A MECHANISM FOR GLUTAMATE TOXICITY IN THE C6 GLIOMA CELLS INVOLVING INHIBITION OF CYSTEINE UPTAKE LEADING TO GLUTATHIONE DEPLETION

S. KATO,*† K. NEGISHI,* K. MAWATARI† and C.-H. KUO§

Molecular Neurobiology Group, *Department of Neurophysiology, Neuroinformation Research Institute (NIRI), School of Medicine, †Department of Medical Technology, School of Allied Medical Professions and §Department of Pharmacology, Cancer Research Institute, University of Kanazawa, 13-1 Takaramachi, Kanazawa 920, Japan

Abstract—We have demonstrated that addition of L-glutamate in millimolar amounts to a culture of C6 glioma cells induced cell death within 24 h. The glutamate-induced toxicity in the C6 glioma cells was completely suppressed by adding L-cystine (0.4–1.0 mM), while the C6 cells degenerated in L-cystine-deprived culture medium. Kinetic studies of [2-3S]cystine and [3H]glutamate uptake showed that cystine competitively inhibited glutamate uptake, and conversely glutamate inhibited cystine uptake competitively, suggesting that C6 cells have a cystine/glutamate antiporter (system CG or XE) similar to that already described in the periphery. Exogenous cystine (1 mM) stimulated a release of endogenous glutamate from C6 cells in a Na+-independent Cl—dependent fashion. Thus, the antiporter normally transports glutamate out of and cystine into the cells. With the glutamate analogues tested, there was a good correlation between cytotoxicity and inhibition of cystine uptake. The de novo synthesis of glutathione was largely dependent upon the uptake of extracellular cystine. Intracellular levels of glutathione were dramatically decreased within 8–10 h by culture in glutamate-added or cystine-free medium. Vitamin E (100 μM), an antioxidant, rescued the death of C6 cells induced by glutamate exposure or by culture in cystine-deprived medium, but did not restore the apparent decrease of intracellular glutathione.

Taken together, the present data strongly indicate that glutamate-induced cell death is initially due to inhibition of cystine uptake through the antiporter XE system; such inhibition leads to glutathione depletion exposing the cells to oxidative stress. Excess of extracellular glutamate introduced from endogenous or exogenous roots might disorder this mechanism, resulting in cell death.

1-L-Glutamate is thought to serve as the major excitatory neurotransmitter in the CNS and produce its neurophysiological effects by acting at three subtypes of glutamate receptors, each commonly referred to by preferred pharmacological agonists: quisqualate, N-methyl-D-aspartate (NMDA) and kainate.37 In fact, glutamate excites virtually all central neurons and is present in nerve terminals at millimolar levels. joe

Another set of experiments, Olney24,26 established that this toxicity, which he later called excitotoxicity, was not unique to glutamate or to retinal neurons, but was a feature common to the actions of all excitatory amino acids on central neurons. He postulated therefore that glutamate or related compounds, might be the cause of the neuronal cell loss found in certain degenerative neurological disorders such as Huntington’s disease or ischemic brain attack, as occurs in a stroke with abrupt deprivation of blood supply.5,31 The toxic changes produced by glutamate or related excitatory amino acids in vivo are of two sorts: acute swelling of neuronal dendrites and cell bodies and a more slowly evolving neuronal degeneration.32 Axons and glia are relatively spared, although high levels of excitatory amino acids can produce some swelling of glia.3,15

