MULT1E-RGD fusion protein drives NK cell-mediated anti-tumor response

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MULT1E-RGD fusion protein drives NK-cell mediated anti-tumor response

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Master of Science
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By
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Accepted by:
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MULT1E-RGD fusion protein drives NK-cell mediated anti-tumor response

Meghan R. Steiner, M.S.
Clemson University, 2010

Abstract

A growing body of evidence indicates that natural killer (NK) cells are paramount to the identification and elimination of cancerous and pre-cancerous cells during normal immunosurveillance. In addition, NK cells provide a vital link between the innate and adaptive immune systems during an anti-tumoral response. In the present study, a novel fusion protein was designed from the extracellular portion of mouse UL16-binding protein-like transcript 1 (MULT1), a ligand for the activating NKG2D receptor on NK cells, and a short arginine-glycine-aspartic acid (RGD) - containing peptide, which binds the integrin $\alpha_v\beta_3$ of tumor-specific neovasculature. In vitro studies showed that the fusion protein gene can be successfully incorporated into the genome of B16 mouse melanoma cells, it can be transcribed and translated efficiently, and it is appropriately secreted into the extracellular milieu. In vitro studies also showed that the MULT1E-RGD fusion protein successfully binds the integrin $\alpha_v\beta_3$, and transfection with the gene does not affected cell growth in B16 cells. In vivo studies indicate that transfected cancer cells, when injected subcutaneously in C57BL mice, take on a new phenotype and a slightly different growth pattern, consistent with an upregulated immune response to the tumor.
Key Words: Natural killer cell; NKG2D; RGD; Integrin αvβ3; MULT1
Dedication:

I dedicate this thesis, and the last two years of my life that I have devoted to cancer research, to my grandmother, Joyce, and my grandfather, Paul. You inspired me to study cancer by facing it valiantly, defeating it heroically, and ultimately surrendering to it with honor and grace. One day I will use you both as examples for my own patients as they face a disease that should no longer be a death sentence.
Acknowledgments:

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1.0 Introduction

1.1 OVERVIEW

Cancer is defined as the uncontrolled replication of formerly ‘normal’ cells, coupled with the adoption of malignant behavior, including invasion and metastasis. This definition, however, is undeniably simplified. Cancer relies upon infinitely complex genetic and epigenetic changes in transformed cell populations, along with intricate and highly variable interactions between the developing tumor and its environment. In their seminal article, “The Hallmarks of Cancer,” Hanahan and Weinberg attempted to organize the immeasurable complexities of cancer into a six major categories of acquired capabilities (Fig. 1), which they deemed the six hallmarks of cancer.
First, cancer cells develop the ability to replicate in the absence of mitogenic growth signals. Some cancers accomplish this by producing their own growth factors, which initiate replication by autocrine signaling loops. For example, glioblastomas have been shown to produce their own platelet-derived growth factor (Fedi, et al., 1997). Yet another mechanism for self-sufficiency in growth signals is the tendency of some cancers to overexpress growth factor receptors, such as HER2/neu overexpression...
in the case of breast cancer (Yarden & Ullrich, 1988). This allows receptors to become overly responsive, sometimes reacting even to ambient levels of extrinsic growth factor (Fedi, et al., 1997). Finally, many cancers avoid the need for extrinsic growth signals by altering their intracellular signaling pathways. It has been estimated that about 25% of human tumors have mutated Ras proteins, which are involved in the extremely complicated SOS-Ras-Raf cascade. When present in structurally altered forms, Ras proteins enable the release of a flux of mitogenic signals regardless of extracellular factors (Giancotti & Rouslahti, 1999).

Like in the case of growth signals, normal tissues also rely on the presence of extrinsic anti-growth signals to remain in equilibrium with their surroundings. Cancer cells, on the other hand, develop an insensitivity to these anti-growth signals. One way many cancers accomplish this is to downregulate expression of receptors for growth-inhibiting factors, such as TGF-β (Moses, et al., 1990). Additionally, cancer cells may alter the particular cell adhesion molecules they express so as to replicate regardless of tethering to extracellular matrix or to other cells. Specifically, tumor cells often alter their expression of integrins so as to transduce mitogenic signals rather than growth-inhibiting signals (Desgroisellier & Cheresh, 2010).

Following the acquisition of self-sufficiency in growth signals and insensitivity to anti-growth signals, cancer cells develop mechanisms for evading apoptosis. Some cancers do this by upregulating anti-apoptotic genes such as PI3-kinase and Akt
(Evan & Littlewood, 1998). Others lose expression of tumor suppressor genes such as PTEN (Downward, 1998). Both of these mechanisms for evasion of apoptosis interfere in intracellular processes. Other mechanisms may evolve to evade extracellular pro-apoptotic signaling. Many ways of abrogating the FAS-mediated cell death pathway have been identified in tumors (Pitti, et al., 1998).

Next in the path toward carcinogenesis, cancer cells adopt limitless replicative potential. Even with the previous three acquired capabilities, normal cell will only replicate a given number of times before they enter a ‘crisis state’ and ultimately undergo cell death due to karyotypic disarray. The means by which cancer cells acquire their hallmark immortal character has to do with their ability to prevent the degradation of their chromosomes during replication. All cells have telomeres on the end of their chromosomes, which are gradually degraded with every cell division. When telomeres become completely degraded over a period of time due to replication, the chromosome becomes compromised. The cell can no longer divide and undergoes apoptosis. Cancer cells, however, are able to activate an enzyme called telomerase, which effectively prevents this cap to replication by adding trinucleotide repeats to the end of chromosomes during every mitotic division (Shay & Barcchetti, 1997).

The final steps toward carcinogenesis are sustained angiogenesis and tissue invasion and metastasis. Tumors require sustained angiogenesis in order to grow to
a diameter of any more than about 2cm. The ability to recruit and harbor a complex circuit of tumor-associated vasculature, therefore, is vital for tumor growth and for eventual metastasis. Tumors may accomplish this task by a number of different mechanisms, including the upregulation of soluble growth factors such as vascular endothelial growth factor (VEGF) and fibroblasts growth factors 1 and 2 (FGF1/2). Tumors may also downregulate inhibitors such as thrombospondin and B-interferon. Tissue invasion and metastasis occurs by some of the same mechanisms as angiogenesis. In addition, tumors lose adhesion molecules, such as N-CAM and E-cadherin, that bind them to their surrounding environment and ECM. Tumors also change integrin expression from those that prefer normal epithelial basement membrane to those that prefer degraded stromal components, which in turn guide them toward blood vessels and eventually into the blood stream (Desgro sellier & Cheres h, 2010).

In addition to Hanahan and Weinberg’s proposed paradigm for cancer, it was proposed in 2009 that a seventh Hallmark of Cancer be added to Hanahan and Weinberg’s paradigm: inflammation. A growing body of research indicates that inflammation plays a pivotal role in the progression of cancer. These claims have been made for several different reasons. First, inflammation provides a unique setting for cancer because of the tissue damage that is often associated with it. Inflammation is marked by proteases, which degrade the stroma around the developing tumor. In addition, reactive oxygen and nitrogen species released by
macrophages during inflammation not only contribute to tissue damage, but also provide a mechanism for the development of further genetic instability, associated with cancer. Next, inflammation provides a wealth of growth signals as well as angiogenesis mediators, which the developing tumor readily utilizes. Finally, the inflammation around a tumor works to recruit an immune response to that tumor. Although immune system involvement in a tumor may sound like a positive effect of inflammation, some propose that the presence of immune cells in a developing tumor may in fact promote its progression (Colotta, et al., 2009). Specifically, it has been argued that the inflammatory microenvironment surrounding a growing tumor places selective pressures on the tumor, allowing certain cells to develop preferentially, based on their ability to evade the immune system. This concept is often referred to as ‘immunoediting’ (Fig. 2).

