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Role of Glutamate Transporters in Redox Homeostasis of the Brain

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Abstract

Redox homeostasis is especially important in the brain where high oxygen consumption produces an abundance of harmful oxidative by-products. Glutathione (GSH) is a tripeptide non-protein thiol. It is the central nervous system's most abundant antioxidant, and the master controller of brain redox homeostasis. The glutamate transporters, System x_c^- (SXC) and the Excitatory Amino Acid Transporters (EAAT), play important, synergistic roles in the synthesis of GSH. In glial cells, SXC mediates the uptake of cystine, which after intracellular reduction to cysteine, reacts with glutamate during the rate-limiting step of GSH synthesis. EAAT3 mediates direct cysteine uptake for neuronal GSH synthesis. SXC and EAAT work in concert in glial cells to provide two intracellular substrates for GSH synthesis, cystine and glutamate. Their cyclical basal function also prevents a buildup of extracellular glutamate, which SXC releases extracellularly in exchange for cystine uptake. Maintaining extracellular glutamate homeostasis is critical to prevent neuronal toxicity, as well as glutamate-mediated SXC inhibition, of which each could lead to a depletion of intracellular GSH and loss of cellular redox control. Many neurological diseases show evidence of GSH dysfunction, and increased GSH has been widely associated with chemotherapy and radiotherapy resistance of gliomas. We present evidence suggesting that gliomas expressing elevated levels of SXC are more reliant on GSH for growth and survival. They have an increased inherent radiation resistance, yet inhibition of SXC can increase tumor sensitivity at low radiation doses. GSH depletion through SXC inhibition may be a viable mechanism to enhance current glioma treatment strategies and make tumors more sensitive to radiation and chemotherapy protocols.

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Keywords

Redox; Glutathione (GSH); System x_c^- (SXC); Excitatory Amino Acid Transporter (EAAT); Reactive Oxygen Species (ROS); Glioma

Introduction

Many important biological processes involve redox reactions, and as a result, produce potentially dangerous byproducts. Oxidative phosphorylation, or oxidative metabolism, provides brain cells with most of their energy requirements. In fact, human brain cells use approximately 20% of the total oxygen consumed by the body, although the brain only makes up 2% of body weight¹. Reactive oxygen species (ROS) are continuously generated as a result of this large usage of oxygen, and therefore mechanisms are required to regulate the redox state of the brain. An unbalanced redox state and buildup of reactive oxygen species results in cell death and detrimental consequences for the brain.

Oxidative stress occurs as a result of an imbalance between oxidant production and neutralization by cellular antioxidants. ROS, as free radical species, are highly reactive compounds. If uncontrolled, they create DNA damage and protein/enzyme oxidation, which leads to cellular dysfunction and death². A number of characteristics of the brain make it more vulnerable to oxidative stress. In addition to the amount of ROS produced by its high oxygen consumption¹, some areas of the brain contain a high content of iron³, which catalyzes ROS generation⁴, and the large amount of unsaturated fatty acid lipids found in the brain are targets for lipid peroxidation^{5,6}. Surprisingly, the levels of activity of common enzymes that catalyze the neutralization of free radicals, specifically superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), are lower in the brain than in other organs such as the liver and kidney⁷.

Common pathologies including neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (ALS) involve ROS-mediated oxidative stress as part of their etiology^{4,8-12}. Even normal aging may be, at least in part, due to ROS. The "free radical theory of aging" hypothesizes that free radical induced cellular damage builds up over time, ultimately resulting in aging and death [see ¹³ for more on this topic]. Many mechanisms exist to regulate redox homeostasis. Here, we focus on the main mechanisms of redox control in the brain. We review the role of glutamate transporters in the production of the intracellular antioxidant glutathione (GSH) and its importance in redox homeostasis. We also discuss the role of glutamate transporter mediated redox imbalance in disease, and present new evidence suggesting their involvement in the radiation resistance of gliomas.

Redox Regulation in the Brain

Reactive oxygen species are generated in cells by the mitochondrial respiratory chain, which occurs on the inner mitochondrial membrane¹⁴⁻¹⁶. Interestingly, although most cancer cells are thought to primarily rely on glycolysis for energy production¹⁷, they produce an even higher level of ROS than normal cells¹⁸. Different theories exist as to how cancer cells

produce ROS, including enhanced metabolism¹⁹, mitochondrial mutations and malfunction²⁰, and chronic inflammation²¹. Regardless, the production of ROS in both non-malignant and malignant cells necessitates the production of intracellular antioxidants to neutralize these free radicals to prevent cell death.

Glutathione

Glutathione (GSH; γ -L-glutamyl-L-cysteinylglycine) is an important non-protein thiol that is found in all organs including the brain^{22–25}. It is the most abundant antioxidant in the central nervous system (CNS), and is found in the brain at concentrations around 1–3 mM⁴. In addition to its antioxidant role, GSH has many non-antioxidant functions. Some of these roles include storage and transport of cysteine²⁶, regulation of apoptosis^{27–29}, cell proliferation³⁰, signal transduction³¹, and immune response³². As a thiol, it consists of three amino acids: L-cysteine (Cys), L-glutamate (Glu), and L-glycine (Gly). γ -glutamylcysteine synthetase mediates the rate-limiting step of GSH synthesis, combining Glu and Cys to form γ -glutamylcysteine (γ GluCys). Glutathione synthetase then incorporates Gly to form GSH. Adenosine triphosphate (ATP) is required for the activity of both of these enzymes^{10,26,33}. Regulation of GSH synthesis is accomplished through a feedback mechanism, where GSH regulates the synthesis of γ -glutamylcysteine synthetase¹³, and, in turn maintains balance of GSH synthesis and consumption.

