Accumulation of amyloid beta in human glioblastomas

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Accepted for publication 2 July 2020
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Summary

Many cancer types are intrinsically associated with specific types of amyloidosis, in which amyloid is accumulated locally inside tumors or systemically. Usually, this condition relates to the hyperproduction of specific amylogenic proteins. Recently, we found that the accumulation of amyloid beta (Aβ) peptide immunofluorescence is linked to glioma cells in mouse tumors. Here we report that amyloid-specific histochemical dyes reveal amyloid accumulation in all human glioma samples. Application of two different antibodies against Aβ peptide (a polyclonal antibody against human Aβ1–42 and a monoclonal pan-specific mAb-2 antibody against Aβ) showed that the amyloid in glioma samples contains Aβ. Amyloid was linked to glioma cells expressing glial-specific fibrillary acidic protein (GFAP) and to glioma blood vessels. Astrocytes close to the glioma site and to affected vessels also accumulated Aβ. We discuss whether amyloid is produced by glioma cells or is the result of systemic production of Aβ in response to glioma development due to an innate immunity reaction. We conclude that amyloid build-up in glioma tumors is a part of the tumor environment, and may be used as a target for developing a novel class of anti-tumor drugs and as an antigen for glioma visualization.

Keywords: brain, cancer, human, microscopy, tumor immunology

Introduction

A build-up of amyloid has been reported in many cancers in tumors, and some cancers are intrinsically connected with specific types of amyloidosis. For example, multiple myeloma, as well as malignant lymphoproliferative diseases, such as chronic lymphocytic leukemia, hairy cell leukemia, Hodgkin lymphoma and Waldenström's disease, is associated with the hypersecretion of Bence Jones [free immunoglobulin (Ig) light chain] protein, generating a particular form of systemic amyloid light-chain (AL) amyloidosis [1-4]. Unlike systemic amyloidosis, breast cancer exhibits localized light-chain amyloidosis, with amyloid bodies limited to the inside of the tumor [5,6]. Many carcinomas have stroma containing AL-type amyloid [7,8]. Localized AL-type amyloidosis was also found in early-stage non-small-cell adenocarcinomas in kidney and lung [9]. It is important to mention that localized AL amyloidosis is related to many cancer types forming amyloid tumors – the condition known as amyloid tumor disease. Such local amyloid deposition may be explained by over-expression of the receptors for amyloid protein oligomers. For example, amyloid accumulation in lung cancer is a consequence of the binding of amyloid oligomers to over-expressed amyloid-binding receptor for advanced glycation end products (RAGE) receptors present in lung tumors [10]. However, AL-type amyloid is not the only amyloid found in tumors, and these others are probably of mixed type, as is common for other amyloid diseases, such as Alzheimer’s disease (AD) [11]. Many proteins form fibrous aggregates due to cross-β polymerization of β-pleated sheets and co-aggregate with other proteins at elevated concentrations, forming a mixed misfolded amyloid [12,13]. An additional systemic amyloidogenic protein (besides light chain protein) involved in multiple myeloma (and in some other cancers) is β2-microglobulin (β2M), an anti-microbial protein with anti-bacterial activity and a light chain of the major histocompatibility complex. β2M protein hyperproduction is so common...
in many cancers (while reduced in gliomas) that it can be used as a tumor marker [14]. These proteins are produced systemically and may accumulate systemically or locally. Conversely, some tissue-specific cancerous tumor types overproduce tissue-specific, aggregation-prone proteins. For example, the amyloid in odontogenic tumors is immunopositive for the enamel matrix protein ameloblastin [15–17]. Unlike odontogenic tumors, the tumor amylloidosis of bone is usually of β2M origin and almost always progresses to multiple myeloma [18].

There is another systemic protein that has a β-pleated sheet structure and is prone to aggregation: the amyloid beta (Aβ) peptide. While many cells produce Aβ, in amyloidosis it usually accumulates in specific tissues. Aβ is a major component of amyloid deposition in AD, in brain trauma (brain tissue), glaucoma (retina), pre-eclampsia (placenta) and many other health conditions and has anti-microbial activity, which may be related to innate immunity (see review: [19,20]). Aβ is known to be elevated in many cancers. For example, the plasma levels of Aβ peptides in esophageal cancer, colorectal cancer, kidney cancer, hepatic cancer and lung cancer patients were significantly higher than in normal controls [21].