Fig. 2: Immunoediting. Normal cells undergo oncogenic transformation, then immune system places selective pressure to produce immune-evasive subpopulation. (Raulet & Guerra, 2009)
Currently, the link between cancer and inflammation is a subject of hot debate. Although the role of inflammation in tumor progression and metastasis is increasingly accepted, controversy surrounds the idea of exactly how the immune system, as a whole, may contribute to cancer versus how the immune system may combat it.

Traditionally, cancer has been treated by one or both of two major classes of therapy: chemotherapy and radiation therapy. These two types of cancer treatment aim to stymie the proliferative nature of cancer by directly killing the cells that compose it. Radiation is a more localized approach to cancer treatment, wherein radioactive fields are applied to the malignant tumor and a small margin of the surrounding tissue, often including the draining lymph nodes (Camphausen & Lawrence, 2008). Chemotherapy, on the other hand, is a more systemic approach to cancer treatment. Since one of the main characteristics of cancer is its ability to disseminate microscopic portions of the primary tumor, which can subsequently invade distant regions of the body, it is often necessary for patients to undergo this kind of universal treatment. The word chemotherapy refers to a wide range of cytotoxic drugs and drug combinations that work by exterminating rapidly dividing cells. Although chemotherapy is often effective against malignancies, the side effects of any therapy with such potent cytotoxic characteristics are often devastating. Specifically, since chemotherapy aims to eradicate growing cell populations, it often attacks non-transformed cell and tissue types that naturally grow constantly, such
as hair follicles, nail beds, bone marrow, and gut epithelium. This non-specific nature of chemotherapy explains the common side effects of chemotherapy: alopecia (hair loss), mucositis (degradation and inflammation of the gut lining), and myelosuppression (decreased population of blood cells) (Hirsch, 2006).

Of particular interest, the myelosuppressive effects of chemotherapy may have more injurious consequences than was originally suspected. Depletion of the blood cell population implies a depletion of the immune system. Accordingly, cancer patients are significantly more susceptible to infection – a fact that has been well recognized since the beginning of chemotherapy. However, recent research indicates a significant role of the immune system in preventing and combating cancer (Chabner & Longo, 2006).

1.2 IMMUNOTHERAPY

In light of growing evidence supporting the effectiveness of the immune system in fighting cancer, much of the medical community has begun to explore new avenues for cancer treatment. Many strategies work to employ the immune system, rather than attack it. These treatments often fall under the heading of immunotherapy. Immunotherapy is defined as “treatment of disease by inducing, enhancing, or suppressing an immune response” (American Heritage Medical Dictionary. 2007). Within the context of cancer treatment, it has been proposed that strategies such as Coley’s toxin may stimulate the immune system in a broad sense, thereby eliciting
an anti-cancer response by indirect means (MD Anderson, 2006). Although Coley’s results may have provided the foundation for immunotherapy for cancer, current research focuses on much more specific, targeted therapies. With increasing exploration of the immune system’s role in fighting cancer, as well as the progressive characterization of the cancer cell phenotype, the potential for driving a specific, anti-cancer immune response is becoming more and more plausible.

Current immunotherapy research relies heavily on the concept of immune surveillance. Immune surveillance is the term used to describe the body’s natural ability to scan and eliminate “abnormal cells.” These cells may be invaders such as bacteria, and will therefore be recognized by immune cells and eliminated due to their non-self nature. In the context of cancer, however, the immune system must recognize cells that are self, but have been altered. The major player in tumor surveillance is the natural killer cell (NK cell) (Smyth, et al., 2001).

1.3 NATURAL KILLER CELLS

1.3.1 Natural killer cells in innate immunity

Natural killer cells are a class of lymphoid cells that respond to viruses and intracellular pathogens. They make up around 15% of the peripheral blood lymphocytes and are also resident in peripheral tissues such as the liver, peritoneal cavity and the placenta. Although they develop from the common lymphoid progenitor cell, NK cells can be distinguished from B and T cells under the
microscope by their larger size and distinctive cytoplasmic granules. Functionally, NK cells are markedly different from their other lymphoid relatives as well. Namely, NK cells can be activated without prior immunization or stimulation, indicating their role in the innate rather than the adaptive immune system. Rather than the later onset cytokines such as interferon gamma (IFN-γ), NK cells are stimulated by cytokines such as IFN-α, IFN-β, TNF-α, and IL-12. Adding to the evidence that NK cells are in fact part of the innate immune system is their lack of variant receptors. Although the mechanism that NK cells employ to attack their target cells is similar to that employed by cytotoxic T cells, it is triggered by invariant receptors that recognize hallmarks of virally infected or transformed target cells, unlike the highly specific interaction that takes between the T cell receptor and its particular target cell. This makes NK cells more effective against infected and transformed cells in the early stages of infection (Murphy, et al., 2008).

In addition to their established role in eliminating viruses, NK cells appear to be a first line of defense against tumor formation. Several studies have found that depletion of NK cells in vivo makes mice significantly more susceptible to tumor formation (Smyth, et al., 2001). Over the last decade, wealth of research has emerged focusing on the role of NK cells in tumor immunity.

1.3.2 Natural killer cells in cancer recognition
NK cells are typically able to recognize and attack tumor cells on the basis of two features: tumor cells often lack specific ‘self’ molecules, such as class-I MHC proteins, which prevent NK cell activation through specific inhibiting receptors on the NK cell surface (Long E, 1999). In addition, tumor cells sometimes upregulate certain molecules that indicate cell stress, transformation or infection. These molecules interact with specific activating receptors on the natural killer cell membrane, and initiate a stimulatory intracellular response (Viver, et al., 2008). NK cells are activated or inhibited based on a collective balance of activating and inhibiting signals. For an NK cell to become active, typically the inhibitory signal – which includes ‘self’ or healthy cell markers like MHC class I molecules – must be removed (Colotta et al. 2009). Many tumor cells shed or downregulate MHC class I proteins in an effort to prevent cytotoxic T cell response. Without MHC class I molecules expressed on the surface of the cancer cell, however, NK cells are released from the inhibitory signal transduced by Ly49 (in mice), KIR (humans), and the CD49/NKG2A receptor (Raulet & Nadia, 2009). Thus, NK activation status hinges upon a delicate balance between activating and inhibiting signals transduced from the various receptors on the NK cell surface (Fig. 3)
The second feature of the NK cell that allows it to be so active against cancer has only recently been explained. In addition to the delicate balance that works to maintain NK cells in an active or passive state, there exists a potent activating receptor on the surface of NK cells: the NKG2D receptor. NKG2D may also be expressed by other cell types such as activated cytotoxic CD8\(^+\) T lymphocytes and NKT cells. NKG2D recognizes various markers that are upregulated on tumor cells and virally infected cells, but not well represented on uninfected cells (Raulet & Nadia, 2009). Among these ligands is the protein MULT1 (in the mouse), which this particular study has utilized. Upon recognition of its ligands, NKG2D drives a potent cytotoxic response, immediately rejecting cells of transformed nature. (Fig. 4)
Although many other populations of cells are important for immune surveillance, the necessity of the NK cell population was made obvious when NK cell deficient mice received transplanted tumors. In comparison with immunologically normal mice, NK cell deficient-mice were significantly more susceptible to the tumors (Cerwenka & Lanier, 2001; Ljunggren & Karre, 1985; Seaman, et al., 1987). Interestingly, mice deficient in T and B lymphocytes did not show similar susceptibility, indicating that NK cells are in fact the major players in immune surveillance against cancer.

