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**The effects of p53 status and EGF on TIMP-2 and other proteins
related to migration in human glioblastoma cell cultures**

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Honors Thesis

Department of Biology

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This thesis has been accepted as part of the honors
requirement in the Department of Biology.

Abstract

The effects of epidermal growth factor (EGF) on proteins that have been implicated in the migration of the Grade IV brain tumor, glioblastoma multiforme, were investigated in this study. Differential expression levels of TIMP-2, MMP-2, and MT1-MMP in response to EGF treatment were compared by immunoprecipitation, and immunoblotting. This study involved two different cultured glioma cell lines, U87, which expresses wild-type p53, and T98, which has a mutation in the *p53* gene, in order to determine if the status of the *p53* tumor suppression gene is a factor in the regulation of proteins involved in this migration pathway. It was determined that the only response of mutant cells to EGF treatment was the putative upregulation of the level of TIMP-2 protein. Treatment of the U87 cells resulted in increased phosphorylation of MT1-MMP. It was believed that phosphorylation of MT1-MMP correlated with activity and ability to cleave the proenzyme of MMP-2, however, the level of MMP-2 activity actually decreased. These data highlight the complex interaction of *p53* status and protein expression and activation that corresponds to the ability of tumor cells to migrate.

Introduction

Glioblastoma multiforme (GBM) is a Grade IV invasive astrocytoma that is among the most aggressive brain tumors. It originates from astrocytes, a type of glial cell normally responsible for support and environmental regulation of neuronal cells. When these cells enter the cancer pathway, large tumors can amass within matter of months and typically kill patients within a year. Between 20,000 and 25,000 cases of glioblastoma, nearly 65% of all malignant adult brain tumors, are diagnosed yearly (Goldman et al., 1993; Furata et al., 2004).

Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) can show the location and relative size of tumors but cannot precisely determine the definitive boundary between tumor and healthy tissue (Black and Ciacci, 1993). Consequently the indistinct boundary makes complete surgical resection of the tumor with limited damage of healthy tissue very difficult. Indeed, nearly 80% of tumor surgeries that initially indicate no post-operative gliomas eventually recur within 2 cm of the resection boundary (Black and Ciacci, 1993). Due to the high rate of tumor recurrence, patients with GBM often undergo multimodal treatment regimens including chemotherapy and radiotherapy (Nagane et al., 1998). Even combined, however, current methods of treatment remain ineffectual at improving the prognosis of GBM patients.

Although the precise cause of transformation of healthy glial cells into gliomas remains under investigation, the *p53* tumor suppressor gene has been implicated as an important player in tumor progression (Ueba et al., 1994). The *p53* gene codes for a nuclear phosphoprotein composed of a β -sandwich, which provides structural support for two large loops and a loop-sheet-helix motif (Cho et al., 1994). These structures form the DNA binding domain that is critical to normal *p53* function because DNA binding is the means by which *p53* regulates the expression of other genes that induce cell cycle arrest or apoptosis (Frebourg and Friend, 1992;

Cho et al., 1994). In order to insure proper function in these important pathways, p53 binds to DNA in a tetrameric conformation that bends the DNA to enforce promoter sequence specificity and proper transcription of genes involved in downstream pathways (Nagaich et al., 1999).

Approximately 1,000 mutations in the *p53* gene sequence have been identified, that typically but do not exclusively affect the DNA binding domain of p53 (Nagaich et al., 1999). While mutation is neither necessary nor sufficient to induce tumorigenesis, mutations in the *p53* tumor suppressor gene have been identified in upwards of 30% of malignant gliomas (Ueba et al., 1994; Van Meir, 1994). Furthermore, experimental evidence suggests that silencing of *p53* by proteins like MDM2 or loss of functional downstream effectors in wild-type p53 tumors may simulate the loss of p53 function without the need for sequence variation. Indeed, mice that do not express any p53 are highly prone to *de novo* tumorigenesis and transfection of these mice with mutant variants of *p53* further enhances tumor potential (Levine et al., 1991; Cho et al., 1994). *In vitro* and *in vivo* studies indicating that the transformed phenotype in mutant *p53* cells can be recovered by suppression of mutant *p53* expression also suggest that mutations in *p53* confer tumorigenic potential (Levine, 1991; Li et al., 1999). Recent trials of p53 gene therapy are taking advantage of the ability of wild-type p53 to suppress tumors and revert cells from the transformed phenotype (Lang et al., 2003). This approach of treating GBM patients appears promising.

