EXPRESSION AND FUNCTION OF AQUAPORINS IN MALIGNANT AND NON-MALIGNANT ASTROCYTES

by

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A DISSERTATION
Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

BIRMINGHAM, ALABAMA
2008
Aquaporins (AQP) constitute the primary pathway for water movement across cellular membranes. As a result, their expression and function are important for regulating cell volume. Both gliomas and astrocytes have highly effective volume regulatory mechanisms capable of rapidly moving ions and water into and out of cells following edemic episodes. In this dissertation, we examined the expression of AQPs in both gliomas and astrocytes in order to understand their function role as well as their regulation. We have shown that primary gliomas highly express AQP1 and AQP4 whereas cell lines express no AQP4 and variable AQP1. We reintroduced AQP1 and AQP4 into D54MG cell line lacking AQPs and examined their function. AQP1 enhanced tumor swelling and migration while AQP4 enhanced cell swelling and adhesion. PKC activation is known to regulate AQP function. Using PKC modulators, we found that while PKC did not regulate AQP1, AQP4 function was highly dependent on PKC activity. Specifically, phorbol 12-myristate 13-acetate, PKC activator, reduced cell swelling in AQP4-expressing tumors as well as migration. Chelerythrine, PKC inhibitor, enhanced both cell swelling and migration. Additionally, knocking out the PKC phosphorylation site at S180 eliminated the regulation of AQP4 water permeability and tumor migration. We verified this data by implanting AQP1, AQP4 and S180-AQP4 intracranially into mice and examined glioma invasion. We found that AQP1 and S180-AQP4 invaded on average significantly further than either AQP4 or control tumor cells.
lacking AQPs. AQP1 and S180-AQP4 invaded much further distances (>1500 µm) suggesting that AQP1 expressing tumors invade into tissue to set up satellite colonies characteristic of gliomas whereas AQP4 mediated invasion is regulated by PKC activity. Finally, we wanted to understand the regulation of AQP1 expression in astrocytes following injury. AQP1 is upregulated following cortical stab injury, and we could mimic this upregulation using an in vitro wound assay. We found that pMEK1/2 and pERK1/2 was increased up to 60 min post-injury but then subsequently decreased. AQP1 expression was increased by 16hrs. MEK1/2 and ERK1/2 activity as well as AQP1 expression was completely inhibited by U0126, a MAPK inhibitor. Astrocyte upregulation following injury is regulated by MAPK signaling.
DEDICATION

This dissertation is dedicated to my wife, Portia, and my son, Seamus. Their love and support has helped me succeed and overcome all obstacles.
ACKNOWLEDGEMENTS

I would like to thank all of my family and friends for their love and support throughout the years. I would also like to thank all of the students, postdocs, faculty and staff that I have worked with during my time here who have made it all worthwhile. I would like to thank my committee, Dr. Dale Benos, Dr. Michael Brenner, Dr. Scott Wilson, and Dr. Bradley Yoder for their time, advice and dedication.

Most importantly, I would like to Dr. Harald Sontheimer for providing me with much support and guidance that I needed to succeed while allowing me to take on a project which required much freedom and independence (and many mistakes to be made).
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INTRODUCTION

Aquaporins

Water channels, or aquaporins (AQP), are the primary route for water movement across cellular membranes. For decades, studies have suggested the presence of water channels, yet they were not confirmed until the 1990s when the first aquaporin was cloned by Peter Agre from erythrocytes (Preston, G.M. and Agre, P., 1991). Thirteen different aquaporins (AQP0-AQP12) have been identified thus far appear to be expressed in virtually all tissue and cell types with each having a distinct cellular and subcellular distribution. In the plasma membrane, AQPs form homotetramers, and each monomer is capable of fluxing water. AQPs transport water across the membrane bidirectionally dependent on the osmotic gradient. They are divided into two groups: the strict water channels, which flux only water, and the aquaglyceroporins, which can transport glycerol, urea and other small solutes. The structure of an AQP is similar to a shaker K\(^+\) channel in that they each have 6 transmembrane spanning domains and 5 connecting loops, with loops B and E folding into the membrane to form a single aqueous pore. The B and E loops contain an arginine-proline-alanine region that acts as the selectivity filter for the channel.

In the central nervous system (CNS) AQP4 is the predominate AQP expressed. It localizes to astrocytic endfeet (Papadopoulos, M.C. et. al., 2002). Transcripts for AQP1, AQP5, AQP8, AQP9 and AQP11 are also known to be expressed (Gorelick, D.A. et. al., 2006;Lehmann, G.L. et. al., 2004;Venero, J.L. et. al., 2001), but protein has been detected for only AQP1, AQP4, AQP9 and AQP11 (Gorelick, D.A. et. al., 2006;Satoh, J.
AQP11 is expressed by neurons (Gorelick, D.A. *et. al.*, 2006), while AQP1 and AQP9 are found in both neurons and astrocytes (Satoh, J. *et. al.*, 2007).

**Aquaporins in Cell Volume Regulation**

Eukaryotic cells lack rigid cell membranes making it necessary to have highly effective volume regulatory mechanisms to prevent excessive cell swelling and shrinkage, which leads to cell lysis. The cell’s ability to regulate its volume following changes in the osmotic environment is a fundamental feature ubiquitous across all species and cell types. Cell volume changes have been linked to many cellular functions, specifically cell proliferation (Bussolati, O. *et. al.*, 1996; Iwamoto, L.M. *et. al.*, 2004; McManus, M.L. *et. al.*, 1995; Muller, R. *et. al.*, 1993), migration (Kim, M.J. *et. al.*, 2004; Shen, M.R. *et. al.*, 2000; Shen, M.R. *et. al.*, 2003), gene expression (Haussinger, D., 1996) and vesicle fusion (Lang, F. *et. al.*, 1998). Cells have complex mechanisms designed to control cell volume, such as regulatory volume decrease (RVD). RVD is important for removing excess intracellular water, which involves the efflux of organic and inorganic osmolytes into the extracellular space followed by obligated water movement (Hoffmann, E.K., 1992; Lang, F. *et. al.*, 1998).

AQPs have a direct role in volume regulation. Hyperosmotic conditions resulted in increased AQP5 protein expression levels in submandibular acinar cell line (Hansen, A.K. and Galtung, H.K., 2007). Following induction of AQP5 protein, cells exposed to additional hyposmotic challenges showed enhanced cell swelling and complete volume recovery compared to control cells that did not. AQP protein expression creates a more
effective volume regulatory mechanism, which is important for astrocytes when regulating edema associated with brain injury.

**Astrocytes**

Glial cells were first discovered by Rudolf Virchow (Virchow, RLK 1856) and were initially believed to be the connective or adhesive cells of the brain. In the CNS, astrocytes and oligodendrocytes are the most abundant glial cell types found. While oligodendrocytes are required for neuronal myelination, astrocytes serve numerous functions throughout the CNS, including nutritive support, injury repair, ion homeostasis, and initiating the inflammatory response.

Astrocytes undergo morphological changes in a process called reactive gliosis in response to injury becoming more proliferative and migratory compared to normal astrocytes. They are able to migrate to the injured area (Lim, J.H. *et. al.*, 2007) and form a glial scar. The glial scar suppresses and controls further trauma to the CNS by creating a barrier to the injured area (Fitch, M.T. *et. al.*, 1999; Myer, D.J. *et. al.*, 2006). In addition, the glial scar acts to inhibit neuronal regeneration (Kerschensteiner, M. *et. al.*, 2005) by preventing neurons from extending axons and reforming connections in the CNS. Further, they secrete cytokines that recruit microglia (Ghirnikar, R.S. *et. al.*, 1996), which invade and phagocytose debris and apoptotic cells (Bell, M.D. *et. al.*, 1994; Christie, R.H. *et. al.*, 1996; Streit, W.J. *et. al.*, 1999). Injury induces astrocytes to shift from a quiescent to reactive state, altering their protein expression to include AQPs.
Aquaporin Expression in Astrocytes

As stated previously, AQP4 is the predominant AQP found in the CNS, and is localized to astrocytic endfeet where it is believed to play a role in K⁺ buffering (Kofuji, P. and Newman, E.A., 2004; Nagelhus, E.A. et. al., 2004). High levels of K⁺ are released into the extracellular space following action potentials. Astrocytes are extremely permeable to K⁺ and have high expression of K⁺ channels (Kir4.1), which allows the astrocytes to siphon K⁺ from synapses toward regions of lower K⁺ concentrations. The diffusion of K⁺ ions, which are osmotically active, necessitates the movement of water, which is greatly facilitated by AQPs. In this vein, AQP4 protein expression has also been detected along synapses to function in maintaining ion homeostasis following action potentials (Vitellaro-Zuccarello, L. et. al., 2005), a particularly important function (Simard, M. and Nedergaard, M., 2004). The failure of astrocytes to reduce the extracellular K⁺ after action potentials results in epileptiform activity, excitotoxicity and neuronal death (Binder, D.K. et. al., 2006). This ionic imbalance alters the osmotic environment leading to changes in fluid content, intra- and extracellularly. Therefore, maintaining proper water balance in the brain is particularly critical due to the size constraints placed by the cranium. This limits the amount of swelling (edema) that can occur following injury, where the regulation of ion and water movement is of utmost importance.

Edema is an uncontrolled flux of ions and water across cellular membranes (cytotoxic edema) or into the surrounding parenchyma (vasogenic edema). Extensive studies have demonstrated a vital role for AQP4 in regulating edema following brain injury (Badaut, J. et. al., 2007; Manley, G.T. et. al., 2000; Papadopoulos, M.C. et. al.,
Unfortunately, the exact role of AQP4 is not completely understood. Different brain injury models show different AQP expression. In stroke models using adult mice, astrocytic AQP4 expression increased at 1 hr and 24 hrs after injury (Ribeiro, M.C. et. al., 2006). In contrast in neonatal rat pups, AQP4 expression was decreased at 1 hr and 24 hrs after hypoxic-ischemic injury. These changes were associated with decreased cytotoxic edema and increased vasogenic edema (Badaut, J. et. al., 2007). Studies also examined the impact of removing AQP4 from astrocytes to understand their role in edema formation. The loss of astrocytic AQP4 in AQP4 knockout (KO) mice resulted in reduced cytotoxic edema (Manley, G.T. et. al., 2000). Vasogenic edema studies using AQP4 KO mice revealed increased edema (Papadopoulos, M.C. et. al., 2004). More specifically, these studies demonstrate that AQP4 expression levels are dependent on the type of injury that occurs and the age of the animal and also, the astrocytic response to edema formation and its removal.

The primary function of AQP1 in the CNS is to form and secrete CSF from the choroid plexus (Bondy, C. et. al., 1993;Hasegawa, H. et. al., 1994). However, AQP1 is also known to be expressed in the axons and cell bodies of primary afferent neurons (Shields, S.D. et. al., 2007), amacrine cells (Kang, T.H. et. al., 2005) and photoreceptor cells in the retina (Iandiev, I. et. al., 2005). AQP1 is upregulated in reactive astrocytes surrounding the injured site following brain contusion (Suzuki, R. et. al., 2006) and in peritumoral regions (Badaut, J. et. al., 2003). AQP1 expression is also increased in reactive astrocytes in Alzheimer’s disease (Perez, E. et. al., 2006), Creutzfeldt-Jakob and bovine spongiform encephalopathy (Rodriguez, A. et. al., 2006). Though AQP1 is upregulated in these astrocytes, the signaling pathways involved in upregulating AQP1
expression have not yet been elucidated. However, in models examining osmotic changes, mitogen-activated protein kinase (MAPK) is also linked to AQP expression (Arima, H. et. al., 2003; Hoffert, J.D. et. al., 2000; Jenq, W. et. al., 1999; Umenishi, F. and Schrier, R.W., 2003).

**MAPK Regulation of Aquaporins**

MAPK is a family of serine/threonine kinases involved in the regulation of mitosis, cell differentiation, gene expression and cell survival. The classical MAPK signaling pathway uses extracellular signal-regulated kinases (ERK), which regulates cell proliferation and differentiation through activation of growth factor receptors. C-Jun N-terminal kinases and p38 are other common MAPK signaling pathways, induced following cell stress, apoptotic stimuli, and heat and osmotic shock. The MAPK signaling pathways are known involved in the expression of AQP1, AQP4, AQP5, and AQP9 following chronic changes in osmolarity (Arima, H. et. al., 2003; Hoffert, J.D. et. al., 2000; Jenq, W. et. al., 1999; Umenishi, F. and Schrier, R.W., 2002; Umenishi, F. and Schrier, R.W., 2003), and blocking MAPK signaling prevented this upregulation. One aim of my thesis was to determine if the increased expression of AQP1 found in reactive astrocytes was dependent upon MAPK signaling.

**Gliomas**

Primary brain tumors are created by abnormal and uncontrolled cell proliferation. On average, 80% of primary brain tumors consist of cells of glial origin including oligodendrocytes, astrocytes and ependymal cells. Approximately, 44,000 people will be
diagnosed with a primary brain tumor each year (Central Brain Tumor Registry of the United States) that are graded according to severity from grades I-IV by the World Health Organization (WHO). Grade I is the least severe and is most often applied to non-infiltrating tumors that occur in childhood. These tumors can usually be cured with surgery alone. Low grade gliomas are composed of homogeneous cells and feature a lack of cell differentiation, and over 90% of patients survive beyond 5 years from diagnosis.

In contrast, grade IV tumors are the most malignant and invasive and are characterized by increased growth rates, heterogeneity, and multiple cell populations. Grade IV glioma (glioblastoma multiforme; GBM) is the most malignant and aggressive brain tumor, which accounts for approximately 20% of all gliomas (CBTRUS, 2005). Patient survival approaches 1 year and less than 5% of patients survive past 5 years even with standard treatments such as radiation, chemotherapy, and surgical resection.

Grade IV gliomas are highly proliferative and commonly feature areas of tissue necrosis within the tumor mass, increased vascularization, undefined borders between normal and tumor tissue, and extensive tumor infiltration into the normal brain parenchyma. Typically, gliomas migrate along the white fiber tracts on blood vessels and invade into the surrounding areas. They are often found crossing the corpus callosum and creating “satellite” colonies great distances from the tumor origin, making them difficult to eradicate with surgery alone and recurrence is almost inevitable.

Genetic mutations associated with gliomas often involve the regulation of cell survival or the process of programmed cell death. A common mutation involves p53, a protein that initiates programmed cell death following DNA damage (Hulleman, E. and Helin, K., 2005). Approximately 60% of all gliomas have increased levels of epidermal
growth factor receptor (EGFR) (Ekstrand, A.J. et. al., 1991), which leads to a constitutive activation of EGFR, greater cell proliferation, and apoptotic pathway blockades (Ekstrand, A.J. et. al., 1994). In addition, a mutation of PTEN, a protein in the PI3K/AKT pathway, plays a major role in regulating cell migration and invasion (Hulleman, E. and Helin, K., 2005).

Gliomas survive and thrive in edematous tissue regions that have accumulated fluid and ions by regulating their cell volume. The malignant features of glioblastoma cells, such as proliferation (Dubois, J.M. and Rouzaire-Dubois, B., 2004; Kimelberg, H.K. et. al., 1990), cell death (Ernest, N.J. et. al., 2008), and invasion (Huang, K.C. et. al., 2005) into normal tissue are all thought to involve volume regulatory mechanisms. We have previously shown that tumors have a well-developed volume regulatory mechanism capable of recovering volume in the presence of significant osmotic changes (Ernest, N.J. et. al., 2005). It is important to understand the expression and function of AQPs in basic tumor biology.

Aquaporins in Gliomas

It is not unexpected that gliomas would show a similar composition of AQPs as non-malignant glial cells. Astrocytes normally express high levels of AQP4 with intense localization near astrocytic endfeet at the blood-brain barrier (Papadopoulos, M.C. et. al., 2002). In injury and in certain disease states, AQP1 has been shown to be upregulated (Badaut, J. et. al., 2003; Badaut, J. et. al., 2008; Hwang, I.K. et. al., 2007; Perez, E. et. al., 2006; Rodriguez, A. et. al., 2006; Suzuki, R. et. al., 2006). However, AQP expression is very different in gliomas as compared to astrocytes. Both AQP4 and AQP1 are highly
expressed in primary gliomas (Markert, J.M. et. al., 2001; Oshio, K. et. al., 2003; Saadoun, S. et. al., 2002b; Saadoun, S. et. al., 2002a) but with very diffuse localization throughout the cell (Warth, A. et. al., 2004) and unlike the distinct AQP4 localization found in astrocytic endfeet. AQP4 and AQP1 are also heterogeneously expressed throughout the tumor mass (Saadoun, S. et. al., 2002a; Saadoun, S. et. al., 2002b). There are regions with high expression of either AQP1 or AQP4 and regions where there is very low protein expression indicating that gliomas require distinct physiological functions from each type of AQP expressed. Thus far, the cellular function of AQPs in gliomas has not been determined. The purpose of these studies was to assess AQP expression in glioma cells, determine their relative contribution to regulating water permeability and cell migration, and finally to understand the signaling mechanisms involved in regulating AQP-mediated water permeability.