**Fig. 4:** Ligands for the NKG2D activating receptor are expressed on cells in response to infection, transformation, or stress. Binding of NKG2D to its ligands causes signaling cascade that results in NK cell activation: release of perforin and granzyme, secretion of cytokines IFN-γ, TNF-α, and GM-CSF. 
*Cerwenka & Lanier, 2001*
A demonstration of the importance of NK cells, and specifically of the NKG2D receptor, was established when NKG2D-deficient mice were studied (Guerra, et al., 2008). In the TRAMP mouse model, a specific form of highly aggressive prostate adenocarcinoma was found to spontaneously arise at an alarming frequency. In comparing the adenocaromas in NKG2D-deficient mice to the adenocarcinomas of mice with normal NKG2D expression, it was found that deficient mice developed tumors that expressed one or more NKG2D ligands, whereas tumors in NKG2D-sufficient mice commonly expressed no NKG2D ligands. It was proposed that those adenocarcinomas that developed in NKG2D-sufficient mice were targeted by NKG2D-mediated immune surveillance. This resulted in the selection of variant tumor cells that lost expression of NKG2D ligands (Fig. 5) (Raulet & Guerra, 2009).
1.3.3 Natural killer cell activation

Fig. 5: mouse model for immunoediting via NKG2D. a) The highly malignant tumors in the NKG2D-deficient TRAMP mice generally express NKG2D ligands, whereas the rarer tumors of this type in NKG2D-sufficient mice generally lack NKG2D ligands, suggesting that the immune response modifies these tumors by selecting for variant tumor cell lines that fail to express NKG2D ligands. b) by contrast, the less aggressive, late developing tumors express NKG2D ligands regardless of whether NKG2D is expressed, suggesting that these tumors evade NKG2D-dependent elimination by a distinct mechanism. (Raulet & Guerra, 2009).
In this study, the extracellular portion of a mouse NKG2D ligand, mouse UL16-binding like protein 1 (MULT1), was fused with a short peptide sequence, arginine-glutamine-aspartic acid (RGD), which binds integrin $\alpha_v\beta_3$. The purpose of the MULT1E portion of the MULT1E-RGD fusion protein is to direct an NK cell-mediated immune response, which will be localized to the tumor microenvironment by way of RGD interaction with the surrounding tumor vasculature.

MULT1E interaction with the NKG2D receptor inspires a signal cascade beginning on the cytosolic domain of the NK cell receptor. NKG2D interacts with the adaptor protein DAP10. Subsequent recruitment and activation of phosphatidyl inositol-3 kinase results in the activation of ERK and Akt, which ultimately result in the production of cytokines, increased NK cell survival, and the initiation of cytotoxicity (Cerwenka & Lanier, 2001).

Cell killing by NK cells requires direct cell...
contact between effector and target. Killing may occur by two pathways: in the first, a membrane puncturing protein called perforin and a group of serine proteases called granzymes are secreted by exocytosis into the fluid surrounding the target cell. As perforin pierces the membrane, granzyme enters and begins to digest the intracellular contents. Between the disruption of the membrane and the toxic environment within, the cell is under a great deal of stress. In addition, granzyme may actually directly activate cell death pathways through interaction with apoptotic caspases. Thus, the perforin granzyme pathway induces rapid apoptosis of target cells (Fig. 6). The second cell death pathway occurs when FAS ligand on the killer-cell membrane causes target cell death receptors, such as FAS (CD59) to aggregate on target cell membrane. The result is the initiation of classical caspase-dependent apoptosis (Trapani & Smyth, 2002).

1.3.4 Natural killer cells link innate and adaptive immunity
In addition to direct attack of target cells, activated NK cells also secrete the pleiotropic cytokine interferon gamma (IFN-γ), which is indispensable for the activation of antigen-presenting cells (Walzer, et al., 2005) and also for the installment of Th1 responses (Martin-Fontecha, et al., 2004). In addition to lymphocyte recruitment and activation, IFN-γ has been implicated in angiogenesis inhibition (Qin & Blankenstein, 2000). Thus, activation of NK cells in the tumor microenvironment would not only elicit an NK-cell mediated immune response against the tumor and its associated vasculature, but it would recruit an adaptive immune response as well (Wendel et al., 2008) (Fig. 7).
In line with the hypotheses that a strong immune response to tumors would improve the overall outcome of the patient's disease, and that a strong anti-tumor response is initiated by NK cell involvement in the tumor microenvironment, significant research supports that NK cell infiltration in tumors is directly correlated with patient survival. A 1997 study found that colorectal carcinoma patients with...
little to moderate NK cell tumor-infiltration had significantly shorter survival rates than those with extensive infiltration (Coca, et al., 1997). A subsequent study in 2000 found similar results in patients with gastric carcinoma. In this study, patients with high NK cell infiltration not only had longer survival, but they had fewer metastases and less lymph node involvement (Ishigami, et al., 2000). In 2001 and 2002, two studies found similar results with lung cancer (Takanami, et al., 2001; Villegas, et al., 2002). These clinical results indicate that NK cell activation is paramount to a strong anti-tumor immune response, and that such an immune response is paramount to overall disease progression and patient survival.

1.4 INTEGRIN $\alpha_v\beta_3$

1.4.1 Angiogenesis and cancer

The RGD portion of the MULT1E-RGD fusion protein is designed to target the tumor-associated vasculature. A requirement for the development of tumors – both solid and hematologic (Padro, et al., 2007) – is the recruitment and growth of new vasculature. In addition to supplying tumors with nutrients, blood vessels also supply tumors with a receptacle for waste products which would otherwise build up on the core of the mass and cause necrotic cell death (Risau, 1997). Importantly, angiogenesis is also vital for the progression of primary cancer to a metastatic state (Zetter, 1998). Thus, it is postulated that blocking angiogenesis would not only starve tumors individually, but it could stop their spread as well (Bergers, et al.,
1999). Angiogenesis involves complex molecular processes of cell recruitment, differentiation and secretion and teathering to the extracellular matrix surrounding forming blood vessels (Risau, 1997). Because this process is so specific, it provides researchers with several tumor angiogenesis-associated markers, including VEGF, integrin $\alpha_v\beta_3$ and integrin $\alpha_v\beta_3$.

The potential exploitation of the tumor-specific angiogenic process has become a hot topic for cancer research over the last 10 years. Many studies have focused on inhibiting the growth of new blood vessels by employing antiangiogenic agents such as angiostatin and endostatin (O’rielly , et al., 1997; Bergers, et al., 1999). These processes, however, require that the therapy directly interfere with the intricate processes of angiogenesis. In this study angiogenesis is indirectly inhibited by targeting an immune-inspring agent, MULT1, to markers on the tumor-associated vasculature. The RGD portion of MULT1E-RGD targets with high affinity a family of integrins, deemed $\alpha_v\beta_3$, which have been found to be tumor specific (Brooks, et al., 1994).