In addition to induction of apoptosis and repression of angiogenesis, p53 may also play a role in the progression of glioblastoma via a pathway that involves enzymes that excavate the extracellular matrix (ECM) to facilitate cell migration. Matrix metalloproteinases (MMPs) are one such family of twenty-eight zinc-dependent enzymes that degrade the ECM in numerous developmental and physiological pathways and may be exploited for tumorigenesis (Somerville

et al., 2003). MMPs are secreted as inactive zymogens subject to highly regulated cleavage reactions in order to control their activity and may be either soluble or membrane bound. (Somerville et al., 2003) One of the MMPs studied here, Membrane Type-1(MT1-MMP), is an integral protein that is activated by furin and is necessary for activation of MMP-2/pro-gelatinaseA (Somerville et al., 2003). The MT1-MMP-dependent activation of MMP-2 is mediated by tissue inhibitor of metalloproteinase-2 (TIMP-2) (Bernardo and Fridman, 2003). TIMP-2 mediates the interaction between MT1-MMP and MMP-2 by forming non-covalent bonds with each MMP at two different binding sites. The N-terminus domain of TIMP-2 complexes with the extracellular N-terminus of MT1-MMP simultaneously as the C-terminus of TIMP-2 associates with a haemopexin-like domain of pro-MMP-2 (Bernardo and Fridman, 2003). A second MT1-MMP protein unbound to TIMP-2 is then recruited to the complex to cleave and activate the bound MMP-2. Additionally, the N-terminal inhibitory region may bind to the active sites of either MT1-MMP or MMP-2 and inhibit their activity (Kurschat et al., 1999). Clearly, TIMP-2 is a key regulatory molecule in both the activation and inhibition of MMP-2; however, the implication of this role in the cancer pathway has not been fully determined.

The regulation of MT1-MMP and subsequent MMP-2 activation in murine lung development was found to involve Epidermal Growth Factor Receptor (EGFR). EGFR is a transmembrane tyrosine kinase receptor that dimerizes and becomes phosphorylated on a tyrosine residue upon ligand binding (Kheradmand et al., 2001; Yen et al., 2002). This phosphorylation is necessary to phosphorylate effector proteins or secondary messengers to conduct external signals to the cellular machinery, particularly in pathways involving tumorigenesis (Connolly et al., 1994. Yen et al., 2002). Its ligands include EGF, a mitogen,

VEGF, a factor that induces angiogenesis, transforming growth factor-beta, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin, and epiregulin (Goldman et al., 1993).

Many experiments have been performed to determine the cellular role of EGFR and show the varied and complex function of this protein. In humans, EGFR has been implicated in positive regulation of angiogenesis, apoptotic evasion, and drug resistance (Nagane et al., 1998; Yen et al., 2002). Experiments using animal models have also demonstrated the role of EGFR during development and other physiological processes. For example, the effects of EGFR inhibition in rat corneal healing indicates that EGFR plays a crucial role in the promotion of wound healing by epithelial cell proliferation (Nakamura et al., 2001). The high cross-species conservation and widespread expression of EGFR suggests that EGFR may also positively regulate cellular proliferation in several areas of human physiology, such as the highly proliferative gliomal cells. Furthermore, EGFR has also been implicated in upstream regulation of cellular migration *in vivo* because inhibition of EGFR or dominant negative mutations of EGFR resulted in the loss of migration during embryogenesis (Duchek and Rorth, 2001). This study proposes that EGFR may also play a role in the migration of other cells, possibly tumor cells. Although the specific downstream components of the migratory pathway have not been determined, experimental evidence suggests that EGFR regulates pathways believed to promote migration via MMP-2 activation (Kheramand et al., 2002).

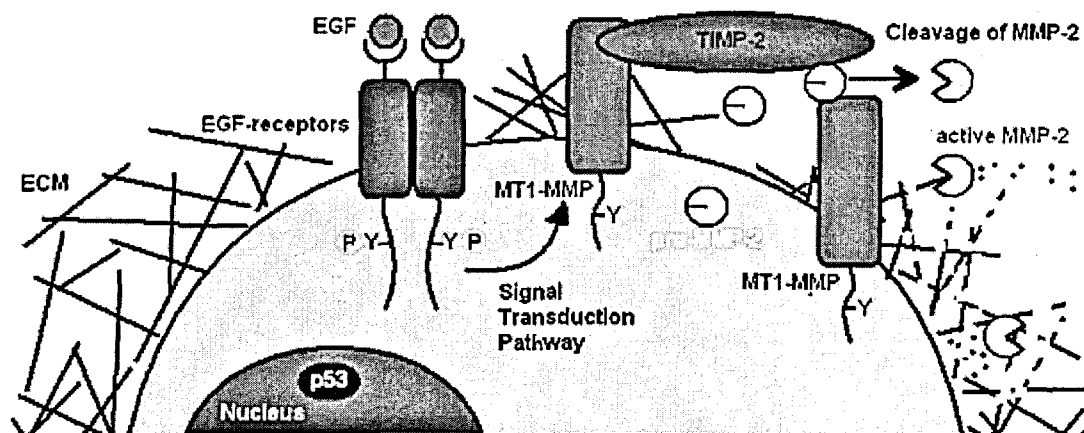


Figure 1: Proposed signal transduction pathway for MMP-2 activation and ECM degradation mediated by EGFR, MT1-MMP, and TIMP-2.