Intracellularly, GSH undergoes both enzymatic and non-enzymatic reactions during the detoxification of ROS. Non-enzymatically, GSH interacts directly with superoxide anions, hydroxyl radicals, and nitric oxide⁴. Enzymatically, GSH detoxifies peroxidases in a reaction mediated by glutathione peroxidase (Fig. 1). During this reaction GSH is oxidized to glutathione disulfide (GSSG). Glutathione reductase catalyzes the regeneration of GSH, forming a glutathione redox cycle³⁴. In turn, glutathione reductase relies on nicotinamide adenine dinucleotide phosphate (NADPH) to reduce GSSG back to GSH. The redox status of cells depends on the ratio of reduced and oxidized glutathione (GSH/GSSG). Under normal conditions the reduced form exceeds the oxidized form by a ratio of nearly 1:100, however, under oxidative stress conditions, this ratio can be reduced to values as low as 1:1²⁵. As oxidative stress is neutralized, GSSG is regenerated to GSH, and the homeostatic ratio is re-established.

GSH is a critical molecule in the CNS that protects against oxidative stress induced cellular damage. Its loss is associated with brain cell death³⁵. Glutathione is found in neurons and glia^{4,36–38}, including astrocytes⁴, oligodendrocytes³⁹ and microglia⁴⁰. Evidence suggests neurons and glial cells are all vulnerable to ROS damage and that they all rely upon glutathione-mediate detoxification for protection [see ⁴ for a review on the role of glutathione in different brain cells]. Astrocytes are thought to contain the highest concentrations of glutathione in the brain⁴¹, and are therefore considered to play the largest role in ROS detoxification^{42–44}. In culture, astrocytes protect neurons and oligodendrocytes against many toxic compounds^{45–48}, further supporting the idea that astrocytes are the main regulators of oxidative stress in the brain.

Astrocytes extend their protection to neuronal cells through the export of GSH into the extracellular space. Neurons rely on extracellular cysteine for their synthesis of GSH^{22,49,50}

and this cysteine is provided by cysteine-precursors from astrocytic glutathione release⁵¹. The export of GSH is mediated by a family of multidrug resistance proteins (MRPs)^{52–54}. Once released, astroglial ectoenzyme γ -glutamyl transpeptidase (γ GT) acts on GSH, creating a CysGly compound⁵⁵. Aminopeptidase N (ApN) then hydrolyzes CysGly to individual amino acids Cys and Gly⁵⁶, which are both then available for import into neurons.

Glutamate Transporters

System x_c^- —The GSH/GSSG redox cycle uses and recycles intracellular GSH. However, since some intracellular reactions consume GSH and it is released from cells for extracellular functions, intracellular GSH levels would become depleted if not regularly synthesized⁴. Intracellular GSH synthesis requires availability of its amino acid precursors, with the incorporation of cysteine being rate limiting. Cystine (Cys₂) is a dimeric amino acid that is formed by a disulfide bond between two cysteine molecules. Cysteine is not stable extracellularly due to oxidizing conditions that favor cystine formation. However, once cystine is imported, intracellular conditions support the rapid reduction of Cys₂ to Cys⁵⁷, providing substrate for GSH synthesis.

Cystine is taken up into cells by a glutamate transporter called System x_c^- (SXC) (Fig. 2). SXC is a Na⁺-independent, Cl[−]-dependent cystine/glutamate exchanger. The uptake of cystine is coupled to the release of glutamate extracellularly in a 1:1, electroneutral manner⁵⁸. Belonging to the heteromeric amino acid transporter (HAT) family, SXC is composed of two subunits coupled by a disulfide bridge. The light subunit, xCT, confers transporter activity and the heavy subunit, CD98 (or 4F2hc), regulates its trafficking to the cell membrane. CD98 is constitutively membrane-expressed, and both subunits must be on the membrane and coupled to comprise a functional transporter⁵⁹.

In the brain, astrocytes predominately express SXC, however, oligodendrocytes⁶⁰, microglia⁶¹, and immature cortical neurons⁶², have also been found to have SXC expression as well. The main function of SXC is importing cystine for GSH synthesis. As an inducible transporter⁶³, cells are able to increase SXC expression and activity when increased intracellular redox control is required²⁴. Oxidative stress or electrophiles induce xCT expression by activating an Antioxidant Response Element (ARE) or Electrophile Reponse Element (EpRE) promoter region, and xCT can then be shuttled to the membrane, allowing increased cystine uptake⁵⁹. SXC may also protect cells against oxidative stress by driving an efficient cysteine/cystine redox cycle. This cycle changes the extracellular redox state from an oxidized to reduced state by releasing cysteine⁶⁴. Studies suggesting that this cycle is important in redox control found xCT overexpression increases cysteine concentrations both intra- and extra-cellularly^{64,65}. These studies argue that the cystine/cysteine cycle is itself an important redox system. However, more work needs to be done on this topic to determine its true significance in redox control, especially in the brain.

Excitatory Amino Acid Transporters—The Excitatory Amino Acid Transporters (EAAT) are a family of high-affinity Na⁺-dependent glutamate transporters. Electrogenic transport of glutamate is coupled to the movement of 3 Na⁺ and 1 H⁺ inward, and K⁺ outward (Fig. 2). There are five cloned members: EAAT1/GLAST, EAAT2/GLT1, EAAT3/

EAAC1, EAAT4 and EAAT5. These transporters are found on neurons and glia, and are responsible for maintaining low extracellular glutamate levels to prevent aberrant glutamate signaling and toxicity⁶⁶. EAAT2/GLT1 is the primary astrocytic glutamate transporter and accounts for nearly 1% of protein in the brain⁶⁷. As the most abundant glutamate transporter, it is responsible for most of the glutamate uptake in the brain^{68,69}. EAAT1/GLAST is also astrocytic, and EAAT5 is primarily located in retinal cells. EAAT3/EAAC1 and EAAT4 are both neuronal glutamate transporters, however, EAAT4 is mainly expressed in Cerebellar Purkinje cells^{66,70,71}.