PKC Regulation of Aquaporins

It is known that AQP-mediated water permeability can be modulated by protein kinase C (PKC) (Yamamoto, N. et. al., 2001; Zelenina, M. et. al., 2002), a serine/threonine kinase from a family of 10 isoforms, 9 of which are found in the CNS. Each individual isoform has its own distinct cellular and subcellular localization. Conventional PKC activation requires Ca$^{2+}$ and diacylglycerol. Once activated, PKC is translocated to the plasma membrane where it remains active for extensive periods of time even after the original signal has faded. PKC alters the function of many cellular proteins through phosphorylation, with each effect specific to a particular cell type, but effects include cell migration regulation, cell proliferation and differentiation. In
gliomas, PKC is upregulated and can regulate tumor cell migration and proliferation (Cho, K.K. et. al., 1999; Couldwell, W.T. et. al., 1990).

Modulating proteins by phosphorylation can change their function. Both AQP1 and AQP9 have consensus phosphorylation sites for PKC (Loitto, V.M. et. al., 2007). PKC activation in astrocytes reduced AQP4-mediated water permeability (Yamamoto, N. et. al., 2001; Zelenina, M. et. al., 2002) through phosphorylation of serine 180 (Zelenina, M. et. al., 2002). Hypothetically, reducing water permeability through AQPs should slow migration and invasion.

Aquaporins in Tumor Migration

Cell migration is a fundamental process that is essential to tissue formation, inflammatory and injury responses, and tumor invasion. Cell migration involves the coordinated movement of ions and water to alter cell size and propel the cell forward. Migrating cells have a clear membrane polarity, a front and a back. Cells utilize an efflux of both K$^+$ and Cl$^-$ followed by obligated water movement in order to decrease cell volume for the invasion of narrow spaces {review (McFerrin M.B. and Sontheimer, H., 2006)}. Aquaporins mediate the rapid water flux across cell membranes, and studies have shown that they are important for stimulating rapid cell migration.

In AQP1 KO mice, epithelial cell migration in vitro is reduced in kidney proximal tubules as compared to wildtype mice (Hara-Chikuma, M. and Verkman, A.S., 2006). Astrocytes removed from AQP4 KO mice (Saadoun, S. et. al., 2005) showed slowed migration towards the site of a cortical stab injury leading to a reduction in the formation of the glial scar (Saadoun, S. et. al., 2005; Loitto, V. M. et. al., 2002). Roles for AQPs in
tumor cell migration have only recently been demonstrated. In melanoma cells, AQP1 expression leads to enhanced migration \textit{in vitro} and enhanced tissue extravasation \textit{in vivo} (Hu, J. and Verkman, A.S., 2006). However, the AQPs involved in mediating glioma migration are not known.

\textbf{Hypothesis}

The overarching hypothesis of this thesis is that gliomas undergo profound changes in cell volume that support distinct aspects of their biology. Specifically, cell migration requires changes in overall cell volume. This necessitates the ability to move water across the cell membrane. I hypothesize that gliomas will utilize AQPs to accomplish these basic functions. In particular, each subtype will have a distinct function in glioma biology. Further, activation of cell signaling pathways will control the function of AQPs in gliomas and their expression in normal astrocytes.
EXPRESSION AND FUNCTION OF WATER CHANNELS (AQUAPORINS) IN MIGRATING MALIGNANT ASTROCYTES

by

ERIC S. McCOY AND HARALD SONTHEIMER

Glia 55(10): 1034-43

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ABSTRACT

Aquaporins (AQP) constitute the principal pathway for water movement across biological membranes. Consequently, their expression and function is important for cell volume regulation. Glioma cells quickly adjust their cell volume in response to osmotic challenges or spontaneously as they invade into the narrow and tortuous extracellular spaces of the brain. These cell volume changes are likely to engage water movements across the cell membrane through aquaporins. Aquaporin expression in glioma cells is poorly understood. In this study, we examined the expression of aquaporins in several commonly used human glioma cell lines (D54, D65, STTG1, U87, U251) and in numerous acute patient biopsies by PCR, Western blot, and immunocytochemistry and compared them to non-malignant astrocytes and normal brain. All glioma patient biopsies expressed AQP1, AQP4 and some expressed AQP5. However, when isolated and grown as cell lines they lose all AQP proteins except a few cell lines that maintain expression of AQP1 (D65, U251, GBM62). Re-introducing either AQP1 or AQP4 stably into glioma cell lines allowed us to show that each AQP is sufficient to restore water permeability. Yet, only the presence of AQP1, but not AQP4, enhanced cell growth and migration, typical properties of gliomas, while AQP4 enhanced cell adhesion suggesting differential biological roles for AQP1 and AQP4 in glioma cell biology.
INTRODUCTION

The ability of cells to control their cell volume in the face of a changing osmotic environment is a fundamental property shared by many cells across species (Lang, F. et. al., 1998). Of particular importance is regulatory volume decrease (RVD) which allows cells to remove excess water from their cytoplasm in an attempt to maintain a proper water balance. RVD has been shown to involve the release of organic and inorganic osmolytes into the extracellular space which in turn leads to an obligatory efflux of water (Parkerson, K.A. and Sontheimer, H., 2003; Parkerson, K.A. and Sontheimer, H., 2004). While the pathways for osmolyte release have been studied for several decades, the pathways for water movement across cell membranes have only recently been elucidated. These studies have demonstrated that water extrusion occurs through specialized water channels or aquaporins (Preston, G.M. et. al., 1992), although in some cells water permeates lipid membranes directly (Zeidel, M.L. et. al., 1992; Zhang, R. et. al., 1993) or passes the membrane through unrelated channels (Fischbarg, J. et. al., 1990; MacAulay, N. et. al., 2001).

Under physiological conditions the brain and spinal cord are thought to encounter few osmotic challenges since the composition of the cerebral spinal fluid fluctuates much less than the blood plasma from which it is separated by the blood-brain barrier. This is important as the tight packaging of neurons, glial cells and blood vessels in brain would not permit significant changes in the volume occupied by individual cells. However, acute injury (Ke, C. et. al., 2001), stroke (Taniguchi, M. et. al., 2000) and inflammation (Alexander, J.J. et. al., 2003) each present with significant water uptake by brain cells resulting in significant brain swelling or edema which confound the neurological

Peritumoral edema occurs as a consequence of a disrupted blood-brain barrier (Bothe, H.W. et. al., 1984; Hossman, K.A. and Bloink, M., 1981). However, tumor growth appears to be unaffected by peritumoral edema and glioma cells actually appear to thrive in this edematous environment. We previously noted that glioma cells have a well-developed volume regulatory response that restores the cell volume even in the presence of significant osmotic challenges (Ernest, N.J. et. al., 2005). These studies suggest a major role for Cl\(^-\) release through ion channels in this process. Pathways for water movement, however, had not been previously investigated and this study set out to fill this void. Water permeability and its role in glioma volume are also important in the context of glioma invasion. Unlike other cancers that spread hematogenously, gliomas invade the brain by active cell migration. We previously hypothesized that glioma cells may have an unusual ability to regulate their volume in order to fit through the tortuous extracellular brain spaces as they invade, which would require enhanced water permeability.

In this study, we examined AQP expression by PCR and Western blot in a panel of frequently used glioma cell lines as well as in acute patient derived biopsies and cells derived from them. We show prominent expression of AQP1 and AQP4, and to a lesser extent AQP5. Surprisingly only AQP1 expression was maintained in cultured glioma cells many of whom lose AQP expression altogether. Re-introducing either AQP1 or AQP4 stable into glioma cell lines showed that each AQP is sufficient to restore water permeability such that cells expressing either AQP behave like perfect osmometers when
challenged osmotically. On the other hand, the absence of AQP1 but not AQP4 impedes the ability of cells to invade. These studies therefore suggest differential roles for AQP1 and AQP4 in glioma cell biology.
MATERIALS AND METHODS

Cell Culture. U87-MG [astrocytoma, World Health Organization (WHO) grade III], STTG-1 (anaplastic astrocytoma, WHO grade III), and D65-MG (WHO grade IV) were all obtained from American Type Culture Collection (ATCC; Rockville, MD). D54-MG (WHO grade IV) and U251-MG (WHO grade IV) were a gift from Dr. D.D. Bigner (Duke University, Durham, NC). Glioblastoma multiforme 010701062 (GBM62; WHO grade IV) and glioblastoma multiforme 010601050 (GBM50; WHO grade IV) were glioblastoma cell lines cultured from patient biopsies (University of Alabama at Birmingham). Cells were grown in Dulbecco’s modified Eagle medium (DMEM/F12; Media Tech, University of Alabama at Birmingham Media Preparation Facility) and supplemented with 2 mM glutamine (Media Tech) and 7% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT) at 37°C and 90% O₂/10% CO₂ humidified environment. Human glioma tissue, astrocytoma tissue and normal brain blocks were obtained from Cooperative Human Tissue Network (CHTN). Representative tissue samples were used in each of the following experiments.

RNA Isolation and PCR. Messenger RNA was extracted from the various cell lines following the RNAqueous protocol (Ambion, Austin, TX). Briefly, the cells were lysed, homogenized, and centrifuged. The supernatant was removed and an equal volume of 64% ethanol was added. The mixture was filtered via centrifugation and the filters were washed. The mRNA was eluted from the filter and DNA-free (Ambion) was used to remove contaminating DNA. RNA quality was evaluated by electrophoresis through 1.5% agarose gels.

The cDNA was synthesized and amplified using the OneStep RT-PCR kit.
(Qiagen, Valencia, CA), per manufacturer’s instructions, by the Eppendorf Mastercycler gradient (Brinkmann Instruments, Westbury, NY). Oligonucleotide primers (Invitrogen, Carlsbad, CA) were designed to amplify specific AQPs and used the sequences described previously (Wang, W. et. al., 2003) and listed in Table 1, and 2 ng of mRNA was loaded per reaction. PCR conditions were as follows; denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 2 min, with a final extension at 72°C for 10 min. Amplified products were electrophoresed through 1.5% agarose gels to determine size. Controls were used for all aquaporins. Kidney cDNA was used for AQP1, 2, 3, 4, 6, 7, 8, and lung cDNA was used for AQP5. Placental, small intestine, and liver cDNA were used for AQP0, AQP10, and AQP9, respectively. Actin was used as loading controls.

Western Blot Analysis. A confluent dish of cells was lysed using RIPA buffer [(50 mM TrisCl, pH 7.5, 150 mM NaCl, 1% Nondet P-40 (NP-40), 0.5% sodium deoxycholate, 1% sodium dodecyl sulfate (SDS)] supplemented with protease inhibitor cocktail (Sigma). Cells were sonicated for 10 s and centrifuged at 14,000 rpm for 10 min, and the supernatant was transferred to a new tube. Protein quantification was performed using a DC protein assay kit (BioRad, Hercules, CA). An equal amount of 6× sample buffer containing 600 mM β-mercaptoethanol was added to the 20-30 µg/ml of cell lysate per lane. Samples were loaded into a 10% precast SDS-PAGE gel (BioRad). Protein separation was obtained using a constant 100 V for 80 min, and the gels were transferred at 200 mA for 2 hrs at room temperature onto polyvinylidene difluoride (PVDF) paper (Millipore, Bedford, MA). Membranes were blocked in blocking buffer (3% nonfat dried milk in TBS plus 0.1% Tween 20). All antibodies were obtained from Chemicon
(Temecula, CA) and used following manufacturer’s instructions. Membranes were incubated in primary antibody for 1 hr at room temperature and washed 3× for 10 min. The membranes were then incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma) at 1:1000 for 1 hr followed by another round of washing (3× 10 min) and developed using Luminol (Santa Cruz, Santa Cruz, CA) on Hyperfilm (Amersham, Arlington Heights, IL).

**Immunocytochemistry.** Cells plated on coverslips were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 10 min. Cells were washed 2× 10 min in PBS, blocked and then permeabilized in PBS containing 0.3% Triton X-100 and 5% normal goat serum (NGS) for 30 min. Cells were incubated overnight at 4ºC in primary AQP antibodies at 1:200. On the following day, glioma cells were washed in PBS (4× 5 min), blocked again in PBS with 5% NGS, and then incubated with phalloidin (Molecular Probes, Eugene, OR) at 1:100 and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (Molecular Probes) diluted at 1:500 in the dark for 1 hr. Cells were washed in PBS (2× 5 min) and incubated with DAPI, a fluorescent nuclear label, (1:2000, Sigma) for 5 min. Cells were washed 2 more times with PBS and mounted on slides with GelMount (Biomedia, Foster City, CA). Images were acquired using an inverted Olympus IX-81 spinning disk confocal microscope (Olympus, Center Valley, PA).

Cryostat sections of human glioma tissue, astrocytoma tissue and normal brain were cut at 8 µm and fixed with 4% paraformaldehyde. Cells were washed 2× in PBS and permeabilized in permeabilization solution (3% goat serum, 0.03% Triton X-100 in PBS). Sections were washed 2× for 5 min in PBS and blocked for 1 hr in blocking solution (5%
goat serum in PBS). Sections were then incubated overnight at 4°C in primary AQP (1:100; Chemicon) and GFAP (1:1000; Sigma) antibodies in incubation solution (1% goat serum, 0.25% Triton X-100 in PBS). After overnight incubation, sections were washed 3× for 10 min in PBS and incubated for 2 hr at RT in the dark in incubation solution. Sections were then washed 2× for 10 min in PBS, DAPI (1:2000; Sigma) was applied for 5 min, and followed by a final wash in PBS for 10 min. Sections were mounted on slides using GelMount (Biomega).

**Transfections.** The AQP1-GFP plasmid was a gift from Dr. Nicholas LaRusso (Mayo Clinic, Minneapolis, MN), and AQP4-DsRed was a gift from Dr. Ken-ichi Nakahama (Tokyo Medical and Dental University, Tokyo, Japan). AQP1 and AQP4 constructs were created as discussed in published work, i.e. (Tietz, P.S. et. al., 2006) for AQP1 and (Nakahama, K. et. al., 2002) for AQP4. D54 tumor cells were transfected with AQP1-GFP or eGFP-N1 (Clontech, Mountain View, CA) and AQP4-DsRed or DsRed-N1 (Clontech) using Nucleofector Kit T (Amaxa, Gaithersburg, MD). On the day of transfection, cells were harvested and $2 \times 10^6$ cells were mixed with 2 µg of plasmid in 100 µl of the nucleofector solution and then electroporated with the Amaxa Nucleofector (Amaxa). Electroporation of cells was completed with Amaxa program T-27. This suspension was transferred to an eppendorf tube and diluted appropriately for plating. After 48 hrs, cells were treated with 1 mg/ml genetinic (Sigma) and each week the concentration was reduced until reaching 250 µg/ml. Individual cells were sorted into 96-well plates by FACS and colonies were selected from single clones. Stable cells were maintained using 250 µg/ml of genetinic.
AQP1-eGFP was constructed by ligating annealed oligonucleotides 5'-
GATCCCGGGTTGGAGATGAAGCCCAAATTTCAAGAGAATTTGGGCTTCATCT
CCACCCCTTTTG-3' and 3'-
GGGCCCACCTCTACTTTCGGGTTTAAAGTTCTCTTAACCCGAAGTAGAGGTTG
GGAAAAACAGCT-5' at BglII and SalI restriction sites of the pZOFF-EGFP plasmid.
AQP1-shRNA was transfected using FuGene (Roche Diagnostics, Indianapolis, IN).
FuGene was incubated for 5 min in 100 µl serum-free media. DNA was added, incubated
for 35 min, dispersed onto 1 × 10^6 cells and cells were allowed to recover for 48 hrs.

*Volume Regulation.* Cell volume measurements were performed using a Coulter Counter
Multisizer 3 (Beckman-Coulter, Miami, FL) as described previously (Parkerson, K.A.
and Sontheimer, H., 2003). Cells were washed in PBS and lifted from the dish using
0.05% trypsin and 0.53mM EDTA. Trypsin was inactivated with the addition of an equal
volume of serum-containing media and cells were briefly centrifuged to pellet. Cells were
resuspended in bath solution [125 mM NaCl, 5.0 mM KCl, 1.2 mM MgSO₄, 1.6 mM
Na₂HPO₄, 0.4 mM NaH₂PO₄, 10.5 mM glucose, 32.5 mM HEPES (acid), 1.0 mM CaCl₂,
pH 7.4, 300 ± 10 mosmol]. Osmolarity for solutions was measured by a freeze point
osmometer (Fiske Micro-Osmometer 210; Fiske-Associates, Norwood, MA). Cells were
equilibrated for approximately 5 min before first reading, and readings were taken
continuously for 3 min. All experiments contained 200 µM 5-nitro-2-(3-
phenylpropylamino)-benzoic acid (NPPB, Sigma) and 250 µM CdCl₂. Bath solution was
made hyposmotic with the addition of water. Mercuric chloride (HgCl₂, Sigma) was
made at 300 µM in bath solution and cells were preincubated for 5 min. Data were
collected by Multisizer 3 software, and 5000 pulse listings were exported to EXCEL as
the average of 40-50 cells for each 20 ms timepoint. Data were collected as mean
diameter and were converted to mean cell volume. Mean cell volumes were normalized
to baseline values. Data were plotted in Origin 7.0 (MicroCal, Northhampton, MA) ± se
with (n) experiments performed. Each time point graphed is an average of the mean cell
volume for 40-50 cells per 20 ms. Each graph for a 3 min experiment contained 5000
data points.

