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Glioblastoma development in mouse brain: general reduction of OCTs and mislocalization of OCT3 transporter and subsequent uptake of ASP⁺ substrate to the nuclei

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Abstract

Organic cation transporters (OCTs) were first found and then isolated from cultured glioma cells. When glioma cells are implanted into brain the fate of OCTs varies with time after implantation and transporter type. Here we report that OCT1, OCT2 and OCT3 immunofluorescence is significantly reduced over time in implanted GL261 glioma cells, during tumor development in the brain. By day 21 after glioma implantation, OCT1, OCT2 and OCT3 immunofluorescence was reduced more than 10-fold in the cytoplasm of glioma cells, while OCT3 immunofluorescence became concentrated in the nucleus. The well-known fluorescent substrate for OCT transporters,

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Competing interests

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4-(4-(dimethylamino)-styryl)-N-methylpyridinium iodide (ASP⁺), previously shown to accumulate in glioma-cell cytoplasm in *in vivo* slices, began to accumulate in the nucleus of these cells, but not in cytoplasm, after 21 days post-implantation. Considering this mislocalization phenomenon, and other literature on similar nuclear mislocalization of different transporters, receptors and channels in glioma cells, we suggest that it is one of the “omens” preceding the motility and aggressivity changes in glioma behavior.

Keywords

Organic cation transporters; glioma; nuclear mislocalization; fluorescent substrate

Introduction

The human organic cation transporter (OCT) type 1 was first isolated from the cultured human glioma cell line SK-MG-1 [1]. Later, the presence of OCT1, OCT2, and OCT3 were confirmed in different cultured glioma cell lines [2]. This corresponded to the early data that cultured gliomas possess well-expressed, Na⁺-independent, monoamine transporters [3, 4]. As was previously shown that 4-(4-(dimethylamino)-styryl)-N-methylpyridinium iodide (ASP⁺), MPP and polyamines are substrates for polyspecific OCT transporters of SLC22A family [5, 6, 7]. ASP⁺ is a fluorescent marker of glioma cell cytoplasm *in vivo* in tumors during early developmental stages [8]. Intriguingly, ASP⁺ fails to stain the cytoplasm of some glioma cells in tumors that were more than two weeks post-implantation. Instead, these non-staining cells were specifically associated with areas where tumor cells had become proliferative and started to migrate [8]. The literature on OCTs is largely focused on studying cultured glioma; thus, the fate of OCT expression in glioma cells inside the growing brain tumors remains generally unknown. It was shown using RNA arrays, that OCT1 RNA are reduced in the brains with malignant gliomas [9]. This corresponds to the finding that OCT1 transporter gene (SLC22A1) is under negative control by SOX2 transcription protein. The SLC22A1 gene became suppressed, while the SOX2 gene was reactivated and overexpressed in malignant glioma cells [10, 9]. Similarly, low expression of another transporter gene, SLC22A18, was found to correlate with poor prognosis in patients with glioma [11]. There is a positive correlation between SOX2 expression and malignancy grade in gliomas, and hypercellular and hyperproliferative areas of glioblastomas are the areas with the highest SOX2 expression [12,13]. Generally, the presence of OCT transporters in glioma cells of developed tumors are of special medical interest, since OCTs are implicated in the transport of several anticancer agents [14, 15, 16, 17, 11] and polyamines [7].

In this study, we investigated the time course of OCT1, OCT2 and OCT3 expression in glioma cells implanted into the brains of C57Bl/6 mice for 7, 10, 14 or 21 days. We also report here the mislocalization of OCT3 to the nucleus in a subset of the implanted GL261 glioma cells in areas where the tumors became hyperproliferative and start to migrate away from main implanted glioma tumor mass. It usually happened after 2.5–3 weeks of local growth of cells implanted as a single mass tumor. We also demonstrated the mislocalization of OCT-substrate transport. After 2–3 weeks, OCT's fluorescent substrate, ASP⁺, starts to

accumulate in the nucleus instead of the cytoplasm of migrating glioma cells in living brain slices. During time, ASP⁺ fluorescence was mainly detected inside the glioma cells nuclei in the tumor center. This suggests OCT3 remains functional at least for some time after mislocalization to the nucleus, and that the mislocalization is somehow related to the proliferative state of the tumor.

Methods

Animals

Male C57Bl/6 mice (Charles River Laboratories, Wilmington, MA, USA), 12–16 weeks old and weighing 25–30 g were used for these experiments. Mice were maintained on a normal phase, 12-h light/dark schedule in a USDA-approved Animal Resources Center and had free access to food and water. The Universidad Central del Caribe Institutional Animal Care and Use Committee approved all protocols.

