EXPRESSION OF VEGF, CXCR4, CD133, AND HIF-1 ALPHA IN OSTEOSARCOMA

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The following faculty members have examined the final copy of this thesis for form and content, and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science, with a major in Biological Sciences.

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ABSTRACT

Osteosarcoma is the most common primary malignant bone tumor seen in orthopaedic practice. Despite intensive treatments of chemotherapy and surgical excision, the current five-year survival rate is only 60% to 78%. We investigated the correlation of vascular endothelial growth factor (VEGF) expression with osteosarcoma growth and metastasis and explored a therapeutic strategy by blocking VEGF. Recent studies suggest that a subpopulation of cancer stem cells (CSCs) plays a role in cancer survival and metastasis. We *hypothesized* that CD133+ osteosarcoma stem cells are present within the osteosarcoma cell lines and they will be less responsive to VEGF blockage treatment than other tumor cells. Using polymerase chain reaction and immunocytochemistry techniques, we studied the expression of VEGF, CXCR4, and cancer stem cell marker CD133 at transcriptional and post-translational levels among different VEGF-expressing osteosarcoma cells. We examined the tumor cell growth patterns and compared the subpopulation ratio of CD133+ cells after VEGF blockage with VEGF inhibitor SU4312.

We also investigated the effects of hypoxia on the proliferation and apoptosis in 1547 osteosarcoma cell line. We chose 1547 cells because of their rapid growth compared to the other osteosarcoma cell lines we used. Hypoxia-inducible factor-1 (HIF-1) activates a series of genes including VEGF which contribute to tumor aggressiveness. Thus we examined the relationship between the expression of HIF-1α or VEGF and osteosarcoma metabolism. Results showed a decline in cell proliferation and an increase in apoptosis under hypoxia. There were more significant fluctuations after inhibition of HIF-1a and/or VEGF which suggested that HIF-1a and VEGF were involved in the promotion of proliferation and anti-apoptosis of 1547 cells under hypoxia or normoxia.

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LIST OF ABBREVIATIONS / NOMENCLATURE

CD133	Cluster of Differentiation 133
cDNA	Complementary DNA
CO_2	Carbon Dioxide
CREB	cAMP-Responsive Element Binding Protein
CSC	Cancer Stem Cells
CXCL12	C-X-C Ligand 12
CXCR4	C-X-C Chemokine Receptor Type 4
dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytidine Triphosphate
dGTP	Deoxguanosine Triphosphate
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
dUTP	Deoxyuridine 5-Triphosphate
ELISA	Enzyme Linked Immunosorbent Assay
HIF-1	Hypoxia Inducible Factor 1
HRE	Hypoxia Responsive Element
ICC	Immunocytochemistry
mRNA	Messenger RNA
MVD	Microvessel density
OS	Overall Survival
OSJ	Osteosarcoma of the Jaw
PBS	Phosphate-Buffered Saline

PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT	Room Temperature
SDF-1	Stromal Cell Derived Factor-1
siRNA	Synthetic Small Interfering RNA
TUNEL	Terminal DNA Breakpoints In Situ 3-Hydroxyl End Labeling
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

LIST OF SYMBOLS

α	Alpha
°C	Degree Celsius
µ	Micro
L	1 Liter
UV	Ultraviolet
m	Milli
g	Gram
H_2O	Water
w/v	Weight/Volume
β	Beta
5’	Five Prime
3’	Three Prime
nm	Nanometer (Wavelength)

CHAPTER 1

INTRODUCTION

Osteosarcoma is the most common primary malignant bone tumor that affects adolescents and young adults during periods of rapid skeletal growth [1-5]. Despite the relative high prevalence and mortality associated with osteosarcoma [1], the precise cell origin of osteosarcoma is not known, but it may be derived from mesenchymal stem cells with partial osteoblastic lineage. Osteosarcoma normally develops in the distal femur, proximal tibia, or proximal humerus [3]. Current treatments involving aggressive chemotherapy and wide surgical excision of the tumor significantly improve the prognosis of osteosarcoma patients. However, the 5-year overall survival (OS) rate associated with this disease remains only 60-78%, and patients with metastases at time of diagnosis suffer poorer prognosis such that the OS rate declines to 30% [3, 4, 6]. Additionally, about half of osteosarcoma patients become resistant to chemotherapy and thus their clinical outlook is challenging [3, 6]. Therefore, new therapies for osteosarcoma are needed.

After decades of clinical chemotherapy treatment for osteosarcoma, the common agents used are methotrexate, doxorubicin, and cisplatin with or without ifosfamide [7]. Osteosarcoma therapy also involves surgical excision of the primary and metastatic tumor. Most patients receive neo-adjuvant chemotherapy followed by surgery to save the limb. As mentioned above, the five-year survival rate for patients with lung metastasis is only 25-50%. New directions for the treatment strategies are presently being explored.

Current research on osteosarcoma focused on known molecular pathways tends to identify molecular markers with the potential to develop new treatment strategies. Studies show that a high metastatic potential and the relapse rate of osteosarcoma are correlated with high

levels of vascularization [3, 8]. In order for the tumor to grow and metastasize it must create its own blood supply. One process that is responsible for this is angiogenesis. One major angiogenic factor is overexpression of vascular endothelial growth factor (VEGF), which causes endothelial cell proliferation. Another target is chemokine receptor type 4 (CXCR4) due to its involvement in tumorigenesis and metastasis [22]. CD133, known as a cancer stem cell marker in many types of solid cancers, may also serve as a prognostic factor in cancer [25, 26]. Lastly, HIF-1 regulates the cellular response to hypoxia and is involved in tumor growth [9]. Our ***hypothesis*** is that these growth factors and mediators play critical roles in osteosarcoma growth and metastasis and therefore a better understanding of their interactions may lead to specific therapies to treat this deadly cancer.

CHAPTER 2

LITERATURE REVIEW

2.1 Angiogenesis in Cancer Development

Research suggests that cancer development can be modified by various proteins that are involved in angiogenesis [4]. Angiogenesis, the development of blood vessels, is responsible for delivering nutrients, oxygen, and growth factors to the tumor and provides a route for metastasis [10]. It is understood that tumor angiogenesis depends on the balance between promoters and inhibitors of angiogenesis. Pro-angiogenic factors are up-regulated in most tumors and with poor prognosis [3, 11]. Many pro-angiogenic factors are known [12], and the most important one is vascular endothelial growth factor A (VEGF-A), a member of the VEGF family. [4].

2.2 VEGF

The VEGF family of cytokines includes VEGF-A, VEGF-B, VEGF-C, and VEGF-D, each operating in different tissues to regulate angiogenesis and/or lymphangiogenesis [13]. Each member has specific functions and effects that are facilitated by the receptor tyrosine kinases VEGFR-1, VEGFR-2, and VEGFR-3. The founding member, VEGF-A, is involved in vasculogenesis and angiogenesis [14]. Its key role in tumor angiogenesis and metastasis has been acknowledged in many cancers [14]. VEGF-A is the most potent among these cytokines with angiogenic, mitogenic, and vascular permeability-enhancing functions specific for endothelial cells [15]. While the expression of VEGF-A in cancer has been comprehensively studied, the roles of the other VEGF family members in tumor angiogenesis and metastasis are poorly understood [14]. VEGF-C was originally recognized as a ligand of VEGFR-3, which exhibited sequence similarity to VEGFR-1 and VEGFR-2. Because expression of VEGFR-3 is limited to lymphatic endothelium, the main function of VEGF-C seems to be in regulating the growth of

lymphatic vessels. New studies propose that VEGF-C might stimulate the spread of cancer cells through lymphatic channels [42, 43]. VEGF-C and VEGF-D are believed to have similar biological functions because, through database searches for sequence homology, they share similar sequences.

VEGF binding to its receptor initiates a tyrosine kinase cascade. As a result, new blood vessels infiltrate into the tumor [16]. Since solid tumor formation depends on angiogenesis [2], various approaches have been established to target VEGF pathways as possible cancer therapies [3]. In epithelial cancers such as breast, lung, or colon, high VEGF expression is associated with negative treatment outcomes [4]. Consequently, VEGF or VEGF receptor (VEGFR) are targeted in anticancer therapies. Bevacizumab is an FDA-approved recombinant humanized monoclonal antibody that blocks angiogenesis by inhibiting vascular endothelial growth factor A (VEGF-A) [17], while another FDA approved chemo-drug sunitinib targets multiple receptor tyrosine kinases (RTKs) including VEGF receptors to treat various types of cancers [18].

In osteosarcoma, mechanisms that control the invasion of tumor cells into nearby tissues, the microcirculatory system, and lymphogenic spread, followed by development of secondary tumors are not well understood. Although high metastasis and recurrence rate [3, 8] are linked to increased vascularization levels. Thus, research high VEGF expression and high vascularization in osteosarcoma are associated with poor prognosis.

2.3 VEGF in Osteosarcoma

The role of VEGF in osteosarcoma was assessed in numerous studies. Research by Lugowska evaluated the prognostic role of VEGF-A in biopsy samples of osteosarcoma patients that were followed up for a 5 year period [4]. Immunohistochemistry was used to assess VEGF-A in pre-treatment biopsy samples of 91 patients with primary, high grade, non-metastatic

osteosarcoma. Lugowska's results showed that high expression of VEGF-A was detected in 39% of cases and was correlated with patients under 14 years of age and with tumor size larger than 8 cm. VEGF-A expression was also linked with decreased survival and progression-free survival. This data suggests that VEGF expression in osteosarcoma cells triggers angiogenesis and leads to more aggressive tumor behavior in clinical situations. This study concluded that VEGF-A expression in biopsy samples serves as a prognostic marker for predicting the progression and outcome of osteosarcoma. Further assessment of VEGF-A may determine different risk levels and define specific target therapies.

A previous study in our laboratory tried to define whether or not there was a correlation between VEGF-A expression and clinical osteosarcoma tumor development and metastasis [3]. It involved fifty- four cases of osteosarcoma patients who were treated during a ten-year period and assessed potential connections between VEGF expression levels and tumor prognosis. We found a positive correlation between VEGF overexpression and stage of the tumor [19, 20]. Additionally, the data suggested a higher tumor recurrence and increased frequency of metastasis in patients with high VEGF expression compared to those with low VEGF expression. These results highlight the role of VEGF in angiogenesis and tumor development and that development of new therapies targeting the VEGF signaling pathway in osteosarcoma may improve survival in conjunction with current treatments.