Cell Migration. Migration was assessed using a modified Boyden Chamber. 8 \( \mu \)m
FluorBlok filters (Fisher Scientific, Pittsburgh, PA) were coated with 2.5 mg/ml
vitronectin (Sigma) overnight at room temperature. Filters were washed twice with PBS
and blocked for 1 hr at 37°C with 1% fatty acid free BSA in PBS. Cells were trypsinized
using 0.5 mM EGTA and centrifuged. Cells were washed twice with PBS and brought up
in migration assay buffer (serum-free DMEM/F12 with 0.1% fatty acid free BSA). Cells
were counted using a hemocytometer and 40,000 cells were plated per filter and allowed
to migrate for 5 hrs. Cells were fixed in 4% paraformaldehyde for 10 min followed by 2
washes in PBS. DAPI was applied at 1:2000 for 5 min followed by an additional 2
washes in PBS. For shRNA experiments, the top and the bottom of each filter were
counted and a comparison was made between migrated and non-migrated cells. Images
of five random fields were taken using Zeiss Axiovert 200M (München, Germany). All
experiments were performed in triplicate.

Cell Adhesion Assay. Coverslips were coated overnight at 4°C with various matrices: 10
\( \mu \)g/ml collagen I (Sigma), 10 \( \mu \)g/ml fibronectin III (Sigma), 20 \( \mu \)g/ml laminin (Sigma),
20 \( \mu \)g/ml vitronectin (Sigma) or 1% BSA (Sigma). 200,000 cells were seeded per well
and allowed to adhere for 1 hr at 37°C. The non-adherent cells were washed away gently
using PBS and cells were fixed using 4% paraformaldehyde. Images of five random fields were taken using Zeiss Axiovert 200M (München, Germany) at 10x magnification.
RESULTS

RT-PCR demonstrates distinct AQP transcripts in glioma cells.

The principal objective of this study was to examine the contribution of aquaporins to trans-membrane water transport and hence cell volume changes in glioma cells. As a first step towards this goal we used RT-PCR to search for the complement of AQP genes expressed. We examined a rather broad range of cells and tissues including 5 commonly used glioma cell lines (D54, D65, STTG1, U87, U251), glioma cultures from 2 patient samples (GBM50 and GBM62) and a selection of acute patient biopsy tissues (Normal Brain, Astrocytoma and Glioblastoma, Fig 1). Primers for each aquaporin are listed in Table 1. The most abundant transcripts were those for AQP1, 3, 5 and 6, which were found in most cell types. AQP4 mRNA similar appeared in most cell lines, but

Table 1. Oligonucleotide primer sequences used to amplify human aquaporins via RT-PCR.

<table>
<thead>
<tr>
<th>GENE</th>
<th>PRIMER SEQUENCE (5’-3’)</th>
<th>LENGTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQP0</td>
<td>Forward: ATT CTC ACT GGG AAC TTC ACT AAC</td>
<td>203</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGG GCC TGG GAG TTC AGT TCA ACA</td>
<td></td>
</tr>
<tr>
<td>AQP1</td>
<td>Forward: GGC CAC GAC CCT CTT TGT CTT CAT</td>
<td>514</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCC CAC AGC CAG TGT AGT CAA TAG</td>
<td></td>
</tr>
<tr>
<td>AQP2</td>
<td>Forward: AGC CGC TCT GCT CCA TGA GAT CAC</td>
<td>375</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGC GGA AAC AGC AGC TAG TGT TAG</td>
<td></td>
</tr>
<tr>
<td>AQP3</td>
<td>Forward: TCA ATG GCT TCT TTG ACC AGT TCA</td>
<td>389</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTT CAC ATG GGC CAG CCT CAC ATT</td>
<td></td>
</tr>
<tr>
<td>AQP4</td>
<td>Forward: CAT CGC CAA GTC TGT CTT CTA CAT</td>
<td>237</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCT ATT GAG CCA GTG ACA TCA GTC</td>
<td></td>
</tr>
<tr>
<td>AQP5</td>
<td>Forward: CTC GTC CAT TGG CCT GTC TGT CAC</td>
<td>225</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGC TCA TAC GTG CCT TTG ATG ATG</td>
<td></td>
</tr>
<tr>
<td>AQP6</td>
<td>Forward: GCA TCA TCA TTG GGA AGT TCA CAG</td>
<td>251</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCG TAG GCT GAT TCA CAC ACT CTC</td>
<td></td>
</tr>
<tr>
<td>AQP7</td>
<td>Forward: AAA TGG TCT CCT GTG CCG TGA TAG</td>
<td>178</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACA CCA AGG TAG CTC CCA AAT GTT</td>
<td></td>
</tr>
<tr>
<td>AQP8</td>
<td>Forward: TGG TGC CAT CTG ATC CTG ATG TCT</td>
<td>520</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCA GCG TCG TCA GGA TGA TCT CGT</td>
<td></td>
</tr>
<tr>
<td>AQP9</td>
<td>Forward: CGG CAT TTG TAC AGT CAG AGA CTC</td>
<td>632</td>
</tr>
<tr>
<td></td>
<td>Reverse: AAT GCG TCC GCC AGA GAT AGA TAC</td>
<td></td>
</tr>
<tr>
<td>AQP10</td>
<td>Forward: TGC GGT TCC TTC TAA ATA TCA A</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTC GCA GGT TCT GGC ACA TTA ACA</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Aquaporin mRNA expression in astrocytes, several commonly used human glioma cell lines and acute patient biopsies (n=6). RT-PCR was used to screen for all AQPs present in each cell type. All panels show a 1.5% agarose gel stained with ethidium bromide following amplification. Primers for each AQP are described in Table I. AQP1, AQP5, and AQP6 were ubiquitously expressed in all cell types, but there was variable distribution of all other AQPs. There was no expression of AQP7 in any of the different cell types, but only the D54-WT showed expression of AQP2. Positive controls for each AQP were used and are described in Materials and Methods.
highest levels were detected in normal brain and glioma patient biopsies (Fig 1). As for other AQPs, expression varied from cell type to cell type. AQP7 mRNA was not detected in any glioma cell type (Fig 1) and only the D54-MG cells showed mRNA expression of AQP2 albeit at a low level. We did not see significant variations in the mRNA expression between the anaplastic astrocytoma and glioblastoma patient biopsies with the exception of AQP1 (Fig 1). Expression levels of AQP1 in GBM tissue was significantly higher than any other tissue sample we examined confirming the findings from previous work (Oshio, K. et. al., 2005). All AQP transcripts found in the glioblastoma biopsies were also found in at least one of the glioma cell lines; of these U87 and U251 had an expression pattern more similar to patient biopsies.

*Differences in aquaporin protein expression between glioma cell lines and patient biopsies.*

We next sought to examine which of the aquaporin transcripts actually yielded aquaporin protein by performing Western blot analyses (Fig 2). Each human glioma cell line was probed with the AQP antibodies to the candidate genes determined by RT-PCR. Surprisingly, AQP1 was absent in GBM50, D54, STTG1, and U87 cells (Fig 2A) but expressed in GBM62 and D65 cells where it occurred as a 28 kD band and a second presumably glycosylated protein band around 38 kD. In U251 cells, AQP1 antibodies only identified the 28 kD band. All cell lines lacked detectable levels of AQP4 and AQP5. For individual patient biopsies (Fig 2B) expression pattern followed much more closely that predicted by RT-PCR with human anaplastic astrocytomas and glioblastomas each expressing AQP1 and AQP4 at high levels. A majority of glioblastoma tissues
Figure 2. Western blot analysis of aquaporin recognized by RT-PCR. Cell lysates were separated using 10% SDS-PAGE gels and transferred onto PVDF paper and probed with antibodies to AQPs previously detected from RT-PCR experiments. (A) Only AQP1 was expressed in the human glioma cell lines, GBM62, D65, and U251. (B) Several tissue biopsies and tumor grades were examined showing expression of AQP1, AQP4 and AQP5 (NB, normal brain n=5, lanes 1, 2; GBM, glioblastoma multiforme n=6, lanes 3-5; AA, anaplastic astrocytoma n=6, lanes 6-8).
samples examined expressed only the 32kD band of AQP4 indicating a high expression of M23 isoform. Normal brain showed similar prominent expression of AQP1 and AQP4. This was in stark contrast to the glioma cell lines, which did not express AQP4 and showed variable AQP1 expression. While low levels of AQP5 was observed in normal brain tissue, two of the three primary glioblastomas revealed high AQP5 expression and one of three astrocytomas showed high expression levels.

Aquaporins show distinct subcellular localizations.

Aquaporin expression on the plasma membrane is necessary for a participating role in water homeostatic mechanisms. In order to determine aquaporin localization, cellular and subcellular, we immunostained glioma cell cultures and patient tissue sections with antibodies to AQP1 and AQP4. Representative fluorescent images were taken at 60x using a spinning disk confocal microscope and z-stack images are illustrated in Figures 3&4. Moderate AQP1 staining was found throughout GBM62 cells (Fig 3A) with prominent AQP1 expression on the surface membrane, especially at the ruffled processes (Fig 3B). Conversely, expression of AQP1 was not observed in D54 cells (Fig 3A), and neither GBM62 nor D54 cultures showed expression of AQP4 (not shown), which is consistent with Western blot (Fig 2A). In contrast and in accordance with Western blot data (Fig 2B), acute patient glioblastoma tissue showed expression of AQP1, AQP4 and AQP5, which was localized throughout the tissue sample with regions of high expression (Fig 4). AQP5 showed expression along fibrous tracts, possibly along the cytoskeleton, which was not found in either AQP1 or AQP4 stained tissue sections. However, all AQPs were found to be heterogeneously expressed throughout the tissue
Figure 3. Localization of AQP1 using immunofluorescence. (A) Glioma cell lines were stained with anti-AQP and DAP1 as described in Materials and Methods. D54 cells do not show any expression of AQP1. GBM62 also shows expression of AQP1 throughout the cell body with distinct localization at the leading process (B).
Figure 4. Localization of AQP1, AQP4 and AQP5 using immunofluorescence as described in Fig 3. (A) Glioma tissue sections show expression of AQP1 throughout the tissue with regions of high expression levels. (B) AQP4 shows little or no expression in any glioma cell line. As with AQP1, AQP4 is expressed throughout the glioma tissue sections. (C) AQP5 expression pattern is similar to AQP4.
The distribution of AQP1 and AQP4 were similar with expression throughout the tissue section consistent with previous studies (Endo, M. et. al., 1999; Saadoun, S. et. al., 2002a; Saadoun, S. et. al., 2002b).

**AQP1 & AQP4 enhance water permeability in glioma cells.**

Taken the above data together suggests prominent expression of AQP1 and AQP4 in glioma biopsies but loss of AQP proteins in long-term glioma cultures and cell lines. While unexpected, it gives us an opportunity to examine, through over-expression of recombinant AQPs, their contribution to water transport and their possible role in cell functions engaging water movement. In agreement with previous studies (Endo, M. et. al., 1999; Markert, J.M. et. al., 2001; Saadoun, S. et. al., 2002a; Saadoun, S. et. al., 2002b), we have determined that AQP1 and AQP4 are both upregulated in glioma tissue. To this end we obtained clones for AQP1 & AQP4, the two proteins consistently expressed in patient derived biopsies and expressed them individually in D54 glioma cells that lack endogenous AQPs. Following transfection with a N1-plasmid containing AQP1-GFP or AQP4-DsRed, we generated stable cell lines (Fig 5) that selectively express AQP1 and AQP4, respectively, henceforth termed D54-AQP1 and D54-AQP4. These showed prominent expression of the respective protein by Western blot and immuno staining (Fig 5A,B) which was absent in D54-WT, wildtype, cells. We next examined water transport directly by measuring cell swelling in response to a hypoosmotic challenge with a Coulter-Counter Cell Sizer. Cell size was measured continuously, with 20 msec time resolution over a 3 min period during which a 60 s baseline volume was followed by the addition of a 50% hypo osmotic challenge and recorded for an additional 2 min (see
Representative examples are shown in Figure 5 in which the average cell volume +/- S.E.M. for 1000 cells each was plotted every 20 msec. It was difficult to quantitatively assess the water permeability in D54-MG cells as the maximal swelling response is counteracted by RVD. To prevent RVD in D54-MG cells, we used Cl⁻ channel blockers, 200 µM NPPB and 250 µM Cd²⁺ at 15 °C since in glioma cells RVD engages a combination of Cl⁻ channels and KCC transport (Ernest, N.J. et. al., 2005). This resulted in cells behaving like perfect osmometers and hence allowing us to isolate and compare more quantitatively the water transport in cell expressing either AQP1 or AQP4 respectively. Figure 5C&D show volume changes caused by water movement in D54 glioma cells expressing either AQP1 or AQP4. From these data we derived the time constant (τ) of the exponential of the rise in volume and plotted the reciprocal, which would be proportional to the water permeability (Fig 5E). This data suggests that D54-AQP1 cells have enhanced water permeability over wildtype D54 by 98% similar to D54-AQP4 expressing cells (96%, Fig 5E). D54-AQP1 cells in which AQP function was inhibited by 300 µM HgCl₂ (Fig 5C, (Jung, J.S. et. al., 1994), showed a reduced rate of cell swelling comparable to that of D54-WT that lack AQP1 (Fig 5E). The rate of swelling seen in D54-AQP1 cells was similar to patient derived GBM62 cells containing endogenous AQP1 and these cells also show mercury sensitivity (data not shown). As expected, HgCl₂ did not inhibit swelling in AQP4-expressing cells (Fig 5D&E), which is expected since AQP4 is insensitive to mercury. Both D54-AQP1 and D54-AQP4 cells did not show much temperature dependence of water transport unless cooled to 4°C (data not shown) which is in line with previous studies (Folkesson, H.G. et. al., 1994; Roberts,
Figure 5. Function and expression of AQP1 and AQP4 in D54 glioma cells. (A,B) Western blot and 20x image of AQP1 and AQP4 expressing D54 cells. (C,D) Mean cell volume was measured for 3 min in D54-WT and AQP1-D54 cells (n=5-7) and AQP4-D54 cells (n=4-7) following a 50% hyposmotic challenge in the presence of 200 µM NPPB and 250 µM CdCl$_2$. HgCl$_2$ was used at 300 µM. (E) Reciprocal exponential time constant ($\tau^{-1}$) which is proportional to osmotic water permeability. (Significance was assessed using an ANOVA *p<0.05 as compared to control. **p<0.05 as compared to AQP1)
S.K. et. al., 1994; Zhang, R. et. al., 1993). Note that differences in the degree of swelling were only observed in the initial minutes following the challenge as ultimately, as expected, both cell types reached similar cell volumes.

**D54-AQP1 show enhanced migration.**

In light of the hypothesized role of water channels in volume regulation associated with cell migration, we next set out to examine the relative role of AQP1 & AQP4 in migration. Transwell assays are a convenient assay system to mimic the spatial constraints of the extracellular space. We plated 40,000 cells onto each 8 µm filter and cells were allowed to migrate for 5 hrs at which time the cells were fixed and images taken for analysis. We found that compared to wildtype cells ~ twice as many AQP1 overexpressing cells had migrated across the filters (Fig 6A). Indeed, the absolute number of cells that successfully migrated was comparable to the patient derived GBM62 cells that maintained endogenous AQP1 (Av. 162+/− versus 181+/− migrated cells/5h). If this were related to AQP1 function, one would expect that a decrease in AQP1 expression in the latter cell lines should cause a decrease in cell migration. Knock down of AQP1 expression using specific shRNA constructs (see methods) indeed caused a 55% reduction in protein expression and a 70% reduction in cell migration (Fig 6C,D). This data suggests that AQP1 function enhances migration of glioma cells. When we more closely examined AQP1 localization in actively migrating cells, we found the protein to localize to the leading edge of the cells as shown for two representative cells at different stages of penetrating the Transwell pores (Fig. 6B). We subjected AQP4-expressing D54-AQP4 cells to the same migration assay and surprisingly found a reduction in migration
Figure 6. The role of AQP5s in invasion. (A) Transwell migration assay comparing GFP-D54 cells and AQP1-D54 cells. 40,000 cells were allowed to migrate for 5 hrs through 8 µm FluorBlok filters. (B) Western blot showing knockdown of AQP1 using shRNA and (C) 40x image of migrating AQP1-expressing D54 cell. (D) shRNA knockdown of AQP1 in D65 and GBM62 glioma cell lines (n=3). (E) Comparison of DsRed-D54 and AQP4-D54 cells following migration. (F) 40x image of AQP4-expressing D54 cell. (Significance was determined using a Student’s t-test; p<0.05)
of approximately 40% (Fig 6E). However, as with D54-AQP1 cells AQP4 still localized to the leading edge of migrating cells (Fig 6F). This supports the findings from a recent study where migrating grade IV glioblastoma cells showed reduced AQP4 expression (Warth, A. et. al., 2007).

