ABSTRACT

Carroll R. Smith III. ERYTHROPOIETIN AND ITS RELATIONSHIP WITH TUMOR GROWTH. (Under the direction of Dr. George Sigounas). Department of Biology, January, 2008

Erythropoietin (EPO) is a glycoprotein widely used to correct the disease-related and drug-induced anemia observed in cancer patients. Several studies have reported that EPO is a pleiotropic cytokine. Recently, a number of investigators have reported that various human cancer cell lines express EPO and the EPO receptor (EPOR), raising suspicion for the presence of an autocrine-paracrine EPO-EPOR system. In this study, we assessed whether or not EPO enhances tumor metastasis of Lewis lung carcinoma (LCC) cells. Furthermore, we assessed the effect of EPO on cancer cell growth both in vivo and in vitro. The levels of VEGF and SDF-1 in plasma and cultures were also determined. We found that there was a statistically significant difference in tumor growth between saline-treated and EPO-treated animals ($p<0.05$). However, the number of lung metastases derived from primary tumors was similar in both groups. The average volume of lung nodules was 24% higher in saline-injected animals compared to EPO-treated mice. The plasma VEGF level in tumor-bearing animals treated with EPO was reduced by 20% compared to the control mice. LLC cells cultured in the presence of EPO secreted lower levels of VEGF compared to the controls. In conclusion, this study shows that the growth of tumors was slower in EPO-treated animals, while EPO had no effect on the number of metastases. The tumor inhibitory effect of EPO may be mediated through the down-regulation of VEGF via a VEGF/EPO negative regulation loop.

ERYTHROPOIETIN AND ITS RELATIONSHIP WITH TUMOR GROWTH

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ERYTHROPOIETIN AND ITS RELATIONSHIP WITH TUMOR GROWTH

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LIST OF ABBREVIATIONS

AKT	Protein Kinase B
BFU-E	Blast Forming Unit-Erythroid
CFU-E	Colony Forming Unit-Erythroid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPO	Erythropoietin
EPOR	Erythropoietin Receptor
ERP	Erythropoietin Receptor Peptide
ESP	Erythropoietin Stimulating Proteins
FBS	Fetal Bovine Serum
G-CSF	Granulocyte Colony-Stimulating Factor
GM-CSF	Granulocyte/Monocyte Colony-Stimulating Factor
JAK	Janus Tyrosine Kinase
HAF	Hypoxia Associated Factor
Hb	Hemoglobin
Hct	Hematocrit
HIF-1	Hypoxia-Inducible Factor 1

HGF	Hepatocyte Growth Factor
HRE	Hypoxia-Responsive-Enhancer
LLC	Lewis Lung Carcinoma
MAPK	Mitogen-Activated Protein Kinase
MMP2	Matrix Metalloproteinase 2
mRNA	Messenger Ribonucleic Acid
PBS	Phosphate Buffered Saline
PMS	Phenazine Methosulfate
PI	Propidium Iodide
PI3K	Phosphatidylinositide-3-Kinase
RBC	Red Blood Cells
rHuEPO	Recombinant Human Erythropoietin
SCID	Severe Combined Immunodeficiency
SDF-1	Stromal Derived Factor-1
STAT	Signal Transducer and Activator of Transcription
uPAR	Urokinase Receptor
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VPF	Vascular Permeability Factor

INTRODUCTION

In 1906 Carnot and Deflandre suggested that red blood cell production is regulated by a hormone. Today this hormone is known as erythropoietin (EPO). EPO is a 30.4 kDa glycoprotein encoded by a gene located on chromosome 7. The gene that encodes EPO consists of five exons and four introns (Beck, 1991). The ability of EPO to govern its biological half-life in the blood is derived from the four glycosylation sites it exhibits. In addition, EPO is commonly produced in the adult kidney and the fetal liver (Jacobson *et al.*, 1957; Fischer and Birdwell, 1961; Kuratowska *et al.*, 1961). This hormone plays a major role in regulation of erythropoiesis, which results in the survival, proliferation and differentiation of erythroid progenitors, and is responsible for EPO receptor (EPOR) maturation. Recently the importance of EPO has been portrayed in studies by generating lines of mutant mice lacking either the EPO or the EPOR gene. Each line of mice died as a result of severe anemia between embryonic days 13 and 15 (Lian *et al.*, 1995).

Erythropoietin Activation

The primary function of EPO is mediated through the EPO receptor (EPOR) found on erythroid progenitor cells (Jelkmann, 1992; Lian *et al.*, 1995). "Erythropoiesis, which normally proceeds at a low basal level to replace aged red blood cells, is highly induced by loss of red blood cells, decreased ambient oxygen tension, increased oxygen affinity for hemoglobin, and other stimuli that decrease delivery of oxygen to the tissues" (Ebert and Bunn, 1999). In states of severe hypoxia, production of EPO is increased up to 1,000-fold in the kidney. Once the secreted EPO is circulating in the blood it will then

bind to its receptors which are specifically expressed on erythroid progenitor cells and will thereby promote viability, proliferation, and terminal differentiation of erythroid precursors, which will result in an increase in red blood cell mass (Ebert and Bunn, 1999). The elevated level of red blood cells leads to an increase in oxygen supply followed by downregulation of EPO expression and inhibition of erythropoiesis (Fig 1). The oxygen carrying capacity of the blood is thus enhanced, increasing tissue oxygen tension, thereby completing a negative feedback loop. Two classes of erythroid progenitor cells have been identified, blast forming unit-erythroid (BFU-E) and colony forming unit-erythroid (CFU-E). Both types of cell have erythropoietin receptors. On BFU-E cells, EPO will bind to its expressed receptor and will then proliferate into CFU-E cells (proerythroblasts) (Lappin, 2003). The newly formed proerythroblasts will then proliferate and develop into erythroblasts and finally into reticulocytes which will enter the peripheral circulation and mature into red blood cells (Lappin, 2003).

The hypoxic conditions that lead to erythropoiesis are based on transcriptional regulation by hypoxia-inducible factor (HIF-1) (Wang and Semenza, 1993). HIF-1 is activated at physiologically relevant oxygen levels, ensuring fast and adequate response. Both EPO and VEGF are oxygen-regulated genes and respond to hypoxia related events. "In addition to EPO and VEGF expression, hypoxia can upregulate other genes, including tyrosine hydroxylase, platelet-derived growth factor B chain, phosphoglycerate kinase 1, and lactate dehydrogenase A" (Bunn and Poyton, 1996).

The transcriptional regulation of EPO can only be achieved when several factors interact with the EPO gene's proximal promoter region, and with its 3' untranslated region (Beru *et al.*, 1990; Beck *et al.*, 1991; Pugh *et al.*, 1991; Semenza *et al.*, 1991;

Blanchard *et al.*, 1992). "In the 3' untranslated region of the EPO gene, there is a 50-bp hypoxia-responsive-enhancer (HRE) element located approximately 120 bp 3' of the polyadenylation site, this site binds the hypoxia-inducible factor (HIF)" (Semenza and Wang, 1992). HIF-1 is also involved with the activation of a related chemokine VEGF. VEGF gene transcription occurs through its interaction with a 47-bp sequence located at nucleotides 985 to 939 5' of the transcription start site (Forsythe *et al.*, 1998). Additional similarities in the gene regulation of EPO and VEGF include their induction by cobalt chloride ($CoCl_2$) and suppression of this induction by carbon monoxide (Goldberg and Schneider, 1994; Liu *et al.*, 1998). Cobalt chloride has been shown to induce HIF-1 (Piret, 2002). In addition to HIF-1, a protein termed hypoxia associated factor (HAF) shows a "sequence-specific interaction with a 17-bp sequence (EP17) in the proximal promoter region of the EPO gene and modulates expression of EPO and VEGF mRNA in response to hypoxia" (Gupta *et al.*, 2000).

When HIF-1 is upregulated in tumor cells, there will also be an observed upregulation of VEGF by HIF-1. This VEGF increase will cause the promotion of tumor growth. Therefore, one can assume that by causing an interruption of the HIF pathway there would be a decrease in angiogenesis and the growth of the tumor would be suppressed (Maxwell *et al.*, 1997; Ryan *et al.*, 1998; Kung *et al.*, 2000; Maxwell *et al.*, 2001; Giantonio *et al.*, 2003). It is plausible that correction of anemia from an addition of EPO would decrease the accumulation of HIF-1 or its upregulation. This may lead to reduction in VEGF secretion, angiogenesis, and the VEGF enhanced potential for increased tumor growth and aggressiveness (Blackwell *et al.*, 2004).

Erythropoietin Receptor

The EPO receptor gene was cloned by *D'Andrea* and coworkers in 1989 from murine erythroleukemia cells (D'Andrea *et al.*, 1989). The EPO receptor is part of a family of cytokine receptors. Some examples of additional members found in this family include various interleukins, granulocyte/monocyte colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), growth hormone and prolactin (Yoshimura and Arai, 1996). "The special characteristic of this family of receptors is that they are switched on and transduce signals to the interior of the cell by the formation of homo- or hetero-oligomers, and the hetero-oligomers of these receptors share a common subunit" (Yoshimura and Aria, 1996). EPOR and the previously mentioned receptors are all homodimerizing receptors that form a 2:1 complex between receptor chains and the hormone. Originally it was believed that in order to trigger EPO's biological responses the EPOR had to go through dimerization. However, recent reports concluded that the EPO receptor was preformed as a dimer, and when EPO was bound to its receptor, the result was a tight association between the two chains and intracellular signaling (Naranda *et al.*, 2002). EPOR activation is now explained to be the result of EPOR-peptide (ERP) binding in the region where the two receptor chains normally interact in a scissor-like model in response to hormone binding. This peptide binding will then cause receptor-mediated signal transduction, cell proliferation, and Colony Forming Unit-Erythroid formation (Naranda *et al.*, 2002).

Erythropoietin acts by binding to a specific erythropoietin receptor on the surface of red cell precursors in the bone marrow, stimulating them to differentiate into mature red blood cells. As a result, the oxygen level in blood reaching the kidney rises, and the

amount of EPO produced decreases. "In this process, the EPO-EPOR signal is mediated by Janus tyrosine kinase (JAK), signal transducer and activator of transcription (STAT), mitogen-activated protein kinase (MAPK), and phosphatidylinositide-3-kinase (PI3K)-protein kinase B (Akt)" (Bittorf *et al.*,1994; Haseyama *et al.*,1999, Bittorf *et al.*,2000). JAK and the downstream signaling molecule STAT5 join together in the role of regulating *in vivo* erythropoiesis (Parganas *et al.*, 1998; Socolovsky *et al.*, 1999) (Fig 2). EPO's activation pathway starts as EPO binds to its receptor. This binding occurs along with the subsequent association of JAK2 to the EPOR cytoplasmic region near the plasma membrane. What occurs next is a sequence of phosphorylation events in which JAK2 will phosphorylate both the EPO receptor and the transcription factor STAT5 (Witthuhn, 1993). Once STAT5 translocates to the nucleus it will recognize a specific base sequence in the promoter region of its target gene, and undergo the beginning of transcription (Yoshimura and Arai, 1996).

EPO and its receptor are also synthesized by neoplastic and tumor endothelial cells. The EPO-EPO receptor system may promote tumorigenesis by acting either directly on the tumor cells, or indirectly on enhancing tumor angiogenesis. Blocking of the EPO receptor may delay tumor growth in mice and reduce angiogenesis (Arcasoy M *et al.*, 2002; Yasuda *et al.*, 2003).

Erythropoiesis was once thought to be the only function which EPO and its receptor had. Other than the adult liver and kidney where EPO/EPOR is normally expressed, additional sites have been discovered. Some of these extra sites include brain endothelial cells, microglia, oliogodendrocytes, astrocytes, neurons, bone marrow macrophages, and trophoblast cells found in the human placenta (Vogt *et al.*, 1989;

Buemi *et al.*, 2003; Conrad *et al.*, 1996). Considerable amounts of EPO are also present in human milk (Kling *et al.*, 1998).

Previous Erythropoietin Studies Associated with Cancer

Experiments have been conducted to determine EPO's role in tumor regression. A systemic regimen of EPO administration was given to mice to establish optimal treatment modalities affecting tumor development. The effects of different regimens with various recombinant human EPO (rHuEPO) doses (10, 20, 30, 40, 50, and 100 units per injection) and time schedules (daily treatment for 10 or 20 consecutive days, or three injections per week for 4 weeks) were tested (Mittelman *et al.*, 2001). When the mice were injected with a progressively growing myeloma and afterwards treated with EPO, there was an observed although limited period where tumor regression had occurred in 30-60% of the mice (Mittelman *et al.*, 2001). The tumor regression seen from EPO's addition was accredited to an effective anti-tumor immune response (Mittelman *et al.*, 2001). Once tumor regression was established, EPO treatment was no longer required for maintenance of regression. In addition, T cells were found to be involved in EPO triggered tumor rejection. T cell involvement with EPO's tumor rejection was determined by comparing the responses of normal, SCID, or nude mice to EPO treatment after the tumor-cell challenge (Mittleman *et al.*, 2001). Immunologically impaired SCID and nude mice were found to be unresponsive to EPO treatment. "Negation of the antitumor response induced by EPO treatment in these T cell-deficient mice ruled out the possibility that EPO has a direct cytotoxic or cytostatic effect on tumor cells" (Mittelman *et al.*, 2001). This shows that EPO may play an immunodulatory role in tumor regression.

Similar studies have determined that a combination of EPO with radiochemotherapies may be the key to what ultimately regresses and/or inhibits tumor growth. EPO alone does not affect tumor growth as shown in the previous experiment. In order to fully enhance drug's effectiveness, EPO may need to be added to the treatment regimen. Therefore, EPO combined with different chemotherapeutic drugs (cisplatin, mitomycin C, cyclophosphamide) was used (Sigounas *et al.*, 2004). Animals injected with a combination of EPO and cisplatin developed tumors with the smallest average volume and weight. However, the mechanism through which EPO modifies the effectiveness of chemotherapeutic drugs was unclear.

Clinical studies have shown that the effectiveness of irradiation with regards to the suppression of tumor growth is influenced by hypoxia (Sigounas *et al.*, 2004). It is assumed that by increasing the oxygen, hematocrit, and hemoglobin levels, EPO makes tumors more susceptible to irradiation (Sigounas *et al.*, 2004).

VEGF

In 1983, Donald Senger, along with a group of scientists, studied the physiological properties of blood vessels. From their studies, they reported the identification and partial purification of a protein that induced vascular leakage. The protein identified was first named vascular permeability factor (VPF) (Senger *et al.*, 1983). In 1989, this protein portrayed growth-promoting activity only towards vascular endothelial cells and was therefore named vascular endothelial growth factor (VEGF) (Lueng *et al.*, 1989).

