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Immunostimulation of C6 Glioma Cells Induces Nitric Oxide-Dependent Cell Death in Serum-Free, Glucose-Deprived Condition

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ABSTRACT– Recently, we reported that immunostimulation of primary rat cortical astrocyte caused stimulation of glucose deprivation induced apoptotic cell death. To enhance the understanding of the mechanism of the potentiated cell death of glucose-deprived astrocyte by immunostimulation, we investigated the effect of immunostimulation on the glucose deprivation induced cell death of rat C6 glioma cells. Co-treatment of C6 glioma cells with lipopolysaccharide (LPS, 1 μg/ml) and interferon γ (IFNγ, 100U/ml) in serum free condition caused marked elevation of nitric oxide production (>50 μM). In this condition, glucose deprivation caused significant release of lactate dehydrogenase (LDH) from C6 glioma cells while control cells did not show LDH release. To investigate whether elevated level of nitric oxide is responsible for the enhanced LDH release in glucose-deprived condition, C6 glioma cells were treated with 3-morphorinosydnonimine (SIN-1) and it was observed that SIN-1 caused increase in LDH release from glucose-deprived C6 glioma cells. Treatment of C6 glioma cells with 25 μM of pyrrolidinedithiocarbamate (PDTC) which inhibit Nuclear factor κB (NF-kB) activation, caused complete inhibition of nitric oxide production. Treatment of C6 glioma cells with NO synthase inhibitors, N^G-nitro-L-arginine (NNA) or L-Nω-nitro-L-arginine methyl ester (L-NAME), caused inhibition of nitric oxide production and also glucose deprivation induced cell death of cytokine-stimulated C6 glioma cells. In addition, diaminohydroxypyrimidine (DAHP, 5 mM) which inhibits the synthesis of tetrahydrobiopterine (BH4), one of essential cofactors for iNOS activity, caused complete inhibition of NO production from immunostimulated C6 glioma cells. The results from the present study suggest that immunostimulation causes potentiation of glucose deprivation induced death of C6 glioma cells which is mediated at least in part by the increased production of nitric oxide. The vulnerability of immunostimulated C6 glioma cells to hypoglycemic insults may implicate that the elevated level of cytokines in various ischemic and neurodegenerative diseases may play a role in their pathogenesis.

Key words □ C6 glioma cell, cytokine, nitric oxide, glucose deprivation, LDH release

Astrocytes play essential roles in the maintenance of normal neuronal function. Recently, we and other researchers have reported that immunostimulated astrocytes and microglia synergistically enhanced n-methyl-D-aspartic acid (NMDA)- or glucose deprivation- mediated neuronal cell death via production of nitric oxide (NO; Kim and Ko, 1998; Hewett *et al.*, 1994, 1996). We have also reported that rat cortical primary astrocyte itself show potentiated glucose-deprivation-induced cell death upon induction of iNOS by the co-treatment with lipopolysaccharide (LPS) and interferon-γ (IFNγ) (Choi and Kim, 1998).

The primary culture of rat astrocyte often contaminate small population of microglial cells which is usually less than 5%. However, the presence of even a small population of microglial cells in astrocyte cultures may complicate the study of NO production and NO-induced toxicity by producing substantial amount of NO and cytokines (Murphy *et al.*, 1993). Several researchers have used rat C6 glioma cells, which is a homogeneous cell line with astrocyte-like property, for the study of NO production and NO-induced toxicity (Tra-

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jkovic *et al.*, 1999; Park and Murphy, 1994; D'sa *et al.*, 1996; Liu *et al.*, 1998).

In this study, we investigated the role of immunostimulation-mediated increase in NO production from C6 glioma cells on the glucose deprivation-induced cell death to further enhance the understanding about the mechanism of the potentiation of glucose deprivation-induced cell death of astrocyte by NO.

MATERIALS AND METHODS

Materials

Lipopolysaccharide (LPS), Nω-nitro-L-arginine methyl ester (L-NAME), pyrrolidinedithiocarbamate (PDTC) were purchased from Sigma (St. Louis, MO). Recombinant rat IFN-r, DMEM/F12, glucose-free DMEM and fetal bovine serum were from Gibco BRL (Grand Island, NY). N^G-nitro-L-arginine (NNA) and 2,4-Diamino-6-hydroxypyrimidine (DAHP) were obtained from Research Biochemical International (Natick, MA). 3-Morpholinosydnonimine (SIN-1) and S-nitroso-N-acetylpenicillamine (SNAP) was obtained from Calbiochem (La Jolla, CA).