**D54-AQP4 show enhanced cell adhesion.**

Migration requires cells to dynamically alter their adhesiveness to their growth substrate (Uhm, J.H. et. al., 1999) hence any changes in migration may be secondary to altered cell adhesiveness. To question the possibility that AQP1 expression may enhance cell migration by reducing cell adhesion, we examined this question directly by coating coverslips with several common extracellular matrices (collagen, fibronectin, laminin, and vitronectin), allowing cells to adhere for 1 hr, and gently washing away any non-adherent cells. Adherent cells were quantified and compared to control D54 cells. As illustrated in Figure 7, there was no significant difference in adhesion when comparing the D54-AQP1 to control D54-WT on the various substrates. However, there was an overall increase in cell adhesion for D54-AQP4 cells for all substrates examined, which may at least in part explain the reduced difference of the cells to migrate (see above).
Figure 7. The role of AQPs in cell adhesion. (A) To measure adherence, 200,000 cells were plated onto various matrices [1% BSA (Con), 10 µg/ml collagen I (CN), 10 µg/ml fibronectin III (FN), 20 µg/ml laminin (LN), 20 µg/ml vitronectin (VN)] for 1 hr. Cells were gently washed away and fixed. Five random images were taken and counted. (Significance was determined using ANOVA; p<0.05)
DISCUSSION

In this investigation, we were able to demonstrate the expression of AQP1 and AQP4 in all glioma patient biopsies examined by PCR, Western blot and immunostaining. This finding largely supports previous studies showing expression of AQP1 and AQP4 (Endo, M. et. al., 1999; Markert, J.M. et. al., 2001; Saadoun, S. et. al., 2002a; Saadoun, S. et. al., 2002b). Surprisingly, we observed the loss of one or all of the AQPs in cell lines established from human gliomas including those cell lines frequently investigated in glioma research. Importantly, AQP1 expression is retained in many of the glioma cultures, but AQP4 was lost in all glioma lines, even in cell lines derived from acute glioblastoma tissue. This loss is likely a result of culture condition. For example, it has been shown by several groups that some aquaporins are regulated via osmotic response elements and hypertonicity (Herrlich, A. et. al., 2004; Hoffert, J.D. et. al., 2000; Jenq, W. et. al., 1999; Umenishi, F. and Schrier, R.W., 2002; Umenishi, F. and Schrier, R.W., 2003). Cells not stimulated by constant changes in osmolarity may selectively downregulate aquaporins. AQP1 is upregulated by hypertonic challenge in kidney cells lacking endogenous expression of AQP1 (Umenishi, F. and Schrier, R.W., 2003). In the absence of osmotic challenges, AQP1 expression may become superfluous in cultured cells and is downregulated. While AQP4 does not contain an osmotic response element (ORE), it may be affected by other artificial culture conditions.

The lack of AQP expression in many cell lines offered us an opportunity to study their relative contribution to water permeability in gliomas. By re-constituting the expression of AQPs through recombinant expression we were able to examine the role each individual AQP. This technique was important because functional studies of AQPs
in primary cells have been difficult since specific drugs that modulate their function are not available. The use of high-speed real-time volumetric measurements confirmed that expression of either AQP1 or AQP4 resulted in a dramatic increase in water permeability. This allowed us to question the importance of AQP mediated water transport in important cell biological functions, specifically cell migration, a pronounced feature of glioblastomas.

We previously hypothesized that the invasive migration of glioma cells requires cells to undergo coordinated cell volume changes, most notably shrinkage as cells invade into narrow extracellular spaces in brain (Sontheimer, H., 2004). Specifically, we showed that secretion of Cl\(^-\) and K\(^+\) is required for cell invasion (McFerrin M.B. and Sontheimer, H., 2005; Ransom, C.B. et. al., 2001). Furthermore, it was demonstrated that Ca\(^{2+}\)-activated K\(^+\) channels, i.e. BK (Weaver, A.K. et. al., 2004) colocalize to lipid raft domains on invadipodia (McFerrin M.B. and Sontheimer, H., 2005). Our immunohistochemical studies also localize AQP1 and AQP4 to the leading edge of migrating tumor cells. We suggest that while Cl\(^-\) and K\(^+\) channels provide the pathways for KCl secretion, AQP1 enhances the release of obligated water, which in turn causes the invading process of the cell to shrink. Consistent with this hypothesis, overexpression of AQP1 doubled the rate of cell migration through a transwell barrier that mimics the spatial constraints of brain. In further support of this argument, previous observations show that AQP1 expression also enhances the migration of melanoma cells (Hu, J. and Verkman, A.S., 2006). Although AQP4 is also localized to invadipodia and similarly increases water permeability in glioma cells, we were surprised to find that it actually reduced the cell’s ability to migrate. This discrepancy may be due to our use of
the M23 splice variant of AQP4, which has been suggested to increase cell adhesiveness (Hiroaki, Y. et al., 2006). As our GBM tissue endogenously expressed high levels of the M23 splice variant as compared to the M1, it is possible that AQP4 itself is not utilized for motility. Another possible hypothesis is that AQP4 colocalizes with a different subset of ion channels than AQP1. We know that D54 gliomas express several K$^+$ channels (Bordey, A. et al., 2000; Olsen, M.L. and Sontheimer, H., 2004; Ransom, C.B. and Sontheimer, H., 2001) and Cl$^-$ channels (Olsen M.L. et al., 2003). AQP1 expression may act in concert with one set of ion channels to enhance migration while AQP4 may interact with a different subset important for maintaining proper cell size. Of note, AQP1 and AQP4 may have similar differential roles in non-malignant glia. For example, during development, non-malignant astrocytes lose expression of AQP1 and express only AQP4. The development of this expression pattern correlates with differentiated astrocytes also becoming stationary cells. Similarly, aquaporins play equally varied roles in many cell types (Echevarria, M. et al., 1994; Nielsen, S. et al., 1993).

Another hypothesis that may explain the differing functionality AQP4 and AQP1 may be a separation of domains. Each AQP may localize within certain isolated regions, along with their respective ion channels, which are necessary to promote a particular cellular function, i.e. migration or adhesion. Specifically, AQP1 has a heterogeneous expression in primary glioblastoma tissue (Endo, M. et al., 1999) indicating regions for rapid water movement. These regions may be indicative of areas of high migration. If AQP1 and AQP4 are indeed localized to different domains, then it is possible that they may also interact with different growth factor receptors. We know that the EGFR is upregulated in glioblastomas (Di Carlo, A. et al., 1992), and EGF has been shown to
increase VEGF secretion (Goldman, C.K. et. al., 1993; Tsai, J.C. et. al., 1995) and regulate proliferation (Engebraaten, O. et. al., 1993). It is possible that gliomas containing high expression of AQP1 may have higher levels of certain growth factors and their respective receptors leading to a switch between docile tumor growth (AQP4-expressing), and a highly migratory and rapidly growing (AQP1-expressing) tumor.

The differential effects of AQP1 and AQP4 expression on glioma cell biology are surprising. Clearly further studies are needed to examine a mechanistic link between AQP function and the underlying biology. While we hypothesize that water flux is the primary function in migrating or proliferating cells, AQPs may have alternative, less obvious functions in this regard.

ACKNOWLEDGEMENTS

We would like to thank Dr. Yancey Gillespie (University of Alabama-Birmingham) for providing us with cells from patient biopsies, Dr. Nicholas LaRusso (Mayo Clinic) for providing the AQP1-eGFP construct and Dr. Ken-ichi Nakahama for providing AQP4 construct. This work was supported by NIH-RO1-NS36692.
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WATER PERMEABILITY THROUGH AQUAPORIN-4 IS REGULATED BY PROTEIN KINASE C AND BECOMES RATE-LIMITING FOR GLIOMA INVASION

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Submitted to Cancer Research, 2008

Format adapted for thesis
ABSTRACT

Glioma cells are highly invasive, a feature requiring dynamic changes in cell volume. Volume changes require water flux through aquaporins (AQPs), and AQP1 and AQP4 are highly expressed in primary brain tumor biopsies. AQP4 localizes to the leading edge of the invadipodia with ClC2 and KCC1, believed to provide the pathways for Cl⁻ and K⁺ secretion to accomplish the cell volume changes required for invasion. Functional studies determined that AQP1 enhances tumor migration whereas AQP4 enhances cell adhesion suggesting that the relative contribution of each may dictate a particular biological state. Since AQPs have a consensus phosphorylation site for PKC, a known regulator of cell invasion, we examined the possibility that AQP function and hence invasion, may be regulated via PKC. Using D54MG glioma cells stably transfected with AQP4, we show that enhanced PKC activity, activated via phorbol 12-myristate 13-acetate, reduced water permeability and cell migration. Chelerythrine, PKC inhibitor, reduces AQP4 phosphorylation leading to enhanced water permeability and tumor invasion. S180A mutation of the consensus PKC phosphorylation site, causes a loss of AQP4 regulation by PKC. Glioma cells selectively expressing AQP1, AQP4 or S180A-AQP4 were implanted intracranially into SCID mice. AQP1 and S180 expressing tumors invaded significantly further into tissue than either AQP4 expressing or control gliomas. This data suggests that PKC regulation of AQP4 is ultimately rate limiting in regards to the rate of tumor invasion in vivo possibly opening new therapeutic avenues.
INTRODUCTION

Under physiological conditions, the brain and spinal cord rarely encounter any significant change in the osmolarity of the extracellular fluid environment. However, acute brain injuries and neurological diseases can lead to an increase in brain water content and swelling, or edema. Edema is also a prominent comorbidity of gliomas, primary brain tumors that often disrupt the blood-brain barrier. Surprisingly, gliomas thrive and expand rapidly in this edematous environment where normal brain cells die. Gliomas have an unusual ability to regulate cell volume if challenged by osmotic changes. Indeed, it has been shown that glioma cells can restore the cell volume even in the presence of significant osmotic challenges through the release of osmotically active anions primarily Cl$^-$ through ion channels. Any reduction in water content therefore requires the redistribution of water and in all likelihood invokes the activity of water channels also called aquaporins (AQP). Aquaporins form a superfamily of 13 members (AQP0-12) and are the principle pathway for water movement across most cellular membranes. Of these AQP1, AQP4, AQP9 and AQP11 have been shown to be expressed in normal brain and AQP1, AQP3, AQP4, AQP5, and AQP9 have been documented in gliomas by PCR. Of these, AQP1 and AQP4 have been shown to be highly expressed at the protein level in the most malignant gliomas called glioblastomas. Functional studies using recombinant over expression delineate a differential role for these two AQP$\cdot$s. AQP1 confers enhanced invasiveness whereas AQP4 over expression enhances cell adhesion thereby reducing invasiveness. Since both AQP$\cdot$s are prominently expressed across human gliomas, we hypothesize that their relative contribution to the cell’s biology may be regulated to best support a particular biological state. More
specifically, during tumor dissemination, the activity of AQP4 may be suppressed to enhance invasiveness, whereas during times of growth and tumor expansion, adhesion to neighboring cells may be enhanced hence displaying enhanced AQP4 activity.

Protein kinase C is known to regulate tumor invasion and has recently been shown to be a regulator of aquaporins in other systems. PKCs constitute a family of serine/threonine kinases with 9 isoforms being expressed with distinct cellular and subcellular localization in the brain and spinal cord. Generally, PKC is recruited to the cellular membrane in response to a rise in Ca\textsuperscript{2+} and diacylglycerol. Upon activation, PKCs phosphorylate a number of proteins that regulate cell proliferation and differentiation. In gliomas, PKC has been shown to be an important modulator of tumor proliferation and migration\textsuperscript{14,15}. More specifically, PKC activity has been shown to enhance tumor invasion in U87\textsuperscript{14} and proliferation in U138 tumor cell lines\textsuperscript{16}.

In this study, we set out to examine whether PKC regulates AQP4 function in glioma cells in ways that enhance the two important biological states of these tumors, growth and invasion. Using D54-MG glioma cells stably transfected with AQP4, we show that the enhanced PKC activity, activated via phorbol 12-myristate 13-acetate (PMA), caused a phosphorylation of AQP4 resulting in reduced water permeability, cell adhesion and inhibition of cell migration. By contrast, chelerythrine, a PKC inhibitor, reduces AQP4 phosphorylation leading to enhanced water permeability and tumor invasion. AQP4 localizes to the leading edge of the invadipodia of migrating cells and colocalizes with ClC2 and KCC1, which are believed to provide the pathways for Cl\textsuperscript{−} and K\textsuperscript{+} release in the context of cell migration\textsuperscript{17} proposing further support for a role for KCl-water extrusion as an enhancer of cell invasion.
MATERIALS AND METHODS

Cell Culture. D54-MG (WHO grade IV) were a gift from Dr. D.D. Bigner (Duke University, Durham, NC). Cells were grown in Dulbecco’s modified Eagle medium (DMEM/F12; Media Tech, University of Alabama at Birmingham Media Preparation Facility) and supplemented with 2mM glutamine (Media Tech) and 7% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) at 37°C and 90% O₂/10% CO₂ humidified environment. AQP4 and AQP1 stable cells lines were made as described previously ⁸.

Western Blot Analysis. Western blot procedure has been described previously ⁸. All antibodies were obtained from Chemicon (Temecula, CA) and used following manufacturer’s instructions. Images were taken on Kodak Imager (Rochester, NY) and analyzed using Kodak Imager software.

Immunoprecipitation. Cells were grown to confluency in a 10 cm² dish. Cells were washed 2× in ice cold PBS. Cells were scraped and collected in 500µl ice cold PBS and centrifuged for 5min at 10,000rpm. Supernatant was removed and pellet resuspended in 500µl RIPA buffer [(50mM TrisCl, pH 7.5, 150mM NaCl, 1% Nondet P-40 (NP-40), 0.5% sodium deoxycholate, 1% sodium dodecyl sulfate (SDS)] containing protease and phosphatase inhibitors. Lysates were rotated for 30min at 4°C followed by a brief sonication. Protein quantification was performed using a DC protein assay kit (BioRad, Hercules, CA) and lysates diluted to 1mg/ml. Lysates were cleared with 50µl agarose-conjugated protein A beads (Roche, Indianapolis, IN) for 30min at 4°C and centrifuged briefly to pellet beads. Supernatant was transferred to a new tube and incubated for 1hr with mouse anti-phosphoserine (Sigma) at 4°C. Protein A beads (50µl) were added and allowed to rotate overnight. On the following day, immunoprecipitates were pelleted at
low speed at 4°C and supernatant removed as unbound fraction. Immunoprecipitates were rinsed 3× with ice cold RIPA and released from beads using 25µl of 100mM glycine and incubated for 3min. Five µl of 6× sample buffer was added and spun down to pellet beads. Supernatant was removed and ran onto 10% gel as described in previously ⁸.

Immunocytochemistry. Cells plated on coverslips and grown to a confluent monolayer and were scratched using a 200µl pipette tip. Cells were allowed to recover for 5hr, washed with PBS and fixed in 4% paraformaldehyde for 10min. Staining procedure has been described previously ⁸. Images of migrating cells were acquired using an inverted Olympus IX-81 spinning disk confocal microscope (Olympus, Center Valley, PA).

Site-Directed Mutagenesis and Transfections. AQP4 was excised from DsRed plasmid using restriction enzymes Apa1 and EcoRI (New England Biolabs). Two sets of primers were created. 5’-CGA ATT CTG ATG GTG GCT TTC AAA GGG G -3’ and 5’-AAC ATC AGT CCG TTT GGC ATC ACA GCT GGC-3’ and 5’-GCC AGC TGT GAT GCC AAA CGG ACT GAT GTT-3’ and 5’-CCG GGC CCG TAC AGA AGA TAA TAC CTC TCC-3’ were used in two separate PCR reactions with the mutation site (bolded). A final PCR reaction was done using primers 5’-CGA ATT CTG ATG GTG GCT TTC AAA GGG G-3’ and 5’-CCG GGC CCG TAC AGA AGA TAA TAC CTC TCC-3’ with PCR products from the previous reactions with annealing occurring at the mutation site. PCR reactions used Phusion High-Fidelity Polymerase (New England Biolabs) and were done as follows: denaturation at 98°C for 2min followed by 35 cycles of 98°C for 10s, 60°C for 10s, 72°C for 45s and a final extension at 72°C for 7min. PCR product was
purified and inserted into the DsRed-Monomer-N1 (Clontech) plasmid using T4 ligase. Stable cell lines were made as described previously (McCoy and Sontheimer).