Recent studies indicate that quisqualate interacts with Na+-independent Cl—sensitive glutamate transporter enriched in brain sonicates and synaptosomes, which was originally misunderstood to be a quisqualate subtype of glutamate receptor,3,38 and L-cystine inhibits glutamate sequestration at this quisqualate-sensitive glutamate transporter.16,40 This carrier site may be an expression for the cystine/glutamate antiporter, designated as system XC, in amino acid transporters as already shown in the peripheral fibroblasts.1,2 Recently, a possible suggestion of such an antiporter system has been presented in a neural and glial cell line.5,42
In a previous paper,12 we have demonstrated that glutamate (in the mM range) induced a delayed cell death of rat clonal C6 glioma cells in culture. Some anionic amino acids of longer chain length, such as homocysteate, \( \alpha \)-aminoacidipate and \( \alpha \)-aminomipimelate were also toxic to the cells while asparatate, the shorter anionic amino acid, failed to destroy the cells. Only quisqualate, but not NMDA and kainate, among the three classes of glutamate receptor agonist was toxic to the C6 cells. Moreover, neither competitive nor non-competitive glutamate antagonists blocked the glutamate toxicity. These pharmacological characteristics of glutamate toxicity on C6 glioma cells were quite similar to those of cystine transport inhibition by glutamate or analogues in the peripheral fibroblasts and in the neuronal cell line as mentioned above.12 These data strongly suggest that the glutamate toxicity in the C6 cells is not mediated through glutamate receptors. Therefore, using the C6 glioma cells, we now test this hypothesis of Coyle's laboratory to our glutamate damage of C6 glioma cells and report a glial expression of the cystine/glutamate antiporter (Xc) and a vital role of cystine uptake through this antiporter system for maintaining a cellular level of glutathione (\( \gamma \)-glutamylcysteinylglycine). Glutathione is an important substrate against oxidative stress in the living cells. The inhibition of cystine uptake by excess extracellular glutamate ultimately reduces the levels of cellular glutathione, resulting in a delayed cell death. Depletion of cellular glutathione by some drugs also induces cell death as occurs with glutamate exposure, while vitamin E, an antioxidant, powerfully blocks the cell death by glutamate. All of the data obtained can be linked to the glutamate-induced cell death by oxidative stress. A preliminary form of this work has been published elsewhere.11

**EXPERIMENTAL PROCEDURES**

**Chemicals**

Monosodium L-glutamate, L-cystine, L-cysteine, maleic acid diethyl ester and vitamin E were purchased from Wako Pure Chemicals (Osaka). Monosodium L-aspartate and \( \alpha \)-aminoimipic acid were purchased from Nakarai Chemicals (Kyoto). DL-Homocysteate acid was purchased from Calbiochem-Behring Corp. (La Jolla). Quiescual acid, kainic acid, DL-\( \alpha \)-aminoacidipic acid, L-buthionine (S, R) sulphoximine, glutathione and glutathione disulphide were purchased from Sigma Chemicals (St Louis). \( \gamma \)-Glutamylcysteine was generously supplied by Dr N. Higuchi and were cultured in standard DMEM. T24 cells were a generous gift from Dr Y. Higuchi and were cultured in standard DMEM. Cell viability (cytotoxicity) was measured by the Trypan Blue dye exclusion test and by the release of the cytosolic enzyme, lactate dehydrogenase (LDH), into the culture medium. After exposure to toxic conditions, the medium was collected from each culture plate and centrifuged at 200 g for 5 min. The supernatant was transferred to a fresh tube. LDH activity in the supernatant was assayed spectrophotometrically.39

**Cystine/glutamate uptake studies**

For measurement of \( ^{35} \)Systeine and \( ^{3} \)Hglutamate uptake by C6 glioma cells, a filtration method was used as previously described.11 Cells were harvested at the stationary phase, washed twice with phosphate-buffered saline (PBS), and suspended in Krebs-Ringer solution (pH 7.4). About 2.2 x 10⁶ cells/tube were incubated with various concentrations of radiochemical (0.4 \( \mu \text{Ci/tube} \)) in the absence (for measuring total uptake) or presence (for measuring non-specific uptake) of 2 mM unlabelled compound in a final volume of 1.0 ml. Cystine was dissolved with hydrochloric acid and the pH was adjusted with NaOH. After incubation at 37°C for 10 min (cystine) or 15 min (glutamate), the uptake was terminated by filtration through a Whatman GF/B glass fiber filter, followed by washing three times with 4 ml of ice-cold Krebs-Ringer solution. The filters were soaked overnight in 1 ml of tissue solubilizer (NCS, Amer sham) and radioactivity was then counted in a liquid scintillation counter. Specific uptake was determined by calculating the difference between the c.p.m. bound to filters in the absence or in the presence of 2 mM unlabelled compound.