EAAT glutamate transporters directly influence redox homeostasis in a similar manner to SXC. As the primary neuronal glutamate transporter, EAAT3 reclaims extracellular glutamate after its release during synaptic signaling⁷². In addition however, EAAT3 mediates cysteine uptake⁷³ and has been shown to do so in neuronal cultures⁷⁴. Cysteine is present at much lower levels than glutamate or glycine in neurons⁷⁵; therefore EAAT3 supplies neurons with the rate limiting substrate for GSH synthesis (Fig. 2; inset). EAAT3 knockout mice have decreased neuronal GSH and increased neuronal oxidative stress, which can be reversed with supplementation of the membrane-permeate cysteine pro-drug N-acetylcysteine (NAC)^{76,77}.

Although some studies have detected xCT expression in cerebral cortex neurons^{60,78}, it is thought that neurons preferentially utilize cysteine, not cystine, for synthesis of intracellular GSH^{10,22,49,79}. Cysteine is rate limiting for neuronal GSH synthesis and down-regulation of EAAT3 function increases neuronal vulnerability to oxidative stress, both *in vitro* and *in vivo*⁷⁵. In one study, EAAT3 knockout mice demonstrated a greater number of degenerating neurons three days after induced ischemia, which was normalized if NAC was administered as a pre-treatment, further supporting the importance of EAAT3-mediated cysteine uptake in neuronal antioxidant function⁸⁰. Additionally, EAAT3 has a much higher affinity for cysteine than cystine⁷³, which supports the finding that neurons rely upon cysteine, not cystine, import for GSH synthesis. Therefore, in the non-pathological brain, EAATs mediate glutamate uptake in astrocytes and neurons, and EAAT3 mediates cysteine uptake for GSH synthesis in neurons. EAAT3 serves as the link between astrocytic GSH release and neuronal GSH synthesis, by importing cysteine released from glutathione breakdown extracellularly.

In addition to this direct effect of glutamate transporters on glutathione production, they also work in concert with SXC to create a cycling of cystine and glutamate that supports redox homeostasis (Fig. 2). The uptake of cystine through SXC requires glutamate release, which is balanced by glutamate uptake^{58,81}. This synergism creates a resting-state cycling of cystine and glutamate, which prevents intracellular cystine depletion and ensures no net glutamate release due to SXC activity⁷². Since glutamate acts as an inhibitor of SXC⁶², a buildup of extracellular glutamate can deplete cells of GSH. Therefore EAAT support SXC function by reducing extracellular glutamate to prevent inhibition of cystine transport into cells⁷².

Glutamate & Calcium Homeostasis

Glutamate is an important signaling molecule in the CNS and is abundant in the brain. However, it has the potential to be toxic and therefore the majority of it is contained intracellularly⁶⁶. Glutamatergic synapses are surrounded by astrocytic processes expressing EAAT2 and can quickly remove the glutamate released from the extracellular space^{82,83}. Glutamate transporters are also located pre- and extra-synaptically to contain any glutamate escaping the synaptic cleft⁸³.

Additionally, astrocytic glutamine transporters, or the sodium-coupled neutral amino acid transporters (SNAT), also play an important role in maintaining glutamate homeostasis by transporting glutamine to neurons, from which they are able to re-synthesize glutamate⁸⁴. Since neurons lack pyruvate carboxylase, the enzyme required to synthesize glutamate from lactate or glucose⁸⁵, they depend on astrocytic recycling of glutamate. Astrocytes convert glutamate, using glutamine synthetase, to glutamine⁸⁶, which is then released through SNATs. Glutamine can be taken up by neurons and converted back to glutamate through neuronal glutaminase^{87,88}. SNAT transporters are tightly coupled to EAAT function, allowing astrocytes to carefully regulate glutamate recycling without increasing extracellular concentrations.

Increased extracellular glutamate creates neurotoxicity by overstimulation of the *N*-methyl-D-aspartic acid (NMDA) receptors on neurons, causing sustained elevations in intracellular Ca^{2+} ⁴⁰. Increases in intracellular Ca^{2+} initiates downstream signaling cascades⁸⁹ that form ROS such as superoxide anions (O_2^-), hydroxyl radicals ($\text{OH}^\bullet$), and hydrogen peroxide (H_2O_2)^{90,91}. Additionally, dysregulation of Ca^{2+} signaling and oxidative stress enhance each other, ultimately leading to Ca^{2+} -dependent activation of intracellular proteases and phospholipases, and disruption of the mitochondrial membrane and release of more ROS and Ca^{2+} ⁸⁹. The rapid and overwhelming increase in ROS during glutamate-mediated neurotoxicity can overwhelm intracellular redox mechanisms and lead to dysfunction and apoptosis of neurons. Interestingly, recent research is uncovering a link between ROS and Ca^{2+} that suggests ROS may act as second messengers that crosstalk with Ca^{2+} [for more on this topic refer to^{26,92}]. These findings strengthen the idea that uncontrolled glutamate-mediated Ca^{2+} influx upsets the intracellular redox homeostasis, lead to disruption of cellular processes and production of excess ROS. When expression of EAAT glutamate transporters is upregulated by administration of Ceftriaxone in mice exposed to hypoxia, they show less oxidative stress than control mice. This study confirmed glutamate-mediated calcium overload causing oxidative stress in the animals⁴⁰. Therefore EAAT glutamate transporters indirectly function in redox homeostasis by maintaining glutamate homeostasis and preventing glutamate-induced oxidative stress and resulting neuronal death.