Microarray analysis of gene expression in several individual tumors indicates amplification of the EGFR gene (Liang et al., 2005). Immunoblotting and RNA hybridizations correlate *EGFR* gene amplification with over-expression of the receptor protein (Wong et al., 2005). Both gene amplification and receptor overexpression are characteristics of one-third of primary glioblastomas (Furata et al., 1994). Tissue analysis of these tumors indicate 50%-70% more expression of EGF-receptors on the cell surface in conjunction with an eight-fold increase in gene amplification compared to normal cells (Sung et al., 2000; Shir et al., 2006). This upregulation in cancer cells, which is absent in normal cells, suggests that EGFR upregulation may be involved in the cancer pathway. Cox survival statistics correlate EGFR upregulation with a peak on the index indicative of poor prognosis and low patient survival (Liang et al., 2005). Consequently, the role of differential EGFR expression in cancer is currently under investigation. Such studies selectively target gene therapy by targeting cells that overexpress EGFR with dsRNA or EGFR specific antibodies to induce apoptosis and reduce tumor growth (Shir et al., 2006).

The objective of this experiment was to determine what effect EGF has on the proteins of interest. MT1-MMP, TIMP-2, and MMP-2 were immunoprecipitated from two cultured

glioblastoma cell lines and immunoblotted to determine if EGF treatment affected the relative amounts of protein, phosphorylation of tyrosine residues in MT1-MMP, or the activation state of MMP-2.

Furthermore, this study took advantage of the availability of *p53* mutant and wild-type cultured glioblastoma tumor cell lines, T98 and U87 respectively, to examine the role of *p53* in gliomal proliferation. Experimentation with the U87 and T98 cell lines provides the opportunity to study how *p53* affects the cellular response of glioblastoma to EGF treatment. Clinical studies of glioblastoma indicate that >95% of pediatric astrocytomas and between 35 and 60% of adult gliomas involve the inactivation of the *p53* tumor suppressor pathway, rather than the Rb/CDK4/p16 tumor suppressor pathway (Sung et al., 2000; Kanzawa et al., 2003). Studying both of these GBM cell lines is particularly pertinent due to previous research that indicates that many glioblastoma tumors are composed of a combination of both wild-type cells and *p53* mutant cells (Kanzawa et al., 2003).

Materials and Methods

Cell Treatment:

T25 flask cultures of U87MG or T98G cells were treated at 90% confluency with 20ng or 80ng EGF (Oncogene, cat# PF011) in 5mL of media. An additional T25 flask was reserved without EGF treatment to serve as a control. Sterile Phosphate Buffered Saline (PBS; 137mM NaCl, 2.7mM KCl, 1.5mM KH₂PO₄, 48.1mM Na₂HPO₄) and Dulbecco's Modified Eagle Medium (DMEM) without FBS (Fetal Bovine Serum) were heated in a 37°C water bath. Cells were rinsed with 6mL of PBS three times and the conditioned media was replaced with 5mL of fresh DMEM without FBS. The specified EGF treatments were added to the flasks and the cells

were incubated at 37 °C for at least 36 hours and up to 48 hours, with exact time being dependent on scheduling conflicts.

Harvesting Cells and Conditioned Media:

The conditioned media was reserved for further testing before the cells were rinsed with sterile PBS three times and drained. Cells were rocked on ice for 1 hour in 2mL Proteinase Inhibitor Solution (PIS; 50mM n-octyl, 1ug/mL Aprotinin, 1mM PMSF, 0.5mg/mL Pepstatin A, 1mM CaCl₂, 1mM MgCl₂, 1mg/mL Leupeptin). The bottoms of the flasks were scraped to ensure a complete harvest of the cells. The cell lysate solution was transferred to 1.5mL eppendorf tubes and centrifuged at 14,000 rpm, 4°C for 15 minutes. The supernatant was collected for use in immunoprecipitation as cellular lysates.

Immunoprecipitation:

Fresh PIS (1.5mL) was added to 150uL of Protein Agarose A (Calbiochem, cat# IP06) and centrifuged for one minute to wash the agarose beads. Supernatant was removed and replaced with 200uL of fresh PIS. Either 200uL conditioned media or 100uL of cellular lysate was combined with 30uL of washed Protein Agarose A in a microcentrifuge tube and nutated for 2 hours at 4°C. After nutation, samples were centrifuged at 14,000 RPM at 4°C for 15 minutes and treated with the appropriate antibody. For samples originating from conditioned media 2ug of TIMP-2 mouse antibody (Chemicon International, cat# MAB13441) were added to 150uL of precleared conditioned media supernatant. The remaining 50uL of precleared conditioned media was preserved at -20°C. Samples from cellular lysates were treated with 6ug of MT1-MMP rabbit antibody (Chemicon, cat# AB815). All samples were nutated at 4°C overnight.