Vascular endothelial growth factor is a specific mitogen for vascular endothelial cells. This growth factor contains a family of many different members with assorted functions, including VEGF-A through VEGF-F and placental growth factor (Tam *et al.*, 2006). All members of this family share a common structure of eight cysteine residues in a VEGF homology domain. A specific VEGF isoform, VEGF-A promotes the angiogenesis of tumors (Ferrera, 2003). Two VEGF receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1) are associated with VEGF-A. VEGFR-2 has been shown to be the major mediator of the angiogenic, mitogenic and permeability-enhancing effects of VEGF-A (Ferrara and Kerbel, 2005). VEGF has a very important correlation with EPO. Recent data shows that "vertebrate erythropoiesis is negatively regulated by endogenous VEGF, and identifies erythrocytosis and EPO level as potential noninvasive markers for high-grade inhibition of VEGF *in vivo*" (Tam *et al.*,2006). This described effect was proposed after *Tam et al.* showed that when using soluble VEGF receptors and antibodies specific to VEGFR-2 they were able to elicit a novel phenotype of erythrocytosis in both mice and primates. Furthermore, "VEGF may act as a physiological regulator of both adult erythropoiesis and synthesis of EPO with inhibition of EPO through VEGF-A and VEGFR-2 dependant endothelial-hepatocyte cross-talk" (Tam *et al.*, 2006). This inhibition may have caused VEGF to indirectly regulate EPO production through the release of an unknown suppressor of EPO production (Fig. 3). In addition, this study raises questions on whether or not a regulatory loop of EPO and VEGF is plausible. The hepatic synthesis of EPO and erythropoiesis may only take place when VEGF inhibition has occurred, or when VEGF has been blocked nearly all the way.

As mentioned earlier HIF-1 plays a role in VEGF expression. In response to

hypoxia, HIF-1 binds to specific enhancer elements which upregulates transcription of the gene (Dor *et al.*, 2001). This situation occurs when hypoxia induces the binding of the HIF-1 to the hypoxia responsive element in the VEGF-A gene promoter region, which will in turn cause VEGF-A transcription (Dor *et al.*, 2001).

Tumor cells are known to release VEGF in order to stimulate tumor angiogenesis. This trait can act as potential target for cancer therapy. Tumors of the lung, gastrointestinal tract, and breast will often metastasize through the lymphatic vessels (Tam *et al.*, 2006). Lymphangiogenic growth factors such as VEGF-C, VEGF-D and the related receptor VEGFR-3 have been shown to encourage lymphatic growth in tumor regions, which would then aid the cancer cells to use the lymphatic vessels for metastasis (Stacker *et al.*, 2002). VEGF-A has been also associated with metastatic spread of tumors.

When VEGF is inhibited, vasculature will regress by almost 70% in some organs (Fischer *et al.*, 2006). The regression of vasculature may cause diminished oxygen delivery, which could trigger the stimulation of endothelial cells as well as the production of EPO (Fischer *et al.*, 2006). The subsequent stimulation of EPO would improve oxygen delivery through its erythropoietic activity, but also stimulate endothelial cell maintenance through its angiogenic activity. What is interesting about VEGF inhibitors is that they have not yet demonstrated the ability to raise RBC counts in treated patients. The only documented occurrence was in a subset population of patients with hemangioblastoma and Von Hippel-Lindau disease, who were treated with VEGF inhibitors, and were able to display a rise in RBC counts (Fischer *et al.*, 2006). The EPO level, however, was not measured.

Stromal Derived Factor-1

Stromal cell-derived factor- 1a (SDF-1) is expressed by a number of cells which include endothelial cells as well as fibroblasts (Muller *et al.*, 2001, Salvucci *et al.*, 2002). The SDF-1 chemokine has been shown to interact with a G protein-coupled receptor named CXCR4 (Federsppiel *et al.*, 1993). CXCR4 is involved in increasing the metastatic potential of colon and breast cancer cells. Also, it has been shown that the SDF-1/CXCR4 axis plays an important role in the neovascularization and tumor progression. When this interaction was inhibited an observed blockage of growth in metastatic lesions was shown. This blockage had partially occurred from suppression of angiogenesis (Guleng *et al.*, 2005). This antigrowth effect seemed to be independent of CXCR4 expression by cancer cells. In murine tumor models CXCR4 was neutralized, but there was no observed change in the amount VEGF in the models (Guleng *et al.*, 2005). The SDF-1/CXCR4 axis might contribute to functional vascular establishment by the regulation of endothelial tube formation. Normal human megakaryoblasts experienced an increase in VEGF secretion from the addition of SDF-1 (Kijowski *et al.*, 2001). Alternatively, when SDF-1 blockade occurred, neovascularization via VEGF was inhibited. Immunochemical analysis was also able to show that SDF-1 is induced downstream of VEGF (Grunewald *et al.*, 2006). Another reported similarity in the expression of VEGF and SDF-1 showed SDF-1 expression *in vivo* in ischemic tissues and CXCR4-positive progenitor recruitment was enhanced by the transcription factor HIF-1 (Fig. 4) (Guleng *et al.*, 2005).

Erythropoietin is a pleiotropic hormone which may affect the proliferation and survival of various non-hematopoietic cell lineages. Furthermore, recent reports have

raised concerns suggesting that EPO might affect the growth and survival of EPOR-expressing neoplastic cells. Using both in vivo and in vitro experimental models in this study, we assessed the role of EPO on tumor growth. We found that EPO may have a positive effect on the suppression of tumor growth via downregulation of VEGF. In addition, we have shown that EPO has little to no effect on the cell proliferation of LLC cells, and no effect on SDF-1 secretion. We propose that the interaction between VEGF/EPO may be a negative regulation loop in which both angiogenic factors may decrease the other's upregulation.

Figure 1: Feedback Loop of Erythropoietin. Erythropoietin activation mechanism showing how EPO expression is turned on and off in a feedback loop. This process starts via a hypoxic response being sent to the kidney which in turn produces EPO. EPO production is shut down once there is an increased level of oxygen circulating in the blood stream (Ebert, 1999).

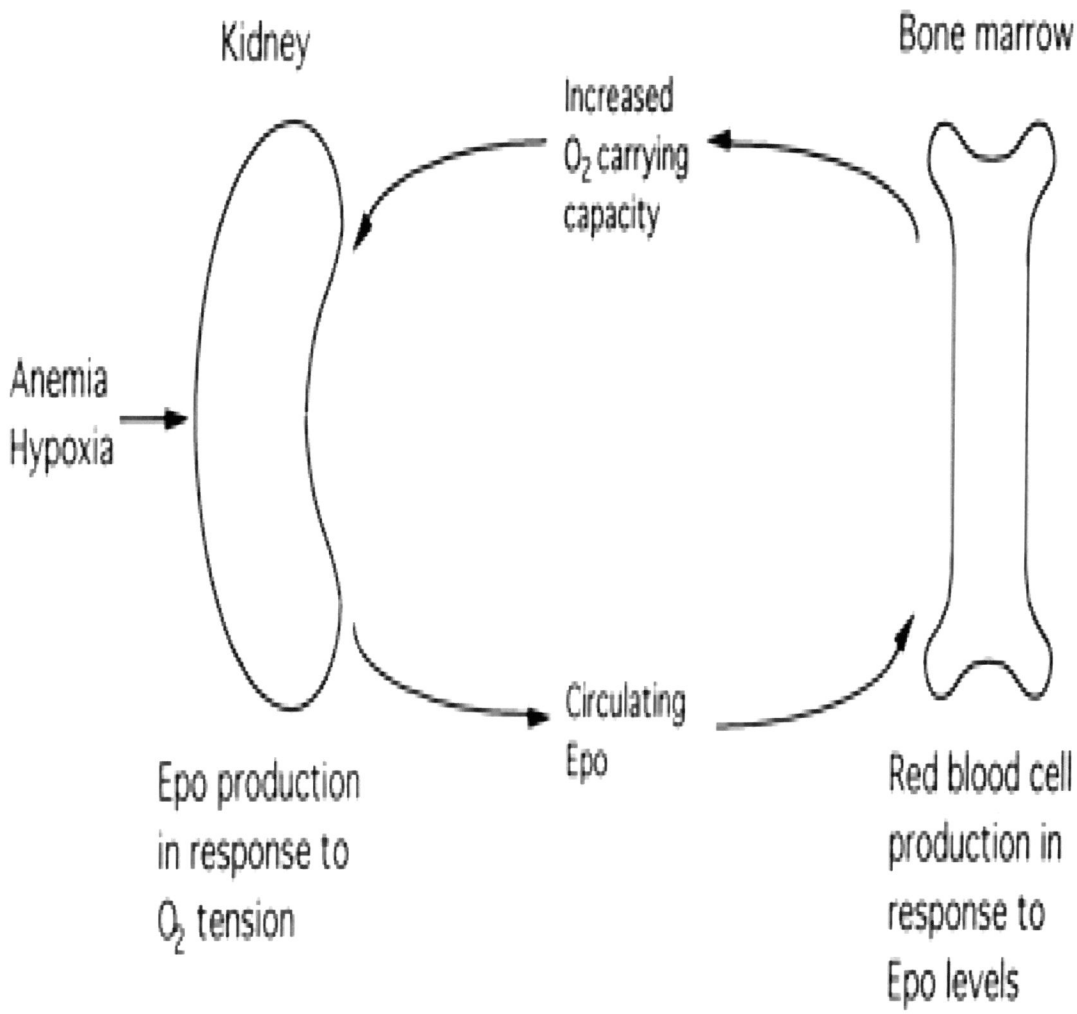

Kidney
Bone marrow
Increased
O_2 carrying
capacity
Anemia
Hypoxia
Circulating
Epo
Epo production
in response to
O_2 tension
Red blood cell
production in
response to
Epo levels

Figure 2: Erythropoietin interaction with its receptor. Once Erythropoietin binds with its receptor a series of pathways will be activated, the receptor will mature, and erythroid progenitor cell will be produced. EPO's binding will promote the viability, proliferation, and terminal differentiation of erythroid precursors, resulting in an increase in red blood cell mass. Image adapted from http://kugi.kribb.re.kr/KUGI/Pathways/mBioCarta/m_epoPathway/, EPO Signaling Pathway, Novemeber 10, 2007.

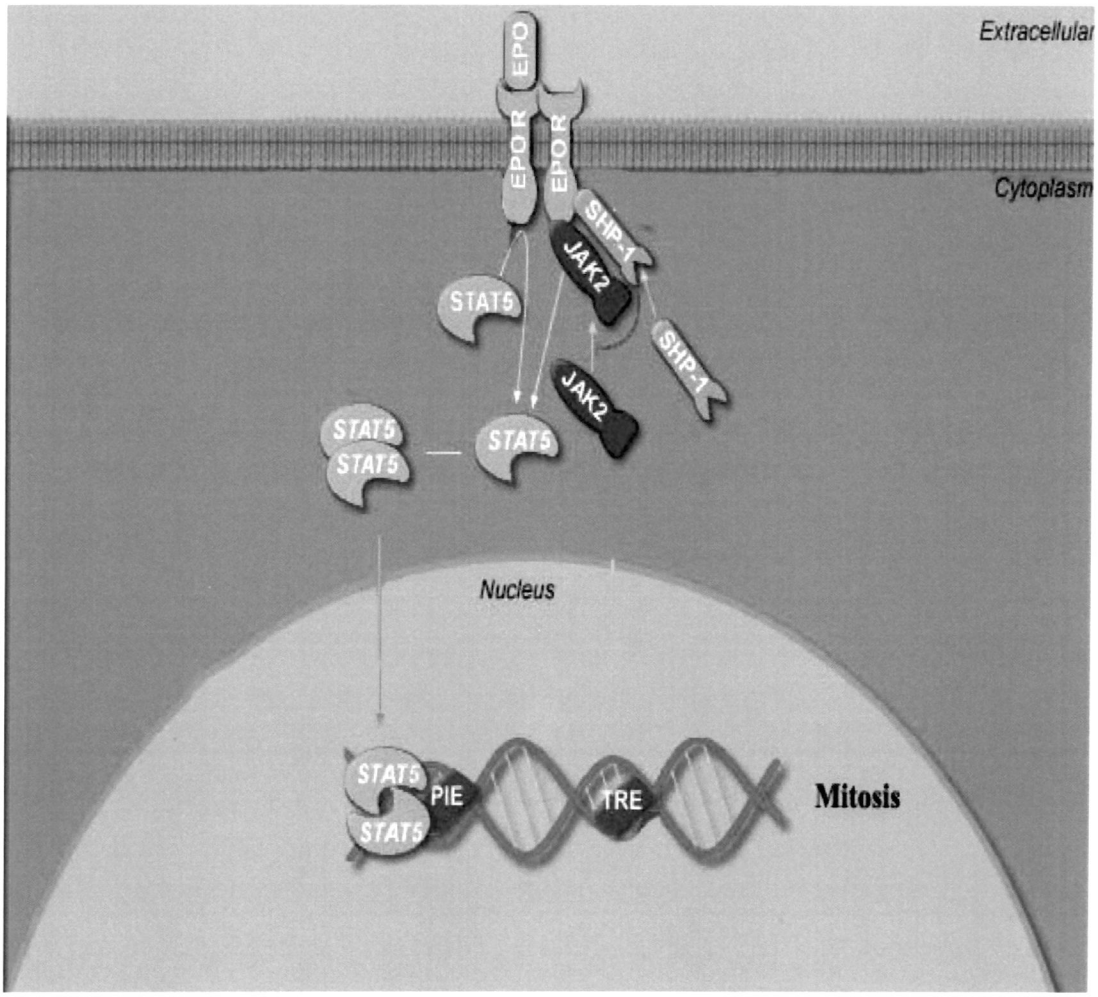

Extracellular
Cytoplasm
EPO
EPOR
EPOR
SHP-1
JAK2
STAT5
SHP-1
JAK2
STAT5
STAT5
STAT5
Nucleus
STAT5
STAT5
PIE
TRE
Mitosis

Figure 3: Proposed VEGF/EPO interaction in vivo. Inhibition of VEGF can be accomplished by using antibodies specific to VEGF receptors. This inhibition results in erythrocytosis. VEGF might indirectly regulate EPO production through release of an unknown suppressor of EPO production (Tam *et al*, 2006). In this figure, VEGF is released and binds to endothelial cells. This binding causes a release of an unknown "x-factor" which will bind to hepatocyte cells and block the production of EPO.