Redox Imbalance in Neurological Disease

Many diseases have been found to either cause or result from oxidative stress and altered glutathione levels. Some of these include atherosclerotic vascular, heart, liver, kidney, and neurological disease, among a host of others^{4,11}. Multiple sclerosis, a chronic inflammatory disease of the CNS, has characteristic white matter lesions, which have been found to contain oxidized lipids and DNA, suggesting profound oxidative damage in

oligodendrocytes⁹³. Reduced glutathione levels and increasing GSSG/GSH ratios are seen in Wilson disease, a disorder in which copper aberrantly accumulates in different tissues, causing hepatotoxicity and neurological pathologies⁹⁴.

Neurodegenerative disorders have been increasingly associated with redox imbalance and oxidative stress. Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis (ALS), and Huntington's are the most well studied disorders regarding pathology associated with loss of redox homeostasis^{4,11}. Familial ALS was traced to a mutation in the Superoxide Dismutase (SOD-1) gene responsible for encoding the enzyme copper-zinc superoxide dismutase (SOD)^{95,96}. SOD is an important enzyme that catalyzes the neutralization of the superoxide anion (O_2^-) to prevent cellular damage. Additionally, one theory revolves around the interaction of ROS and Ca^{2+} signaling. A combination of oxidative stress and perturbed energy metabolism may compromise Ca^{2+} -regulating mechanisms, leading to brain cell death^{12,97}. In Parkinson's disease, sustained intracellular calcium in dopaminergic neurons has been detected, which increases mitochondrial oxidant stress⁸. Increased ROS generation is detected in Alzheimer's disease and Parkinson's disease, along with many other neural disorders, ultimately leading to brain cell dysfunction and death. For a more in depth review on oxidative stress's role in neurodegenerative diseases see ¹¹.

The Role of Glutamate Transporters and Glutathione in the Biology and Treatment of Glioma

Cancer is one of the most widely studied diseases in terms of oxidative stress, redox homeostasis, and glutathione. As rapidly dividing cells, they undergo continuous cellular metabolism and therefore produce higher than normal levels of ROS. Yet, they manage to flourish under these conditions. By altering the glutathione and redox systems in cells, they not only enhance their own survival, but detoxify our treatment interventions as well.

Clinically, we take advantage of exogenous ROS production for the treatment of cancer. Ionizing radiation is used to kill cancer cells by two mechanisms. The direct mechanism of cell death is achieved when ionizing radiation directly interacts with DNA, creating DNA damage and ultimately leading to cellular apoptosis. The most common mechanism of irradiation-induced cell death is through the indirect method, whereby radiation interacts with water molecules, which are abundant in cells, creating hydroxyl free radicals ($\text{OH}^\bullet$), that then interact with and damage DNA, causing cell death⁹⁸.

Glutathione is an important player in cancer biology and treatment resistance. In fact, GSH is increased in many human cancers including breast, colon, pancreas, and brain, among many others^{99–104}. Studies have found a link between increased GSH levels and chemotherapy and radiation resistance in many cancer types^{105,106} and it seems that cancer cells may respond to radiation therapy by increasing their production and utilization of GSH^{107,108}. Additionally, loss of GSH is thought to either allow or trigger the activation of the apoptotic cascade in cells, and there seems to be a correlation between GSH depletion and apoptosis in some cell types^{109–112}. Cells with higher levels of GSH tend to have more resistance to apoptosis^{113,114}.

In glioma, or glial-derived primary brain tumors, as in other cancers, glutathione is an important survival mechanism. Glioma cells are dependent upon SXC-mediated cystine uptake for viability, as they require intracellular cystine/cysteine for GSH synthesis^{115,116}. Removal of cystine from the medium, as well as inhibition of GSH synthesis with the γ -glutamylcysteine synthetase inhibitor buthionine sulfoximine (BSO) results in cell death. Similarly, treatment with Sulfasalazine to inhibit SXC depletes intracellular cystine and GSH, and inhibits glioma cell growth^{117,118}. Additionally, as tumor cells outgrow their blood supply, areas of the tumor experience low oxygen tension, or hypoxia. Under hypoxic conditions, the dependence of glioma cells upon GSH increases, and they increase xCT cell-surface expression and SXC-mediated cystine uptake to meet this higher demand for GSH^{115,119}. Expression of SLC7A11, the gene that encodes xCT, negatively correlates with chemotherapeutic resistance in glioma cells and inhibition of SXC activity or GSH synthesis increases sensitivity^{120,121}.

Chemotherapy resistance in glioma is associated with elevated GSH levels^{122,123}. GSH is able to bind xenobiotics, such as chemotherapy drugs, and export them out of cells through this family of proteins¹²⁴. Expression of MRP1, one of the MRPs that mediate cellular export of GSH, is elevated in glioma¹²⁵, and has also been found to be higher in higher-grade gliomas versus lower-grade gliomas¹²⁶. Administration of Indomethacin, an MRP inhibitor, is able to increase the cytotoxic effect of etoposide in glioma cell lines¹²⁵. The higher expression of these proteins may be at least one cause of multidrug resistance in human gliomas.