Fresh PIS was prepared to wash fresh Protein Agarose A. Microcentrifuge tubes used in immunoprecipitation of TIMP-2 and MT1-MMP were treated with 30uL of washed Agarose and

nutated at room temperature for 2 hours. Samples were centrifuged at 14,000 RPM at 4 °C for 15 minutes. Supernatant was replaced with 1mL of IP Wash Solution (20mL stock solution [0.5M NaCl, 50mM Tris-HCl, pH 7.4] and 100uL of Tween-20). Tubes were centrifuged at 10,000 RPM at 4 °C for 5 minutes and supernatant was removed with a vacuum aspirator. IP wash solution was added to the pellet and centrifuged for a total of six washes.

Bradford Assay:

The Bradford Assay was performed on precleared lysate in order to estimate the total protein concentration in the samples for adequate and equal loading into the SDS gel. In cuvettes, 3mL of the Coomassie Protein Assay Reagent (Pierce, cat# 1856209) was combined with 75uL of precleared lysate and 25uL superwater and incubated at room temperature for 10 minutes. A spectrophotometer was used to measure the absorbance of each sample at 595nm. Absorbance values were correlated to protein concentration by a standard curve determined for known concentrations of BSA. (Figure 2)

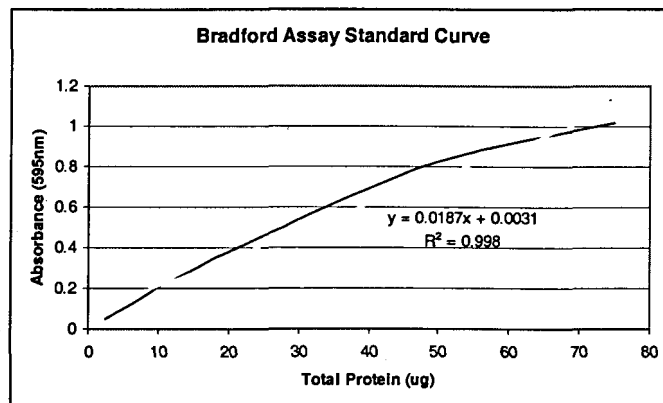


Figure 2: Bradford Assay Standard Curve. The mathematical relationship between the absorbance value of a sample at 595nm and the total protein content has been determined for several different known concentrations of BSA protein (Pierce). When plotted on an axes, the curve provides means of estimating the protein concentration of unknown samples.

SDS-PAGE:

Each washed IP pellet was treated with 40uL of 2X NuPage LDS Sample Buffer (Invitrogen, cat# NP0007) and incubated at 90°C for 5 minutes then centrifuged for 2 minutes. The appropriate volume of the supernatant from the IP pellet or samples of precleared lysates with 2mg of protein was combined with 1.3uL of NuPage Reducing Agent (Invitrogen NP0004), 3uL of 2X NuPage LDS Sample Buffer, and superwater to a total volume of 20ul. A molecular standard was prepared with 10uL of SeeBlue Plus2 (Invitrogen, cat#LC5925), 9ul superwater, 3uL of 4X NuPage LDS Sample Buffer, and 1.3uL of NuPage Reducing Agent. All samples and standards were incubated at 70°C for 10 minutes and loaded into NuPage polyacrylamide gel (Invitrogen, cat# NP0321BOX). The running buffer used in the outer chamber was 1X NuPage MES SDS Running Buffer (Invitrogen, cat# NP0002) and the running buffer in the inner chamber included 5mL NuPage sample antioxidant (Invitrogen, cat# NP0005) combined with 200mL 1X running buffer (Invitrogen, cat# NP0002). Gel was run at 200volts for 35 minutes or until bands were adequately separated. Proteins were then transferred to nitrocellulose membrane (Bio-Rad, cat# 162-0115) in transfer buffer (1L 1X NuPage Transfer Buffer [Invitrogen, cat# NP0006-1], 1mL NuPage sample antioxidant [Invitrogen, NP0005], and 100mL 10% methanol per gel) for 1 hour at 30 volts.