**Quantitation of releasable glutamate from C6 cells**

Cellular or releasable levels of glutamate were assayed by a high performance liquid chromatography (HPLC) method with postcolumn derivatization using o-phthalaldehyde.14 The measurement of endogenous glutamate release from C6 cells was done as follows: the C6 cells (5.75 x 10⁶) cultured in standard DMEM for four days were collected and incubated in 200; µl of Krebs-Ringer solution without Ca²⁺ ions (Ca²⁺-free) for 5 min at 37°C and then centrifuged at 2,000 g for 1 min at 4°C. Then the resulting supernatant was directly loaded to HPLC for a measurement of spontaneous glutamate release. This 5-min incubation was repeated three times. At the fourth incubation, 1 mCi of cystine in the normal Krebs-Ringer solution was added to the C6 cells for 5 min at 37°C. After centrifugation, the resulting supernatant was filtered and loaded to HPLC for a measurement of glutamate release evoked by cystine. If required, the normal Krebs-Ringer solution containing cystine was replaced by Ca²⁺-free (simple removal of CaCl₂ from normal Ringer solution), Na⁺-free (replacement of NaCl by L-tartrate or choline chloride) or Cl⁻-free (replacement of Cl⁻ by gluconates from NaCl, KCl and CaCl₂) Krebs-Ringer solution containing cystine. Effects of quisqualate on the cystine induced glutamate release from C6 cells were also studied in this manner.

**Glutathione separation by high performance liquid chromatography**

Thiols and disulphides such as cysteine, \( \gamma \)-glutamylcysteine, glutathione and cystine, glutathione disulphide
cells were then loaded onto 0.8% (w/v) agarose horizontal gels. Electrophoresis was performed in 0.5 Tris-acetate (pH 8.0)/25 mM EDTA and incubated for 30 min

Incorporation of [13S]cystine into glutathione fraction

Incorporation of [35S]cystine by C6 glioma cells was linearly increased until 15 min and then saturated. It was also dependent on 10 mM Tris-HCl, 20 mM Na-acetate, 2 mM EDTA, pH 7.5) buffer at 50 V. Genomic DNAs on the agarose gels were stained with ethidium bromide and photographed under UV light with Polaroid type 667 film. Hind III digested bacteriophage λ DNA fragments were used as marker DNAs.

RESULTS

Suppressive effect of cystine to glutamate-induced cell death in C6 glioma cells

In control culture, 1.5 x 10^6 C6 glioma cells were plated in 60-mm dishes and incubated with the standard DMEM. The cells covered the whole surface of the dish as a confluent monolayer which formed two days after culture (Fig. 1A). When 10 mM glutamate was added to the medium at the beginning of the incubation, morphological degeneration of the cells progressed 14–16 h after culture and almost all cells died 24 h after culture, resulting in detachment from the surface of the dish and flotation of cells in the medium (Fig. 1B). The ED50 of glutamate on the cell death was about 4 mM. The surviving fraction of the glutamate-treated cells on day 2 was less than 0.1% of the untreated cell population. The glutamate toxicity was not attenuated by any glutamate receptor antagonists or uptake inhibitors. As such, we examined the role that the quisqualate-sensitive Cl−-dependent glutamate transport system played in the resulting glutamate damage to C6 cells. Addition of cystine (0.5 mM) with glutamate at the beginning of the culture completely suppressed the glutamate-induced cell death, and the morphology on day 2 was similar to that of a control culture (Fig. 1C). A range of 0.4–1.0 mM cystine effectively suppressed the glutamate (7.5 mM)-induced cytotoxicity of C6 cells (Fig. 2). When cystine was removed from the standard DMEM (containing 0.25 mM cystine), C6 cells rapidly degenerated within 12 h even in the absence of glutamate (Fig. 1D). The cell death in the cystine-free medium was more severe and prominent than that induced by glutamate.

Cystine/glutamate uptake in the C6 cells

The above data clearly showed that addition of cystine rescued C6 cells from the glutamate-induced death, and deprivation of cystine from the culture medium resulted in a cell death similar to that seen by glutamate exposure. Therefore, we examined cystine uptake by C6 cells. The cystine uptake by C6 cells cultured in standard DMEM was performed at 37°C for 10 min in Krebs-Ringer solution with [35S]cystine (10 μM to 1 mM). The uptake of [35S]cystine by C6 cells was linearly increased until 15 min and then saturated. It was also dependent upon the number of C6 cells and the temperature of incubation. Lineweaver–Burk analysis of cystine uptake into C6 cells cultured for four days shows that the apparent K_m and V_max for cystine transport were 192 μM and 9.1 nmol/2.2 x 10^6 cells/10 min, respectively (Fig. 3). Glutamate (50–100 μM) clearly inhibi-
Fig. 1. Glioma C6 cells cultured under various conditions. 1.5 × 10^6 cells were plated and incubated for 48 h (A, C and E) and 24 h (B, D and F), respectively. (A) Control culture in standard DMEM; (B) with 10 mM glutamate; (C) with 10 mM glutamate plus 1 mM cystine; (D) in cystine-free medium; (E) with 10 mM glutamate plus 100 μM vitamin E; (F) with 0.5 mM buthionine sulfoximine.