Furthermore, our laboratory assessed a retrovirus-mediated soluble Flt-1 (a VEGF receptor) gene insertion to cancer cells as a potential therapy to inhibit the growth and metastasis of osteosarcoma [19, 20]. VEGF exerts its biological effects on endothelial cells by binding to its cell surface receptors, Flk-1 (fetal liver kinase-1, or VEGFR-2) and Flt-1 (fms-like-tyrosine kinase-1, or VEGFR-1). Soluble Flt-1 is an alternatively spliced form of the Flt-1 VEGF receptor

that only contains extra-cellular domain of the receptor without trans- and inter-cellular domains, which has a same affinity to compete with the physiological receptors to bine VEGF yet lack of the capacity to transduce its physiological signals [20]. To assess the gene modification of soluble Flt-1, human osteosarcoma G-292 cells were transduced with retroviral vectors encoding sFlt-1. Then, the cells were transplanted into the proximal tibia of 4 week old immune deficient SCID mice and later compared with the controls: wild type G292 cells and LacZ-transduced cells. The mice were checked every day for tumor growth. After 6 weeks, a palpable tumor was discovered in the control mice. MicroCT analysis revealed early osteoblastic and osteolytic changes within 3 weeks of tumor cell injection and severe osteolytic lesions after 3 weeks. After 4, 6 and 8 weeks, the tumors with sFlt-1 gene modification were much smaller than the G-292 and LacZ transduced groups. Immunohistochemistry data confirmed orthotopic tumor development in the proximal tibia at 6 weeks, and pulmonary metastastic lesions at 8 weeks in all control mice with wild type or LacZ-transduced osteosarcoma cells, but none in sFlt-1 transduced mice. Overall, the data suggested that retrovirus-mediated sFlt-1 gene alteration halts osteosarcoma tumor growth in this mouse model. Therefore, targeting the VEGF pathway could be a potential anti-osteosarcoma treatment.

2.4 Role of CXCR4 in Osteosarcoma

Studies also indicate that chemokines and their receptors play an important role in determining the metastatic site of tumor cells. Chemokines are a superfamily of small, cytokine-like proteins that stimulate cytoskeletal rearrangement, bind to endothelial cells, and direct cell migration [21]. These proteins work along with cell-surface proteins, including integrins, to direct the specific homing of numerous subgroups of haematopoietic cells to particular locations.

One example is CXCR4 (C-X-C chemokine receptor type 4), a seven-transmembrane G protein-coupled receptor, which regulates hematopoietic and tumor cell bone localization [21, 22].

Müller *et al.* proposed that tumor cells may use chemokine-controlled mechanisms, such as those controlling leukocyte trafficking, during metastasis [21]. Their study found that chemokines and their associated receptors such as CXCL12 and its receptor CXCR4 are involved in determining the metastatic site of tumor cells. Their analysis suggested that small molecule chemokine receptor antagonists may interfere with tumor progression and metastasis. Their findings in breast cancer and malignant melanoma suggest that malignant cells express specific patterns of chemokine receptors.

While the role of CXCL12 and CXCR4 in metastasis has been confirmed [23], not much is known about the expression of CXCR4 at primary and/or metastatic sites. However, it is established that neovascularization is important in metastatic progression because it allows tumor cells to invade neighboring tissues, get into the circulation, proliferate, and form secondary tumors. VEGF regulates many endothelial cell functions, such as mitogensis. Research has revealed that VEGF expression at the primary tumor site is linked with metastasis of osteosarcoma. Thus, research by Lin *et al.* sought to determine whether CXCR4 expression was associated with VEGF expression in osteosarcoma. Additionally, they wanted to know if the expression of these factors was linked to metastasis. Using tissue microarrays, they found a significant positive correlation between the expression of VEGF and CXCR4. Their results suggested that CXCR4 can serve as a novel anti-angiogenic therapy to suppress primary and metastatic tumor formation. Research in our laboratory showed that CXCR4 expression in osteosarcoma was significantly reduced following VEGF blockade[24]. The mechanism of

blocked VEGF function by enhanced sFlt-1 expression possibly blocks microvessel development and leads to low CXCR4 expression.

On the other hand, it is well known that solid tumors contain heterogeneous cell subpopulations with different proliferation abilities and differentiation potential [5]. One of these is a small population of cancer stem cells or tumor-initiating cells that can self-renew, proliferate, differentiate, and sustain malignancy. New therapies targeting these cells may improve cancer treatment. Hence it is important to identify such cell subpopulation within tumors.

2.5 CD133

One potential cancer stem cell marker and cancer-initiating subpopulation of cancer cells is CD133. CD133 is a penta-membrane spanning glycoprotein [25] that is found on plasma membrane projections of embryonic epithelial structures [26]. Currently CD133 is considered a stem cell marker in many normal tissues and solid tumors because studies report that tumors originate from transformed normal stem cells [25]. Thus, the purpose of Tirino's study was to observe the possible expression of CD133 antigen in osteosarcoma cell lines and understand their biological function and features [5]. They identified and characterized CD133+ cells in three human sarcoma cell lines and observed that the CD133+ cells formed sphere-clusters in a small fraction of the cell population and had high proliferation rates. Furthermore, these cells were extremely clonogenic and tumorigenic. This was the initial discovery of CD133+ cells in osteosarcoma cell lines with stem cell properties and may lead to the design of new therapies against osteosarcoma.

Many studies state that tumors commonly originate from transformed stem cells [25, 26]. It is postulated that related signaling pathways control self-renewal in stem cells and cancer cells and that cancer cell populations may include cancer stem cells that can self-renew. Therefore,

cancer stem cells, possibly CD133+ cells, may be involved in the "formation, growth, infiltration, metastasis, and recurrence of tumors" [6, 27, 28].

2.6 Hypoxia

Additionally, hypoxia affects various physiological and developmental functions of the cell [29]. Hypoxia, referring to low oxygen state, is a characteristic of many solid cancers [30] and stimulates the expression of genes involved in proliferation, glycolysis, and angiogenesis [31]. Tumor cells adapt to the hypoxic environment, resulting in an aggressive and metastatic tumor phenotype that is linked with a poor treatment results to radiation and chemotherapies. Malignant tumors can grow to be several cubic millimeters in the absence of neoangiogenesis due to the shortage of oxygen and glucose diffusion from blood vessels [30]. Thus, neoangiogenesis and cellular adaptation to hypoxia are crucial for cancer development. Hypoxia may also be involved in various growth modulating effects that could give malignant cells a growth advantage.

The key transcription factor accountable for the cellular adaptation to hypoxia is HIF-1 [32]. HIF-1 has two subunits, a beta unit (HIF-1b) and an oxygen regulated alpha unit (HIF-1α or HIF-2α). When oxygen is present, the alpha subunits of HIF-1 are hydroxylated, which allows the binding to the Von Hippel Lindau (VHL) protein and subsequent targeting for ubiquitination and degradation. In hypoxia, hydroxylation does not occur and the alpha subunits stabilize and bind with HIF-1b. The complex enters the nucleus, initiating the transcription of various target genes involved in drug resistance. Thus inhibiting HIF-1α could be a good target for drug treatment in hypoxia and reverse drug resistance.

HIF-1α expression is controlled by changes in concentration of both oxygen and growth factors [31]. In normoxia, HIF-1α is quickly degraded through ubiquitination and degradation by

the proteasome. In response to hypoxia, HIF-1α stabilizes and enters the nucleus to bind to the HIF-1, thus forming the HIF-1 complex. HIF-1 binds to the hypoxia responsive element (HRE), recruits the coactivator cAMP-responsive element binding protein (CREB) binding protein and other proteins to initiate transcription.

HIF-1 is associated with the activation of many hypoxia-response genes, including VEGF. VEGF binds to transmembrane VEGF receptor tyrosine kinases on endothelial cells to activate intracellular signal transduction pathways that facilitate angiogenesis and vascular permeability. Hypoxia markers, such as HIF-1 and VEGF can be identified in osteosarcomas and their detection is associated with poor patient outcome; thus hypoxia plays an important role in osteosarcoma [32]. Under hypoxic conditions, tumor cells grow and feature a more aggressive phenotype by triggering a cascade of molecular events that are partially controlled by the markers HIF-1α and VEGF [33]. High VEGF expression is linked with tumorigenesis and metastasis. Likewise high VEGF gene expression has been linked to in many cancer cell lines and primary tumor tissues such as the breast, lung, ovary, liver, and colon. Osteosarcoma cell lines and primary tumor samples also show higher VEGF expression compared to normal tissues. Cancer cells respond similarly to normal cells under hypoxia by initiating signaling pathways that cause cell proliferation, angiogenesis, apoptosis, and other processes. Under hypoxia, more of the pathways initiated in neoplastic cells promote tumor growth than apoptosis. Analysis of many tumor types has found tumor hypoxia and proliferation rate as potential determinants of clinical outcome. Hypoxic conditions may be due to an imbalance between tumor cell proliferation and new endothelial cell formation, and disorganization of new vascularization.

Cytotoxic drug resistance in hypoxia can differ between tumor type and treatment [32]. Research has shown the importance of hypoxia in osteosarcoma but the influence of hypoxia on

the response of osteosarcoma cells to cytotoxic drugs has not been reported. In Adamski's analysis [32], hypoxia induced resistance to cytotoxic drugs by suppressing apoptosis in all osteosarcoma cell lines. The significant hypoxia-induced drug resistance in the osteosarcoma cell lines suggested that hypoxia is a possible target.

2.7 HIF-1α

The role of HIF-1α subunit in cancer and neoplastic growth have been determined by measuring protein expression levels [33]. Studies have found HIF-1α subunit to be overexpressed in colon, breast, gastric, lung, skin, ovarian, pancreatic, prostate, and renal carcinomas and has also been linked with cell proliferation.

