TARGETING ANGIOGENESIS WITH PLASMINOGEN KRINGLE 5

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TARGETING ANGIOGENESIS WITH PLASMINOGEN KRINGLE 5

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NEUROBIOLOGY

ABSTRACT

The recombinant fifth kringle domain of plasminogen (rK5) has been shown to induce apoptosis of dermal microvessel endothelial cells (MvEC), and this pro-apoptotic effect required rK5 binding to cell surface glucose-regulated protein 78 (GRP78). GRP78 is a member of the heat shock protein family and under certain conditions is expressed on the cell surface. I am interested in identifying new anti-angiogenic therapy for glioblastoma tumors. The efficacy of certain anti-angiogenic therapy can be improved when combined with radiation, and radiation is a standard therapy for glioblastoma tumors; therefore, I investigated the pro-apoptotic effect of rK5 combined with radiation on primary human brain MvEC. I found that treatment of brain MvEC with rK5 induced apoptosis in a dose- and time-dependent manner, and that prior irradiation significantly sensitized (500-fold) the cells to the pro-apoptotic effect of rK5. In both the unirradiated and irradiated MvEC, the rK5-induced apoptosis required the expression of GRP78 and the low density lipoprotein receptor-related protein 1 (LRP1), a scavenger receptor. This was determined by blocking studies with an antibody directed toward GRP78 and with a competitive inhibitor of ligand binding to LRP1, as well as by downregulation studies with small interfering RNA. Also, I found p38 MAP kinase to be a necessary downstream effector of rK5-induced apoptosis, in contrast to Erk and JNK. These data suggest that irradiation sensitizes brain MvEC to rK5-induced apoptosis and that this signal requires LRP1 internalization of GRP78 and the activation of p38 MAP
kinase. The physiologic relevance of these findings are supported by my observation that expression of GRP78 protein is upregulated on the brain MvEC in glioblastoma tumor biopsies as compared to the normal brain, suggesting a potential tumor-specific effect of rK5. In addition, in an orthotopic intracerebral xenograft mouse model of malignant glioma, I found that treatment with rK5 significantly decreased tumor volume. Taken together, these in vitro and in vivo data potentially present an important new therapeutic role for rK5 in the treatment of malignant gliomas.
DEDICATION

To my family.
ACKNOWLEDGMENTS

To Brad. Thank you for being such a wonderful husband and my support through these past 4 years. Thank you for always encouraging me in my career and helping me make the best decisions for our future together.

To my parents. Thank you for the endless support and encouragement you have given me. I am blessed to have been raised in such a loving home. You strived for better things for us, rather than yourself, and I cannot thank you enough. To my brother Robert, it has been nice having you here in Birmingham these last few years. I wish you all the luck in your studies and I know you will succeed.

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# INTRODUCTION

## NEW CONCEPTS REGARDING FOCAL ADHESION KINASE PROMOTION OF CELL MIGRATION AND PROLIFERATION

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<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<tr>
<td>CAS</td>
<td>Crk associated substrate</td>
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<tr>
<td>Cdk5</td>
<td>cyclin-dependent kinase 5</td>
</tr>
<tr>
<td>DISC</td>
<td>death inducing signaling complex</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>EGF receptor</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>Erk</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
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<tr>
<td>FAT</td>
<td>focal adhesion targeting</td>
</tr>
<tr>
<td>FERM</td>
<td>domain with homology to the ezrin, radixin and moesin family of proteins</td>
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<tr>
<td>FIP200</td>
<td>FAK interacting protein of 200-kDa</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphosphate dehydrogenase</td>
</tr>
<tr>
<td>GRP78</td>
<td>glucose regulated protein 78</td>
</tr>
<tr>
<td>GSK3</td>
<td>glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HEF1</td>
<td>human enhancer of filamentation 1</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSP70</td>
<td>heat shock protein 70</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>JSAP1</td>
<td>JNK stress-activated protein kinase-associated protein-1</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>K5</td>
<td>kringle 5 domain of plasminogen</td>
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<tr>
<td>LRP1</td>
<td>low density lipoprotein receptor-related protein 1</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryo fibroblasts</td>
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<tr>
<td>MvEC</td>
<td>microvessel endothelial cells</td>
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<tr>
<td>p38MAPK</td>
<td>p38 mitogen activated protein kinase</td>
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<tr>
<td>PARP</td>
<td>poly (ADP-ribose) polymerase</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>PDGFR</td>
<td>PDGF receptor</td>
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<tr>
<td>PI3-kinase</td>
<td>phosphatidylinositol 3-hydroxy kinase</td>
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<td>PLC-γ</td>
<td>phospholipase-C γ</td>
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<tr>
<td>PR</td>
<td>proline rich</td>
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<td>RAP</td>
<td>receptor associated protein</td>
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<tr>
<td>rec</td>
<td>recombinant</td>
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<tr>
<td>rK5</td>
<td>rec kringle 5 domain of plasminogen</td>
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<tr>
<td>si</td>
<td>small interfering</td>
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<tr>
<td>SBD</td>
<td>Src binding domain</td>
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<tr>
<td>Scid</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>Sin/Efs</td>
<td>Src interacting protein/embryonal Fyn substrate</td>
</tr>
<tr>
<td>Skp2</td>
<td>S phase kinase-associated protein-2</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor α</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal 3’-dexoynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>UPR</td>
<td>unfolded protein response</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>vWf</td>
<td>von Willebrand factor</td>
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<td>WHO</td>
<td>World Health Organization</td>
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INTRODUCTION

Glioma Tumors

Patients with malignant glioma tumors have a dismal prognosis due to the high morbidity and mortality associated with these tumors. Malignant glioma tumors that exhibit immunohistochemical expression of glial fibrillary acidic protein (GFAP), an intermediate protein found in astrocytes, are termed malignant astrocytomas (Kleihues et al., 2002). The highest grade of malignant astrocytoma tumors is highly angiogenic and frequently contains areas of necrosis. In addition, malignant astrocytoma cells are highly invasive, and migrate through the tight extracellular space of the brain parenchyma as well as along white matter tracks generating satellite tumors. Current therapy for malignant astrocytoma tumors includes partial surgical resection, irradiation, and chemotherapy. Despite these therapies malignant astrocytomas regrow rapidly, highlighting the need for new more effective therapy.

Histology and Grading

There are four relatively common types of astrocytoma tumors that are graded from I to IV according to the World Health Organization (WHO) histologic classification. A common feature of all grades of astrocytoma tumors is the expression of GFAP. Grade I astrocytomas are typically successfully treated and predominately occur in children. The most common of the Grade I astrocytomas is termed a pilocytic
astrocytoma. The Grade II astrocytomas are also known as fibrillary astrocytomas and hypercellularity is a key histologic characteristic. The two malignant astrocytomas are the Grade III, also known as anaplastic astrocytoma, and the Grade IV, also known as glioblastoma or GBM. Hypercellularity is also found in the Grade III and IV tumors and is accompanied by pleomorphic nuclei and mitotic figures. The additional histologic features of angiogenesis and/or necrosis distinguish the Grade IV tumors from the Grade III tumors (Kleihues et al., 2002). The median survival for an anaplastic astrocytoma is three years and for a GBM tumor 12-18 months (Nabors, 2004; Ohgaki and Kleihues, 2007). Mouse modeling studies suggest that most glioma tumors are probably derived from glial progenitor cells (Singh et al., 2004). Mouse models are now available that replicate in large part the histologic characteristics of human malignant astrocytomas.

Genetic Alterations

There are multiple genetic alterations found in glioblastoma tumors. In some instances these molecular alterations are also observed in other types of malignancy. Amplification of the epidermal growth factor receptor (EGFR) or expression of the mutated receptor (EGFRvIII) is found in roughly 40% glioblastoma tumors (reviewed in Smith and Jenkins, 2000). Other alterations include p53 and PTEN mutations (40% and 30% frequency respectively), loss of cell cycle checkpoint kinases, alterations in the phosphatidyl inositol 3-OH kinase (PI3-K) pathway, and loss of the pRb and p16 genes, all of which contribute to the highly proliferative phenotype of these tumors (reviewed in Shapiro and Shapiro, 1998; Smith and Jenkins, 2000; Brat et al., 2003; Ohgaki and Kleihues, 2007). Certain genetic alterations can be used to predict response to therapy,
for example, methylation status of the MGMT promoter gene predicts a better response to temozolomide therapy in gliomas (Everhard et al., 2006).

GBM tumors can be separated into those that arise *de novo* (termed primary tumors), and those that progress from a lower grade tumor (termed secondary GBM tumors). Although both primary and secondary GBM tumors appear similar on histologic examination, they have different genetic alterations. For example, primary GBM tumors frequently have EGFR amplification or mutation, PTEN mutations, and chromosome 10q loss, whereas secondary GBM tumors frequently have mutations in the p53 and Rb genes, as well as overexpression of platelet derived growth factor receptor (PDGFR) (Smith and Jenkins, 2000).

*Current Treatments*

The treatment of GBM tumors remains complex and largely unsuccessful. Complete surgical resection is frequently avoided due to the neurologic deficit that would be created and due to the difficulty in detecting the tumor margin. If possible, tumor resection is attempted, but frequently a 1-2 cm cuff of tumor cells remain. A partial resection of these tumors frequently relieves the impact of mass effect on the surrounding brain thus improving certain symptoms, such as edema. Once malignant glioma cells have escaped the primary tumor mass they eventually reappear as satellite tumors at a later time point (Chamberlain and Kormanik, 1998).

Following surgery, radiation therapy is typically administered along with chemotherapy (temozolomide). Radiation therapy is not withheld from patients with GBM tumors as it has been shown to increase survival by ~3 months (Nabors, 2004).
Radiation is typically administered over a 4-6 week period at a dosage of 50-60 Gy that is targeted to the tumor area and to a 3 cm cuff around the tumor. The time frame of chemotherapy administration is variable; it can be administered immediately after surgery and prior to radiotherapy, or concurrently with radiation therapy to potentially sensitize the tumor to the effects of radiotherapy. Despite these therapeutic approaches, GBM tumors re-grow rapidly (3-6 months) (Nabors, 2004), thus the critical need for new more effective therapy.

Angiogenesis

*Overall Process*

Angiogenesis is an increased density of small highly permeable blood vessels that are derived from the existing vasculature (Hanahan and Folkman, 1996). Angiogenesis occurs when the ratio or balance of pro-angiogenic to anti-angiogenic factors in a tumor favors the pro-angiogenic factors (Browder et al., 2000). The newly formed vessels in a malignant tumor are abnormal (see below); they grow in response to pro-angiogenic factors secreted by the tumor cells. One well characterized pro-angiogenic factor is vascular endothelial growth factor (VEGF). VEGF is secreted by tumor cells and binds to its cognate receptor on nearby endothelial cells, resulting in the activation of the endothelial cells. Activated endothelial cells proliferate, secrete matrix metalloproteinases that degrade the basement membrane and then migrate through the degraded matrix (termed sprouting). Ultimately tube formation and formation of a partial basement membrane occur along with pericyte recruitment. Angiogenesis also occurs through the sprouting of endothelial cells into the lumen of an existing vessel and the
subsequent bridging of the sprouted endothelial cells. This is known as intussusception (reviewed in Berger and Song, 2005). The process of angiogenesis is complex and its regulation is still being characterized.

**Glioma Angiogenesis**

The neovasculature in glioblastoma tumors is abnormal due to reduced pericytic coverage, an abnormal basement membrane, and reduced numbers of astrocytic endfeet on the pial-glial membrane surrounding the vessels, as well as other alterations (Bello et al., 2004; Berger and Song, 2003; Jain, 2005). These abnormalities result in leaky vessels and the breakdown of the blood brain barrier. An important new therapeutic approach for GBM tumors is the targeting of the neovasculature, especially when this therapy is combined with cytotoxic tumor therapy (Jain, 2005; Rege et al., 2005). As radiation is always included in GBM tumor therapy due to the clear but small increase in survival with radiation therapy, combining radiation with anti-angiogenic agents has gained favor in the neuro-oncology discipline. Radiation is thought to enhance the efficacy of certain anti-angiogenic agents (Li et al., 2005; reviewed in O’Reilly, 2006), for example, the multi-targeted kinase inhibitor ZD6474 shows enhanced anti-glioma activity when combined with radiation (Damiano et al, 2005). Other agents, such as Anginex, which is an anti-angiogenic peptide, also shows enhanced anti-tumor and anti-angiogenic activity when combined with radiation (Dings et al., 2005).

**Apoptosis Signaling**

Apoptosis is the process of programmed cell death. The morphologic characteristics of a cell undergoing programmed cell death include cytoplasmic blebbing
and nuclear condensation. Apoptosis is a complex and multistep process with multiple areas of cross talk and feedback loops. The two basic pathways of apoptosis are the so-called extrinsic and intrinsic pathways (Figure 1). The extrinsic pathway, also known as the death receptor pathway, is activated by death receptor ligands, such as FasL, TNFα, and TRAIL. Binding of the death receptor ligand to the death receptor induces the death inducing signaling complex (DISC), followed by the activation of caspases. Caspases are serine proteases that become active when cleaved, and they in turn activate additional molecules involved in the cell death process. Cleavage of the initiator caspases (caspase 8, 9-, and 10) results in their cleavage/activation of the effector caspases (caspase 3, 6-, and 7). The intrinsic pathway can be activated by multiple stimuli, including cellular stress and DNA damage. This causes cytochrome c release from the mitochondria, which then activates initiator caspases, followed by effector caspase activation. Cytochrome c release from the mitochondria is regulated by the Bcl2 family of pro- and anti-apoptotic proteins that regulate mitochondrial membrane integrity. Both the intrinsic and the extrinsic pathways converge at the level of the effector caspases. In addition, there is crosstalk between these two pathways via caspase 8 cleavage of Bid, generating a truncated Bid, which then activates the intrinsic pathway. As a measure of cell death, quantification of effector caspase cleavage is frequently used as an endpoint (reviewed in Boatright and Salvensen, 2003; Cory and Adams, 2002).

There is an additional pathway that can lead to cell death called autophagy. Autophagy, which means “eat oneself,” is a pro-survival pathway in which the cell will consume and degrade its own organelles. In this pathway the lysosomal machinery is involved in degrading organelles. There is a basal level of autophagy that occurs in cells
and functions as the balance of synthesis and degradation, or recycling of certain organelles. Autophagy is known to be activated under certain conditions, such as nutrient stress or other harmful stimuli. If a cell undergoes an excessive amount of autophagy, the apoptotic pathway can be activated (reviewed in Stromhaug and Klionsky 2001).

**Kringle 5**

Plasminogen is a precursor to the anti-coagulant enzyme plasmin; plasmin promotes degradation of the fibrin matrix at blood clots (Cao et al., 1996; Castellino et al., 1997). Plasminogen is synthesized by the liver and a few other organs and is found in serum. The domain structure of plasminogen contains 5 kringle (K) domains that share ~50% homology. Each kringle domain contains a triple-looped structure formed through three intramolecular disulfide bonds (Castellino et al., 1997; Chang et al., 1998). Proteolytic cleavage of the first four kringle domains (K1-4) of plasminogen occurs in vivo and yields angiostatin (O’Reilly et al., 1994). Angiostatin is an endogenous angiogenesis inhibitor that is currently being tested as an anti-cancer agent (O'Reilly et al., 1994; Jain, 2005). The recombinant fifth kringle domain of plasminogen (rK5), also has anti-angiogenic activity (Davidson et al., 2005; Lu et al., 1999; Cao et al., 1997). There is no evidence that cleavage of plasminogen to yield K5 occurs in vivo and intact plasminogen has no known anti-angiogenic activity. The rK5 used in this thesis project was provided by Abbott Laboratories as part of a collaboration.