**Volume Regulation.** Cell volume measurements were performed using a Coulter Counter Multisizer 3 (Beckman-Coulter, Miami, FL) as described previously and modified. Data were collected by Multisizer 3 software, and 5000 pulse listings were exported to EXCEL as the average of 40-50 cells for each 20ms timepoint. Data were collected as mean diameter and were converted to mean cell volume. Mean cell volumes were normalized to baseline values. Data were plotted in Origin 7.0 (MicroCal, Northhampton, MA) ± se with (n) experiments performed. Each time point graphed is an average of the mean cell volume for 40-50 cells per 20ms.

**Cell Migration.** Migration was assessed using a modified Boyden Chamber and described previously. Images of five random fields were taken using Zeiss Axiovert 200M (München, Germany). All experiments were performed in triplicate.

**Cell Adhesion Assay.** Coverslips were coated overnight at 4°C with various matrices: 10µg/ml collagen I (Sigma), 10µg/ml fibronectin III (Sigma), 20µg/ml laminin (Sigma), 20µg/ml vitronectin (Sigma) or 1% BSA (Sigma). 200,000 cells were seeded per well and allowed to adhere for 1hr at 37°C. The non-adherent cells were washed away gently using PBS and cells were fixed using 4% paraformaldehyde. Images of five random fields were taken using Zeiss Axiovert 200M (München, Germany) at 20x magnification.

**Tumor Implantation.** We obtained 6 wk old CB17 SCID mice. Animals were anesthetized using isofluorane. An incision was made along the midline and a hole was drilled ~2mm post bregma and 2mm to the right of the midline where a 30-gauge syringe was steroetactically inserted 2mm. 500,000 cells were injected and animals were sutured
and allowed to recover for 2 weeks. Mice were transcardially perfused with 4% paraformaldehyde and incubated overnight at 4°C. Brains were placed in 10% sucrose for 1 hr at 4°C followed by an overnight incubation with 30% sucrose. Brains were fixed in OCT and placed in -80°C then sectioned in cryostat at 20µm. For imaging cell migration, sections were dried overnight at 37°C, washed with 0.1M PB for 45s followed by 4X 10s in dH2O and incubated overnight at 37°C. Sections were rehydrated by incubating for 5 min in each ethanol: 100%, 95% and 70% followed by 2X washes in dH2O, mounted and imaged on Zeiss Axiovert 200M.
RESULTS

**PKC phosphorylates AQP4.**

The primary objective of this study was to determine whether regulation of AQP4 function may alter the degree of tumor migration or invasiveness. This objective is predicated on our previous observation that overexpression of AQP4 in D54 glioma cells causes a marked reduction in cell migration. Furthermore, astrocytes treated with dopamine and thrombin show a reduction in AQP4 water permeability as a direct result of PKC phosphorylation. Therefore, it appears to be a logical first step to examine whether PKC phosphorylates AQP4 and whether this in turn may affect the degree to which these cells migrate. To achieve this goal we first examined the level of endogenous PKC expression in AQP4 expressing glioma cells (AQP4-D54) as compared to nonmalignant astrocytes (Fig 1A). Consistent with previous studies, we found much enhanced expression of PKC in tumor cells by Western blot. Importantly, AQP4 and PKC were found colocalized at the leading edge of migrating cells (Fig 1B). To examine whether the relative activity of PKC alters the degree of AQP4 phosphorylation, we used immunoprecipitation experiments in which anti-phosphoserine antibodies were used to precipitate proteins that show phosphoserine phosphorylation and then probed these with antibodies to AQP4. These experiments were done prior to (control) and following activation [phorbol 12-myristate 13-acetate (PMA)] or inhibition (chelerythrine) of PKC. As illustrated in Fig. 1C, AQP4 showed constitutive phosphorylation which could be further enhanced by exposure of cells for 45min to 1µM PMA, a PKC activator. Conversely, 45min exposure to 1µM chelerythrine, a broad spectrum PKC inhibitor, resulted in significantly decreased AQP4 phosphorylation. Importantly, neither treatment
Figure 1. PKC expression and phosphorylation of AQP4. Cell lysates were separated using 10% SDS-PAGE gels and transferred onto PVDF paper and probed with antibodies. Western blot analysis was used to examine PKC expression levels in AQP4-D54 cells as compared to primary astrocyte cultures (A) and localization in migrating tumor cells (B) using PKC antibodies as described in Materials and Methods. (C) Immunoprecipitation showed that pretreatment with either Chelerythrine or PMA modulated AQP4 phosphorylation levels but did not alter basal AQP4 expression levels (D). (Green-AQP4, Red- PKC, Blue- DAPI)
altered the total protein level for AQP4 as illustrated in Fig. 1C (right). To examine whether a more physiological activator of PKC could similarly enhance AQP4 phosphorylation we used the known PKC activator thrombin. Thirty minute exposure of glioma cells to 0.5U thrombin showed significantly enhanced AQP4 phosphorylation (Fig. 1D). Taken together this data shows that AQP4 in gliomas is the target of PKC phosphorylation, and albeit significantly phosphorylated at rest, phosphorylation can be further enhanced by PKC activation.

*PKC modulators alter AQP4 water permeability.*

Previous work using astrocytes has shown that activation of PKC leads to a reduction in water permeability via AQP4, and this occurs through direct phosphorylation of AQP4 at the S180 site. Hence the expectation would be that exposure to PKC activators would reduce while PKC inhibitors should enhance water permeability. To examine water transport directly, we measured the rate of cell swelling in response to a hypo osmotic challenge using a Coulter-Counter Cell Sizer as previously described. This instrument allowed us to size populations of cells in real time with a 20msec time resolution. Recordings were obtained over a 3min period during which a 60s baseline volume was acquired followed by the addition of a 50% hypo osmotic challenge for an additional 2min (see methods for detail). The average cell volume +/- S.E.M. for 100 cells each was plotted every 20msec. Figure 2A shows representative examples of volume changes caused by water influx into AQP4 expressing D54 glioma cells treated with either chelerythrine or PMA as compared to untreated controls. From this data, we derived the time constant (τ) of the exponential rise in volume and plotted the reciprocal,
Figure 2. Functional regulation of AQP4 by PKC. Mean cell volume was measured for 3 min in AQP4-D54 cells (A, n=4-7) and S180 mutant AQP4 (B) following a 50% hyposmotic challenge. Representative graphs show water permeability over the first few seconds following hyposmotic challenge. Reciprocal exponential time constant (τ⁻¹) is proportional to osmotic water permeability (C). (Significance was assessed using an ANOVA *p<0.05 as compared to control.)
which is directly proportional to the water permeability. Relative changes compared to untreated control AQP4-D54 cells are given in (Fig 2C). This data suggests that inhibition of PKC using chelerythrine results in a ~2-fold enhancement of water permeability when exposed to a 50% hypo osmotic challenge. By contrast, cells treated with PMA showed ~2-fold decrease. Comparable results to PMA were obtained with thrombin (data not shown). To rule out non-specific effects of these drugs we next mutated the phosphorylation site, S180A, of AQP4 and created sister cells that expressed the mutated form of AQP4 which should now be insensitive to PKC. Indeed, as illustrated in Fig. 2B/C, these cells showed no difference in water permeability when treated with either chelerythrine or PMA. This data suggests that in gliomas water permeability through AQP4 is under the regulation of PKC via phosphorylation of S180.

**PKC regulates tumor migration in AQP4 expressing cells.**

It has been hypothesized that water channels aid cell volume changes required by tumor cells as they invade brain tissue \(^{17}\). To examine whether PKC regulation of AQP4 may alter the ability of cells to undergo volume changes as they invade, we used Transwell assays which mimic the spatial constraints of the extracellular space. We plated 40,000 cells into the top compartment of a Transwell insert containing 8μm pores and cells were allowed to migrate for 5hrs at which time the cells were fixed and images taken for analysis. Cells were continuously treated with specific inhibitors for defined signaling steps associated with PKC signaling. These included the PKC inhibitor chelerythrine, a PLC inhibitor, U73122, and a MEK1/2 inhibitor, U0126. As illustrated in Fig. 3A, a dephosphorylation of AQP4 with chelerythrine not only enhanced water
Figure 3. Role of PKC in modulating migration and adhesion in AQP4-expressing tumor cells. (A) Transwell migration assay comparing AQP4-D54 cells treated with inhibitors of various signaling molecules. 40,000 cells were allowed to migrate for 5 hrs through 8 µm FluorBlok filters. Cells were treated with PKC modulators, chelerythrine and PMA, U73122, a PLC inhibitor and U0126 as a MAPK inhibitor. (B) To measure adherence, 100,000 cells were plated onto various matrices [1% BSA (Control), 10 µg/ml collagen I (CN), 10 µg/ml fibronectin III (FN), 20 µg/ml laminin (LN), 20 µg/ml vitronectin (VN)] for 1 hr. Cells were gently washed away and fixed. Five random images were taken and counted.
permeability but also showed ~75% enhancement in tumor cell migration as compared to untreated AQP4 cells (Fig 3A). Conversely, PMA, which reduced water permeability in AQP4-D54 cells, also reduced tumor migration by ~40%. Once again, the specificity of these effects for AQP4 mediated phosphorylation was validated by the fact that glioma cells containing the mutated serine residue that eliminated PKC phosphorylation, S180A, showed migration rates similar to those of chelerythrine treated cells and were insensitive to treatment with PMA or chelerythrine. Also, MAPK signaling appeared to have little effect since inhibition of MEK1/2 activation with U0126 did not affect the migration of AQP4-D54 glioma cells. By contrast, in all cell types tested, the PLC inhibition by U73122 completely eliminated cell migration (Fig 3A). This finding is consistent with previous studies showing an absolute requirement for PLC in chemotactic migration since PLC is important for increasing the Ca\(^{2+}\) concentration necessary for inducing migration\(^{21,22}\) and cytoskeletal reorganization\(^{23}\). These effects were of course unrelated to AQP4 as the drug also inhibited the migration of glioma cells that lack AQP4 expression (data not shown). Taken together, the above data suggests that AQP4-D54 cell migration is effectively modulated by PKC signaling through altering the cells water permeability and in turn the cells ability to adjust their shape as they migrate.

*_AQP4 modulation does not alter cell adhesion._*

Although the above data are consistent with the hypothesized role of aquaporins in volume changes, it is possible that PKC effects may be explained by other mechanism. For example, PKC has been shown to modulate cellular adhesion through phosphorylation of focal adhesion kinase\(^{24}\). This is important in light of previous
findings demonstrating that AQP4 cells expressing the M23 isoform show enhanced cellular adhesion \(^{25}\). To rule out changes in adhesiveness as mediators of the observed changes in cell migration, we examined this alternative possibility directly. Therefore, we conducted traditional cell adhesion studies in which we coated coverslips with several common extracellular matrices (collagen, fibronectin, laminin, and vitronectin), allowing cells to adhere for 1hr and gently washing away any non-adherent cells. Adherent cells were quantified and compared to controls. We found that although expression of AQP4 per se enhanced cell adhesion compared to that of glioma cells lacking AQP4, there was no change in the cellular adhesion when AQP4 expressing cells were treated with chelerythrine and this was true for all matrices examined (Fig 3B). This data suggests that the observed changes in AQP4-D54 migration following PKC modulation, is not due to an alteration in the cell’s ability to adhere to various matrix molecules.

**AQP1 is not regulated by PKC.**

Previous work suggested that PKC regulates AQP1 whereby increasing PKC activity increases AQP1 mediated water permeability \(^{26}\). Since glioma *in vivo* express both AQP1 and AQP4, we wanted to determine if AQP1 was also under regulation by PKC. We examined AQP1-D54 cells in regards to phosphorylation, volume regulation and migration as performed on AQP4-D54. Immunoprecipitations revealed no change in phosphorylation of AQP1 by PKC modulators PMA and chelerythrine (Fig. 4A). Further, neither cell swelling nor cell migration were altered by chelerythrine in AQP1 expressing tumors (Fig 4B/C). This data suggests that AQP1 is not functionally regulated by PKC activity, which is in stark contrast to AQP4.
**AQP4 colocalizes with KCC1 and ClC2 in migrating cells.**

While water permeability is required for cell shape changes, the directionality of the water movement as well as the energetic driving force is the consequence of salt movements. In this context it is thought that the efflux of Cl\(^-\) and K\(^+\), i.e. KCl, provide the driving force for cell shrinkage at the leading invading edges of the cells. Important candidates that have been implicated in the release of Cl\(^-\) are the K\(^+\)-Cl\(^-\) cotransporter 1 (KCC1) and the chloride channel (ClC2). Interestingly, phosphorylation by PKC regulates these proteins in a similar fashion as AQP4 and with the same directionality. Specifically, phosphorylation by PKC reduces the outward Cl\(^-\) movement of both the transporter and the channel\(^{27,28}\) and reduces water permeability. This would work hand-in-hand to reduce the overall release of cytoplasm and hence reduce shrinkage, whereas a decrease in phosphorylation affects each protein in the opposite manner hence enhancing cytoplasmic release. In light of this, we examined the possibility that these proteins may colocalize, and in order to address this question, we wished to examine cells that were in the process of extending a leading process and were actually migration. This can be conveniently accomplished using an *in vitro* wound assay. Therefore using a sterile 200µl pipette tip a confluent monolayer of glioma cells was scratched, and cells were allowed to migrate for 5hrs into the bare regions of the coverslip. Following fixation images of cells that appeared to be migrating into the lesion were triple labeled with antibodies to ClC-2, AQP4 and DAPI or KCC1, AQP4 and DAPI and representative examples of individual and merged images are shown in Fig. 4A each showing that AQP4 colocalized with both ClC2 and KCC1 along the leading edge of the cell.
Figure 4. PKC does not regulate AQP1. Using immunoprecipitation as described in Fig. 1, chelerythrine and PMA treatment did not alter AQP1 phosphorylation (A). Neither water permeability (B) nor cell migration (C) was altered by PKC activity.
**Chelerythrine reduces phosphorylation of ClC2 and KCC1.**

We next examined if ClC2 and KCC1 were indeed also subject to regulation via PKC phosphorylation in glioma. We performed immunoprecipitation experiments to determine the degree of phosphorylation of ClC2 and KCC1 in AQP4-D54 cells treated with chelerythrine. As illustrated in Fig. 5B, cells treated with the PKC inhibitor, chelerythrine, showed reduced phosphorylation for both KCC1 and ClC2. Additionally, chelerythrine did not alter expression of total protein of either KCC1 or ClC2 (Fig. 5B) similar to what we demonstrated for AQP4 in Fig. 1C.

**PKC modulation of chloride channels and transporters does not alter migration.**

Unlike other cancers that spread hematogenously, gliomas invade the brain by active cell migration. As alluded to above, and more extensively discussed in the literature \(^{17,29,30}\), migrating tumor cells exude K\(^+\) and Cl\(^-\) along with obligated water in order to undergo the shape changes required of migrating cells. Since de-phosphorylation enhances migration while also enhancing water permeability and K\(^+\) and Cl\(^-\) secretion, we were interested to delineate whether these phosphorylation effects required all of these constituents to be simultaneously regulated or whether water permeability may be the rate limiting step. To do so, we used D54MG glioma cells that lack expression of all aquaporins while maintaining expression of ClC2 and KCC1 to ask whether altering the phosphorylation status of ClC2 and KCC1 via a PKC inhibitor would suffice to change the rate of migration. Using Transwell assays, we show in Fig. 5C that in the presence of chelerythrine or PMA glioma migration was unaltered suggesting that PKC modulation of Cl\(^-\) movement alone does not alter tumor migration.
Figure 5. PKC modulates phosphorylation of ClC2 and KCC1 but not migration. Using immunofluorescence (A) and immunoprecipitation as described in Fig. 1, chelerythrine reduced PKC mediated ClC2 and KCC1 phosphorylation without altering the basal expression levels (B). PKC does not modulate migration in D54 control cells (C). Migrating cells were treated acutely with 40 µM DIOA, 12.5 µM CdCl₂, or both (D). (Significance was determined using ANOVA; p<0.05) (Green-ClC2 or KCC1, Red-AQP4, Blue- DAPI)
However, we further tested whether AQP4 cells, with enhanced water permeability, were more sensitive to chloride channel and transporter inhibitors than control cells. We then seeded AQP4 and control D54 cells onto a Transwell migration assay filter in the presence of a KCC inhibitor (40 µM DIOA), a ClC inhibitor (12.5 µM CdCl₂) or both and allowed the cells to migrate for 5hrs. We found that both control cells and AQP4-D54 cells were inhibited to comparable levels under all conditions (Fig 5D). This data implies that enhanced water permeability does not increase the tumor cells sensitivity to inhibitors of Cl⁻ movement.