Western and ECL:

Nitrocellulose membranes were rolled in 10mL blocking solution (0.3g powdered milk in 10mL 1X Tris Buffered Saline [TBS: 1.54M NaCl and 0.10M Tris-HCl, pH 7.4]) for 1 hour then 10mL 1X TBS for 10 minutes. An additional 0.2g powdered milk was added to the phosphotyrosine blots to reduce background on the blots. Blots were then rolled in 1X TBST (99.8mL 1x TBS, 0.2g milk powder (0.2%), and 200µl Tween-20) and the appropriate dilution of primary

rabbit antibodies for one hour (TIMP-2, MT1-MMP, pro-MMP-2 [Chemicon, cat# AB809], and MMP-2 [Chemicon, cat# AB808], 1:5000; phospho-tyrosine [Chemicon, cat# AB15991] :8000). All blots were washed 3 times with 10uL of TBST for 10 minutes then rolled in TBST with a 1:5000 concentration of secondary goat-anti-rabbit antibodies for one hour (Santa Cruz, cat# SC-2004). Unbound secondary antibodies were rinsed from the blot with four five-minute wash cycles in 10uL of TBST. Blots were immediately treated with 3.5 mL of both ECL Immunoblotting Detection Reagents (Amersham Biosciences, cat#RPN2209) for one minute. Blots were then exposed to Kodak X-OMAT AR Film (Kodak, cat#165-1496) and automatically developed in order to observed relative amounts of protein. Densitometry was performed on the resulting bands with Adobe Photoshop in order to quantify the relative levels of protein.

Zymography:

Samples were prepared to observed MMP-2 activity by combining 12 uL of conditioned media with 12 uL of 2X Tris-Glycine Sample Buffer (Invitrogen, cat# LC2676). A zymography Standard was prepared with 1uL of stock standard (0.1 mg/mL, Chemicon, cat# CC073) and 99uL 2X Tris-Glycine Sample Buffer. Samples and standard were incubated at room temperature for 10 minutes loaded into Tris-Gly Zymography Gel (Invitrogen, cat# EC6175BOX). Gel was run for 90 minutes at 120 volts in 1X Novex TrisGlys SDS Running Buffer (Invitrogen, cat# LC2675-5). The gel was removed from the plastic casing and treated with 1X Novex Zymogram Renaturing Buffer (Invitrogen, cat# LC2670) and 1X Novex Zymogram Developing Buffer(Invitrogen, cat# LC2671), each for 30 minutes with agitation. Gel was incubated at 37°C overnight, stained with 0.5% Coomassie blue for an hour with agitation. It was destained (10% isopropanol, 10% HOAc, 80% superwater) for 3 hours with agitation then transferred to superwater.

Results

The effects of EGF treatment on the relative amount of TIMP-2 protein in U87 and T98 glioma cell lines

Previous research in this lab suggested a positive correlation between the amount of TIMP-2 protein and the concentration of EGF treatment in the U87 cell line. However, this data was refuted by more recent data with U87 that suggested the amount of TIMP-2 protein was unaffected by either 20ng or 80ng of EGF (Figure 3). Comparison of the intensity of the 24kDa bands between the control and the treatments of 20ng and 80ng yields no significant difference in the amount of TIMP-2 across the different treatment regimens.

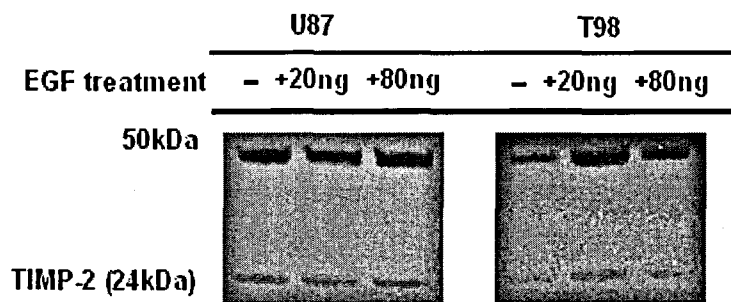


Figure 3: Immunoblot of the relative amount of TIMP-2 protein in the conditioned media of U87 and T98 cells following EGF treatments. U87 or T98 cells were treated with either 20ng or 80ng of EGF for 36 to 48 hours. Conditioned media was harvested for immunoprecipitation and SDS-PAGE of TIMP-2 proteins. Immunoblotting and ECL indicated no response in the relative amount of TIMP-2 to EGF treatment in U87 cells treated with EGF; however, T98 cells treated with 20ng EGF suggests a positive response. An unidentified band that migrated at 50kDa co-immunoprecipitated with TIMP-2 reflects this trend.

Experimentation with the T98 cell line suggests that a mutation in the *p53* gene may be involved in the response of the levels of TIMP-2 protein expression (Figure 3). Treatment of T98 cells with 20ng of EGF resulted in a visible increase in the intensity of the bands at 24kDa representative of TIMP-2 when compared to the untreated sample. The positive response to EGF, however, was only observed in the +20ng samples, with the +80ng EGF eliciting no

response to EGF treatment. Another replication of this experiment did not indicate a difference in TIMP-2 levels across the treatments.