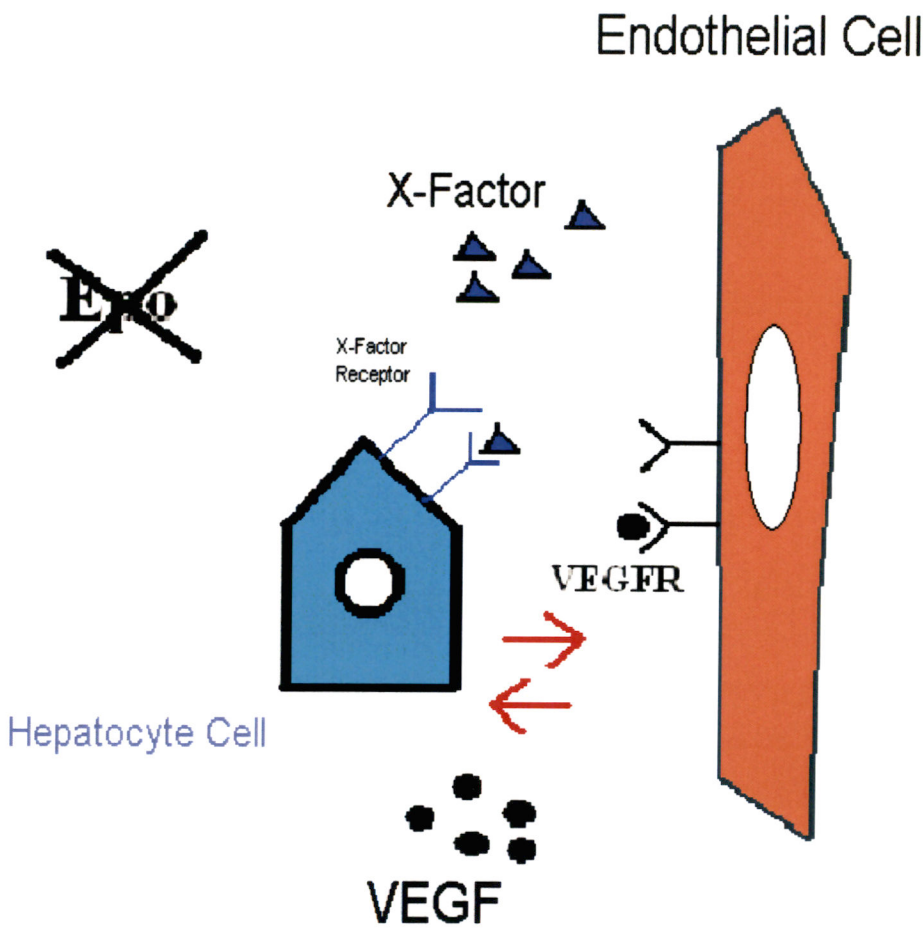
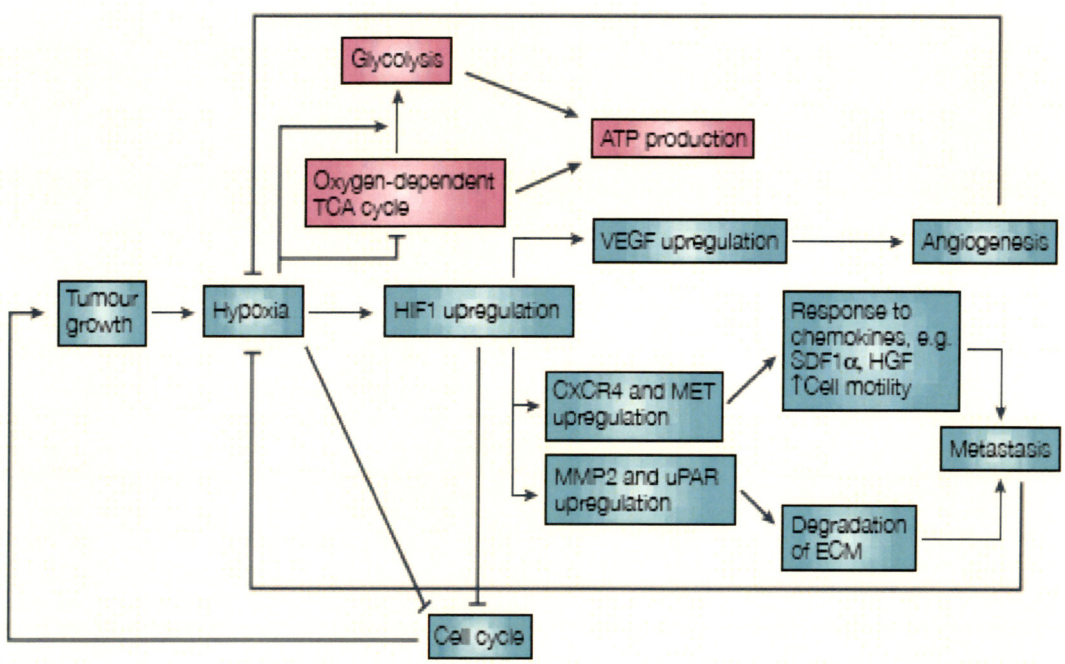

Endothelial Cell
X-Factor
X-Factor
Receptor
VEGFR
Hepatocyte Cell
VEGF

Figure 4: Hypoxic Responses of Tumor Cells. Rapid increase in tumor mass outpaces angiogenesis and results in tumor hypoxia. Hypoxia induces hypoxia-inducible-factor 1 (HIF1), which upregulates various genes. At the same time, progression through the cell cycle is inhibited by HIF1-dependent and -independent mechanisms. VEGF upregulation promotes angiogenesis, so that hypoxia of tumor cells can be resolved by vascularization. Simultaneously, chemokine receptors such as CXCR4 and MET are upregulated, so that tumor cells can respond to chemokines in the environment (SDF-1, HGF). MMP2 and uPAR are upregulated, leading to degradation of the extracellular matrix (ECM), so that tumor cells can migrate away from the hypoxic region and metastasize. When hypoxia is resolved in this way, cell-cycle arrest is released and further proliferation is initiated. (Kitano, 2004)) HGF, Hepatocyte growth factor; SDF1α, Stromal-derived factor 1α., uPAR, Urokinase receptor, MMP2, Matrix metalloproteinase 2


Glycolysis
ATP production
Oxygen-dependent TCA cycle
VEGF upregulation
Angiogenesis
Tumour growth
Hypoxia
HIF1 upregulation
Response to chemokines, e.g. SDF1α, HGF ↑Cell motility
CXCR4 and MET upregulation
Metastasis
MMP2 and uPAR upregulation
Degradation of ECM
Cell cycle

HYPOTHESIS

We hypothesize that EPO suppresses tumor growth through a reduction of VEGF secretion by cancerous cells and through downregulation of VEGF in tumor-bearing animals.

MATERIALS AND METHODS

Chemicals

Lewis Lung Carcinoma (LLC) cells used in this study were obtained from American Type Culture Collection (Rockville, MD). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, VT). All other chemicals were obtained from Sigma Chemicals (St Louis, MO) and Fisher Scientific (Fair Lawn, NJ).

Cell Cultures

LLC cells were grown in 75 cm^2 T-flasks in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 2% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cell cultures were incubated at 37° C in a humidified 5% CO_2 incubator. Cell concentrations were maintained below $2x10^4$ cells/cm^2 (Sigounas *et al.*, 2004). This was achieved by doing cell counts and splitting the cells to the desired concentration. Viability of cells was assessed by staining with trypan blue and cell counts were determined using a hemocytometer.

Animal Treatment

Animals:

Female, 7-8 weeks old, C57BL/6 mice, obtained from Harlan (Indianapolis, IN) or Jackson (Bar Harbor, ME) Laboratories, were used in this study. Groups of 4-8 mice were housed in plastic cages and allowed *ad libitum* access to mouse food and water. Animal procedures were approved by the ECU Animal Care Committee.

Injections:

For the axilla injection studies eleven experiments were performed. Animals were randomly divided into two treatment groups with 6-8 mice per group. Single cell suspensions of LLC cells were injected subcutaneously in the right frontal axilla. The animals of each group were treated as follows: group 1 (saline), injected with PBS; group 2 (EPO), injected with EPO alone. EPO was used at a concentration of 60 units/mouse.

Analysis of Tumors, Metastasis and Lung Seeding:

High-dosage anesthetic was used to euthanize the animals 13 days after LLC cell injections. Surrounding muscles and dermis were then separated from the primary tumors. The tumors were excised, weighed, and fixed in formalin for histological analysis. A small incision was made in the trachea, and, using a blunt-end 18-gauge needle, the lungs were perfused through the incision with 1 ml of 5% India ink. After the lungs were removed, they were rinsed with tap water for 5 minutes to remove excess stain, and preserved in Fehetes solution for macroscopic and histological analysis. Surface lung metastasis were recognized as white spots (nodules) on a black-stained background, and counted with a dissected microscope. An eyepiece with micrometer was used to determine the size of individual pulmonary metastases.

Blood Analysis:

Direct cardiac puncture was used to obtain whole blood from the anesthetized mice. The blood was collected in EDTA-containing tubes, and White Blood Cell, Red Blood Cell, hematocrit, and hemoglobin quantities were determined using a cell counter

(Cell-dyn, Abbott Laboratories, Abbott Park, IL).

rhEPO Treatment of LLC cells in vitro

Subconfluent cultures were exposed to different concentrations of rhEPO diluted in 1x PBS. The difference in concentrations depended on the experiment being performed. For the cell proliferation assay, we used EPO concentrations ranging from 0 units (U) to 10U, or 0U to 100U. For VEGF and SDF-1 ELISA assays, we used EPO concentrations ranging from 0U to 100U. A control group from the same initial culture alone was not treated with EPO and will be known as (0 units EPO).

Cell Proliferation Assay

A cell proliferation assay was conducted to assess the effect of rhEPO on the proliferative capacity of LLC cell line. Untreated cells were harvested, centrifuged and resuspended in medium at a concentration of 4 x 10^4 cells/ml. Approximately 2,000 cells (50µl) were added to each well of a 96-well culture plate (Corning, Corning, NY). Cells were then treated in triplicates with 50µl of medium containing the appropriate concentration of rhEPO to give the intended final concentration. For each of the eight groups, 3 wells were designated blanks and contained only medium and the corresponding treatment. The plate was then incubated for 3 days in a 5% CO_2 humidified environment at 37°C. After 3 days incubation, cell proliferation was assessed by a CellTiter 96® A_{queous} Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI) using a colorimetric assay that determines the number of viable cells. Two solutions were included in the assay kit: 1) a tetrazolium compound (3-(4,5-

dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and 2) phenazine methosulfate; PMS). Phenazine methosulfate is an electron coupling reagent, while MTS is a solution that is converted to a soluble formazan product by bioreduction. The MTS and PMS solutions were mixed immediately before application to the cell cultures by adding 100µl of PMS to 2.0ml of MTS. The MTS/PMS solution (20µl) was then added to each well and the plate was incubated for 3 hours in a 5% CO_2 fully humidified environment at 37°C. Absorbance at an optical density of 490nm was determined using a VICTOR^3™ 1420 Multilabel Counter and accompanying software (PerkinElmer™, Turku, Finland), measuring the quantity of formazon, which is directly proportional to the number of viable cells in each well. The average absorbance was calculated for each group as well as for the blanks. The absorbance for each corresponding blank was subtracted from the average absorbance to give a corrected average absorbance. The corrected average absorbance for each of the six groups was expressed as a percent of the control group, with the control group being 100 percent cell proliferation.

VEGF Assay

The VEGF assay was used to quantitatively determine the concentrations of mouse Vascular Endothelial Growth Factor in supernatant of in vitro grown LLC cells, and plasma of tumor-bearing mice injected with LLC cells. For supernatant collection, Lewis lung carcinoma cells were seeded in six-well plates (1.0×10^5 cells per well) and incubated at 37°C. After 24 h, the cells were incubated in medium supplemented with a predetermined concentration of EPO for 24h to 72 h. The cell culture supernatant was

harvested and centrifuged at 800 x g for 3 min at 4°C to remove cell debris. The supernatant was either frozen at -20°C for VEGF assay later or assayed immediately using ELISA (Babak, 2000). ELISA was performed by using a pre-coated 96 well plate that is coated with monoclonal VEGF antibody. Assay diluent was added to each well in preparation for sample addition. Once diluent was added supernatant samples from LLC cells were put into each well, and the plate was then incubated for 2 h at room temperature. The plate was then washed five times using a wash buffer. Afterwards 100 µl of prepared VEGF conjugate was added to each well and left to incubate for 2 h at room temperature. Solution was then discarded and washed five times with PBS. Next 100 µl substrate solution or hydrogen peroxide/chromogen was added to each well for 30 min in the dark at room temperature. Finally a stop solution of 100 µl of 1 M H_3PO_4 was added to each well. The plate was then read at 450 nm and absorbance was determined using an ELISA plate reader. Absorbencies were compared to VEGF standard curve for the determination of actual VEGF amount (Fig. 5). For analysis of plasma VEGF, plasma samples from tumor-bearing animals were diluted 1:3 with calibrator diluent. VEGF in these samples was then assessed by ELISA as previously described.

SDF-1 Assay

The SDF-1 assay was used to quantitatively determine the concentrations of mouse Stromal Derived Factor-1 in supernatant of in vitro grown LLC cells, and plasma of tumor-bearing mice injected with LLC cells. For supernatant collection, Lewis lung carcinoma cells were seeded in six-well plates (1.0×10^5 cells per well) and incubated at 37°C. After 24 h, the cells were incubated in medium supplemented with a predetermined

concentration of EPO for 24h to 72 h. The cell culture supernatant was harvested and centrifuged at 800 x g for 3 min at 4°C to remove cell debris. The supernatant was either frozen at -20°C for SDF-1 analysis later or assayed immediately using ELISA (Babak, 2000). ELISA was performed by using a pre-coated 96 well plate that is coated with monoclonal SDF-1 antibody. Before the addition of supernatant samples, samples were diluted 1:10 with calibrator diluent. Assay diluent was added to each well in preparation for sample addition. Once diluent was added, supernatant samples from LLC cells were put into each well, and the plate was then incubated for 2.5 h at room temperature on a horizontal orbital microplate set at 500 rpm. To generate a standard curve, r-m-SDF-1 was used at a concentration range of 0.156 ng/ml to 10 ng/ml through 7 serial dilutions in calibrator diluent. After 2 h incubation at room temperature, plates were washed and 100 µl of a SDF-1 conjugate were added to each well. After 2 h incubation at room temperature on the shaker, plates were thoroughly washed of unbound antibody and 200 µl of substrate solution of a hydrogen peroxide/ chromogen solution were added into each well and plates were incubated at room temperature for 30 min. The reaction was stopped with 100 µl of stop solution or 1 M H_3PO_4. The plate was read at 450 nm and absorbance was determined using an automated ELISA plate reader. Absorbencies were compared to SDF-1 standard curve for the determination of actual SDF-1 values (Fig. 6). Assay background was determined as was indicated for flt3-L. For analysis of plasma SDF-1, plasma samples from tumor-bearing animals were diluted 1:2 with calibrator diluent. These samples were then assessed by ELISA as previously described.

