2 through internalization and degradation of the receptor (Hinoda et *al*., 2004). Down-regulation of ErbB2 receptors by Herceptin has been shown to increase the effectiveness of TRAIL, thus it appears to sensitize cancer cells to apoptosis (Cuello et *al*., 2001). Herceptin exerts additive or synergistic effects on tumor cell growth inhibition in combination with a range of cytotoxic cancer drugs (Hinoda et *al*., 2004). Herceptin may be an effective anti-cancer therapy under hypoxic conditions as well, as it sensitizes MCF-7 breast cancer cells to hypoxia-induced apoptosis through an incompletely

understood mechanism (Kothari et *al*., 2003). In regards to VEGF signaling, the humanized monoclonal antibody bevacizumab reduces VEGF levels. Clinical studies of bevacizumab treatment have shown decreased disease progression. Further, patients exhibited a substantially higher response rate and quality of life when treated with high or low doses of bevacizumab than when treated with chemotherapy alone (Bergsland et *al*., 2000). Thus, bevacizumab sensitizes cancer cells to apoptosis by blocking angiogenic and anti-apoptotic signaling by VEGF. As a whole, this suggests that targeting growth factor signaling with tyrosine kinase inhibitors or monoclonal antibodies could sensitize cancer cells under hypoxic stress to programmed cell death.

In order to develop new treatments, it is necessary to identify the aberrant biochemical and molecular pathways that differentiate malignant and nonmalignant cells. A major limitation of the current study is that the anti-apoptotic pathways activated by EGF and VEGF were not characterized. Thus, future directions of this research should focus on performing Western blots for hypoxic cancer cells treated with VEGF or EGF versus untreated cells. Determining the precise mechanisms by which EGF and VEGF protect hypoxic cancer cells from apoptosis would be critical in the design or improvement of anti-cancer agents against EGF and VEGF signaling. For instance, it would be valuable to examine the expression levels of proteins involved in survival or apoptotic signaling. Potential targets for investigation are the phosphorylation state of Akt following growth factor stimulation as an indication of Akt activation, and caspase-3, an effector molecule in apoptotic cascades. From the findings presented here, it would be expected that Akt would be in its active phosphorylated form more often in hypoxic cancer cells treated with EGF/VEGF than in untreated hypoxic cells. This would indicate that EGF and VEGF have a net positive impact

on the growth and survival of cells in oxygen depleted malignant tumors. In terms of caspase-3, the results suggest that a greater proportion should be present in its inactive, uncleaved state when hypoxic cancer cells are treated with VEGF/EGF. A greater relative quantity of uncleaved caspase-3 would denote that apoptotic signaling cascades had not been activated to as great of an extent in cells treated with growth factors. This would be consistent with the findings that fewer treated cells died under hypoxic stress. Additionally, it would be beneficial for future research to examine the effects of EGF and VEGF *in vivo*. This is because tumor progression is not independent of surrounding healthy cells (Hanahan and Weinberg, 2000). For example, VEGF induces angiogenesis by stimulating endothelial cells in addition to reducing apoptosis. Thus, *in vivo* studies would be beneficial. This may be accomplished by injecting cancer cells in to immuno-deficient mice. Subsequently, growing tumors in the presence or absence of growth factors and examining the survival of cells in hypoxic regions within the tumors would provide a more complete comprehension of the role growth factors have in cancer progression.

There is a multitude of evidence that both EGF and VEGF serve as protective agents against wide variety of apoptotic stimuli. This study has provided further evidence that EGF and VEGF positively affect the progression of cancer, as they reduce apoptosis in cancer cells grown under conditions of low oxygenation. Further characterization of the mechanisms by which EGF and VEGF signaling allow malignant cells to subvert apoptosis in hypoxia is required. This may enable the development of more specific drugs targeting EGF and VEGF signaling, which could sensitize aggressive cancers to hypoxia-induced apoptosis *in vivo*, and ultimately allow for a better prognosis of survival and quality of life.

Conclusions

1. Treating hypoxic U251 cancer cells and HEK 293 transformed cells with various EGF dosages for 72 hours showed that the greatest protective effect was exerted when EGF concentrations were 0.1μg/ml, or greater.

2. Treatment of U251 and HEK293 cells under hypoxic stress with 0.1μg/ml EGF over a 72 hour time course reduced apoptosis relative to untreated cells, especially after 48 and 72 hours. Conversely, the levels of apoptosis associated with EGF-treated and untreated normoxic cancer cells did not depend on EGF treatment.

3. After 72 hours of hypoxic stress in various concentrations of VEGF, the proportion of apoptotic U251 and HEK293 cells was reduced the most by VEGF concentrations of 0.1μg/ml.

4. Treatment of U251 and HEK293 cells with 0.1μg/ml VEGF over a 72 hour time course reduced the proportion of apoptotic cells, especially in HEK293 cells. As with the EGF time response trials, apoptosis in VEGF-treated and untreated cells did not depend on VEGF treatment.

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