Poliovirus Receptor CD155 Over-Expression Effect on Migration in C6 Glioma Cells

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Abstract

Cell migration plays a key role in brain cancer invasion, an early step in metastasis, and proteins that regulate migration are often up-regulated in tumor cells. The poliovirus receptor CD155 has recently been shown to affect migration levels of select malignant glioma strains, fueling the exploration of treating brain cancer with oncolytic virus recombinants. In the beginning phase of experimentation, we analyzed the migratory behavior of control C6 gliomas through two rounds of transfection assays. In order to explore the function of CD155 in glioma migration, we then conducted two over-expression experiments with the use of a full-length cDNA expression vector and compared the resulting migration rates to control data. We found that an average of 304.25 control cells cross our transfectants after 5.5 hours, and an average of 354.5 cells transfected with the PVR expression vector. Our results reflected a 16.5% increase in cell migration due to an increased presence of the protein CD155. In order to confirm that CD155 was expressed in the rat C6s, we subsequently conducted two Western Blots: one comparing control C6s and transfected C6s, and the other running control C6s next to a human U87 glioma cell control line. The blots establish that CD155 is indeed present in both of these human and rat strains of glioma, and that our over-expression was successful.

Key words: Glioblastoma multiforme; tumor migration; poliovirus receptor CD155; protein over-expression.

Introduction

After the human brain completes its development so that it has the capacity of its cells to enter the G0 phase, in which they never divide again. One exception to this rule is when a brain tumor develops as a result of abnormal, unregulated cell division. The glioma cells re-enter the cell cycle because of alterations in any of a large number of genes that control cell division and growth. Astrocytomas and oligodendrogliomas are types of gliomas that are the most common primary tumors of the adult brain. Primary brain tumors arise from cells of the brain itself rather than traveling, or metastasizing, to the brain from another location in the body like other known forms of cancer. Tumors are generally classified in four grades, with grade 1 being the most benign and grade 4 being the most malignant. Signs that the tumor is growing rapidly include cells undergoing division (mitosis), the presence of newly-formed blood vessels (angiogenesis), and evidence that the tumor is outgrowing its blood supply (necrosis). Typically, malignant gliomas show an area of necrosis, called a glioma, around which the tumor is highly unlikely. Unfortunately, high doses of radiation are not recommended for treatment, as this may cause too much damage to the normal brain tissue (Giese, 2003).

Having an agent that blocks migration is key to managing glioblastoma multiforme. Interventions to control the spreading of glioblastoma multiforme have the potential to slow the clinical course of the disease and improve overall survival rates. Preventing glioma invasion has the potential to convert this highly malignant tumor into a focal disease, which could then be effectively treated with focal therapies, such as radiation and surgery (Madsen, 2003; Gillespie, 1999).

Invasion and Migration of Malignant Gliomas

Tissue invasion by malignant gliomas is a multi-step process. Central to this is the ability of transformed cells to crawl through the extracellular matrix. The initial step requires receptor-mediated adhesion of tumor cells to matrix proteins, followed by a second phase of degradation of the matrix by tumor-secreted proteases. The ability of invasive glioma cells to navigate these diverse anatomic structures and molecular substrates raises the question of whether specific mechanisms and phenotypes of invasive cells are involved in dispersion following specific pathways (Gillespie, 1999).