Glutamate receptor agonists or related compounds were then tested for their effect on the cystine uptake system. Of these compounds, DL-homocysteic acid, D,L-α-aminoadipic acid, D,L-α-aminopimelic acid and quisqualate competitively inhibited the [35S]cystine uptake. Quisqualate was the most effective and the IC₅₀ for cystine uptake was 50 μM (Table 1). In contrast, aspartic acid, L-glutamine, GABA, NMDA, kainate and AMPA (a powerful agonist for quisqualate receptor) produced less than 10% inhibition of cystine uptake. There was a good correlation between inhibition of cystine uptake and cytotoxicity by glutamate analogues tested (Table 1; see also Kato et al.2).

We further examined glutamate uptake by C6 glioma cells in the same way for cystine uptake as shown in Fig. 3. Glutamate uptake was performed with [3H]glutamate (10 μM–1 mM) at 37°C for 15 min. A Lineweaver–Burk plot of glutamate uptake
Glutamate toxicity and glutathione in the C6 glioma cells

Fig. 2. Suppressive effect of additional cystine on glutamate (L-Glu) damage in two-day culture of C6 glioma cells. An addition of cystine (0.1–1.0 mM) to the culture medium at beginning completely blocked the glutamate-induced cell death in C6 cells. The C6 cells were proliferated as seen as control culture (cf. Fig. 1C). Each value is the mean of three different experiments with duplicate samples. Vertical bars show S.D.

Fig. 3. Lineweaver-Burk plots of the rate of [35S]cystine uptake by C6 glioma cells as a function of exogenous cystine concentrations. The C6 cells were cultured for four days and then collected. Uptake of cystine was measured after 10-min incubation at 37°C in Krebs-Ringer solution supplemented with 0.01–1 mM [35S]cystine. Glutamate (L-Glu; 50 μM, —O— and 100 μM, —■—) clearly inhibited this cystine uptake in a competitive fashion. Each value is the mean of three different experiments with duplicate samples. Vertical bars show S.D.

Table 1. Cytotoxicity and inhibition of [35S]cystine uptake by glutamate or related substances on the glioma C6 cells

<table>
<thead>
<tr>
<th>Substances</th>
<th>Toxicity ED50 (mM)</th>
<th>Inhibition IC50 (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Glutamic acid</td>
<td>4 mM</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DL-Homocysteic acid</td>
<td>2 mM</td>
<td>0.08 mM</td>
</tr>
<tr>
<td>GABA</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DL-α-Aminoadipic acid</td>
<td>4 mM</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>DL-α-Aminopimelic acid</td>
<td>4 mM</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>Ș-N-methyl-ns-aspartic acid</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Kainic acid</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Quisqualic acid</td>
<td>1 mM</td>
<td>0.05 mM</td>
</tr>
<tr>
<td>AMPA</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

C6 glioma cells were cultured for two days with various substances and cytotoxicity was assayed by counting the number of viable cells (Kato et al.). Cystine uptake experiment was done with [35S]cystine for C6 glioma cells (2.2 × 10⁶). The inhibition of cystine uptake was estimated by counting the radioactivity in the presence or absence of various substances.
Uptake of glutamate was measured for 15-min incubation at 37°C in Krebs-Ringer solution supplemented with 0.01-1 mM [3H]glutamate. Cystine (L-Cys; 200 μM) competitively inhibited the glutamate uptake. Each value is the mean of three different experiments with duplicate samples. Vertical bars show S.D. Therefore, cystine uptake is thought to be a rate-limiting process in glutathione synthesis. A simultaneous separation of cystine, cysteine, γ-glutamylcysteine, glutathione or GSSG was performed by an HPLC method with SH group specific fluorogenic reagents, ABD-F and SBD-F. The retention times in our HPLC system were as follows: cystine, 4.0 ± 0.2 min; cysteine, 5.5 ± 0.2 min; GSSG, 6.7 ± 0.1 min; glutathione, 11.9 ± 0.1 min; γ-Glutamylcysteine, 13.3 ± 0.1 min. C6 glioma cells (6 x 10⁶) were incubated with 10 μM [35S]cystine (2 μCi/tube) for 15 min–6 h, and the cells were pelleted and suspended in PCA. After deproteination, incorporation of [35S]cystine into cysteine, γ-glutamylcysteine and glutathione in the PCA-soluble fractions were measured by counting the radioactivity in each peak fraction separated with HPLC method.