Statistical Analysis

The Student's t-test, ANOVA, Kolmogorov-Smirnov test analyses were used to evaluate differences between groups. Statistically significant differences between groups were considered to have a p value of <0.05. Results are expressed as the mean $\pm$ standard error of more than 3 experiments. The Student's t-test was performed using Microsoft Excel. ANOVA was performed using the automated program from the physics department of The College of Saint Benedict and Saint John's University. The Kolmogorov-Smirnov test was used for analysis of tumor weight.

Figure 5. Vascular Endothelial Growth Factor ELISA Assay Standard Curve.

Known samples of VEGF ranged from 0 to 250 (pg/ml) were ran in duplicate. The absorbencies of these specimens were determined at 450 nm and a standard curve was established. Based on this graph, the (a) and (b) values of the equation ($y= ax + b$) which provides the correlation between absorbance and VEGF concentration were determined. Using this equation, we were able to determine the amount of VEGF present in each sample. The graph also gave us an R^2 value (0.9997) which showed us that the known samples were close in accuracy.

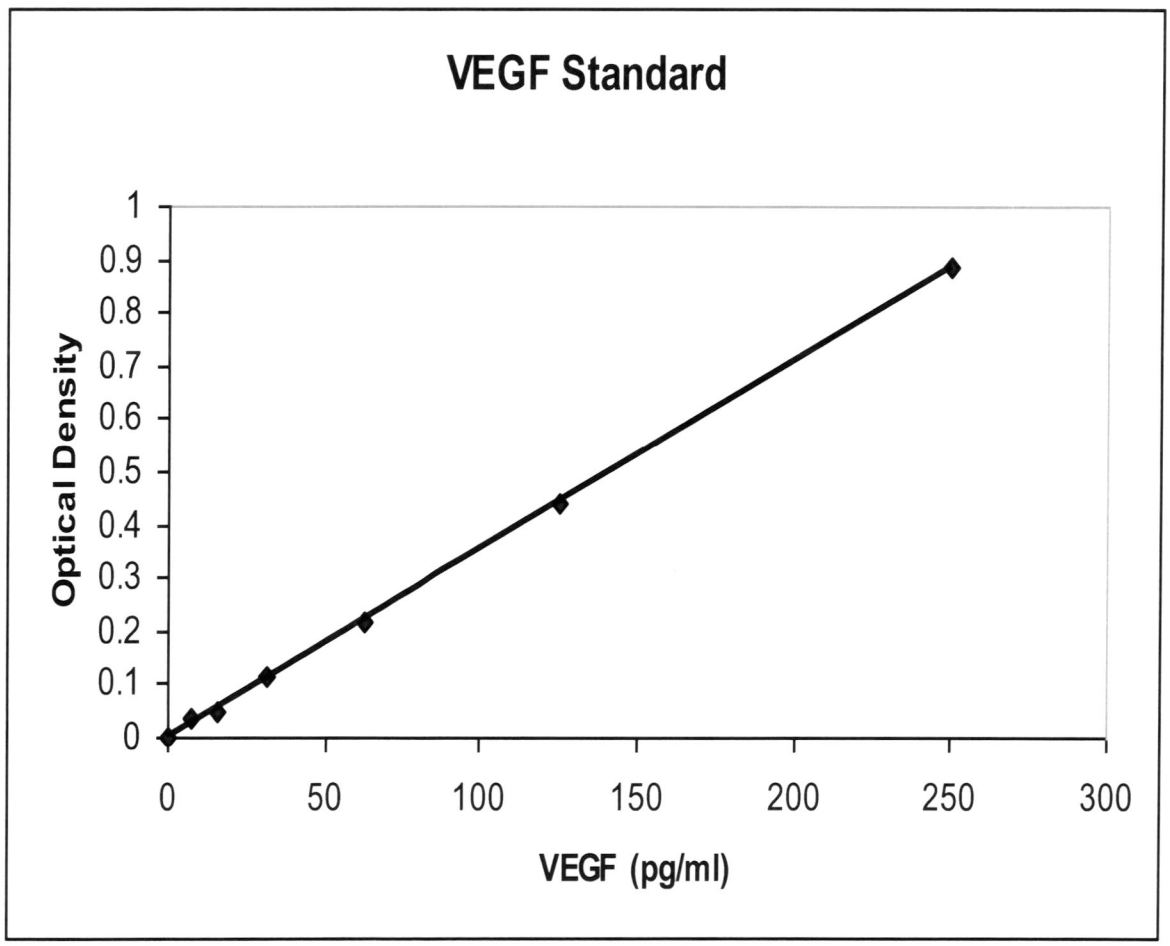
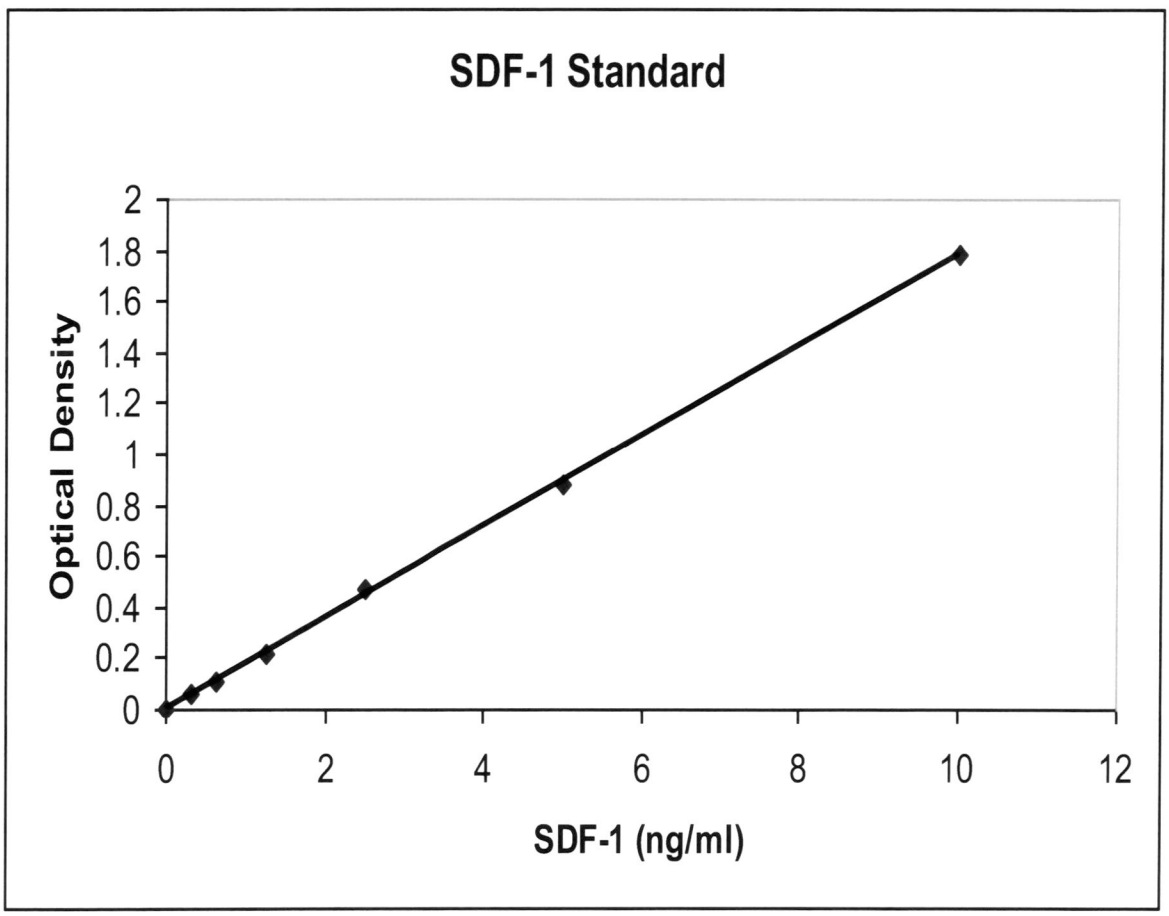

VEGF Standard
Optical Density
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
50
100
150
200
250
300
VEGF (pg/ml)

Figure 6. Stromal Derived Factor-1 ELISA Assay Standard Curve Known samples of SDF-1 (ng/ml) ranged from 0 to 10 (ng/ml) were ran in duplicate. The absorbencies of these specimens were determined at 450 nm and a standard curve was established. Based on this graph, the (a) and (b) values of the equation (y= ax+ b) which provides the correlation between absorbance and SDF-1 concentration were determined. Using this equation, we were able to determine the amount of SDF-1 present in each sample. The graph also gave us an R^2 value (0.9995) which showed us that the known samples were close in accuracy.


SDF-1 Standard
Optical Density
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
SDF-1 (ng/ml)

RESULTS

To determine the effects of Erythropoietin on in vivo grown tumors and in vitro grown cancer cells we used the Lewis Lung Carcinoma cell line. This cell line was chosen based on its growth potential. LLC cells have characteristics of being highly metastatic towards the lungs, being highly aggressive, and they express the EPO receptor.

In Vivo Studies

Effect of Erythropoietin on Red Blood Cells, Hematocrit and Hemoglobin Levels

We conducted blood analysis on blood samples from tumor-bearing mice treated with EPO and saline. Blood was analyzed using a cell counter program for Red Blood Cells (RBC), Hematocrit (Hct), and Hemoglobin (Hb) levels in both groups of mice. There were 80 samples of blood tested from the saline-treated group and 79 samples tested from the EPO-treated group. When EPO was injected into tumor-bearing mice there was an observed increase in RBC, Hct, and Hb levels (19.4%, 18.9%, 18.3%), respectively, compared to saline-treated mice (Fig. 7). This increase suggests that when EPO is added there will be a rise in the oxygen delivery capability of the test subject.

Effect of Erythropoietin on Tumor Weight

After 13 days of treatment the excised tumors of both groups were weighted and averaged. Overall, mice treated with EPO grew smaller tumors than the saline-treated animals (Fig. 8). Kolmogorov-Smirnov analysis indicated that there was a statistically significant difference in tumor growth between the two animal groups bearing tumors

ranged from 1g to 2.5g ($p<0.04$). However, there was no difference between the two experimental groups when tumor size was smaller than 1g or larger than 2.5g.

Effect of Erythropoietin on Pulmonary Metastases

In order to determine the effect of the EPO on pulmonary metastases stained lungs were examined for white spots (nodules) examined against a black stained background. The number of nodules for each animal were counted and averaged separately according to group. The EPO-treated group had an average of 3.9 nodules per lung, while the saline-treated group had an average of 4.15 nodules per lung. The number of lung metastases (tumor nodules) was similar among the EPO and Saline treatment groups. Tumor-bearing mice treated with saline had a very small increase in the number of metastases, but the difference between the two groups was not statistically significant ($p>0.05$, n.s.) (Fig. 9).

Effect of Erythropoietin on Tumor Volume of Lung Metastases

The volume of each metastatic nodule was individually measured in each animal of the experimental groups. These tumor volumes were added together for each mouse and averaged. The averaged numbers were used to determine a final tumor volume for the two groups. In assessing size of the metastatic tumors, we found that the average volume of lung nodules was 24% higher in saline-injected animals compared to EPO-treated mice (Fig. 10). However, this difference was not statistically significant ($p>0.05$).

Effect of Erythropoietin on Vascular Endothelial Growth Factor

Plasma from the collected blood sample was used to determine the amount of VEGF (pg/ml) in each tumor-bearing mouse. The VEGF level in each sample was determined by ELISA. Samples were run in duplicate for each individual mouse. In order to determine the amount of VEGF found in each sample a standard curve was created that allowed us to compare the absorbencies of our samples to the known amounts of VEGF (0 pg/ml -250 pg/ml) as described in Figure 5.The amount of VEGF present in the EPO-treated group was 18.02 pg/ml compared to the saline-treated group which had 22.08 pg/ml of VEGF (Fig. 11). This 18%.VEGF reduction was statistically significant ($p<0.02$).

Effect of Erythropoietin on SDF-1

Plasma from the collected blood sample was used to determine the amount of SDF-1 (ng/ml) in each mouse. SDF-1 in each sample was determined by ELISA.. Samples were run in duplicate for each individual mouse. In order to determine the amount of SDF-1 found in each sample a standard curve was created that allowed us to compare the absorbencies of our samples to the known amounts of SDF-1 (0-10 ng/ml) (see Fig. 6). The amount of SDF-1 found in the EPO-treated group was 0.6 ng/ml compared to the saline-treated group which had 0.56 ng/ml (Fig. 12). This difference was not statistically significant ($p>0.05$). The detection levels of SDF-1 were within the sensitivity range of the assay and our results were confirmed after performing multiple tests.

In Vitro Studies

Effect of Erythropoietin (0-100 units/ml) on Cell Proliferation

We conducted five independent cell proliferation assays to ascertain the effect of EPO on the proliferative capacity of LLC cells. Cells were plated in triplicate and treated with 0u, 1u, 2.5u, 5u, 10u, 25u, 50u, or 100u per ml of EPO for 3 days. Proliferation analysis indicated that the high concentrations of EPO had little or no effect on cell growth (Fig. 13). We observed a slight proliferative increase of 5% with 5u of EPO ($p>0.05$, n.s.).

Effect of Erythropoietin (0-10 units/ml) on Cell Proliferation

We conducted eight independent cell proliferation assay to investigate the slight increase observed during the cell proliferation assay that used (0-100 units/ml) of EPO. Cells were plated in cell triplicate and treated with 0u, 0.3u, 0.6u, 1.25u, 2.5u, 5u, 7.5u, or 10u per ml of EPO for 3 days. Proliferative analysis indicated that the low concentrations of EPO induced a slight increase (10%) in proliferation of the LLC cells when compared to the 0u control ($p>0.05$, n.s.) (Fig. 14).