It has also been shown that gliomas are extensively infiltrated by platelets [22,23], which are the main source of Aβ in the blood [20,24,25]. These small, anuclear blood cells and their Aβ-containing microvesicles easily penetrate the blood–brain barrier, especially in the glioma-affected zone [22,26,27]. As an important part of innate immunity, platelets respond to bacterial invasions and malignant cancers releasing many factors necessary to recruit other immune cells, but also releasing their own defense peptides, including Aβ [28,29]. The anti-tumor effects of Aβ peptides are well known [30,31], and recently we found an accumulation of Aβ on the surface of glioma cells within glioma tumors in mice. Visible Aβ immunofluorescence in nearby vessels suggests that the most possible source of Aβ in mouse tumor is blood, and we propose that this amyloid buildup could be a target for developing a novel class of drugs for glioma visualization and treatment [32]. The question then is: do human glioma tumors also accumulate amyloid and have Aβ present within the tumor?

Here we report the accumulation of amyloid in human glioma, showing that amyloid is linked to glioma cells of glial origin, both intra- and extracellularly, and to glioma blood vessels. In this study we used various dyes known for specific amyloid staining to visualize amyloid in tumors from surgically removed brain tissue from patients of different ages diagnosed with glioblastoma. Immunostaining revealed that the amyloid in these tumors contains Aβ. We also used double immunostaining against human Aβ and specific co-staining of different cell types, as we found that, as well as extracellular deposition of amyloid, many cells in glioma specifically accumulate this substance. This double staining allowed us to determine which cells in glioma accumulate Aβ.

**Material and methods**

Paraformaldehyde-fixed human glioma tissue samples were obtained with patient consent from collaborators at the Universidad Central del Caribe for future use as unidentifiable tissue samples, as approved by the Institutional Review Board (IRB) Human Research Subject Protection Office (protocol no. 2012-12B). In total, we analyzed glioblastoma samples from five patients.

**Tissue preparation and cryosectioning**

The tissue was fixed in a solution of 4% paraformaldehyde and incubated overnight (16–24 h) in the same fixative solution. After incubation, the tissue was transferred to phosphate-buffered saline (PBS: NaCl, 137 mM; KCl, 2.70 mM; Na₂HPO₄, 1.77 mM; pH 7.4). After rinsing the tissue three times for 10 min per rinse at room temperature (RT) in PBS with 5% sucrose (with gentle rotation), the samples were cryoprotected with increasing concentrations of sucrose in PBS, which were obtained by mixing 5% sucrose and 20% sucrose at ratios of 2 : 1, 1 : 1 and 1 : 2. The tissue was incubated for 30 min at each step in the ascending series of sucrose solutions at RT and then finally placed in 20% sucrose at 4°C, allowing preservation of the tissue over a long period. Before staining, 30-μm slides were prepared from the samples using a cryomicrotome (CM 1850; Leica Microsystems GmbH, Wetzlar, Germany) at –23°C.

**Immunostaining, histochemistry and fluorescence confocal microscopy**

The slides were air-dried for 30 min and immunostained using a protocol previously established in our laboratory. First, sections were treated for 20 min with a permeabilization solution consisting of 0.03% Triton X-100 and 1% dimethyl sulfoxide (DMSO; MP Biomedicals, Santa Ana, CA, USA) in PBS under gentle agitation on an orbital shaker. Secondly, the sections were treated for 60 min with a blocking solution containing 5% normal goat serum, 5% normal horse serum (Vector Laboratories, Burlingame, CA, USA) and 2% w/v bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO, USA) in permeabilization solution. Following the blocking step, the sections were processed separately using two different antibodies against Aβ. Slices were incubated with a mouse monoclonal antibody against glial fibrillary acidic protein-cyanin 3 (GFAP-Cy3) (1 : 200; Abcam, Cambridge, MA, USA, cat. no. ab49874), a rabbit polyclonal antibody against Aβ1–42 (1 : 100; Abcam, cat. no. ab216504), a rabbit monoclonal antibody against neuronal...
Accumulation of amyloid beta in human glioblastomas

