Neurodegeneration in the Brain Tumor Microenvironment: Glutamate in the Limelight

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Abstract: Malignant brain tumors are characterized by destructive growth and neuronal cell death making them one of the most devastating diseases. Neurodegenerative actions of malignant gliomas resemble mechanisms also found in many neurodegenerative diseases such as Alzheimer's and Parkinson's diseases and amyotrophic lateral sclerosis. Recent data demonstrate that gliomas seize neuronal glutamate signaling for their own growth advantage. Excessive glutamate release via the glutamate/cystine antiporter xCT (system xc-, SLC7a11) renders cancer cells resistant to chemotherapeutics and create the tumor microenvironment toxic for neurons. In particular the glutamate/cystine antiporter xCT takes center stage in neurodegenerative processes and sets this transporter a potential prime target for cancer therapy. Noteworthy is the finding, that reactive oxygen species (ROS) activate transient receptor potential (TRP) channels and thereby TRP channels can potentiate glutamate release. Yet another important biological feature of the xCT/glutamate system is its modulatory effect on the tumor microenvironment with impact on host cells and the cancer stem cell niche. The EMA and FDA-approved drug sulfasalazine (SAS) presents a lead compound for xCT inhibition, although so far clinical trials on glioblastomas with SAS were ambiguous. Here, we critically analyze the mechanisms of action of xCT antiporter on malignant gliomas and in the tumor microenvironment. Deciphering the impact of xCT and glutamate and its relation to TRP channels in brain tumors pave the way for developing important cancer microenvironmental modulators and drugable lead targets.

Keywords: GBM, glutamate, neuron, NMDA, ROS, systemX, TRP, xCT.

INTRODUCTION

Primary brain tumors are derived from glial cells or glial progenitors or stem cells. Malignant or high grade gliomas such as the glioblastoma are the most malignant human entities [1, 2]. Despite multimodal therapy regimens including cytoreduction, radiotherapy and cytotoxic chemotherapy (i.e. temozolomide), the prognosis of glioma patients remains poor, i.e. less than 3% of patients survive more than five years [3, 4]. Rapid proliferation, diffuse brain invasion as well as tumor-induced brain edema and neurodegeneration are pathological hallmarks of these tumors and determine the unfavorable prognosis [5]. A common concept concerning the development of cancer considers deregulation of specifically metabolic-associated genes with link to the redox system [6, 7]. Generally, metabolic processes are associated with the formation of reactive oxygen species (ROS) such as hydroxyl radical (-OH), hydrogen peroxide (H₂O₂), superoxide radical (O₂⁻) and oxygen (O₂) which contribute to TRP channel activation, Ca²⁺ shifts and oncogenic challenges. Oncogenic transformation via oncogenic activation and loss of tumor suppressor genes conversely drives metabolism and thereby initiate a vicious circle with fostered aerobic glycolysis at the expense of the host organism. The price cancer cells have to pay for increased cell growth and survival is their thirst for glucose due to high consumption of carbon skeletons, NADPH, and ATP. To a great extent nutrient transporters meet this task and in addition are also involved in regulating redox homeostasis and cell metabolism [8, 9]. Current knowledge on nutrient transporters in cancer points to the fact that cancer cells adjust their increased demand for nutrients and excessive glucose metabolism via these transporters [10-13]. To keep up with the increased energy demand, cancer cells express nutrient transporters for lactate and amino acids which in particular are primarily related to augmentation of ROS and redox cycle regulation [14, 15]. Another clinical feature of increased transporter action is the influence on the host tissue. Nutrient transporters such as the monocarboxylate/lactate transporters have direct impact on the tumor microenvironment influencing various processes such as cell survival, immune response and angiogenesis [13, 16].