Effect of Erythropoietin on VEGF secretion by LLC cells in culture

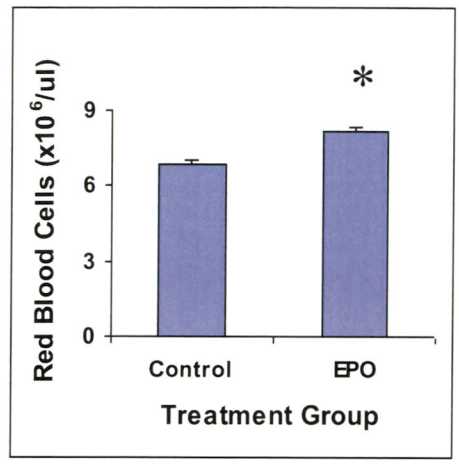
We conducted 4 independent experiments to test VEGF production by LLC cells after EPO treatment. In order to collect the supernatant, cells were plated in triplicate for either 24 hr, 48 hr, or 72 hr in 0u, 1u, 2.5u, 5u, 10u, 25u, 50u, or 100u per ml concentrations of EPO. ELISA assay was used to determine the amount of VEGF present

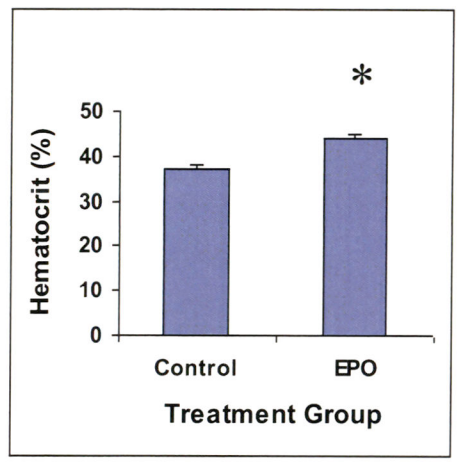
in the supernatant samples. Samples were run in duplicate. All LLC cell cultures treated with EPO for 24 hours had reduced levels of VEGF (Fig. 15). The largest decrease was observed when 50u of EPO were added and it was 22% ($p>0.05$, n.s.). Supernatant collected from day 2 showed an average decrease of 40.88 pg/ml or 24% of VEGF when compared to the 0u control ($p<0.05$) (Fig. 16). The largest decrease came when 50u of EPO were added. When using an ANOVA test it was determined that there was a statistically significant difference between 0u of EPO when compared to 1u ($p<0.05$), 2.5 ($p<0.04$), 50u ($p<0.03$), or 100u ($p<0.02$). The observed changes in VEGF appear to be dose-dependant as the levels of VEGF change varied.

Effect of Erythropoietin on SDF-1 from LLC cell supernatant

We conducted 2 independent SDF-1 ELISA assays to test SDF-1 production by LLC cells after EPO treatment. Supernatant was collected from LLC cells that were plated in triplicate for 24 hr, 48 hr, or 72 hr in the presence of various concentrations of EPO, 0u, 1u, 2.5u, 5u, 10u, 25u, 50u, or 100u per ml. ELISA analysis showed that there was no SDF-1 present in any of the samples showing that LLC cells did not secrete SDF-1 (Table 1). Furthermore, these results indicate that EPO was not capable of inducing SDF-1 expression. Clearly we were able to detect SDF-1 in the serum, but not in the supernatant (Fig. 12). Even though no SDF-1 was detected the assay used was highly sensitive, and after multiple tests run on the samples our results were confirmed.

Figure 7: Effect of Erythropoietin on Red Blood Cell, Hematocrit, and Hemoglobin levels of Tumor-bearing mice. Tumor-bearing C57BL/6 mice were treated either with saline (control) or erythropoietin (EPO) as described in Materials and Methods. Animals were sacrificed 13 days after subcutaneous injections of LLC cells. Blood was collected by cardiac puncture. Analysis of blood was done using Cell Dyn cell counter program. Graphs represent averages ± standard errors of 60-80 samples from each group. The EPO group was statistically different from the control group with a p-value < 0.0001 as denoted by (*).


Red Blood Cells (x10 6/ul)
9
6
3
0
*
Control
EPO
Treatment Group


Hematocrit (%)
50
40
30
20
10
0
*
Control
EPO
Treatment Group

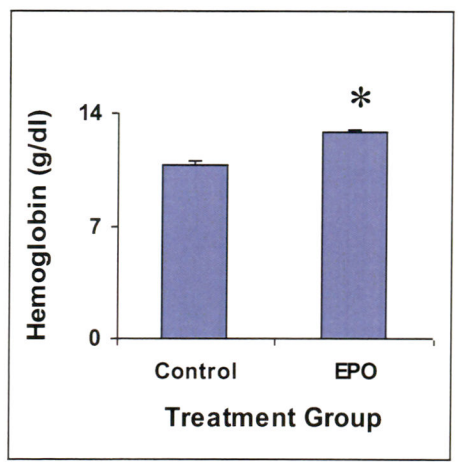

Hemoglobin (g/dl)
14
7
0
*
Control
EPO
Treatment Group

Figure 8. Effect of Erythropoietin on Tumor Weight. Tumor-bearing C57BL/6 mice were treated either with saline (control) or erythropoietin (EPO) as described in Materials and Methods. Animals were sacrificed 13 days after subcutaneous injections of LLC cells. Tumors were excised and excess dermis and muscle was removed. Once cleaned tumors were weighted and recorded. Data was represented using Kolmogorov-Smirnov Graphs of 60-80 samples from each group. The EPO group was statistically different from the control group with a p-value ($p < 0.05$).

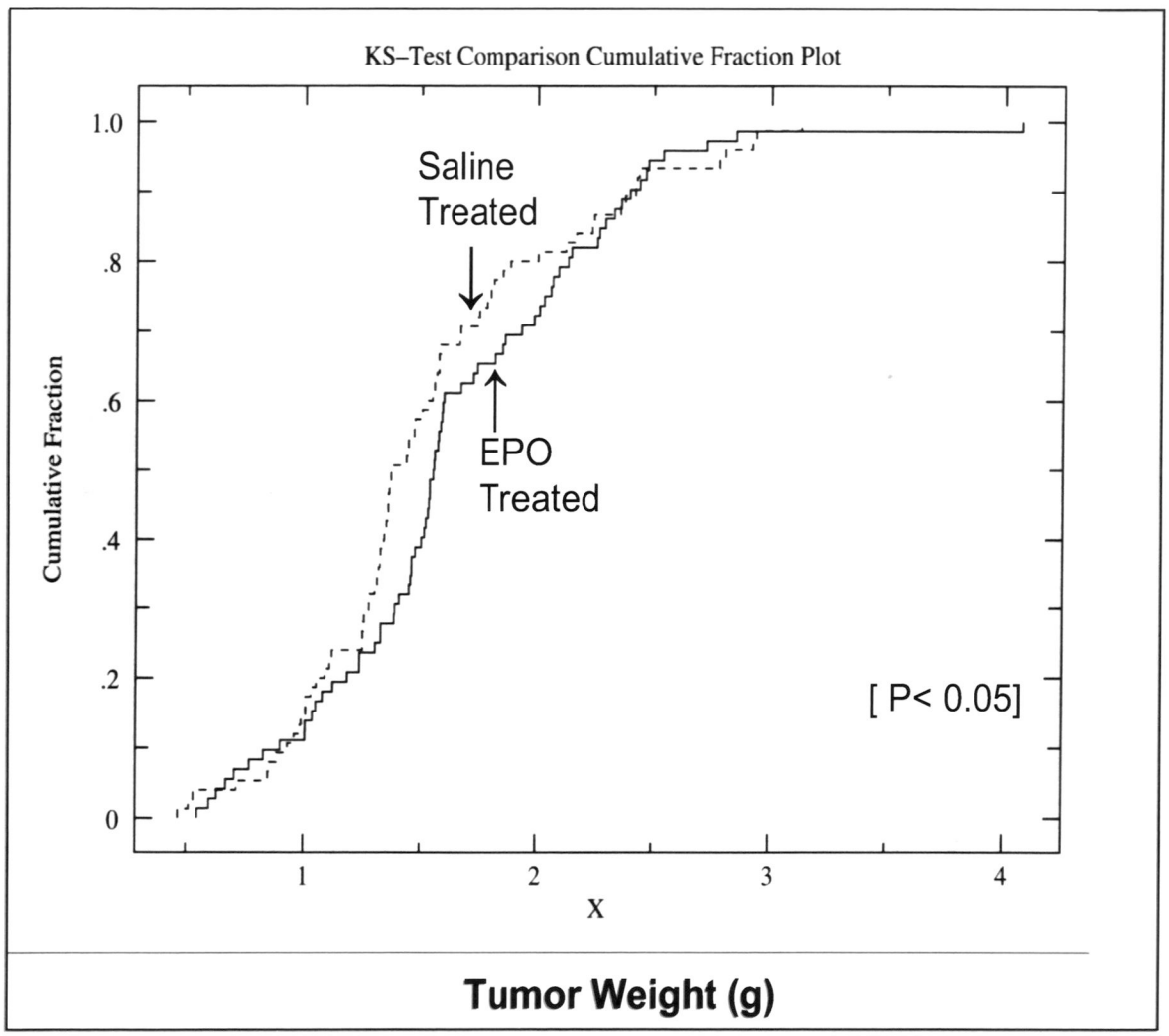

KS–Test Comparison Cumulative Fraction Plot
Cumulative Fraction
1.0
.8
.6
.4
.2
0
Saline
Treated
EPO
Treated
[P< 0.05]
1
2
3
4
X
Tumor Weight (g)

Figure 9. Effect of Erythropoietin on Pulmonary Metastases. Tumor-bearing C57BL/6 mice were treated either with saline (control) or erythropoietin (EPO) as described in Materials and Methods. Animals were sacrificed 13 days after subcutaneous injections of LLC cells. Lungs were injected with 5% India ink, and excised. Surface lung metastases were recognized as white spots (nodules) on a black-stained background. Nodules were counted and averaged for each mouse. Graphs represent averages ± standard errors of metastases per lung of 60-80 samples from each group. The EPO group was not statistically significant when compared to the saline treated group ($p>0.05$).

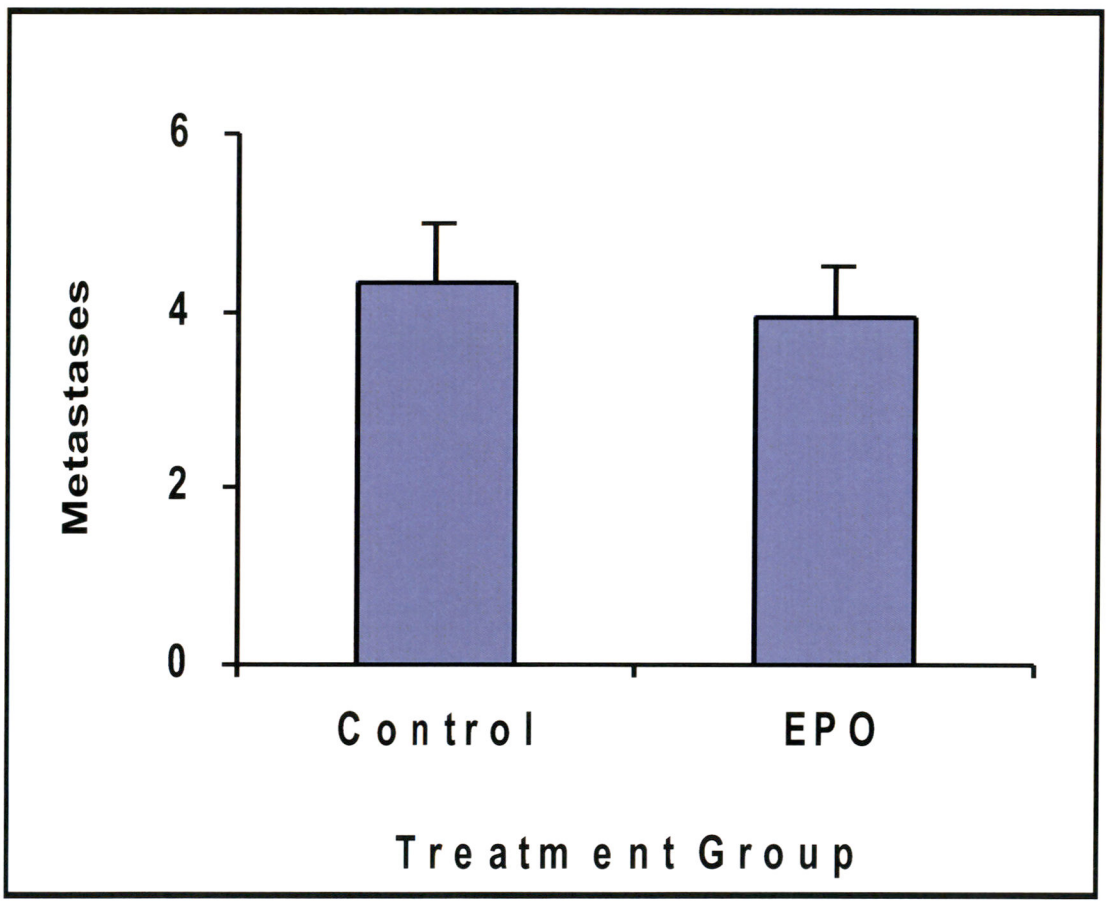

Metastases
6
4
2
0
Control
EPO
Treatment Group

Figure 10. Effect of Erythropoietin on Tumor Volume in Lung Metastases. Tumor-bearing C57BL/6 mice were treated either with saline (control) or erythropoietin (EPO) as described in Materials and Methods. Animals were sacrificed 13 days after subcutaneous injections of LLC cells. Lungs were injected with 5% India ink, and excised. Surface lung metastases were recognized as white spots (nodules) on a black-stained background. Nodules were measured under a dissecting microscope using a micrometer. Tumor volume was calculated using the formula =0.5 x Long diameter x Short Diameter x Short Diameter. Graphs represent averages ± standard errors of 60-80 samples from each group. The EPO group was not statistically significant different when compared to the saline treated group ($p>0.05$).