Glioma implantation

For the *in vitro* slice experiments, GL261 mouse glioma cells were implanted into the right cerebral hemisphere of mice using previously published methods [8]. Briefly, mice were anesthetized with isoflurane and a midline incision was made on the scalp. At the stereotaxic coordinates [18] from bregma—2 mm lateral, 1 mm caudal and 2 mm ventral—a small burr hole was made in the skull. One μL of cell suspension (1×100 cells/ μL in PBS) was delivered at a depth of 3 mm using a 10 μL Hamilton microsyringe with a point style 2 needle. On days 7, 10, 14 or 21 following implantation, animals were sacrificed. Brains were removed and used for brain-slice immunostaining and for live brain-slice preparations.

Immunostaining and histomorphometric measurement of staining intensity

Animals were anesthetized with pentobarbital (50 mg/kg) and transcardially perfused with PBS followed by 4% paraformaldehyde (PFA). Brains were removed and postfixed in 4% PFA/PBS for 24 h at 4°C, followed by serial incubation in 0.15M, 0.5M, and 0.8M sucrose at 4°C until fully dehydrated. Brains were then frozen-embedded in Cryo-M-Bed embedding compound (Bright Instrument, Huntingdon, England) and cut using a UltraPro 5000 cryostat (Vibrotome-SIMS Co., LTD., Haverhill, MA, USA) to 25 μm slices.

Immunostaining was performed using our previously established protocol [19]. Frozen, 25- μm coronal sections encompassing the entire tumor were generated from each mouse brain. The sections were blocked with 5% normal goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS containing 0.3% Triton X-100 and 0.05% phenylhydrazine for 30 min and then immunostained with OCT1, OCT2, or OCT3 antibody produced in rabbit (Sigma-Aldrich; St. Louis, MO, USA) and fluorescently-tagged anti-GFAP antibody (Sigma-Aldrich) to detect glioma and astrocytes. The sections were then incubated with the secondary antibody for each respective OCT (Fluorescein anti-rabbit IgG; Vector Laboratories) and stained with DAPI (D3571; Invitrogen Life Technologies, Grand Island, NY, USA). Sections were visualized using a laser Olympus Fluoview FV1000 confocal microscope with 40 $\times$ oil-immersion objectives. To facilitate better visual discrimination of the immunostaining GFAP fluorescence was pseudo-colored red, OCT fluorescence was

pseudo-colored green, and DAPI fluorescence was pseudo-colored blue. The fluorescent images were processed measuring mean optical density of OCT immunostaining of 12-bit images of respective parts of glioma cells at 400x magnification using Image J standard function (NIH, <http://rsbweb.nih.gov/ij/download.html>) and LOCI plugin (<http://www.openmicroscopy.org/site/support/bio-formats>).

ASP⁺ and DAPI staining in live slices

To study the active accumulation of fluorescent dyes in living cells we used a protocol that has been previously published [8]. Briefly, for the preparation of brain slices 13–21 days after implantation of GL261 cells, mice were decapitated and brains removed in ice-cold (2–4°C), artificial cerebrospinal fluid (ACSF) composed of 126 mM NaCl; 2.5 mM KCl; 1.2 mM NaH₂PO₄; 1.0 mM MgCl₂; 2.0 mM CaCl₂; 25 mM Glucose; 25 mM NaHCO₃; saturated with 95% O₂ and 5% CO₂. Slices, 250 µm thick, were cut from the tumor-containing tissue using a vibroslicer (Leica VT1000S, Leica Microsystems, Wetzlar, Germany). Slices intended for ASP⁺ staining were placed in an incubation chamber containing ACSF at 35°C. ASP⁺ stock solution (2 mM dissolved in water), was added to the incubation chamber at a final concentration of 1 µM, for direct staining for 10 min. DAPI was added in the same way, to similar final concentration.

For the fluorescent confocal microscopy, live ASP⁺- and DAPI-stained brain slices were placed on microscope slides and covered with glass coverslips (#1.5; Warner Instruments, Hamden, CT, USA). Slices were immediately visualized using an Olympus Fluoview FV1000 confocal microscope with 4×–10× objectives or 40×–60× oil-immersion objectives. Fluorescent cells that had accumulated ASP⁺ were identified using a FITC excitation-emission filter set (absorption maximum at 494 nm and emission maximum of 521 nm), and a standard DAPI filter set in the second channel. Images were captured starting from a depth of 50 µm to avoid surface areas damaged during slice preparation and finished at a depth of 200 µm (1.5 µm step size, 100 steps). In the presented experiments we visualized ASP⁺ fluorescence in live brain slices within 30 min after ASP⁺ application. Images were captured with a black and white camera and ASP⁺ fluorescence was pseudo-colored green, whereas DAPI fluorescence was pseudo-colored in red for better visual discrimination of the two signals. The fluorescent images were processed using NIH Image J with LOCI plugin.