BRAIN TUMORS HIJACK THE GLUTAMATE SIGNALING SYSTEM FOR THEIR GROWTH ADVANTAGE

There exist now ample evidence that the cystine/glutamate antiporter xCT is expressed in various malignant tumors such as leukemias [17, 18], lymphomas [19-21],...
Karposi’s sarcoma [21, 22], pancreatic cancer [23], breast cancer [24, 25], squamous cell carcinoma/epithelial carcinomas [26, 27] and brain tumors [5, 28, 29]. There, the cystine/glutamate antiporter system xct is composed of the catalytic domain xCT (solute carrier family 7 member 11, SLC7a11) and its heavy chain chaperone CD98/4F2hc (SLC3A2) (Fig. 1). In this complex, xCT functions as a Na-independent, electro-neutral exchange system for cystine/glutamate with cystine entry and glutamate efflux in a 1:1 molar ratio [30, 31]. Intracellular cystine becomes reduced to cysteine required for the tripeptide glutathione (GSH) and protein biosynthetic pathways (Fig. 1). Importantly, cysteine is the rate-limiting substrate for GSH synthesis and GSH is required for proliferation, redox cycling and antioxidative defense [32, 33]. This dual function of system xct (xCT) and its interrelation to cell proliferation led to the concept of targeting xCT in cancer. The idea on which inhibition of xCT is based was to reduce intracellular glutathione levels and thereby subsequently disturbing cellular redox balance leading eventually to cancer cell death. Such cytotoxic strategy is especially appealing when the drug target acts specifically on cancer cells and target inhibition does not awry normal cell functioning within the therapeutic window.

Indeed, many cancer cells express elevated levels of xCT and one explanation for this phenomenon is that cancer cells facilitate xCT for their glutathione (GSH) demands required for recovering the increased glutathione consumptions [28, 34, 35]. However, recent data indicate an alternative scenario which opens another biological function apart from the ‘glutathione-only’ concept (Fig. 1). Independent experimental evidences show that increased xCT expression in tumor cells does not necessarily recover intracellular glutathione levels even though xCT overexpression gains cell survival under glutathione and glutamine depletion [19, 24, 36]. This discloses another biological role of the antiporter xCT with respect to the extracellular space. In addition to the intracellular cis-action xCT modulates also the extracellular space by releasing high amounts of glutamate (glutamate-microenvironment hypothesis) [6, 36].

This overlooked aspect of the xCT antiporter biology comes from the perception that the metabolite glutamate which is exported in exchange to cystine has been viewed solely as a byproduct of the GSH cycle. In fact the amino acid glutamate represents a potent signaling molecule and neurotransmitter acting on ionotropic and metabotropic glutamate receptors [37-39]. While in the brain glutamate...
signaling evokes $\text{Ca}^{2+}$-dependent depolarization of the membrane, excessive glutamate release and hence glutamate receptor activation can lead to excitatory neuronal cell death [40, 41]. In particular in primary brain tumors elevated xCT expression is interconnected with increased extracellular glutamate levels in the peritumoral zone [5, 42] resulting in neuronal damage, brain swelling and tumor-associated seizures [5, 43]. Numerous studies demonstrated in experimental and clinical settings that glioma cells secrete high levels of the neurotransmitter glutamate, resulting in neuronal damage [5, 44-46]. Conversely, antagonizing ionotropic glutamate receptors alleviates neuronal degeneration in the tumor vicinity and lessens glioma growth in vivo, suggesting that neurodegeneration is a prerequisite for rapid glioma progression [42]. Excessive glutamate release by glioma cells has also been linked to decreased activities of glial glutamate transporters such as EAAT1 (GLAST/SLC1A3) and EAAT2 (GLT-1/SLC1A2). This can in addition result in decreased glutamate incorporation and consequently reduced extracellular glutamate clearance.

In consequence, reduced EAAT1 and EAAT2 expression and concurrently abundant xCT (system Xc\textsuperscript{-}) activity lead to a net balance shifted towards extracellular glutamate release in favor of glioma progression. In line with these findings, enhancing EAAT2 expression in C6 rat glioma cell grafts reduced tumor size and elevated the lifespan of tumor-bearing animals [47]. Moreover, inhibition of glutamate release via system Xc\textsuperscript{-} (old term for the xCT (SLC7A1) and CD98 (SLC3A2, 4F2HC) heterodimer), profoundly decelerates the malignant phenotype of gliomas in vivo [5, 48, 49] and in addition mitigates tumor-induced brain swelling and microenvironmental disturbances [5]. Even though the glioma-promoting properties of glutamate release by glioma cells appear to be without doubt, the underlying mechanisms are still under investigation. Besides neurotoxic effects, there is good evidence for extracellular glutamate as a promoter for glioma and cancer growth [50, 51]. The mode of action of the growth promoting effects of glutamate could possibly be in an autocrine or paracrine fashion via activation of the fast transmitting ionotropic glutamate receptors on gliomas.