A recent study evaluated the expression HIF-1α in osteosarcoma of the jaw (OSJ) to reveal the relationships between HIF-1α and clinic-pathologic features of OSJ and examined the influence of HIF-1α on OSJ incidence and development [34]. Their results showed an overexpression of HIF-1α in OSJ. HIF-1α expression in OSJ was linked with tumor size, pathologic grade, clinical stage, and primary occurrence or recurrence but was not linked with metastasis.

Another study explored the impact of HIF-1α overexpression on prognosis and the association with clinicopathological features in human osteosarcoma [30]. HIF-1α protein expression in histologic sections from 39 treated patients was analyzed by immunochemistry. HIF-1α protein expression was identified in 31 of 39 cases (79%), predominantly within the tumor cell nuclei. Their study indicated that HIF-1α expression was associated with surgical stage and percentage of dead cells, which had previously been described as prognostic factors in osteosarcoma. High expression of HIF-1α forecasts a poor outcome but it is not the only factor at work. We suggest that HIF-1α could be a novel therapeutic target in osteosarcoma.

Further, HIF-1α and HIF-2α were both found expressed in normal and malignant bone cell lines. However, the expression was greater in osteosarcoma lines (5.5 fold more under normoxia and 20.7-fold more under hypoxia) [29]. This was more evident under hypoxic conditions as there was a noticeable activation of HIF-1α at the mRNA and protein levels in osteosarcoma cell lines. Through western blotting, they verified that it was the overexpression of HIF-1α under hypoxia that seems to protect malignant cells from undergoing apoptotic cell death. Their data suggested that HIF-1α is involved in protecting osteaosarcoma cells from hypoxic-induced apoptosis.

2.8 VEGF and HIF-1α

Neoangiogenesis is necessary for tumor growth, metastases development, and invasive cancer progression [30]. When cancer cell proliferation outpaces the rate of angiogenesis, tumor cells must adapt to tissue hypoxia, which is linked with resistance to chemotherapy, radiotherapy and overall poor survival. HIF-1α is involved in the cellular adaptation to hypoxia and the activation of genes such as the VEGF that is associated with tumor progression. The regulatory mechanisms of HIF-1α action on VEGF are slowly coming to light [34]. When HIF-1α binds to hypoxic-response elements on the VEGF enhancer, HIF-1 induces VEGF transcription while stabilizing the VEGF mRNA. VEGF then binds to vascular endothelial cells, causing growth, vascular permeability, and therefore promotes angiogenesis. The antiangiogenic effects produced by blocking the HIF target, VEGF, also highlight the significance of this protein for tumor angiogenesis [35]. Furthermore, HIFs participate in drug-resistance and radiation-resistance which are problematic in cancer treatment. Inhibition of the HIF pathway could offer a therapeutic advantage and HIF-1α may be a key target in tumor antiangiogenic therapy [34].

HIF-1α expression is correlated with a more aggressive phenotype of cancer cells and with poor clinical outcome in many human cancers [30]. Prior to Yang's study, the prognostic significance of HIF-1α in human osteosarcoma had not been assessed. Yang's analysis confirmed a strong association between HIF-1α expression and increased microvessel density (MVD) in human osteosarcoma. Furthermore, it is known that increased MVD and VEGF expression are correlated with poor prognosis in human osteosarcoma.

In osteosarcoma, VEGF and neovascularization have been investigated, but the correlation of these with cell proliferation and HIF-1α expression had not. Hence, Mizobuchi determined that increased HIF-1α expression, VEGF expression, vascularization, and cell proliferation in osteosarcoma patients are good predictors of the clinical outcome [33].

Neoplastic cell growth under hypoxic conditions becomes more aggressive by triggering molecular events partially controlled by HIF-1α and VEGF [33]. Thus, Mizobuchi hypothesized that a correlation between hypoxia markers and osteosarcoma prognosis could also be used to determine chemotherapy response and metastatic phenotype. Hence, they determined HIF-1α expression, VEGF expression, vascularization, and cell proliferation in osteosarcoma patients to predict the clinical outcome by correlations with chemotherapy response and metastases.

Their results showed metastases were present in 61% of the HIF-1α-positive patients which suggested that increased HIF-1α expression is a critical sign of metastases in osteosarcoma patients [33]. The data confirmed previous work that reporting a higher rate of HIF-1α overexpression in metastases in other cancers. A research study on OSJ revealed HIF-1α positively correlated with tumor growth and invasion [34]. The analysis showed a significant difference in micro-vascular density between HIF-1α–negative and HIF-1α–positive OSJ, thus suggesting the involvement of HIF-1α in OSJ angiogenesis.

2.9 CXCR4 and HIF-1α

Previous studies established a correlation between CXCR4 and HIF-1α expression in different cancers [9]. HIF-1α regulates the cellular response to hypoxia and is involved in tumor growth. HIF-1α upregulates CXCR4 expression; therefore chemokine receptors in hypoxic regions of tumors may increase to allow survival and metastasis.

Mingjun Guo and others first examined whether CXCR4 plays a role in increased migration ability and increased expression/activation of CXCR4 was associated with cancer metastases [9]. Data established that CXCR4 expression levels were significantly different in numerous human osteosarcoma cell lines with distinct metastatic potentials. Cells were treated with hypoxic and normoxic conditions and CXCR4 mRNA and protein levels were measured. Their results showed that the mRNA and protein levels of CXCR4 increased under hypoxia. They also evaluated the expression of HIF-1α at both the mRNA and protein levels and found that the level of HIF-1α protein increased under hypoxia. Moreover, immunocytochemistry analysis revealed that HIF-1α and CXCR4 expression were significantly increased under hypoxia.

Subsequently Guo et al investigated whether HIF-1α is linked with CXCR4 expression by transfecting synthetic siHIF-1α (or negative control siRNA [NC-siRNA]) into SOSP-9607 cells [9]. In normoxia and hypoxic conditions, CXCR4 and HIF-1α protein levels significantly decreased by siHIF-1α transfection compared with control. Similarly, the mRNA expression levels of CXCR4 and HIF-1α were also diminished following siHIF-1α treatment. HIF-1α expression levels in SOSP-9607 cells significantly increased under hypoxic environments. As a result, they concluded that hypoxia-induced CXCR4 expression was controlled by HIF-1α in human SOSP-9607 cells. Following these results, they examined the connection between

CXCR4 and HIF-1α expression in 98 osteosarcoma cases. The patients with tumors expressing CXCR4 and HIF-1α had poorer overall survival rates than patients with tumors that did not express CXCR4 or HIF-1α. This showed that CXCR4 and HIF-1α expression is highly correlated with metastasis in patients with OS and is important in the survival prognosis of OS patients. Their findings confirmed that many OS specimens express HIF-1α, thus they proposed that hypoxia is an attribute of OS.

CHAPTER 3

OBJECTIVES

The ***purpose*** of my project was to examine if regulating the function of VEGF can change the population of CSCs in osteosarcoma. We ***hypothesized*** that CD133+ osteosarcoma stem cells are present within the osteosarcoma cell lines and they will be less responsive to VEGF blockage treatment than other tumor cells. Using polymerase chain reaction and immunocytochemistry techniques, we studied the expression of VEGF, CXCR4, and cancer stem cell marker CD133 at transcriptional and post-translational levels among different VEGF-expressing osteosarcoma cells. We also examined the tumor cell growth patterns and compare the subpopulation ratio of the CD133+ stem-like cells after VEGF blockage.

Additionally, we wanted to understand the roles of VEGF and HIF-1α in osteosarcoma. Our ***hypothesis*** is that these two growth factors may act interactively to regulate the tumor cell survival and growth. Through application of relevant inhibitors, we analyzed the effect of hypoxia on these regulator-involved cell proliferations. Using polymerase chain reaction and ELISA techniques, we also examined the expressions of VEGF and HIF-1α at transcriptional and post-translational levels of VEGF-expressing 1547 osteosarcoma cells.

CHAPTER 4

MATERIALS AND METHODS

4.1 VEGF, CXCR4 and CD133

4.1.1 Cell Culture

Cell culture is a process by which cells are grown under controlled conditions outside of their natural environment. This technique must be performed under sterile conditions to avoid bacterial contamination. In our experiment, osteosarcoma cells were used for *in vitro* culture. Cells are maintained in DMEM culture medium containing 10% fetal calf serum, 100 U/mL penicillin, and L-glutamine. Flasks are incubated at 37 °C in a 5% CO_2 incubator and the medium is changed twice a week. After confluence, culture medium is removed, the cell layer is rinsed thoroughly with phosphate-buffered saline (PBS), digested with 0.25% trypsin, and subdivided into new flasks until the end of each experiment. Before RNA extraction, 3 cell lines were grown in 12 well plates for 24 hours (control vs. treated). The next day, the labeled treated wells received VEGF inhibitor (SU 4312) for 4, 24 and 72 hours.

4.1.2 RNA Extraction and Real-Time Polymerase Chain Reaction (PCR) for Gene Expression

PCR is a technique that amplifies a fragment of DNA and therefore produces many copies of a particular DNA sequence. In this experiment, PCR was performed to detect the expression of VEGF, CXCR4, and CD133. First, RNAzol (1 mL) is added to the collected cell suspension ($2x10^6$ cells). Following homogenization, chloroform (0.2 mL/1mL of homogenate) is added. Subsequently, the tubes are agitated by hand for 20 seconds and then placed on ice for 5 minutes. The tubes are centrifuged at 12,000 x g for 15 minutes at 4 °C. Following centrifugation, the solution is separated into 3 phases in which RNA was in the clear aqueous

layer. The aqueous layer is transferred to a new tube and mixed with an equal volume of isopropanol to completely precipitate the RNA. RNA is kept frozen at -20 °C overnight.

The next day, the RNA is centrifuged at 12,000 x g for 15 minutes at 4 °C. A white precipitate forms at the bottom and the side wall of the tube. The supernatant is removed and the precipitate was washed in 800 uL of 70% ethanol, agitated and centrifuged at 7,500 x g for 8 minutes at 4 °C. The ethanol is removed and the RNA precipitate is allowed to dry at room temperature for 20 minutes. Lastly, RNA is dissolved in RNAse-free water.

