

Ketamine suppresses the proliferation of rat C6 glioma cells

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Abstract. The present study investigated the effects of N-methyl-D-aspartate receptor (NMDAR) antagonist ketamine, on the growth of gliomas. To analyze the effects of ketamine treatment, rat C6 glioma cells arising from astrocytes, and RNB cells representing non-malignant astrocytes, were examined. In ketamine-treated C6 cells, the gene expression changes associated with cell proliferation following ketamine treatment were evaluated using a cDNA microarray. A cell proliferation assay was performed to analyze the dose-dependent proliferation of C6 glioma and RNB cells following culture (72 h) with ketamine treatment (0-100 μ M). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were performed following cell incubation with/without ketamine, to confirm if the ketamine-induced cell death of C6 glioma and RNB cells were due to apoptosis. In addition, cell proliferation and TUNEL assays were performed following cell incubations with a selective NMDAR antagonist, D-2-amino-5-phosphonovaleric acid (D-AP5). Analysis of the cDNA microarray indicated that the growth of C6 glioma cells were suppressed by the effects of ketamine. Furthermore, results of the proliferation assay confirmed that ketamine treatment inhibited C6 cell proliferation, most notably at a dose of 30 μ M (n=7, 66.4%; P<0.001). The TUNEL assay results revealed that ketamine induced an apoptotic effect on C6 glioma cells, with a significant effect on the rate of death observed at all tested concentrations (3, 10, 30 and 100 μ M). Results of the aforementioned proliferation and TUNEL assay experiments were reproduced when ketamine was replaced with a selective NMDAR antagonist,

D-AP5. However, the NMDAR antagonist-induced effects were not observed in RNB cell cultures. Although it would be premature to apply the results from the present study to human cases, these results indicated that ketamine is an anesthetic candidate providing potential benefit for glioma resection.

Introduction

Glutamate, a major excitatory neurotransmitter in the brain that activates glutamate receptors including N-methyl-D-aspartate receptors (NMDARs), is primarily associated with neuronal communication; and it is involved in normal brain functions including cognition, memory and learning (1). Glutamate-secreting brain tumors have been reported to exhibit enhanced growth (2). Thus, it was speculated that the blockade of NMDARs may be a beneficial approach to the treatment of such brain tumors. Indeed, among NMDAR antagonists, MK801 and memantine were demonstrated to inhibit the growth of glutamate-secreting tumors (2). MK801 is used only for basic research due to its psychotomimetic side effects, including schizophrenic symptoms (3). In contrast, memantine is the agent approved for moderately severe to severe Alzheimer disease in Europe and for moderate-to-severe Alzheimer disease in the United States (4). However, neither MK801 or memantine can be used for anticancer treatment in the clinical setting.

In contrast, the NMDAR antagonist ketamine is widely used in clinical practice as an intravenous or intramuscular anesthetic in humans as well as animals, and it may be an anesthetic candidate for use in tumor resection. However, even basic data on the effects of ketamine in this regard are lacking. The present study hypothesized that ketamine can also inhibit the growth of glutamate-secreting tumors in a similar fashion to MK801 and memantine. To test our hypothesis, the present study thus evaluated the effects of ketamine on the proliferation of gliomas, known as glutamate-secreting brain tumors, using a cell line culture model. The objective of this study is to provide basic data on the anticancer effect of NMDAR antagonist, ketamine.

Materials and methods

Cell culture. The rat brain glioma C6 cell line and the neonatal rat astrocyte RNB cell line were obtained from The Health Science Research Resources Bank (Tokyo, Japan). Since

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Abbreviations: NMDAR, N-methyl-D-aspartate receptor; DIDS, 4,4'-diisothiocyanate-2,2'-disulfonic acid stilbene; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling

Key words: ketamine, glioma, apoptosis, N-methyl-D-aspartate receptor antagonist

gliomas arise from astrocytes, the RNB cells were selected for use in the present study as representative non-malignant astrocytes for comparison. For C6 and RNB cell cultures, cells were seeded in 100-mm culture plates at 1×10^5 cells/well and cultured in Hams F10 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 15% horse serum (ICN Flow 2070033; MTX Lab Systems, Inc., Vienna VA, USA) and 2.5% fetal bovine serum (ICN Flow 101083; MTX Lab Systems, Inc.), and grown in a humidified atmosphere of 95% air and 5% CO₂ at 37°C for 72 h. Following a 72 h-incubation, the cells were harvested from the dishes using trypsin/EDTA in each culture medium for further passages.

Cell proliferation assay following ketamine treatment. The C6 and RNB cell lines were detached using trypsin/EDTA and seeded in 6-well plates, and cultured at 37°C in a humidified incubator containing 95% air and 5% CO₂ with medium as aforementioned. Cell cultures were maintained for 24 h to allow cells to adhere, at which time cultures were incubated for a further 72 h in medium containing ketamine at a range of concentrations (0, 3, 10, 30 and 100 μ M; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). To assess the dose-response of ketamine at 72 h of culture, cell proliferation was determined from each treatment group by counting the numbers of cells using a cell counter (Nihon Kohden, Tokyo, Japan). To determine the time-response of cell proliferation to ketamine treatment, cells were incubated in medium with/without ketamine (30 μ M; Sigma-Aldrich; Merck KGaA), and cell proliferation was assessed using the aforementioned method at 0, 24, 48 and 72 h time points.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) detection of apoptotic cells following ketamine treatment. The C6 and RNB cell lines were detached using trypsin/EDTA and seeded in 24-well plates, and incubated at 37°C in a humidified incubator containing 95% air and 5% CO₂ with Hams F10 medium (Thermo Fisher Scientific, Inc.). Cell cultures were maintained for 24 h to allow cells to adhere, at which time cells were incubated for a further 72 h in medium containing ketamine at a range of concentrations (0, 3, 10, 30 and 100 μ M; Sigma-Aldrich; Merck KGaA), renewed each day. At the termination point of 72 h of culture, the cells were subjected to *in situ* labeling and a quantitative analysis of late-phase apoptotic cells using the TUNEL method (5). TUNEL staining, which detects fragmented DNA, was performed using an In Situ Apoptosis Detection kit, according to the manufacturers protocol (Takara Biotechnology Co., Ltd., Dalian, China). Calcium ionophore A23187-induced apoptosis in each cell line and was used as a positive control for TUNEL staining. Apoptotic cells were visualized with 3,3'-diaminobenzidine at 15–25°C-incubation for 20 min and detected by light microscopy (12 fields of view and magnification, $\times 400$). Cell nuclei were counterstained with methyl green. at 15–25°C-incubation for 15 min The numbers of apoptotic cells were counted, and the percentages of apoptotic cells relative to the total cells were calculated.