Radiation resistance in glioma also revolves around increased GSH. One study found that by depleting nuclear GSH before irradiation, nuclear fragmentation and apoptosis was increased¹²⁷. A radioresistant clone of U251, a commonly used glioma cell line, has a 5-fold increase in antioxidant enzymes, including glutathione peroxidase and glutathione reductase. This resistant cell line showed cross-resistance to the commonly used chemotherapeutic cisplatin as well¹²⁸. Similar findings are seen in other cancer cells. Sertoli cells demonstrate an increase in GSH after irradiation that is dose dependent¹⁰⁸, and decreasing intracellular thiols in a lymphoma cell line increases radiation-induced apoptosis¹²⁹. Even photodynamic therapy (PDT), which works in a similar manner to radiation therapy by producing ROS to kill cancer cells, is enhanced through GSH inhibition using BSO. PDT plus BSO enhances the ability of this treatment modality to kill tumor cells and decrease their migration¹³⁰.

Taken together, much of the research today on cancer biology and therapy suggests GSH is an important therapeutic target. Many current treatment strategies are focused on GSH inhibition as a way to sensitize glioma and other cancer cells to current chemo- and radiotherapy [see ¹²⁴ for an overview of some of these therapies]. Surprisingly however, little has been published regarding SXC inhibition in the context of sensitizing glioma cells to treatment. Recent studies have found that SXC inhibition using Sulfasalazine decreases chemotherapy and radiation resistance in pancreatic cancer¹⁰³ and many *in vitro* and *in vivo* studies showing GSH dependence on SXC activity strongly suggest that SXC inhibition may be a viable mechanism to enhance current treatment of glioma^{116,117,131,132}. In this study, we have investigated the consequences of SXC expression on radiation resistance in human derived glioma tissue. Using a xenograft model of glioma, where patient-derived

glioblastoma tissue is propagated in the flank of nude mice, we were able to study tumors with high and low SXC expression and function. We found that high SXC expressing tumors are more radiation resistant than low SXC expressing tumors, and SXC inhibition with Sulfasalazine increases the sensitivity of high SXC expressing tumors. These results are presented and discussed in the following Results & Discussion section.

Methods

Drugs

All drugs were purchased from Sigma unless otherwise specified (St. Louis, MO). (S)-4-Carboxyphenylglycine was purchased from Tocris Bioscience (Ellisville, MO).

Xenografts

Xenografts were derived from primary brain tumors of patients and maintained by serial passage in mice, as previously described¹³³. Flank tumor xenografts were harvested, mechanically disaggregated, and maintained in culture as 'gliospheres' in Neurobasal-A medium (Invitrogen) supplemented with 10mg/ml of EGF and FGF (Invitrogen), 250 µM/ml amphotericin, 50mg/ml gentamycin (Fisher), 260mM L-glutamine (Invitrogen), and 10 ml B-27 Supplement w/o Vitamin A (Invitrogen). Cells were used within 2–3 weeks after harvesting from the animals.

Cell Culture

For experiments requiring a monolayer of cells, gliospheres were dissociated into single cell suspensions using accutase (Sigma) and plated using DMEM/F-12 supplemented with 7% fetal bovine serum and either 1% penicillin/streptomycin or 1% gentamycin (Fisher). Experiments were completed within 7 days of cells being plated.

Cell Proliferation

Proliferation was measured by seeding either 10,000 cells/well into each well of a 12-well plate, or 100,000 cells/well into each well of a 6-well plate. Cells were harvested using either 0.05% trypsin or accutase and re-suspended in 10 ml bath solution (125 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1 CaCl₂, 1.6 mM Na₂HPO₄, 0.4 NaH₂PO₄, 10.5 mM Glucose, and 32.5 mM HEPES acid). The pH was adjusted to 7.4 using NaOH and osmolarity was measured at ~300 mOsm. Cell number readings were made using a Coulter-Counter Cell Sizer (Beckman-Coulter, Miami, FL) and cell number was recorded per 500 µl. Mean cell number was normalized to either Day 0 or Day 4 control.

Western Blot

Radio-Immunoprecipitation Assay Buffer (RIPA) was used to lyse tissue as previously described¹¹⁵. Western blots were probed with the primary antibodies goat anti-xCT and CD98 (0.06 µg/ml, Abcam, Cambridge, MA), overnight at 4°C, and mouse anti-GAPDH (0.05 µg/ml, Abcam, Cambridge, MA) for 45 min at room temperature (RT). Blots were then washed in TBST 4 × 5 min and transferred to horseradish peroxidase (HRP) – conjugated secondary antibodies (2 µg/0.5 ml, Santa Cruz Biotechnology Inc, Santa Cruz,

CA) for 1 h at RT, followed by another wash (4×5 min in TBST. Enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL) was used to develop blots and the Kodak Image Station 4000MM (Kodak, New Haven, CT) was used to expose and image membranes.

³H-L-Glutamate Uptake

Glutamate uptake assays were performed as described previously¹³¹. Na⁺-independent uptake solution consisted of (in mM): 125.0 NaCl, 1.75 KCl, 1.25 mM KH₂PO₄, 23 triethylammounium (TEA) bicarbonate, 10 Glucose, 2.0 mM MgSO₄, warmed to 37°C and bubbled with 5% CO₂/20% O₂ to maintain pH at 7.4. Cells were washed twice with uptake solution, then uptake solution containing 0.1 mM glutamate and 2 µCi ³H-glutamate (American Radiolabeled Chemicals, Saint Louis, MO) was added for 3 minutes. Cells were washed twice with ice cold PBS to terminate uptake and lysed with 0.3 N NaOH for 30 min. 0.3 N HCl was then added to neutralize the solution. ³H activity was detected using a liquid scintillation counter (Beckman Instruments, Fullerton, CA) and counts were normalized to protein contents, as measured using the Better Bradford Protein Assay (Thermo Fisher Scientific, Rockford, IL).