C6 glioma and primary astrocyte cell culture treatment with LPS and IFNγ, and glucose deprivation

C6 glioma cells were derived from American Type Culture collection (ATCC) and were cultured in DMEM/F12 with 10% heat inactivated fetal bovine serum (FBS). Confluent cultures of C6 glioma cells were harvested by brief trypsinization and were adapted to serum free condition for 48hr. During the adaptation period, cell mortality was less than 5% as evidenced by trypan blue exclusion. The adapted cells, which were grown on 12-well plates (NUNC, Denmark) were pretreated for 48 hours with IFNγ (100U/ml) and LPS (1 μg/ml) alone or in combination. To induce alterations in NO production from immunostimulated C6 glioma cells, L-NAME (1 mM), L-NNA (800 μM), PDTC (25 μM), diaminohydroxypyrimidine (DAHP, 5 μM) were treated with IFNγ and LPS. Rat primary astrocytes were cultured from the prefrontal cortices of 2- to 4-day-old Sprague-Dawley rat pups as previously described (Kim *et al.*, 1999; Kim and Pae 1996). Cells (12-13 days in culture) were pretreated for 48 hours with IFNγ and LPS alone or in combination.

After the immunostimulation, glucose deprivation was achieved by repeated rinsing and incubation in glucose free Dulbeccos modified Eagles medium (DMEM, Gibco BRL, Grand island, NY) that was not supplemented with serum, which interfered with the LDH assay.

To investigate the effect of exogenous NO on glucose deprivation induced death of C6 glioma cells, the cells were treated with 200 μM of SIN-1 during the glucose deprivation period.

Measurement of NO

NO production was determined by measuring nitrite, a stable oxidation product of NO, as described previously (Green *et al.*, 1990). In brief, nitrite levels were determined by adding the Greiss reagent (mixing equal volumes of 0.1% napthylethylenediamine dihydrochlroride and 1% sulfanilamide in 5% phosphoric acid). After 10 min, the absorbance at 550 nm was determined using an UV spectrophotometer (Beckmann DU-650, Fullerton, CA).

Measurement of cell death

Cell death was assessed by morphological examination of cells using phase-contrast microscopy and quantified by measuring LDH release into the medium at various time points after starting glucose deprivation. The LDH amount corresponding to complete glial damage/death was measured in sister cultures treated with 0.1% Triton X-100 for 30 min at 37°C. Basal LDH levels (generally less than 10% of total LDH release) were determined in sister cultures subjected to sham wash with 5 mM glucose containing DMEM and subtracted from the levels in experimental conditions to yield the LDH signal specific to experimental injury.

Nuclear staining with Hoechst 33258

After glucose deprivation, C6 glioma cells were fixed with 4% paraformaldehyde, permeabilized with ice-cold-ethanol, and stained with 50 ng/ml Hoechst 33258 as described previously (Jacobson and Raff, 1995). Stained nuclei were observed and photographed with Olympus BH2 fluorescent microscope (Olympus, Japan).

Statistical analysis

Data are expressed as the mean ± standard error of mean (S.E.M) and analyzed for statistical significance by using one way analysis of variance (ANOVA) followed by Kneuman-Keul's test as a *post hoc* test and a P value <0.01 was considered significant.

