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Glutamate/glutamine metabolism coupling between astrocytes and glioma cells: Neuroprotection and inhibition of glioma growth

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ABSTRACT

Glioma glutamate release has been shown to promote the growth of glioma cells and induce neuronal injuries from epilepsy to neuronal death. However, potential counteractions from normal astrocytes against glioma glutamate release have not been fully evaluated. In this study, we investigated the glutamate/glutamine cycling between glioma cells and astrocytes and their impact on neuronal function. Co-cultures of glioma cells with astrocytes (CGA) in direct contact were established under different mix ratio of astrocyte/glioma. Culture medium conditioned in these CGAs were sampled for HPLC measurement, for neuronal ratiometric calcium imaging, and for neuronal survival assay. We found: (1) High levels of glutaminase expression in glioma cells, but not in astrocytes, glutaminase enables glioma cells to release large amount of glutamate in the presence of glutamine. (2) Glutamate levels in CGAs were directly determined by the astrocyte/glioma ratios, indicating a balance between glioma glutamate release and astrocyte glutamate uptake. (3) Culture media from CGAs of higher glioma/astrocyte ratios induced stronger neuronal Ca^{2+} response and more severe neuronal death. (4) Co-culturing with astrocytes significantly reduced the growth rate of glioma cells. These results indicate that normal astrocytes in the brain play pivotal roles in glioma growth inhibition and in reducing neuronal injuries from glioma glutamate release. However, as tumor growth, the protective role of astrocytes gradually succumb to glioma cells.

1. Introduction

Glioblastomas are the most common and aggressive brain tumors. Malignant gliomas have been shown to release high amount of glutamate [1], which can promote the growth of gliomas and mediate neuronal cell death [2–4]. In normal brain, glutamate is released from neurons and predominantly taken up by astrocytes, forming a glutamate/glutamine metabolism coupling between astrocytes and neurons [5–8]. Similarly, we speculate that glioma glutamate release and astrocyte glutamate uptake can also form a glutamate/glutamine metabolism coupling, with glioma cells replacing neurons. In an early-stage glioma, glioma cells are surrounded by a large number of astrocytes, glutamate released from glioma cells can be near completely eliminated by astrocytic glutamate uptake. This can prevent neuronal death and reduce the stimulatory role of glutamate on glioma growth, leading to inhibition of glioma expansion. Previous studies demonstrated that inhibition of glioma glutamate release or blockade of glutamate receptors could limit tumor growth [9,10]. However, ammonia is produced when glutamine is deamidated to glutamate by glutaminase, which could induce astrocyte swelling and dysfunction of glutamate uptake via excessive oxidative stress [5,11,12]. Ammonia-induced dysfunction of astrocytic glutamate uptake may contribute to glioma-induced neuronal damage in advanced glioma. In addition, increased glutamate concentration in gliomas has been shown to be associated with a higher risk of glioma-induced seizures [13,14].

Abbreviations: CGA, co-culture of glioma cells with astrocytes; HPLC, high performance liquid chromatography; GFAP, glial fibrillary acidic protein; GDM, glutamate depleted medium; TFB-TBOA, (2S,3S)-3-[3-[4-(trifluoromethyl)benzoylamino]benzyloxy]aspartate; OPA, o-phthalaldehyde; ACSF, artificial cerebrospinal fluid; HBSS, HEPES-buffered saline solution.

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Currently, little is known about the setting of extracellular glutamate concentration by the balance between glioma glutamate release and astrocyte glutamate uptake. We established a co-culture experimental model to evaluate the following hypotheses: (1) Glioma cells express abundant glutaminase, while astrocytes do not. (2) Glioma cells release a large amount of glutamate depending on extracellular glutamine. (3) At an early stage of glioma, glioma cells were outnumbered by surrounding astrocytes and extracellular glutamate could be clamped at low levels to minimize neuronal injury and proliferation of glioma cells. However, at an advanced stage of glioma, glutamate uptake will be compromised by glioma cells: (1) Accompanying glutamate release, glioma cells released large amounts of ammonia may damage astrocytic glutamate uptake. (2) Furthermore, excessive extracellular glutamate can induce neuronal injury.