Glutathione Assay

Reduced glutathione was measured using the QuantiChrom™ Glutathione Assay Kit (BioAssay Systems, Hayward, CA). Manufacturer protocol was followed. Briefly, cells were collected and sonicated in a solution containing 50 mM NaH₂PO₄ and 1 mM EDTA. Lysates were centrifuged at 10,000 g for 15 min at 4°C and the supernatant collected. Equal volumes of Reagent A was added to the supernatant, vortex, and centrifuged for 5 min at 14,000 rpm. 200 µl of sample/Reagent A mixture was aliquoted into wells of a 96-well plate and 100 µl of Reagent B was added to each well. After a 25 min incubation at RT, the samples were read at OD_{450nm}. GSH concentration was calculated using the formula $(OD_{\text{sample}} - OD_{\text{blank}}) / (OD_{\text{calibrator}} - OD_{\text{blank}}) \times 100 \times n = \text{GSH } (\mu\text{M})$. GSH concentration was normalized to protein concentration, which was measured with the Bio-Rad DC protein assay kit (BioRad, Hercules, CA).

Radiation

Irradiation of cells was performed using an X-RAD 320 Irradiator (Precision X-ray Inc, North Branford, CT). The X-ray tube potential was set at 320 KV, and the mA was set at 12.50. Cells were irradiated at predetermined dose of Gy, as measured by a dosimeter. Cells were pretreated with drug for 1 h before irradiation, and fresh medium was added $\pm$ drug 24 h after irradiation.

Data Analysis

Data was graphed using Origin (v.8.5.1, MicroCal Software, North Hampton, MA) and analyzed using GraphPad InStat 3 (GraphPad Software, San Diego, CA, USA Copyright 1992–1998 GraphPad Software, Inc.). A two-way ANOVA followed by a Tukey post hoc test or unpaired t-test was used to determine significance, unless stated differently in the figure legend.

Results & Discussion

Protein analysis of commonly used cell lines (C6, D54-MG, U87, U251 and STTG1) reveals high expression of SXC^{79,131,134–136}. Since protein expression can be altered over time in cell lines, we have adopted a xenograft model of glioma in which patient tissue is propagated in the flank of nude mice¹³³. Using this model, we found that SXC expression in patient tumors is heterogeneous, and as a result we chose to functionally study tumors that express high and low levels of SXC. Figure 3A shows the xCT and CD98 expression of four of these ‘xenolines’, with two expressing high levels (GBM22 and GBM59) and two expressing low levels (GBM14 and GBM39) of the catalytic xCT subunit. We first tested functionality of SXC by performing radiolabeled ³[H]-glutamate uptake assays, a commonly used method of testing SXC function¹³¹. High SXC expressing GBM22 xenolines showed Na⁺-independent, Cl⁻-dependent uptake, which was inhibited with the SXC inhibitors Sulfasalazine (SAS) and (S)-4-Carboxyphenylglycine (S4CPG), confirming SXC-mediated uptake in high SXC expressing GBM22, but not in low SXC expressing GBM14 (Fig. 3B).

Glioma Cells Expressing High SXC Depend on L-Cystine and Glutathione for Growth

Previous studies have found that glioma cells rely on L-cystine for growth. Inhibition of SXC-mediated uptake results in cell death^{115,117}. Glioma cell dependency on cystine uptake is due to their need to maintain intracellular GSH synthesis to detoxify ROS. Direct inhibition of GSH synthesis using buthionine sulfoximine (BSO), an inhibitor of γ -glutamylcysteine synthetase, the enzyme mediating the rate-limiting reaction for GSH synthesis, significantly reduces glioma growth¹¹⁵. We therefore examined the growth dependency of GBM22 and GBM14 on L-cystine and GSH. Measuring cell number after four days of growth in the presence of increasing L-cystine concentration (0 – 100 μ M) indicated that GBM22, but not GBM14, requires cystine for growth (Fig. 4A). At day 4, GBM22 showed a maximum increase of 7.52 ± 1.64 fold over control, versus 1.99 ± 0.38 for GBM14. The greatest increase in cell growth for GBM22 was reached with 10 μ M L-cystine, which increased growth 6.58 ± 1.91 fold over day 0. In the absence of L-cystine, the growth of GBM22 cells was reduced to 0.83 ± 0.14 fold, which is significantly lower than at 10 μ M L-cystine ($p < 0.005$) (Fig. 4A). Some cells are able to use a transsulfuration pathway to synthesize GSH from L-methionine in the absence of L-cystine¹³⁷ and certain cancers are dependent exclusively on L-methionine availability for GSH synthesis and growth¹³⁸. Therefore we also measured growth of these cells in the presence of increasing concentration (0 – 100 μ M) of L-methionine. GBM22 cells showed no reliance upon this alternative GSH synthesis pathway, as their growth did not exceed 0.72 ± 0.11 fold from day 0 (Fig. 4B).

These findings suggest that GBM14 cells behave differently than GBM22 cells. Overall, growth of GBM14 cells did not exceed ~2 fold under control conditions (Fig. 4A). Growth of cells in the presence of L-cystine or L-methionine at any concentration did not exceed 1.99 ± 0.38 and 1.44 ± 0.17 fold, respectively, which was not significant (Fig. 4C). To determine the dependence of these xenolines on GSH, GBM22 and GBM14 cells were treated with increasing concentrations (0 – 300 μ M) of BSO for four days. Growth of GBM14 cells when treated with 300 μ M BSO only decreased from 1.97 ± 0.67 to $1.33 \pm$

0.20 fold (Fig. 4D). However, the growth of GBM22 cells decreased from 7.28 ± 1.13 to 3.81 ± 0.80 fold, a difference which is significant when compared with a one-tailed paired t test ($p = 0.0301$) (two-tailed paired t test $p = 0.0603$).