Gliomas typically invade the brain by migrating long distances through white matter tracts and by infiltrating cortex and subcortical gray matter structures. When migrating, the brain white matter, gliomas move in a manner remarkably similar to that previously described for neural progenitor cells. Given the submicrometer size of the extracellular spaces that are present in the tightly packed intercellular spaces of the brain, most cells would likely find it impossible to migrate because there would be no room to allow a lamellipodium to form. The unique morphologic behavior of glioma and neural progenitor cells generated from rodents indicates adaptations that these cells have to make to a uniquely challenging environment with small effective pore sizes (Beadle, 2008). One of these adaptations is the absence of myosin II. The major source of cytoplasmic contractile force (Gillespie, 1999). Myosin II is absolutely required for migration in the brain, where other cancers do not require myosin II for the invasive behavior expressed by all levels of grading gliomas. Significantly, gliomas typically invade the brain by migrating long distances through white matter tracts and by infiltrating cortex and subcortical gray matter structures. When migrating, the brain white matter, gliomas move in a manner remarkably similar to that previously described for neural progenitor cells. Given the submicrometer size of the extracellular spaces that are present in the tightly packed intercellular spaces of the brain, most cells would likely find it impossible to migrate because there would be no room to allow a lamellipodium to form. The unique morphologic behavior of glioma and neural progenitor cells generated from rodents indicates adaptations that these cells have to make to a uniquely challenging environment with small effective pore sizes (Beadle, 2008). 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of its host cell 4 to 6 hours following initiation of infection in cultured mammalian cells. The mechanism of viral release from the cell is unclear, but each dying cell can release up to 10,000 polio virions (Merrill, 2004).

**CD155/PVR**

The cellular life cycle of poliovirus is initiated by binding to the cell surface molecule CD155 (PVR). CD155 is a transmembrane glycoprotein. The viral RNA is taken up by endocytosis, and the viral RNA is released. The translation of the viral RNA occurs by an IRES-mediated mechanism. The polymerase is then cleaved, yielding mature viral proteins. The positive-sense RNA serves as template for complementary negative-strand synthesis, producing double-stranded replicative form (RF) RNA. Many positive strand RNA copies are produced from the single negative strand. The newly synthesized positive sense RNA molecules can serve as templates for translation of more viral proteins, or can be enclosed in a capsid to form progeny virions. Lysis of the infected cell results in release of progeny viroplasts.

The presence of CD155 is thought to define the animal that can be infected by poliovirus. CD155 has only been found on the cells of humans, higher primates, and Old World monkeys. Do not naturally infect any other species. CD155 is a Type I transmembrane glycoprotein in the immunoglobulin superfamily. Commonly known as Poliovirus Receptor (PVR) due to its involvement in the binding and entry of poliovirus into the cell. PVR is a Type I transmembrane protein with four extracellular Ig-like domains. The main function of PVR is thought to be the selective binding of the virus to the cell membrane, thereby allowing the virus to enter the cell. PVR is also involved in the regulation of cell motility and has prompted studies of its role in cancer cell proliferation. PVR is over-expressed in cancer cells, leading to increased cell migration and invasion. PVR has also been implicated in the development of brain tumors and gliomas, where it plays a role in promoting glioma cell survival and proliferation.

**Oncolytic Applications**

Viral oncolysis has been recently recognized as a new treatment in the development of malignant glioma. Oncolytic viruses must specifically target tumor cells, a property related to the fact that tumor cells often have aberrant immune responses. The first reported incidences of viral oncolysis, over a century ago, were due to non-intended exposure to naturally occurring viruses or after administration of live attenuated vaccine strains. In the last twenty years, new prospects for genetically manipulating viruses have opened possibilities for increasing the tumor specificity and lowering the toxicity of oncolytic viral agents. The oncolytic properties of adeno- viruses, herpesviruses, reoviruses, vesicular stomatitis virus, and most recently, polioviruses. The antineoplastic effects of oncolytic viruses are subject to multifaceted mechanisms, including immune-mediated effects of oncolysis and induction of immune responses against the patient's tumors. Viruses for viral oncolysis are characterized by their ability to induce cell death, or apoptosis, in target cells and to elicit an immune response against the virus and the tumor cells. These effects are mediated by the virus itself and by the immune system.

**Materials and Methods**

**Cell Culture**

C6 rat glioma cell lines (Canoll Lab, Columbia University) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, L-glutamine, and sodium pyruvate supplemented with 10% Fetal Bovine Serum and 1% Pen Gent (Mediatech). Cells were incubated at 37°C in a NACPO Series 8000 W2 CO2 incubator. Cells were passaged every seven days onto fresh poly-L-lysine coated T75 flasks, and medium was changed every three days.