hexaribonucleotide binding protein 3 (NeuN)-Alexa Fluor 647 (1: 100; Abcam, cat. no. ab190565) and a mouse monoclonal antibody (mAb-2) against Aβ (1: 100; Abcam, cat. no. 126649), all incubated overnight at 4°C. After three washes with permeabilization solution for 10 min, the secondary antibodies (fluorescein-labeled goat anti-rabbit IgG and fluorescein-labeled horse anti-mouse IgG) were added at a dilution of 1: 200 with shaking for 2 h at room temperature and protected from light. The slices were then washed three times with PBS for 10 min and once with distilled water before being transferred onto a glass slide containing Fluoroshield mounting medium (Sigma-Aldrich, cat. no. F6057) with 4′,6-diamidino-2-phenylindole (DAPI).

For thioflavin staining we used thioflavin S (Th-S). Human brain slices (30 μm) containing tumors were completely air-dried prior to staining. The slides were first washed with 70% ethyl alcohol (EtOH) for 1 min, followed by 80% EtOH for 1 min, and then incubated for 15 min in a filtered solution of Th-S (0.2-μm filter). A solution of 1% Th-S was prepared in 80% EtOH. After incubation, the slides were washed with 80% EtOH for 1 min followed by 70% EtOH for 1 min, washed twice with distilled water and air-dried again. The coverslip was mounted with a drop of Vectashield® mounting medium for visualization of fluorescence (Vector Laboratories, Burlingame, CA, USA, cat. no. H-1000).

Brilliant blue (BBG) staining was performed as follows. The human brain slices (30 μm)-containing tumors were allowed to air-dry completely, stained with a drop of 0.25 mg/ml BBG in distilled water (0.2-μm filter) for 5 min, washed twice with distilled water, and air-dried again. The glass slides were mounted with Fluoroshield mounting medium containing DAPI (Sigma-Aldrich, cat. no. F6057).

Congo red staining was performed as follows. A 0.5% Congo red solution was prepared in 50% alcohol. Human brain slices (30 μm) containing tumors were air-dried completely, then washed twice with absolute alcohol for 2 min. The slices were then washed with 90% alcohol for 2 min, followed by 70% alcohol for 2 min, washed once with running water for 2 min and incubated for 20 min in a filtered solution of Congo red (0.2-μm filter). After incubation, the slides were rinsed with distilled water once, rinsed repeatedly in alkaline alcohol solution (five to 10 times), and rinsed in tap water for 2 min. Finally, the slices were dehydrated through immersion in 95% alcohol, followed by two immersions in 100% alcohol for 3 min each. The glass slides were mounted with Fluoroshield mounting medium containing DAPI.

Fluorescein isothiocyanate (FITC) excitation/emission filters were used to visualize specific amyloid-associated Th-S and Congo red fluorescence, whereas tetramethylrhodamine isothiocyanate (TRITC) filters were used to reveal non-specific Congo red fluorescence, and TRITC excitation/emission filters were used to visualize BBG fluorescence.

Images were acquired using an Olympus Fluoview FV1000 scanning inverted confocal microscope system equipped with a 4×, 10×, 20× or 40×/1.43 oil objective (Olympus, Melville, NY, USA). The images were analyzed using ImageJ software (version 1.8.0_112, http://imagej.nih.gov/ij) with the Open Microscopy Environment Bio-Formats library and plugin (http://www.openmicroscopy.org/site/support/bio-formats5.4/), allowing for the opening of Olympus files. The images were evaluated using custom colorization.

Results

Standard histological dyes for amyloid show its accumulation in human glioma tumors extracellularly and in certain tumor-related cells

Glioma tissue samples from patients of different ages (aged 35–73 years) were studied. To visualize amyloid, we used two standard histological dyes, Congo red and Th-S, known for their staining of protein amyloid aggregates. Aggregation produces a fluorescence shift, with an increase proportional to the extent of aggregation [33,34]. Coomassie brilliant blue G (BBG) was used to stain amyloid fibrils and prefibrillar amyloid intermediates [35]. We studied samples of glioma tissue (removed from four different patients: patients 17, 11, 7 and 1), and in one patient (patient 1) there were two tissue samples from the glioma zone, one containing glioma only and the other from the border zone containing glioma cells but also small fragments of normal tissue. This allowed us to use the normal tissue in the second sample as an internal reference (control). When stained with Th-S, all glioma samples examined showed a specific amyloid staining inside the tumor. In all cases, the amyloid was present in or around some glioma cells (distributed compactly or sparsely in the glioma tumor) as well as extracellularly (Fig. 1). Interestingly, the size of glioma cells stained with Th-S in samples of different patients varied by two to threefold (Fig. 1a–d).