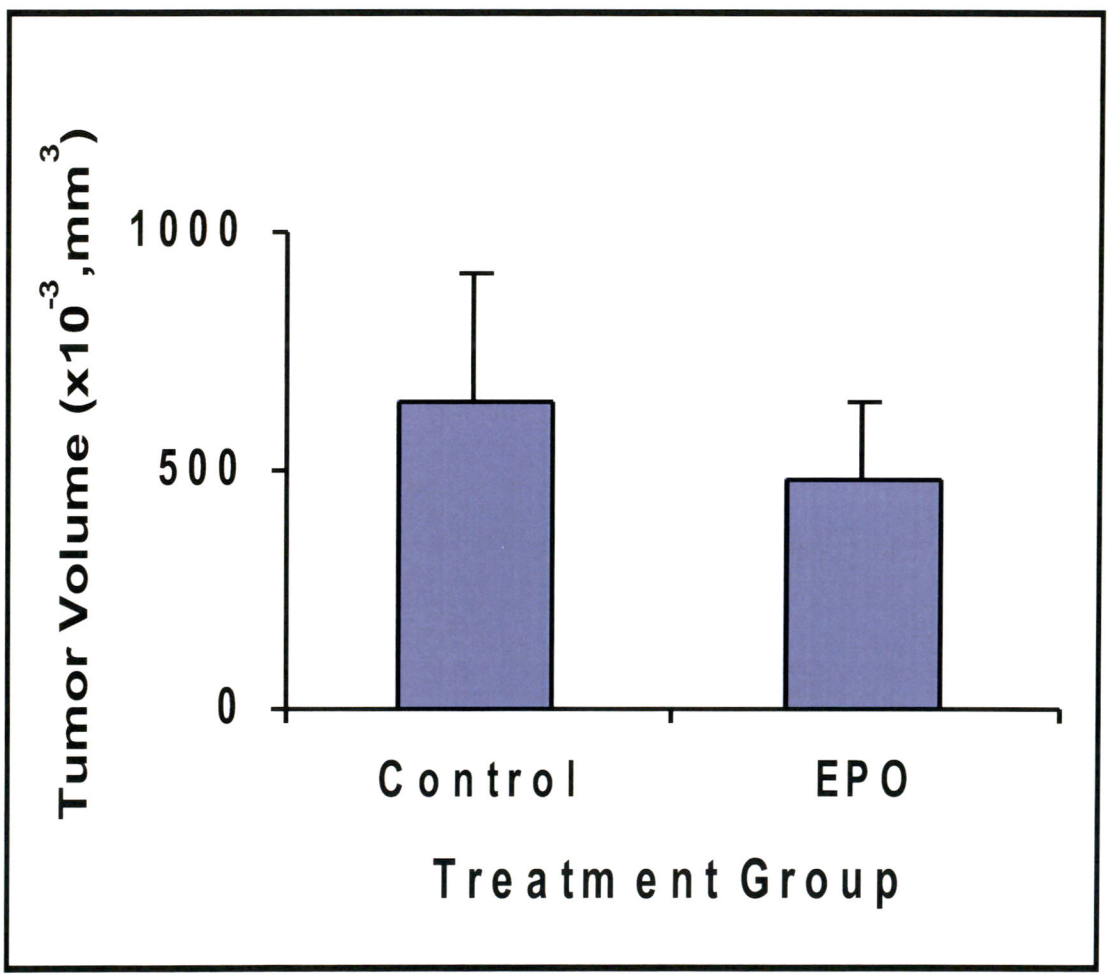

Tumor Volume (x10^{-3},mm^{3})
1000
500
0
Control
EPO
Treatment Group

Figure 11. Effect of Erythropoietin on Plasma VEGF Levels in Tumor-bearing Animals. Tumor-bearing C57BL/6 mice were treated either with saline (control) or erythropoietin (EPO) as described in Materials and Methods. Animals were sacrificed 13 days after subcutaneous injections of LLC cells. Blood was collected by cardiac puncture. Blood was centrifuged and plasma was removed for analysis. Plasma was tested for presence of VEGF using an ELISA assay. Graphs represent averages ± standard errors of 60-80 samples from each group. The EPO-treated group was statistically significant when compared to the saline treated group ($p<0.02$) as denoted by asterisk (*).

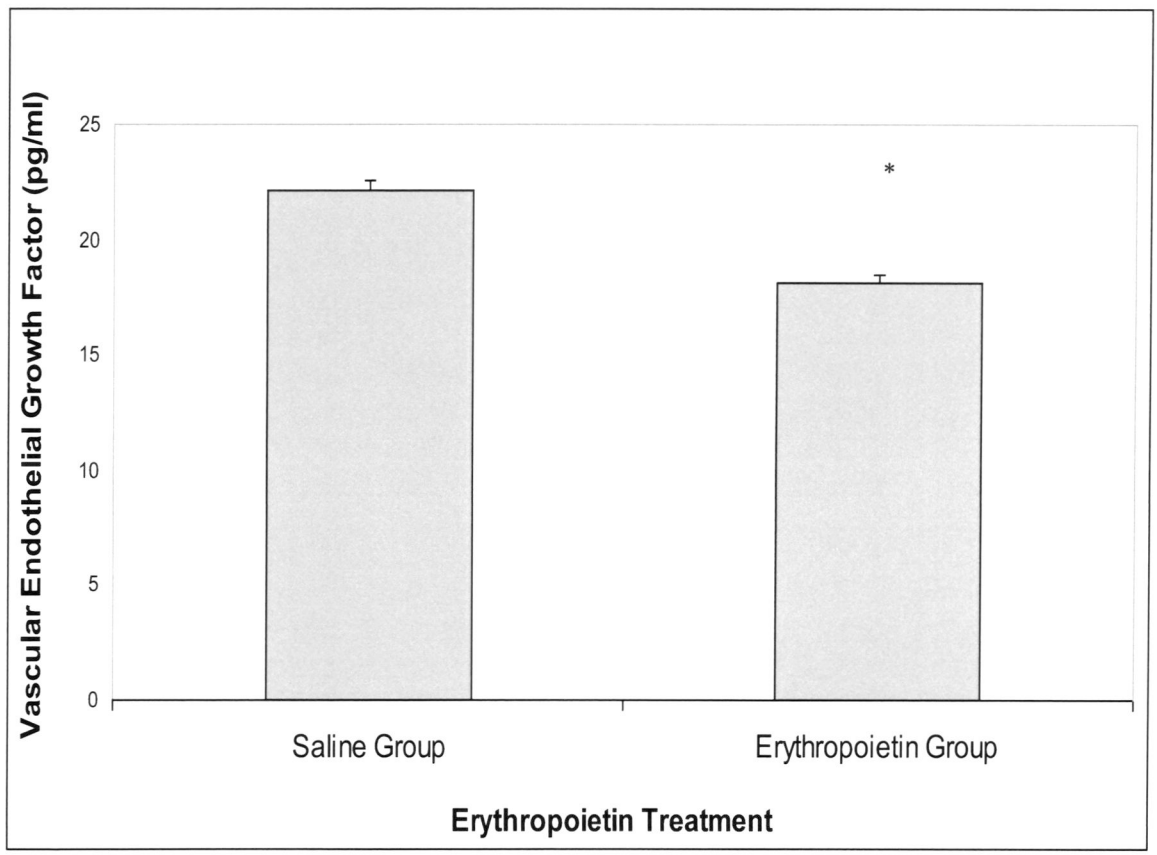

Vascular Endothelial Growth Factor (pg/ml)
25
20
15
10
5
0
*
Saline Group
Erythropoietin Group
Erythropoietin Treatment

Fig. 12. Effect of Erythropoietin on Plasma SDF-1 in Tumor-bearing animals. Tumor-bearing C57BL/6 mice were treated either with saline (control) or erythropoietin (EPO) as described in Materials and Methods. Animals were sacrificed 13 days after subcutaneous injections of LLC cells. Blood was collected by cardiac puncture. Blood was centrifuged and plasma was removed for analysis. Plasma was tested for presence of SDF-1 using SDF-1 ELISA assay. Graphs represent averages ± standard errors of 60-80 samples from each group. The EPO-treated group was not statistically significant when compared to the saline treated group ($p>0.05$).

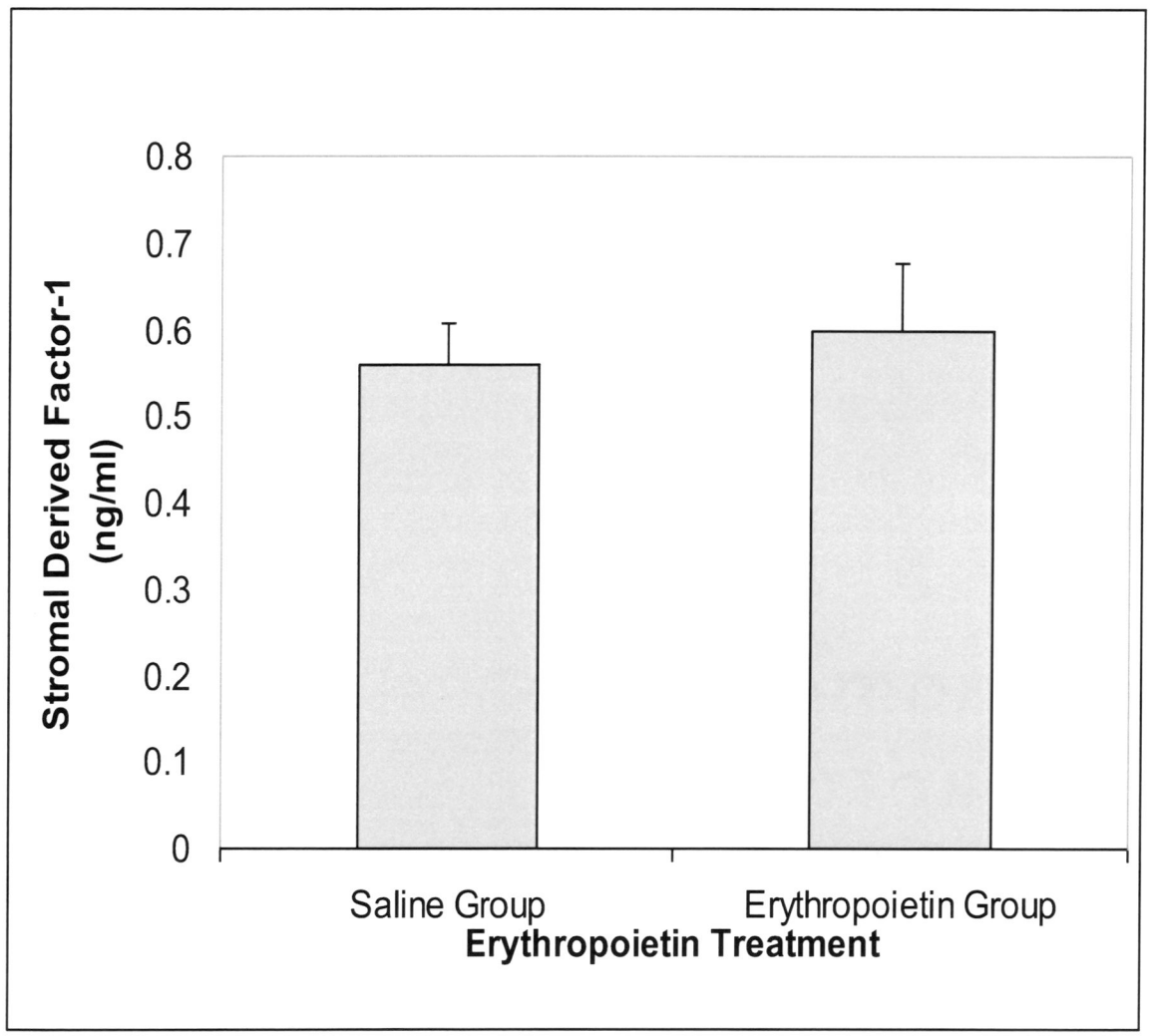

Stromal Derived Factor-1 (ng/ml)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Saline Group
Erythropoietin Group
Erythropoietin Treatment

Figure 13. Proliferation of LLC cells treated with 0-100 units/ml of Erythropoietin. Eight treatment groups (0u, 1u, 2.5u, 5u, 10u, 25u, 50u, 100u) were used to assess the effect of Erythropoietin on cell proliferation. Cells were treated for 3 days with EPO. Using the Promega Non-Radioactive colorimetric assay, the quantity of formazon (which is directly proportional to the number of viable cells in each well) was determined by measuring the optical density of the cultures at 490nm. Results are expressed as percent of the control group. Graphs represent averages $\pm$ standard errors of five independent experiments. None of the groups represented were statistically significant different when compared with each other ($p>0.05$).

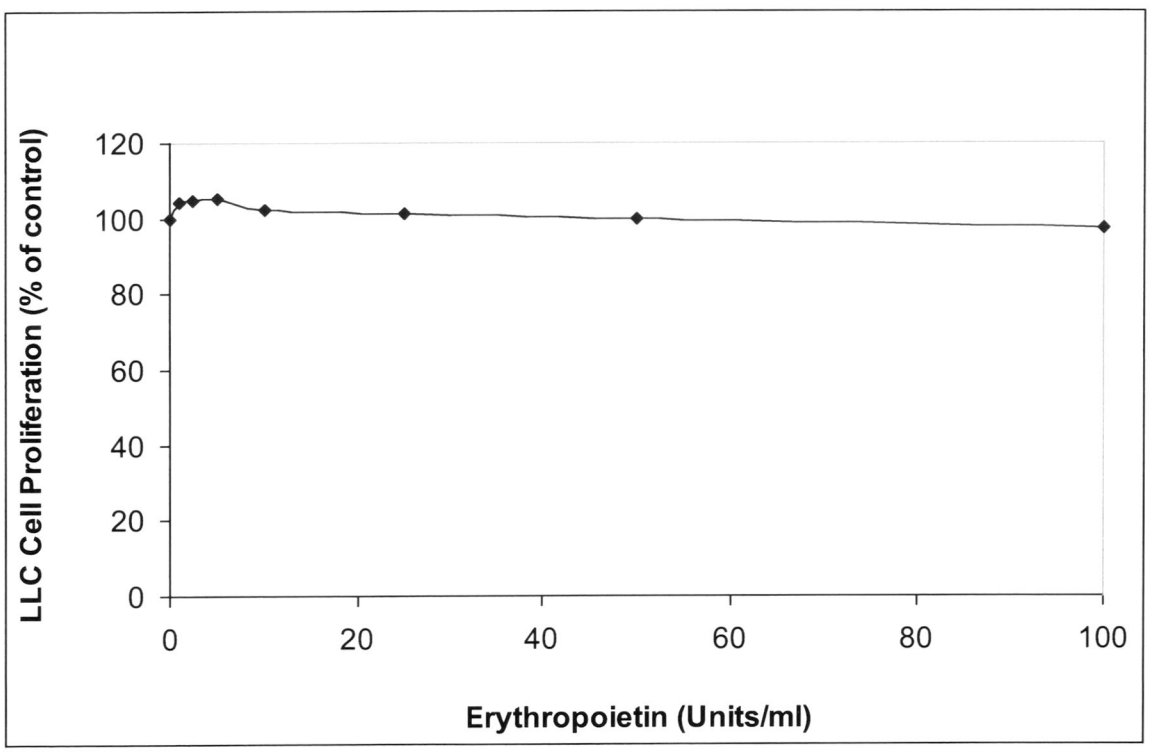

LLC Cell Proliferation (% of control)
120
100
80
60
40
20
0
0
20
40
60
80
100
Erythropoietin (Units/ml)

Figure 14. Proliferation of LLC cells treated with 0-10 units/ml of Erythropoietin. Eight treatment groups (0u, 0.3u, 0.6u, 1.25u, 2.5u, 5u, 7.5u, 10u) were used to assess the effect of Erythropoietin on cell proliferation. Cells were treated for 3 days with EPO. Using the Promega Non-Radioactive colorimetric assay, the quantity of formazon (which is directly proportional to the number of viable cells in each well) was determined by measuring optical density of the cultures at 490nm. Results are expressed as percent of the control group. Graphs represent averages $\pm$ standard errors of eight independent experiments. None of the groups represented were statistically significant different when compared with each other ($p>0.05$).