Here, especially the NMDA and AMPA receptor subtypes are in focus of current research activities [52].

**CROSSTALK BETWEEN TRP CHANNELS AND GLUTAMATE RELEASE**

Recent data indicate that TRP channels can modulate and potentiate glutamate release in a ROS dependent manner [53-55]. In particular this has been demonstrated for TRPA1, TRPM3 and TRPV1 in spinal cord and brain. Although this connection between TRP channels and glutamate release has been shown for pre-synaptic terminals, it is likely that such regulatory mechanisms exist also in glial-derived primary brain tumors (Fig. 1). These data point to an orphan role of TRP channels in glutamate signaling with wide ranging implications. Since xCT and subsequently glutamate signaling in gliomas is associated with resistance towards chemotherapeutic agents (such as temozolomide and carmustine), tumor invasion and neuronal cell death, TRP channels may provide a new starting point for targeting this system (Table 1). This is in particular relevant since TRP channels have been considered as targets of chemotherapeutics and drug carriers enabling the efficacy of tamoxifen or alltrans retinoic acid on cancer cells.

Apart from glutamate release, the xCT antiporter is involved in cysteine uptake, which is further metabolized into glutathione (GSH). GSH belongs to the main cellular reactive oxygen scavenging systems balancing cytosolic reactive oxygen species (ROS, $\text{H}_2\text{O}_2$) and gaseous messenger molecules ($\text{O}_2$, $\text{H}_2\text{C}, \text{CO}_2$). Noteworthy is the fact that TRP channels such as TRPA1, TRPV1, TRPV4, TRPM2, TRPM7 and TRPC5 can act as sensors for ROS and molecular oxygens and subsequently can be activated upon GSH and ROS shifts [56]. Thus, the glutamate-cysteine antiporter xCT could potentially regulate TRP channel activation by its ROS regulating impact in the cytosol. Vice versa, TRP channels may impact on xCT and drive glutamate release thereby modulating glutamate signaling. The basis for such ROS sensitivity lays in the redox sensitive cysteine residues present in TRP channels. It has been shown that ROS can modify covalently cysteine residues and thereby alters the

| TRP Subfamily | Organ Distribution | Cancer Types | Expression in Tumors | Relevance to Glutamate |
|---------------|-------------------|--------------|----------------------|-----------------------|
| TRPV1         | Brain, Skin, Prostate | Glioma       | ↑↑                   | ++                    |
| TRPV2         | Brain, Prostate,   | Glioma       | ↓↓                   | ?                     |
| TRPA1         | Skin, Brain,       | ?            | ?                    | ++                    |
| TRPC5         | Breast, Brain      | ?            | ?                    | ?                     |
| TRPC6         | Breast, Brain, Prostate | Glioma    | ↑                    | ?                     |
| TRPM2         | Prostate           | Prostate cancer | ↑↑                  | ?                     |
| TRPM3         | Brain              | Ependymoma   | ↑↑                   | ++                    |
| TRPM7         | Breast             | Breast cancer | ↑↑                   | ?                     |

*Overview of the expression pattern of TRP channels in relation to brain and glutamate signalling. Boxes with question marks indicate missing published data.*
responsiveness to stimuli [55]. Alternatively, ROS challenges in the extracellular space can affect agonists of TRP channels and therefore influence the TRP activation in a paracrine mode.

In conclusion, there is now increasing evidence for a role of TRP channels in malignant brain tumors (Table 1). First, TRPV1, TRPV2, and TRPC6 have been found to be expressed and up-regulated in gliomas [53, 57, 58]. Second, TRP channels can transit directly glioma cell cycle progression and thereby contribute to glioma development as shown for TRPC6 [58].

The relation of TRP and brain tumors becomes less linear when considering TRP vanilloid type channels. Noteworthy, it has been shown that TRPV1 and TRPV2 stimulation can cause cell death in malignant glioma [59, 60]. Moreover, there is a role for TRPV channels also in neuro-inflammation (Table 1). Thus, future studies will unravel whether TRP channels are involved in tumor-immune responses and tumor-associated microglial modulation.