**Transfection of cDNA expression vector**

We obtained glycerol stocks of E. coli transformed with a 3.9-kb plasmid containing an expression vector (Image ID: 3902226, American Type Culture Collection), which was used a 50 µg/µl ampicillin marker and was stored indefinitely at -80°C. DNA was isolated according to standard plasmid preparation procedures and absorption was measured at 260 nm via spectrophotometer (BioMate).

One day prior to transfection, the cells were trypsinized from the T75 flask and added to 10 mL of growth medium. The mixture was then spun in a tabletop centrifuge (2000 rpm for 5 minutes) and resuspended in 5 mL of antibiotic-free growth medium. Cells were trypsinized again and then diluted in 50 µl of serum-free medium. Both cell types were then adjusted to a density of 1×10⁶ cells/mL. One microgram of DNA was mixed with 20 µl of Lipofectamine 2000 reagent and 200 µl mixture. Cells were left in the incubator to run on medium for 24 hours. The next day, media were changed, and the cells were incubated for an additional 48 hours.

**Transfection of DNA expression vector**

The viral genome that eliminates functions that are dispensable in cancer cells, but not in normal cells. Gromeier et al. replaced the normal poliovirus IRES with a thymidine IRES, altering tissue specificity (Gromeier, 2000). The resulting PV1(RIO) virus was able to selectively destroy malignant glioma cells, while leaving normal neuronal cells untouched.

Although the poliovirus itself has been demonstrated in previous studies to have an oncolytic effect in certain types of gliomas, it is still somewhat unclear what the actual role of the receptor CD155 might have, if any, in this relatively new discovery of cancer treatment through viral infection. In glioblastoma, the most aggressive form of glioma, tumor cells disperse so extensively that current treatment approaches such as surgical resection or radiation therapy have little effect in checking progression. The survival of patients with malignant gliomas, and glioblastoma in particular, is poor, with a median survival time of patients with malignant glioma and a median survival of 14 months. Invasive cells remaining after the surgical resection significantly contributes to the patient's outcome. Successful treatment will have to treat the invasive portion of the tumor and the core lesion. Specifically targeting invasive glioma cells remains an interesting concept because molecules expressed in invasive glioma cells may be unique to most tumor cells remaining after surgical resection. Because it has been increasingly shown that glioma invasion is regulated by distinct trigger mechanisms, downstream effector molecules of the invasion process represent the best treatment targets. It has been previously found that CD155/PVR was highly expressed in both U87 human glioma cells and primary glioblastoma tumor tissue, and that inactivation of CD155/PVR reduced cell migration in vitro (Sloan, 2005). These findings suggest a novel role for CD155/PVR in regulating motility and has prompted me to investigate the possibility of using CD155/PVR in glioma cells we have here in lab by conducting an over-expression experiment to mirror the relationship that has been found in the knockdown experiments.

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hydro at 4°C for 20 minutes. After 3 washes with phosphate-buffered saline (PBS) at room temperature, the cells were stained with the nuclei stain Hoechst (Calbio-chem/EMD) at a 1000X dilution of 10µg/10µl for 30 minutes at 4°C. The cells were washed again 3 times with PBS and then plated onto glass coverslips for analysis.

Data Analysis
The number of nuclei on the bottom of each of both control filters was counted in two low power fields (25X) of a Zeiss Axiosplan microscope and averaged. The nuclei on the top of the filters was also counted in two low power fields and averaged in order to further assess the rate of migration in normal C6 cell lines.

Lysates
Lysis Buffer (RIPA buffer) was prepared (1 M pH 8 Tris, 5 M NaCl, 20% NP40, 10% SDS, distilled H2O up to 50 mL) and stored at 4°C. 5X Running Buffer (7.5 g Tris, 47 g Glycine, 25 mL 10% SDS, distilled H2O up to 500 mL), 5X Transfer Buffer (9.5 g Tris, 47 g Glycine, distilled H2O up to 500 mL), and 10X TBS (6 g Tris, 44 g NaCl, distilled H2O up to 500 mL, pH 7.4) were prepared and kept at room temperature.