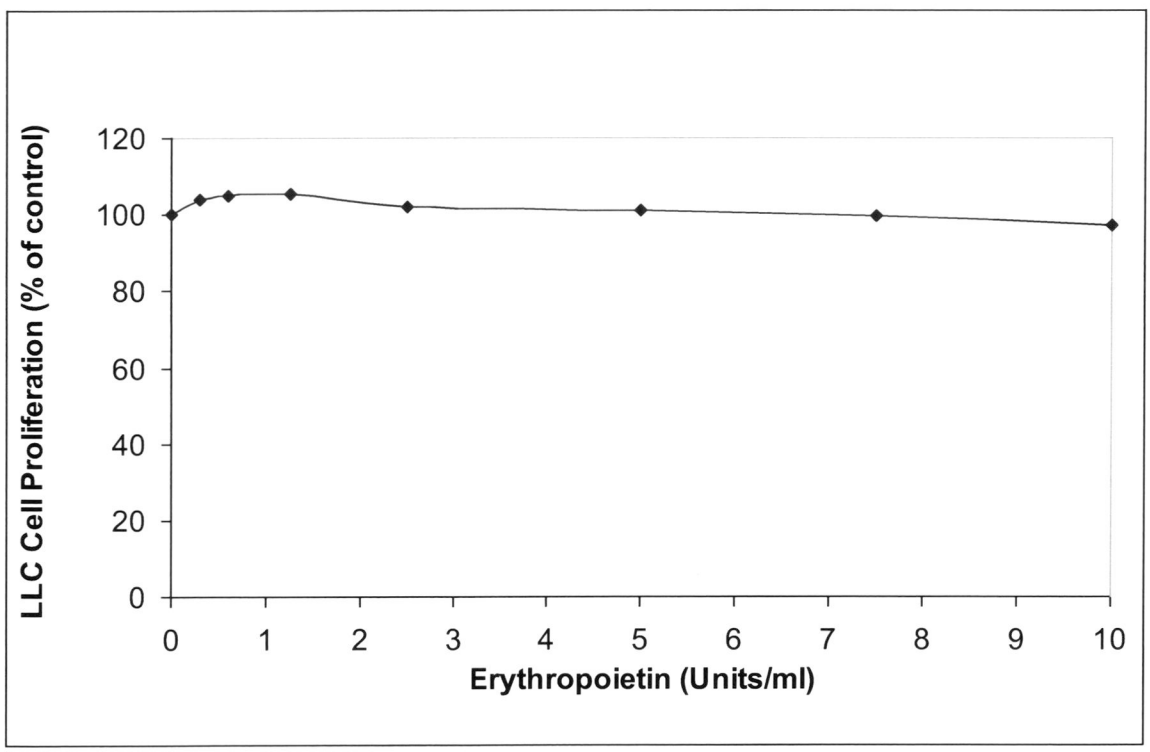

LLC Cell Proliferation (% of control)
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
10
Erythropoietin (Units/ml)

Figure 15. VEGF levels in Supernatant of LLC cells Treated with Erythropoietin for 1 Day. Eight treatment groups (0u, 1u, 2.5u, 5u, 10u, 25u, 50u, 100u) were used to assess the effect of Erythropoietin on the amount of VEGF (pg/ml) found in LLC cell supernatant 1 day following exposure to the hormone. ELISA assay was used to determine the amount of VEGF present in each sample. Supernatant samples were run in duplicate for each test. A standard curve was used to compare known amounts absorbencies to our sample absorbencies which allowed us to determine the amount of VEGF present. Graphs represent averages $\pm$ standard errors of four independent experiments. None of the groups represented were statistically significant different when compared with each other ($p>0.05$).

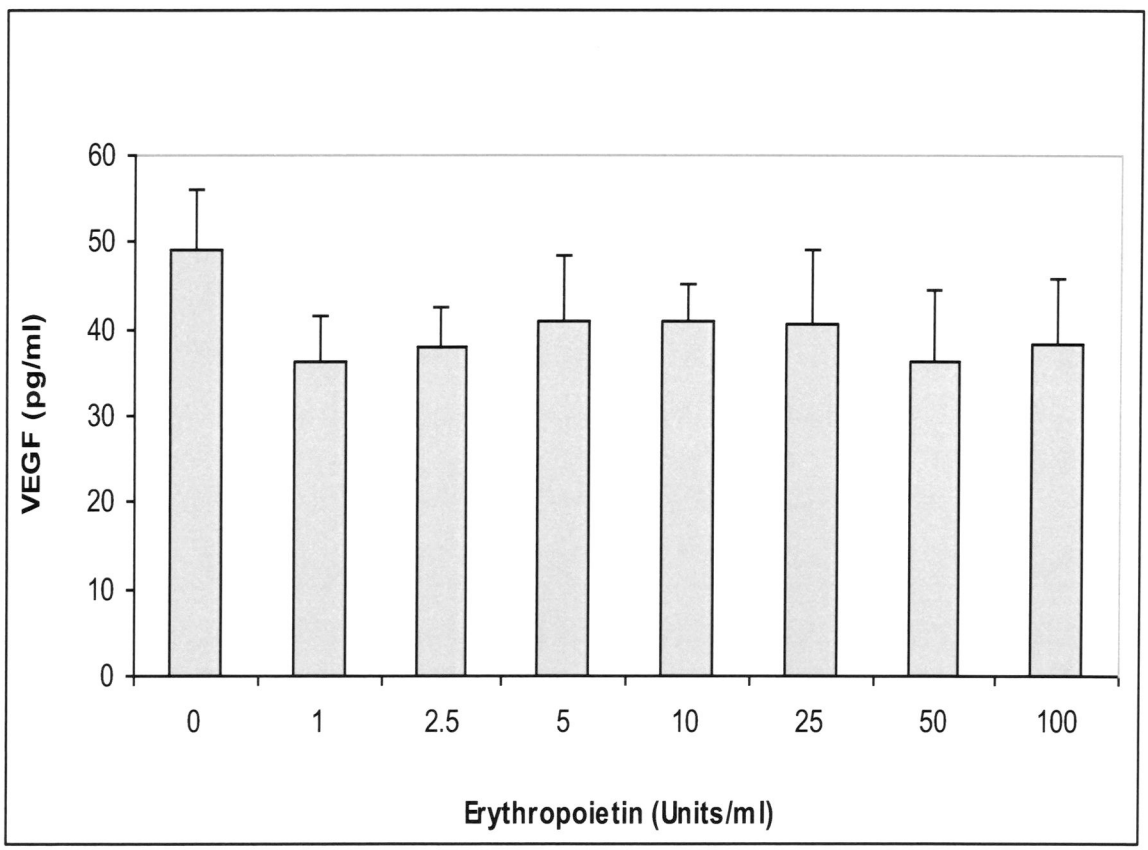

60
50
40
30
20
10
0
VEGF (pg/ml)
0
1
2.5
5
10
25
50
100
Erythropoietin (Units/ml)

Figure 16. VEGF levels in Supernatant of LLC cells Treated with Erythropoietin for 2 Days.. Eight treatment groups (0u, 1u, 2.5u, 5u, 10u, 25u, 50u, 100u) were used to assess the effect of Erythropoietin on the amount of VEGF (pg/ml) found in 2-Day LLC cell supernatant. ELISA assay was used to determine the amount of VEGF present in each sample. Supernatant samples were run in duplicate for each test. A standard curve was used to compare known amounts absorbencies to our sample absorbencies which allowed us to determine the amount of VEGF present. Graphs represent averages $\pm$ standard errors of four independent experiments. Some EPO-treated groups were statistically significant different when compared to the 0u EPO treated group ($p<0.05$) as denoted by asterisk (*).

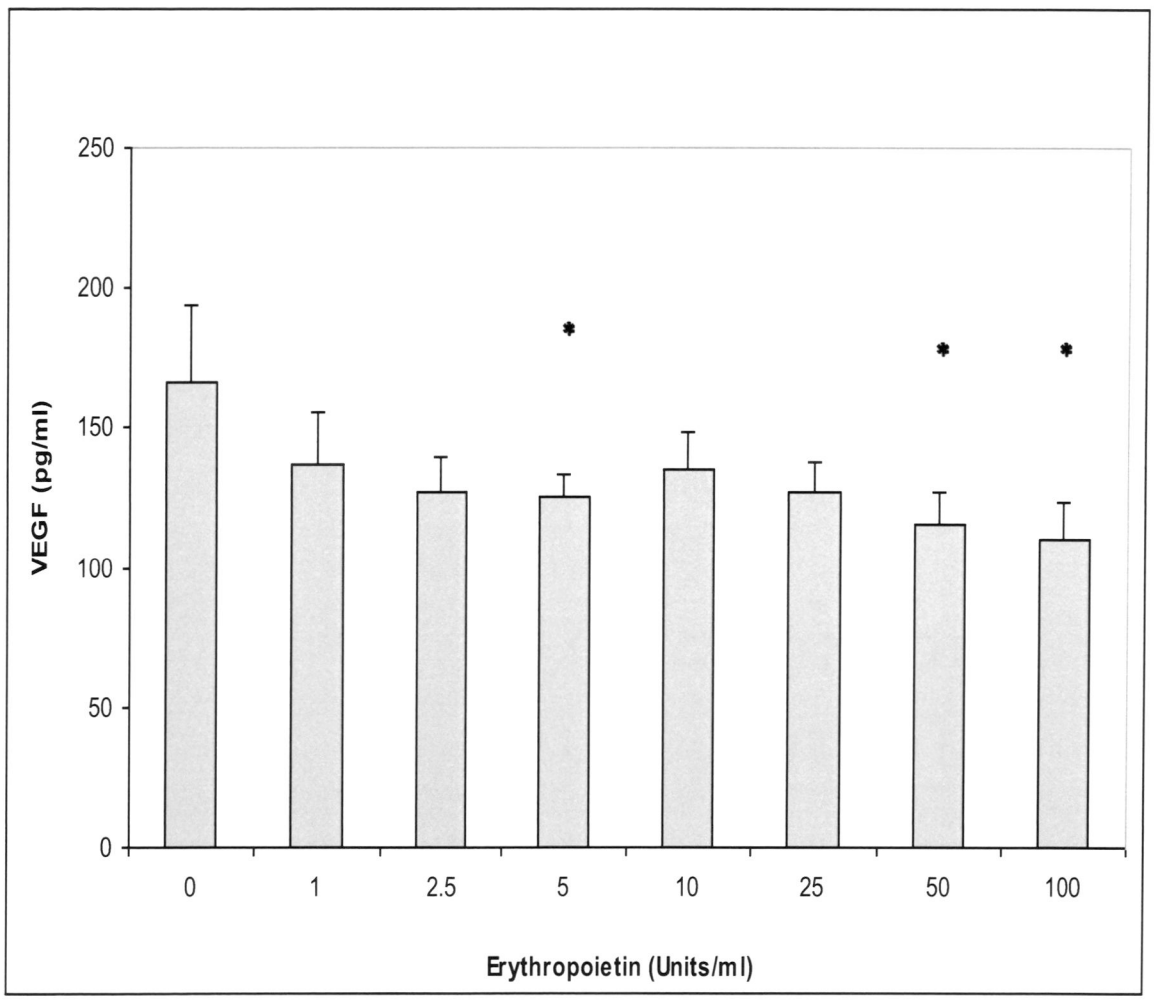

250
200
150
100
50
0
VEGF (pg/ml)
0
1
2.5
5
10
25
50
100
Erythropoietin (Units/ml)

Table 1: Effect of Erythropoietin on SDF-1 from LLC cell supernatant

Erythropoietin (Units/ml)	SDF-1 (ng/ml) present in supernatant
100u	0
50u	0
25u	0
10u	0
5u	0
2.5u	0
1.0u	0
0u	0

Eight treatment groups (0u, 1u, 2.5u, 5u, 10u, 25u, 50u, 100u) were used to assess the effect of Erythropoietin on the amount of SDF-1 (ng/ml) found in LLC cell supernatant. SDF-1 ELISA assay was used to determine the amount of SDF-1 present in each sample. Supernatant samples were run in duplicate for each test. A standard curve was used to compare known amounts absorbencies to our sample absorbencies which allowed us to determine the amount of SDF-1 present. Table represents averages of two independent experiments. Values were below the level of the assay's sensitivity for the detection of SDF-1.

DISCUSSION

The pleiotropic properties of Erythropoietin vary having effects on both hematopoietic and non-hematopoietic cells (Sigounas *et al.*, 2007). EPO's regulation of hematopoietic cells includes erythroid cell differentiation, EPOR maturation, and erythroid cell proliferation. These effects are observed as HIF-1 is released and EPO gene activation occurs in response to hypoxic stimulus. Transcriptional activation and mRNA stabilization of the EPO gene can be activated by low tissue oxygen tension (Hardee *et al.*, 2006). The action of EPO on non-hematopoietic endothelial cells appears to be two-fold; both promoting cell survival and growth as it does in erythroid cells, and directly promoting angiogenesis, a function unique to endothelial cells (Carlini, *et al.*, 1995). Previous studies on EPO have shown that its' addition can have a positive effect on tumor suppression (Mittleman *et al.*, 2001).

The role of vascular endothelial growth factor in angiogenesis has been extensively proven in previous research (Turner *et al.*, 2003). Hypoxia inducible factor-1, a key mediator of hypoxic responses is responsible for VEGF mRNA expression (Roy *et al.*, 2006). Neovascularization is required for adequate blood supply and availability of nutrients by tissues. For this reason, it can be all but assumed that both EPO and VEGF must have some type of interaction.

Adenovirus titration studies and dose-dependant effects with soluble VEGFR's and DC101 have shown that VEGF is required for the induction of erythropoiesis and hepatic synthesis of EPO. However, this effect was only observed when a near-complete blockade of VEGF had been established (Tam *et al*, 2006). Furthermore, hepatocyte-

produced VEGF contributes to the ongoing repression of adult hepatic EPO production. VEGF inhibitors may cause oxygen delivery and trophic endothelial activities to diminish, which might trigger compensatory production of EPO. Oxygen delivery would improve as a result of EPO production, and additionally an increase in endothelial cell maintenance could be observed as a result of its angiogenic activity (Fischer *et al.*, 2006). This study has raised the question as to how VEGF regulates EPO production. The authors speculated that VEGF might indirectly regulate EPO production through release of an unknown suppressor of EPO expression. In addition, this study raises questions on whether or not a regulatory loop of EPO and VEGF is plausible.

The data in this study supports our hypothesis that EPO may suppress tumor growth through a reduction of VEGF secretion by cancerous cells and through downregulation of VEGF in tumor-bearing animals. This was indicated by both our in vivo and in vitro data. First, we set out to test the effect of EPO on tumor-bearing mice injected with Lewis Lung Carcinoma cells. This highly metastatic and aggressive tumor line was used in order to emphasize that EPO's effects were not merely derived because of a weak tumor cell line. We first analyzed the effect that EPO would have when added for 13 days on the RBC, Hct, and Hb levels in the tumor-bearing mice. When compared against the tumor-bearing saline treated group, it was shown that there was a significant increase of RBC (19.4%), Hct (18.9%), and Hb (18.3%) levels when EPO was added ($p<0.05$). These results suggest that when EPO was added to the tumor-bearing animals, an increase of erythroid cell proliferation occurred, which would lead to the subsequent increase of RBC, Hct, and Hb levels. Independent experimentation performed on the effect of EPO to RBC and Hct provides similar evidence. When EPO was added for 15

days to A431 human epidermoid carcinoma tumor-bearing severe combined immunodeficient mice, a similar increase of RBC (17.8%) was shown, as well as an increase in Hct levels (14.8) ($p<0.05$) compared to tumor-bearing mice control (Tovari *et al.*, 2005). Additionally, the increase on the RBC, Hct, and Hb levels was also observed when saline-treated tumor-bearing mice were compared to EPO-treated tumor-bearing mice in another study testing the effect of EPO on Lewis Lung Carcinoma tumors (Sigounas *et al.*, 2004). These findings suggest that external EPO will increase the levels of these hematological parameters in tumor-bearing mice.