_Prior In Vitro Studies with rK5_

It has been shown previously that rK5 induces apoptosis of dermal MvEC (Davidson et al., 2005) and of rat pulmonary endothelial cells (measured as Annexin V
positivity and DNA fragmentation) (Lu et al., 1999). rK5 treatment has also been shown
to block the proliferation of calf pulmonary arterial endothelial cells and of bovine
adrenal capillary endothelial cells, although the mechanism was not determined (Lu et al.,
1999; Cao et al., 1997). Consistent with these findings, other investigators have shown that
rK5 inhibits proliferation of basic fibroblast growth factor (bFGF)-stimulated calf pulmonary
arterial endothelial cells and of bovine adrenal capillary endothelial cells, although again the
mechanism was not determined (Lu et al., 1999). Similar results have been observed with
cell lines established from umbilical vein endothelial cells (Ji et al., 1998). More recently, the
chemotactic migration of non-brain MvEC, of human umbilical vein endothelial cells, and of
macrophages was shown to be inhibited by rK5 (Davidson et al., 2005; Perri et al., 2005).

In Vivo Studies with rK5

The potential in vivo anti-angiogenic effect of rK5 was initially tested in a rat
model of retinal neovascularization induced by hyperoxia; in this model treatment with rK5
inhibited neovascularization and the proliferation of human retinal capillary endothelial
cells propagated in vitro (Zhang et al., 2001). Also, angiogenesis in colorectal carcinoma
and glioma tumors propagated s.c. in immunocompromised mice was inhibited by stable
K5 expression in the tumor cell lines (Liu et al., 2005; Perri et al., 2005). These studies
suggest that rK5 has an anti-angiogenic effect in vivo and supports further studies in
glioma models.

Glucose Regulated Protein 78

The cell surface binding protein for rK5 in dermal MvEC has been shown to be
glucose regulated protein 78 (GRP78) (Davidson et al., 2005). GRP78 is a member of
the heat shock protein family, and is upregulated during the unfolded protein response which is part of the general cellular defense mechanism of stressed cells (reviewed in Kleizen and Braakman, 2005; Li and Lee, 2006; Lee, 2005). GRP78 and other ER-resident stress proteins have been shown to be upregulated in a number of cancers (i.e. lung, breast, and colon) (Lee, 2005; Dong et al., 2004; Mintz et al., 2003; Arap et al., 2004; Fernandez et al., 2000; Gazit et al., 1999; Wang et al., 2005; Chen et al., 2000). In addition, the expression of GRP78 and other members of the unfolded protein response on tumor cells has led to a significant scientific interest in targeting members of the unfolded protein response in cancer (Arap et al., 2004; Fernandez et al., 2000; Lee, 2005; Mintz et al., 2003; Gazit et al., 1999; Dong et al., 2004). In these papers no mention of the tumor endothelial cell GRP78 expression was reported.

Chaperone Signaling

A characteristic of the unfolded protein response (UPR) is the induction of endoplasmic reticulum (ER)-resident stress proteins, which are referred to as glucose-regulated proteins (GRPs) (Lee, 2005). GRPs are chaperone proteins that normally have protective effects on the cell. The best characterized of these proteins is GRP78, a 78-kDa protein also known as BiP. When GRP78 functions as a chaperone protein, it forms complexes with heterologous proteins that are processed through the ER (Lee, 2005). Under normal non-stress conditions, GRP78 directly binds to IRE, PERK, and ATF6 on the luminal side of the ER. During stress, GRP78 is released from IRE, PERK, and ATF6, which then allows for the induction of the UPR signal through IRE, PERK, and ATF6 signaling (reviewed in Quinones et al., 2008).
Role in Cancer

GRP78 expression is highly induced in tumor cells in vivo, in both human tumor tissue and mouse models of tumors (Dong et al., 2004; Chen et al., 2000; Mintz et al., 2003; Barnes and Smoak, 2000; Li and Lee, 2006). In contrast, GRP78 expression in normal organs is very low (brain, lung, heart, breast, liver, spleen, kidney and pancreas) (Dong et al., 2004; Bini et al., 1997; Shin et al., 2003). Recent studies suggest that the increased expression of GRP78 in cancer cells provides a pro-survival effect and a chemoresistance effect (Li and Lee, 2006). Thus, GRP78 may represent a clinically relevant target in multiple tumors.

Cell Surface Interactions

Cell surface GRP78 can promote a pro-apoptotic or a pro-proliferative signal, depending on the "ligand" binding or associating with GRP78, the cell type and other experimental conditions (Davidson et al., 2005; Misra et al., 2002, 2003, 2005; Reddy et al., 2003). Davidson et al. (2005) demonstrated recently that rK5 binds specifically to GRP78 on the surface of dermal MvEC, that GRP78 is necessary for the pro-apoptotic effects of rK5 in these cells and that rK5 binds to a recombinant GRP78 protein in vitro (Figure 2). Furthermore, the peptide sequence, PRKLYDY, in rK5 is required for the interaction of rK5 with GRP78 (Davidson et al., 2005). GRP78 is likely not the only binding partner for rK5. Other cell surface binding partners of rK5 have been reported, such as the voltage-dependent ion channel (Gonzalez-Gronow et al., 2003) and endothelial cell ATP synthase (Veitonmaki et al., 2004).

Low Density Lipoprotein Receptor Related Protein 1

Structure and Function
LRP1 (low density lipoprotein receptor related protein 1) is a cell surface binding partner for GRP78 (Misra et al., 2002). LRP1 was initially identified as a scavenger receptor associated with lipid metabolism (Boucher and Gotthardt, 2004; Herz and Strickland, 2001). LRP1 binds its cell surface ligand and once internalized, LRP1 ultimately targets its ligand to the lysosome for degradation. LRP1 is composed of two subunits, a heavy subunit (515-kDa) and a light subunit (85-kDa), that are noncovalently associated (Ellgaard et al., 1997; Strickland et al., 1990). The heavy subunit contains four extracellular ligand binding domains, and the majority of its ligands bind to domains 2 and 4. Thirty different cell surface ligands for LRP1 have been identified. The 39-kDa receptor-associated protein (RAP) is a competitive inhibitor of ligand binding to LRP1 and is frequently used in studies probing LRP1 function (Medh et al., 1995; Herz et al., 1991; Willnow et al., 1996). LRP1 is abundantly expressed in the normal brain, particularly in neurons, with minimal expression observed in the endothelium (reviewed in Lillis et al., 2008). In contrast, one study indicates LRP1 expression is elevated on the angiogenic endothelial cells in Grade IV malignant astrocytoma (GBM) tumor biopsies (Yamamoto et al., 1997).

Signaling

The biologic effects of LRP1 signaling are dependent on multiple factors, including the cell surface ligand, the cell type, and the experimental conditions (Davidson et al., 2005; Gotthardt et al., 2002; Misra et al., 2002, 2004; Weaver et al., 1997). Signaling is known to occur when cell surface molecules associate with LRP1, such as the platelet derived growth factor receptor, or cell surface calreticulin (an ER chaperone protein) (Newton et al., 2005; Orr et al., 2003a&b). Ligand binding results in the phosphorylation of the LRP1
cytoplasmic tail on serine/threonine and tyrosine residues (Boucher et al., 2002; Loukinova et al., 2002; Boucher and Gotthardt, 2004). These phosphorylation events determine in part the adaptor protein that binds to the cytoplasmic tail and the signal generated. Multiple adaptor proteins have been reported to bind the LRP1 cytoplasmic tail (Boucher and Gotthardt, 2004; Herz and Strickland, 2001). For example, PDGF-BB stimulation can result in phosphorylation of the LRP1 cytoplasmic tail, causing association of the adaptor molecule Shc, and signaling to the ERK MAP kinase pathway (Loukinova et al., 2002). In other conditions, the adaptor molecule Dab1 or Feb65 can associate with the LRP1 cytoplasmic tail and send different signals (Boucher and Gotthardt, 2004).

Focal Adhesion Kinase Family

Focal Adhesion Kinase Family Signaling

Focal adhesion kinase (FAK) is a non-receptor cytoplasmic tyrosine kinase that promotes survival or proliferation and migration/invasion of normal and tumor cells. FAK is part of a signaling complex located on the submembranous portion of the cell membrane and referred to as a focal adhesion or focal contact. Focal adhesions transmit signals from the extracellular matrix to the cell cytoskeleton through cell membrane integrin receptors. These signaling complexes (focal adhesions or focal contacts) are created when integrin receptors recognize their ligand in the extracellular matrix and cluster in the cell membrane. Multiple kinases, adaptor molecules and cytoskeletal proteins are recruited to focal adhesions and focal contacts. FAK promotes cell cycle progression through transcriptional activation of the cyclin D1 promoter (Ding et al., 2005; reviewed in Parsons 2003). In contrast, Pyk2, which is a FAK family member that shares a similar domain homology, can promote apoptosis or inhibit
cell cycle progression when expressed in multiple cell types, suggesting FAK family members may have opposing functions depending on the cell type, level of FAK expression relative to Pyk2, and the experimental conditions (Melendez et al., 2004; Xiong et al., 1998; Xiong and Parsons, 1997).

Regulation of Apoptosis by Pyk2

FAK is primarily involved in pro-survival pathways whereas the FAK family member Pyk2 has been shown to promote apoptosis in certain conditions. Pyk2 can promote apoptosis not only when it is overexpressed but also upon activation by certain cell stimuli. For example, Pyk2 is activated during TNFα stimulation of neutrophils and is thought to play a role in inducing apoptosis (Avdi et al., 2001). Also, methylmethane sulfonate (MMS) induction of apoptosis in cells requires Pyk2 activation of JNK (Pandey et al., 1999), and in MDA-MB468 breast cancer cells the expression of the transcription factor SMAD4 increases Pyk2 expression and induces apoptosis (Ramachandra et al., 2002).

Pyk2 can also inhibit cell cycle progression by competing with endogenous FAK at focal adhesions for binding partners and through the differential activation of MAP kinases (Zhao et al., 2000). Pyk2 can compete with FAK for binding to c-Src and/or Fyn and thereby inhibit ERK activation (Zhao et al., 2000). FAK promotes cell cycle progression through the activation of ERK, and induction of the transcription factor kruppel-like factor 8 (KLF8) that activates transcription of cyclin D1 (Zhao et al., 2000, 2001, and 2003). In glioblastoma cells FAK also promotes cell cycle progression through an inhibition of p27Kip1 expression (Ding et al., 2005)
Figure 1. Simplified Signaling Schematic of Apoptosis Illustrating the Two Basic Pathways (Extrinsic and Intrinsic).
Figure 2. Previously Proposed Mechanism by Which rK5 Induces Apoptosis of Dermal MvEC (Davidson et al., *Cancer Research* 2005).
NEW CONCEPTS REGARDING FOCAL ADHESION KINASE PROMOTION OF CELL MIGRATION AND PROLIFERATION

by

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I. ABSTRACT

Focal adhesion kinase (FAK) is a non-receptor cytoplasmic tyrosine kinase that plays a key role in the regulation of proliferation and migration of normal and tumor cells. FAK associates with integrin receptors and recruits other molecules to the site of this interaction thus forming a signaling complex that transmits signals from the extracellular matrix to the cell cytoskeleton. Crk associated substrate (CAS) family members appear to play a pivotal role in FAK regulation of cell migration. Cellular Src bound to FAK phosphorylates CAS proteins leading to the recruitment of a Crk family adaptor molecule and activation of a small GTPase and c-Jun N-terminal kinase (JNK) promoting membrane protrusion and cell migration. The relocalization of CAS and signaling through specific CAS family members appears to determine the outcome of this pathway. FAK also plays an important role in regulating cell cycle progression through transcriptional control of the cyclin D1 promoter by the Ets B and KLF8 transcription factors. FAK regulation of cell cycle progression in tumor cells requires Erk activity, cyclin D1 transcription, and the cyclin-dependent kinase (cdk) inhibitor p27\(^{Kip1}\). The ability of FAK to integrate integrin and growth factor signals resulting in synergistic promotion of cell migration and proliferation, and its potential regulation by NFkB and p53 and a ubiquitously expressed inhibitory protein, suggest that it is remarkable in its capacity to integrate multiple extracellular and intracellular stimuli.

Abbreviations: CAS, Crk associated substrate; Cdk5, cyclin-dependent kinase 5; EGF, epidermal growth factor; EGFr, EGF receptor; FAK, focal adhesion kinase; FAT, focal adhesion targeting; FERM, domain with homology to the ezrin, radixin and moesin
family of proteins; FIP200, FAK interacting protein of 200-kDa; GSK3, glycogen synthase kinase 3; HEF1, human enhancer of filamentation 1; JNK, c-Jun N-terminal kinase; JSAP1, JNK stress-activated protein kinase-associated protein-1; MEF, mouse embryo fibroblasts; PDGF, platelet-derived growth factor; PDGFr, PDGF receptor; PI3-kinase, phosphatidylinositol 3-hydroxyl kinase; PLC-γ, phospholipase-C γ; PR, proline rich; SBD, Src binding domain; Scid, severe combined immunodeficiency; si, small interfering; Sin/Efs, Src interacting protein/embryonal Fyn substrate; Skp2, S phase kinase-associated protein-2; TNFα, tumor necrosis factor α; VEGF, vascular endothelial cell growth factor.

II. INTRODUCTION

Focal adhesion kinase (FAK) is a non-receptor cytoplasmic tyrosine kinase that plays a key role in several different cell processes largely through its function as a scaffolding molecule (reviewed in Parsons, 2003; Hanks et al., 2003; Craven et al., 2003; and Schlaepfer and Mitra, 2004). This review focus is limited to its role in promotion of cell proliferation and migration. The reader is referred to other recent reviews that cover the recent progress in the inter-related issues of its roles in cell survival and differentiation, as well as its key role during development. In its role as a scaffolding molecule, FAK recruits other signaling molecules to the sub-membranous region of the cell where integrin receptors (also known as cell adhesion receptors) cluster. The signaling complex formed then transmits signals from the extracellular matrix to the cell cytoskeleton through the integrin receptor.
**FAK domain structure.** FAK contains an amino-terminal domain that mediates its interactions with membrane proteins, a centrally-located kinase domain, three proline rich (PR) regions (two in the carboxyl-terminal half of the molecule and one in the amino-terminal half), and the carboxyl-terminal focal adhesion targeting (FAT) domain (see Fig. 1A) (Schaller et al., 1992; reviewed in Parsons, 2003; Hanks et al., 2003; and Schlaepfer and Mitra, 2004). The amino-terminal domain plays a role in the interactions of FAK with integrin receptors, as well as with the platelet-derived growth factor receptor (PDGFr) and the epidermal growth factor receptor (EGFr) (Sieg et al., 2000; Dunty et al., 2004). This domain has homology with the band 4.1 protein as well as with the ezrin, radixin and moesin family of proteins and thus is known as the FERM domain. The association of the FERM domain of FAK with integrins is likely mediated through another protein whereas the interaction with the EGFr appears to be direct (Sieg et al., 2000). It is thought that the FERM domain of FAK likely interacts directly with the PDGFr. The FERM domain also is thought to modulate the kinase activity of FAK; in cell conditions in which FAK is not activated (such as non-tumor cells held in suspension) the FERM domain interacts directly with the kinase domain potentially inhibiting its function (Cooper et al., 2003). The carboxyl-terminal FAT domain contains binding sites for paxillin and talin which aid in the recruitment of FAK to adhesion complexes (reviewed in Parsons, 2003). Adhesion complexes form when cells adhere as a monolayer to a matrix protein substrate. The immature adhesion complexes are known as focal contacts and the mature complexes are known as focal adhesions. Paxillin and talin act together with FAK to link integrin receptors to the cytoskeleton (reviewed in Parsons, 2003; Hanks et al., 2003; and Schlaepfer and Mitra, 2004). The FAT domain of
FAK also interacts with p190RhoGEF, a RhoA specific GDP/GTP exchange factor, and this promotes the phosphorylation of p190RhoGEF and is associated with enhanced RhoA activity (Zhai et al., 2003). A larger region of the carboxyl-terminus, which includes PR1 and PR2 and the FAT domain, is known as the FAK-related-non-kinase domain (FRNK). This domain can be expressed independently of the entire FAK protein through alternative splicing of the FAK gene in certain mammalian organs, such as the lung and testes (reviewed in Parsons, 2003). When expressed in cells propagated in vitro, FRNK acts as a negative regulator of FAK. The two proline-rich regions located in the carboxyl-terminal half of the molecule (known as PR1 and PR2) interact with SH3-domain containing molecules, such as the CAS family of docking molecules (Polte and Hanks, 1995; reviewed in Hanks et al., 2003; and O’Neill et al., 2000).