0.5 mL of EDTA was added to 4.5 mL of lysis buffer to make a 10X stock of protease inhibitor cocktail. The cocktail was then diluted 1:10 in lysis buffer (50µl to 450µl). Cells were washed twice with PBS and 250µl of the lysis buffer mixture was added directly to the tissue culture dish. Cells were scraped and pipeted to an epiphelo tube, vortexed for 10 seconds and returned to ice, and then vortexed for 30 seconds. Cells were then spun for ten minutes at 1600 rpm and the supernatant was pulled off into a new epipol. The lysates were snap-frozen with liquid nitrogen and stored at -80°C until quantitation.

Western Blot Analysis
CD155 expression levels were determined by using a Western blot procedure. 24 µl protein lysate was mixed with 8 µl 4X sample buffer (20 mL 4% Glyc- erol, 4 g SDS, 0.1 g, 0.2% Bromophenol-blue, 5 mL beta-mercaptoethanol (BME), 45mL ddH2O) and boiled for ten minutes. 10% stacking and separating acrylamide gels were prepared according to PAGE Recipe Calculator (Chang Bioscience) and placed into the chamber (VWR), followed by the addition of 1X running buffer up to the brim of the apparatus. The protein mixture, along with Pre-stained Standard (BioRad) was then loaded into the wells and run at 110V for approximately two hours. After the gel was run, it was placed directly into 1X transfer buffer. The nitrocellulose was preret in sterile water to activate and the gels were subjected to overnight electro- phoretic transfer (~03amp) following standard procedures. Membranes were preret in sterile water and then blocked with 50 mL of Tris-buffered saline TWEEN-20 with 0.25 g BSA (TBSTB) + 5% milk for one hour on the shaker at room temperature. After soaking, the blots were treated with Anti-Nec-1 rat monoclonal antibody Clone 1A8-8 (Gift of Dr. Yoshimi Takai, stored at -80°C) diluted 1:1000 in TBSTB and incubated overnight at 4°C. After the removal of the primary antibody and 3 consecutive ten-minute washes of TBST, the last with TBSTB + 5% milk, the membranes were treated with secondary Anti-Mouse IgG-Alkaline phosphatase antibody produced in goat (SIGMA, stored at 4°C) diluted 1:10,000 in TBSTB and set on the shaker for 1 hour. After three thorough rinses of the blots with TBST, they were treated with BM Purple (Roche) until the appearance of protein bands—after which they were stored at 4°C in TBST.

Results
With two successful Western Blots (See Figures 5 and 6), we were able to formulate a comprehensive picture of C6 glioma migration influenced by transfection of PVR. To determine if increased synthesis of CD155 affects the migration of C6 glioma cells, we captured two images of each control and transfection well, as well as one field each of the cells remaining on the top of the filter (See Figures 1, 2, 3). It was not immediately prevalent through qualitatively assessing cell numbers that a substantial increase in cell migration occurred between the control and transfected cells, but a detailed count revealed an increase.

The nuclei counts for the control fields were 315, 355, 320, and 287, respectively. The numbers of cells transfected with the PVR expression vector that transversed the matrix were 327, 367, 332, and 392. The average of migrating control cells was 304.25, in comparison with the transfection average of 354.5. Our data reflect an average percent increase of 16.5%. By conventional standards according to a statistical T-test (two-tailed P-value of 0.057), this difference is not considered to be remarkably significant. It is important to note, however, that the second round of transfilter assays yielded a 32.4% increase in glioma migration as a result of the transfection (See Figure 4).