2. Materials and methods

2.1. Culturing of glioma cells, primary astrocytes and neurons

Glioma cell lines (STTG1, U251, T98G) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen), at 37 °C in humidified atmosphere (95% air/5% CO_2).

Primary cultures of mouse cortical astrocytes were prepared from neonatal C57BL/6 pups as previously described [15]. Briefly, cortices were separated, minced and incubated in papain for 15–20 min. After digestion, dissociated cells were suspended in culture media consisting of DMEM supplemented with 10% FBS and 0.1% penicillin/streptomycin (Gibico). Cells were typically utilized after 2–3 weeks in culture. Greater than 90% of the cultured cells were glial fibrillary acidic protein (GFAP)-positive and cultures were essentially free of neurons. Culture medium was changed every 4–5 days.

Primary cultures of mouse cortical neurons were prepared similarly to astrocytes, except the digested tissues were triturated by pipettes in glutamate depleted medium (GDM) [1] and plated at a density of 1×10^5 cells/ml into 24-well plates or 6-well plates previously coated with poly-lysine, 10 μM 1-β-D-arabinofuranosylcytosine was added to kill off proliferating cells.

2.2. Co-Culture

Glioma cells were co-cultured in direct contact with astrocytes in 96-well plates and 6-well plates. $0.5–1 \times 10^4$ glioma cells/well (U251, T98G, and STTG1) were seeded in 96-well plates, and $1–2 \times 10^5$ glioma cells/well in 6-well plates. All the glioma cells were then mixed with astrocytes at different ratios. In order to eliminate the influence of glutamate contained in serum, cells were cultured in serum-free DMEM medium containing 4 mM glutamine. After incubation for 18 h, cultured media were sampled for HPLC measurement, or for testing effects on cultured neurons.

To evaluate whether or not astrocytes co-cultured with glioma cells remove glioma-released glutamate, TFB-TBOA, a high affinity inhibitor of glutamate transporters, was used for inhibiting astrocytic glutamate uptake.

To assess effects of ammonia on astrocytic glutamate uptake, astrocytes were seeded on 96-well plates with DMEM supplemented with 10% FBS overnight, subsequently medium was replaced by ACSF (115.75 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 23 mM $NaHCO_3$, 10 mM Glucose, 2 mM $MgSO_4$, 2 mM $CaCl_2$) supplemented with 100 μM glutamate and NH_4Cl at indicated concentrations. After a 5 h incubation, extracellular media were collected for HPLC measurement.

2.3. Measurement of extracellular glutamate levels

Glutamate concentration was measured by a breeze HPLC system (Waters, Milford, MA) as previously described [15]. Briefly, samples (10–20 μL) were collected and derivatized with o-phthalaldehyde (OPA), followed by separation through a C18 reverse-phase adsorbosphere OPA-HR column (Alltech, Deerfield, IL) at 37 °C. Mobile phase A consisted of 25 mM sodium acetate, 0.4% 1,4-dioxane and 4.3% 2-isopropanol with pH adjusted to 5.90 by acetic acid. Mobile phase B was a mixture of methanol (97%), 1,4-dioxane (1.5%) and 2-isopropanol (1.5%). Fluorescent signals were excited at 338 nm and detected at 450 nm by a 2475 scanning fluorescent detector (Waters).

2.4. Proliferation of glioma cells and astrocytes in co-culture models

To assess the effects of co-culturing with astrocytes on the proliferation of glioma cells, glioma cells were co-cultured in direct contact with astrocytes in 96-well plates in serum-free medium, control groups were sister astrocytes and glioma cells grown in separate wells. After 4 days, cultured cells were dissolved with 0.05 M NaOH and subsequently neutralized with HCl. The protein concentration of the lysate was measured with the BCA protein assay kit (Pierce).