RESULTS

C6 glioma cells were treated with LPS (1 μg/ml), IFN-γ

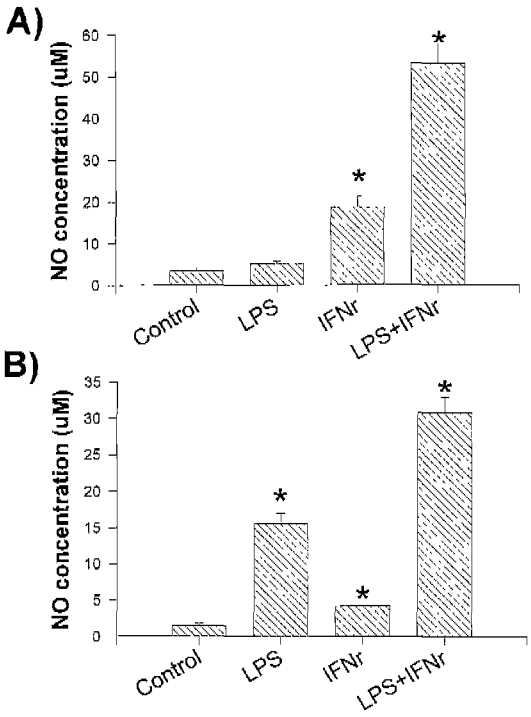

Fig. 1. Production of NO from C6 glioma and primary rat cortical astrocytes. C6 glioma cells (A) and primary rat cortical astrocytes (B) were stimulated for 48 hr with LPS (1 μg/ml), IFNγ (100 U/ml) or LPS plus IFNγ in serum-free DMEM. Culture media was analyzed for NO production by Griess reaction as described in Materials and Methods. Each bar indicates the mean ±S.D. (n=4). *Significant difference from untreated control (*: P<0.01).

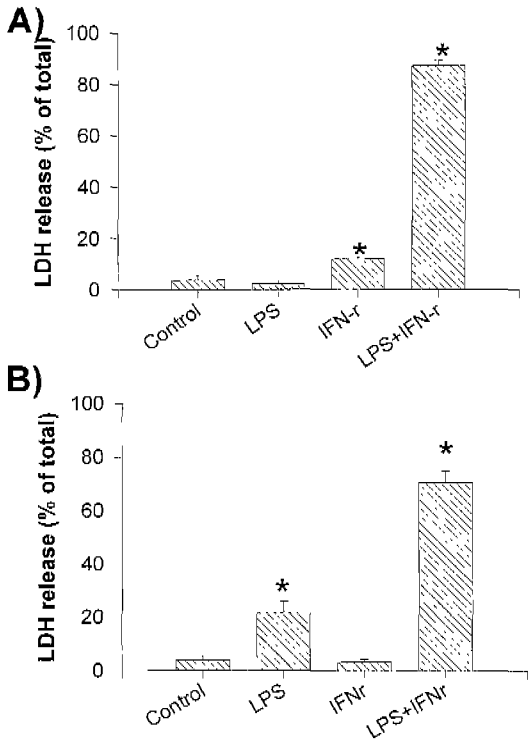

Fig. 2. Potentiated death of glucose-deprived cells after induction of iNOS. C6 glioma cells (A) and primary rat cortical astrocytes (B) were stimulated for 48hr with LPS (1 μg/ml), IFNγ (100 U/ml) or LPS plus IFNγ in serum-free DMEM. Immunostimulated cells were incubated in the absence of glucose. The amounts of LDH released in the medium from C6 glioma cells and primary rat cortical astrocytes were determined at 8 and 6 hr, respectively, after starting the glucose deprivation. *Significant difference from untreated control (*: P< 0.01).

(100 U/ml) or the combination of LPS and IFN-γ for 48 hr. Both LPS and IFNγ slightly increased the NO production from C6 glioma cells and also from primary rat cortical astrocyte. Co-treatment with LPS and IFN-γ synergistically stimulated the NO production from the cells (Fig. 1).

When these cells were deprived of glucose, only LPS and IFN-γ co-treated C6 glioma and primary astrocyte cells showed time-dependent release of cellular LDH contents. In case of LPS or IFN-γ, LDH release was only marginal at all time points investigated (Fig. 2). Longer periods of glucose deprivation (typically, more than 12 hr) caused significant LDH release from the control C6 cells or C6 cells treated with LPS or IFN-γ alone (data not shown). The death of C6 glioma cells were apoptotic by nature when the cells were observed by light microscopy as evidenced by cell shrinkage and occasional apoptotic body. In addition, chromatin condensation, which was commonly used as one of the whole-mark of apoptotic cell death, was observed when the cell was stained with Hoechst dye (Fig. 3).