1.4.2 Integrin $\alpha_v\beta_3$ in tumor-associated angiogenesis

The word integrin refers to a diverse family of cell adhesion receptors which mediate a myriad of cellular functions. Research has shown that integrins are vital to the initiation, progression and metastasis of solid tumors (Desgrosellier & Cheresh, 2010). Integrins have been implicated in tumor cell proliferation,
migration and survival. Their role in cell migration relies on their ability to directly bind the components of the extracellular matrix (ECM). They act by way of a ratcheting mechanism that allows tumor cells to “crawl” along, effectively invading the ECM and tumor microenvironment, often leading to blood vessel intrusion and subsequent metastasis (Guo & Giacotti, 2004). Integrins also mediate invasion of the microenvironment through their ability to aid in the remodeling of the ECM. They do this by regulating the localization and activity of proteases (Guo & Giacotti, 2004). Integrins have also been shown to regulate tumor cell proliferation, even in the absence of adhesion-dependent replication control mechanisms (Vellon, et al., 2005, Assoian & Klein, 2008) (Fig. 8). Finally, almost all of the currently identified integrins are expressed by endothelial cells, especially during angiogenesis (Brooks, Clark & Cheresh, 1994). Because of the profound role of integrins in tumor initiation, progression, and ultimate metastasis, they have been proposed as potential targets for therapy as well as diagnosis of the malignant potential of a given tumor. However, the integrin family is very broad, and the majority of integrins are expressed in many normal tissues, if not ubiquitously (Desgrosellier &
The integrin $\alpha_v\beta_3$, which this study targets with the RGD portion of the fusion protein, has been specifically implicated in several different cancers (Fig. 9). The $\alpha_v\beta_3$ integrin is essential for tumor associated angiogenesis, lymphangiogenesis, and desmoplasia. In addition, $\alpha_v\beta_3$ is expressed at low or undetectable levels in normal, non-tumor-associated tissues (Brooks, Clark & Cheresh, 1994). Therefore, this particular integrin presents a promising means of targeting tumor neovasculature.

Of particular interest, however,
identification of antagonists to the $\alpha_v\beta_3$ integrin versus agonists. It appears that the ability to activate the receptor, as opposed to inhibit it, may rely many characteristics of the ligand. For example, some research has found that certain extracellular matrix proteins such as vitronectin and fibronectin may activate integrin $\alpha_v\beta_3$ signaling, however similar proteins in soluble form may, in fact, inhibit it (Berrier & Yamada, 2007). Interestingly, integrin $\alpha_v\beta_3$ cross-talk in tumor-associated endothelial cells with angiogenesis-associated receptors, fibroblast growth factor receptor (FGFR) and vascular endothelial growth factor receptor 2 (VEGFR2), has been found to prevent intrinsic and extrinsic apoptosis, respectively (Hood, et al., 2003; Alavi, et al., 2003). This indicates that integrin $\alpha_v\beta_3$, and perhaps other integrins, may act synergistically with various oncogenes to further promote tumor progression. Given the specificity of integrin $\alpha_v\beta_3$ to the tumor-associated vasculature, and the potent effects observed when the integrin is activated and inhibited (Fig. 10), much research has focused on exploiting it for its potential therapeutic value.
**Fig. 10:** Integrins expressed on many cancer-related cell types. Step 1: integrins in endothelial cells regulate migration, proliferation and survival necessary for angiogenesis. Step 2: integrins mediate the binding of endothelial cells to pericytes, which aids in subsequent pericyte recruitment. Step 3: cytokines and growth factors from myeloid cells and monocytes help initiate angiogenesis and tumor cell migration. Step 4: integrins aid in directing myeloid cells and monocytes to tumors, as well as desmoplasia. Step 5: invading fibroblasts deposit collagen that may make tumor resistant to treatment. Step 6: integrins regulate growth factor secretion by tumor associated fibroblasts. Integrins may also control platelet expression, which may ultimately aid in metastatic dissemination. *Desgrosellier & Cheresh, 2010*
1.4.3 Exploitation of Integrin $\alpha_v\beta_3$ - RGD

RGD was first identified as a major player in cell adhesion in 1985 (Pytel et al., 1985), and its interaction with integrin $\alpha_v\beta_3$ was characterized by 1988 (Smyth & Cheresh, 1988). By 1994, use of RGD as an $\alpha_v\beta_3$ antagonist was confirmed to directly inhibit angiogenesis in a tumor model (Brooks et al., 1994). Over the last 10 years, RGD has become a well-recognized tool for targeting anti-tumoral and anti-angiogenic agents to tumors and tumor-associated vasculature by way of its interaction with integrin $\alpha_v\beta_3$. RGD has been utilized as an integrin antagonist in several studies ranging from in vitro experiments all the way to pre-clinical and clinical studies. Consistently, it demonstrates potent inhibition of tumor progression. Currently, Phase III clinical trials on an RGD-containing drug called Cilengitide are under way, and showing promising results and few side effects (Reardon et al., 2008).

1.5 THIS STUDY

Given the proven success of RGD in targeting tumors, and the well-established ability of MULT1 to activate natural killer cells by way of their NKG2D receptors, the following study tested the effects of transfecting mouse B16 melanoma cells with the gene for a fusion protein, MULT1E-RGD, on in vitro and in vivo tumor growth. In addition, the study tested the efficacy of the MULT1E-RGD fusion protein in binding immobilized integrin $\alpha_v\beta_3$. The portion of the MULT1 gene encoding the
extracellular domain of the protein was reverse-transcribed from mouse thymocytes, then linked to the gene for the hinge domain of mouse IgG. The gene encoding the RGD peptide (ACDCRGDCF) was included in a reverse primer, to be employed in a polymerase chain reaction. PCR successfully amplified the MULT1E-RGD gene, which was inserted into a vector that inserted a signal sequence on the end of the RGD peptide, such that the ultimate protein would be secreted by the cell. In accordance with predictions, MULT1E-RGD was secreted effectively bound integrin αvβ3 in vitro. Relative to B16 cell lines not expressing the fusion protein, MULT1E-RGD-expressing B16’s did not grow at a significantly different rate in vitro, however a significant difference in tumor growth and progression was observed in vivo.
2.0 Materials and Methods

2.1 CONSTRUCTION OF RECOMBINANT MULT1E-RGD VECTOR:

The extracellular domain of the murine ULBP-like transcript 1, MULT1E, was fused with a short peptide sequence incorporating the arginine-glutamine-aspartic acid (RGD) motif. RGD was embedded within the peptide sequence ACDCRGDCF (Li J, et al., 2004). The MULT1E-RGD gene construct was then inserted into a recombinant expression vector.