Presently, there is not an adequate means of normalizing the protein in the conditioned media so it must be assumed that the total protein content of each sample was the same for the purpose of cross comparison.

Additionally, there was an unidentifiable band migrating at ~50kDa in both the U87 and T98 cell lines and may be an artifact of the heavy chain of the TIMP-2 antibody.

	U87			T98		
EGF Treatment	-	+20ng	+80ng	-	+20ng	+80ng
MT1-MMP	[Blacked out immunoblot data]					
P-Tyr						

Figure 4: Immunoblot of the relative amount of MT1-MMP protein and phosphorylated tyrosine residues of MT1-MMP in the cellular lysates of U87 and T98 cells following EGF treatments. U87 or T98 cells were treated with either 20ng or 80ng of EGF for 36 to 48 hours. Cellular lysates were harvested for immunoprecipitation and SDS-PAGE of MT1-MMP protein. Immunoblotting and ECL indicated no response in the relative amount of MT1-MMP to EGF treatment in U87 cells. However, immunoblotting for phospho-tyrosine on MT1-MMP proteins in U87 cells treated with 80ng of EGF indicates a positive response.

The effects of EGF treatment on the relative amount of TIMP-2 related proteins

In addition to TIMP-2, the levels of proteins that interact with TIMP-2 and may play a role in the pathway were also analyzed. Immunoblotting was used to determine the relative amounts of MT1-MMP protein and phosphorylated tyrosine residues on MT1-MMP as well pro-MMP-2 and active MMP-2 in response to EGF treatment. While analysis of the protein amounts of pro-MMP-2, active MMP-2, MT1-MMP protein and phosphorylated tyrosine residues on MT1-MMP in the T98 cells showed no significant response to either 20ng or 80ng of EGF treatment, EGF did elicit some response in the U87 cell line (Figures 4 and 5). A Bradford Assay performed to determine total protein in each sample of lysate blotted for MT1-MMP and

phospho-tyrosine indicated that samples contained very little overall protein. While normal amounts of protein are expected to have total protein concentrations of 0.054 ug/ul, the control, +20ng, and +80ng samples were estimated to contain 6.10×10^2 ug/ul, 3.21×10^2 ug/ul, and 4.01×10^2 ug/ul, respectively (Table 1). Despite the significantly lower than average levels of total protein, the experiments are routinely performed with similar low protein concentrations with no apparent complications.

Treatment	Volume Assayed	Absorbance Values (595 nm)	Average Absorbance Values (595 nm)	Protein Concentration (ug/ul)
0 EGF	75ul	0.013	0.015	0.0107 ug/ul
	75ul	0.016		
+20 EGF	75ul	0.001	0.003	0.0021 ug/ul
	75ul	0.005		
+80 EGF	75ul	0.006	0.008	0.0057 ug/ul
	75ul	0.009		

Table 1: Total Protein concentration of cellular lysates as determined by Bradford Assay. The absorbance of samples at 595nm was measured after 75ul of pre-clear cell lysate and 3mL Coomassie Protein Assay Reagent were combined and incubated at room temperature for ten minutes. A standard curve was prepared using BSA and used to correlate observed absorbance values to protein concentration. Bradford data was used to normalize proteins in the gel blotted for MT1-MMP and phospho-tyrosine (Figure 3) to the maximum loadable amount for these samples, 0.042ug. The Bradford assay could not be applied to the TIMP-2 or MMP-2 data (Figures 2 and 4) that was obtained from conditioned media, so 10uL of each sample was loaded into the gel.

In the U87 cell line, the intensity of the 62 kDa bands immunoblotted for MT1-MMP showed no significant difference between either 20ng or 80ng EGF and the control treatment. However, samples blotted for phospho-tyrosine residues (P-Tyr) in U87 cells showed a marked increase in the amount of phosphorylated tyrosine residues on the MT1-MMP tail (62kDa) in the 80ng EGF treatment when normalized to the total amount of MT1-MMP protein. The intensity of bands corresponding to phospho-tyrosine was unaltered by treatment with 20ng of EGF.