Applying different amyloid-specific dyes, Th-S, Congo red and BBG, showed very similar staining patterns in glioma samples from patient 1, indicating the accumulation at (on or inside) some glioma cells as well as in extracellular aggregates (Fig. 1a–f). As Congo red and Th-S are known to bind only to aggregated amyloid, while BBG binds to the prefibrillar/fibrillar forms of amyloid [35], the same pattern of staining suggests that amyloid is present in glioma in both fully aggregated and pre-aggregated forms. As a control, the application of Th-S, Congo red and BBG to areas of normal tissue (Supporting information, Fig. S1a–c) showed no specific staining.
Staining with amyloid-specific dyes showed that some amyloid deposition is connected with glioma cells (accumulating on or inside the cell). This finding raises questions: which specific cells have the properties that lead to accumulation of amyloid in glioma tumors, and which type of amyloid is it? In mice, we found that glioma amyloid has Aβ peptide as a mandatory component and accumulates in association with GFAP-expressing glioma cells, and we suggest that this may also be the case in humans. To study this possibility we used immunostaining for human Aβ combined with antibodies against specific cell-type markers.

Tumor cells of glial origin and some astrocytes close to blood vessels within the tumor site accumulate Aβ

It is known that the main cell type in glioma tumors is malignant glioma cells of glial origin, and these have low-level but clear expression of GFAP filaments [36]. Antibodies against GFAP label glioma cells as well as astrocytes, which become reactive near the tumor, with high expression of GFAP [37]. In addition, we recently showed that glioma cells expressing low levels of GFAP accumulate Aβ in mice [32]. Therefore, we decided to apply antibodies against GFAP, thereby labeling glioma cells and astrocytes, as well as an antibody specifically against NeuN, in order to distinguish between these two cell types. As mentioned above, in one patient we had two samples, one containing a highly developed glioma and another of more peripheral tissue with small insertions of normal brain tissue, allowing its use as a normal tissue reference.

To visualize Aβ we used two different types of antibodies highly specific for Aβ peptide (Figs. 2 and 3) with no cross-reaction with its precursor [38,39]. Both antibodies yielded a similar pattern of staining, showing clusters of glioma cells containing Aβ (Fig. 2a,d1). In addition, simultaneous application of anti-Aβ and anti-NeuN staining showed that cells containing Aβ (Fig. 2a) have no neuron-specific staining. Neuronal staining was obvious only in the sample of normal tissue as far away from the glioma as possible (Fig. 2b), and was practically non-existent in

Fig. 1. Top row: glioma samples from three patients stained with thioflavin S (Th-S, green fluorescence). (a) Glioma patient 17; (b) glioma patient 11; (c) glioma patient 7. These images were acquired at the same amplification. Cells with Th-S fluorescence have different sizes in different patients. Some extracellular staining is also present, showing possible extracellular deposition. Bottom row: glioma removed from patient 1 stained with different amyloid-detecting dyes: D = thioflavin S (Th-S, green); E = Congo red (red); F = Coomassie brilliant blue G (BBG, red). All stains for amyloid beta peptides (Aβ) aggregates yield the same pattern of sparsely or compactly distributed cells as well as extracellular aggregates. White arrow = extracellular aggregates; black arrow = cells with amyloid; scale bar = 40 μm.
the glioma. Conversely, simultaneous application of anti-Aβ and anti-GFAP staining showed that reactive astrocytes, which express GFAP, are visible on the margins of the sample containing normal tissue (Fig. 2c, upper quadrant), with no associated amyloid. This figure shows also that Aβ is found in the blood vessel wall (green + red = yellow) and in some astrocytes having endfeet extending to the blood vessel wall (Fig. 2c, lower quadrant).