To directly determine the dependence of cell growth on GSH, glioma cells were treated with 100 μM L-cystine, 0 μM L-cystine, or 0 μM L-cystine + 5 mM GSH ethyl ester (GSHee) for four days. GSHee is a membrane permeable GSH-derivative that can supplement intracellular GSH. As in Figure 4A, 0 μM L-cystine decreased growth in GBM22 but not GBM14 xenolines. When GSHee was added to the medium, growth of GBM22 cells was restored, but had no effect on the growth of GBM14 cells (Fig. 5A).

To further investigate the differences in GSH dependence between GBM22 and GBM14 xenolines, we directly determined intracellular reduced GSH levels in the cells. Surprisingly, there was no difference in the basal levels of GSH between GBM14 ($1.63 \pm 0.18 \mu\text{M}/\mu\text{g}$) and GBM22 ($2.29 \pm 0.04 \mu\text{M}/\mu\text{g}$) (Fig. 5B). However, when the cells were treated with 100 μM L-cystine, 0 μM L-cystine, or 0 μM L-cystine + 5 mM GSH ethyl ester (GSHee) for 24 hours, intracellular GSH in GBM22 changed dramatically, unlike GBM14 GSH concentrations. In the GBM22 cells, intracellular GSH levels decreased from $7.52 \pm 1.64 \mu\text{M}/\mu\text{g}$ with 100 μM L-cystine, to $0.83 \pm 0.14 \mu\text{M}/\mu\text{g}$ with 0 μM L-cystine. When GSHee was added however, intracellular GSH concentration was rescued ($7.31 \pm 1.00 \mu\text{M}/\mu\text{g}$) (Fig. 5C). Intracellular GSH concentrations in GBM14 changed very little, and no rescue of GSH was seen with GSHee treatment (Fig. 5D). The changes in GSH follow the same trend as the changes in cell number in Figure 5A exposure to the same treatments.

High SXC Expressing Cells are more Resistant at Low Radiation Doses

As a result of their ability to utilize extracellular cystine to increase intracellular GSH, we hypothesized that tumors with higher levels of SXC are inherently more radiation resistant than low SXC expressing tumors. To test this hypothesis, GBM22 and GBM14 cells were irradiated with 0, 1, 5, and 10 gray (Gy). Four days after irradiation, the cells were counted and percent survival was determined compared to day 4 control 0 Gy. GBM22 tumors were significantly more radiation resistant at 1 Gy, ($p < 0.001$). Survival of GBM14 cells was decreased approximately 50% versus GBM22 cells, with percent survival being $49.5 \pm 2.6\%$ and $106.4 \pm 8.5\%$, respectively (Fig. 6A). The LD_{50} radiation dose for GBM22 was ~5 Gy versus 1 Gy for GBM14, a 5x increase in the lethal dose required to kill 50% of the GBM22 tumor cells. At higher radiation doses, once 50% cell death occurred, the radiation resistance was eliminated for high SXC tumors. To test the effect of SXC inhibition on radiation resistance, we treated cells with 0.5 mM SAS before and after irradiation at 1 Gy. SAS + 1 Gy radiation decreased cell survival by approximately 50% [GBM22 % Survival at 1 Gy was 113.50 ± 11.33 versus GBM22 + 0.5mM SAS at 1Gy was 65.32 ± 9.81], showing an increase in sensitivity to SAS plus radiation, at low doses (Fig. 6B). Fig. 6C illustrates the dose response of GBM22 tumor cells to 1 – 1000 μM SAS at 1 Gy of radiation. SAS treatment did not alter GBM14 radiation sensitivity (Fig. 6B).

Conclusions

High SXC expressing tumors exhibit a growth advantage over low SXC expressing tumors, as seen by the growth differences between the GBM22 and GBM14 xenolines. These findings are also replicated *in vivo*, as GBM22 xenolines that are implanted intracranially into mice kill the animals ~2x faster than implanted GBM14 tumors (unpublished data). This enhanced growth of high SXC expressing tumors is a result of their ability to utilize extracellular cystine for intracellular GSH synthesis. Our data suggests that high SXC expressing tumors are more radiation resistant, at least at lower radiation doses, due to their ability to increase their synthesis of GSH to neutralize the free radicals formed as a consequence of radiation exposure. SXC inhibition reduced the resistance of GBM22 cells to 1 Gy radiation, bringing their radiation sensitivity closer to that seen in the GBM14 cells. We found that the GBM14 cells grew much slower in culture than the GBM22 cells, which may be masking a difference in radiation sensitivity at higher doses. Since radiation more efficiently damages cells that are actively dividing, one could argue that the GBM14 cells are not being exposed to equivalent doses of radiation due to their slower growth. Treating these cells with a fractionated radiation dose schedule (ex. spreading a dose of 5 Gy over 5 days, administering 1 Gy/day), may uncover a greater separation of radiation sensitivity in these xenolines. Additionally, we hypothesize that at least under *in vitro* conditions, higher levels of radiation may cause cellular damage too quickly for GSH to neutralize and therefore these experiments should be repeated using an *in vivo* model to more accurately determine radiation dose and tumor resistance with and without SAS at higher doses.