**FAK activation.** The activation of FAK requires autophosphorylation of residue Y397. Such autophosphorylation has been shown to be temporally-related to the clustering of integrin receptors in the cell membrane that occurs when these receptors bind their ligand (reviewed in Hynes, 2002 and Hanks et al., 2003). The integrin receptor clustering triggers a conformational change in the associated FAK that alters the interaction of the FERM domain with the kinase domain (Cooper et al., 2003). FAK also undergoes autophosphorylation with the activation of certain growth factor receptors in adherent cells (reviewed in Parsons, 2003; Hanks et al., 2003; Craven et al., 2003; and Schlaepfer and Mitra 2004). Phosphorylated (p) Y397 is a high-affinity binding site for Src, and the autophosphorylation of FAK results in the recruitment and binding of cellular Src to pY397 (Schaller et al., 1994). Cellular Src can then phosphorylate Y576/Y577 in the kinase domain of FAK, in the second step of the autoactivation loop.
that is essential for maximal FAK kinase activity and activation (Calalb et al., 1995; reviewed in Hanks et al., 2003). Recent analysis of the crystal structure of the FERM domain suggests that Src binding to the linker region of FAK (adjacent to the FERM domain) through its SH3 and SH2 domains could possibly regulate the protein interactions of the FERM domain (Ceccarelli et al., 2005).

**FAK substrates.** Although FAK function appears to be mediated largely through its action as a scaffolding molecule, FAK also directly phosphorylates several proteins, such as the N-Wiskott Aldrich syndrome protein (N-WASP) (Wu et al, 2004), as well as talin, paxillin, and CAS family proteins (reviewed in Parsons, 2003 and O’Neill et al., 2000). N-WASP is a member of the WASP family of proteins that modulate actin cytoskeletal remodeling. Cellular Src also phosphorylates paxillin, and most likely, cellular Src is the predominant kinase that phosphorylates the tyrosine residues of paxillin (reviewed in Parsons, 2003).

**SH2 domain binding sites in FAK.** In addition to phosphorylating Y576/577 of FAK, cellular Src can phosphorylate several other tyrosine residues that, when phosphorylated, serve as docking sites for SH2 domain-containing molecules (reviewed in Parsons, 2003; Hanks et al., 2003; and Schlaepfer and Mitra, 2004). These include Y407, Y861, and Y925. To date, however, the autophosphorylation site (Y397) appears to be the main binding site for the molecules that associate with FAK through an SH2 domain, including Src, Shc, phosphatidylinositol-3 hydroxyl kinase (PI-3 kinase), phospholipase C (PLC)-γ, and Grb7. Although the binding partners of pY407, pY861, and pY925 are not entirely clear, the phosphorylation of these residues may be important for specific cell events. For example, it has been reported that phosphorylation of Y861
is necessary for cell migration in Ras-transformed NIH3T3 cells (Lim et al., 2004) and for the TGF-β1-induced epithelial-mesenchymal transition of NMuMG murine mammary epithelial cells (Nakamura et al., 2001). In addition, it has been reported that phosphorylation of Y925 is associated with integrin adhesion and E-cadherin deregulation during Src-induced epithelial-mesenchymal transition in colon cancer cells (Brunton et al., 2005), and with Erk activation in human 293 kidney epithelial cells (Schlaepfer and Hunter, 1997).

Sites of serine phosphorylation. FAK contains four sites of potential serine phosphorylation, S722, S732, S843 and S910. S722 is phosphorylated by glycogen synthase kinase 3 (GSK3) during cell spreading thereby decreasing the kinase activity of FAK; conversely, inhibition of GSK3 decreases phosphorylation of S722 and increases FAK activity in spreading cells (Bianchi et al., 2005). In adherent and spreading cells, S722 is targeted for dephosphorylation by serine/threonine protein phosphatase type 1 (Bianchi et al., 2005). Consistent with these findings, in migrating cells phosphorylation of S722 is decreased and GSK3β is inactive. These data suggest a mechanism by which FAK activity is regulated during cell spreading and migration, i.e., the phosphorylation of S722, and hence the activity of FAK is dictated by the competing actions of GSK3β and serine/threonine phosphatase type 1. Phosphorylation of S732 by cyclin-dependent kinase 5 (Cdk5) is important for microtubule organization, nuclear movement, and neuronal migration in cultured neocortical neurons (Xie et al., 2003). It has been shown that FAK phosphorylated on S732 (pS732) is enriched along a centrosome-associated microtubule fork that abuts the nucleus in these cells (Xie et al., 2003). Expression of a mutant FAK(S732A) results in disorganization of the microtubule fork and impairment of
nuclear movement in vitro, and neuronal position defects in vivo (Xie et al., 2003). These observations suggest that Cdk5 phosphorylation of FAK is critical for neuronal migration and acts through regulation of a microtubule fork important for nuclear translocation. Phosphorylation of S843 and S910 has been reported to occur during cell mitosis and is further discussed in Part IV below (Ma et al., 2001).

**Cellular Src, a functional partner of FAK.** Like FAK, cellular Src is a non-receptor cytoplasmic tyrosine kinase that plays a key role in regulating multiple cellular functions, including cell migration and proliferation. Cellular Src associates with multiple cell surface receptors, including some integrins, and with the organized actin cytoskeleton (reviewed in Thomas and Brugge, 1997), as well as with FAK. There are nine known Src family members: c-Src, Fyn, Lyn, c-Yes, Hck, Fgr, Blk, Lck, and Yrk (reviewed in Thomas and Brugge, 1997). All of the Src family members share a similar domain structure which consists of six distinct functional domains (see Fig. 1B). As with FAK, it has been shown that the activation of cellular Src occurs upon ligand binding and clustering of the integrin, and with the activation of certain growth factor receptors in adherent cells (Thomas and Brugge, 1997). FAK and cellular Src act to activate each other. Thus, the association of the SH2 domain of cellular Src with pY397 of FAK and cellular Src phosphorylation of Y576/577 in the autoactivation loop of FAK described above also increases cellular Src activity by opening the closed auto-inhibited conformation of cellular Src resulting in increased autophosphorylation (reviewed in Hanks et al., 2003). These reciprocal roles of FAK and cellular Src in mutual activation is supported by studies of MEFs derived from the FAK-null mouse in which reduced cellular Src activity is found (reviewed in Hanks et al., 2003). Maximal activation of
cellular Src promotes its interaction with, and phosphorylation, of two major targets, CAS family members and paxillin, both of which bind FAK (reviewed in Hanks et al., 2003). Cellular Src also may play a direct role in phosphorylating Y397 (the autophosphorylation site) of FAK, as cells lacking Src family kinases exhibit reduced phosphorylation of Y397 of FAK, and v-Src has been shown to phosphorylate Y397 of FAK (reviewed in Hanks et al., 2003). Thus, cellular Src may function both upstream and downstream of FAK.

**Specific functions of Src family members.** A growing body of evidence indicates that individual Src family members have distinct or specific functions. The specificity of the function of the Src family members is likely regulated by multiple factors, including the cell type, the receptor engaged, the cooperation of receptors, and the subcellular localization of the Src family member, as well as other as yet unidentified factors (Osterhout et al., 1999; Colognato et al., 2004; Ding et al., 2003; Thomas and Brugge, 1997). For example, Fyn promotes the differentiation of neuroglial progenitor cells into oligodendrocytes (Osterhout et al., 1999), whereas Lyn promotes the proliferation of the neuroglial progenitor cells (Colognato et al., 2004). Also, we have shown that Lyn, but not Fyn, is necessary for the migration promoted by the cooperation of integrin αvβ3 and the PDGFrβ in human glioblastoma cells (Ding et al., 2003). Notably, we found a specific requirement for Lyn in this interaction despite the fact that Fyn mRNA and protein are expressed at higher levels than Lyn in these cells (Ding et al., 2003).

**The regulation of FAK.** The regulation of FAK can occur through several different mechanisms including amplification of the gene, alternative splicing, and
dephosphorylation by phosphatases (reviewed in Parsons, 2003; Hanks et al., 2003; Craven et al., 2003; and Schlaepfer and Mitra, 2004;). Most recently, a novel inhibitor known as FAK inhibitory protein of 200-kDa (FIP200) and transcriptional regulation of the promoter also have been shown to regulate the activity of FAK. FIP200, a cellular protein that was identified by yeast two-hybrid screening (Abbi et al., 2002), binds to the kinase domains of FAK and Pyk2 (a FAK family member) and inhibits their functions, although the inhibitory mechanism is not yet entirely clear. As it is expressed in many tissues and cell lines, it may play an important role in inhibiting some cellular processes involving FAK. Studies of the function of the various domains of FIP200 have shown that the amino-terminal domain associates with the kinase domain of FAK and inhibits its kinase activity, as well as inhibiting FAK promotion of cell migration and proliferation in fibroblasts propagated in vitro (Abbi et. al., 2002). The middle domain of FIP200 also associates with the kinase domain of FAK but inhibits FAK activity to a lesser extent than the amino-terminal domain, whereas the carboxyl-terminal domain of FIP200 binds to the amino-terminal (FERM) domain of FAK but does not affect FAK activity (Abbi et. al., 2002). Recently, FIP200 overexpression in MCF-7 breast cancer cells was shown to inhibit cell cycle progression, proliferation, and clonogenic survival (Melkoumian et. al., 2005). The overexpression of FIP200 decreased the level of cyclin D1 protein, likely through inhibition of FAK function (Melkoumian et. al., 2005). These data suggest the possibility FIP200 may function as a tumor suppressor molecule in normal cells. The second recently identified regulatory mechanism was revealed on cloning of the 5’ promoter region of the FAK gene when a 600 base pair region was shown to be necessary for maximal FAK promoter activity (Golubovskaya et al., 2004). Sites for the binding of
several transcriptional factors, including nuclear factor kappa B (NfkB) and p53, have been identified (Golubovskaya et al., 2004). The NfkB transcription factor likely plays a role in regulating FAK transcription, as inhibition of NfkB function was shown to decrease FAK luciferase activity, whereas stimulation with tumor necrosis factor α (TNFα) increased FAK luciferase activity.

III. NEW FINDINGS REGARDING FAK AND CELL MIGRATION (CAS family member specific signaling)

FAK is known to regulate cell migration through its promotion of membrane protrusion and focal adhesion turnover and, potentially, other mechanisms (reviewed in Parsons, 2003; Hanks et al., 2003; Craven et al., 2003; and Schlaepfer and Mitra, 2004). Membrane protrusion and focal adhesion turnover are necessary for cell migration and invasion. The focal adhesions are highly dynamic and heterogenous structures that occur at sites of close membrane contact with the extracellular matrix substrate and anchor a highly bundled and cross-linked actin stress fiber network. Key studies indicating a role for FAK in promotion of cell migration include those demonstrating that FAK-null MEFs migrate poorly in response to chemotactic and haptotactic signals and exhibit an increased number of focal adhesions in adherent cells (Ilic et al., 1995), with re-expression of wild-type FAK restoring the migratory responses (reviewed in Hanks et al., 2003). Similarly, expression of FAT, FRNK or mutant FAK(397F) constructs, which typically act as dominant interfering forms of FAK, in various cell types usually results in partial inhibition of FAK activation and partial inhibition of cell migration (Hauck et al., 2001; Ding et al., 2005; reviewed in Hanks et al., 2003; and Schlaepfer and Mitra, 2004). Conversely, the overexpression of wild-type FAK in various different cell types enhances
cell migration (Wang et al., 2000; Natarajan et al., 2005; reviewed in Schlaepfer and Mitra, 2004).

**CAS protein structure and family members.** FAK promotes cell migration through more than one signaling pathway (reviewed in Hanks et al., 2003). One signaling pathway utilizes the CAS family of proteins as downstream effectors of FAK. The CAS family of proteins include p130CAS, identified initially as a highly tyrosine phosphorylated protein in v-Src and v-Crk transformed cells (Sakai et al, 1994; reviewed in Parsons, 2003), human enhancer of filamentsation 1 (HEF1) identified in a yeast two-hybrid screen as a protein homologous to p130CAS that promotes a morphologic change (Law et al., 1996; Minegishi et al. 1996), and Src interacting protein/embryonal Fyn substrate (Sin/Efs) that was first identified as a Fyn interacting protein (Ishino et al., 1995; Alexandropoulos and Baltimore 1996). p130CAS, HEF1, and Sin/Efs share a relatively conserved architecture, with an amino-terminal SH3 domain, followed by a substrate domain, a serine-rich region, and a carboxyl-terminal region that promotes dimerization of HEF1 (reviewed in Hanks et al., 2003 and O’Neill et al., 2000) (see Fig. 1C). The substrate domain contains multiple tyrosine residues that are a consensus binding site for the SH2 domain of the docking molecule Crk. In the carboxyl-terminal half of the molecule is a peptide containing a tyrosine residue that, when phosphorylated by FAK, is recognized by the SH2 domain of cellular Src and is known as the Src binding domain (SBD) (Tachibana et al., 1997). In addition to the domains described above, p130CAS and Sin/Efs contain two PR regions that are not found in HEF1. Although there is substantial domain homology among the three CAS family members, their
expression, utilization, and function differ (Law et al., 2000; Manie et al., 1997; Almeida et al., 2000; Cho and Klemke, 2000; Natarajan et al., 2005).

The FAK/CAS signaling pathway. CAS proteins bind through their SH3 domain to the PR1 and/or PR2 region(s) of FAK or Pyk2 (a FAK family member) (Polte and Hanks, 1995; reviewed in Hanks et al., 2003; and O’Neill et al., 2000). Cellular Src optimally phosphorylates CAS proteins in the substrate domain when bound to FAK, leading to the recruitment of a Crk family adaptor molecule, activation of a small GTPase, such as Rac1 or Cdc42 and JNK, and the subsequent promotion of membrane protrusion and cell migration (Klemke et al., 1998; Kiyaokawa et al., 1998; Dolfi et al., 1998; Cary et al., 1998; Cho and Klemke, 2000; Fashena et al., 2002; Natarajan et al., 2005). Consistent with a pivotal role for CAS family proteins in cell motility, fibroblasts isolated from p130CAS null mice exhibit defects in cell migration (Honda et al., 1998). Knockout mouse models of HEF1 and Sin/Efs, have not yet been reported. Recently it was shown that a scaffold protein for c-Jun N-terminal kinase (JNK) known as JNK stress-activated protein kinase-associated protein-1 (JSAP1) cooperates with FAK to regulate JNK activation and cell migration (Takino et al., 2005). JSAP1 complexes with FAK and p130CAS in the U-87MG glioblastoma cells upon fibronectin stimulation, resulting in increased FAK activity and the phosphorylation of JSAP1 and p130CAS (Takino et al., 2005).