2.5. Ratiometric $[Ca^{2+}]_i$ measurements

To evaluate their impacts on neuronal Ca^{2+} influx, CGA-conditioned media were applied to cultured neurons, and recordings were obtained and analyzed as previously described with some modifications [1]. Briefly, neurons were loaded with ratiometric Ca^{2+} dye Fura-2-acetoxymethylester (10 μM; Invitrogen) in cultured medium for 45 min. Subsequently, neurons were rinsed with HBSS (126.25 mM NaCl, 2.0 mM $CaCl_2$, 3.0 mM KCl, 1.25 mM NaH_2PO_4, 2.0 mM $MgSO_4$, 10 mM glucose, and 25 mM HEPES, pH 7.40) and placed in a series 20 microperfusion chamber (Harvard Apparatus). To reduce the impact of non-glutamate factors on inducing basal Ca^{2+} response in neurons, CGA-conditioned medium was diluted 1:4 with HBSS, and used to perfuse cultured neurons at a rate of 2.0 ml/min. Neurons were excited at 340 and 380 nm, digitized images were collected at 0.1 Hz for calculating 340:380 ratio by Metafluor program (Molecular Devices).

2.6. Effects of CGA-conditioned medium on neuronal survival

To assess the effects of CGA-conditioned medium on neuronal survival, CGA-conditioned medium was applied to cultured neurons for 48 h, and neuronal survival was evaluated by cell counting on a Leica microscope.

2.7. Immunohistochemistry

Cells grown on glass coverslips were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After three washes in PBS, cells were blocked with PBS + 0.1% Triton X-100 + 5% normal goat serum (Invitrogen) for 90 min. Cells were then incubated with rabbit anti-glutaminase (abcam, ab156876, 1:500) overnight followed by rinses and incubation with fluorescent conjugated secondary antibodies. Monoclonal antibody for GFAP (Cy3 conjugated, sigma, C9205, 1:10,000) was used for co-staining with rabbit anti-glutaminase by incubation together with its compatible secondary antibody. All coverslips were then rinsed, counterstained with 1 μM DAPI (Invitrogen), and mounted onto glass slides with Prolong Gold Antifade (Invitrogen). Immunofluorescence images were collected with a CoolSNAP HQ2 camera (Photometric, Tucson, AZ)

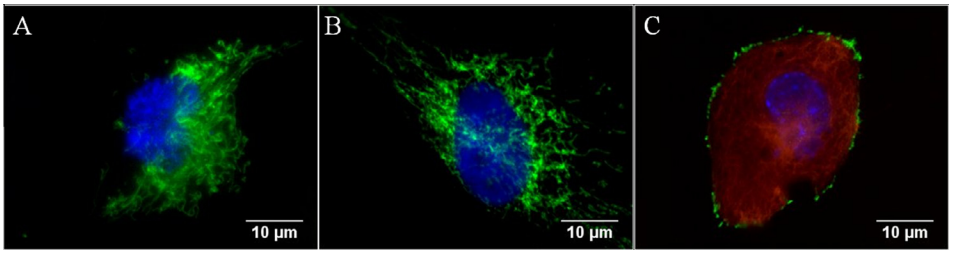

Fig. 1. (A and B) Enriched glutaminase expression in glioma cells (A, STTG1; B, T98G. DAPI: Blue. Glutaminase: green). (C) Small amount of glutaminase immunoreactivity in an astrocyte co-stained with GFAP (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

controlled by MetaMorph program (Molecualr Devices) on a Leica DMI 6000B microscope.

2.8. Statistical analysis

All data were expressed as mean ± SD. Analysis of variance (ANOVA) or Students' test were used to assess the differences between groups, with $P < 0.05$ considered statistically significant. Each experiment was repeated at least 6 times.

3. Results

3.1. Glioma cells expressed glutaminase much more abundantly than astrocytes

All tested glioma cells, including STTG1 (Fig. 1A), T98G (Fig. 1B), and U251 (data not shown), expressed glutaminase, with the high expression levels of glutaminase brightly outlining the mitochondria network. To the contrast, astrocytes do not have significant glutaminase expression, except the small amount in the edge of astrocytes, not overlapping with GFAP and unlikely to be associated with mitochondria (Fig. 1C).

3.2. Glioma cells release large amounts of glutamate in the presence of extracellular glutamine

In the presence of extracellular glutamine, glioma cells released glutamate exceeding 30 μM after 18 h (Fig. 2A–C). In the presence of 20 μM TFB-TBOA in the co-culture systems, extracellular glutamate remained at high levels regardless of the presence of increasing number of astrocytes, indicating a complete blockage of astrocytic glutamate uptake by 20 μM TFB-TBOA without affecting glioma glutamate release (Fig. 2C).