GLUTAMATE MODULATES THE TUMOR MICROENVIRONMENT

Malignant gliomas can disturb the extracellular glutamate homeostasis in the brain via enhanced xCT antiporter activity. Interestingly, in contrast to glial cells or neurons, gliomas can cope with toxic glutamate levels and utilize the amino acid for their growth advantage [6]. Conversely, brain cells are sensitive even to subtle glutamate challenges and are prone to excessive glutamate levels. Neurons, microglial cells and macroglial cells (i.e. astrocytes and oligodendrocytes) have been shown to express ionotropic and metabotropic glutamate receptors in a temporal and spatial manner [61-63]. Neurons and oligodendrocytes are in particular sensitive to micro-molar elevations of glutamate and enduring stimulation leads to neuronal cell death around the initial ischemic area in stroke, a process also termed as penumbra. A recent investigation revealed that peri-infarct depolarization in ischemia is driven by elevated xCT expression [64]. This study by Soria and colleagues uncovered for the first time a role of xCT in stroke contributing to neurodegeneration as has been shown in brain tumors [5]. Although the penumbra in stroke and peritumoral zone in gliomas has different underlying mechanisms the events of neurodegeneration are in both cases glutamate-driven. Astrocytes can buffer neurotoxic glutamate concentrations to a certain extent and provide protection for neurons. However, exposure of excessive glutamate levels to astrocytes eventually leads to glitoxicity as well [65]. Microglial cells express ionotropic and metabotropic glutamate receptors which are implicated in microglial activation and motility [66]. Up to now the precise microglial activation status (M1 or M2) upon glutamate needs yet to be defined. Following brain damage glutamate levels burst potentially from dying neurons and astrocytes and activated microglial cells migrate to the lesion site and subsequently proliferate and phagocytose damaged cells and debris. There, the spatial and temporal activation of microglial cells is decisive whether microglia contributes in a beneficial or detrimental manner. Under chronic conditions microglial function is less unambiguous indicating a correlation of microglial activation and ongoing neurodegenerative diseases. There, microglial cells have the capacity to release large amount of detrimental factors and ROS thereby poisoning their microenvironment. Interestingly, microglial redox balance has been indicated to be controlled by the induction of xCT [67].

What is the biological advantage for malignant gliomas to release high amounts of glutamate?

First, malignant gliomas show destructive characteristics to their environment and are hallmarked by the induction of cell death and neurodegeneration. Second, this tumor entity is surrounded by a unique environment which is restricted in space due to the bony skull. This makes the environment of primary brain tumors so different to those of non-CNS tumors.

Thus, the physical constrains in space and the hallmark of massive neuronal destruction made the hypothesis appealing that glioma-derived glutamate release is implicated in the mechanisms for creating space for tumor growth (Fig. 2). The level of tumor-derived extracellular glutamate has been determined in vitro and in vivo and glutamate levels above 250 µM have been monitored [44, 45, 68], a concentration known to be neurotoxic. Also, there are independent evidences that malignant gliomas destroy the peritumoral area which is also reflected by the development of cytotoxic edema. Moreover, these data are confirmed by studies utilizing the competitive NMDA receptor antagonist MK801 or uncompetitive NMDA receptor antagonist memantine in gliomas where neuronal cell death could be inhibited. However, pharmacological or genetic inhibition of xCT revealed that glioma cells grow unlimited albeit their neurodegenerative potential is restricted. Thus, the concept that the neurodegenerative potential of gliomas creates space for unrestricted tumor growth in the brain is thus not likely to be the main biological role of xCT in brain tumors. In favor for alternative explanation is the finding that tumor-derived glutamate induces a plethora of signaling events beside neuronal damage (Fig. 2).

These may have not yet been recognized such as effects on the blood-brain barrier, blood flow and metabolism. The not well perceived effects are focus of ongoing research. Up to now there is solid evidence that xCT is the essential glutamate transporter in malignant gliomas related to excessive glutamate release.

XCT AND GLUTAMATE: DRUGABLE TARGETS FOR THERAPY

Now, there exist robust data on the consequences of xCT expression in gliomas which call for clinical interventions. So how can we block xCT in tumors and what is the expected outcome for patients?