The quantity and purity of the RNA were determined by a spectrophotometer at 260 nm and 280 nm. First, in a UV plate, 198 uL of water and 2 uL of RNA are added in each well. The plate is placed in the ELISA reader, which measures the RNA concentration. The OD reading for OD_{260} and OD_{280} are recorded. The primers used to amplify the mRNA were 18s, VEGF, CXCR4, and CD133. VEGF primer sequences: forward 5'- cccactgaggagtccaacat - 3' and reverse 5'- tttcttgcgctttcgttttt – 3'; CXCR4 sequences: forward 5' – ccgtggcaaactggtacttt – 3' and reverse 5' – ccttttcagccaacagcttc – 3'; and CD133 sequences: forward 5' – tcagtgagaaagtggcatcg – 3' and reverse 5' – gcttttcctatgccaaacca – 3'. The targets and the house-keeping gene 18s of each sample were analyzed by real-time PCR to calculate gene concentrations by standard curve.

4.1.3 Immumocytochemistry (ICC)

ICC is a technique used to assess the presence of a specific antigen in a cell using a specific antibody. The antibody binds to the antigen, thus allowing visualization and analysis under a microscope. In our experiment, three cell lines were plated and grown in 8 well chamber slides (control vs. treated) for 24 hours. The next day, the labeled treated chamber slides received VEGF inhibitor SU4312 (6.4 μM) and then the cells were harvested after 1, 4, 24, and 72 hours. Pictures were taken before and after treatment. After treatment, the slides are fixed with formalin

fixation solution then washed 3 times for 5 minutes in PBS. Slides were incubated in 3% normal goat serum in PBS to block nonspecific binding. After 1 hour, blocking serum from each slide is removed then the slides are incubated with primary antibody CD133 (2 ug/mL diluted in PBS with 3% normal goat serum) for 1 hour at 37 °C. Slides are washed 3 times for 5 minutes in PBS then incubated with biotin-conjugated anti-rabbit IgG secondary antibody (1 ug/mL diluted in PBS with 3% normal goat serum) for 1 hour at 37 °C. Slides are then incubated with avidin biotin enzyme reagent for 30 minutes at 37 °C. DAB (1 DAB tablet + Urea Hydrogen Peroxide + ultrapure H_2O) is added to develop staining. After being stained for 5 minutes with DAB at room temperature, the tissues are counterstained with hematoxylin for 2 minutes and immediately washed with several changes of deionized water, dehydrated through alcohols and xylenes as follows: soaked in 95% ethanol 2 times for 1 minute, then 100% ethanol twice for 1 minute, then xylenes three times for 1 minute each. Immediately after dehydration, tissues are mounted (1-2 drops of permanent mounting medium), cover slipped, and observed by light microscopy. Sections were screened for CD133 expression under x100 magnification.

4.1.4 Statistics

Photomicrographs were taken and a computerized image analysis system (ImagePro+) was used to quantify the positive stains. Statistical analysis between different VEGF expressing cells was performed using SPSS software. A P-value <0.05 was considered as significant. The data was expressed as mean ± standard deviation.

4.2 HIF-1α

4.2.1 Cell Culture

Human osteosarcoma cell line ATCC-1547 was cultured in 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37 °C in a 5%

CO_2 incubator. The medium was changed every 3 days. When 90% confluence was reached, culture medium was removed, the cell layer rinsed thoroughly with phosphate buffered saline (PBS), and enzymatically dissociated by adding 0.25% (w/v) trypsin-0.03% (w/v) EDTA. Cells were maintained by subculture at a ratio of 1:8. Prior to implantation, the cell suspensions were diluted with 0.5% (w/v) trypan blue in 0.16 mol/L ammonium chloride to assess cell viability and number. Cell cultures with 40 uM HIF-1a inhibitor—KC7F2(4324/10, R&D,USA)(named HIF group), 10uM VEGFR inhibitor —SU4312(58567,Sigma,USA)(named VEGF group) and these two inhibitors (named BOTH group) respectively, were regulated under either normoxia (20% O_2 concentration) or hypoxia (GasPak EZ Gas Generating Sachet-induce hypoxia) condition for 72 hours.

4.2.2 Proliferation of the Osteosarcoma Cells

The effect of hypoxia on cell proliferation of ATCC-1547 was evaluated by MTT assay. 1547 cells at $1x10^5$ per well were seeded into 96-well plates and allowed to adhere overnight. The next day, cells were exposed to different inhibitors and oxygen tension for 72 hours. Subsequently, 20 mL of MTT (5 mg/ml) were added and incubated at 37 °C for 4 hours. The medium was removed, 200 ul 10% SDS was added to each well and cultured overnight to dissolve the dark blue crystals. Optical density readings were obtained at 590 nm.

4.2.3 Terminal DNA Breakpoints In Situ 3-Hydroxyl End Labeling (TUNEL)

ATCC-1547 cells at $2x10^4$ per well were seeded into chamber slide and allowed to adhere overnight at 37 °C. The next day, the cells were incubated with different inhibitors and oxygen tension for 72 hours. In Situ Cell Death Detection kit (11684809910, Roche, Germany) was purchased from Roche. Staining procedures were as following: (1) cell samples were fixed with a freshly prepared fixation solute for 60 minutes; (2) the slides were rinsed with PBS twice for 5

minutes each, (3) incubated in permeabilization solution for 2 minutes on ice, and (4) then rinsed with 1X PBS twice for 5 minutes each. Then, 50 uL of TUNEL reaction mixture was added to the sample. The slides were incubated for 60 minutes at 37 °C in a humidified chamber in the dark and then rinsed with PBS three times for 5 minutes each. After rinsing, 50 uL of converter-AP was added and then the slides were incubated in a humidified chamber in the dark at 37 °C for 30 minutes. The slides were rinsed with PBS three times for 5 minutes each. During this time, fast red solution was prepared. After rinsing, 100 µl of substrate solution was added and the slides were then incubated in a humidified chamber in the dark for 10 minutes at room temperature (RT). The slides were rinsed with PBS three times for 5 minutes each. Slides were mounted with glass coverslips and analyzed under a microscope.

4.2.4 Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (Real-Time RT-PCR)

Total RNA extraction was performed using a commercial kit (Tel-Test Inc, Friendswood, Tex, USA) in accordance with the manufacturer instructions. The precipitated RNA was then treated with DNase and passed through a spin column (Rneasy mini kit, Qiagen) for further purification. Briefly, cDNA was reverse transcribed from 0.5 µg of total RNA in 40 µL reaction mixture (containing 1×PCR buffer, 500 µM each of deoxynucleotide triphosphates (dNTP), 0.5 U/µL of RNase inhibitor, 2.5 µM random hexamers, 5.5 mM $MgCl_2$, and 1.25 U/µL of reverse transcriptase (Perkin Elmer, Conn, USA)) and then incubated in a DNA Thermal Cycler (Veriti 96 well Thermal Cycler, AB Applied Biosystems, USA) at 25°C for 10 minutes, 48 °C for 5 minutes followed by 95 °C for 5 minutes. Primers were designed using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) and synthesized by Life Biotech. HIF-1α sequences: forward 5'- actagccgaggaagaactatgaa-3' and backward 5'-tacccacactgaggttggtta-3'. for the HIF-

cDNA(Life, USA); Sense 5'-cccactgaggagtccaacat-3'and antisense 5'-tttcttgcgctttcgttttt-3' for the VEGF165 cDNA(Life, USA). To standardize target gene level with respect to variability in RNA and cDNA quality, housekeeping gene 18S was co-amplified as an internal control. Reaction mixtures of 25 μL contained 12.5 μL of 2XSYBR Green PCR Master Mix (5mM MgCl2, 200μM dATP, dCTP, dGTP, 400μM dUTP, 1.25U AmpliTaq Gold DNA polymerase, 0.5U AmpErase uracil N-glycosylase), 0.5 μL each of 0.4 μM target primers and 2 μL of cDNA. The PCR reactions were set in MicroAmp optical 96- well reaction plates with MicroAmp optical caps and amplified in the Step One Plus Real-Time PCR System(AB Applied Biosystems, USA) for 40 cycles (95 °C for 15seconds, 60 °C for 1minute). The fluorescent signals were recorded dynamically. The values of threshold cycle (Ct) at which a statistically significant increase in reporter-dye signals (ΔRn) is first detected, were imported into Microsoft Excel program and used to calculate the relative quantification of the target gene expression. The Ct values of 18S were the internal control samples and the mean Ct values of target gene expression from the cells without inhibitor in normoxia or hypoxia condition were the calibrator samples.

4.2.5 Enzyme Linked Immunosorbent Assay (ELISA) for Cytokine Quantification

Human VEGF Mini ELISA Development Kit (PEPROTECH, 1012010-M) was used to quantitate the level of VEGF in the conditioned medium 1547 cells and the Human/Mouse Total HIF-1a ELISA Kit (R&D,DYC1935-2) for HIF-1a. First, 100 μL of capture antibody was added with a working concentration to each ELISA plate well. The plate was sealed and incubated overnight at 4 °C. The wells were aspirated to remove liquid and the plate was washed 4 times using 300 μL of wash buffer per well. After the last wash, the plate was inverted to remove residual buffer and blotted on a paper towel. Then, 300 μL of block buffer was added to each

well and incubated for at least an hour at room temperature. The plate was aspirated and washed 4 times. Standard form 10000 pg/ml was diluted by half to zero in diluents. These were the working concentrations. Then, 100 µl of standard or sample was added to each well in duplicate and incubated overnight at 4 °C. The next day, the plate was aspirated and washed 4 times. The detection antibody was diluted in diluent to a working concentration and 100 µl was added to each well. The plate was incubated at room temperature for 2 hours. Then, 100 µl of 1x streptavidin-HRP was added to each well and kept at room temperature for 40 minutes. The plate was aspirated and washed 4 times. Then, 100 µl of 1xTBM solution was added into each well and kept at room temperature in the dark for 5 minutes. Finally, 50 µL of stop solution (1N HCl) was added to quench the reaction. OD was measured at 450 nm using a micro-plate reader, and cytokine levels were calculated based on standard curves. Each experiment was repeated in triplicates.

4.2.6 Statistical Analysis

Statistical analysis between different groups was performed by Student t-test or the one-way ANOVA test using the SPSS (Version 17.1). A p-value of less than 0.05 was considered significant. The data was expressed as mean ± standard error of the mean.