The ratio of [35S]cystine into cysteine and γ-glutamylcysteine was very low and was maximal after 60 min incubation followed by the low constant level (Fig. 6A). In contrast, the ratio of [35S]cystine into glutathione was very high and increased linearly until 2-h-incubation (Fig. 6A). The elution profile of cystine, cysteine, glutathione and γ-glutamylcysteine labeled with [35S]cystine for 4 h is shown in Fig. 6B. The largest peak (retention time = 4 min) was that of cystine taken up into the C6 cells. The incorporation of [35S]cystine measured in PCA-insoluble fraction is also shown in Fig. 6A (a dashed line). The ratio (radioactivity) into the PCA insoluble fraction was 3-4 times higher than that into glutathione. A similar experiment of [35S]methionine incorporation into glutathione was also performed in C6 cells. The ratio of conversion into glutathione from [35S]methionine was less than one tenth of that with [35S]cystine (data not shown).

Reduction of cellular glutathione levels in the C6 cells after treatment with glutamate

We further examined effects of glutamate exposure on the cellular levels of glutathione and GSSG in the C6 cells. A marked decrease in cellular levels of glutathione and GSSG by treatment with 10 mM glutamate is illustrated in Fig. 7. After 4 h incubation, the cellular glutathione gradually decreased and a significant decrease was obtained at 8-10 h after exposure. It reached a level of about 1.5 pmol/μg protein (one tenth of control) at 24 h after exposure. The GSSG content transiently increased at 2-3 h culture and then gradually decreased to an almost undetectable level at 24 h (Fig. 7). The total protein

Cystine clearly induced an endogenous glutamate release from C6 cells in a Cl--dependent manner (B, C). *P < 0.01 compared with control. This cystine-induced glutamate release was blocked by co-existence of quisqualate (D). Experiments were repeated quadricated and a representative chromatogram was shown. **P < 0.01 compared with B. Scale shows 250 pmol for glutamate.
Glutamate toxicity and glutathione in the C6 glioma cells

Fig. 6. Incorporation of \[^{35}\text{S}\]cystine into glutathione (GSH) in the C6 cells. C6 cells (6 x 10^6) cultured for four days were collected and incubated with 10 \(\mu\)M of \[^{35}\text{S}\]cystine at 37°C for 15 min - 6 h. The cells were washed twice with cold PBS, suspended with 0.6 N PCA and sonicated for 1 min. After centrifugation, the PCA-soluble fraction was loaded to HPLC with a reversed phase column. Thiols in the supernatant from C6 cells incubated for various time-periods were separated. Incorporation of \[^{35}\text{S}\]cystine into each thiol was estimated by measuring radioactivities of peak fractions separated by HPLC method. (A) A time-course of \[^{35}\text{S}\]cystine incorporation into thiols (GSH, ---○--; cysteine, ---O--; \(\gamma\)-glutamylcysteine, ---A--). Incorporations into PCA insoluble fraction of C6 cells were also shown in a dashed line (---n---). (B) An elution profile of thiols in the PCA soluble fraction from C6 cells incubated with \[^{35}\text{S}\]cystine for 4 h. The peak of cystine was confirmed by using authentic \[^{35}\text{S}\]cystine.

content was not significantly affected during this period (8-10 h), although it was slightly reduced at 24 h after glutamate exposure (Fig. 7; dashed line). A dose-response curve for glutamate’s effect on cellular glutathione levels by 24-h treatment is shown in Fig. 8. The IC\(_{50}\) of glutamate for reduction of glutathione levels was about 4 mM. The value is the same as seen in the case of glutamate toxicity (see Table 1). Effects of other compounds on GSH content in the C6 glioma cells are also summarized in Table 2.