Together, this data suggests that SXC is an important transporter for redox control in glioma cells and offers a growth and survival advantage to tumors that upregulate the expression of this transporter. Interestingly, it seems that low SXC expressing tumors may have alternative mechanisms that they rely upon to synthesize baseline GSH, as attested to by our finding that GBM14 cells have equivalent GSH concentrations to GBM22 under non-stressed, control conditions. However, these mechanisms do not appear to be as robust as SXC-mediate cystine uptake for GSH production, which may explain the sensitivity to radiation and decreased growth ability seen in the GBM14 xenolines. Other antioxidants such as superoxide dismutase and catalase may play a larger role in ROS detoxification in low SXC expressing tumors, since they are unable to increase their synthesis of GSH when greater antioxidant function is required. These findings are important especially for tumors expressing higher levels of SXC, as SAS may be a useful adjuvant to radiation therapy that may allow more effective therapy at lower radiation doses.

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Highlights

- Redox homeostasis is important in the brain due to high O₂ use and ROS production.
- Glutathione is an important mediator of redox homeostasis in the brain.
- System x_c⁻ and EAAT provide intracellular substrates for glutathione synthesis.
- Gliomas cells with increased system x_c⁻ have a growth survival and advantage.
- System x_c⁻ and glutathione may be important players in tumor treatment resistance.

Figure 1. Intracellular GSH/GSSG Redox Cycle

Glutathione (GSH) mediates the detoxification of reactive oxygen species (represented by the generic peroxide, ROOH) through an enzymatic reaction involving glutathione peroxidase. The sulfhydryl, or thiol, group (SH) serves as the proton donor in these reactions. GSH donates a reducing equivalent (H^+ and e^-) and in turn is converted to its oxidized form, glutathione disulfide (GSSG), which is created by the formation of a disulfide bond between two GSH molecules. GSSG is then reduced back to GSH by the enzyme glutathione reductase, which relies on NADPH as an electron donor. The GSH:GSSG ratio indicates the redox state of cells. In healthy cells, greater than 90% of the glutathione is in the reduced GSH form. Oxidative stress can be indicated by a decrease in this ratio.

Figure 2. SXC and EAAT Cystine/Glutamate Cycle

System x_c^- (SXC) and the Excitatory Amino Acid Transporter (EAAT) glutamate transporters work together to provide substrate for glutathione (GSH) synthesis. SXC mediates cystine (Cys-Cys) uptake in exchange for glutamate (Glu) release. Cys-Cys is reduced by the intracellular environment to cysteine (Cys), and is can then react with Glu and Gly to form GSH. EAAT mediate glutamate uptake, providing both substrate for GSH synthesis and substrate for SXC-mediated Cys-Cys uptake. These transporters synergize to provide intracellular Glu and Cys for GSH-mediate redox control. Direct cysteine uptake through the EAAT3 (EAAC1) glutamate transporter provides the rate limiting substrate for intracellular GSH in neurons.

Figure 3.

A xenograft model of glioma, where human tissue is maintained *in vivo*, indicates heterogeneous expression of the two System x_c⁻ (SXC) subunits, xCT and CD98. A) Sample of four xenograft glioma tissues (labeled as GBM#) show two high and two low SXC expressing tumors. B) ³H-glutamate uptake assays indicate the high SXC expressing GBM22 tumor has Na⁺-independent, Sulfasalazine (SAS) and (S)-4-Carboxyphenylglycine (S4CPG) sensitive uptake, indicating SXC activity. Low SXC expressing tumors GBM14 have no measurable SXC activity. GBM14 and GBM22 were chosen as low and high SXC expressing tumors, respectively, for this study. (mean ± SEM; *p<0.05)

Figure 4.

High System x_c^- (SXC) expressing cells are more dependent upon cystine and glutathione (GSH) for growth. A) GBM22 cells, but not GBM14 cells, are dependent on L-cystine in the medium for growth. Significant increases in cell number occur at 10, 30, and 100 μ M, with the greatest change in growth being at 10 μ M L-cystine. Low SXC GBM14 cells do not show the same dependence on L-cystine, with no change in cell number with added L-cystine. B–C) Neither GBM22 nor GBM14 cells rely upon methionine for growth. D) GSH synthesis inhibition using BSO decreases cell number of GBM22 cells, but not GBM14 cells, suggesting that GBM22 cells are much more reliant upon GSH for cell growth and survival. (mean $\pm$ SEM; ** p <0.01, *** p <0.001).

Figure 5.

High SXC expressing cells depend on GSH for growth and require extracellular cystine for GSH synthesis. A) 0 mM cystine decreases GBM22 cell number almost 8-fold, and supplementing GSH ethyl ester, a membrane permeable GSH-derivative, restores cell growth. No significant change was seen in cell number for GBM14 with cystine (Cys₂) depletion or GSHee supplementation. B) Reduced intracellular GSH concentrations are not significantly different between GBM22 and GBM14 at baseline, non-stressed conditions. C & D) GBM22 cells, but not GBM14 cells increase intracellular GSH concentrations when medium Cys₂ are increased to 100 μM. GSH is depleted when Cys₂ is removed from the medium, and rescued with GSHee supplementation, whereas GBM14 cell number remains unchanged. (mean ± SEM; **p<0.01, ***p<0.001).

Figure 6.

System x_c^- (SXC) increases the resistance of glioma cells to low dose radiation. A) High SXC expressing GBM22 cells are >50% more resistant to 1 Gy radiation than low SXC expressing GBM14 cells. The LD₅₀ (dotted horizontal bar) is 1 Gy for GBM14 cells versus 4.7 Gy for GBM22 cells, approximately a 5x higher lethal dose required for high SXC expressing cells. B) Inhibition of SXC using 0.5mM Sulfasalazine (SAS) did not affect the radiation response of GBM14 cells; however, it did decrease the percent of surviving cells exposed to 1 Gy. (mean $\pm$ SEM; **p<0.01, ***p<0.001). C) Dose response curve of GBM22 to 1 – 1000 μ M SAS at 1 Gy.