3.3. Astrocyte/glioma ratios determined extracellular glutamate concentrations in mixed cultures

In co-culture systems, increase in astrocyte/glioma ratio was accompanied by reduction of extracellular glutamate level (Fig. 2A–C). At astrocyte/glioma ratios of 1:1 or greater, extracellular glutamate levels were reduced to lower than 1–5 μM (Fig. 2A–C).

However, high concentration of ammonia is produced when glutamine is deamidated to glutamate by glutaminase [5,11,12]. On cultured astrocytes, ammonia dose-dependently slowed down astrocytic glutamate uptake, resulted in higher remaining gluta-

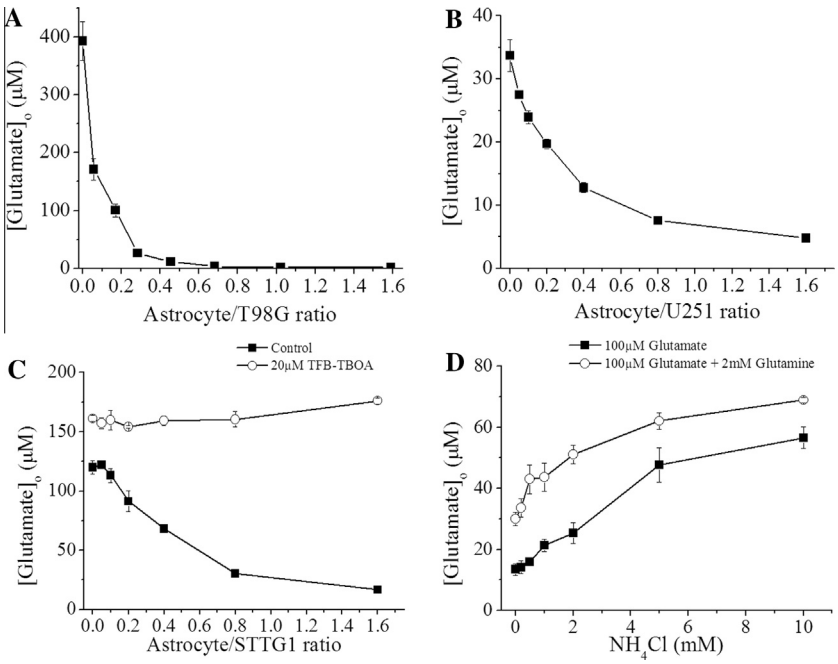

Fig. 2. (A–C) Determination of extracellular glutamate in astrocytes-gliomas co-culture systems after incubation for 18 h. (C) Extracellular glutamate increased after treatment of 20 μM TFB-TBOA ($p < 0.05$). (D) NH_4Cl-compromised astrocytic glutamate uptake (5 h). The inhibitory effects of NH_4Cl on astrocyte glutamate uptake increased in the presence of 2 mM glutamine ($p < 0.05$).