However, the Aβ/GFAP double staining revealed that Aβ accumulates mainly in cells of glial origin expressing GFAP (Fig. 2d). There was good coincidence of GFAP-expressing cells (Fig. 2d1, red) and Aβ-containing cells (Fig. 2d2, green, also Fig. 2d3, composite). These cells have much less arborization than astrocytes and sometimes less pronounced GFAP expression in the cell body (Fig. 2d2). The majority of these GFAP-expressing cells have associated amyloid, although it is difficult to ascertain whether there is also extracellular amyloid present.

**Amyloid in glioma cells is mainly present in cells around blood vessels and in perivascular spaces**

It is known that glioma is highly vascular, and tumor invasion along blood vessels facilitates its spread in the brain [40]. Similarly, we found that there is a substantial accumulation of Aβ in blood vessel walls and in the cells around blood vessels (Fig. 3a–c). Green fluorescence of Congo red (FITC filter) at low magnification (Fig. 3a) shows that amyloid aggregates mainly in cells around and inside the blood vessels. The red fluorescence of Congo red (TRITC filter) enables visualization of blood vessel walls in general, because this non-specific red fluorescence stain labels mainly the vessel walls [41]. A very similar result was found by
immunostaining with an antibody against Aβ (green), which is concentrated in glioma cells around the blood vessels but especially in the vessel walls and in the perivascular space (Fig. 3b, arrow). In a marginal sample (Fig. 3c) there was a layer of normal reactive astrocytes (red) lying on top of the glioma cell layer (green). In this figure (Fig. 3c), Aβ fluorescence is also present in the blood vessel walls and in the endfeet of astrocytes on the surface of the vessels. We also present this figure as a series of confocal images to clarify the three-dimensional positions of different cell layers (Supporting information, Fig. S2).

Discussion

Recently, we found that glioma in mice accumulate Aβ, mainly in glioma cells expressing GFAP and around nearby blood vessels [32]. We suggested that the amyloid in this tumor might be used as a target to develop a novel class of drugs for visualization and treatment of glioma. This possibility has inspired us to determine whether a similar accumulation is found in human glioma.

Here we report that amyloid-specific dyes (Th-S, Congo red, BBG) show amyloid accumulation in all human glioma samples that we received from different patients. Some amyloid was present extracellularly, but was mainly concentrated in glioma cells (Fig. 1). Application of two different antibodies against Aβ peptide (a polyclonal antibody against human Aβ1–42 and a monoclonal pan-specific mAb-2 antibody against Aβ residues 1–4) revealed that the amyloid in glioma samples contains Aβ and that both antibodies show a similar staining pattern (compare Fig. 2a with Fig. 2d1). Double staining with antibodies to cell-specific antigens showed that Aβ accumulation is present mainly in glioma cells of glial origin that express GFAP in blood vessel walls and in some astrocytes close to the glioma body. No correlation between Aβ and a specific neuronal marker was found (Fig. 2b). These results fully support our previous study in a mouse model of glioma [32].

Our decision to first investigate neurons and astrocytes as possible sources of Aβ was motivated by the fact that these cell types are known for their ability to produce large amounts of Aβ peptide [42,43]. It was previously shown that astrocytes may also take up Aβ generated by other cell types [44]. We also suspected glioma cells, as the production of Aβ by cultured malignant glioma cells was shown: these cells produce a 4-kDa peptide that co-migrates with synthetic Aβ1–40 (also known as Aβ40) and is specifically recognized by antibodies raised against terminal domains of the Aβ peptide, and the cells release this peptide into the medium [45]. These results and our previous experience with a mouse glioma model allow us to suggest which types of cells are involved. There are many other cell types associated with glioma; for example, myeloid-derived suppressor cells (MDSCs), regulatory T cells and tumor-associated macrophages (TAMs) [46], as well as microglia [47], but their presence is stage-dependent, and they constitute a smaller population of cells. Therefore, we decided to first study neuronal/glial markers and return to other...
Accumulation of amyloid beta in human glioblastomas

cells if these markers failed to reveal which cells accumulate amyloid. This approach was successful in confirming that GFAP-expressing glioma cells of glial origin show Aβ fluorescence (Figs. 2a and 2d1), as do some astrocytes (Fig. 2d3) and the blood vessel walls (Fig. 3). This result may be interpreted in different ways: either (1) glioma cells produce Aβ or (2) Aβ is produced systemically and migrates to glioma. This last possibility can explain why amyloid is concentrated around blood vessels (Fig. 3a,b).