The interactions of FAK, cellular Src, p130CAS and paxillin promote focal contact/focal adhesion turnover at the leading edge of the cell in fibroblasts (Webb et al., 2004). The promotion of focal contact/focal adhesion turnover by FAK in these cells is due, at least in part, to the ability of FAK to promote the localization of calpain to focal
contacts/focal adhesions (Carragher et al., 2003). Calpain cleaves talin and FAK (reviewed in Schlaepfer and Mitra, 2004). Focal adhesion disassembly appears to be triggered by localized elevation of the levels of calcium (Giannone et al., 2004), and by the localization of microtubules to focal adhesions (Ezratty et al., 2005). Both FAK and the GTPase dynamin are necessary for focal adhesion disassembly induced by microtubules (Ezratty et al., 2005).

**CAS phosphorylation and localization.** CAS proteins are modified by tyrosine kinases in response to integrin ligation, cell adhesion and oncogenic transformation (Sakai et al., 1994; Polte and Hanks, 1995; Petch et al., 1995; Minegishi et al., 1996; Harte et al., 1996; Manie et al., 1997; Vuori et al., 1996; Fashena et al., 2002). The phosphorylation status of CAS proteins contributes to their subcellular localization. Thus, p130CAS and HEF1 localize to focal adhesions (Sakai et al., 1994; Polte and Hanks, 1995; Fashena et al., 2002), with the SH3 domain of p130CAS being necessary for its localization in focal adhesions (Nakamoto et al, 1997). Non-phosphorylated p130CAS is localized mainly in the cytosol, but when phosphorylated p130CAS is localized predominantly in an insoluble fraction associated with focal adhesions, membranous regions and the nucleus (Sakai et al., 1994; reviewed in Hanks et al., 2003).

**CAS family tissue distribution.** Although the CAS family of proteins exhibits high levels of homology, the tissue distribution of the individual family members differs. p130CAS is expressed ubiquitously (Sakai et al 1994), whereas HEF1 is expressed most abundantly in epithelial and lymphoid cells and in the developing hindbrain (Law et al, 1996; Minegishi et al, 1996; Merrill et al., 2004), and Sin/Efs is expressed in embryonic tissues, the thymus, adult muscle and brain (Ishino et al., 1995). The ubiquitous nature of
p130CAS suggests a fundamental role for this molecule in normal cell function, whereas the apparently restricted tissue distribution of HEF1 and Sin/Efs may reflect their specific functions.

**CAS family member specific signaling downstream of FAK.** We have shown recently that HEF1 acts as a necessary and specific downstream effector of FAK in the migration and invasion of glioblastoma cells (Natarajan et al., 2005). In studies aimed at determining whether a CAS family member is a downstream effector of FAK in promoting glioblastoma cell migration, we utilized conditions in which cells overexpressing FAK were stimulated with PDGF. These conditions simulate the in vivo situation in glioblastoma tumor biopsies, as FAK expression is elevated and a PDGF autocrine loop has been identified (Wang et al, 2000; Hecker et al., 2002; Zagzag et al., 2000; Hermansson et al., 1992). We found that both FAK overexpression and PDGF stimulation promoted the phosphorylation of HEF1 on tyrosine residues but not of p130CAS or Sin/Efs, in glioblastoma cells adherent to rec-osteopontin or vitronectin (Natarajan, et al., 2005). Similarly, FAK overexpression and PDGF stimulation promoted the migration of glioblastoma cells adherent to rec-osteopontin or vitronectin, and the invasion of glioblastoma cells through detergent-free normal brain homogenates. To determine the requirement for HEF1, we downregulated HEF1 or p130CAS using small interfering (si) RNA. We found that the downregulation of HEF1, but not of p130CAS, inhibited the basal, FAK-promoted and PDGF-stimulated migration of the glioblastoma cells as well as their invasive potential (Natarajan et al., 2005) (see Fig 2). Using a second glioblastoma cell line, we confirmed that HEF1 was necessary for basal and PDGF-stimulated migration (Natarajan et al., 2005) and further demonstrated that
FAK acts to promote migration through the activation of the Rac1 effector molecule. Consistent with the proposed role of HEF1 in glioblastoma cell migration, we found that in glioblastoma cells adherent to rec-osteopontin or vitronectin, HEF1 was localized predominately in the 0.1% Triton X-100 insoluble fraction. This is consistent with a localization at focal adhesions. In the two glioblastoma cell lines used in these studies (U-251MG and U-87MG) the relative levels of p130CAS, HEF1 and Sin/Efs proteins were not significantly different; thus, assuming that the polyclonal antibodies used were of relatively similar affinity, the differences in the relative levels of expression cannot account for the specific phosphorylation of HEF1 under these cell conditions. Taken together, these data suggest that signaling through CAS family members, in this case HEF1, promotes migration and invasion of tumor cells. FAK overexpression was shown previously to promote the phosphorylation of p130CAS and cell migration in fibroblasts, CHO cells and COS cells (Klemke et al., 1998; Cary et al., 1998) and the phosphorylation of HEF1 and cell migration in T cells (van Seventer et al., 2001).

Signaling through specific CAS family members had previously been reported in B cells. In this case, stimulation of the β1 integrin resulted in HEF1 and p130CAS phosphorylation, whereas stimulation with anti-IgM or anti-IgG F(ab’)_2 antibody resulted in HEF1 (not p130CAS) phosphorylation (Manie et al., 1997). Similarly, the response to apoptotic stimuli appears to be affected by the CAS family member activated. The overexpression of p130CAS in fibroblasts protects cells from apoptosis induced by serum starvation, whereas the overexpression of HEF1 in MCF7 breast epithelial cells increases their sensitivity to TNFα, which is a pro-apoptotic stimulus (Almeida et al., 2000; Cho and Klemke, 2000; Law et al., 2000).
IV. NEW FINDINGS REGARDING FAK AND CELL PROLIFERATION (A Role For p27\textsuperscript{kip1})

FAK promotes cell proliferation by promoting cyclin D1 transcription.

Accumulating evidence suggests that FAK plays an important role in regulating cell cycle progression by integrating signals from integrins and growth factor receptors. Previous studies have shown that inhibition of FAK function by microinjection of the dominant-interfering FAK construct-FRNK results in decreased cell proliferation, as measured by BrdU labeling, in non-tumor cells (reviewed in Parsons, 2003; and Craven et al., 2003). Building on these studies, Zhao et al. (1998) using NIH3T3 fibroblasts demonstrated that the overexpression of wild-type FAK increased cell proliferation by accelerating cell cycle progression through the G\textsubscript{1} to S phase, whereas the expression of a FAK mutant construct lacking the 14 carboxyl-terminal amino acids (\textDelta C14) and acting as a dominant interfering construct inhibited cell cycle progression. Cell cycle progression at the G\textsubscript{1} to S phase is regulated by cdk inhibitors, cyclins, and the Ink4 family of tumor suppressor genes (reviewed in Slingerland and Pagano, 2000; Pietempol and Stewart, 2002; and Ivanchuk et al., 2001). In studies dissecting the mechanism whereby FAK regulates cell cycle progression in NIH3T3 fibroblasts, Zhao et al. (1998, 2001) demonstrated that expression of the mutant FAK (\textDelta C14) construct resulted in a decrease in cyclin D1 expression, whereas overexpression of wild-type FAK increased cyclin D1 expression. Also, cyclin D1 expression was necessary for wild-type FAK promotion of cell cycle progression, and FAK increased transcription of the \textit{cyclin D1} gene by enhancing the binding activity of an Ets transcription factor to the Ets B element in the cyclin D1 promoter (Zhao et al., 2001). Furthermore, Erk activity was necessary for FAK
promotion of cell proliferation. These results are consistent with previous reports that active Erk phosphorylates Ets transcription factors (Wasylyk et al., 1998) and induces transcription of cyclin D1 (Lavoie et al., 1996).

**The transcription factor-Kruppel-like factor 8 (KLF8) is also a downstream target of FAK.** Zhao et al. (2003) went on to show that FAK regulates expression of KLF8 in NIH3T3 cells; FAK overexpression increased KLF8 expression whereas expression of the mutant FAK (ΔC14) inhibited KLF8 expression. KLF8 directly activates cyclin D1 transcription by binding to the GT box in the cyclin D1 promoter. FAK promotion of cyclin D1 expression in the NIH3T3 cells required KLF8, as the downregulation of KLF8 with siRNA inhibited FAK-induced cyclin D1 expression and cell cycle progression (Zhao et al., 2003). These studies suggest that FAK regulates cell cycle progression in normal or non-tumor cells through the transcriptional control of the cyclin D1 promoter by enhancing the binding activity or expression of the Ets B and KLF8 transcription factors, respectively.

**Other molecules associated with FAK promotion of cell proliferation in non-tumor cells.** Recent studies have shown that the overexpression of FAK in smooth muscle cells is associated with increased expression of S phase kinase-associated protein 2 (Skp2) and increased proliferation (Bond et al., 2004). Skp2 has been reported previously to be necessary for the ubiquitination and degradation of p27^Kip1 (Carrano et al. 1999; Sutterluty et al. 1999). Consistent with these findings, inhibition of Skp2 function by microinjection with an anti-Skp2 antibody resulted in cell cycle arrest at the G1 phase (Zhang et al. 1995). These results suggest that Skp2 also may be involved in FAK regulation of cell cycle progression in non-tumor cells.
FAK regulates the cell cycle through cyclin D1 and p27\textsuperscript{Kip1} in tumor cells propagated in vitro. Cell cycle progression through the G\textsubscript{1} phase requires upregulation of cyclins and downregulation of cdk inhibitors, allowing cells to pass through the G\textsubscript{1} restriction point, enter S phase, and proliferate (Slingerland and Pagano, 2000; Pietempol and Stewart, 2002). Cyclin-cdk complexes are regulated by two families of proteins, the INK4 family of tumor suppressor genes (p16, p15, p18, and p19) and the Cip/Kip family of cdk inhibitors (p21\textsuperscript{Waf1}, p27\textsuperscript{Kip1}, and p57\textsuperscript{Kip2}) (Ivanchuk et al., 2001; Slingerland and Pagano 2000). In non-tumor cells, specifically NIH3T3 fibroblasts, the ability of the mutant FAK (\Delta C14) construct to inhibit cell cycle progression did not require the cdk inhibitor p21\textsuperscript{Waf1}, and the level of p27\textsuperscript{Kip1} protein was not altered with overexpression of wild-type FAK or expression of the mutant FAK; thus the necessity of p27\textsuperscript{Kip1} was not tested (Zhao et al., 1998). However, the activity and regulation of signal transduction pathways controlling cell cycle progression and cell proliferation are frequently aberrant in tumors and this is thought to contribute to their malignancy (Slingerland and Pagano, 2000; Nho and Sheaff, 2003). We have shown previously that overexpression of wild-type FAK promotes soft agar growth in the U-251MG glioblastoma cells (Wang et al., 2000), whereas expression of mutant FAK(397F) inhibits soft agar growth of these cells (Hecker et al., 2004). In vivo, we have demonstrated that glioblastoma cells overexpressing wild-type FAK propoagated in the brains of C.B.17 severe combined immunodeficiency (scid) mice exhibited higher cell proliferation (~2-fold) than wild-type U-251MG glioblastoma cells or control clones propagated similarly (Wang et al., 2000). Expression of the mutant FAK(397F) in glioblastoma cells inhibited proliferation of these cells on propagation in the brains of C.B.17 scid mice (Ding et al., 2005). These studies
suggest that FAK promotes cell proliferation in malignant astrocytomas/glioblastoma tumors. These animal studies simulate the elevated expression of FAK that has been demonstrated in tumor cells of human tumor biopsies from various types of malignant tumors (reviewed in Craven et al., 2003), including human malignant astrocytoma/glioblastoma (Wang et al., 2000; Zagzag et al., 2000).

In experiments to determine whether FAK promotes cell cycle progression in these tumor cells, we found that overexpression of FAK decreased the expression of p27\textsuperscript{Kip1} and p21\textsuperscript{Waf1} and increased the expression of cyclins D1 and E, whereas the expression of mutant FAK(397F) enhanced the expression of p27\textsuperscript{Kip1} and p21\textsuperscript{Waf1} and reduced the expression of cyclins D1 and E (Ding et al., 2005). Furthermore, the expression of p27\textsuperscript{Kip1} was necessary for the inhibition of cell cycle progression that occurs on expression of the mutant FAK(397F), as downregulation of p27\textsuperscript{Kip1} with siRNA blocked the inhibitory effect of the mutant FAK(397F) (Ding et al., 2005) (see Fig 3). In contrast, although increased expression of p21\textsuperscript{Waf1} protein was seen on expression of the mutant FAK(397F), downregulation of p21\textsuperscript{Waf1} with siRNA did not block the inhibition of cell cycle progression associated with the expression of mutant FAK(397F) (Ding et al., 2005) (see Fig 3). These data indicate that p27\textsuperscript{Kip1} is required for the inhibition of cell cycle progression by mutant FAK(397F). Similar to results reported for NIH3T3 cells, we also found that cyclin D1, not cyclin E, was necessary for the promotion of cell cycle progression observed on overexpression of wild-type FAK in the glioblastoma cells, and that the overexpression of wild-type FAK enhanced KLF8 expression whereas expression of the mutant FAK(397F) had the opposite effect (Ding et al., 2005).
Importantly, we had shown previously that the overexpression of FAK promoted Ras and Erk activity in the U-251MG glioblastoma cells (Hecker et al., 2002, 2004). In support of these studies and those of Zhao et al. (1998), we also found that Erk activity was necessary for FAK-promoted glioblastoma cell proliferation (Ding et al., 2005). Our studies suggest that FAK regulation of cell cycle progression in glioblastoma tumor cells, and perhaps tumor cells in general, requires Erk activity, cyclin D1 transcription, and p27\textsuperscript{Kip1}.

**FAK promotes cell cycle progression in vivo- Role for p27\textsuperscript{Kip1} and cyclin D1.**

As all prior work regarding FAK regulation of the cell cycle had been performed on cells propagated in vitro, we examined whether FAK also regulated the cell cycle in vivo using an intracerebral xenograft model of human glioblastoma cells propagated in the C.B.17 scid mouse brain. We found that, as our in vitro findings had suggested, the overexpression of wild-type FAK in the glioblastoma cells propagated in vivo promoted cell cycle progression, whereas expression of the mutant FAK(397F) inhibited cell cycle progression (Ding et al., 2005). The mechanism by which FAK regulates the cell cycle in vivo appeared similar to that observed in vitro, as FAK overexpression inhibited p27\textsuperscript{Kip1} and p21\textsuperscript{Waf1} expression and increased cyclin D1 and E expression, whereas expression of the mutant FAK(397F) increased p27\textsuperscript{Kip1} and p21\textsuperscript{Waf1} expression and reduced cyclin D1 and E expression (Ding et al., 2005). Also, FAK overexpression in vivo promoted Erk activity and increased KLF8 expression, whereas expression of the mutant FAK(397F) inhibited Erk activity and reduced KLF8 expression. These data taken together with our in vitro findings suggest that FAK regulation of the cell cycle is a physiologic event, and that in tumor cells it requires not only Erk activity and cyclin D1, but also p27\textsuperscript{Kip1}. 