Briefly, the MULT1E-RGD gene construct was created by a series of steps. First, mRNA encoding the extracellular portion of MULT1 was reverse transcribed from mouse thymocytes. A hinge domain taken from a gene for mouse IgG was then added to the cDNA by way of PCR (Kotturi, et al., 2008). Finally, PCR was run using the MULT1E + hinge DNA construct as the DNA template. Primers were designed such that the final gene construct would include restriction enzyme recognition sites at either end of the gene. In addition, the reverse primer included the sequence encoding the RGD peptide, along with a piece of the hinge portion of the template DNA. Primers used looked like:

MULT1E-5’ HindIII Forward primer:

5’ CCAAAGCTT ATG GAG CTG ACT GCC AGT AAC AAG GTG C 3’
RGD-hinge-3’ BamHI Reverse primer:

5’ CG GGA TCC TCA GCC GCA GAA ACA ATC TCC TCG GCA GTC GCA GGC TC CGG CCT
GTA CAT ATG CAA GGC 3’

The final gene construct (Fig. 11 A & B) would include, in the 5’ to 3’ direction, a restriction enzyme recognition site for HindIII, the MUL1E gene, the gene for the hinge domain of the protein, the RGD-encoding sequence, and finally a restriction enzyme recognition site for BamHI.

PCR product was purified by gel electrophoresis on a 1% agarose gel and subsequent gel extraction (Qiagen). Next, the gene construct was inserted into the recombinant vector, pcDNA3.1 (+) (Invitrogen) (Fig. 11C). Briefly, both the vector and the PCR product were digested with the restriction enzymes BamHI and HindIII. Following gel purification (Qiagen), a ligation reaction (Epicentre Fast-Link™) was run so as to insert the gene construct into the recombinant vector (Figure 11D), which included a signal sequence at the end of the MUL1 portion of the protein.
Figure 11: Construction of the MULT1E-RGD-containing plasmid. A: putative gene construct containing extracellular portion of MULT1 (MULT1E), IgG hinge region, and RGD peptide, plus restriction enzyme digest regions for BamHI and HindIII. B: gene sequence for MULT1E-RGD, following PCR to fuse RGD to MULT1E-hinge. C: pcDNA3.1 recombinant expression vector, into which MULT1E-RGD is inserted, containing sites for restriction enzyme digest and antibiotic resistance. D: resultant plasmid containing MULT1E-RGD fusion protein gene.
2.2 TRANSFORMATION OF COMPETENT CELLS WITH pMULT1E-RGD:

Invitrogen Max Efficacy DH5α™ chemically competent cells were thawed and incubated on ice for 30 minutes in the presence of 2ul of ligation reaction product. Cells were then transformed by heat shock and subsequently shaken for one hour in S.O.C. media at 37°C, 225 g-force. Finally, cells were spread on ampicillan-containing LB plates and incubated overnight at 37°C. Colonies were isolated from the plates after 24 hours, cultured in a selective ampicillin-containing LB broth overnight, and finally harvested for DNA extraction (Quiagen). After DNA extraction by miniprep (Zymo Research™ Miniprep protocol), plasmid DNA was digested with BamHI and HindIII restriction enzymes and run on a .7% gel to identify colonies which appeared to have replicated plasmid DNA of the appropriate size. Six colonies were chosen for sequencing, and resultant sequences were compared to the fusion protein DNA construct.

Two pMULT1E-RGD-positive colonies (as confirmed by sequencing) were chosen for further amplification of the plasmid DNA. Colonies 1a and 13c were cultured and plasmid DNA was extracted according to the Qiagen Midiprep protocol. Quantity of plasmid DNA was then determined by UV absorbance specrometry and appropriate gene sequence length was re-confirmed by restriction enzyme digest with BamHI and HindIII.
2.3 TRANSFECTION OF B16 MOUSE MELANOMA CELLS WITH pMULT1E-RGD:
The mouse B16-F0 cell line was used for transfection studies. Briefly, pMULT1E-RGD isolated from midiprep 1a was linearized using the restriction enzyme BstB1. B16 cells were cultured on a 6-well plate for 48 hours prior to transfection with linearized pMULT1E-RGD conjugated with Lipofectamine 2000™ transfection reagent (GIBCO). Transfection was performed on 2 wells of ~85% confluent B16 cells. One well would ultimately be used for selection of stable clones, whereas the other would be analyzed for mRNA analysis forty-eight hours after transfection.

2.3.1 Transient Transfection:
Forty-eight hours after transfection, one of the two wells of the 6-well-plate treated with trypsin to harvest B16’s, and RT-PCR was performed to analyze expression of MULT1E-RGD fusion protein gene (MasterAmp™ High-Fidelity RT-PCR Kit, Epicentre). PCR product was run on a 1.5% gel for visualization of RNA expression.

2.3.2 Establishment of Stable Cell Lines:
Transfected B16 cells were cultured in selective medium containing G418 sulfate (Geneticin®, by Invitrogen) at a concentration of 1mg/mL. When cultured at a very low initial concentration, it was possible to observe the formation of specific and visible clones after about 1.5 weeks in culture. Clones were then transferred individually to separate wells, by way of cloning papers, for subsequent expression analysis. Following the establishment of stable cell lines, mRNA expression of the
MULT1E-RGD gene was analyzed by way of RT-PCR (MasterAmp™ High-Fidelity RT-PCR Kit, Epicentre). PCR products for each clone were run on a 1.5% gel (Fig. 17B) to visualize presence or absence of MULT1E-RGD gene, as well as presence of β-actin gene in all cell lines.

2.4 MULT1E-RGD PROTEIN CONFIRMATION:

By way of a specially designed enzyme-linked immunosorbent assay (ELISA) protocol (Stilletti, et al., 2001), translation of the MULT1E-RGD gene to protein was confirmed. Forty eight hours before protein analysis, cells were seeded on 6 well plates in either regular medium containing 10% FBS, or in minimal-serum medium. Briefly, microtiter wells were coated with integrin αvβ3 (Millipore™). Fifty µL of integrin αvβ3, at a concentration of 2.5 µg/mL, were added to wells of a 96-well plate. Plates were incubated at 37°C in 5% CO₂ overnight. Prior to incubation with medium, wells were blocked with 1% BSA. 100µl medium from established cell lines either positive for MULT1E-RGD transcription or negative for MULT1E-RGD transcription (Fig. 17B) was added to appropriate wells and incubated overnight at 37°C. Following thorough washes of wells, primary antibody against the MULT1 portion of the protein, rat anti-mouse MULT1 antibody, was added to wells and incubated at room temperature for 30 minutes. Finally, a secondary antibody: goat anti-rat IgG conjugated to a fluorescent reporter, fluorescein isothiocyanate (FITC), was applied to the wells and incubated for 30 minutes. Following thorough washes,
the plate was read for fluorescence on a Gemini XS™ fluorescence microplate reader (Molecular Devices). High fluorescence values indicated presence of secreted fusion protein in medium. Each cell line was tested for protein translation in regular and minimal-serum medium, and each treatment group was tested in triplicate. **Figure 12** gives a schematic representation of the protein confirmation assay.

![Diagram](https://via.placeholder.com/150)

**Fig. 12**: MULT1E-RGD protein confirmation. Purified integrin α₅β₃ is immobilized on surface of microtiter plate wells. Serum from culture of stable cell lines is then added to wells and incubated overnight. Presence of protein is confirmed following ELISA protocol, using 1º antibody against MULT1 portion of protein, then 2º antibody conjugated to FITC reporter indicates relative protein concentration by fluorescence value.