Finally, we examined whether AQP4-D54 tumor cells in which chelerythrine was used to maximize water permeability through AQP4 would now show an enhanced susceptibility to Cl⁻ channel/transporter inhibitors with regards to their rate of Transwell migration. As illustrated in Fig. 5D, this was not the case as there was only a marginal and non-significant enhancement in tumor migration in chelerythrine treated cells compared to control cells. Taking this data together suggests that, while Cl⁻ and K⁺ release is essential for cell volume changes to support glioma migration/invasion, expression levels of the KCC1 and ClC channels that mediate electrolyte release are not rate limiting but instead, water permeability is ultimately rate limiting with regards to the rate of tumor migration.

*AQP1 expression leads to a more aggressive tumor in vivo.*

While previous reports have shown AQP1 and AQP4 expression in vivo in gliomas, our lab has shown individual functions for both AQP1 and AQP4 in vitro, specifically that AQP1 is more migratory than AQP4. We aimed to determine if a
Figure 6. AQP1 expression enhances tumor invasion. AQP1, AQP4 and S180A-AQP4 expressing D54MG were injected intracranially into 6 weeks old mice. (A) Representative images of tumor growth. Images showing tumor cell invasion away from the major tumor mass (A). Arrows indicate tumor and migrating cells. AQP1 expression increased tumor migration by ~2 fold over control and AQP4 expressing D54-MG (B). AQP1 tumors migrated over a wider range of distances as compared to AQP4 (C).
similar result could be found in vivo and if so then we would hypothesize that the reduced rate of migration found in AQP4 tumor cells would be dependent on its regulation by PKC activity. In order to accomplish this, we intracranially injected 500,000 cells expressing AQP1, AQP4, S180A-AQP4 or control cells and allowed the tumors to grow for 2 weeks in 6 week old SCID mice and then examined cell invasion. AQP1 expressing tumor cells invaded ~2 fold further than either control cells or AQP4 tumors cells (Fig 6A). Even though the number of AQP1 tumor cells invading <500µm was lower than either AQP4 or control cells when cell distances were binned into segments of 500µm, AQP1 tumor cells showed increased cell numbers for distances >1000µm (Fig 6B) and these tumors formed more distant satellite colonies (data not shown). However, AQP4 expressing tumors and controls showed highest cell numbers <500µm being close to the tumor, which was reduced over increasing distances. S180A-AQP4 mutants had increased invasion as compared to the AQP4 expressing gliomas (Fig 6A). Additionally, S180A-AQP4 tumors showed enhanced tumor migration for distance greater than 1000µm (Fig 6B). Representative images in Figure 6C demonstrate individual cells that have invaded away from the main tumor mass. Taken together, the data suggests that AQP1 tumors display a more invasive phenotype and suggests that these tumor cells are forming the satellite colonies characteristic of gliomas. Further, AQP4 mediated invasion is regulated by PKC and its invasion is limited in vivo due to an increase in PKC activity upregulated in gliomas.
DISCUSSION

In this study, we were able to demonstrate that PKC regulates AQP4 function in glioma cells through phosphorylation on S180 leading to reduced water permeability and enhanced water permeability when de-phosphorylated. Our findings agree with previous work in astrocytes where exposure to dopamine \(^{19}\) or thrombin \(^{20}\) activates PKC and results in a reduction in water permeability. Our studies mutating S180 on AQP4 in gliomas unequivocally suggest that PKC signaling is biologically important for this regulation. Interestingly, our immunoprecipitation and Western blotting experiments suggest a significant degree of constitutive phosphorylation at this site in unstimulated gliomas. This leads us to conclude that water permeability is maintained at a reduced level in resting cells. Experimentally, we were able to greatly enhance water permeability and the rate at which cell changes their cell volume using chelerythrine to reduce AQP4 phosphorylation. This may be important in invading cells that are exposed to environmental signal that cause a reduction in PKC activity.

We hypothesized that the invasive migration of glioma cells requires cells to undergo coordinated cell volume changes, most notably shrinkage as cells invade into narrow extracellular spaces in brain \(^{29}\). Previously we showed localization of AQP4 to the leading edge of migrating tumor cells \(^{8}\) and our immunohistochemical data reveals localization of Cl\(^-\) channels/transporters to the leading edge of migrating cells. Furthermore, we showed that secretion of Cl\(^-\) and K\(^+\) is required for cell invasion \(^{17,31}\). Although, Cl\(^-\) and K\(^+\) channels provide the pathways for KCl secretion, water release is ultimately required to achieve any volume change in the context of cell migration with AQP4 in the leading edge of an invading cell possibly serving this role. Intuitively, we
had assumed that water permeability would not be limiting in this context but our experimental data refute this. Our data suggests that modulating the phosphorylation state of chloride channels or KCC transporters which are known to be enhanced in their activity following dephosphorylation does not alter tumor migration. Yet, inhibiting chloride movement also inhibits invasion. Hence, the data suggest that while Cl\textsuperscript{−} movement is necessary for tumor invasion, an enhancement of water permeability also correlated with enhanced tumor invasion leads us to conclude that water permeability does become the rate limiting step. A recent study suggests that the related AQP1 is also regulated by PKC but with opposite effects namely PKC inhibition would lead to a reduction in water permeability\textsuperscript{26}. This would lead us to conclude that since tumor cells exhibit high protein levels of PKC, AQP4 water permeability would be highly depressed while tumor cells expressing high levels of AQP1 would be able to utilize rapid water flux to invade into the surrounding tissue. However, we saw only small if any changes in water permeability in AQP1-D54 cells treated with chelerythrine and no difference in tumor migration. Since PKC is highly expressed in tumor cells, we may not see an additional increase in AQP1 water permeability with PMA treatment as this protein may be maximally phosphorylated. Clearly further studies are needed to further elucidate the role of PKC in regulating AQP1.

PKC activity promotes migration in several cell types. Specifically, PKC activity enhances migration in endothelial cells\textsuperscript{32}, CHO cell spreading\textsuperscript{33}, and vascular smooth muscle cell migration\textsuperscript{34}. In addition, other PKC isoforms have been implicated in regulating cell migration. PKC reduces chemotaxic migration in monocytes\textsuperscript{35}, PDGF-stimulated migration of smooth muscle cells\textsuperscript{36} and EGF-stimulated migration of
fibroblasts. PKC activity also plays a role in regulating cell invasiveness in several different tumor systems, i.e. breast carcinoma, mammary carcinoma and colon carcinoma. This is consistent in primary glioblastomas where glioma invasion is modulated by PKC. However, this study suggests that while PKC modulates tumor migration and invasion, water movement is the limiting factor for regulating migration/invasion. Based on previous studies showing that PKC modulates AQP4 water permeability, we hypothesize that reducing water permeability should slow glioma migration. AQP4 KO mice show reduced astrocyte migration in vitro as well as in vivo following injury, which leads to a reduction in the formation of the glial scar. This suggests that altering water permeability by eliminating the channel alone can prevent migration. Therefore, reducing water permeability should also reduce cell migration. Further, Warth et al (2007) showed that while AQP4 is upregulated in primary glioblastomas obtained from patient biopsies, the cells located in the infiltration zone contain very low levels of AQP4. Our in vivo data is consistent with these results where by AQP4 cells are less invasive than AQP1 expressing gliomas. The implication would be that while AQP4 is upregulated AQP1 cells are infiltrating the surrounding areas establishing satellite colonies. Our data suggests that while AQP4 is highly expressed in primary glioblastomas, it is not the primary AQP necessary for tumor migration. It does suggest that enhanced phosphorylation via PKC regulates invasiveness providing a novel target to reduce glioma invasion.
ACKNOWLEDGEMENTS

I would like to thank UAB Neuroscience NINDS Protein Core-C for creation of site mutation and UAB Neuroscience Blueprint Cellular and Molecular Neuropathology Core-C for use of stereotactic microscope and cryostat. This work was supported by NIH Neuroscience Blueprint Core Grant NS57098, NINDS Core Grant P30 NS47466, and NIH 5R01NS031234 and NIH 2R01NS036692.
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MAPK INDUCES AQP1 EXPRESSION IN ASTROCYTES FOLLOWING INJURY

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Submitted to Journal of Neurochemistry, 2008

Format adapted for thesis
ABSTRACT

Aquaporin-4 (AQP4) is the principle water channel in astrocytes and the primary route for water transport across astrocytic membranes. AQP4 co-localizes with Kir4.1 channels at astrocytic endfeet and it has been suggested that these channels cooperate in $K^+$ and water homeostasis. In response to injury, two additional aquaporins, AQP1 and AQP9, can be detected in astrocytes, yet neither is found in cultured astrocytes and therefore their contribution to astrocyte water uptake and biology is poorly investigated. In this study, we used a cortical stab wound assay to demonstrate an upregulation of AQP1 following injury in reactive glia. We were able to mimic such injury in astrocytic cultures and show that AQP1 expression can be induced within 16h following injury in vitro. This induction could be blocked by inhibition of MEK1/2 using U0126 suggests that AQP1 is specifically induced in reactive astrocytes via the MAPK signaling pathway.
INTRODUCTION

Brain edema presents with marked astrocytic swelling whereby individual cells reach nearly twice their normal size. The associated cellular water uptake is believed to occur via AQP4 water channels, the principle aquaporin expressed in astrocytes (Papadopoulos et al. 2002). Immuno-gold electron microscopic studies show high levels of AQP4 on astrocytic endfeet associated with blood vessels (Nielsen et al. 1997; Rash et al. 2004), and AQP4 colocalizes with Kir4.1. It has been suggested that Kir4.1 and AQP4 cooperate in the context of extracellular K⁺ buffering (Kofuji and Newman 2004; Chen and Nicholson 2000; Amiry-Moghaddam et al. 2003). Consistent with this notion, several studies have shown that the loss of AQP4 in astrocytes plays a role in the brain following injury (Zhao et al. 2005; Manley et al. 2000; Papadopoulos et al. 2004). AQP4 appears to play either a beneficial or pathological role in the formation of brain edema following injury. Specifically, following acute water intoxication, AQP4 KO mice showed a reduction in cytotoxic edema suggesting that AQP4 is the mediator of cytotoxic edema (Manley et al. 2000), whereas following vasogenic edema, AQP4 KO mice showed increased edema formation and a worse neurological outcome, implicating astrocytic AQP4 in removal of excess fluid from the surrounding parenchyma (Papadopoulos et al. 2004).

AQP4 levels fluctuate over the time course for edema formation suggesting that there are factors regulating its expression (Badaut et al. 2007; Ribeiro et al. 2006). It has recently been shown that hyperosmolarity leads to an increase in AQP4 expression similar to edemic conditions (Arima et al. 2003) implying a potential role for changes in osmolarity as a mediator of AQP4 protein expression. However, little is known about the
role other AQPs play in the formation of edema. As with AQP4, osmotic changes alter the expression levels of both AQP9 and AQP1 (Arima et al. 2003; Jenq et al. 1999; Umenishi and Schrier 2002; Umenishi and Schrier 2003).

In the CNS, AQP1 expression is highest in the choroid plexus where it functions in the formation of cerebral spinal fluid (Bondy et al. 1993; Hasegawa et al. 1994). Recent studies have shown that AQP1 colocalizes with nociceptors in dorsal root ganglia, along the axon as well as at the synapse (Shields et al. 2007). This suggests AQP1 plays a role in regulating the osmotic changes associated with rapid ionic changes found during neuronal firing. Further, chronic osmotic changes have been shown to upregulate AQP1 expression in kidney cells (Jenq et al. 1999; Umenishi and Schrier 2002; Umenishi and Schrier 2003). The activation of an osmotic response element (ORE) located in the promoter region leads to gene transcription and AQP1 upregulation (Umenishi and Schrier 2002), and these changes were shown to be regulated by mitogen-activated protein kinases (Umenishi and Schrier 2003).

Mitogen-activated protein kinases (MAPK) are serine/threonine specific protein kinases activated by extracellular factors and function in various cellular roles, i.e. gene expression, cell survival, apoptosis (Carriere et al. 2008; Hebert and O'Callaghan 2000; Castigli et al. 2000). Some growth factor stimulated responses are mediated through the MAPK signaling pathway, i.e. (Santos et al. 2007; Katsura et al. 2008). During injury, MAPK signaling is upregulated within the first few minutes of insult, spreading to surrounding areas and is important in regulating the process of reactive gliosis (Lim et al. 2007; Mandell et al. 2001).
In this study, we investigated the hypothesis that changes in AQP1 expression following injury are regulated by MAP kinases. Consistent with previous results (Suzuki et al. 2006), we found an increase in AQP1 expression in astrocytes in rats following cortical stab injury. Through Western blotting, immunostaining and cell volume experiments, the expression and upregulation of AQP1 in astrocyte cultures was examined to determine the role of MAPK in its expression. We found that AQP1 is expressed in primary astrocyte cultures and is lost following the first passage. Consistent with these results, primary astrocyte cultures exhibit a HgCl$_2$ sensitive water permeability indicative of AQP1 expression. This expression can be rescued using the \textit{in vitro} wound assay in passaged astrocytes. The upregulation can be prevented by using U0126, a MEK1/2 inhibitor suggesting that an increase in AQP1 expression following injury is due to signaling through MAPK.
MATERIALS AND METHODS

Cell Culture. Primary cortical astrocytes were obtained from Sprague-Dawley rats at postnatal day 0-1 by modification of the technique described previously (McCarthy and deVellis 1980). The brain tissue was dissected in ice-cold DMEM (Media Tech), supplemented with 20mM glucose, L-glutamine, and antibiotics and fungicide (ABF), and the cortex was removed from surrounding regions. The meninges were then removed, and the tissue was tritured in O_2-saturated papain (Worthington, Lakewood, NJ) with 2-5mg DNase (Worthington) and placed in 37°C for 20min. The cells were kept at 37°C in a 95% O_2/5% CO_2 atmosphere. Medium was replaced the next day and every third day thereafter. Cells were allowed to grow for 7-10 days. For passaged astrocytes primary astrocytes were shaken overnight at 37°C at 200 rpm, trypsinized and replated. Secondary astrocyte cultures were used one week after plating. Cells were grown in Dulbecco’s modified Eagle medium (DMEM; Media Tech, University of Alabama at Birmingham Media Preparation Facility) and supplemented with 2 mM glutamine (Media Tech) and 7% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT) at 37°C and 95% O_2/5% CO_2 humidified environment.

RNA Isolation and PCR. Messenger RNA was extracted from the various cell lines following the RNAqueous protocol (Ambion, Austin, TX). Briefly, the cells were lysed, homogenized, and centrifuged. The supernatant was removed and an equal volume of 64% ethanol was added. The mixture was filtered via centrifugation and the filters were washed. The mRNA was eluted from the filter and DNA-free (Ambion) was used to remove contaminating DNA. RNA quality was evaluated by electrophoresis through 1.5% agarose gels.
The cDNA was synthesized and amplified using the OneStep RT-PCR kit (Qiagen, Valencia, CA), per manufacturer’s instructions, by the Eppendorf Mastercycler gradient (Brinkmann Instruments, Westbury, NY). Oligonucleotide primers (Invitrogen, Carlsbad, CA) were designed to amplify specific AQPs and used the sequences described previously (Wang et al. 2003) and listed in Table 1, and 2 ng of mRNA was loaded per reaction. PCR conditions were as follows; denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 2 min, with a final extension at 72°C for 10 min. Amplified products were electrophoresed through 1.5% agarose gels to determine size. Controls were used for all aquaporins. Kidney cDNA was used for AQP1 and AQP4. Actin was used as loading controls.

*Western Blot Analysis.* A confluent dish of cells was lysed using RIPA buffer [(50 mM TrisCl, pH 7.5, 150 mM NaCl, 1% Nondet P-40 (NP-40), 0.5% sodium deoxycholate, 1% sodium dodecyl sulfate (SDS)] supplemented with protease inhibitor cocktail (Sigma). Cells were sonicated for 10 s and centrifuged at 14,000 rpm for 10 min, and the supernatant was transferred to a new tube. Protein quantification was performed using a DC protein assay kit (BioRad, Hercules, CA). An equal amount of 6× sample buffer containing 600 mM β-mercaptoethanol was added to the 20-30 µg/ml of cell lysate per lane. Samples were loaded into a 10% precast SDS-PAGE gel (BioRad). Protein separation was obtained using a constant 100 V for 80 min, and the gels were transferred at 200 mA for 2 hrs at room temperature onto polyvinylidene difluoride (PVDF) paper (Millipore, Bedford, MA). Membranes were blocked in blocking buffer (3% nonfat dried milk in TBS plus 0.1% Tween 20). All antibodies were obtained from Chemicon (Temecula, CA) and used following manufacturer’s instructions. Membranes were
incubated in primary antibody for 1 hr at room temperature and washed 3× for 10 min. The membranes were then incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma) at 1:1000 for 1 hr followed by another round of washing (3× 10 min) and developed using Luminol (Santa Cruz, Santa Cruz, CA). Images were taken on Kodak Imager (Rochester, NY) and analyzed using Kodak Imager software.