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During mitosis FAK is phosphorylated on serine residues. Investigators have shown that FAK is heavily phosphorylated on serine residues during mitosis in rat and chicken embryo fibroblasts (Yamakita et al., 1999; Ma et al., 2001). This increase in serine phosphorylation is found on residues 843 and 910 and correlates with a decrease in FAK activation and overall tyrosine phosphorylation (Yamakita et al., 1999; Ma et al., 2001). In addition, FAK re-distributes to the mitotic spindle, the microtubule-organizing center or centrosomes during mitosis of T lymphocytes (Rodriguez-Fernandez et al., 1999). These data taken together with the above discussed role of FAK in promoting exit from the G_1 phase of the cell cycle, suggests that FAK is active early on in cell cycle progression and that its localization and function are altered with progression through the G_2/M phase of the cell cycle.

Serine phosphorylation of FAK may help to determine whether FAK promotes cell migration or proliferation. Phosphorylation of the S722 residue inhibits the rec-SH3 domain of p130CAS from binding to the PR1 region in FAK (Yamakita et al., 1999; Ma et al., 2001). Consistent with this observation serine phosphorylation of FAK correlates with FAK dissociation from p130CAS (Yamakita et al., 1999; Ma et al., 2001).

HEF1 regulates the activation of kinases necessary for centrosome function. HEF1 redistributes from focal adhesions to mitotic spindle asters in MCF7 breast epithelial cells in the G_2/M phase (reviewed in O’Neill et al., 2000). Most recently, Pugacheva and Golemis (2005) reported that HEF1 was necessary for the activation of two kinases, AuroraA and Nek2, at the centrosome. This suggests that the cell adhesion protein HEF1 can help to regulate both cell migration and cell division.
V. FUTURE DIRECTIONS REGARDING FAK-PROMOTED MIGRATION THROUGH CAS AND FAK-PROMOTED PROLIFERATION

What determines CAS family member specificity in the signaling downstream of FAK that promotes cell migration? There is some evidence to suggest that the functions of the CAS family members are cell-type specific; for example, the aforementioned differences in the distribution of the individual family members in various different tissues and cell-types (reviewed in O’Neill et al., 2000). Further tissue and cell distribution studies regarding the CAS family members need to be performed; however, the preferential utilization of CAS family members cannot be explained completely in terms of cell type as we observed preferential utilization in cells in which all three CAS family members were expressed at relatively similar levels. There is evidence to suggest that CAS family member signaling could be dependent on the microenvironment, including the specific cytokine or growth factor stimuli. Law et al. (2000) reported that the overexpression of HEF1 in MCF7 breast epithelial cells increased their sensitivity to the pro-apoptotic effect of TNFα stimulation. This concept needs further exploration, as we found that EGF stimulation of the glioblastoma cells adherent to a rec-osteopontin substrate did not promote increased HEF1 phosphorylation, nor did it promote the phosphorylation of p130CAS or Sin (Natarajan et al., 2005). Consistent with these findings no increase in migration was observed on EGF stimulation of these cells. Rather, we found that PDGF stimulation promoted HEF1-specific phosphorylation in the cells adherent to rec-osteopontin or vitronectin and that this correlated with increased cell migration (Natarajan et al., 2005). Thus, further defining
how changes in the microenvironment affect specific CAS family member activation will likely lead to a better understanding of CAS family member specific functions. Also, importantly, in vivo studies in animal models need to be performed examining the effect of conditionally downregulating a specific CAS family member, followed by studies determining the effect on cell migration or invasion.

**How does FAK regulate p27^Kip1?** The mechanism by which FAK regulates p27^Kip1 has not yet been elucidated and it will be intriguing to determine whether FAK regulation of p27^Kip1 occurs through FAK regulation of Skp2. Notably, p27^Kip1 is aberrantly regulated in many cancer cells. This aberrant regulation is associated with an alteration in its protein binding partners, causing it to be sequestered in the cytoplasm, and increased ubiquination, which leads to enhanced degradation (Slingerland and Pagano, 2000; Nho and Sheaff, 2003). Thus, the effect of reduced levels of p27^Kip1 on FAK-mediated proliferation in cancer cells is also of interest. Similarly, as a p53 binding site has been identified in the FAK promoter (Golubovskaya et al., 2004) and an absence of p53 function is observed in many tumors (Peitenpol and Stweart, 2002), the effects of p53 on FAK regulation of the cell cycle is of interest.

In conclusion, FAK appears to play a key role in cell migration and proliferation and to coordinate the activities of several molecules that are considered to be associated closely with pathophysiologic proliferative conditions, including cancer, inflammation, and atherosclerosis. As is apparent from this prospect/review, the effects of FAK on cell migration and the pathways associated with these activities have been analyzed in several normal cell types and multiple tumors, whereas the effects of FAK on cell proliferation have been analyzed in only a few normal cell types and a few tumors. The results of the
studies to date suggest that analysis of other cell types, tumors that originate from these cells, and pathophysiologic conditions in animal models could be very informative. Importantly, as FAK serves to integrate the effects of signals generated by several different components of the extracellular environment (such as matrix proteins and growth factors/cytokines), such studies should yield data that is of relevance to the behavior of cells in vivo and could lead to the further development and clinical application of FAK specific inhibitors in various diseases.

**Acknowledgements:** This work was supported by grants #CA97110 and CA109748 from the National Institutes of Health-National Cancer Institute to CLG. We thank Mrs. Joe Self for assistance in preparing this manuscript.
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Brunton VG, Avizienyte E, Fincham VJ, Serrels B, Metcalf CA 3rd, Sawyer TK, Frame MC. 2005. Identification of Src-specific phosphorylation site on focal adhesion kinase:


Xie Z, Sanada K, Samuels BA, Shih H, Tsai LH. 2003. Serine 732 phosphorylation of FAK by Cdk5 is important for microtubule organization, nuclear movement, and neuronal migration. Cell 114:469-482.


Fig. 1. Domain organization of FAK, Cellular Src, and CAS. A, The domain organization of focal adhesion kinase (FAK). The FERM domain shares homology to the band 4.1 protein, and the ezrin, radixin, moesin family of proteins. The kinase domain indicates the region of catalytic activity. PR1 and PR2, denotes proline-rich regions 1 and 2 in the carboxyl-terminus. FAT denotes the focal adhesion targeting domain. FRNK denotes the FAK-related non-kinase domain. Sites of tyrosine and serine phosphorylation are indicated. The amino-terminal proline-rich domain is not shown. B, The domain organization of cellular Src. The catalytic domain contains the autophosphorylation site Y416/418 in c-Src. The carboxyl-terminal negative regulatory peptide contains Y527/529 which is negatively regulated in mouse/human c-Src. C, The domain organization of CAS family members.
A. FAK

B. Cellular Src

C. CAS

% Homology in the Various Domains Between Cas Family Members
**Fig. 2.** Downregulation of HEF1, but not p130CAS, inhibits basal, FAK-promoted and PDGF-stimulated migration, as well as invasion of glioblastoma cells. (a–d) OFAK5 cells and U-87MG cells were transfected with 200 nM siHEF1 or sip130CAS, the transfection repeated at 24 h, and at 48 or 72 h, the cells lysed in RIPA lysis buffer and the lysates (40 µg) from each condition subjected to SDS–PAGE, and immunoblotted with the indicated antibodies. (e) OFAK5 cells were transfected with 200 nM siHEF1 or sip130CAS, the transfection repeated at 24 h, and at 48 h, treatment with doxycycline or vehicle was begun and continued throughout the experiment. At 72 h, the cells were replated onto rec-osteopontin-coated wells in DMEM-0.4% FBS-1% BSA and subject to the scratch assay. (f) U-87MG cells were similarly transfected with siHEF1 or sip130CAS, the cells harvested with buffered EDTA, resuspended in DMEM-0.8% FBS-1% BSA and plated onto vitronectin-coated wells. At 24 h the monolayer was subject to the scratch assay and photographed at 48 h. (g) OFAK5 cells were treated, harvested with buffered EDTA, resuspended in DMEM-0.4% FBS-1% BSA, and plated onto 8 mm pore filters coated with 50 µg/ml normal brain homogenate and allowed to invade for 24 h (37°C, 5% CO2). Cells on the bottom filter surface were fixed, stained, and counted. The data were analysed and presented as the mean ± S.E.M. for each condition. This figure is reprinted with permission from Oncogene 2005;25:1721-32.
a) siRNA HEF1 (h)  OFA5 Cells  
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b) siRNA p130CAS (h)  OFA5 Cells  
| Anti-p130CAS IgG | 1 | 2 | 3 | 130 kDa |
| mAb anti-HEF1 | 1 | 2 | 3 | 110 kDa |
| mAb anti-Actin | 1 | 2 | 3 | 42 kDa |

c) siRNA HEF1 (h)  U-87MG Cells  
| mAb anti-HEF1 | 1 | 2 | 3 | 110 kDa |
| Anti-p130CAS IgG | 1 | 2 | 3 | 130 kDa |
| mAb anti-Actin | 1 | 2 | 3 | 42 kDa |

d) siRNA p130CAS (h)  U-87MG Cells  
| Anti-p130CAS IgG | 1 | 2 | 3 | 130 kDa |
| mAb anti-HEF1 | 1 | 2 | 3 | 110 kDa |
| mAb anti-Actin | 1 | 2 | 3 | 42 kDa |

e) OFA5 Cells Adherent to rec-Osteopontin  
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</tr>
<tr>
<td>PDGF</td>
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* The average number of cells migrated in all four conditions with siHEF1 is significantly smaller (p=0.003) than the average number of cells migrated in all four conditions with sip130 CAS.

f) U-87 MG Cells Adherent to Vitronectin  
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<td>PDGF</td>
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p=0.008

g) OFA5 Cells  
| Number of Cells Invading |
| DOX | siHEF1 | sip130 CAS |
| + | - | + |
| + | + | - |
| + | + | + |

p<0.01
**Fig. 3. Down-regulation of p27^{kip1} blocks the inhibitory effect of mutant FAK397F on the cell cycle progression of glioblastoma cells.** Parallel cultures of the OFAK397F-18 clone propagated in complete medium were administered 2 µg/ml doxycycline (Dox) or vehicle for 4 days and then transfected with duplex siRNA directed toward p27^{kip1}, p21^{Waf1}, or p57^{kip2}. **A**, post-transfection (48 h) the cells were harvested and RNA extracted for real time RT-PCR using forward and reverse primers directed specifically toward p27^{kip1}, p21^{Waf1}, or p57^{kip2}. The relative expression of these mRNAs was normalized to actin. **B**, cells were detergent lysed and the lysate subjected to immunoblotting with the indicated antibodies. **C** and **D**, the cells were harvested, propidium iodide labeled, and the DNA content analyzed by FACS analysis. In **D**, the percentage of cells in G0/G1, S, and G2/M phases is plotted as a histogram, and the statistical significance of differences in each phase when cells were Dox treated versus vehicle treated is indicated by the p value; p < 0.05 was considered significant. This figure is reprinted with permission from the Journal of Biological Chemistry 2005; 280:6802-6815.
**A.**

Relative Expression

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**B.**

*siRNA: p27*

- Anti-p27 IgG blot
- Anti-p21 IgG blot
- mAb anti-actin blot

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*siRNA: p21*

- Anti-p21 IgG blot
- Anti-p27 IgG blot
- mAb anti-actin blot

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**C.**

*OFAK397F-18; siRNA: p27*

Cell Number

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**D.**

*OFAK397F-18; siRNA: p27*

% of Cells positive

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PLASMINOGEN KRINGLE 5 INDUCES APOPTOSIS OF BRAIN MICROVESSEL ENDOThELIAL CELLS: SENSITIZATION BY RADIATION AND REQUIREMENT FOR GRP78 AND LRP1

by

BRADEN C. MCFARLAND, JERRY STEWART JR., AMAL HAMZA, ROBERT NORDAL, DONALD J. DAVIDSON, JACK HENKIN, AND CANDECE L. GLADSON

Submitted to Cancer Research

Format adapted for dissertation

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ABSTRACT

Recombinant plasminogen kringle 5 (rK5) has been shown to induce apoptosis of dermal microvessel endothelial cells (MvEC) in a manner that requires glucose-regulated protein 78 (GRP78). As we are interested in anti-angiogenic therapy for glioblastoma tumors, and the effectiveness of anti-angiogenic therapy can be enhanced when combined with radiation, we investigated the pro-apoptotic effects of rK5 combined with radiation on brain MvEC. We found that rK5 treatment of brain MvEC induced apoptosis in a dose- and time-dependent manner, and that prior irradiation significantly sensitized (500-fold) the cells to rK5-induced apoptosis. The rK5-induced apoptosis of both unirradiated and irradiated MvEC required expression of GRP78 and the low density lipoprotein receptor-related protein 1 (LRP1), a scavenger receptor, based on downregulation studies with small interfering RNA, and blocking studies with either a GRP78 antibody or a competitive inhibitor of ligand binding to LRP1. Furthermore, p38 MAP kinase was found to be a necessary downstream effector for rK5-induced apoptosis. These data suggest that irradiation sensitizes brain MvEC to the rK5-induced apoptosis and that this signal requires LRP1 internalization of GRP78 and the activation of p38 MAP kinase. Our findings suggest that prior irradiation would have a dose-sparing effect on rK5 anti-angiogenic therapy for brain tumors and further suggest that the effects of rK5 would be tumor-specific as the expression of GRP78 protein is upregulated on the brain MvEC in glioblastoma tumor biopsies as compared to the normal brain.
INTRODUCTION

Glioblastoma tumors have a dismal prognosis with a median survival of 12 to 15 months. As the tumors typically exhibit angiogenesis (1-3), anti-angiogenic therapy may represent an effective therapeutic strategy. In this study we investigated the pro-apoptotic effect of the recombinant form of the fifth kringle domain of plasminogen (rK5) on human brain microvessel endothelial cells (MvEC). Irradiation is known to promote the ability of other anti-angiogenic agents to inhibit tumor growth (4-6) and irradiation is a standard initial therapy for patients with glioblastoma tumors (2); therefore, we also investigated the potential promotion of rK5-induced apoptosis of brain MvEC by irradiation.

It has been shown previously that rK5 induces changes indicative of apoptosis in non-brain MvEC (7,8) and inhibits proliferation of basic fibroblast growth factor (bFGF)-stimulated calf pulmonary arterial endothelial cells and bovine adrenal capillary endothelial cells (8,9). The ability of rK5 to inhibit neovascularization has been demonstrated directly in a rat model of hyperoxia-induced retinal neovascularization (10). Moreover, stable expression of K5 in U-87MG glioblastoma cells prior to their s.c. propagation in nude mice resulted in inhibition of angiogenesis and tumor growth (11) and inhibition of angiogenesis was seen when colorectal carcinoma cells stably expressing K5 were propagated s.c. in athymic nude mice (12).