Up to now two compounds are commonly in use for the pharmacological disruption of xCT. First, the phenylglycine derivate (S)-4-carboxyphenylglycine (S-4CPG) has been described as competitive antagonist for Group I metabotropic glutamate receptors with an approx. IC_{50} of 4x10^{-5} M [69-71]. Later, S4CPG was discovered to competitively inhibit system xc- in a non-substrate dependent manner with a K_{i} of 5x10^{-6} M and IC_{50} of 5x10^{-5} M [31]. In primary brain tumor cells xCT-drug targeting with S-4CPG in micro molar range
can potently inhibit glutamate secretion [49]. In vivo, S-4CPG reduces tumor-induced neuronal cell death [5]. In addition, S-4CPG can exert antiproliferative and cytotoxic effects at higher concentrations in some glioma cell lines, pointing to the fact that xCT inhibition does not act generally cytotoxic for cancer. Indeed, genetic deletion of xCT by homologous recombination (xCT\(^{-}\)) or by naturally occurring mutations (in subtle grey [Sut] mice) display no malformation or alterations in cell proliferation in vivo [72-74]. This indicates that xCT is dispensable for normal cell proliferation and stem cell growth. Notably, the xCT knockout mouse and the spontaneous occurring xCT mutant sut differ in particular behavioral and chemical tests and thus further investigations are required for valid xCT mouse models [75].

Second, Sulfasalazine (brand name Azulfidine in the U.S., Salazopyrin in Europe) is a FDA approved drug for the treatment of inflammatory bowel disease and rheumatoid arthritis. In addition Sulfasalazine (SAS) has been described for the inhibition of xCT/system xc\(^{-}\) in normal and tumor cells [30, 76]. However, antiproliferative effects of SAS on tumor cells are not likely to be exclusively attributable to xCT inhibition since SAS actions are generally pleiotropic. It has been reported that SAS can inhibit NF\(\kappa\)B [77, 78] and glutathione transferase [79], and also shows a wealth of immunomodulatory actions [80]. Thus, so far with the two available drugs in hand information on xCT in tumor biology is limited since off-target effects and xCT-independent effects cannot clearly be distinguished from sole xCT inhibitory effects.

This may be able to be overcome by more recently developed small molecule inhibitors. Currently, one study reported on erastin and sorafenib (Nexavar) as potential xCT inhibitors in the micromolar range [81]. This is in particular interesting since erastin is known as an antitumor agent selective for tumor cells bearing oncogenic RAS. Formerly erastin was supposed to be acting on mitochondrial voltage-dependent anion channel (VDAC) gating.

Recently, histone deacetylase inhibitors (HDACi) as an entirely new drug family have been shown to specifically target the glutamate transporter xCT [82]. The HDACi vorinostat (suberoylanilide hydroxamic acid or SAHA) is a clinically established drug especially used for chemotherapeutically resistant tumors. High xCT expression in tumors is associated with chemoresistance [36]. The finding that epigenetic modulation by histone deacetylase inhibitors impacts xCT expression opens new avenues for modulating the brain tumor microenvironment and treating neurodegeneration.

Thus, it can be expected in the future that better drugs and eventually small molecule inhibitors for xCT will expanding the experimental tools to further decipher xCT function in physiology and in cancer. Moreover, the relation of TRP channel activation and glutamate release opens a new path to control glioma-derived glutamate signaling. Future investigations will decipher whether TRP channels and their pharmacological targets offer an option for regulating tumor-induced glutamate-dependent excitotoxicity.

**CONCLUSION AND FUTURE SUBJECT**

What can we expect as an outcome when blocking the xCT transporter in cancer?

The initial ‘glutathione-only’ hypothesis that xCT biology is primarily based on the role of glutathione in cell
proliferation is probably only one side of the coin. Evidences exist that equally important for the clinical settings are the effects of xCT on the tumor microenvironment. There, xCT regulates extracellular glutamate levels as in the case of brain tumors gives cancer cells a survival advantage over neurons. Another important aspect is the regulation of the extracellular cysteine concentration by xCT. Here, xCT directly operates on the net lipid peroxidation level at the plasma membrane and thus can lead to resistance towards glutathione depleting anticancer drugs [23]. Thus, inhibition of xCT transporter in cancer extent the cytotoxic approach as xCT inhibition can reduce intracellular glutathione levels and at the same time modulates the tumor microenvironment. Dependent on the type of cancer one or the other effect may dominate the outcome of treatment and hence determines its efficacy in cancer therapy. Another rising topic is the modulation of xCT which could be in principle achieved by TRP channels. Whether this is a valid path also in brain tumors will be deciphered in future investigations.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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