CHAPTER 5

RESULTS

5.1 Expression of VEGF, CXCR4, and CD133 in Osteosarcoma Cell Lines

5.1.1 Real-Time PCR

Using cell culture and real time PCR, we analyzed expression of VEGF, CXCR4, and CD133 (as shown in Table 1) in three osteosarcoma cell lines (Fig. 1). These three osteosarcoma cell lines were chosen because they represent different VEGF expression levels (low, moderate, high expression).Thus, these cells represent different stages of cancer development. For example, during rapid growth, as observed in 1423 cells, the expression of CD133 decreases as VEGF expression increases. However, in the early initial stage, or stem cell phase, as observed in 1543 cells, there is high CD133 expression but low VEGF expression.

Table 1

PRIMERS UTILIZED FOR REAL-TIME PCR

Target Primer	Forward Sequence	Reverse Sequence
VEGF	cccactgaggagtccaacat	Tttcttgcgctttcgttttt
CXCR4	ccgtggcaaactggtacttt	Ccttttcagccaacagcttc
CD133	tcagtgagaaagtggcatcg	Gcttttcctatgccaaacca
HIF-1α	actagccgaggaagaactatgaa	Tacccacactgaggttggtta
18s	cggctaccacatccaaggaa	Gctggaattaccgcggct

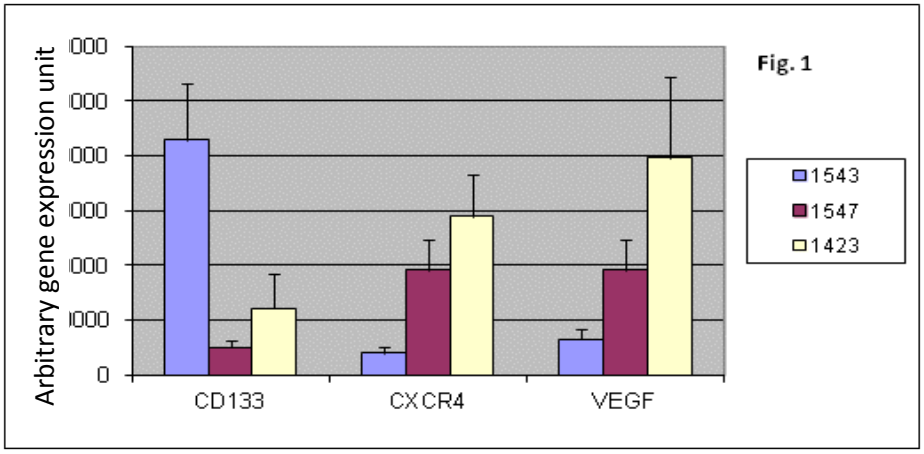

Fig. 1. Gene expression of VEGF, CXCR4, and CD133 in osteosarcoma cells 1547, 1543, and 1423 cell lines.

5.1.2 Detection of CD133+ Osteosarcoma Cells by ICC

Immunocytochemical analysis on osteosarcoma cells was performed in this study to examine the post-translational expression of CD133. Our results confirmed the presence of CD133+ cells in osteosarcoma cells. By visual detection, 1423 cells had more positive signal (greater expression of CD133) in the VEGF inhibitor treated groups compared to the control groups at all time points (Fig. 2). Over time, cell number dramatically decreased in the treatment groups and changes in cell shape (shriveling) were evident (Fig. 2 E-H). Additionally in 1423 cells, CD133 expression increased from 1 hour through 72 hours when VEGF was blocked. In 1547 cells, it appeared that at all time points, cell number was not affected by the VEGF inhibitor (Fig. 3). In 1543 cells, the cell number was not affected by the drug at 1 hour and 4 hours (Fig. 4 E-F). However, at 24 hours, cell number decreased (Fig. 4 G). At 72 hours, the amount of cells in control and. treatment group was relatively the same (Fig. 4 D, H). Additionally, at 1 hour and 4 hours, the 1543 control groups had more CD133+ cells compared to the treated cells. However, at 24 hours and 72 hours, CD133+ cells are relatively the same in control and treated groups.

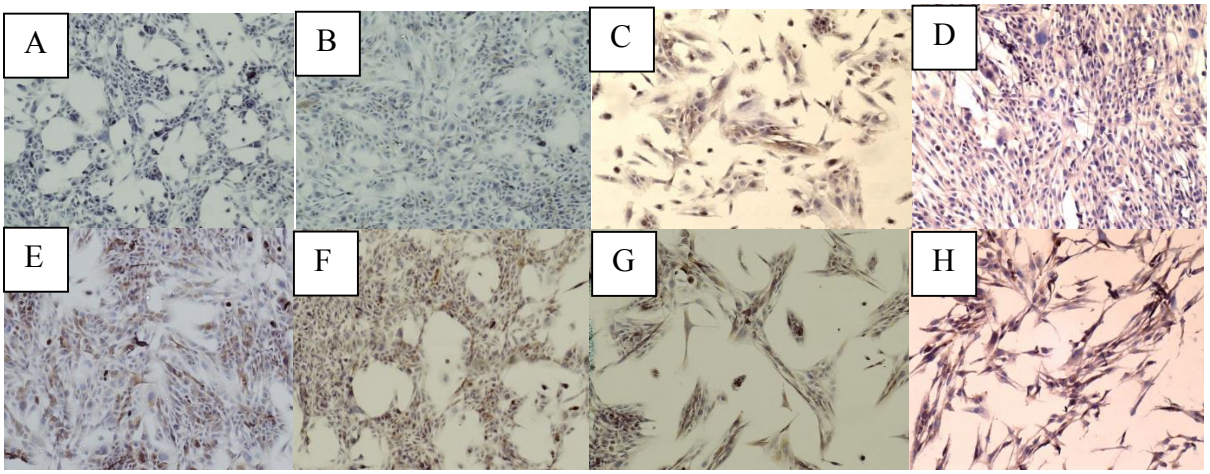

Fig. 2. Immunocytochemical analysis of CD133 staining [1423 control groups at 1 (A), 4 (B), 24 (C), and 72 (D) hours vs. 1423 VEGF inhibitor SU4312 treated groups at 1 (E), 4 (F), 24 (G) and 72 (H) hours].

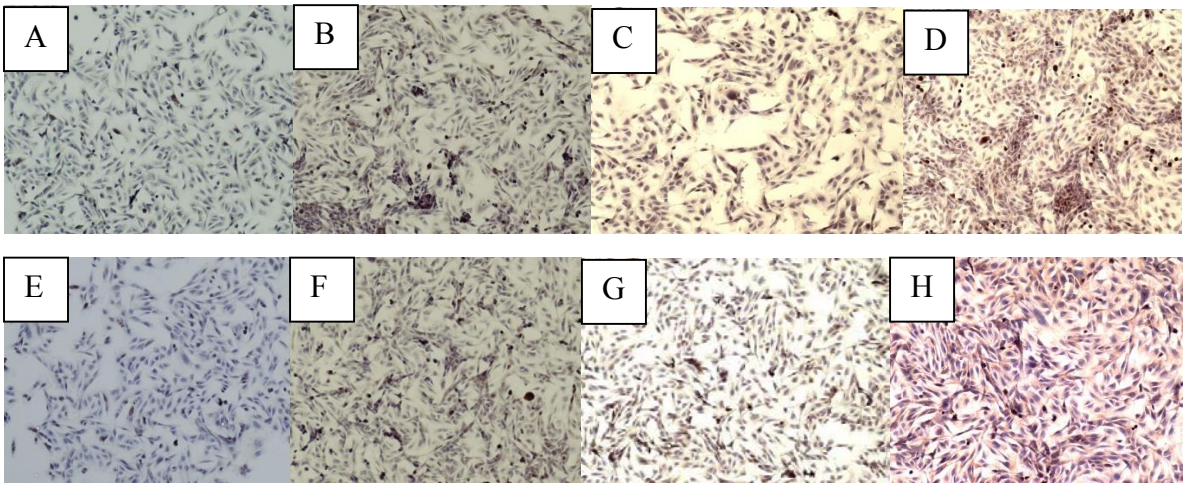

Fig. 3. Immunocytochemical analysis of CD133 staining [1547 control groups at 1 (A), 4 (B), 24 (C), and 72 (D) hours vs. 1547 VEGF inhibitor SU4312 treated groups at 1 (E), 4 (F), 24 (G) and 72 (H) hours].

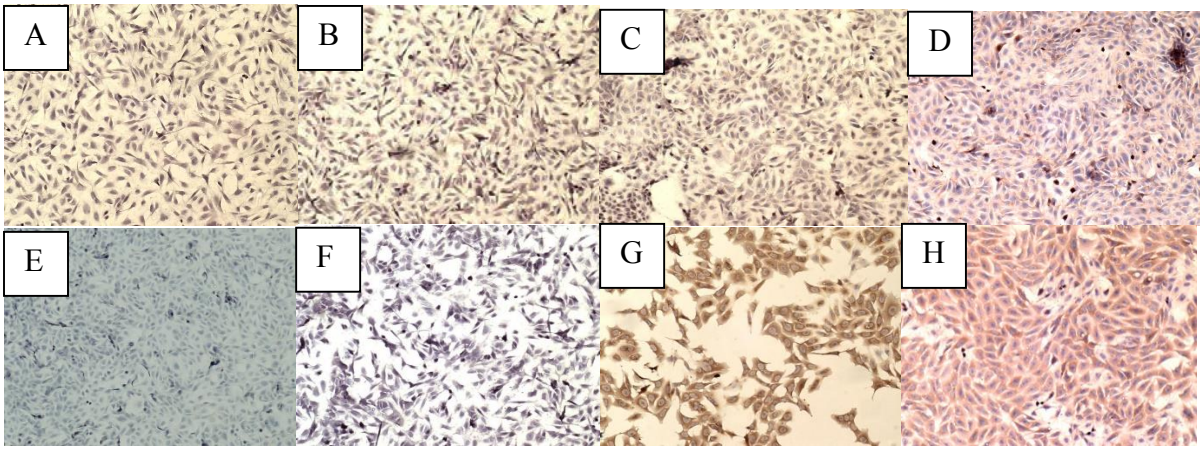

Fig. 4. Immunocytochemical analysis of CD133 staining [1543 control groups at 1 (A), 4 (B), 24 (C), and 72 (D) hours vs. 1543 VEGF inhibitor SU4312 treated groups at 1 (E), 4 (F), 24 (G) and 72 (H) hours].