The cell surface binding protein for rK5 on dermal MvEC, is glucose-regulated protein 78 (GRP78) (7). GRP78 is a member of the heat shock protein (HSP) 70 family, and its upregulation is part of the general cellular defense mechanism of stressed cells that is
referred to as the unfolded protein response (reviewed in 13); (14). The increased expression of members of the unfolded protein response in tumors suggests they may be promising therapeutic targets (reviewed in 15). GRP78 associates with the scavenger receptor low density lipoprotein receptor-related protein 1 (LRP1) on the cell surface (16). LRP1 is known to signal upon associating with and internalizing its ligands (17-20). The biologic consequences of its internalization are varied and depend on which one of approximately 30 different ligands it binds (reviewed in 21,22); (23), as well as its association with other cell surface proteins, the cell type, and the experimental conditions (16,20,24,25). As the rK5 inhibition of retinal capillary endothelial cell proliferation was unaffected by RGD-containing peptides (10), it likely occurs in an integrin receptor-independent manner.

We found that rK5 induces apoptosis of brain MvEC and that prior irradiation significantly sensitizes the cells to rK5-induced apoptosis resulting in a dose-sparing effect. In both unirradiated and irradiated brain MvEC, the rK5-induced apoptosis requires expression of GRP78 and LRP1, as well as the activation of p38 mitogen-activated protein (MAP) kinase.
MATERIAL AND METHODS

Materials: Recombinant K5 (rK5) (ABT-828) expressed in yeast was provided by Abbott Laboratories (Abbott Park, IL); its native folded structure was verified by NMR as being comparable to K5 naturally derived from human plasminogen by elastase cleavage. Curcumin and PD98059 were purchased from Calbiochem, and SB202190 and SP600125 from A.G. Scientific Inc.. Recombinant receptor-associated protein (RAP) was purchased from Maine Biotechnology Services Inc. (Portland, ME), and dialyzed prior to use.

Cell culture: Primary human brain MvEC were purchased from Cell Systems, utilized at passages 2 to 8 and propagated as recommended in 70% CSC media and 30% M199 media, with 10% low-endotoxin FBS (BD Biosciences). Unless otherwise indicated, cells were split the day before the experiment, and then re-plated onto collagen-coated wells in M199 media with 10% low endotoxin FBS, 5 ng/ml bFGF and 10 ng/ml vascular endothelial growth factor (VEGF) 4 h prior to treatment with rK5. Cells were subconfluent at the time of treatment.

Analysis of cell surface expression: For analysis of the cell surface expression of GRP78 and LRP-1, cells were labeled with biotin (26), lysed in 1% NP40 lysis buffer (20 mM TrisBase, 137 mM NaCl, 2 mM EDTA, pH 8.0 with 10% glycerol and with protease inhibitors), immunoprecipitated with mAb anti-LRP1 or goat anti-GRP78 IgG, the immunoprecipitates harvested by centrifugation, washed, subjected to SDS-PAGE,
transferred to an Immobilon-P membrane, reacted with HRP-conjugated streptavidin, and developed using chemiluminescence (Amersham). Alternatively, cells were incubated with 10 µg/ml of primary antibody (30 min, 4°C), washed, incubated with 10 µg/ml Alexa-488-conjugated secondary antibody (30 min, 4°C), washed, fixed, and subjected to FACS analysis using a FACScan instrument, as described previously (27). Goat anti-GRP78 (directed toward the N-and C-terminus) was purchased from Santa Cruz Biotechnology and mAb anti-LRP1 from Calbiochem.

**Immunoblot analysis:** Cells and tissue samples were lysed in RIPA lysis buffer (0.05 M Hepes, pH 7.4, 0.15M NaCl, 1% deoxycholate, 1% Triton X-100 and 0.1% SDS) with protease inhibitors, as described previously (28). Equivalent amounts of lysate (typically 100-130 µg) were separated on SDS-PAGE then transferred to an Immobilon-P membrane, probed with primary antibody (4°C, overnight), washed, reacted with a horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad), and developed using enhanced chemiluminescence (Amersham) (28). Antibodies were purchased as follows: rabbit anti-p38 MAP kinase, anti-phospho-p38 MAP kinase (Y182), anti-HSP70, anti-c-terminal Jun kinase (JNK), and anti-calreticulin (Santa Cruz Biotechnology); rabbit anti-cleaved caspase-3 or 7 (Calbiochem); rabbit anti-von Willebrand factor (vWF IgG) (Chemicon); mAb anti-actin (Sigma), mouse anti-caspase-3 (Cell Signaling); and mAb anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Research Diagnostics Inc.).

**siRNA downregulation:** SMARTpool small interfering (si)RNA consisting of a pool of four SMARTselection-designed siRNA duplexes directed toward GRP78, HSP70,
calreticulin, p38 MAPK, and extracellular signal-regulated kinase 1 (Erk1) were purchased from Dharmacon, and siRNA directed towards LRP1 was purchased from Santa Cruz Biotechnology. HP-validated siRNA directed toward JNK2 was purchased from Qiagen. Two nonsense mutations were introduced into the JNK2 siRNA sequence as a custom control purchased from Qiagen. Transient transfections were carried out using the HiPerFect Transfection Reagent (Qiagen) according to the manufacturer’s guidelines.

**Apoptosis assays:** Terminal 3’-dexoynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were carried out using the ApopTag peroxidase in situ apoptosis detection kit (Chemicon) as per the manufacturer’s instructions and as described previously (28). Tumor necrosis factor α (TNFα) induction of apoptosis was used as a positive control (29). For determination of caspase 3/7 activity, a caspase-3/7 luminescent activity assay was performed using the Caspase-Glo 3/7 Assay kit (Promega).

**Analysis of human tissue samples:** Tissue samples were obtained from the Cooperative Human Tissue Network of the National Cancer Institute and the University of Alabama at Birmingham Brain Tumor Bank in accordance with University Human Tissue Committee policies. Tumors were histologically graded according to the World Health Organization classification of brain tumors (1). Frozen normal adult brain (cortex and white matter) and glioblastoma tumor samples obtained at autopsy within 18 h of death were homogenized in RIPA lysis buffer with protease inhibitors as described (30) for western
blot analysis. For immunohistochemical analysis, frozen sections, as well as formalin-fixed and paraffin-embedded, normal brain and glioblastoma tumor samples were prepared from surgical biopsy samples and treated with blocking buffer to inhibit endogenous peroxidases and prevent non-specific protein binding, reacted with the primary antibody in 5% BSA/PBS/0.01% Tween 20 (4°C, 20 h), washed, reacted with a HRP-conjugated secondary antibody (22°C, 1 h), followed by the 3,3’-diaminobenzidine (DAB) substrate (ScyTek) (30), then counterstained with hematoxylin.

Statistics: After determining the data were normally distributed a two-sample t test was used for data analysis and a p < 0.05 was considered significant.
RESULTS

rK5 induces apoptosis of primary human brain MvEC. rK5 treatment has been shown to induce apoptosis of dermal MvEC (7). To determine the potential of rK5 treatment to induce apoptosis of brain MvEC, we treated primary human brain MvEC plated onto collagen type IV and grown in complete media (10% FBS) in the presence of VEGF (10 ng/ml) and bFGF (5 ng/ml) with rK5 at concentrations of 10 to 5000 ng/ml for 17 h. We found that 5000 ng/ml of rK5 was necessary to induce a significant increase in caspase-3/7 activity in the human brain MvEC (Supplemental Fig. 1A). Consistent with this result, 5000 ng/ml of rK5 induced a significant and maximal increase in the numbers of TUNEL-positive human brain MvEC (≈ 8% positive) at 17 h (Fig. 1A). Immunoblotting analysis confirmed that treatment of the cells with 5000 ng/ml rK5 for 17 h resulted in a significant increase in cleaved caspase-7, and that treatment for time periods greater than 17 h (25 and 40 h) did not result in a further increase in the amounts of cleaved caspase-7 (data not shown).

Irradiation sensitizes primary human brain MvEC to rK5-induced apoptosis. To evaluate the effect of prior irradiation on rK5-induced apoptosis of brain MvEC, we irradiated (2 or 5 Gy) primary human brain MvEC, allowed the cells to recover for 20 h, then re-plated them onto collagen-coated wells for 4 h prior to treatment with rK5 for 17 h. In the absence of treatment with rK5, only a low percentage of brain MvEC that were irradiated exhibited TUNEL positivity (Fig. 1B). Notably, the percentage of TUNEL-positive cells in the brain MvEC that had been irradiated prior to treatment with 10 ng/ml of rK5 was similar to the percentage of TUNEL-positive cells in unirradiated MvEC that
were treated with 5000 ng/ml of rK5 (Fig. 1B). Thus, the prior irradiation appeared to increase the sensitivity of the cells to rK5, i.e., the irradiation had a dose sparing effect, but did not seem to increase the numbers of cells that were susceptible to rK5. Similarly, irradiation of the brain MvEC (2 or 5 Gy) followed by rK5 treatment (3 – 5000 ng/ml, 17 h) resulted in a significant increase in the cleavage of caspase-3 or 7 that was maximal at 10 ng/ml rK5 (Fig. 1C, and data not shown) confirming that irradiation plus rK5 induces apoptosis. Consistent with the results of the TUNEL assays, irradiation alone (5 Gy) did not induce a significant increase in cleaved caspase-3 (Supplemental Fig. 1B). The time course of rK5-induced caspase-7 cleavage in the irradiated brain MvEC was maximal at 17 h and detectable as early as 3 h post-rK5 treatment (Fig 1D). Collectively, these data establish that prior irradiation of the brain MvEC resulted in a significant reduction in the dose of rK5 required for optimal induction of apoptosis.

**GRP78 is necessary for rK5-induced apoptosis of unirradiated and irradiated primary human brain MvEC.** Analysis of the expression of GRP78 on the cell surface of the MvEC used in the analyses of rK5-induced apoptosis by cell surface biotinylation followed by detergent lysis, immunoprecipitation with anti-GRP78 antibody and SDS-PAGE analysis indicated that GRP78 was expressed on both unirradiated and irradiated human brain MvEC (Fig. 2A). Although irradiation has been reported to upregulate GRP78 (31), we found that the surface expression was equivalent in the irradiated and unirradiated brain MvEC suggesting that an upregulation of GRP78 does not contribute to the enhanced sensitivity of the irradiated brain MvEC to low doses of rK5 in our experiments.
After further confirming the cell surface expression of GRP78 on the brain MvEC by FACS analysis (data not shown), we determined the requirement for GRP78 in rK5-induced apoptosis by downregulating GRP78 with specific duplex siRNA. As a control, we downregulated the endoplasmic reticulum chaperone protein calreticulin, which is expressed, in part, on the cell surface (19) or we downregulated HSP70. Downregulation of GRP78 and calreticulin was confirmed by western blotting (Supplemental Fig. 1C) and we had established previously that HSP70 protein is downregulated by >70% with 50 nM siHSP70 treatment in the unirradiated human brain MvEC (data not shown). No adverse effects of the siRNAs on cell viability or morphology were detected over the time course of these experiments (data not shown). The downregulation of GRP78 significantly blocked rK5-induced apoptosis (5000 ng/ml; 17 h) of the unirradiated brain MvEC as determined using a TUNEL assay and blotting for cleaved caspase-3 (Fig. 2B), whereas the downregulation of calreticulin or HSP70 had no effect.

Similar results were obtained on downregulation of GRP78 in irradiated cells. Downregulation of GRP78 (> 70%) was achieved on treatment of the irradiated brain MvEC with 50 nM siGRP78 (Supplemental Fig. 1D). In these experiments, the downregulation of HSP70 was used as a control rather than calreticulin as both GRP78 and HSP70 were downregulated at 24 h post-irradiation, whereas optimal downregulation of calreticulin required prolonged treatment (data not shown). Downregulation of GRP78 in the irradiated brain MvEC significantly blocked rK5-induced apoptosis (10 ng/ml rK5; 17 h) as detected using the TUNEL assay and blotting for cleaved caspase-7 (Fig 2C).
The downregulation of HSP70 had no effect. Further support for a role for GRP78 in rK5-induced apoptosis of the irradiated brain MvEC was obtained by the results of treatment with an anti-GRP78 antibody (10 µg/ml) that is directed toward the N-terminus. This inhibited rK5-induced apoptosis (data not shown) as described previously for unirradiated dermal MvEC (7). These data indicate that GRP78 is necessary for rK5-induced apoptosis of both unirradiated and irradiated primary human brain MvEC.

**LRP1 is necessary for rK5-induced apoptosis of unirradiated and irradiated human brain MvEC.** GRP78 is known to associate with LRP1 on the cell surface (16); therefore we examined the potential role of LRP1 in rK5-induced apoptosis of MvEC. The expression of LRP1 on the cell surface was verified using cell surface biotinylation, followed by detergent lysis and immunoprecipitation with a mAb anti-LRP1, which recognizes the 85-kDa light chain. SDS-PAGE analysis of the immunoprecipitates indicated that LRP1 is expressed on the surface of both the unirradiated and irradiated MvEC, and further indicated the levels of expression of LRP1 on the cell surface were not altered by irradiation (Supplemental Fig. 2A). To determine whether LRP1 is required for the rK5-induced apoptosis of the human brain MvEC, we pretreated the cells with receptor associated protein (RAP), a competitive inhibitor of ligand binding to LRP1 (32). Prior addition of 60 nM rec-RAP blocked rK5-induced apoptosis in the irradiated and unirradiated cells as detected using the TUNEL assay and cleavage of caspase-3 (Fig 3A&B, respectively). These results were confirmed by downregulation of LRP1 with pooled specific duplex siRNA. Downregulation was confirmed by western blotting of the irradiated and unirradiated cells (Supplemental Fig. 2B&C, respectively) and no effect of
siLRP1 administration on cell viability or morphology was detected over the time course of these experiments. Downregulation of LRP1 post-irradiation blocked rK5-induced apoptosis (10 ng/ml), whereas the downregulation of HSP70 as a control had no effect (Fig 3C). In the unirradiated cells, downregulation of LRP1 significantly blocked the rK5-induced apoptosis (5000 ng/ml), whereas the downregulation of calreticulin had no effect (Fig. 3D). These data suggest that LRP1 internalization of GRP78 is likely necessary for rK5-induced apoptosis in both the irradiated and the unirradiated brain MvEC.

**p38 MAP kinase is necessary for rK5-induced apoptosis of the irradiated human brain MvEC.** Stress is known to induce the activation of p38 MAP kinase (33) and p38 MAP kinase can promote a pro-apoptotic signal (34-36). We found a time-dependent increase in phosphorylated p38 MAP kinase in the irradiated cells treated with rK5 (Fig. 4A). Treatment with a small molecule inhibitor of p38 MAP kinase (SB202190) at 2.5-fold the IC$_{50}$ (1.5 µM), significantly blocked the pro-apoptotic effect of rK5 in the irradiated brain MvEC (Fig. 4B). In contrast, the pro-apoptotic effects of rK5 were not blocked by treatment with inhibitors of other kinases at 2.5-fold the IC$_{50}$: 5 µM PD98059 (MEK inhibitor), 7.5µM curcumin (JNK inhibitor), SP600125 (JNK inhibitor), and FR180204 (Erk inhibitor) (Fig 4B). To further evaluate the requirement for p38 MAPK in the irradiated MvEC we downregulated p38 MAPK with pooled specific duplex siRNA and downregulated Erk1 as a control (Supplemental Fig. 3A). TUNEL analysis indicated that the downregulation of p38 MAPK in the irradiated brain MvEC blocked
the pro-apoptotic effect of rK5 (Fig. 4C), whereas the downregulation of Erk1 had no effect.