Substances and Materials

All other substances and materials, including ASP⁺ and antibodies against OCT1, OCT2 and OCT3 were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Results

1. OCT1, OCT2 and OCT3 immuno-fluorescence was significantly reduced in the cytoplasmic region of glioma cells across 21 days of tumor development

Using immunostaining for OCT1, OCT2 and OCT3 transporters at days 7, 10, 14 and 21 (n=4) after the implantation of GL261 mouse glioma cells, we determined how the density of OCTs changes with time after implantation. Fine slices were prepared from the zone of cortex (right hemisphere) of the mouse brain containing the tumor. In order to positively

recognize glioma cells not only by shape and position, additional GFAP immunostaining (glioma and astrocyte marker) were applied to the same slices. Additionally, DAPI staining was used to identify the nuclear region of the cells (see Methods). Fig. 1 shows the mean density of OCT1, OCT2 and OCT3 immunofluorescence in the cytoplasmic and nuclear regions of glioma cell images, assessed on day 7, 10, 14 and 21 after implantation. The OCTs immunofluorescence, recorded from images in cytoplasmic region decreased with time from the implantation for OCT1, OCT2 and OCT 3 by more than 10 fold. Interestingly, all OCTs show low immunofluorescence in nuclear zone of the glioma cell at all time points, except for OCT3. For OCT3, the immunofluorescence presence in nuclear zone of glioma cell images was dramatically augmented in many cells at day 21 after the implantation of glioma to the brain (Figs. 1 and 2). To further study this mislocalization phenomenon we used immunostaining for OCT3 in coincidence with DAPI as a nuclear marker. We found that in the margin of the 3-weeks-old tumor the majority of OCT3-associated fluorescence coincided with nucleus marked with DAPI fluorescence (Fig. 2, margin of tumor zone marked by circle). This mostly happens at the invasive areas of the tumor mass, at the edges of the tumor. In the central part of the tumor the OCT3 immunofluorescence is not in the nucleus, but still is mainly allocated to zones in cytoplasm around cell nucleus.

2. The fluorescent substrate for OCT3 transporter, ASP⁺, accumulates in the nucleus, instead of cytoplasm, in some glioma cells during tumor development with time

As previously reported, the fluorescent substrate for OCT, ASP⁺, accumulated in the cytoplasm of implanted live glioma cells [8]. However, within 2–2.5 weeks after implantation, special zones, usually on tumor margin, started to appear within the growing tumor mass (n=6) that had different characteristics from a main cell type. Unlike cells in the main tumor that accumulated fluorescent ASP⁺ mainly in cytoplasm so that the nucleus was less fluorescent then the rest of the cell body, these latter stage tumor cells had low concentrations of ASP⁺ in the cytoplasm, and instead had ASP⁺-dye accumulated in the nuclei (cells marked with arrows in Fig. 3). It can be seen, that ASP⁺ staining in this case coincides with staining by the nuclear marker, DAPI (Composite, Fig. 3), while in main tumor mostly cell cytoplasm was marked by ASP⁺ (See insert A in Fig. 3). Often cells with nuclear accumulation of ASP⁺ appeared forming an islet inside main tumor or appeared on the margin of the main tumor and supposedly starting to spread away (Fig. 3). After 3.5 weeks the majority of cells within the center of the rapidly growing tumor stopped accumulating ASP⁺, while some continued to accumulate it mainly in the nucleus, similarly, like it is shown for the early migrating cell (See Fig. 3, insert A).

Discussion

OCT transporters are synthesized in the cytoplasmic region and with time, due to constant intracellular trafficking, are partially moved and inserted to the external membrane [6]. How many of the OCT proteins remain in the cytoplasm during this process is not known, but if there is a similarity to other solute carriers, about 60 stays in cytoplasm, while the rest are attached to the external membrane [20]. Usually it is possible to determine if the protein is attached to external membrane by employing biotinylation and sucrose-gradient

ultracentrifugation methods [20] or modern multimodal imaging techniques [21,22]. In our experiments we used different methods: 1. In immunostaining experiments we used confocal microscopy that allow us to observe the localization of immunofluorescence, but not the functionality of the OCT transporters. 2. We used *in vivo* slices with implanted glioma and ASP⁺ applications to determine if the transporters were functional. Previously, we reported that implanted glioma uptake ASP⁺ during 2.5 weeks, then significantly reduce the uptake [8].