Medium from culture of untransfected B16 cells were acted as a negative control, as well as medium from culture of clones #11 and #16, which showed no transcription
of the MULT1E-RGD gene according to RT-PCR (Fig. 17B). Other controls included wells exposed to medium – both regular and minimal serum – but no integrin, and wells coated in integrin, but exposed to no medium. The purpose of these controls was to rule out non-specific protein binding to well surfaces, and non-specific antibody binding to integrin, respectively, which would give a false-positive fluorescent result. Figure 13 represents the assay setup. Each cell represents one well used of the 96-well microtiter plate.
**Fig. 13: MULT1E-RGD Protein Confirmation Assay**

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<td>Integrin αvβ3 + clone #11 min. serum medium (-)</td>
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<tr>
<td>C</td>
<td>Integrin αvβ3 + clone #16 regular medium (-)</td>
<td>Integrin αvβ3 + clone #16 min. serum medium (-)</td>
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<td>D</td>
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<td>Integrin αvβ3 + clone #3 minimal serum medium (+)</td>
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<td>Integrin αvβ3 + clone #8 minimal serum medium (+)</td>
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<td>F</td>
<td>Integrin αvβ3 + clone #26 regular medium (+)</td>
<td>Integrin αvβ3 + clone #26 minimal serum medium (+)</td>
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<td>G</td>
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<td>Integrin αvβ3 + clone #28 minimal serum medium (+)</td>
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<td>H</td>
<td>Integrin αvβ3 + NO medium (-)</td>
<td>untransfected B16 regular medium, NO integrin</td>
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<td>untransfected B16 minimal serum medium, NO integrin (-)</td>
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**Fig. 13:** MULT1E-RGD protein confirmation assay. Each experimental and control group was run in triplicate. Results were taken as average fluorescence of three wells.
2.5 IN VITRO CELL GROWTH STUDY:

In order to determine the relative rates of cell growth of the various cell lines, an *in vitro* cell growth study was performed. Briefly, 10,000 cells of each cell line - untransfected B16 control, #11, #16, #3, #8, #26 and #28 – were seeded on 24-well plates. As such, each cell line was represented by 9 wells of a 24 well plate, such that at 3 different time points, 3 wells could be harvested from each clone and counted to measure replication. Cell growth was measured at 24 hours, 48 hours, and 72 hours. Each time point was measured in triplicate. Results represent the average cell number among the three measured wells of the 24-well plate at each time point.

2.6 IN VIVO TUMOR PROGRESSION:

Sixteen C57BL mice were injected subcutaneously with B16 melanoma cells. Four cages, each housing four mice, were designated as untransfected B16 control, Clone #11 negative control, Clone #8 experimental group and Clone #26 experimental group. Each mouse was then injected subcutaneously with 200,000 cells of the appropriate B16 cell line. Tumor growth and progression was then monitored for the following 4 weeks, taking the first measurement at day 11 and continuing to monitor tumor size and progression every 2 days. Mice were euthanized when tumors abscessed and became necrotic, or when tumors exceeded 2cm in diameter and/or began to impair normal function.
3.0 Results

3.1 CONSTRUCTION OF RECOMBINANT MULT1E-RGD VECTOR:

PCR amplification of the MULT1E-RGD fusion protein gene construct showed successful amplification of a strand of DNA roughly 750 bp in size, according to gel electrophoresis. This length of DNA strand was consistent with the goal gene construct (Fig. 14).

Subsequent insertion of the MULT1E-RGD gene into the pcDNA3.1 (+) vector appeared to have successfully ligated the 750 bp target gene to the 5,427 bp recombinant vector, according to gel electrophoresis (Fig. 15) results obtained after miniprep.

![Image](image_url)

**Fig. 14**: result from PCR amplification of putative MULT1E-RGD gene. Large, bright band at about 750 bp, according to 1Kb ladder, indicates amplification of DNA of appropriate size. 1% agarose gel, supplemented with ethidium bromide.
3.2 TRANSFORMATION OF COMPETENT CELLS WITH pMULT1E-RGD:

Following transformation of competent cells with pMULT1E-RGD, 11 clones were initially identified by gel electrophoresis as replicating a strand of DNA of appropriate size (Fig. 15).

Isolated DNA from 6 of these colonies was sent away for sequencing, which showed that all but one of the 6 colonies had replicated the target MULT1E-RGD gene with 100% accuracy. Two of the 6 positive colonies were chosen for further amplification of the pMULT1E-RGD plasmid. Both showed a large quantity of DNA present in the two resultant midipreps, according to ultraviolet absorbance spectrometry (Fig. 16).
3.3 TRANSFECTION OF B16 MOUSE MELANOMA CELLS WITH pMULT1E-RGD:
Reverse transcription polymerase chain reaction, using primers identical to those used for the initial amplification of the MULT1E-RGD gene, confirmed the expression of the MULT1E-RGD gene at least to the mRNA level (Fig. 17A) in the transient transfection. Upon establishment of stable cell lines, it was found that 4 cell lines: clones #3, #8, #26 and #28 all constitutively transcribed MULT1E-RGD fusion protein gene (Fig. 17B).

![Fig. 16: UV absorbance spec used to determine concentration of DNA from midipreps of clones 1a and 13c, both of which were found to replicate pMULT1E-RGD with 100% accuracy.]

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<td>1627.5</td>
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<td>13c</td>
<td>1.852</td>
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![Fig. 17: RT-PCR results from transfection of B16-F0 mouse melanoma cells with pMULT1E-RGD. Presence of MULT1E-RGD gene represented by bands at 750 bp, β-actin bands present in all cells lines, transfected and not transfected. A: results from transient transfection; B16s are able to incorporate pMULT1E-RGD into genome and transcribe it. B: RT-PCR on stable cell lines following transfection with pMULT1E-RGD and subsequent selection with G418 antibiotic. Clones #3, #8, #26 and #28 show transcription of gene. Untransfected B16’s, clones 11, 13 and 16 did not express the gene.]

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Meg Steiner
3.4 MULT1E-RGD PROTEIN CONFIRMATION:

By way of previously described ELISA techniques (Stilletti et al., 2001), the presence of MULT1E-RGD fusion protein was detected in the medium of 4 cell lines: #3, #8, #26 and #28. In addition, no fusion protein was detected in the medium of cell lines that did not show mRNA transcription of the MULT1E-RGD gene, namely clones #11 and #16, and the untransfected B16 cell line. Culture in minimal serum medium showed no significant difference in protein levels as opposed to regular medium with normal levels of serum. Figure 18 shows average fluorescence levels of each cell line’s culture medium, taken from 3 readings. Results indicate that cell lines that transcribe the fusion protein gene, MULT1E-RGD, also translate it to protein. In addition, the MULT1E-RGD protein is secreted into the extracellular space, or medium. Finally, the results show that the RGD portion of the protein successfully binds the integrin αvβ3. This is supported by the fact that fluorescence depends on binding of the secondary enzyme to the primary enzyme, and primary enzyme must bind to the MULT1E-RGD protein, which can only be present if it is bound to Integrin αvβ3 (Fig. 12).
Fig. 18: ELISA assay to confirm presence of MULT1E-RGD fusion protein in medium of clones transcribing MULT1E-RGD. Clones #3, #8, #26 and #28, which all showed presence of MULT1E-RGD RNA, according to RT-PCR, also showed translation of protein. Clones that did not show transcription of gene also show little or no translation of gene.
3.5 *IN VITRO* CELL GROWTH:

Prior to the animal study, it was necessary to determine the relative rates of cell growth of each of the cell lines to be tested in the animal study. Untransfected B16 cells, Clone #11 (negative for MULT1E-RGD expression), Clone #3, Clone #8, Clone #26 and Clone #28 were analyzed at 3 different time points for replication: 24 hours, 48 hours, and 72 hours. Results of the *in vitro* cell growth experiment showed no significant difference in the rates of cell division of each of the cell lines. **Figure 19** shows representative cell growth curves for each of the cell lines. Each data point is an average of the three wells measured at each time point, due to the protocol requirements.
**Fig. 19: In vitro Cell Growth**

Fig. 19: Results of *In vitro* cell growth assay. Cell counts at three different time points: 24h., 48h., and 72h., show no significant difference in cell growth among the 6 cell lines. Results indicate that transfection with pMULT1E-RGD has no effect on cell proliferation rates.
3.6 IN VIVO TUMOR PROGRESSION:

From weeks 1 to 2, tumors expressing the MULT1E-RGD fusion protein seemed to grow at the same rate, or even faster, than the control tumors expressing no fusion protein. Interestingly, after 2 weeks the experimental tumors’ growth rates seemed to slow significantly, whereas the tumors that did not express MULT1E-RGD continued to grow rapidly and invade the subcutaneous tissue aggressively. Perhaps more interesting than the differences in growth rate between control and experimental tumor cells lines, was the difference in tumor progression. Tumors not expressing MULT1E-RGD were extremely aggressive, and began to abscess within the second week following injection. In addition, control tumors rarely reached a diameter greater than 1.5 cm without becoming noticeably necrotic. Experimental tumors, however, reached much larger sizes without abscessing and becoming necrotic. All mice injected with tumors expressing MULT1E-RGD fusion protein were ultimately euthanized due to the size of their tumors. However, only one of the 8 mice showed any signs of abscess in the tumor, and even this abscess was quite small in relation to the tumor size. Figure 20 gives the day-by-day measurements of the individual tumor growth and progression. Figure 21 shows the 4 growth curves representing the average size of each tumor cell line at the given time points. Figure 22 shows pictures of the tumors of mice injected with untransfected B16 cells, as opposed to B16’s that expressed the MULT1E-RGD fusion protein. Mice with control
tumors developed aggressive abscesses and had to be euthanized early, whereas mice injected with experimental B16 cells which expressed the fusion protein developed markedly different tumors. Experimental mice lived longer than control mice, and tumors did not develop aggressive abscesses like their control counterparts. Results indicate that cells producing the MUL1E-RGD fusion protein interact differently with the mouse system than those no expressing the fusion protein, thereby giving rise to the different tumor morphology.
**Fig. 20: In vivo tumor progression**

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**Fig. 20:** Measurement of tumor sizes in mice injected with untransfected B16 cells, clone #11 cells (not expressing MULT1E-RGD), and clones #8 and #26 (expressing MULT1E-RGD). Notably, mice injected with cells that express MULT1E-RGD develop large tumors, but do not develop necrotic abscesses. Mice injected with negative control cells, however, had to be euthanized early due to necrotic, open tumors.
Fig. 21: Growth curves for tumors either expressing MULT1E-RGD fusion protein (#8 & #26) of not expressing MULT1E-RGD fusion protein (B16 control & #11). Although growth curve indicates possibly faster growth of protein-expressing tumors, the curve is a representation of the average tumor growth all mice injected with the given cell line. In general, mice injected with tumors expressing the fusion protein lived longer, and therefore developed larger tumors, whereas those injected with control tumors were euthanized early, and therefore tumors were smaller.
Fig. 22: Phenotypic difference between tumors expressing MUL1E-RGD fusion protein, and tumors not expressing the protein. A&B: Tumors of mice not expressing MUL1E-RGD. Mouse was euthanized on day 18 due to necrotic hole in tumor. C&D: tumor on mouse injected with clone #8. Tumor is large, but shows no sign of opening. Mouse was euthanized on day 24 due to size of tumor and end of study.
4.0 Discussion

4.1 CONSTRUCTION OF RECOMBINANT MULT1E-RGD VECTOR:

In order to produce a protein that incorporates both the extracellular domain of MULT1 that activates the NKG2D receptor, and the RGD peptide sequence that binds $\alpha_v \beta_3$ integrin, it was necessary to design a DNA template that could generate such a sequence when the correct primers were employed. First, mRNA encoding the extracellular domain of MULT1 was reverse transcribed from mouse thymocytes to produce cDNA. Next, a DNA sequence was added to the MULT1E gene which would code for the hinge portion of mouse IgG. The purpose of the hinge domain of the MULT1E-RGD protein will be to allow for mobility in the protein such that steric hindrance does not interfere with RGD binding to integrin $\alpha_v \beta_3$, or MULT1 binding to NKG2D (Kotturi, et al., 2008). Finally, the MULT1E-RGD gene construct was completed by running a polymerase chain reaction using the MULT1E-hinge sequence as the template DNA, along with specially designed primers. These primers added restriction enzyme digest sites to the ends of the gene construct so as to allow for easy insertion into a recombinant vector. In addition, the reverse primer contained the sequence encoding the RGD peptide motif, thereby adding the RGD portion of the protein onto the 3’ end of the gene construct. The reverse primer also
coded for a small portion of the hinge domain of the template DNA. This portion of the primer ensured that the final DNA product would incorporate all of the necessary components to code for the entire fusion protein.

The MULT1E-RGD gene construct was then inserted into a recombinant vector so as to allow for transformation of bacterial cells with the gene-containing plasmid. The pcDNA3.1 (+) vector, by Invitrogen, was chosen for several reasons. First of all, the vector contains restriction enzyme digest sites for both BamHI and HindIII. Thus, the PCR product could be easily inserted into the linearized vector and subsequently ligated. Secondly, the resultant plasmid would contain genes for ampicillin resistance along with neomycin resistance. These antibiotic resistance genes would allow for selection of positive clones both following bacterial cell transformation and ultimately B16 cell transfection, respectively. Finally, the pcDNA3.1 (+) vector contains a signal sequence, which will tag the protein for secretion immediately following translation.

4.2 TRANSFORMATION OF COMPETENT CELLS WITH pMULT1E-RGD:
In order to confirm that the DNA sequence produced by PCR was, in fact, consistent with the targeted MULT1E-RGD fusion protein gene, competent cells were transformed with pMULT1E-RGD, plasmid DNA was isolated by way of miniprep,
and DNA was sent off for sequencing. Sequencing confirmed that not only was PCR amplification of the MULT1E-RGD gene accurate, but insertion into the pcDNA3.1 (+) recombinant vector was successful, ampicillan-resistance was retained by the competent cells, and the plasmid was easily replicated by the cells. Further culture of positive colonies 1a and 13c allowed for extraction of greater quantities of plasmid DNA, which could ultimately be used to transfect a murine cancer cell line.

4.3 TRANSFECTION OF B16 MOUSE MELANOMA CELLS WITH pMULT1E-RGD:
Two wells of a 6-well plate containing a mouse melanoma cell line were transfected with pMULT1E-RGD. The first of the wells served as a transient transfection study, in which transcription of the fusion protein gene was measured 48 hours after transfection and in the absence of selective medium. The purpose of this procedure was to confirm the ability of B16 cells to express the fusion protein gene. In the absence of selective medium, cells are not pressured to preferentially express antibiotic resistance genes and therefore are likely to express the fusion protein gene more readily. After only 48 hours, though, the fusion protein gene has likely not been stably incorporated into the genomic DNA of most B16 cells. Thus, gene expression is only transient, and may be lost if no pressure is placed on the cell lines to continue expressing the gene. This pressure comes from the application of antibiotic to the medium. mRNA analysis of B16’s from the transient transfection,
therefore, confirmed the ability of B16’s to express the gene, but it did not establish stable cell lines.