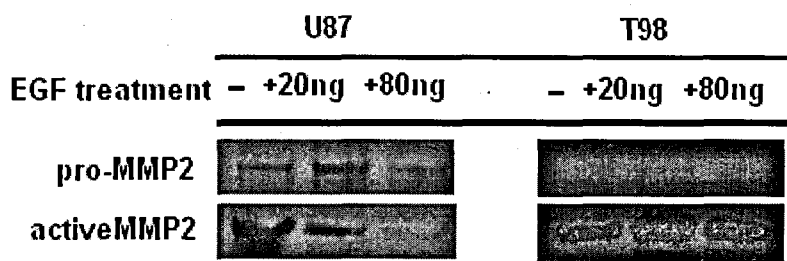


Figure 5: Immunoblots of the relative amount of pro-MMP-2 and active MMP-2 protein in the conditioned media of U87 and T98 cells following EGF treatments. U87 or T98 cells were treated with either 20ng or 80ng of EGF for 36 to 48 hours. Conditioned media was harvested for SDS-PAGE of pro-MMP-2 and active MMP-2 proteins. Immunoblotting and ECL indicates no response in the relative amount of pro-MMP-2 to EGF treatment in the T98 cells treated with either 20ng or 80ng EGF. The T98 cells treated with EGF suggests a negative response of active MMP-2 relative to the proenzyme.

Analysis of immunoblotting for active MMP-2 (72kDa) and pro-MMP-2 (66kDa) in U87s in response to the EGF treatments suggests that amount of active MMP-2 relative to pro-MMP-2 decreased with both 20ng and 80ng treatments of EGF (Figure 5). However, zymography of conditioned media suggested that the activity of MMP-2 in the conditioned media did not change across treatments and MMP-2 was constitutively in the active form (data not shown).

Discussion

The effect of EGF on TIMP-2 expression

The overexpression of EGF-receptors in tumor cells but not the surrounding healthy tissue reported in primary literature suggests that the addition of EGF to the media might play a role in initiating or exacerbating cancer pathways (Kanzawa et al., 2003). This experiment investigated the response of proteins implicated in glioblastoma tumor growth and invasion, specifically TIMP-2, to treatment with EGF. It was determined that although EGF treatment did not generate a difference between band intensity blotted for TIMP-2 in the U87 cell line, a slight increase in the T98 cell line with treatment of 20ng of EGF was observed. However, lack of

adequate means of normalizing the total protein content of conditioned media casts doubt upon the validity of this data. While samples collected from the cellular lysates can be normalized according to total protein determined by the Bradford Assay, the abundance of protein in the conditioned media makes it difficult to obtain accurate measurement of total protein.

Compounding the problem of uniform loading of samples is the current lack of a protein that can be immunoblotted for as a control. While studies of proteins from the lysates can take advantage of the ubiquitous intracellular expression of actin to verify that loaded samples contained similar total protein, an analogous ubiquitous protein in the conditioned media remains to be identified. Furthermore, stripping of antibodies has yet to be successfully performed on the nitrocellulose blots to allow for accurate actin quantifications of lysate-derived samples.

Consequently, the increased intensity of the TIMP-2 band with 20ng EGF may be due solely to the loading of a more concentrated sample and is not necessarily reflective of actual changes in TIMP-2 protein expression. Furthermore, due to suspected mutations in the U87 cells affecting growth patterns and causing clumping in culture, experiments with this particular cell line was halted until the cause of the altered cultures could be determined. As a result, the data that supports conclusions based on the U87 cell line are derived from a single experiment and remains to be replicated and verified.

Presuming that the putative TIMP-2 band results from a genuine increase in the amount of TIMP-2 protein in response to 20ng of EGF, the data suggests that mutation in the p53 gene may influence the response of cells to low doses of EGF. At this time, the dose-dependent mechanism that prevents the positive response in TIMP-2 protein levels in the 80ng of EGF that was observed with 20ng of EGF in T98s has not been investigated.

Moreover, a study of bladder cancer cell lines used immunoblotting to determine that EGFR stimulation correlated with downregulation of TIMP-2, rather than upregulation seen in the T98 glioma cells (Nutt et al., 2004). This downregulation could possibly play a role in prevention of TIMP-2 inhibition of MMP-2 activation. While this study was performed on bladder cells, the inconsistency with the unverified findings of this study raises the question of whether or not EGF affects TIMP-2 protein expression and how EGF might influence TIMP-2 levels in gliomas to remain unanswered.

The effect of EGF on proteins associated with TIMP-2

The initial hypothesis of this experiment supposed that the binding of EGF to the EGF-receptor would influence MMP-2 activation by signaling for the phosphorylation of the tyrosine residues of MT1-MMP. The primary literature states that EGF induces auto-phosphorylation of the cytoplasmic tyrosine kinase domain of EGFR, which may then phosphorylate a number of secondary species (Yamakazi et al., 1988; Honegger et al., 1990). MT1-MMP has not only been identified as a major downstream target of EGFR in lung development by zymography and mRNA analysis in EGFR double negative mice, but also has been implicated in mediation of the EGFR regulated activation of MMP-2 (Kheradmand et al., 2002). The studies of U87 presented here, however, show that the increase of phosphorylated tyrosine was observed only after treatment with 80ng EGF, whereas the MMP-2 activation state was affected with only a 20ng EGF treatment. This data suggests that phosphorylation of MT1-MMP was unrelated to the activation state of MMP-2 and may not be involved in the pathway.