While EPO has been shown to have some angiogenic effects, which could possibly stimulate tumor metastases, increase tumor volume, and increase tumor weight, we observed that EPO could cause a decrease in this area. When compared to the saline-treated tumor-bearing mice group, our EPO-treated group exhibited smaller tumors than the saline-treated animals. However, the suppression of tumor growth was only apparent between tumor weights of 1g to 2.5g ($p<0.05$). This observed suppression indicated that EPO may possibly have a direct/indirect effect. The effect may be caused by a correction of O_2 availability due to EPO's addition (Sigounas *et al.*, 2004). The effect may also be from EPO's downregulation of HIF-1. When looking at the physiology of a solid tumor, one will find necrotic cells. Necrotic areas in tumors are the result of enormous local cell death. An example of this would be when the tumor environment had poor accessibility to oxygen and growth factors which occurs when tumors grow in size and their local vascularization becomes inadequate (Darzynkiewicz, 2000). We believe that there was decrease only in the tumor size of mid-weight tumors because EPO's effect may be tumor-weight dependant. Small-weight tumors may have not been affected because they

may have not been developed enough for EPO to have an effect. In this case, the growth factors that tumors need in order to grow may not have been upregulated to the point which EPO would have an affect. The large-weight tumors were not affected because they may have been too overgrown at that size, and their large center of necrotic cells could not be affected by EPO. Tumor metastasis was similar in both saline-treated and EPO treated groups. When the volume of tumors was analyzed, the data showed a (24%) increase in tumor volume of the saline-treated group when compared to the EPO treated group ($p>0.05$). On the other hand, a recently published study reported that the inhibition of EPO may be the cause of disrupted tumor growth and neovascularization. Hardee *et al* data indicated that when EPO was blocked using anti-EPO mab proteins, they observed an inhibition of tumor angiogenesis and progress as opposed to when EPO was added to the tumors. These data indicate that erythropoietin is an important angiogenic factor that regulates the induction of tumor cell-induced neovascularization and growth during the initial stages of tumorigenesis (Hardee *et al.*, 2007). This data is contrary to ours, as we are reporting that EPO is necessary in order to see the decrease in tumor growth. The differences between the two studies could be the possible explanation for the variance in results. The tumor-cell lines used in each study were different, which can lead to different results, as tumor-cell lines vary in many ways. In addition, the report fails to signify the fact that not only did anti-EPO mab proteins delay tumor angiogenesis and growth, but the addition of EPO did as well.

VEGF is an important angiogenic factor that will be causative in increasing tumor growth. Not only will VEGF promote the proliferation of endothelial cells, but also new blood vessel formation. Each of these steps appears to be critical in the growth regulation

and spread of cancer (Folkman *et al.*, 1995). "High-affinity, anti-VEGF antibodies have demonstrated a direct role for VEGF in tumor development" (Turner *et al.*, 2003). These anti-VEGF antibodies have shown potent inhibition of VEGF in three human tumor cell lines that were injected into nude mice (SK-LMS-1 leiomyosarcoma, G55 glioblastoma multiforme, and A673 rhabdomyosarcoma). VEGF was shown to be inhibited 70-95% after VEGF antibody administration (Kim *et al.*, 1993). A reduction in the growth of established tumors was observed with the use of Anti-VEGF monoclonal antibodies (Melnyk *et al.*, 1999). As we hypothesized, a downregulation of VEGF by addition of EPO may be the observed mechanism in which tumor growth is suppressed. Our in vitro and in vivo VEGF analysis supported this hypothesis. The plasma of EPO-treated tumor-bearing mice showed an 18% decrease in the amount of VEGF present when compared to our saline-treated group. A decrease in this VEGF level could possibly be due to a correction of anemia by EPO. Similar VEGF control levels (25 pg/ml) were observed in a study that injected the Lewis Lung carcinoma cells into mice (Okazaki *et al.*, 2005). Clinical data has shown that an increase in hemoglobin level will not only decrease the amount of VEGF in serum, but also increase survival rates of patients (Dunst, 2004). This increase of hemoglobin could be a direct result of epoetin alfa, which would not only raise the Hb level, but also additionally lower the amount of VEGF present. When accessing the effect of EPO in vitro, we observed similar effects. After treating our cells for one day with EPO of varying concentrations, we found that EPO may have caused a 22% decrease of VEGF levels when compared to the control, which contained no EPO. A similar decrease in VEGF levels was observed when we treated our cells with varying EPO concentrations for two days. EPO addition caused a 24% decrease in the amount of

VEGF secreted by the cells. The effects of EPO could be considered dose-dependant as the levels of VEGF decrease were different when compared to the other concentrations of EPO that were added. Both in vivo and in vitro studies on the effect of EPO on VEGF levels confirm that EPO may have a regulatory role on VEGF expression.

Our in vitro studies indicated that LLC cells do not secrete SDF-1 and the addition of EPO in cultures of these cells does not induce expression of the factor. Also, we found that there was no difference between EPO- and saline-treated animals regarding SDF-1 plasma levels. Nevertheless, intensive research has shown that the SDF-1 receptor, CXCR4, is involved in increasing the metastatic potential in a variety of cancers such as the colon (Zeelenberg *et al.*, 2003), and breast cancer (Muller *et al.*, 2001). Because the exogenous EPO had no effect on plasma SDF-1 production, the observed similarity in the numbers of lung metastases in the two experimental animal groups was expected. SDF-1 has been shown to be related to VEGF in regard to angiogenesis. "Immunohistochemical analysis showed that switching on VEGF expression in either heart or liver resulted in induction of SDF-1 protein, predominantly around blood vessels of the respective organ, and to a lesser extent also in the endothelium" (Grunewald *et al.*, 2006). This data showed that SDF-1 is induced downstream of VEGF. Murine tumor models have shown that the VEGF concentration in tumors will not be changed when CXCR4 was neutralized. This may suggest that additional chemokine systems are involved in the recruitment of VEGF-secreting cells (Guleng *et al.*, 2005).

Concern of EPO's ability to cause cell proliferation, and survival in non-erythroid cells has often been mentioned. It is known that the EPO receptor is not only expressed on hematopoietic cells, but other types as well. Because EPO is found to be expressed on

many types of tumor cell lines, questions are often raised about the possibility of EPO stimulating tumor growth. Additional concern lies with whether or not EPO could interfere with the action of antitumor therapies by either enhancing tumor proliferation rates or interfering with apoptotic cell death (Gewirtz *et al.*, 2006). Our study tested the effects of cell proliferation on Lewis Lung Carcinoma cells, which express the EPO receptor (Shannon *et al.*, 2005). We determined the effect EPO addition at different concentrations on LLC cells. Proliferation analysis indicated that the high concentrations of EPO had little or no effect on cell growth. Low concentrations of EPO (5 u/ml) induced a slight increase (10%) in proliferation of the LLC cells ($p>0.05$). These increases in proliferation were not significant, and it could be concluded that EPO does not affect the proliferation of these cells. This conclusion is supported by another experiment, which tested the proliferation of LLC cells with the addition of darbepoietin alfa, a similar erythropoietic molecule. The study provided the information that even though LLC cells express the EPO receptor, the cells were not stimulated by the addition of darbepoietin (Shannon *et al.*, 2005).

Some studies show that EPO can have a positive effect on the proliferation of tumor cell lines. *Westenfelder and Baranowski* reported an activation of the EPO receptor and increase in cell proliferation after EPO treatment of renal carcinoma cells. These renal carcinoma cells expressed the EPO receptor mRNA and protein (Westenfelder and Baranowski, 2000). According to their studies, these findings suggested that recombinant EPO addition to renal cell carcinoma patients has the potential to modify tumor growth by stimulating cell proliferation and by possibly supporting angiogenesis. Despite this report, many studies have shown that tumor cell lines expressing the EPO receptor do not

have an increase in cell proliferation. One investigation tested the effects of recombinant EPO on 10 different cell lines derived from solid tumors and hematological malignancies. The effects of EPO were illustrated by testing the effects of this factor on clonogenic growth and DNA synthesis (Roset *et al*, 1993). Results of that study showed that recombinant EPO, even at high concentrations, had no effect on either colony growth or DNA synthesis in the cell lines tested, including K-562, HEL, HL-60, PLB 985, KG-1, H69, N417, and OCUM-1 (Roset *et al*, 1993). Other studies done on different tumor cell lines such as R3230 tumors showed no enhancement of growth when recombinant EPO was added over a 3 to 4 week period. One possibility is that more than EPO alone is required in order to see EPO's pro-angiogenic properties and modest growth-promoting effects. EPO may also be required to be sustained at a high local concentration in the tumor microenvironment, which cannot be achieved by systemic, intermittent recombinant EPO administration (Hardee, 2007). These conflicting results are what give rise to the speculation found in the medical community over the use of EPO. Despite this controversy, our studies show that EPO will not promote the growth of LLC cells.

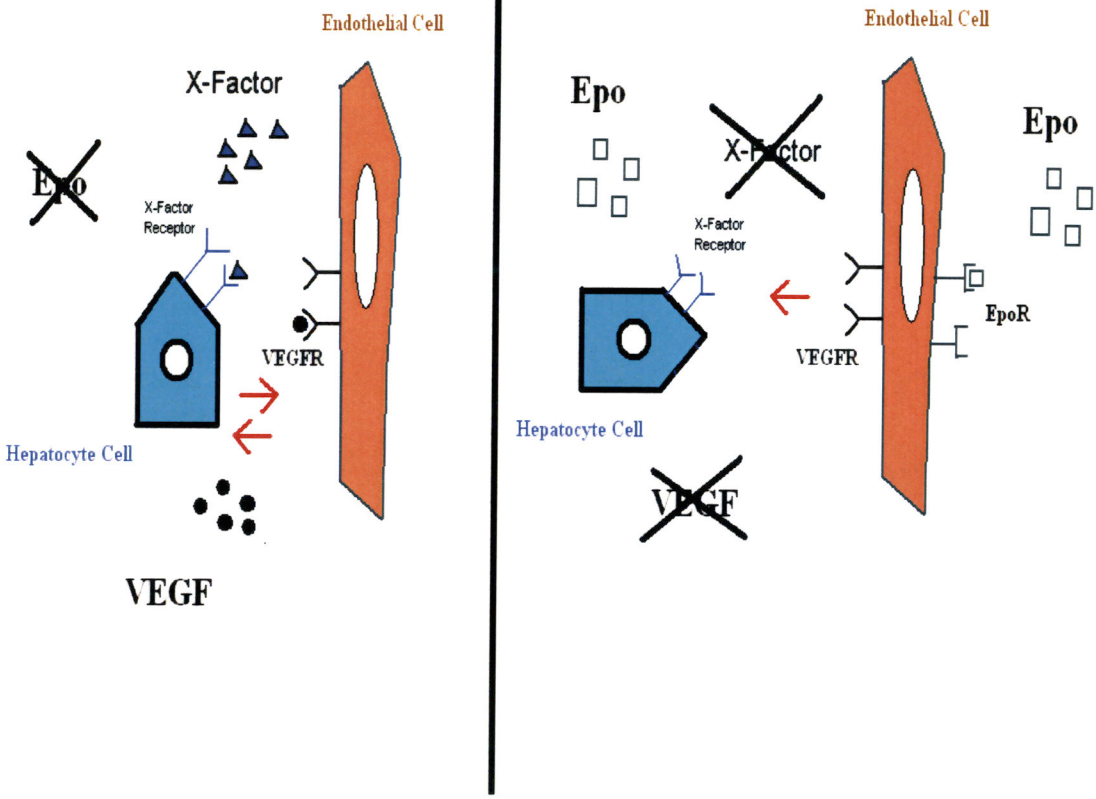
In addition to the previously mentioned concerns about tumor cell proliferation in response to EPO, other questions have been raised. One recent clinical trial with breast cancer patients indicated a deleterious effect of EPO therapy on patient survival (Leyland-Jones, 2003). Harmful effects were observed in one study that looked at the effect of EPO on patients suffering from neck squamous cell carcinoma. This study indicated that EPO therapy for chemotherapy-induced anemia is potentially harmful to cancer patients (Henke, 2003). However, these clinical trials have been criticized in regard to their design in a recent review. Supported by preclinical evidence, the effect of

any erythropoiesis stimulating proteins (ESP) (which were used in previously mentioned studies) on tumor outcomes may depend on baseline hemoglobin levels, with different effects when anemic and non-anemic individuals are treated (Glaspy, 2005). On the other hand, there have been just as many clinical studies showing the advantages of using EPO. One clinical study has shown that the addition of EPO was able to enhance breast cancer patients' response to chemotherapy (Larsson, 2005). Another clinical trial was able to suggest that EPO may reduce the risk of tumor progression in solid tumors (Pronzato, 2005). This study went on to state that the addition was able to improve the quality of life of anemic patients with cancer, decreasing fatigue and improving the ability to perform usual daily activities. In addition, EPO was able to prevent the onset of anemia and reduce the requirements for transfusion in patients with a high risk of developing anemia during chemotherapy, such as those receiving platinum-based regimens (Pronzato, 2005). These studies, in addition to ones about EPO's effect on tumor growth and EPO's effect on chemotherapy, show the importance of understanding the mechanisms by which EPO works. Our studies suggest that EPO affects tumor growth in LLC cells by downregulating the amount of VEGF produced by these cells.

In this study, we have provided information that EPO may have a positive effect on the suppression of tumor growth via downregulation of VEGF. We have proposed that the interaction between VEGF-EPO may be a negative regulation loop in which one angiogenic factor may decrease or increase the expression of the other and vise versa as indicated in Figure 17. Future studies should explore this mechanism as to what other factors may affect it. In addition EPO treatment of patients with tumor growth must be

reevaluated as we have shown that EPO can cause a decrease in the VEGF levels of plasma, and ultimately a decrease in tumor growth.

Figure 17. Proposed VEGF/EPO negative regulation loop. We are proposing that the interaction between VEGF and EPO may be a negative regulation loop in which the two factors may upregulate and downregulate each other. When EPO is added it will bind to endothelial cells and inhibit the release of the unknown X-factor. This will then cause a decrease in the secretion of VEGF.


Endothelial Cell
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Hepatocyte Cell
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Endothelial Cell
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VEGF

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