To investigate whether increased NO production from immunostimulated C6 glioma cells was responsible for the potentiation of glucose deprivation-induced death, we treated C6 glioma cells with several agents which may hinder NO production or iNOS induction. As shown in Fig. 4 the treatment with NO synthase inhibitors, NNA (800 μM) or L-NAME (1 mM), caused significant inhibition of NO production from immunostimulated C6 glioma cells. Also, the treatment with PDTC (25 μM), a NF-κB inhibitor, caused complete abolishment of increase in NO production from C6 glioma cells. In addition, diaminohydroxypyrimidine (DAHP, 5 mM) which inhibits the synthesis of tetrahydrobiopterine (BH4), one of essential cofactors for iNOS activity, caused complete inhibition of NO production from immunostimulated C6 glioma cells (Fig. 4A). At these conditions, glucose deprivation did not show significant LDH release from C6 glioma cells (Fig. 4B).

To further confirm whether the increased NO potentiates the glucose deprivation-induced death of C6 glioma cells, we

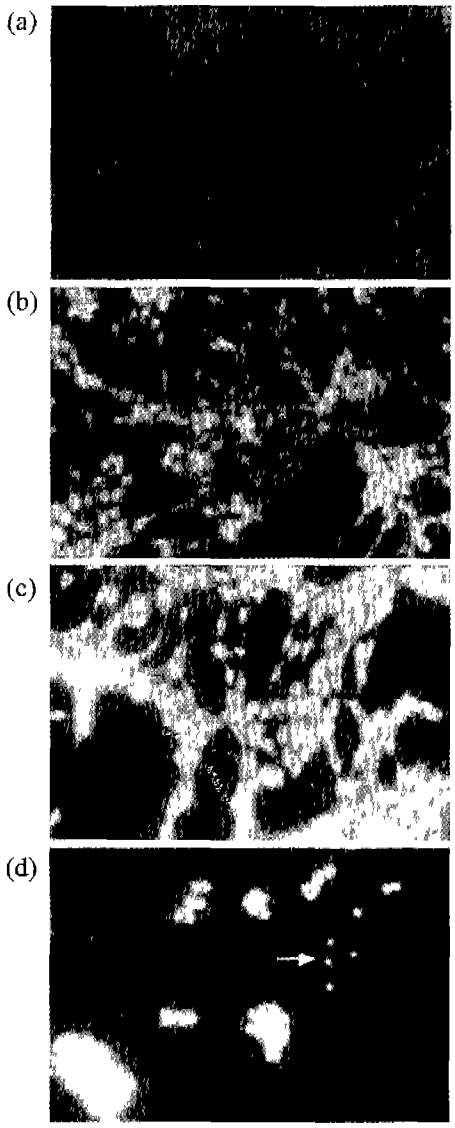

Fig. 3. Glucose deprivation-mediated apoptotic death of immunostimulated C6 glioma cells. Cell nucleus was stained with Hoechst 33258 at 6hr after starting the glucose deprivation from control (a), LPS (1 μg/ml) (b), IFNγ (100 U/ml) (c) or LPS plus IFNγ (d) treated C6 glioma cells. In LPS plus IFNγ treated cells, nuclear condensation was observed, which was indicated by an arrow.

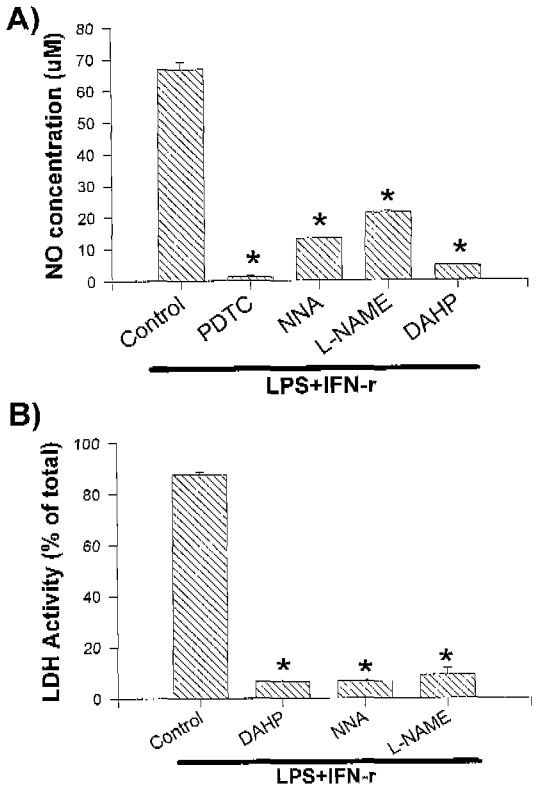

Fig. 4. Inhibition of NO production and cell death from immunostimulated C6 glioma cells by NO inhibitors. C6 glioma cells were co-treated with L-NAME (1 mM), NNA (800 μM), PDTC (25 μM), DHEA (100 μM) or DAHP (5 μM) upon immunostimulation with LPS plus IFNγ. The production of NO (A) and the activity of LDH released into the media (B) were determined as described in figures 1 and 2. *Significant difference from untreated control (*: $P<0.01$).