Further investigation involving site-directed mutagenesis of specific tyrosine residues of MT1-MMP and the affected interaction with MMP-2 activation could help confirm that phosphorylation of MT1-MMP is not an active part of the pathway. Additionally, the expression

and phosphorylation of the EGFR receptor should be investigated with immunoprecipitation and immunoblotting and compared to the primary literature.

Not only was the phosphorylation of MT1-MMP found to be extraneous to the MMP-2 activation state, the level of TIMP-2 protein was also not determined to affect MMP-2 activation. While both the intensity of bands immunoblotted for TIMP-2 and active MMP-2 indicated a response to 20ng of EGF, TIMP-2 levels were unresponsive to any treatment in the U87 cells, which were the only cell line to demonstrate a change in MMP-2 activation. Especially interesting is the trend of decreasing MMP-2 activation in a dose dependent response to EGF, rather than an increased activation seen in other studies of MMP-2 (Kheradmand et al., 2001; Morgunova et al., 2002).

Active MMPs are necessary to degrade the ECM in order to clear a path for migrating tumor cells and EGFR has been found to play a crucial role in cellular migration (Duchek and Rorth, 2001; Somerville et al., 2003; Shir et al., 2006). Thus, it was predicted that the overexpression of EGFR in cancer cells might serve the purpose of increasing the activation of MMPs. Indeed whole tissue zymography studies of the differential MMP-2 activation state of EGFR deficient mice support the hypothesis that EGFR stimulation of the glioma cells would result in increased activation of MMP-2 (Kheramand et al., 2002). Furthermore RNA anti-sense assays which show co-expression of TIMP-2 and MT1-MMP with MMP-2 suggest that TIMP-2 and MT1-MMP may play a role in EGFR regulated MMP-2 activation (Kheramand et al., 2002). Moreover, zymography of cells transfected with a TIMP-2 expressing vector also showed dose dependent increase in MMP-2 activation (Bernardo et al., 2003). Taken together, these experiments indicate that the levels of MMP-2 activity should be positively affected by EGF treatment, however the data suggests rather a downregulation of MMP-2 in response to EGF. In

light of data inconsistent with the literature, it is difficult to determine why EGF treatment of these glioma cells resulted in an apparent inactivation of MMP-2 since it is counterproductive to the proposed pathway of tumor migration.

The results of this experiment are inconsistent with the initial hypothesis and call for an alternate model. In line with the current understanding of molecular mechanisms involved in this study, several explanations of the data are possible. Firstly, the dual role of TIMP-2 as both an inhibitor of the active site of MT1-MMP and MMP-2 and as an intermediary between MT1-MMP-dependent MMP-2 activation may be responsible for these results (Bernardo and Fridman, 2003). The U87 cell line may cause the TIMP-2 molecules to behave preferentially as inhibitors of MMP-2 activation to elicit the observed decrease in active MMP-2, even though the amount of TIMP-2 protein remained constant. Furthermore, several pathways remaining to be described may be responsible for the slight increase of TIMP-2 in the T98 cell line that was not correlated to any change in the state of MMP-2. It is important to also note that treatment of T98 cells with EGF may actually be ineffectual because the over-expression of EGFR is sometimes found only in cells with wild-type p53 (Nagane et al., 1998).

The role of p53 in the regulation of the MT1-MMP/TIMP-2/MMP-2 pathway

The preliminary findings of this study suggest that p53 status may influence the response of the MT1-MMP/TIMP-2/MMP-2 pathway to treatment with epidermal growth factor. While EGF treatment of T98 cells affected only TIMP-2 protein with no correlation to dosage, both the EGF treatment regimens in the U87 cells altered the levels of phosphorylated MT1-MMP and the activation of MMP-2 to variable degrees. Previously, the wild-type p53 gene product was demonstrated to activate reporter constructs of the MMP-2 promoter (Bian and Sun, 1997). This

is inconsistent with the decrease in MMP-2 activation seen the U87 cell line and suggests rather inhibition of MMP-2 activation by p53 either transcriptionally or postranslationally. However, the current data is insufficient to conclusively determine this without a direct quantitative comparison between cells with wild-type and mutant p53 status.

While the results of this experiment have yet to be sufficiently replicated, the inability of EGF to significantly affect the activity of the MT1-MMP and MMP-2 proteins in the T98 cell line studied suggests that the mutation in the p53 may prevent proper relay of the EGFR signals to MMP-2 activation, possibly due to lack of EGFR overexpression in these cells. While, many aspects of this cancer pathway have yet to be determined, future pursuits should certainly consider the role of *p53* status and sensitivity to the effects of EGF.

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