On analysis of the unirradiated cells, we also found that the p38 MAPK inhibitor SB202190 at 2.5 fold the IC$_{50}$ significantly blocked the pro-apoptotic effect of rK5 (Fig 5A, lanes 2&3) whereas the Erk inhibitor FR180204 did not (Fig 5A, lanes 2&4). Similarly, downregulation of p38 MAP kinase in the unirradiated cells (Supplemental Fig. 3B) blocked the pro-apoptotic effect of rK5 as detected by TUNEL assay, whereas the downregulation of Erk1 had no significant effect (Fig 5B). These data suggest that p38 MAP kinase is a necessary downstream signaling effector in the pro-apoptotic effect of rK5 in irradiated and unirradiated human brain MvEC.

Although JNK has been reported to be involved in signaling that promotes apoptosis (34), we found that the JNK inhibitor, SP600125, at 2.5-fold the IC$_{50}$ in the irradiated cells (Fig. 4B) or the unirradiated cells (Fig. 5C) did not block the pro-apoptotic effect of rK5. However, a small increase (30%) in phosphorylation of the 54-kDa JNK2 isoform was detected in a time course analysis of JNK phosphorylation post-rK5 treatment of the unirradiated cells (data not shown). Downregulation of JNK2 with 200 nM specific duplex siJNK2 RNA (70% downregulation of the 54-kDa isoform) (Supplemental Fig. 3C) confirmed that downregulation of JNK2 in the unirradiated cells did not block the pro-apoptotic effect of rK5 (5000 ng/ml) as analyzed by TUNEL assay (Fig. 5D). A mutated JNK2 siRNA (two base pair mutations) was used as a control.
**GRP78 expression is higher on brain MvEC in glioblastoma tumor samples as compared to the normal brain.** As our ultimate goal is to develop a novel therapeutic strategy for glioblastoma tumors, we evaluated the expression of GRP78 in frozen and paraffin sections of 14 glioblastoma tumor biopsy samples by immunohistochemistry. We found expression of GRP78 in an estimated 30% of MvEC in the tumor portion of all glioblastoma samples and in an estimated 30% of tumor cells (Fig. 6A, and Supplemental Table 1). The staining localized to the cell membrane and the cytoplasm. The staining was specific for GRP78 as it was largely competed out by preincubation of the anti-GRP78 antibody with a 50-fold molar excess of GRP78 peptide (data not shown). Staining with rabbit IgG was used as a negative control and staining with anti-vWf antibody was used as a positive control. Using this technique GRP78 expression was below the limit of detection in endothelial and glial cells in the frozen and paraffin sections of normal brain, although expression of GRP78 was detected in neurons in the 13 normal brain (data not shown). To confirm the upregulation of GRP78 in the tumors as compared to the normal brain, we immunoblotted lysates from nine normal brains and nine glioblastoma tumor samples with anti-GRP78 antibody, followed by stripping and reprobing with antibodies directed toward GAPDH and HSP70 (Fig. 6B). The immunoblotting data were quantitated by densitometric analysis and GRP78 expression was normalized to HSP70 and to GAPDH and plotted as scattergrams (Fig. 6C&D). We found on average a 2.0 to 2.5-fold increase in GRP78 expression relative to HSP70 or when normalized to GAPDH in the nine tumor samples as compared to the nine normal brain samples. These data support our immunohistochemistry results indicating that GRP78 expression is upregulated in glioblastoma tumors *in vivo.*
DISCUSSION

In this report we show that rK5 can induce apoptosis of brain MvEC and that irradiation significantly sensitizes primary human brain MvEC to the pro-apoptotic effect of rK5. We found that rK5 treatment of brain MvEC induces apoptosis, as measured by several different assays, when administered at 5000 ng/ml (≈ 500 nM). This is similar to the dosage of rK5 required to induce apoptosis of dermal MvEC (7). Importantly, we found that prior irradiation significantly sensitized the brain MvEC to rK5-induced apoptosis, as a 500-fold lower dose of rK5 (10 ng/ml) induced a similar percentage of TUNEL-positive cells and cleavage of caspase-3 or 7.

Few studies have focused on irradiation as a potential sensitizing agent for anti-angiogenic therapy. Our studies are consistent with the recent report of Jin et al., (37) that rK5 combined with irradiation enhanced the anti-angiogenic effect of rK5 in a mouse tumor model of Lewis lung carcinoma cells propagated s.c.. Under our experimental conditions (10% FBS with 10 ng/ml VEGF and 5 ng/ml bFGF) irradiation alone did not induce significant cell death or caspase 3/7 cleavage. Other investigators have reported a pro-apoptotic effect of irradiation (2 or 5 Gy) alone on dermal MvEC that were propagated and radiated in reduced serum (2% FBS), and this was attributed to reduced levels of the anti-apoptotic protein Bcl2 (38,39). We did not find an altered level of Bcl2 protein post-irradiation of the MvEC at two time points in our experimental conditions (B.C. McFarland and C.L. Gladson, unpublished observation). This may be due to the higher levels of FBS and added VEGF, as the level of Bcl2 expression is known to be
regulated by VEGF (39) or it may reflect the use of brain MvEC rather than dermal MvEC. rK5 has been reported to induce autophagy of non-brain endothelial cells in different experimental conditions (40); therefore, it is possible that rK5-induced autophagy contributes to the apoptosis we have observed in the brain MvEC.

Our finding that GRP78 is required for rK5-induced apoptosis of human brain MvEC is consistent with the report of the requirement for GRP78 in the pro-apoptotic effect of rK5 on dermal MvEC, and of a direct interaction of rK5 with rec-GRP78 (7). GRP78 is an endoplasmic reticulum chaperone protein that is expressed on the cell surface in stress conditions and in tumors (14,15); however, we found no change in the cell surface expression post-irradiation in the time course and conditions of our experiments. This suggests that an increase in GRP78 expression post-irradiation is not the mechanism by which irradiation sensitizes the cells to rK5-induced apoptosis. The ligand binding to cell surface GRP78 appears to determine the signal generated. We and others (7) detect a pro-apoptotic signal when rK5 binds to cell surface GRP78 on MvEC, whereas α2-macroglobulin binding to cell surface GRP78 on human prostate cancer cells and macrophages initiates a pro-proliferation signal (41). The voltage-dependent ion channel also has been reported to be a receptor for rK5 (42).

We found that LRP1 is necessary for the rK5-generated pro-apoptotic signal. This suggests that LRP1 internalization of GRP78 is likely necessary for the pro-apoptotic effect of rK5 in the brain MvEC. The signal generated upon LRP1 binding and internalizing its ligand is dependent on the cell and the environmental context (21). For
example, a pro-migratory signal is generated when LRP1 binds and internalizes the complex of cell surface calreticulin and thrombospondin-1 in coronary artery endothelial cells (19,20). Differential LRP1 signaling is thought to be due to different adaptor molecules that bind to the phosphorylated tyrosine residue(s) in the LRP1 cytoplasmic tail and thereby activate different downstream effectors (21,23,43).

We found that p38 MAP kinase was activated with rK5 treatment post-irradiation and that p38 MAP kinase was necessary for rK5-induced apoptosis of the irradiated and unirradiated brain MvEC. In contrast, neither Erk nor JNK were necessary for the rK5-induced apoptosis of the human brain MvEC. Notably, the LRP1 family member LRP8, expressed on platelets, is known to activate p38 MAP kinase upon binding its ligand, the low density lipoprotein, suggesting that the LRP family of proteins is capable of signaling to p38 MAP kinase (44).

In support of the potential clinical relevance of our findings, we show that the expression of GRP78 is increased on the MvEC in glioblastoma tumor samples as compared to the normal brain. GRP78 expression was also increased on the tumor cells in the glioblastoma biopsy samples, as compared to glial cells in the normal brain. Our results are consistent with those in the literature indicating that GRP78 expression is upregulated in tumors (reviewed in (15); (45,46). Two conditions frequently found in malignant tumors, hypoxia and hypoglycemia, are known to upregulate GRP78 expression (14,47). Recently, GRP78 expression has been associated with chemoresistance making it a promising target for cancer therapy (reviewed in 15); (45,48).
In summary, our data indicate that irradiation sensitizes primary human brain MvEC to the apoptosis-inducing effect of rK5 and that this pro-apoptotic signal requires LRP1 internalization of GRP78 and p38 MAP kinase activity. As the cell surface binding partner for rK5 (GRP78) and its scavenger receptor partner (LRP1) (49) are both expressed on MvEC in glioblastoma tumor biopsy samples, these data suggest that rK5 treatment post-irradiation should be considered in the design of new therapies for patients with glioblastoma tumors.

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Fig 1. Irradiation Sensitizes Primary Human Brain MvEC to rK5-Induced Apoptosis

A, Primary human brain MvEC plated on collagen type IV in M199 media with 10% FBS, 5 ng/ml bFGF and 10 ng/ml VEGF were treated with rK5 (or vehicle) for 17 h, and then subjected to a TUNEL assay. 

B-D, Primary human brain MvEC cultured as described above were irradiated, allowed to recover for 20 h, and then re-plated onto collagen-coated wells in fresh media (4 h), followed by treatment with rK5 or vehicle for 17 h or for the indicated times, and then subjected to a TUNEL assay (B) or detergent lysis, SDS-PAGE, and immunoblotting with the indicated antibodies (C&D).
Fig. 2. GRP78 Is Necessary for rK5-Induced Apoptosis of Human Brain MvEC

A, Unirradiated and irradiated primary human brain MvEC (plated and treated as described in the legend for Fig. 1) were surface biotinylated 24 h post-irradiation, detergent lysed, immunoprecipitated with anti-GRP78 antibody, and subjected to SDS-PAGE. B, Unirradiated primary human brain MvEC were treated with siRNA for 48 h, re-plated onto collagen-coated wells in fresh media, and subjected to a TUNEL assay or detergent lysed and immunoblotted with the indicated antibodies. C, Irradiated primary human brain MvEC were treated immediately with siRNA for 20 h, re-plated onto collagen-coated wells in fresh media, and treated with siRNA for an additional 4 h, and subjected to a TUNEL assay or detergent lysed and immunoblotted with the indicated antibodies.
A

Cell Surface Biotinylation

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<td>GRP78</td>
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Probed with streptavidin-HRP

B

% TUNEL Positive per 20K Field

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C

% TUNEL Positive per 20K Field

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Fig. 3. LRP1 Is Necessary for rK5-Induced Apoptosis of Primary Human Brain MvEC.

**A&B**, Irradiated (A) and unirradiated (B) primary human brain MvEC were treated with rK5 or vehicle (as described in the legend for Fig. 1) or rec-RAP and then subjected to TUNEL assay or detergent lysed and immunoblotted with the indicated antibodies.  

**C**, Primary human brain MvEC were irradiated and immediately treated with siRNA for 20 h, the cells re-plated and treated with siRNA for an additional 4 h, followed by treatment with rK5 or vehicle (17 h) and then subjected to a TUNEL assay.  

**D**, Primary human brain MvEC were treated with siRNA for 48 h, re-plated and treated with rK5 or vehicle (17 h) and then subjected to a TUNEL assay.
Fig. 4. p38 MAP Kinase is Necessary for rK5-Induced Apoptosis of Irradiated Brain MvEC  

**A&B**, Primary human brain MvEC were irradiated, allowed to recover, re-plated and treated with rK5 or vehicle for 17 h, or for the indicated time as described in the legend for Fig. 1, followed by detergent lysis, and immunoblotting with the indicated antibodies (A). Inhibitors were added 30 min prior to rK5 addition (B). **C**, Primary human brain MvEC were treated with siRNA for 48 h, irradiated, treated with siRNA for an additional 20 h, re-plated and treated with siRNA for 4 h then treated with rK5 or vehicle (17 h) followed by a TUNEL assay.
Fig. 5. p38 MAP Kinase is Necessary for rK5-Induced Apoptosis of Unirradiated Brain MvEC. A&C, Primary human brain MvEC were plated and treated with rK5 or vehicle for 17 h, as described in the legend for Fig. 1, followed by detergent lysis, and blotting with the indicated antibodies (A), or subjected to TUNEL Assay (C). Inhibitors were added 30 min prior to rK5 addition. B&D, Primary human brain MvEC were treated with siRNA for 68 h, re-plated and treated with siRNA for an additional 4 h and then treated with rK5 or vehicle (17 h), followed by a TUNEL assay.
Fig. 6. Increased Expression of GRP78 in Glioblastoma Tumor Samples. A, Specific expression of GRP78 on brain MvEC and tumor cells in glioblastoma tumor biopsy samples. Sections of glioblastoma tumor (GBM) or normal brain (NB) were reacted with 15 µg/ml anti-GRP78 IgG, 10 µg/ml anti-vWF IgG, or 15 µg/ml IgG (20 h, 4°C), followed by an HRP-conjugated secondary IgG, substrate, and hematoxylin counterstaining. Arrows denote microvessels. Magnification – 400X. B, Immunoblotting of glioblastoma samples demonstrates increased GRP78 expression. Tissue samples were homogenized in detergent lysis buffer with protease inhibitors, and 100 µg of lysate subjected to 10% SDS-PAGE, followed by blotting with the indicated antibodies. C&D, Bands were subjected to densitometry and the ratio of GRP78 to HSP70 and GRP78 to GAPDH determined and plotted as a scattergram for each sample.
Table I. GRP78 Expression in Glioblastoma Tumors

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<th>Tissue</th>
<th>Endothelial Cells</th>
<th>Tumor Cells</th>
<th>Neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Brain (n = 13)</td>
<td>– (13/13)</td>
<td>N.A.</td>
<td>Weak to 1+ (12/13)</td>
</tr>
<tr>
<td>Glioblastoma Tumors (n = 14)</td>
<td>2+ (30%) (14/14)</td>
<td>1+ (30%) (14/14)</td>
<td>N.A.</td>
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</tbody>
</table>

Table I Legend: Immunohistochemistry was performed as described in the Materials and Methods. The intensity of staining was graded as follows: -, non-detectable staining; 1+, medium intensity staining; 2+, strong intensity staining. N.A. denotes not applicable. In the glioblastoma tumors, an estimated 30% of endothelial cells and an estimated 30% of tumor cells stained positively.
Figure 7. Supplemental Fig 1. A, Primary human brain MvEC plated on collagen type IV in M199 media with 10% FBS, 5 ng/ml bFGF and 10 ng/ml VEGF were treated with rK5 (or vehicle) for 17 h, and then subjected to a luciferase-coupled caspase 3/7 substrate luminescence assay. B, Primary human brain MvEC cultured as described above were irradiated, allowed to recover for 20 h, and then re-plated onto collagen-coated wells in fresh media (4 h), followed by treatment with rK5 or vehicle for 17 h, followed by detergent lysis, SDS-PAGE, and immunoblotting with the indicated antibodies. C, Unirradiated cells (plated as described in the legend for Fig 1) were treated with siRNA for 48 h, lysed in detergent and immunoblotted. D, Irradiated primary human brain MvEC were treated immediately with siRNA for 20 h, re-plated onto collagen-coated wells in fresh media, and treated with siRNA for an additional 4 h, and then lysed and immunoblotted with the indicated antibodies.
A

![Bar graph showing luminescence (% increase from control) vs. rK5 (ng/ml)](image)

B

<table>
<thead>
<tr>
<th>Radiation (Gy)</th>
<th>rK5 (ng/ml)</th>
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<td>5</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
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Anti-cleaved caspase-3
Anti-GAPDH

C

Unirradiated Brain MvECs

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<thead>
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<th>siGRP78 (nM)</th>
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<th>siCacteolin (nM)</th>
<th>Anti-Cacteolin (kDa)</th>
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<td>90</td>
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<tr>
<td>100</td>
<td>42</td>
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D