**Immunocytochemistry.** Cells plated on coverslips were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 10 min. Cells were washed 2× 10 min in PBS, blocked and then permeabilized in PBS containing 0.3% Triton X-100 and 10% horse serum (HS) for 30 min. Cells were incubated overnight at 4ºC in primary AQP antibodies at 1:500 and mouse anti-GFAP. On the following day, astrocytes were washed in PBS (4× 5 min), blocked again and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (Molecular Probes) diluted at 1:500 and tetramethyl rhodamine iso-thiocyanate (TRITC)-conjugated goat anti-mouse at 1:750 in the dark for 1 hr. Cells were washed in PBS (2× 5 min) and incubated with DAPI, a fluorescent nuclear label, (1:2000, Sigma) for 5 min. Cells were washed 2 more times with PBS and mounted on slides with GelMount (Biomedia, Foster City, CA). Images were acquired using an inverted Olympus IX-81 spinning disk confocal microscope (Olympus, Center Valley, PA).

**Volume Regulation.** Cell volume measurements were performed using a Coulter Counter Multisizer 3 (Beckman-Coulter, Miami, FL) as described previously (Parkerson and Sontheimer 2003) and modified (McCoy and Sontheimer 2007). Cells were washed in PBS and lifted from the dish using 0.05% trypsin and 0.53mM EDTA. Trypsin was inactivated with the addition of an equal volume of serum-containing media and cells
were briefly centrifuged to pellet. Cells were resuspended in bath solution [125 mM NaCl, 5.0 mM KCl, 1.2 mM MgSO\(_4\), 1.6 mM Na\(_2\)HPO\(_4\), 0.4 mM NaH\(_2\)PO\(_4\), 10.5 mM glucose, 32.5 mM HEPES (acid), 1.0 mM CaCl\(_2\), pH 7.4, 300 ± 10 mosmol]. Osmolarity for solutions was measured by a freeze point osmometer (Fiske Micro-Osmometer 210; Fiske-Associates, Norwood, MA). Cells were equilibrated for approximately 5 min before first reading, and readings were taken continuously for 3 min. Bath solution was made hyposmotic with the addition of water. Mercuric chloride (HgCl\(_2\), Sigma) was made at 300 µM in bath solution and cells were preincubated for 5 min. Data were collected by Multisizer 3 software, and 5000 pulse listings were exported to EXCEL as the average of 40-50 cells for each 20 ms timepoint. Data were collected as mean diameter and were converted to mean cell volume. Mean cell volumes were normalized to baseline values. Data were plotted in Origin 7.0 (MicroCal, Northhampton, MA) ± se with (n) experiments performed. Each time point graphed is an average of the mean cell volume for 40-50 cells per 20 ms. Each graph for a 3 min experiment contained 5000 data points.

**Wound Assay.** Astrocytes were grown to a confluent monolayer and scratches were made using a 200 µl pipette tip. Cells were allowed to recover and migrate into the wound site for 24 hrs in the presence and absence of 2 µM U0126. Cells were fixed and immunostained for GFAP and either AQP1 or AQP4 as stated previously or for Western blotting, lysates were collected from scratched 10 cm\(^3\) dishes and probed for mouse anti-MEK1/2, phospho-MEK1/2, ERK1, ERK2, or p44/p42 (Cell Signaling Technology, Danvers, MA).
*Cortical Stab Wound.* We obtained 6 week old male Spague-Dawley rats. Animals were anesthetized using isofluorane and a 30-gauge syringe was stereotactically inserted 2 mm intracranially. Animals were transcardially perfused 24 hr post injury with 4% paraformaldehyde. Brains were additionally fixed overnight at 4°C and sectioned coronally at 100 µm using a series 1000 vibratome (Vibratome, St. Louis, MO) and stained as described previously in methods.
RESULTS

*AQP1 is upregulated in reactive glia following cortical stab injuries.*

The primary objective of this study was to examine changes in the expression of AQPs in response to injury, and how the changes may be regulated. Published data suggest that the predominant AQP in astrocytes is AQP4, yet several reports find expression of AQP1 in brain after injury (Suzuki et al. 2006; Badaut et al. 2003). As a starting point for our inquiry, we utilized a cortical stab injury *in vivo* to document the appearance of AQP1 within 24h after injury. As illustrated in Fig. 1, AQP1 expression was localized to cell clusters rather than throughout the gliotic tissue suggesting that AQP1 upregulation may be exclusive to select populations of astrocytes. For comparison, sections from the contralateral uninjured brain do not show detectable AQP1 immunoreactivity (Fig. 1). Similar observations were reported in other injury models, i.e. brain contusion (Suzuki et al. 2006), peritumoral edema and subarachnoid hemorrhage (Badaut et al. 2003).

*Primary astrocytes demonstrate AQP1 protein expression.*

To examine the regulation of AQPs by injury, we sought to mimic an injury condition in primary cultured monolayers of astrocytes. As a first step towards examining this preparation, we used PCR, Western blot and immunohistochemistry to assess the complement of AQPs in these cultures. When examined after 7-10 day primary astrocyte cultures obtained from p0 rats showed prominent expression of AQP1 and AQP4 with all detection methods (Fig. 2). This contradicts previous publications that claim an absence of AQP1 in cultured astrocytes (Nakahama et al. 1999; Nicchia et al. 2000). The latter
Figure 1. AQP1 expression in astrocytes in a cortical stab wound injury. (A) Representative image of the lesion site. (B) Fluorescence images showing GFAP staining at lesion site indicating reactive gliosis. Using immunofluorescence, we found an upregulation of AQP1 in cortical astrocytes around the site of injury but not in the contralateral side (B). (Arrows indicate site of lesion; Green = AQP1, Red = GFAP, Blue = DAPI)
Figure 2. AQP expression in astrocyte cultures. Aquaporin mRNA expression in astrocytes. All panels show a 1.5% agarose gel stained with ethidium bromide following amplification (A). Cell lysates were separated using 10% SDS-PAGE gels and transferred onto PVDF paper and probed with antibodies. Western blot analysis was used to examine AQP1 and AQP4 expression levels in primary and passaged astrocyte cultures (B). Immunofluorescence showed expression of AQP1 in primary but not passaged astrocyte cultures (C). (Green- AQP, Red- GFAP, Blue- DAPI)
studies, as most studies in this field, utilized passaged, secondary or tertiary cultures of astrocytes. We, therefore, passaged our astrocytes and once again ran them side-by-side with primary astrocytes on Western blot and immunostainings. As illustrated in Fig. 2B, AQP1 protein expression was selectively lost upon passaging of astrocytes, though there were detectable mRNA levels suggesting that AQP1 protein is not being translated into protein. Immunostaining in Fig. 2C shows punctuate expression of AQP4 in both the primary and passaged astrocytes, whereas AQP1 shows nice expression in primary astrocyte cultures and is absent in passaged cultures. This, therefore, gives us an opportunity to examine a model system in which both AQP1 and AQP4 are natively expressed versus one in which only AQP4 is present, the latter mimicking adult brain.

Primary astrocytes exhibit mercury sensitive cell swelling following a hypo osmotic challenge.

It also was important to question the degree to which each of these AQPs contributes to homeostasis in astrocytes, i.e. indicative of functional AQPs. To determine if there were changes to the water permeability in passaged astrocytes expressing only AQP4 as compared to primary cultures expressing both AQP1 and AQP4, we examined water transport directly by measuring cell swelling in response to a hypo osmotic challenge with a Coulter-Counter Cell Sizer. Cell size was measured continuously, with 20 msec time resolution over a 3 min period. Here a 60 s baseline volume was followed by the addition of a 50% hypo osmotic challenge and recorded for an additional 2 min (see methods for detail). The average cell volume +/- S.E.M. for 1000 cells each was plotted every 20 msec. Figure 3 shows volume changes caused by water movement in
Figure 3. Functional expression of AQP1 and AQP4 in astrocytes. Mean cell volume was measured for 3 min in primary (n=6) and passaged astrocyte cultures (n= 6) following a 50% hypo osmotic challenge. HgCl$_2$ was used at 300 µM. Primary astrocytes (A) have mercury sensitive water movement that is not found in the passaged astrocytes (B).
primary and passaged astrocyte cultures (Fig 3A,B respectively). Both cell types showed a rapid influx of water following the hypo osmotic challenge indicative of the expression of functional aquaporins. Primary astrocytes in which AQP1 function was inhibited by 300 µM HgCl₂ (Fig 3A; Jung, J.S. et. al., 1994), showed a reduced rate of cell swelling as compared to passaged astrocytes lacking AQP1 expression (Fig 2B). These data indicate that primary astrocytes utilize AQP1 as the predominant aquaporin for water movement. These studies also show that the degree and time-course of swelling was identical in primary and passaged cells suggesting that AQP4 alone can confer the same water permeability as AQP1 and AQP4 together in primary astrocytes.

*Astrocytes upregulate AQP1 following injury.*

Based on the above studies, secondary astrocyte cultures, expressing exclusively AQP4 should be a suitable model system to mimic such injury in vitro. We employed the use of an artificial wound assay in which a 200 µl pipette tip was used to produce multiple scratches through a confluent monolayer of astrocytes. Cells were then, allowed to recover for 10 min, 30 min, 60 min, 16 hr and 24 hr prior to harvesting cell lysates for Western blot. As shown in Fig. 4A, AQP1 became detectable 16h after injury and was prominently expressed at 24h. This could also be seen by immunostaining of astrocytes at the lesion 24h after injury (Fig. 4B) yet no AQP1 positive cells were detectable 30 minutes after a lesion. Cell nuclei are visible by DAPI staining. Mechanical injury and the ensuing gliosis is a hallmark of other neurological diseases including Alzheimer’s disease (Unger 1998), Creutzfeldt-Jakob (Rodriguez et al. 2006), ALS, Parkinson’s disease and multiple sclerosis (Drew et al. 2006). Gliosis has also been shown to activate
Figure 4. AQP1 expression increases in passaged astrocytes following an *in vitro* wound assay. A confluent monolayer of astrocytes was scratched using a 200 µl pipette tip and allowed to recover for 10 min, 30 min, 60 min, 16 hr, and 24 hr and protein expression was examined using Western blotting. AQP1 expression was detectable at 16 hr and was maintained at 24 hr (A). The addition of U0126 abolished the upregulation of AQP1. Immunofluorescence confirms the Western blot results (B,C). AQP1 expression was seen in astrocytes at the lesion at 24 hr but not at 30 min, and U0126 blocked AQP1 expression at 24 hr. (Green- AQP, Blue- DAPI)
multiple signaling cascades in astrocytes including the MAPK signaling pathway, which had been suggested as a possible regulator of AQP s (Umenishi and Schrier 2003; Arima et al. 2003). To examine the possibility that MAPK activation may underlie the observed induction of AQP1 after injury, we repeated the above experiments in the presence of 2 µM U0126, a MEK1/2 inhibitor. As illustrated in Fig. 4 A & C, inhibition of MAPK signaling completely inhibited the induction of AQP1 expression following injury and no AQP1 positive cells could be identified at the lesion by immunohistochemistry. However, western blotting revealed an increase in pMEK1/2 and p44/p42 at 10 min, 30 min and 60 min, which was subsequently lost at later time points and this increase in activity was inhibited by addition of U0126 (Fig 5). Further, immunostaining for MEK1/2, ERK1/2, p44/p42 and pMEK1/2 at the lesion site revealed an increase in pMEK1/2 and p44/p42 at 30 min which was absent by 24 hr (Fig 6) without changes in the overall MEK1/2 and ERK1/2 levels. This data suggests that the MAPK signaling pathway regulates the upregulation of AQP1 expression following injury.

DISCUSSION

In this study, we were able to show by Western blotting and immunostaining that upregulation of AQP1 in astrocytes following injury is due to activation of the MAPK signaling pathway. Previous work has shown that AQP1 protein expression is regulated by MAPK signaling following a chronic hypertonic challenge (Umenishi and Schrier 2003) which occurs in a time-dependent manner similar to the data in this paper. Following injury, we discovered an increase in phosphorylation of MEK1/2, ERK1 and ERK2 which occurs by 30 min, extending to 60 min but was absent by 16 hr. However,
Figure 5. MAPK expression is upregulated immediately following injury. Western blot analysis shows increased expression of pMEK1/2 and p44/p42 within 60 min following injury which can be inhibited by U0126.
Figure 6. Expression of MAPK signaling molecules over 24 hr time course in a wound assay using passaged astrocytes. Immunofluorescence showed expression of MEK1/2 and ERK1/2 at both 30 min and 24 hr time points in migrating astrocytes. However, their phosphorylated forms were only detected at the 30 min time point. (Green-MAPK, Blue-DAPI)
high expression of AQP1 was found to be present at 16 hr and 24 hr time points. These effects could be inhibited by pretreating the cells with U0126, a MEK1/2 inhibitor. Additionally, we discovered that primary astrocytes express both AQP1 and AQP4 while passaged astrocytes express only AQP4. These results are consistent with published work in human astrocytes where AQP1 and AQP4 are both expressed in vitro and in vivo in normal and diseased brain (1). However, water permeability in primary astrocytes was mediated principally through AQP1 as illustrated by its increased sensitivity to HgCl₂ which only blocks AQP1.

MAPK signaling is important for regulating gene transcription and differentiation and cellular response to growth factors. Specifically in astrocytes, GFAP upregulation is dependent on MAPK signaling (O'Callaghan et al. 1998) and studies suggest that the process of reactive gliosis is as well (Mandell et al. 2001; Mandell and VandenBerg 1999). Several AQPs have also been shown to be regulated by MAPK. Specifically, AQP1 and AQP5 upregulation following chronic hypertonicity is due to an increased activation of MAPK (Umenishi and Schrier 2003; Hoffert et al. 2000), and AQP4 and AQP9 through hyperosmolar conditions (Arima et al. 2003). Following subarachnoid hemorrhage, AQP1 expression is increased around blood vessels due to an increased activation of JNK signaling (Yatsushige et al. 2007), and AQP1 upregulation is thought to mediate the increase in vascular permeability found in SAH, which is consistent with this study where AQP1 becomes the predominant water channel for mediating water permeability in primary cortical astrocyte cultures. Therefore, inhibition of MAPK signaling may have both positive and negative therapeutic effects. Specifically, if MAPK is regulating the process of reactive gliosis, then blocking AQP1 expression by blocking
MEK1/2 following injury would prevent proper formation of the glial scar and possibly causing more neurological damage. AQP1 could also indicate a more migratory phenotype for astrocytes necessary for glial scar formation leading to a delay in injury repair. However, if AQP1 expression in reactive astrocytes is leading to increase edema during the later stages, blocking its expression could alleviate the damage that occurs in the later stages of injury as in a stroke.

Previous reports have shown an increased expression of AQP1 in reactive astrocytes following injury (Suzuki et al. 2006) and we were able to show this in response to a cortical stab wound. Importantly, AQP1 can be modulated by changes in hypertonicity. Previous studies have shown an increase in AQP1 protein expression following a chronic hypertonic challenge (Jenq et al. 1999) and this upregulation is due to an osmotic response element (ORE) located in the promoter region (Umenishi and Schrier 2002). This binding domain is common among several other channels and transporters known to be important in regulating cell volume, i.e. sodium/myo-inositol cotransporter, sodium/chloride/betaine cotransporter and aldose reductase {8250}. It is likely that astrocytes associated with gliotic lesions are also exposed to changes in extracellular osmotic milieu, i.e. edema, and the observed upregulation of AQP1 may serve to better equip them with the channels required for proper volume regulation. Since AQP1 expression is not found until around 16 hrs in astrocytes following injury, it would be necessary for controlling the late phases of brain edema while early phase edema water movement would be dictated by AQP4 expression and function. Indeed, our studies suggest that in astrocytes expressing AQP1 and AQP4, the majority of water
transport occurs via AQP1, which may have a preferential role in water movement under edemic conditions.

ACKNOWLEDGEMENTS

This work was supported by NIH 5R01NS031234 and NIH 2R01NS036692.
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DISCUSSION

Cells undergo profound changes in cell volume during stages of their development including during cell migration and proliferation. Aquaporins are specialized water channel that rapidly move water across the plasma membrane and aid in volume regulation. Primary gliomas, which are of glial origin, are characterized as being highly invasive and proliferative, two processes that require profound changes in cell volume, and therefore they would be expected to express high levels of AQPs to support these functions. We examined AQP expression in gliomas by PCR, Western blotting, and immunostaining and determined that primary patient-derived gliomas express AQP1 and AQP4. Interestingly, glioma cell lines used by us and others lacked expression of AQP4 and had variable expression of AQP1. Reintroducing AQP1 into gliomas, by transfection, increased cell migration by increasing water permeability, while AQP4 expression reduced cell migration correlating with an increase in cell adhesion. To understand this decreased migration in AQP4-expressing tumors, we examined several cell signaling pathways that are upregulated in disease and pathological states. Decreased tumor migration in AQP4-expressing gliomas was dependent on the activation of PKC, and its phosphorylation of AQP4 at S180.