Fig. 7. Reduction of cellular levels of glutathione (GSH) after treatment by glutamate (l-Glu). C6 cells were cultured with or without 10 mM glutamate and collected at the time-periods indicated. Cellular levels of glutathione and GSSG were measured by the HPLC method. The levels of glutathione (---○---) were dramatically decreased by this 8-10-h glutamate treatment. GSSG (---O---) was also much decreased by 8-10-h incubation. In contrast, protein concentrations were not so decreased by the 8-10-h glutamate exposure (---■---), although a slight decrease was seen at 24 h after exposure. Each value is the mean of five different experiments. Vertical bars show S.D.

Fig. 8. A dose-response curve of glutamate in reduction of cellular level of glutathione (GSH). Cellular glutathione in C6 cells cultured with various concentrations (1-15 mM) of glutamate for 24 h was measured by the HPLC method. Glutamate clearly induced a decrease in cellular glutathione level in a dose-dependent fashion. The IC\(_{50}\) was about 4 mM. Each value is the mean of three to five different experiments. S.D. value is less than 10% of the corresponding mean.
Table 2. Cellular levels of glutathione in the C6 and T24 cells after various treatments

<table>
<thead>
<tr>
<th>Cellular glutathione levels 24 h after treatment</th>
<th>C6 cells</th>
<th>T24 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.4 ± 2.1 pmol/µg protein</td>
<td>15.1 ± 2.6</td>
</tr>
<tr>
<td>Glutamate (10 mM)</td>
<td>1.5 ± 0.8</td>
<td>13.4 ± 1.8</td>
</tr>
<tr>
<td>+cystine (1 mM)</td>
<td>14.2 ± 1.8</td>
<td></td>
</tr>
<tr>
<td>+vitamin E (100 µM)</td>
<td>1.4 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Quisqualate (4 mM)</td>
<td>11.1 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>NMDA (10 mM)</td>
<td>15.0 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>Kainate (10 mM)</td>
<td>14.9 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>Cystine-free medium</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>L-Buthionine sulfoximine (1 mM)</td>
<td>2.4 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Glutamate (10 mM)</td>
<td>13.4 ± 1.8</td>
<td></td>
</tr>
</tbody>
</table>

C6 glioma cells or T24 cells were cultured for 24 h with various substances and cellular levels of GSH were measured by an HPLC method with fluorogenic reagents (see Experimental Procedures). Each value is the mean ± S.D. of three to five determinations.

Glutathione modifying agents to the C6 cells

Exposure of buthionine sulfoximine (BSO, 0.5 mM), an inhibitor of glutathione synthesis, induced a delayed cell death of C6 cells as seen after glutamate treatment (Fig. 1F). The relationship between C6 cell death and BSO concentration is shown in Fig. 9. More than 0.5 mM BSO is lethal to the cell. By the co-existence of BSO and glutamate, the cell death was further accelerated and the C6 cells degenerated within 10–11 h after exposure of them (data not shown). Any concentrations of cystine tested did not suppress the BSO-induced cell death. This BSO treatment of C6 cells induced a marked reduction of glutathione content at 24 h after incubation (Table 2).

Vitamin E protects the C6 cells against glutamate-induced toxicity

In order to see whether glutamate toxicity in the C6 cells was primarily caused by depletion of glutathione itself or, secondarily, caused by exposing the cells to oxidative stress, we examined effects of an anti-oxidant or reducing agents on the C6 cells treated with glutamate (10 mM). Of these compounds, vitamin E (100 µM) protected the C6 cells against glutamate-induced cell death (Fig. 1E), but did not prevent the reduction of glutathione content by glutamate exposure (Table 2). The protective effect of vitamin E was ascertained by the measurement of releasable LDH into the culture medium (Fig. 10). A significant release of LDH was evoked 13–14 h after glutamate exposure. The release was gradually increased thereafter. The addition of vitamin E clearly blocked the release of LDH even after two-day culture, as in the control culture. The addition of vitamin E was effective not only at the beginning of the culture but also at 10–12 h after glutamate exposure (data not shown). Two reducing agents, glutathione and cysteine (more than 1 mM) did not protect against glutamate toxicity.