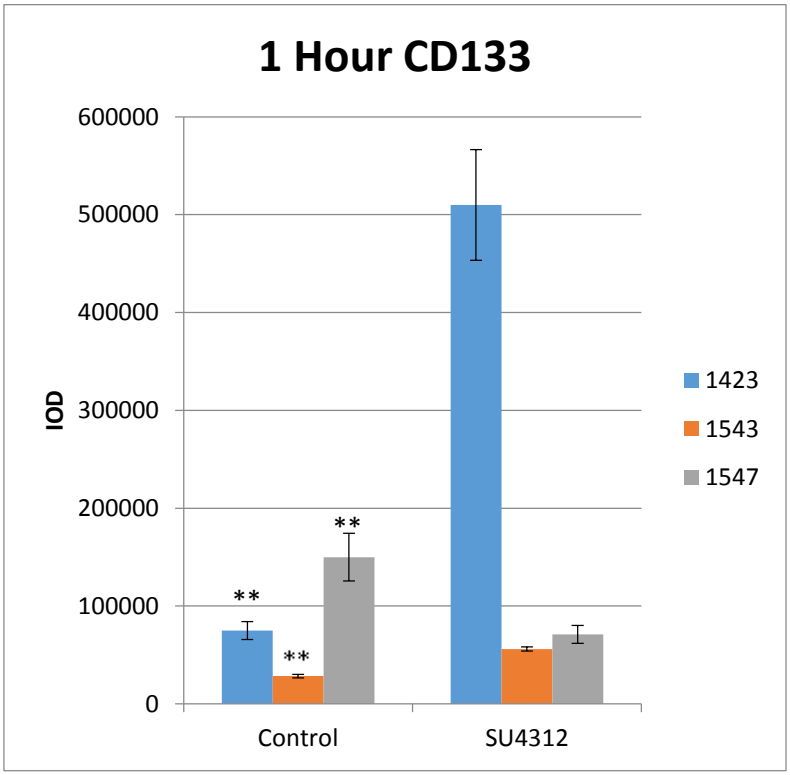

Fig. 5. CD133 expression in 1423, 1543, and 1547 osteosarcoma cells after 1h VEGF inhibitor SU4312 treatment compared to control. This data was analyzed by SPSS.

After 1 hour of treatment with VEGF inhibitor SU4312, VEGF was blocked and CD133 expression increased in 1423 osteosarcoma cells (Fig. 5). However after 72 hours, the drug was

no longer effective, hence CD133 expression decreased (Fig. 6). After 1 hour of treatment with VEGF inhibitor SU4312, there was no influence on the lower/middle range VEGF expressing osteosarcoma cells (1547 & 1543) (Fig. 5). However, after 72 hours, CD133 expression increased (Fig. 6).

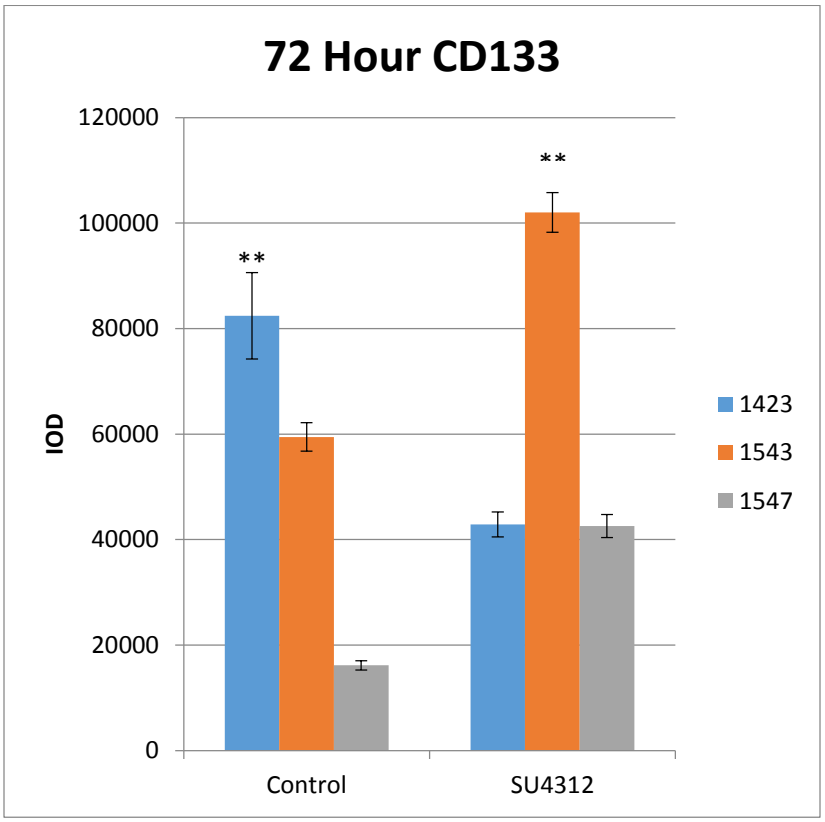

Fig. 6. CD133 expression in 1423, 1543, and 1547 osteosarcoma cells after 72h VEGF inhibitor SU4312 treatment compared to control. This data was analyzed by SPSS.

5.2 Effect of Hypoxia on Cell Proliferation

5.2.1 MTT Assay

MTT assay was performed on 72-hour culture to assess the cell proliferation patterns after treatment under different oxygen conditions. It is apparent that significantly slower osteosarcoma cell growth was observed in hypoxia condition than those in normoxia condition (Fig. 7). Further, addition of chemical inhibitors to block functions of VEGF and/or HIF-1

significantly decreased the cell proliferation patterns in normoxia condition (Fig. 8). Since all groups in hypoxia conditions already exhibited inhibited cell proliferation, further treatment only resulted in a significantly lower cell growth in VEGF inhibition group compared to the controls (Fig. 8).

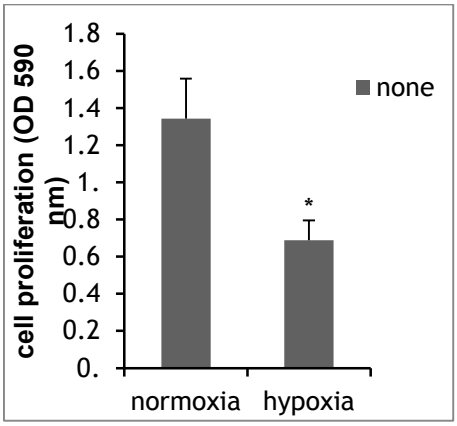

Fig. 7. Hypoxia inhibits ATCC 1547 proliferation*p<0.05 compared to normoxia. This data was analyzed by one-way ANOVA. All experiments were repeated three times.

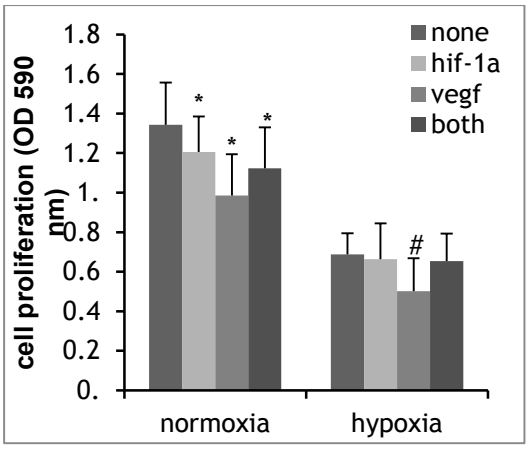

Fig. 8. Inhibits suppress ATCC 1547 proliferation*p<0.05 compared to none under normoxia. #p<0.05 compared to none under hypoxia. This data was analyzed by one-way ANOVA. All experiments were repeated three times.

5.2.2 Terminal DNA Breakpoints In Situ 3 - Hydroxyl End Labeling

TUNEL assay revealed an obvious more apoptotic cells after VEGF and/or HIF-1 inhibition treatments in cell cultures in normoxia condition compare to the non-treated controls (Fig 9). Dramatic cell number decrease was observed in osteosarcoma cell culture under hypoxia (Fig. 10), where addition of the VEGF/HIF-1 inhibitors resulted in further cell apoptosis. Indeed, there was barely any cell survived after combination treatment of VEGF+HIF-1 blockages under hypoxia condition (Fig. 10D). Figure 11 summarizes the computerized image analysis on the TUNEL stains that hypoxia condition significantly increased apoptotic cells compared to the cells in normoxia condition (Fig. 11). Additionally, there was a significant increase in apoptotic cells when HIF-1α, VEGF, or combinations of both were blocked compared to controls (Fig. 12).

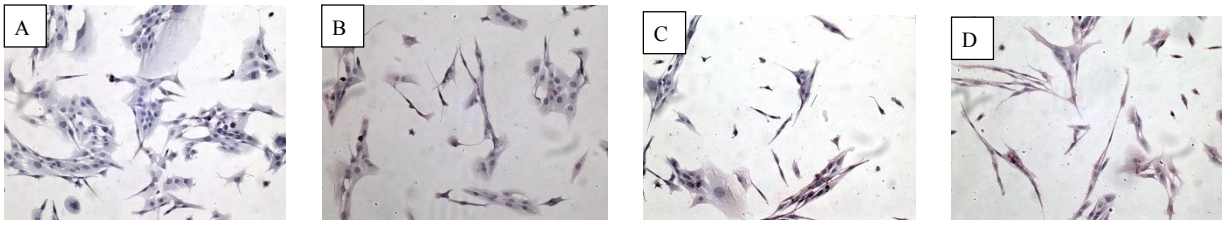

Fig. 9. Apoptosis observed in ATCC 1547 cells under normoxia. (A) No inhibitors; (B) HIF-1a inhibitors; (C) VEGF inhibitors; (D) Both HIF-1a and VEGF inhibitors.