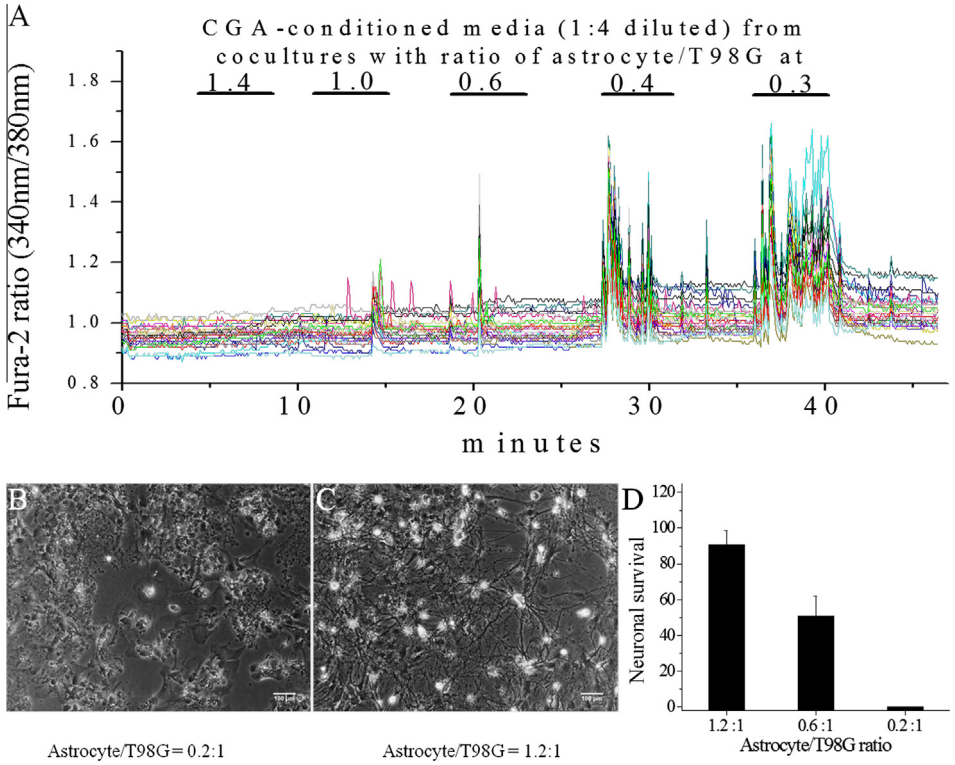

Fig. 3. (A) CGA-conditioned media derived from co-cultures with different astrocytes/glioma ratios induced different amplitudes of Ca^{2+} influx into neurons. (B) Neuronal death in a culture treated with CGA (at astrocyte/glioma ratios of 0.2:1)-conditioned medium (48 h). (C). Most of the neurons survived in CGA (at astrocyte/glioma ratios of 1.2:1)-conditioned medium (48 h). (D). Percentage of neurons survived in CGA (at astrocyte/glioma ratios of 0.2:1, 0.6:1, and 1.2:1)-conditioned medium (48 h).

mate levels after a 5 h incubation in the presence of higher concentration of ammonia (Fig. 2D). It is likely that ammonia-induced dysfunction of astrocytic glutamate uptake may induce elevated glutamate levels in co-culture systems that are dominated by glioma cells (Fig. 2A–C). Interestingly, the toxicity of NH_4Cl on astrocytic glutamate uptake increased remarkably in the presence of millimolar glutamine (Fig. 2D). Perhaps extracellular glutamine in high concentration would inhibit the rapid removal of ammonia and glutamate by astrocytes for synthesizing glutamine [16].

3.4. CGA-conditioned media derived from co-cultures with different astrocyte/glioma ratios induced different amplitudes of Ca^{2+} influx and damage to neurons

Glutamate activation of glutamate receptors can induce Ca^{2+} overload and neuronal death [17]. At astrocyte/glioma ratios of 0.6:1 or lower, a large and persistent increase in $[Ca^{2+}]_i$ (Fig. 3A) was induced, in line with increasing extracellular glutamate concentration, and subsequently led to delayed Ca^{2+} overload and neuronal death after long-term exposure (Fig. 3B and D) [1]. Following the decrease of ratio between astrocytes and glioma cells, cell death of neurons increased (Fig. 3D). However, at astrocyte/glioma ratios of 1:1 or greater, glutamate released by glioma cells can be adequately removed by astrocytes and neuronal death largely prevented in the co-culture systems (Fig. 3C and D).

3.5. Proliferation of glioma cells in co-culture systems containing astrocytes was markedly decreased

In comparison to astrocytes and glioma cells grown in separate wells, proliferation of cells in the co-culture systems was markedly decreased (Fig. 4A). Because glioma cells have profoundly higher