![Fig. 9. Buthionine sulfoximine (BSO)-induced cell death in the C6 glioma cells. Addition of BSO (0.1–0.5 mM) to the culture medium induced a cell death just as seen after glutamate exposure (cf. Fig. 1B and 1F). Each value is the mean of three different experiments with duplicate samples. S.D. value is less than 10% of the corresponding mean.](image)

![Fig. 10. LDH release by glutamate and protection by vitamin E. C6 cells were cultured in standard DMEM (–O–), with 10 mM glutamate (–•–) or with 10 mM glutamate plus 100 µM vitamin E (–○–) and the supernatant of each culture condition was collected at the time-periods indicated (0–36 h). LDH activity was expressed by measuring the reduction of NADH (ΔΔA660/3 min per 20 µl). Glutamate evoked a significant release of LDH at 13–14 h after exposure. In contrast, addition of vitamin E clearly protects against the release of LDH into the culture medium (cytotoxicity) induced by glutamate. Each value is the mean of three different experiments with duplicate samples. Vertical bars show S.D.](image)
Glutamate toxicity and glutathione in the C6 glioma cells

DNA fragmentations by glutamate treatment

In the previous paper,12 we reported decompositions of cellular DNA in glutamate-treated C6 cells by flow cytometric analysis. In the present study, DNA damage of C6 glioma cells caused by glutamate (10 mM) was examined by analysis of genomic DNA prepared from the cells using 0.8% agarose gel electrophoresis. Genomic DNAs from glutamate-treated C6 glioma cells for 24 h show DNA fragmentation as smear bands of 4–20 kbp on a 0.8% agarose gel (Fig. 11; Glu), whereas genomic DNAs from control C6 cells show a single band of more than 20 kbp (Fig. 11; C6). The DNA fragmentation by glutamate was also protected with vitamin E treatment (data not shown).

Lack of effect of glutamate on T24 cells

In order to test whether or not the cytotoxicity of glutamate is universal to any tumor cells, T24 cells (originally derived from human urinary bladder carcinoma cells; Japanese Cancer Research Resources Bank) were cultured with glutamate. The T24 cells did not degenerate in the presence of more than 10 mM glutamate (data not shown). The IC₅₀ of glutamate on the inhibition of [³⁵S]cystine uptake was more than 1 mM for T24 cells, and the cellular glutathione level in T24 cells cultured with 10 mM glutamate for 24 h did not change (Table 2).

DISCUSSION

Cystine/glutamate antiport transporter system in the C6 cells

The glutamate-toxicity in our C6 glioma cells is characterized as follows: (i) the effective concentration of glutamate was very high, at mM levels; (ii) only quisqualate was toxic to the cells, but not kainate, NMDA or even AMPA; (iii) the toxicity was not attenuated by glutamate receptor antagonists (including CNQX) or glutamate uptake inhibitor.

These data suggest that the glutamate toxicity is not mediated through glutamate receptors. And quisqualate does not interact with glutamate receptor

Fig. 11. DNA fragmentations of C6 cells by glutamate treatment. Genomic DNAs (0.5 µg) from C6 cells treated with or without 10 mM glutamate for 24 h were extracted and separated on 0.8% agarose gel electrophoresis. Bacteriophage λ DNA with Hind III digestion was used as marker DNAs. By glutamate treatment (Glu), DNA fragmentations became evident as a smear of 4–20 kbp size as compared with control (C6). An arrow indicates the starting origin of electrophoresis.
sites or with ordinary Na⁺-dependent glutamate carrier sites. According to the recent paper by Cho and Bannai,⁴ glutamate transport in the C6 glioma cells was divided into two components; Na⁺-dependent and Na⁺-independent. The presence of a Na⁺-independent glutamate transport was reported in brain synaptosomes,⁴⁰ neuroblastoma–retina hybrid N18-RE-105 cells²² and in glioma LRM-55 cells.³⁵ The glutamate transport in these cells may reflect the expression of antiporter for cystine/glutamate Nl8-RE-105 cells² and in glioma LRM-55 cells. Therefore, the present paper is elucidating and confirming the presence of cystine/glutamate antiporter system in C6 glioma cells.

The protective effect of cystine (0.4-1.0 mM) on glutamate toxicity in our C6 cells can be reasonably explained by this antiporter system. It is well known that glutamate is the most abundant free amino acid in the central nervous system. The intracellular level of glutamate in our C6 cells cultured for four days is high, 50.6 ± 10.4 nmol/mg protein (the intracellular concentration is around 10 mM). Therefore, the antiporter for cystine/glutamate usually functions to transport glutamate outside and cystine inside. If excess of glutamate is added in the culture medium, it may compete with cystine for the transporter, resulting in cell death. Addition of cystine (0.4-1.0 mM) restores the driving force for cystine uptake against glutamate exposure, while the experiment in the cystine-free medium further supports this idea. The C6 cells cultured in the cystine-free medium degenerated more rapidly within 12 h than after glutamate exposure (24 h). Such a hypothesis was also supported by a good correlation between cytotoxicity and inhibition of cystine uptake (Table 1). In T24 cells which showed a poor expression of the cystine/glutamate antiporter, over 10 mM of glutamate was not cytotoxic to the cells. This suggests that the mechanism for glutamate toxicity in the present study is not generally applied to any tumour cells, but is limited to cells or cell lines having the cystine/glutamate antiporter system.