Interestingly, statistically independent cohort studies found an inverse association between cancers in general and Alzheimer’s disease (AD) [48,49]. Nevertheless, there is a significant positive correlation between AD and the malignant brain tumor mortality rate, suggesting that there are common mechanisms [50–52]. Could it be malignancy or skin and that this release is concentrated near blood vessels? 

While glioma cells produce Aβ, its production is very limited [45]. Systemic production of Aβ is also well known, and recently we showed that platelets produce a massive release of Aβ after thrombosis in the brain or skin and that this release is concentrated near blood vessels [39,53]. Aβ was shown to be an anti-microbial/anti-viral agent and the important part of the systemic innate immunity arsenal [29,54–59]. We also previously suggested that blood platelet activation, as a response to the inflammation, can add to the accumulation of Aβ in AD (reviewed in [19]). It was shown that platelets become hyperactivated in AD [60], and this may lead to excessive release of Aβ in the brain, overwhelming its clearance during the disease. A similar accumulation of Aβ happens during glaucoma, in damaged skin and during pre-eclampsia [20].

It has also been shown that platelets are hyperactivated in cancer patients and form cancer cell-induced aggregates and micro-thrombi in the vasculature near tumors (reviewed in [61]). We suggest that these platelet aggregates can release Aβ. Release of systemic Aβ peptides may also be a response to the development of glioma in the brain and even a weapon against glioma. It was reported that full-length Aβ40 and its shorter fragments suppress human U87 glioblastoma subcutaneous xenografts in nude mice, while systemic delivery of this shorter peptide leads to reductions in glioma proliferation, angiogenesis and invasiveness [62]. The same investigators also found that transgenic mice over-expressing Aβ40 showed reductions in glioma growth, invasion and angiogenesis [63,64]. Recently, there has been another report that Aβ oligomers are an effective weapon against cancer cells [31]. The mechanism of external Aβ accumulation in glioma cells expressing GFAP may also be because of modified RAGE (a receptor for Aβ) isoform expression on their surface and not only in tumor-associated macrophages [65,66]. Generally, high platelet count is associated with poor survival in a large variety of cancers, while thrombocytopenia or anti-platelet drugs can reduce the short-term risk of cancer, cancer mortality and metastasis (reviewed in [67]).

Conclusions

It is now clear that Aβ-containing amyloid in glioma is an obligatory part of the tumor environment, although it is still uncertain whether Aβ build-up is a systemic innate immunity reaction to the tumor, or Aβ peptides are produced locally by glioma cells. Aggregated amyloid and amyloid intermediates were present inside glioma tumors of all studied glioma samples, while Aβ peptide immunofluorescence labeled glioma cells, nearby astrocytes and blood vessels. In any case, Aβ peptides and their amyloid aggregates are highly specific antigens, and their presence in glioma tumors can be used to mark glioma cells for visualization, as there are many substances already approved by the Food and Drug Administration for this purpose [68]. The amyloid build-up in glioma tumors may also be used as a target to develop a novel class of anti-tumor drugs.

Acknowledgements

We would like to thank Dr L. Kucheryavykh and the personnel of her laboratory in Universidad Central del Caribe for their kind help. This research was supported by NIH grants SC2GM111149 to M. I. The funding sources had no role in study design; data collection, analysis or interpretation; or the decision to submit this article.

Disclosures

The authors declare that they have no conflicts of interest.

Author contributions

A. Z.-S. performed experiments, analysed the data, and cowrote the paper; A. D.-G. performed experiments, analyzed the data and co-wrote the paper; R. N.-R. performed experiments and analyzed the data; M. I. designed the study, analyzed the data and co-wrote the paper.

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A. Zayas-Santiago et al.

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Accumulation of amyloid beta in human glioblastomas

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web site:

Fig. S1. Samples of glioma with peripheral (control) tissue removed from Patient 1 and stained with different amyloid-detecting dyes: A, thioflavin S (Th-S, green); B, Congo red (red); C, Coomassie brilliant blue G (BBG, red). These slices show no visible staining. Scale bar, 40 μm.

Fig. S2. see https://inyushinlab.org/accumulation-of-amyloid-beta-in-human-glioblastomas/