We have shown that during glioma development following implantation of GL261 mouse glioma cells into the cortex of mice, OCT1, OCT2 and OCT3 immunofluorescence in cytoplasm steadily declines with time, probably showing a decline in synthesis. Immunostaining also showed that OCT3 was mislocalized to the nucleus in some of the tumor cells in late stages—21 days after implantation. ASP⁺ fluorescence also accumulated in the nucleus of glioma cells in late-stage glioma progression. This process starts (about 2.5 weeks after implantation) from the tumor margin where glioma cells launch to migrate away from main tumor, and with time, the majority of accumulated ASP⁺ localizes in the glioma cell nucleus in living brain slices. This suggests that the mislocalized transporter remains active in the nucleus, pumping fluorescent substrate to the inside of the nucleus of migrating glioma cells. The functionality of transporters has not been studied for other transporters reportedly also mislocalized in gliomas [23]. The only study describing functional mislocalization to the nucleus is the case of CXCR4 mislocalization in prostate-cancer cells [24]. In this case, CXCR4 remains active in the nucleus, producing receptor-stimulated release of intra-nuclear Ca²⁺. Recently, numerous reports have emerged that some important transporters, receptors and channels, normally presented in the plasma membrane can be mislocalized in gliomas and, generally, in cancer cells, to the nuclei. It was shown that glutamate transporters became mislocalized to the nucleus in glioma cells that depends upon cellular confluence or the amount of cells in the tumor (tumor size) [25, 23]. Also, potassium inward-rectifying channels Kir 4.1 and Kir 2.3 in glioma cells are found almost exclusively in either the cell nucleus or endoplasmic reticulum/Golgi apparatus (Kir 3.1) [26]. These data suggest that mislocalization of Kir channel proteins to intracellular compartments is responsible for a lack of appreciable Kir currents in glioma cells, similar to immature glia. While it is known that G-protein coupled receptors can be mislocalized in many diseases due to aberrant trafficking [27, 28], it was specifically shown that Cysteine (C)-X-C Receptor 4 (CXCR4) is associated with the nucleus of malignant prostate-cancer tissues and remains functional [24]. Although shown to be common in malignant cancers, the logics and mechanisms of mislocalization remain unclear. The karyopherin (KPNA) protein family is involved in nucleocytoplasmic trafficking. Increased KPNA levels have been found to predict poor prognosis for a variety of solid tumors, including astrocytic gliomas of WHO grades II–IV [29]. Transportin-β1 molecules are implicated in case of CXCR4 mislocalization [24], but it is unclear if Transportin-β1 is somehow changed in quantities or chemically to start mislocalization.

It was shown that monoubiquitination can act as a signal in nuclear export, and monoubiquitin-mediated protein trafficking can be expanded generally to nuclear-cytoplasmic shuttling of different proteins during carcinogenesis [30]. Similarly, conjugation

of the small ubiquitin-like modifiers (SUMO), to target proteins is linked to the regulation of multiple cellular pathways, including nucleocytoplasmic trafficking and cell-cycle progression, so it can be linked to cell cycling in general [31]. Thus, the phenomenon of mislocalization of proteins usually present on the external cell membrane can reflect general changes of protein localization during cell dedifferentiation with significant increase in cell growth, invasiveness and tumorigenicity. In that case, it is clear that the mislocalization phenomenon constitutes one of the “omens” preceding the motility and aggressivity changes in glioma behavior, while there is still no answer to the question of why some of mislocalized proteins retain their functionality.

In addition, we can conclude that OCT mislocalization is important for polyamine (PA) exchange in glioma. These transporters are widely expressed in the CNS [32, 33] and other major organs in both humans and rodents. They are low-affinity, high-capacity and polyspecific transporters that take up PAs with different structures [7]. PAs are found in healthy glia [34, 35] probably due to their uptake via OCTs [7]. Our current study shows mislocalization of OCT3 from the cell surface to nuclei area in migrating glioblastoma cells. Therefore, this mislocalization can have important consequences for PA metabolism in cancer cells. These cells produce PAs while normal glial cells lack such synthesis [36]. Thus, during cancer development PA synthesis occurs in the cytoplasm and OCTs are probably not necessary for PA uptake from the extracellular space. So, current clinical use of anticancer drugs based on anti-OCT strategy may be partially insufficient due to OCT removal from the cell surface.

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Fig. 1. Density of OCT's mean immunofluorescence (N=4) in cytoplasmic and nuclear zones of glioma cells on different stage of tumor development (day 7,10,14 and 21 after implantation, see Methods).

Fig. 2. Mislocalization of OCT 3 to the nucleus of glioma cells at day 21. OCT3 staining is green (A), DAPI is blue and GFAP is red. In (B) and (C), the composite of OCT-DAPI, and OCT-DAPI-GFAP are present, respectively. The arrows show coincidence of OCT3 and DAPI staining. The margin invasive area of the tumor is marked with circle. Some reactive astrocyte sprouting can be seen nearby.

Fig. 3.

Uptake of 1 μM ASP⁺, a substrate for OCT transporter, into the nucleus of migrating glioma cells. As we reported previously [8](Kucheryavykh et al., 2012), ASP⁺ was accumulating in the cytoplasm of glioma cells forming main tumor after the implantation. But at days 15–20 when glioma cells start to migrate (marked by arrows), they began to accumulate ASP⁺ to the nucleus (see also insert A).