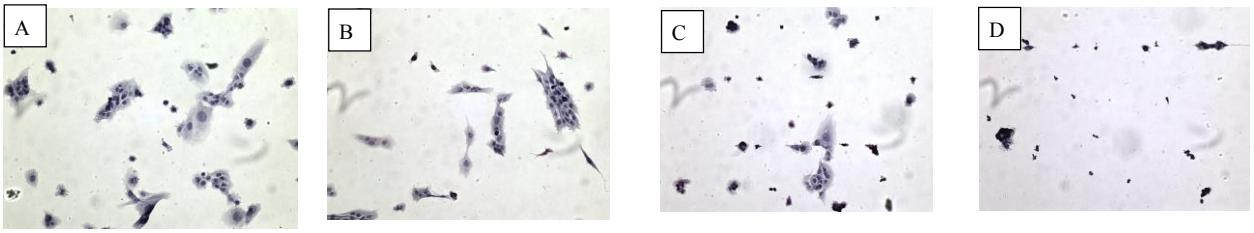

Fig. 10. Apoptosis observed in ATCC 1547 cells under hypoxia. (A) No inhibitors; (B) HIF-1a inhibitors; (C) VEGF inhibitors; (D) Both HIF-1a and VEGF inhibitors.

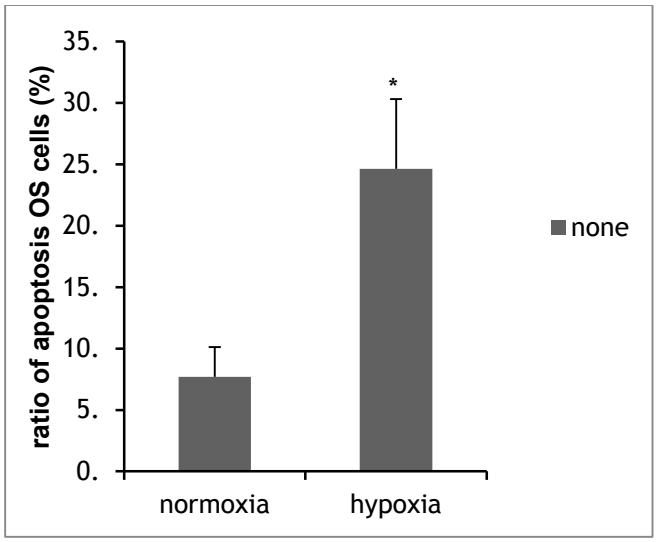

Fig. 11. Hypoxia improves ATCC 1547 cell apoptosis. *p<0.05 compared to normoxia. This data was analyzed by one-way ANOVA. All experiments were repeated three times.

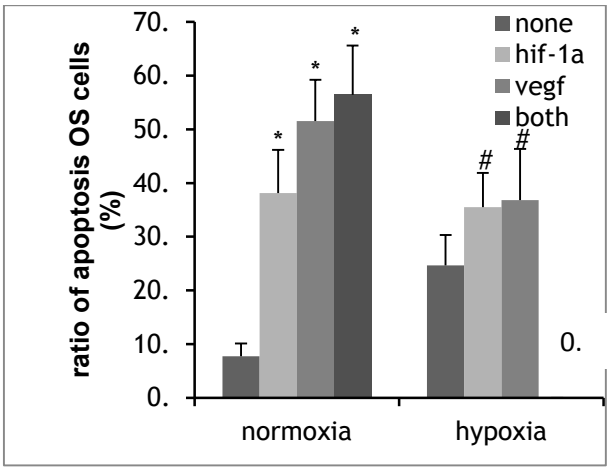

Fig. 12. Inhibition improved ATCC 1547 apoptosis.*p<0.05 compared to none under normoxia. #p<0.05 compared to none under hypoxia. This data was analyzed by one-way ANOVA. All experiments were repeated three times.

5.2.3 Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction

cDNA expression of HIF-1a and VEGF had a significant difference under different oxygen tension. In hypoxia, HIF-1a had higher cDNA expression than in normoxia (Fig. 13). HIF-1a inhibitor did not restrain the cDNA expression of HIF-1a, but VEGFR inhibitor did. When VEGF and BOTH were blocked, HIF-1α was inhibited. In hypoxia conditions, HIF-1α expression is higher in hypoxia-none and hypoxia-HIF-1α compared to normoxia. Therefore,

their inhibition effect was less in hypoxia compared to normoxia. Under hypoxia, HIF expression is upregulated in control and HIF-1α inhibitor did not interfere with HIF gene expression. When VEGF is blocked, HIF-1α expression decreased.

In Figure 14, both HIF-1a inhibitor and VEGFR inhibitor suppressed VEGF cDNA expression in different oxygen conditions. VEGF expression was higher in normoxia compared to hypoxia.

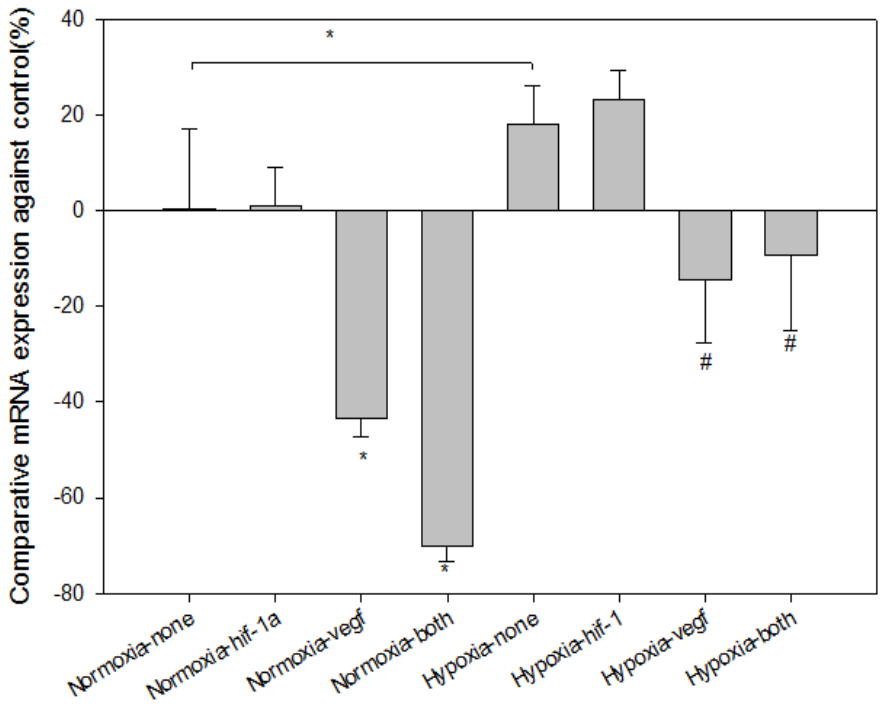

Fig. 13. HIF-1a relative mRNA expression of ATCC1547. *p<0.05 compared to none under normoxia. #p<0.05 compared to none under hypoxia. This data was analyzed by one-way ANOVA. All experiments were repeated three times.

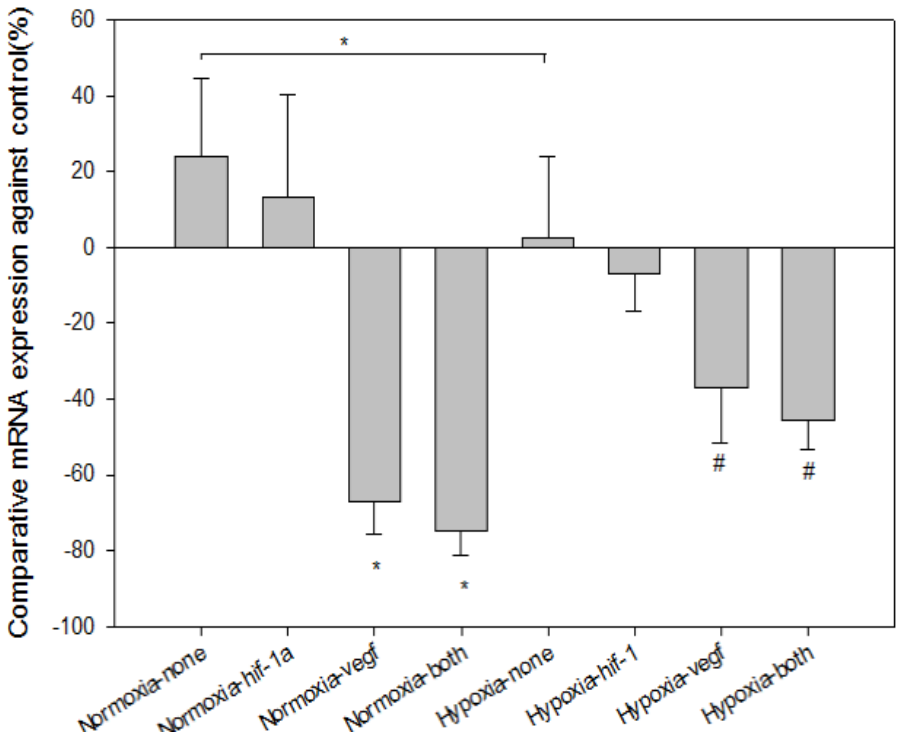

Fig. 14. VEGF relative mRNA expression of ATCC1547 .*p<0.05 compared to none under normoxia . #p<0.05 compared to none under hypoxia. This data was analyzed by one-way ANOVA. All experiments were repeated three times.

5.2.4 Enzyme Linked Immune Sorbent Assay (ELISA): Quantification of the Protein of HIF-1a and VEGF

The data showed an increase in HIF-1a protein level under hypoxia, but no significant difference was defined (Fig. 15). Both HIF-1α and VEGFR inhibitors can suppress the HIF-1α protein expression under different oxygen tension (Fig 16). When VEGF or combination of both VEGF and HIF-1α were blocked, HIF-1α protein expression significantly decreased. There was a lower protein expression of VEGF at hypoxia (Fig. 17), and the inhibitors against VEGF or HIF-1α significantly down-regulated VEGF levels under either normoxia or hypoxia condition (Fig. 18).