Irradiated (5 Gy) Brain MvECs

<table>
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<tr>
<th>siGRP78 (nM)</th>
<th>Anti-GRP78 (kDa)</th>
<th>siHSP70 (nM)</th>
<th>Anti-HSP70 (kDa)</th>
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</tr>
<tr>
<td>10</td>
<td>42</td>
<td>200</td>
<td>42</td>
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</table>

Anti-ACTIN
Fig 8. Supplemental Fig. 2. A, Irradiated or unirradiated primary human brain MvEC (plated and treated as described in the legend for Fig.1) were surface biotinylated 24 h post-irradiation, detergent lysed, immunoprecipitated with mAb anti-LRP1, and subjected to SDS-PAGE. B&C, Primary human brain MvEC were irradiated and immediately treated with siRNA for 24 h (B), or were not irradiated but were treated with siRNA for 48 h (C), both preparations were immunoprecipitated (IP) and blotted with mAb anti-LRP1.
**A**

Cell Surface Biotinylation

<table>
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<th>5 Gy</th>
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<tr>
<td>IgG</td>
<td>![Image 1]</td>
<td>![Image 2]</td>
</tr>
<tr>
<td>LRP1</td>
<td>![Image 3]</td>
<td>![Image 4]</td>
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</table>

Probed with streptavidin-HRP

**B**

Irradiated (5 Gy) Brain MvECs

<table>
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<th>200</th>
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</table>

IP and Blot: Anti-LRP1

85 kDa

**C**

Unirradiated Brain MvECs

<table>
<thead>
<tr>
<th>siLRP1 (nM)</th>
<th>0</th>
<th>100</th>
<th>200</th>
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IP and Blot: Anti-LRP1

85 kDa
Fig 9. Supplemental Fig. 3. A, Primary human brain MvEC were treated with siRNA for 48 h, irradiated, treated with siRNA for an additional 20 h, re-plated and treated with siRNA for 4 h followed by detergent lysis and immunoblotting with the indicated antibodies. B&C, Primary human brain MvEC were treated with siRNA for 68 h, re-plated and treated with siRNA for an additional 4 h and then detergent lysed and immunoblotted with the indicated antibodies.
CONCLUSIONS

Dissertation Summary

rK5 Induces Apoptosis of Primary Brain MvEC

In this project, I tested the ability of rK5 to induce apoptosis of primary human brain MvEC and the effect of prior irradiation. I found that 5000ng/ml of rK5 was necessary to induce apoptosis of primary human brain MvEC, and the dose of rK5 needed to induce apoptosis was significantly decreased when the cells were irradiated prior to rK5 treatment. In the irradiated cells 10ng/ml rK5 was sufficient to induce apoptosis; this is a 500-fold reduction in the concentration of rK5. Also, I demonstrated that GRP78, a cell surface binding partner of rK5, was necessary for the pro-apoptotic effect of rK5 in both the unirradiated and irradiated cells. Furthermore, the pro-apoptotic effect of rK5 required LRP1 internalization of GRP78/rK5 and p38 MAPK activation, neither of which has been shown previously. An illustration of the proposed mechanism by which rK5 induces apoptosis in brain MvEC is shown in Figure 1: rK5 binds to cell surface GPR78, LRP1 binds and internalizes the GRP78/rK5 complex leading to the activation of p38 MAP kinase and the activation of effector caspases and apoptosis.

The Effects of Irradiation

Molecular effects of radiation include double stranded DNA breaks, plasma membrane damage, and the generation of damaging free radicals. These molecular
alterations can contribute to changes in cell function, for example, radiation can induce cell cycle arrest, the cellular stress response, and activation of DNA damage repair enzymes, as well as apoptosis (reviewed in Watters et al., 1999). I did not find an induction of apoptosis in the primary human brain MvEC irradiated with 2 or 5 Gy in my experimental conditions (complete media with addition of VEGF and bFGF); nevertheless it is possible that the effects of radiation may activate other signaling pathways that sensitize the cells to the pro-apoptotic effect of rK5 treatment.

I initially evaluated the possibility that the dose-sparing effect of irradiation could be due to an increase in the amount of GRP78 on the surface of the brain MvEC following irradiation. It has been reported that expression of GRP78 is increased in C3H1OT1/2C18 mouse embryo cells following treatment with ultraviolet light, a form of irradiation, combined with 3-methylcholanthrene (Patierno et al., 1987), suggesting increased GRP78 expression could be occurring in the brain MvEC post-irradiation in my experiments. Photodynamic therapy is known to induce GRP78 expression in tumors (Dong et al., 2004). However, I found no increase in cell surface GRP78 or LRP1 expression following irradiation in the brain MvEC in the time frame and conditions of my rK5 experiments. Also, to my knowledge, there are no prior studies examining the effect of radiation on GRP78 expression in endothelial cells.

Irradiation (2 Gy) is known to induce p38 MAP kinase activation and apoptosis in dermal MvEC under serum-starving conditions and without VEGF stimulation (Kumar et al., 2004). This was blocked by VEGF stimulation, and this pro-survival effect of VEGF was thought to be due to its upregulation of the anti-apoptotic protein Bcl2 through the VEGF activation of the PI3K/Akt signaling pathway (Kumar et al., 2004). Dermal MvEC
show a significant decrease in Bcl2 protein following irradiation in serum starving conditions and in the absence of VEGF (Kumar et al., 2004). Consistent with the findings in the dermal MvEC, I found that the primary human brain MvEC were resistant to apoptosis, when irradiated with 2 or 5 Gy in the presence of 10% FBS, 10ng/ml VEGF, and 5 ng/ml bFGF. However, it is possible that irradiation may be activating p38 MAPK prior to the addition of rK5, thus providing another mechanism of sensitization. The possible activation of p38 MAPK following irradiation and prior to rK5 treatment has yet to be examined in my experimental conditions.

Irradiation has also been shown to induce autophagy. Autophagy, which is primarily a pro-survival mechanism, can be activated under stress. If irradiation is activating autophagy, this would provide another mechanism that would cause the cells vulnerable to a pro-apoptotic stimuli, such as rK5 treatment. The potential induction of autophagy in my experimental conditions needs to be analyzed in future studies.

Another potential mechanism of sensitization by irradiation includes alterations in the Bcl2 family of mitochondrial proteins. My preliminary studies indicate that the anti-apoptotic protein BclXL has a decreased level of expression following irradiation (data not shown). However, this decrease was seen 4 hr after irradiation and would need to be evaluated at the time of rK5 treatment following irradiation. In addition, the entire Bcl2 family of pro- and anti-apoptotic proteins needs to be examined.

Preliminary In Vivo Studies

*rK5 Treatment Shows An Anti-Tumor Effect in an Intracerebral Mouse Model of Glioma*
In order to evaluate the potential anti-tumor effect of rK5 treatment in vivo, I utilized an intracerebral xenograft mouse model of human glioma. Xeno6 human glioblastoma tumor cells (500,000 cells in 5 µl of PBS) were injected intracerebrally using stereotactic assistance. The tumor was allowed to establish for 7 days before beginning rK5 treatment, and then intraperitoneal (i.p) injections of 25 mg/kg of rK5 or saline control were administered twice daily (50 mg/kg rK5 per day total) from day 7 until euthanasia on day 19. At euthanasia the brains were fixed in formalin, paraffin embedded, and serially sectioned (8 µm sections). H&E stained sections at 70 µm intervals were photographed at 1.5 X magnification, the tumor area in each section determined in Adobe Photoshop as a pixel-number and these areas summed to recreate tumor volume as this lab has described previously (Akeela et al., 2006). Microvessel density was also determined on sections immuno-stained for von Willebrand factor (vWf) (an endothelial cell marker) as the number of positive microvessels per 40X field of view in the tumor.

I found that 50mg/kg of rK5 per day significantly decreased tumor volume as analyzed by a one-sample t test (Figure 2). The mean tumor volume for the saline treated mice was 0.062 mm³ ± 0.021 while the mean tumor volume for the rK5 treated animals was 0.034 mm³ ± 0.006. Analysis of microvessel density, one indicator of angiogenesis, showed there was a small but not significant decrease in microvessel density in the rK5 treated group (Figure 3). This suggests that the decrease in tumor volume in the rK5 treated mice may not be due entirely to a pro-apoptotic effect and raises the possibility that there is another mechanisms by which rK5 inhibits tumor growth in vivo in the mouse model. Potential explanations for this lack of a significant reduction in
microvessel density with rK5 treatment could be due to the need for a larger group size (larger n) to achieve statistical significance, rK5 may be inhibiting proliferation of the MvEC in an apoptosis-independent mechanism, or rK5 may be inhibiting tumor cell proliferation.

**rK5 Inhibits Proliferation of Endothelial Cells in Other Systems**

It has been shown previously in other cell types that rK5 treatment can inhibit cell proliferation of cell monolayers propagated in vitro. In the initial characterization of K5, Cao et al. (1997) showed that K5 inhibited proliferation of bFGF stimulated bovine capillary endothelial cells, but the mechanism was not determined. Also, a dose-dependent inhibition of proliferation was reported in dermal endothelial cells and in human umbilical vein endothelial cells (HUVECs) treated with rK5, and in these studies the anti-proliferative effect was attributed to the pro-apoptotic effect of rK5 (Davidson et al., 2005). Most recently, rK5 treatment has been shown to inhibit proliferation and induce autophagy of HUVECs (Nguyen et al., 2007). Autophagy, which is primarily a pro-survival mechanism and is often induced under stressful conditions, could have anti-proliferative effects but the mechanism is still unclear (Klionsky 2007).

**rK5 Treatment has Anti-Tumor Effects In Vitro**

It is also possible that the decrease in tumor volume that I observed with rK5 treatment could be to a direct tumoricidal effect of rK5 on the Xeno6 tumor cells. It has been shown previously that rK5 induces apoptosis of stressed fibrosarcoma cells propagated in vitro (Davidson et al., 2005). Consistent with these findings, Jin et al. (2007) found that rK5 treatment induces apoptosis of Lewis Lung carcinoma cells.
propagated in vitro. Stable expression of rK5 in breast cancer cells followed by propagation in a syngeneic mouse model resulted in both anti-tumor and anti-angiogenic effects. In addition, rK5 appeared to have a pro-inflammatory effect as rK5 treatment generated an infiltration of both neutrophils and natural killer T cells to the site of the breast cancer cell implantation (Perri et al., 2007).

Future Directions

Anti-angiogenic therapy appears to have great promise for treating malignant tumors including malignant glioma. A number of questions remain. In my experimental conditions, does rK5 induces autophagy, and does it activates the so-called “intrinsic” or the “extrinsic” pathway of apoptosis or both through cross-talk? Does rK5 inhibit brain MvEC proliferation in an apoptosis-independent mechanism? Also, the effects of combined radiation and rK5 on proliferation need to be determined and whether the cells need to be in a specific phase of the cell cycle in order to respond to the effect of rK5. In addition, the effect of rK5 treatment on glioma cells needs to be determined as others have demonstrated anti-tumor effects of rK5 in addition to anti-angiogenic effects in vitro.

Another avenue of future study is to determine whether rK5 signaling requires internalization of LRP1. The vast majority of studies suggest LRP1 signaling requires the internalization of its ligand; however, one paper suggests LRP1 can signal independent of internalization (Gotthardt et al., 2000). Phosphorylation of the cytoplasmic tail of LRP1 should also be examined as the phosphorylation of LRP1 has been shown to regulate the binding partner and downstream signal.
An important next step in the preclinical analysis of rK5 would be to combine it with radiation in the animal model studies of glioma. Since certain anti-angiogenic agents have been shown to have additive or synergistic effects when combined with radiation, and my in vitro data with rK5 suggest prior irradiation reduces the dosage of rK5 necessary to induce apoptosis, rK5 may also show efficacy at a lower dosage when combined with radiation in vivo. If prior irradiation caused a dose sparing effect on rK5 in vivo, this could have wide clinical benefit as decreasing the amount of rK5 needed for an effective response could mean wider availability and less cost for patients.
Figure 1. Proposed Mechanism by Which rK5 Induces Apoptosis of Brain MvEC. Once rK5 is administered it binds to cell surface GRP78, LRP1 binds and internalizes the GRP78/rK5 complex leading to the activation of p38 MAP kinase and the activation of effector caspases and apoptosis. It is possible that the internalized GRP78/rK5 could also be activating other signaling mechanisms such as autophagy or could be directly interacting with the effector caspases 3 or 7. Potential mechanisms by which radiation may be sensitizing the cells to the pro-apoptotic effect of rK5 include a low level of activation of p38 MAP kinase prior to rK5 treatment, an inhibition of cell cycle progression, an induction of autophagy, or alterations in the Bcl2 family of mitochondrial membrane proteins that activate the intrinsic pathway.
Figure 2. rK5 Treatment Decreases Tumor Volume in an Intracerebral Xenograft Mouse Model of Human Glioma. Xeno6 human glioma cells (500,000 cells in 5 µl of PBS) were injected intracerebrally using a stereotactic device approximately 2mm anterior and 2mm lateral to the bregma suture at a depth of 2mm. The tumor was allowed to establish for 7 days prior to beginning rK5 treatment. Intraperitoneal (i.p) injections of 50 mg/kg per mouse of rK5 or saline control were administered over two doses daily for 12 days and on day 19 post tumor cell injection all mice were euthanized and the brains harvested, formalin fixed, paraffin embedded, and serially sectioned (8 µm sections), as described previously (Ding et al., 2005). H&E stained sections at 70 µm intervals were photographed at 1.5 X magnification and tumor volume was recreated in Adobe Photoshop as described previously (Akella et al., 2006). In the saline group: n=8, mean tumor volume = 0.062 mm$^3$ ± 0.021 and in the rK5 group: n=9, mean tumor volume = 0.034 mm$^3$ ± 0.006. All procedures were performed in accordance with the guidelines and recommendations of the IACUC with appropriate Animal Welfare Approval.
Figure 3. rK5 Treatment Does Not Significantly Decrease Microvessel Density in an Intracerebral Xenograft Mouse Model of Human Glioma. For the microvessel density analysis, paraffin sections were treated with blocking buffer to inhibit endogenous peroxidases and prevent non-specific protein binding, reacted with the rabbit anti-vWF antibody (20 ug/ml) or rabbit IgG (20 ug/ml) in 5% BSA/PBS/0.01% Tween-20 (4°C, 20 h), washed, reacted with a HRP-conjugated secondary antibody (22°C, 1 h), followed by the 3,3’-diaminobenzidine (DAB) substrate (ScyTek), and then counterstained with hematoxylin. Two sections per animal were stained with anti-vWF antibody and microvessel density was calculated as the number of microvessels stained positive per 40X field of view in the tumor. The microvessel density in the saline group (n=8), mean = 4.8 ± 0.78 and in the rK5 group (n=9), mean = 3.8 ± 0.44. Data were analyzed using a two-sample t test. A p value of 0.3 was obtained and was not considered significant (A p < 0.05 was considered necessary for statistical significance).
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APPENDIX:

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: August 13, 2008

TO: Candece L. Gladson, M.D.
ZRB-434 0007
FAX: 934-7346

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on August 13, 2008.

Title of Application: The Role of Lyn in Glioma Progression and Migration
Fund Source: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW) (Assurance Number A3255.01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: August 13, 2008

TO: Candece L. Gladson, M.D.
ZRB-434 0007
FAX: 934-7346

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: The Role of Lyn in Glioma Progression and Migration
Sponsor: NIH
Animal Project Number: 080807534

On August 13, 2008, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
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Animal use is scheduled for review one year from August 2008. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 080807534 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
B10 Volker Hall
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205.934.7692
FAX 205.934.1188

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