Glutathione depletion and glutamate toxicity in the C6 cells

The incorporation of [³⁵S]cystine into glutathione in the C6 glioma cells was very rapid, and the incorporation ratio was about ten-times higher than that of [³⁵S]methionine into glutathione, indicating that the de novo synthesis of this tripeptide in our C6 cells was largely dependent on the uptake of extracellular cystine but not methionine through the cystine/glutamate antiporter. The deprivation of cystine from the culture medium rapidly reduced the glutathione level in C6 cells and was lethal to the cells, while addition of cystine maintained a normal level of cellular glutathione even with glutamate exposure (Table 2). Furthermore, both glutathione inhibiting and depleting agents induced a delayed death of C6 cells as did glutamate exposure. The BSO and glutamate were additively toxic to the cells. These data together suggest that the inhibition of cystine uptake by glutamate leads to glutathione depletion and finally results in delayed death of C6 cells (16-24 h). The glutamate-induced DNA damage has been also confirmed by DNA fragmentations in agarose gel electrophoretic analysis.

Glutathione is a major non-protein SH compound in the living cells and plays a key role for protection against oxidative stress and the conjugation for various toxins.⁹,²⁰ Therefore, the glutathione depletion by glutamate appears to induce a cell death. After 8-10 h glutamate exposure, the glutathione content of C6 cells significantly declined, although the significant release of LDH into the culture medium was evoked only 13-14 h after glutamate exposure. The data strongly suggest that the decrease in intracellular level of glutathione by glutamate treatment precedes the release of LDH (cytotoxicity). GSSG levels were also decreased by this treatment, although its concentration increased transiently 2-3 h after glutamate exposure. Such an imbalance between glutathione and GSSG contents at the initial period of glutamate exposure has never been reported and could form the basis of future studies. The present vitamin E experiment suggests that the death of C6 cells by glutamate is not simply due to glutathione depletion but is due to the generation of some oxidative processes, such as membrane lipid peroxidation as reported by others.²¹,²² However, questions as to which types of oxidative stress (including active oxygen or free radicals) involved in the glutamate-induced cell death and DNA damage remain unsolved.

CONCLUSION

Glutathione undoubtedly is important in brain metabolism. The intracerebral level is high, ranging from 1 to 3 mM in human and monkey.²² All the enzymes for the γ-glutamyl cycle are present in the brain and choroid plexus.²³ Alterations of glutathione metabolism may accompany derangements of brain function. In particular, hypoxia is associated with a reduction of brain glutathione levels.⁵,³⁰ Furthermore, Raps et al.²⁵ recently observed that glutathione levels in astrocytes are high compared with those in neurons. Yudokoff et al.³⁴ also showed that the glial glutathione pool is quite large and its turnover is high.
Glutamate toxicity and glutathione in the C6 glioma cells

enough to be of potential importance to brain glutamate metabolism. The cellular pools of glutathione must be considered more important with respect to cystine/glutamate antiporter in glial cells, since the antiporter is necessary to support maintenance of cellular level of glutathione. Furthermore, as the existence of such an antiporter system has been recently reported in primary culture of newborn rat brain astrocytes, the gliotoxicity induced by glutamate with high concentrations on CNS glia may be partly mediated through this mechanism.

Acknowledgements—We thank Dr N. Sakura and N. Asano (School of Pharmacy, Hokuriku University) for preparing γ-glutamylcysteine. We also thank Dr Y. F. Mahe for reading our manuscript and Mrs Tami Urano for her secretarial assistance. This study was supported in part by research grants (Nos 61480105 and 01650506 to S.K.) from the Ministry of Education, Science and Culture, Japan and from the Fund for Medical Treatment of the Elderly, School of Medicine Kanazawa University, 1991.

REFERENCES


(Accepted 5 November 1991)