treated the C6 glioma cells with NO releasing reagent 3-morpholinosydnonimine (SIN-1, 200 μM). The treatment with SIN-1 caused time- and dose-dependent release of LDH upon glucose-deprivation (Fig. 5) from the C6 glioma cells. Another NO releasing reagent, S-nitroso-N-acetylpenicillamine (SNAP) showed qualitatively similar results (data not shown).

DISCUSSION

Recently, we have reported that immunostimulated rat primary astrocyte culture showed potentiated death upon glucose deprivation possibly *via* a mechanism involving increased NO production (Choi and Kim, 1998).

Several researchers have reported that although passaging of primary astrocytes eliminates microglia, those secondary culture still contain 1-3% of microglia (Simmons *et al.*, 1992; Galea *et al.*, 1992; Feinstein *et al.*, 1994). Those contaminating microglia showed stronger response than astrocytes upon treatment with LPS and cytokines to produce cytokines (Simmons *et al.*, 1992; Galea *et al.*, 1992). Since those inflammatory mediators including IL-1β and TNF-α could participate in the induction and/or regulation of NO production, it would be of interest to investigate the NO mediated potentiation of glucose deprivation induced cell death upon immunostimulation in C6 glioma cells. C6 glioma cell driven from rat astrocytes is homogeneous and free of microglial contamination.

In this study, LPS and IFNγ alone only slightly increased

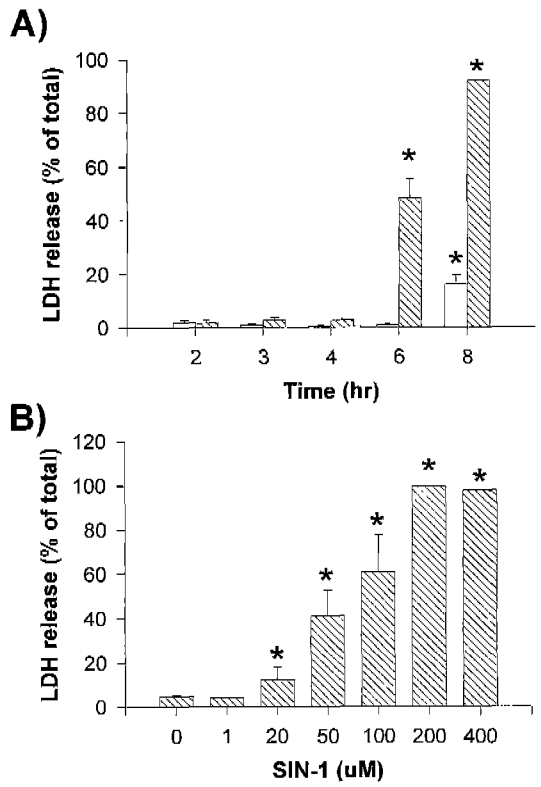

Fig. 5. SIN-1 potentiates glucose deprivation-induced LDH release from C6 glioma cells. A) C6 glioma cells were exposed to SIN-1 in the absence (open bar) or presence (hatched bar) of glucose. The activity of LDH released into the media from cells treated with SIN-1 (200 μM) was determined at various time points after glucose deprivation. *Significant difference from time zero (*: $P<0.01$). B) Activity of LDH released into the media from cells treated with various concentration of SIN-1 was determined at 8 hr after glucose deprivation. *Significant difference from untreated control (*: $P<0.01$).