In the first Western Blot, which compared CD155 protein levels in C6 control cells to C6 cells transfected with the PVR over-expression vector, we could confirm from the location of the bands (around 40 kDA, just like the mass of CD155) that the antibody did recognize the

Image Content | DNA Absorbance (µg/ul) | Nuclei Count | Ave of Assay 1 Control: 335 Tfect: 347 | Ave Percent Increase: 3.5%
|----------------|-----------------|-------------|---------------------------------|----------------|
| Control 1A | 315 | Control 1B | 355 | Avg of Assay 2 Control: 273.5 Tfect: 362 | Ave Percent Increase: 32.4%
| Control 2A | 260 | Control 2B | 287 | | |
| Tfect 1A | 327 | Ave of All Control: 304.25 Tfect: 354.5 | Ave Percent Increase: 16.5%
| Tfect 1B | 376 | | |
| Tfect 2A | 332 | | |
| Tfect 2B | 392 | Two-tailed P-value = 0.0957 | | |
human protein and that it is in fact present in the cells. Because the band on the right, the transfected band, is so much darker, we can also assume that our over-expression last term was successful and that any impact on migration we saw was directly correlated to the up-regulation of CD155 in the C6 cells (Figure 5). In the second Western Blot, which compared C6 and U87 control glioma cells, we can observe two distinct, dark bands (again located around 40 kDA) (Figure 6). This is a strong indication that both C6 and U87 glioma cells naturally contain comparable and substantial levels of CD155 protein.

Discussion

We were attempting to mirror the results of a reputable knockdown study conducted by the Department of Physiology at Tufts University School of Medicine, (Boston, Massachusetts) and the Department of Neurobiology at Yale University School of Medicine (New Haven, Connecticut). This study showed that a knockdown of CD155 by RNAi in U87 MG cells resulted in a significant (16 to 22%) decrease in migration compared with the control (Loa, 2005). Because the siRNA expression vector was not available to conduct a knockdown experiment, our study would be used to perhaps mirror the relationship that has been found previously in the knockdown of CD155.

We expected that over-expression of CD155 would significantly increase migration based on the expression of protein levels indicated by Western Blots, which were conducted to determine if increased synthesis of any other protein, would be needed to add to future experiments. An additional variation observed was large—precluding a sound conclusion on the role of CD155 in migration. An additional challenge we faced during this experiment was the presence of CD155 speeding up the growth of cells. If a knockdown in U87 glioma decreased migration of cells, we could infer that CD155 possibly enhances the tumor protein, and if the mouse antibody would recognize the human homolog. Since the blots confirm that CD155 is indeed present in C6 cells, in an amount comparable to that present in U87 human cells, we can confirm that the mouse and human homologs of the protein are similar. Indeed, CD155 and Necl-5 are referred to in combination within scientific dialogue and have virtually the same structure. Yet if the antibody treats CD155 and Necl-5 as the same protein, why is the poliovirus itself specific to humans? Not only do our findings shed light on the strict nature of specificity characteristic to viruses, but these results suggest that CD155 has more than one function besides being the human receptor for poliovirus, and in contrast to our assay counts, does effect migration on some level. Although some of our counts were not statistically significant, what we do see is that the second assay with a higher concentration of DNA had an over-all much larger percentage difference than the control. If we know that the C6 cells contain the human protein and their migration is influenced by it, we can now potentially use rats as a model for CD155/PVR oncolytic treatment in humans.

A study done by the Department of Molecular Genetics and Microbiology at the State University of New York (Stony Brook, NY) demonstrated for the first time that highly attenuated poliovirus recombinants can infect and propagate in cell lines derived from malignant gliomas and, most interestingly, halted tumor progression and eliminated tumors in athymic mice. They proposed that susceptibility of these malignant cells to poliovirus may be mediated by expression of the CD155 gene in glial neoplasms (Gromeier, 2000). As our understanding of the biology of brain cancer progresses, new knowledge about tumorigenesis and tumor biology can be used to diagnose, treat, and prevent this type of cancer. Our experiment may help support the concept that oncolytic poliovirus recombinants may be the next possible treatment for malignant glioma.

References