As an extension of this study, we examined the expression of AQPs in reactive astrocytes, a second glial pathology. Reactive astrocytes show enhanced migration and proliferation, characteristics commonly found in gliomas. Using a cortical stab injury as a model for reactive gliosis, we demonstrated AQP1 expression is increased in reactive astrocytes within 24 hrs of injury. We examined the signaling mechanisms involved in
the upregulation of AQP1 in reactive astrocytes. MAPK signaling has been shown to be activated during reactive gliosis and injury. Our results demonstrated that AQP1 expression in reactive astrocytes requires activation of the MAPK pathway. Further, inhibition of this pathway prevented expression of AQP1.

Multiple AQPs are Expressed in Reactive and Malignant Astrocytes

Gliomas and astrocytes have a highly evolved volume regulatory mechanism allowing the cell to undergo rapid changes in cell volume. Volume regulation requires the movement of water across the cell membrane, and AQP expression would greatly enhance the cell’s water permeability and therefore, the cell’s ability to volume regulate. Specifically, cell proliferation, migration and apoptosis have all been shown to involve this mechanism (Bussolati, O. et. al., 1996; Dubois, J.M. and Rouzaire-Dubois, B., 2004; Ernest, N.J. et. al., 2008; Iwamoto, L.M. et. al., 2004; McCoy, E. and Sontheimer, H., 2007; McManus, M.L. et. al., 1995; Muller, R. et. al., 1993). Astrocytes become reactive during pathological states and upregulate AQP1 and expressing both AQP1 and AQP4. They also undertake several functions not attributed to normal astrocytes, such as proliferation and migration. Our studies, and others (Badaut, J. et. al., 2003; Endo, M. et. al., 1999; Markert, J.M. et. al., 2001; McCoy, E. and Sontheimer, H., 2007; Saadoun, S. et. al., 2002a; Saadoun, S. et. al., 2002b), have shown that in primary patient-derived gliomas both AQP1 and AQP4 are highly expressed as with reactive astrocytes, yet their expression is heterogeneous throughout the tumor population. This is also consistent with AQP1 expression in reactive astrocytes where it is expressed in clusters of cells (data not shown). Since gliomas are derived from glial cells, it is possible that many of
the features commonly found in gliomas coincide with reactive astrocytes, like enhanced proliferation and invasion.

This opens the possibility that AQP1 and AQP4 may have separate functions in tumor and glial biology. In previous studies examining expression of multiple AQPs in a single cell, when one AQP was knocked out, the second AQP did not recover function of the missing AQP. In some cases, the expression of the second is lost as well (Verkman, A.S. et al., 2000). This suggests that AQPs do not compensate for the loss of another AQP. Therefore, we would predict that in gliomas and reactive astrocytes AQPs would be performing two separate functions independent of the other in regards to proliferation, migration, or volume regulation and edema.

**AQP4 Migration is Limited by PKC Activity and Cell Adhesion in Gliomas**

AQP4 has been extensively studied in the CNS. It is known to be highly expressed in astrocytes where it plays a role in fluid movement associated with the formation and removal of edema, maintaining water balance following action potentials, and astrocyte migration (Manley, G.T. et al., 2000; Papadopoulos, M.C. et al., 2004; Saadoun, S. et al., 2005; Vitellaro-Zuccarello, L. et al., 2005). Unfortunately, little is known about AQP4 function in gliomas. In this thesis, we demonstrated that AQP4 expression enhances glioma water permeability, consistent with previous studies (McCoy, E. and Sontheimer, H., 2007; Saadoun, S. et al., 2005; Tang, Y. et al., 2007; Thi, M.M. et al., 2008). However, our results showed a reduction in tumor cell migration in AQP4-expressing gliomas, where others have shown a reduction in migration in AQP4−/− astrocytes (Saadoun, S. et al., 2005). While their data suggest that AQP4 expression
enhances migration and invasion, astrocytes have lower levels of PKC as compared to primary gliomas. We demonstrated that part of the reduction in migration was due to the increased activity of PKC.

PKC is activated by several G-protein coupled receptor pathways, including growth factor receptors, and this activation involves increases in Ca$^{2+}$. We have demonstrated high expression of PKC in gliomas, and PKC activation reduces AQP4-mediated water permeability. When we pharmacologically inhibited the phosphorylation of AQP4, we could enhance the water permeability of gliomas, which correlated with an increase in the rate of migration. This suggested that PKC activity is directly regulating AQP4-mediated migration. In further support, we found similar results when we removed the phosphorylation site, S180.

The importance of PKC activity in the context of tumor migration is emphasized by examining the effect of the thrombin data. Our data showed that AQP4-expressing tumors treated with thrombin had an increase in the phosphorylation state of AQP4 and a reduction in water permeability and migration. Thrombin is a PKC activator found in high levels in the blood but low levels in the brain. When tumor cells disrupt the BBB, thrombin follows its gradient into the surrounding brain tissue leading to an activation of PKC in tumors. This would in turn lead to a reduction in water permeability and tumor migration. Making it is possible that PKC activity is preventing the tumor from invading into regions of high edema formation. However, if these gliomas are encountering natural PKC activators that could inhibit tumor invasion, they could also encounter PKC inhibitors to enhance glioma invasion allowing tumors to invade into favorable brain regions.
Interestingly, even when the PKC activity is decreased, AQP1-expressing tumors are still more invasive than AQP4-expressing tumors suggesting that there is another mechanism involved in AQP4-mediated tumor migration. It is possible that cell adhesion is regulating tumor migration. In this thesis, we demonstrated that the expression of AQP4 in tumors enhances cell adhesion. PKC is known to regulate cell adhesion through its phosphorylation of focal adhesion kinase. However, we found that inhibiting PKC activity did not alter the enhanced cell adhesion associated with AQP4 suggesting that PKC activity is only responsible for regulating water permeability not cell adhesion. If the adhesion site on AQP4 was eliminated, then I would hypothesize that AQP4 expressing tumors would be more invasive, and may even reach the invasiveness of AQP1.

*AQP1 Expression in Tumor Cells Leads to a More Invasive Phenotype*

Primary gliomas are highly invasive tumors and consist of heterogeneous cell populations. There is no consensus on what particular cell population is responsible for the invasion into the surrounding parenchyma. However, in primary gliomas, AQP1 expression is heterogeneous throughout the tumor mass with variable expression throughout the tumor (Saadoun, S. *et. al.*, 2002a;Saadoun, S. *et. al.*, 2002b). This heterogeneous expression found within tumors may represent two separate phases of tumor biology. One set of cells could be indicative of an invasive state.

As discussed in Chapters 1 and 2 of this thesis, AQP1 expression in gliomas increased tumor migration and invasion. These AQP1-expressing tumor cells were able to migrate/invade nearly twice as far as control tumor cells lacking AQP expression.
They were also more invasive than gliomas expressing AQP4, also shown to be highly expressed in primary gliomas (Saadoun, S. et. al., 2002a; Saadoun, S. et. al., 2002b). This enhanced invasiveness elicited by expression of AQP1 suggests that AQP1-expressing tumors are responsible for forming satellite colonies. These cells were able to migrate great distances (>1500 µm) in a mouse model of glioma tumors, similar to the invasion seen in human patients.

The importance of these findings is the indication that AQP1 expression labels invading tumors, consistent with previous reports demonstrating increased invasion in AQP1-expressing melanoma cells (Hu, J. and Verkman, A.S., 2006). If AQP1 expression is indicative of a more invasive phenotype, these cells would be responsible for establishing the satellite colonies so characteristic of primary glioblastomas. In addition, tumor areas containing high AQP1 expression could be used as a therapeutic target to potentially invasive tumor regions.

*How AQP-Mediated Water Movement Enhances Cell Migration*

How do AQPs enhance cell migration? Several models for cell invasion suggest that salt along with obligated water movement mediates rapid shape/volume changes required for cell migration (Soroceanu, L. et. al., 1999). Although water is capable of moving through the lipid bilayer, expression of AQPs confers enhanced water movement across the plasma membrane. This change in water permeability allows the cell to rapidly move water into and out of the cell in areas of osmotic changes. AQP expression increases the number and turnover rate of filopodia (Loitto, V.M. et. al., 2007). A migrating cell first invades the extracellular environment by extending filopodia,
suggesting that expression of water channels enhances the cell’s ability to invade the local area. AQP expression may also facilitate rapid changes in cell volume required for cell advancement through confined spaces. An invading cell needs to rapidly change its volume. Initially, a cell rapidly effluxes osmolytes and water at the leading edge to shrink and extend into a confined space. As the cell continues to move its body through the confined space, multiple sites of cell volume changes are necessary. First, the leading edge will increase its cell volume by influxing osmolytes and water as it exits the confined spaces, while the next section of the cell entering the confined space will shrink. Also, the lagging edge will be changing its volume when releasing its cell adhesion points to accommodate the moving cell. Therefore, as the cell continues to invade, AQP expression throughout the cell membrane would be necessary so that all parts of the cell will be able to quickly change their volume.

*Other Roles for AQP4 in Gliomas*

If AQP4 expression in tumors may not be involved in cell invasion, it may function in fluid removal associated with edema. Warth *et al* (2007) noted that AQP4 expression was localized to the tumor mass and not to infiltration zones, but AQP4 expression correlated with the level of peritumoral edema in glioma patients (Warth, A. *et. al.*, 2007). This suggests two possibilities in regards to AQP4 and edema. First, AQP4 is regulated by chronic changes in osmolarity (Arima, H. *et. al.*, 2003), which occurs during edema formation. The osmotic changes associated with the tumor mass lead to changes in AQP4 expression allowing the tumor to remove fluid from the interstitial space. However, if infiltration zones are localized to regions of edema
formation, then the absence of AQP4 would indicate that it does not have a role in fluid removal associated with vasogenic edema during invasion and is not being regulated by changes in the osmotic environment. This would confound the idea that AQP4 is regulating peritumoral edemic fluid. If AQP4 is not important in infiltration zones where there is increased edema, then AQP4 expression is not being regulated by osmotic changes and must have an alternate role in tumor biology.

AQP4 expression may be dependent on vascular proliferation. AQP4 expression was found to be highest in WHO grades I, III, and IV gliomas (Warth, A. et. al., 2007). Grades III and IV are different in most aspects of tumor biology as compared to grade I pilocytic astrocytomas, specifically in proliferation and invasion. However, grades I, III, and IV form glomeruloid bodies, and it is possible that AQP4 expression may be associated with the formation of new vessels or glomeruloid bodies where neoplastic cells have been suggested to react to the atypical vessel formation (Davies, D.C., 2002; Saadoun, S. et. al., 2002b). In addition, factors are released during vascularization that may increase AQP4 expression. Recently, in reactive astrocytes, increased expression of AQP1 and AQP4 were associated with increased vascular endothelial growth factor (VEGF) following injury (Suzuki, R. et. al., 2006). VEGF may directly cause AQP4 expression. However, studies from our lab have not been able to increase AQP4 expression in gliomas using VEGF (data not shown). Since VEGF secretion causes opening of gap junctions and BBB breakdown, AQP4 expression may be due to either a change in the local osmotic environment or a cellular reaction to the formation of the new vasculature.
**AQP1 and Edema in Reactive Astrocytes**

AQP1 expression in reactive astrocytes may be necessary to remove vasogenic edema fluid. The role in fluid movement associated with edema has been well established for AQP4 in astrocytes (Manley, G.T. *et. al.*, 2000; Papadopoulos, M.C. *et. al.*, 2004; Ribeiro, M.C. *et. al.*, 2006). As stated previously, AQP4 expression in astrocytes has been demonstrated to be beneficial for alleviating vasogenic edema following cryo-lesion (Papadopoulos, M.C. *et. al.*, 2004), and its expression has been shown to increase within 1 hr of a traumatic injury (Ribeiro, M.C. *et. al.*, 2006). In contrast, our data shows that the upregulation of AQP1 occurs in astrocytes on a much slower time scale (~24 hrs post lesion). Previous reports have shown that AQP1 expression is regulated by osmotic changes (Jenq, W. *et. al.*, 1999; Umenishi, F. and Schrier, R.W., 2002; Umenishi, F. and Schrier, R.W., 2003), which are associated with edema formation. This suggests that the expression of AQP1 in reactive astrocyte may be linked directly to the changing osmotic environment following injury. Interestingly, AQP1 expression is found in clusters of astrocytes around the lesion site. It is possible that these regions may have increased edema. To the detriment of the astrocyte, the increased expression of AQP1 would be important for removing fluid from the interstitial space characteristic of vasogenic edema. AQP1 upregulation has been correlated with an increase in vascular endothelial growth factor (VEGF) expression (Suzuki, R. *et. al.*, 2006). This formation of new blood vessels would cause localized edema, and AQP1 expression could be increased to alleviate the excess fluid.
**AQP1 and Proliferation**

One characteristic of both reactive astrocytes and gliomas is that they are not only invasive, they are also proliferative. Cell cycle progression and proliferation necessitates dynamic changes in cell volume, which requires water movement across the cell membrane. As the cell proceeds towards division, it must reach a certain size (Dubois, J.M. and Rouzaire-Dubois, B., 2004; Habela, C.W. and Sontheimer, H., 2007). AQP expression could enhance the time it takes the cell to reach its optimal size, and therefore enhance the rate of proliferation. Previous work has demonstrated that AQP1 expression enhanced proliferation in lung carcinoma cells (Hoque, M.O. *et al.*, 2006), suggesting that AQP1 could enhance cell proliferation in both gliomas and reactive astrocytes. Consistent with this idea, our data indicates higher proliferation rates in gliomas transfected with AQP1 (data not shown). We have also demonstrated that tumor cells undergo relatively rapid volume decrease just prior to cell division (Habela, C.W. and Sontheimer, H., 2007) that is dependent on Cl$^-$ efflux. AQP1 may be responsible for the obligated water movement associated with the Cl$^-$ efflux, which would allow gliomas and reactive astrocytes to reach their optimal cell volume in order to undergo cell division and therefore, act to increase the rate of division. If AQP1 expression enhances proliferation and cell division, then it would be expected that it is absent in normal astrocytes where there is little cell division, whereas reactive astrocytes need to proliferate rapidly and thereby express AQP1. Interestingly, the rate of proliferation may be linked to the invasive phenotype in gliomas. If AQP1-expressing gliomas are invading into the surrounding tissue and setting up satellite colonies, then having an enhanced proliferative state would be beneficial. Once the cell reaches an advantageous location, the cell could
rapidly divide to establish a colony to exploit the area and begin the formation of a new tumor mass.

Conclusions

The data provided in this thesis suggests that AQP expression and function is tightly regulated in astrocytes following injury and upon neoplastic transformation. The increased expression and activity of PKC that is characteristic of gliomas, limits AQP4-mediated water permeability and cell migration implying an alternate role for AQP4 in tumor biology. Since AQP1 is heterogeneously expressed in primary gliomas and its expression increases tumor invasiveness both in vitro and in vivo, cells that express AQP1 may be responsible for creating the satellite colonies common among glioblastomas. In addition, AQP1 expression could indicate invasive tumor regions, thereby opening the potential for therapeutics targeting of AQP1. It would be interesting to further explore the role PKC plays in modulating AQP4 function. Since gliomas are highly proliferative, PKC may also modulate tumor proliferation in AQP4-expressing cells. It is not known what region of the AQP4 channel is responsible for its adhesive properties. Since AQP0 acts as a gap junction channel moving water between cells, it would be interesting to determine if AQP4 behaves in a similar manner or if AQP4 interacts with integrins and the cytoskeleton. In astrocytes, AQP4 is associated with α-syntrophin in the dystroglycan complex linking it to the actin cytoskeleton. AQP4 may possibly act as an environmental sensor, associating with integrins and the extracellular matrix. Co-immunoprecipitations could detect whether AQP4 localizes with particular
integrins in cultured astrocytes, or with integrins and extracellular matrix molecules in tissue samples taken from human patients.

Finally, MAPK regulation of AQP1 expression is intriguing. There are numerous molecules released following injury that could activate the MAPK pathway and lead to the increase in AQP1 protein expression. The most likely candidates are growth factor receptors or factors that change the osmotic environment. Also, it would be interesting to note if upregulation of AQP1 expression results in a functional change in astrocytes, either leading to the downregulation of AQP4 expression or a decrease in cell water permeability. These changes would be determined using similar methods as described in my study of MAPK regulation of AQP1.
GENERAL REFERENCE LIST


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APPENDIX A: IACUC APPROVAL FORM
NOTICE OF APPROVAL

DATE: April 7, 2008

TO: Harald Sontheimer, Ph.D.
CIRC-425 0021
FAX: 975-5518

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

SUBJECT: Title: Amino-Acid Transport and the Biology of Human Gliomas
Sponsor: NIH
Animal Project Number: 080407391

On February 29, 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.

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Animal use is scheduled for review one year from April 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) 080407391 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.