Accordingly, the second well of the 6-well-plate was transfected with the purpose of establishing stable clones expressing pMULT1E-RGD. By culturing cells at a very low concentration in selective medium containing neomycin, it was possible to isolate specific clones of cells which were resistant to neomycin. Since neomycin resistance is not native to the B16 cell line, it must be delivered by the recombinant vector with which cells were transfected. Thus, in some cases, neomycin resistance should be linked to expression of the fusion protein gene. The establishment of stable cell lines allows for analysis of cancer cells which have incorporated pMULT1E-RGD into their genomes. This is significant, since transfection only ensures that a cell may use the DNA that is introduced, but does not necessarily guarantee that DNA is integrated in the genome. Thus, a cell line that incorporates the gene of interest in its genome and constitutively expresses that gene will maximize the output of fusion protein. These stable cell lines, all with the same genotype, can then be analyzed for protein and RNA expression profile. In addition, stable cell lines are useful in animal studies.

4.4 MULT1E-RGD PROTEIN CONFIRMATION:
As previously described (Stilletti et al., 2001), purified integrin $\alpha_v\beta_3$ can be adsorbed onto 96-well microtiter wells by a simple, overnight incubation. Given that the RGD portion of the MULT1E-RGD fusion protein aims to bind the developing tumor vasculature via a high-affinity interaction with integrin $\alpha_v\beta_3$ expressed on tumor-associated endothelial cells, protein analysis by this particular method serves two major purposes. First of all, a positive ELISA result – namely an increase in fluorescence of specific wells of the microtiter plate – indicates the presence of fusion protein in the medium of the associated cell line. Thus, the four cell lines which showed transcription of the MULT1E-RGD fusion protein gene appear to be translating MULT1E-RGD to protein, as well. This assay also confirms that the protein is in fact being secreted by the B16 cells, since only the medium is used for the assay, and therefore membrane-bound protein could not elicit a positive result here. Interestingly, each of the cell lines expresses the protein at relatively equal levels. In addition to confirming the presence of protein, however, an integrin-based ELISA protocol, like the one employed here, carries functional significance. It shows that not only is the protein of interest present, but the RGD portion of the protein is functioning as it is intended to function: it effectively binds integrin $\alpha_v\beta_3$. This result is paramount to the overall implications of the experiment, since integrin $\alpha_v\beta_3$ is selectively expressed during tumor-associated angiogenesis. Thus, it can be inferred that RGD is an effective tool for targeting MULT1E to the tumor vasculature.
4.5 *IN VITRO* CELL GROWTH:

Because the animal study would be largely focused on how MULT1E-RGD expression affects tumor size and progression in injected B16 tumors, it was necessary to determine if there was a difference in growth rate among the various cell lines to be tested in the animal study. Because there appears to be no significant difference in *in vitro* growth rates among the 4 cell lines, it was assumed that any difference in tumor progression *in vivo* was a result of the mouse system response to the tumor, and not a result of the inherent growth capacities of the various cell lines.

4.6 *IN VIVO* TUMOR PROGRESSION:

For the first two weeks following tumor cell injection, the four cell lines appeared to grow at relatively equal rates, regardless of fusion protein expression. This coincides with the data gathered in the *in vitro* cell growth study. After about two weeks, however, the growth of the experimental tumors seemed to plateau. Unfortunately, it was impossible to determine the long term growth trend of the tumors, since mice had to be euthanized once tumor diameter reached 2 cm. This may indicate that the immune system has waged an attack on the tumor and has begun to stymie its growth. It may also indicate that the tumor vasculature is compromised, and therefore the tumor is beginning to reach its maximum volume. To better determine the pattern of growth in these tumors, a second study should be
done in which fewer B16 cells are injected initially. Tumor growth should then be measured over a longer period of time. The reasons for the discrepancies in tumor growth might be elucidated by histological study of the tumors, as well as cell staining to define what types of cells inhabit the tumor.

In addition to the difference between growth rates is the striking difference in phenotype between the tumors expressing MULT1E-RGD and the tumors not expressing the protein. Interestingly, control tumors rarely reached the 2cm humane endpoint because the mice had to be euthanized earlier due to a large, dark abscess that invariably ruptured from the apex of the tumor. Experimental tumors, however, were allowed to grow much longer because the tumors did not develop this abscess. Thus, the tumors grew to a larger size. B16 tumors typically become obviously necrotic by the time they reach about 1.5 cm in diameter. The point at which mice must be euthanized is denoted the obvious opening that appears at the apex of the tumor. This abscess, which is very dark and deep (~2/3 the depth of the overall tumor) relative to the tumor size, is probably due to the highly aggressive nature of the B16 cell line. The tumors that showed significant expression of MULT1E-RGD fusion protein were visibly different from the control tumors, however. Fusion protein-secreting tumors did not develop necrotic abscesses and almost never opened. Interestingly, these tumors still grew to be quite large, but they seemed to take on a markedly different phenotype. In some instances, tumors
could reach 4cm in diameter without erupting, however most were euthanized by
the time tumor diameter reached 2-3 cm.

This difference in phenotype could be indicative of an anti-tumoral immune
response. This is evidenced by the slight decrease in growth rate of the tumors in
addition to the change in tumor phenotype. More study should be done on this
particular characteristic of the transfected cell lines. Histological exploration of the
tumors and the surrounding tissue might provide indications of the amount of
inflammation caused by the tumor. Cell staining could indicate the amount of tumor-
infiltrating lymphocytes, macrophages, and other immune cells are present in and
around the tumor. Finally, TUNEL assays might reveal a difference in the kind of cell
death that is occurring among the different cell lines. By gross examination alone,
there appears to be less necrotic material in the tumors that expressed MULT1E-
RGD. It might be hypothesized that the fusion protein present in the two positive
cell lines (#8 & #26) is eliciting an immune response, which is causing a higher
infiltration of immune cells (and this could affect the apparent volume of the tumor),
more apoptosis relative to necrosis, and ultimately a slow decline in cell growth
rate. Certainly more in vivo studies should be done concerning the MULT1E-RGD
fusion protein's effect on tumor morphology and the anti-tumoral immune
response.
5.0 Conclusion

According to the results obtained in this study, it can be concluded that fusion of the extracellular portion of MULT1 with a short peptide sequence containing RGD presents a fusion protein with many interesting properties requiring further investigation. First, the fusion protein appears to successfully home to the tumor neovasculature by way of the integrin \( \alpha_v\beta_3 \). Secondly, the protein has some effect on tumor cell growth \textit{in vivo}, which depends on an interaction with the mouse itself, since there appears to be no difference in growth rates of cells \textit{in vitro}. Finally, tumors which express MULT1E-RGD appear to grow at a different rate and undergo morphological changes as a result of transfection with the fusion protein. These changes are only observed in the live mouse model, indicating that phenotypic changes in the tumor are due to some interaction with the mouse system.
References


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