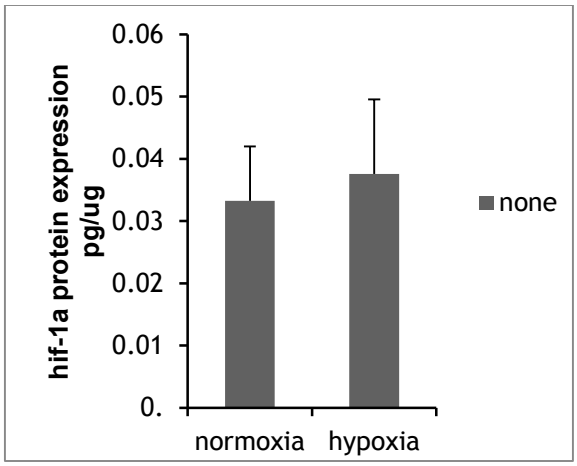

Fig. 15. Hypoxia improves HIF-1α protein expression of ATCC 1547 cells under hypoxia, but there is no significant difference.

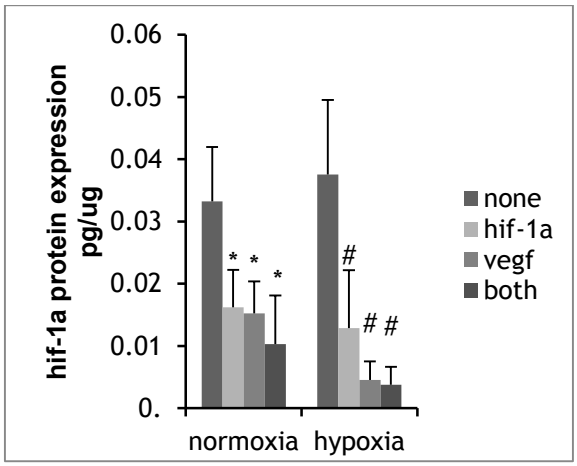

Fig. 16. Inhibition suppresses HIF-1a protein expression of ATCC 1547.*$p<0.05$ compared to none under normoxia. #$p<0.05$ compared to none under hypoxia. This data was analyzed by one-way ANOVA. All experiments were repeated three times.

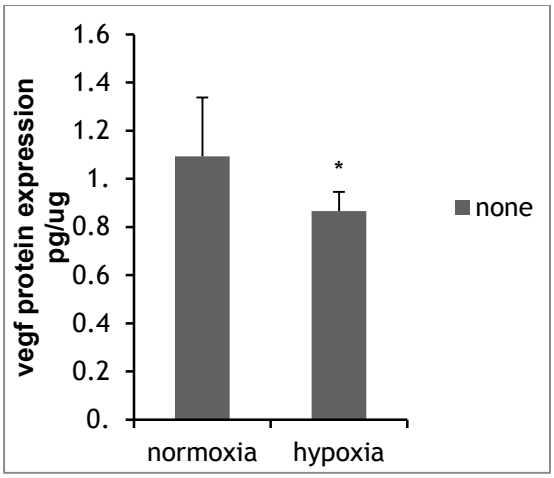

Fig. 17. Hypoxia suppresses VEGF protein expression of ATCC 1547 cells.*p<0.05 compared normoxia

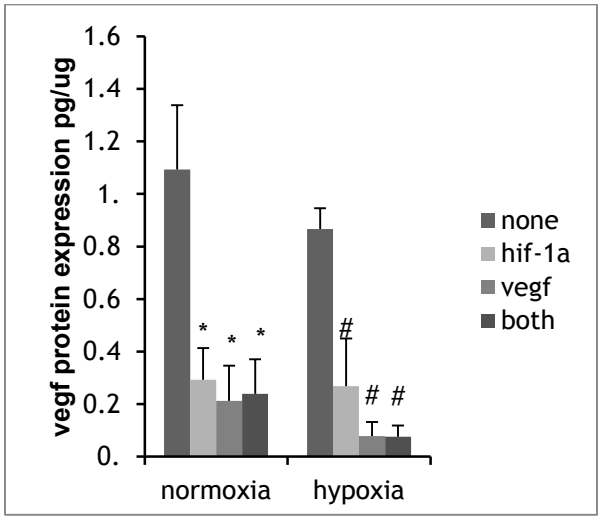

Fig. 18. Inhibition suppresses VEGF protein expression of ATCC 1547.*p<0.05 compared to none under normoxia .#p<0.05 compared to none under hypoxia. These data were analyzed by one-way ANOVA. All experiments were repeated three times.

CHAPTER 6

DISCUSSION

The precise mechanism of osteosarcoma, an aggressive bone cancer, remains unclear. However, research finds that tumor reemergence and metastasis is associated with vascularization. A tumor that can initiate the growth of a blood supply is likely to grow rapidly and become more aggressive, metastasize, and ultimately lead to death. Following several studies supporting the involvement of VEGF in tumor angiogenesis and metastasis, we tried to target the VEGF pathway by blocking it as a means to suppress tumor growth and metastasis. Using a newly developed mouse orthotopic osteosarcoma model, we were able to visually detect a decrease in cell growth when cells were treated with VEGF inhibitor (through a virus-mediated gene therapy) [19, 20].

Another factor that is currently of interest due to its role in the recruitment of leukocytes to sites of inflammation is CXCR4. Research has confirmed that CXCL12, the only recognized ligand for CXCR4, is expressed at high levels in the lung which is the common metastatic site for osteosarcoma. Therefore, we proposed that by targeting the CXCR4/CXCL12 complex, tumor formation and metastasis could be inhibited. Previous studies in Dr. Yang's lab found that expression of CXCR4 dramatically decreased after blocking VEGF function (unpublished data).

As of now, the linkage between CD133 expression and prognosis of osteosarcoma is yet unknown. In this study, we analyzed the correlation of CD133+ cells with VEGF expression. Reports have stated that tumors form from transformed stem cells. It is believed that cancer cell populations contain a small portion of cancer stem cells. Since cancer cells grow slowly, chemotherapeutic drugs are ineffective, thus these cells are potentially more dangerous. In our study, we wanted to find out whether these unresponsive cells are cancer stem cells. If so, they

may be involved in formation, growth, metastasis, and recurrence of tumors in osteosarcoma. These cells may potentially grow fast, metastasize, thus we proposed that VEGF plays a role with CD133+ cells.

Suppressing cell proliferation and/or inducing cell apoptosis in tumors is a crucial step. Almost all solid tumors are dependent on vascular networks that supply nutrition and oxygen. Hypoxia is one of the most important pathological characteristics of solid tumors which is the result of an imbalance between tumor cell proliferation and blood supply [36, 37]. Hypoxia markers, including hypoxia-inducible factor-1 (HIF-1) and vascular endothelial growth factor (VEGF), can be detected in osteosarcomas [30, 33] and the presence of these markers correlates with poor patient outcome, suggesting that hypoxia has an important role in osteosarcoma [32]. Increasing evidence demonstrates that intra-tumoral hypoxia may promote invasive growth and metastasis [38]. However, hypoxia promoting tumor cell apoptosis or having anti-apoptotic affects is controversial. In our study, we used GasPak EZ Gas Generating Sachet to simulate a hypoxic microenvironment in ATCC1547 cells. Cell proliferation was suppressed and more apoptotic cells were observed in the hypoxia groups. More recently, experimental and clinical studies demonstrated that intra-tumor hypoxia might be a key factor in the tumor microenvironment that promotes invasive growth and metastasis [38]. The increased malignancy of hypoxic tumors has been attributed to the ability of hypoxia to select cells with diminished apoptotic potential and to induce their clonal expansion [39]. Under hypoxic conditions, cells must compensate for the lack of oxygen. The adaptive responses of tumor cells to hypoxia apparently results in the more malignant phenotypes of the tumor cells that promotes their abilities of angiogenesis, invasion, metastasis and resistance to chemotherapy and radiotherapy.

Our research showed that, under hypoxia, the transcript activity of HIF-1a increased, which is consistent with other reports. After inhibition of HIF-1a by KC7F2, which is thought to act via down-regulation of HIF-1α protein synthesis, protein expression was suppressed but gene expression was not impacted. In normoxia, cell proliferation decreased when HIF-1α was suppressed. However, cell proliferation was not influenced by the fluctuation of HIF-1a under hypoxia, which suggested that cell proliferation is not correlated with HIF-1a in low oxygen tension. With the inhibition of HIF-1a, cell apoptosis increased as reported by [40], which means inhibition of HIF-1a could promote apoptosis regardless of whether under hypoxia or normoxia. HIF-1a is a critical regulator in osteosarcoma cell survival.

Vascular endothelial growth factor is considered a crucial mediator of angiogenesis and is implicated in carcinogenesis and metastasis [41]. In our study with HIF-1, VEGF gene expression decreased under hypoxia, which coincided with suppressed cell proliferation in lower oxygen tension. Specifically, by blocking VEGFR with SU4312, we observed a significant down-regulation of gene expression in both normoxia and hypoxia. Interestingly, cell proliferation was suppressed at the same time. We deduced that VEGF promotes cell proliferation directly. With the blockage of VEGFR, the quantity of apoptotic cells increased. Especially, more apoptotic cells were observed when HIF-1 and VEGF were blocked.

In conclusion, the involvement of VEGF, CD133, CXCR4, and HIF-1 in osteosarcoma is clearly a very important matter. Our PCR data revealed that VEGF expression is positively correlated with CXCR4 expression, but negatively correlated with CD133 expression. It appears that immunocytochemistry data complements our PCR data showing that blocking VEGF resulted in CD133+ switching. CD133+ cells were identified in osteosarcoma cell population, which may be cancer stem cells and could potentially lead to tumor growth and increased risk of

lung metastasis. Additionally, our study suggests that both cell apoptosis and proliferation occur during tumor development in ATCC1547 cells. HIF-1a significantly induced an anti-apoptotic effect and VEGF promoted cell proliferation. Blocking both HIF-1a and VEGF may be a potential therapy for osteosarcoma. However, further experiments are warranted to determine the clinical significance of such approach. Alternate strategies to analyze the expression of these oncogenes include ELISA and Western blot. Thus, further experiments are needed to confirm the findings and investigate the link between the blockage of VEGF in regulation of other oncogene (CXCR4, CD133, and HIF-1) expressions.

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