NO production if any from both primary astrocytes and C6 glioma cells while combination of the two immuno-stimulants caused synergistic increase of NO production. The synergistic increase in NO production by co-stimulation of LPS and IFNγ was also reported by several other researchers (Gross *et al.*, 1992; Dobashi *et al.*, 1997; Noda *et al.*, 1997). The large increase in NO production induced by co-treatment with LPS and IFNγ caused potentiated cell death upon glucose deprivation in C6 glioma cells which is also observed in primary rat cortical astrocytes (Figs. 1, 2). In this study, LPS alone caused slight increase in NO production from primary astrocytes but the extent of the increase in NO production was much less in case of C6 glioma cells. Glucose deprivation induced slight LDH release from primary astrocytes but C6 glioma cells did not show increase in LDH release when treated with LPS alone (Figs 1,2). Several researchers have indicated that LPS induces substantial amount of cytokine and NO production from microglia which is normally contaminating astrocyte culture and to a lesser extent from astrocyte itself (Galea *et al.*, 1992; Benveniste *et al.*, 1986). It has also been reported that microglia play a pivotal role on the production of oxygen radicals and also on the progression of some forms of neurodegenerative diseases (Chao *et al.*, 1995; Hall *et al.*, 1998). Therefore, the above results may suggest that astrocyte-microglial network is important for the production of NO and other inflammatory mediators, which may be co-operative for the expression of toxicity to primary astrocyte culture upon glucose deprivation. Several researchers reported that the microglia and astrocyte might form a paracrine mode of network for the synergistic activation of the production of cytokines and NO from primary astrocyte culture (Simmons *et al.*, 1992; Pahan *et al.*, 1998). In addition, several researchers reported that coordinated action of oxygen free radicals and NO, both of which is produced from primary astrocytes stimulated with LPS, is important for the neuronal cell death induced by glutamate treatment or glucose deprivation (Kim *et al.*, 1998, 1999; Hewett *et al.*, 1994, 1996).

When the C6 glioma cells were treated with IFNγ alone, 18.7±2.5 μM of NO was produced from the cell and only slight LDH release was observed in this condition (Figs, 1,2). These data may suggest that it would be required a threshold level of NO production for the induction of LDH release from glucose-deprived C6 glioma cells. At this point, it is important to note that although similar level of NO production was achieved by LPS treatment in primary astrocyte (15.5±1.5 μM), significant cell death was observed in this case (21.7±4.2%) as compared with IFNg effects on C6 glioma cell (11.9±0.8%). These data further implicate that in primary astrocyte culture, other factors such as reactive oxygen species rather than NO alone is also responsible for the potentiated cell death in glucose-deprived condition. Several researchers have reported the synergistic increase in cytotoxicity by NO and reactive oxygen species (Bonfoco *et al.*, 1995; Weinberger *et al.*, 1998).

NO synthase inhibitors, L-NAME and L-NNA, decreased the NO production from immunostimulated C6 glioma cells by 80±0.4% and 68±0.9%, respectively and this caused complete inhibition of cell death upon glucose deprivation (Fig. 4). The induction of NO production from immunostimulated C6 glioma cells and primary astrocytes involve the activation of NF-κB, which is inhibited by PDTC (Pahan *et al.*, 1999). In the present study, the treatment with PDTC completely inhibited NO production. These results further demonstrate

the importance of NF-kB for the induction of NO production from C6 glioma. Nitric oxide synthase requires for its maximal enzyme activity several cofactors including tetrahydrobiopterine (BH4) and nicotinamide adenine dinucleotide phosphate (NADPH). It has been reported that immunostimulation of C6 glioma cells or primary astrocytes induces both iNOS and GTP cyclohydrolase I, which is rate limiting enzyme for the synthesis of BH4 and is inhibited by DAHP (Sakai *et al.*, 1995; D'Sa *et al.*, 1996). In this study, we co-treated C6 glioma cells with DAHP during the immunostimulation period, which caused complete inhibition of NO production and glucose deprivation induced cell death of C6 glioma cells (Fig. 4). These data implicate that BH4 availability but not is also an important regulator for NO production from immunostimulated C6 glioma cells as well as iNOS induction.

In conclusion, C6 glioma cells produced large amount of NO upon stimulation with LPS and IFNγ. The increase in NO production from the cell caused potentiation of the glucose deprivation-induced death of C6 glioma cells, as in case of primary astrocytes. It has been suggested that the potentiation of glucose deprivation-induced death of primary astrocytes have some implications in the severe pathogenesis of recurrent ischemia (Choi and Kim, 1998). C6 glioma cell, which is homogeneous and continuous astrocyte-like cell line free of microglial contamination, would be very useful for the study of the mechanism of the potentiated death of astrocyte by immunostimulation.

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