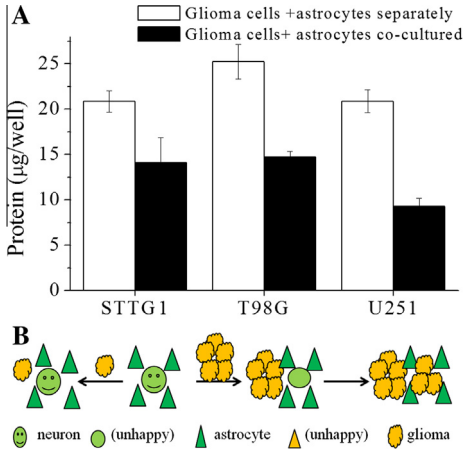

Fig. 4. (A) Glioma cells were co-cultured in direct contact with astrocytes in 96-well plates in serum-free medium, and control groups were astrocytes and glioma cells grown in separate wells. After 4 days incubation, cell proliferation in co-culture systems were markedly less than the sums of astrocytes and glioma cells grown in separate wells ($p < 0.05$). (B). In an early-stage glioma, extracellular glutamate can be controlled by astrocytic glutamate uptake to reduce neuronal injury and glioma expansion. However, in an advanced glioma, glioma-released glutamate in excessive amounts might induce excitotoxic neuronal death and provide space for tumor growth and invasion.

proliferation rate than astrocytes, the reduction of total protein contents in the co-culture system largely suggests inhibition of glioma proliferation. At astrocyte/glioma ratios of 0.3:1 or greater in co-culture systems, astrocytes may inhibit growth of glioma cells in serum-free medium (Fig. 4A). Growth inhibition was detected in co-culture models with direct contact, but not in co-culture models without direct contact (data not shown).

4. Discussion

In this study, the existence of glutamate/glutamine metabolism coupling between glioma cells and astrocytes was proposed: (1) High levels of expression of glutaminase were detected in glioma cells, which was in line with the literature [18]. (2) Glioma cells maintained the ability to release a large amount of glutamate in co-culture systems. (3) Glutamate was promptly removed by astrocytes in co-cultures dominated by astrocytes to prevent neurons from Ca^{2+} overload and cell death, more importantly, proliferation of glioma cells in co-culture systems containing astrocytes was markedly decreased. Giomas glutamate release has been shown to promote glioma growth and to mediate neuronal cell death [1–3]. In the brain, glioma cells are surrounded by large number of astrocytes in an early-stage glioma, extracellular glutamate can be properly controlled by astrocytic glutamate uptake to reduce neuronal injury and inhibit glioma expansion (Fig. 4B). Previous studies indicated that increased tumor cell apoptosis could be induced by increasing glutamate uptake in astrocytes via propentofylline [19]. Furthermore, increased expression of glutamate transporter in peritumoral tissue inhibited glioma growth [20]. Research also demonstrated that seizures were originated close to the tumor mass and patients with small tumor mass were not associated with significant injury to the central nerve system [13,21].

In co-culture systems dominated by glioma cells, markedly increased extracellular concentrations of glutamate were detected. Because ammonia is produced when glutamine is deamidated to glutamate by glutaminase [5,11,12], high concentration of ammonia is produced accompanying excessive glutamate release from glioma cells. Astrocytic glutamate uptake can be compromised by high concentration of ammonia, likely via excessive oxidative stress, and treatment with antioxidants could significantly diminish the ammonia-induced glutamate uptake inhibition [11]. Interestingly, ammonia-induced glutamate uptake inhibition was more obvious in the presence of millimolar glutamine. Perhaps high concentration of extracellular glutamine would exacerbate amonia-induced oxidative stress in astrocytes, in addition to feed-back inhibition of glutamine synthetase [16]. In gliomas at advanced stage, elevated glutamate levels and subsequent neuronal death may be related to ammonia-induced astrocyte dysfunction. Excessive glioma glutamate release could induce excitotoxic neuronal death due to activation of NMDA or AMPA receptors [22]. Recent studies indicated that astrocyte elevated gene-1 (AEG-1) overexpressed in glioma promoted glioma-induced neurodegeneration by increasing glutamate excitotoxicity [23]. In advanced gliomas, glioma-released glutamate could induce excitotoxic neuronal death and claim space for tumor growth and invasion (Fig. 4B).

In summary, glutamate/glutamine metabolism coupling exists between glioma cells and astrocytes, with the extracellular glutamate levels determined by the balance between glioma glutamate release and astrocyte glutamate uptake. Adequate astrocyte glutamate uptake is pivotal to preventing neuronal damage and inhibition of glioma growth.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgments

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