Inhibition of apoptosis using DIDS. Confluent C6 glioma cells were seeded in 24-well plates, and cultured for 24 h to

allow for adherence. C6 cells were incubated for 1 h in Hams F10 medium supplemented with various concentrations of 4,4'-diisothiocyanate-2,2'-disulfonic acid stilbene (DIDS; 0, 10, 30 and 100 μ M; Wako Pure Chemicals Industries, Ltd., Osaka, Japan). Following 1 h incubation, the cells were incubated for a further 72 h with/without various drugs (medium alone as a control, or 30 μ M ketamine +0, 10, 30 or 100 μ M DIDS). Apoptotic cells were detected using the TUNEL method as aforementioned. DIDS was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the experimental tubes did not exceed 0.04% and did not affect the viability of the cells. Cell proliferation was assessed in this assay by counting cells using a cell counter (Nihon Kohden).

Cell proliferation and apoptosis assay following D-AP5 treatment. Confluent C6 glioma cells were seeded in 6-well plates at 2×10^4 cells/well, and incubated in Hams F10 medium (Thermo Fisher Scientific, Inc.) as aforementioned. Following 24 h of culture to allow for adherence, cells were incubated for a further 72 h in medium containing various concentrations (0, 3, 10, 30 and 100 μ M) of D-AP5 (Sigma-Aldrich, Merck KGaA) or ketamine (0–100 μ M; Sigma-Aldrich, Merck KGaA). To assess the dose-dependent response of D-AP5 after 72 h of culture, cell proliferation was determined from each treatment group, by counting the numbers of cells using a cell counter (Nihon Kohden). In addition, to assess the apoptotic response of cells following 72 h of culture with various concentrations of D-AP5 (0–100 μ M), renewed every 3 days, the TUNEL assay was performed as aforementioned.

Large-scale gene expression analysis of tumor growth from a cDNA microarray. To determine an estimate of the effects of ketamine on C6 glioma cell growth, a large-scale gene expression survey of cell growth, using a cDNA microarray was performed, as follows.

RNA isolation. Following incubation of C6 cells for 30 min at 37°C with Hams F10 medium (Thermo Fisher Scientific, Inc.) containing 100 μ M ketamine (Sigma-Aldrich; Merck KGaA), total RNA was extracted from the cell monolayer using an RNeasy Mini kit (Qiagen, Inc. Valencia, CA, USA) according to the manufacturer's protocol. The total RNA was treated with RNase free-DNase (Qiagen, Inc.) for eliminating genomic DNA contamination from RNA samples prior to the cDNA microarray.

cDNA labeling and microarray hybridization. Reverse transcription, labeling and hybridization were performed with LabelStar (Qiagen, Inc.) according to the manufacturer's protocol. Briefly, total RNA was reverse-transcribed into target cDNA using LabelStar with Cy3 and Cy5 for control and ketamine-treated cells, respectively. Labeled cDNAs were purified using MiniElute spin columns (Qiagen, Inc.). Cy3-labeled and Cy5-labeled target cDNAs were combined, dried and resuspended in hybridization buffer. The target cDNA mixture was hybridized with rat ADME cDNA microarrays (Asahi Techno Glass; Tokyo, Japan) that included 1,936 cDNA elements. The microarrays were scanned in the Cy3 and Cy5 channels with an Affymetrix 428 Array Scanner

(Affymetrix; Thermo Fisher Scientific, Inc.), and analyzed using the ImaGene and GeneSight-Lite software 7.0 packages (BioDiscovery, El Segundo, CA, USA). Following background subtraction and dye bias normalization, poor-quality features were excluded from further analysis. Features with low signal intensity in the reference channel were filtered if the signal-to-noise ratio was <2.5 . The fold-change in the expression of each gene is expressed as the logarithm of the ratio (Cy5 fluorescence/Cy3 fluorescence). The log ratio ≥ 0.3 or ≤ -0.3 (i.e., normalized intensity ≥ 2 or ≤ 0.5) was used to define genes with significant changes in expression.

Statistical analysis. Multiple group comparisons were performed by one-way analysis of variance, followed by a Tukey-Kramer post hoc test. Data were presented as mean \pm SEM. Student's unpaired t-tests were performed to compare differences between two groups. $P < 0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using the software KyPlot 5.0 (KyensLab, Inc., Tokyo, Japan).

Results

Large-scale gene expression survey of tumor growth using the cDNA microarray. Analysis of the large-scale gene expression survey obtained from the cDNA microarray, revealed that the expression of multiple growth factor genes [epidermal growth factor, transforming growth factor (TGF) β 1-induced transcript 1 and TGF β 3] and transcriptional regulators (wilms tumor 1), tumor protein p53 and signal transducer and activator of transcription 3) of C6 glioma cells remained unchanged; although several genes were downregulated in response to ketamine treatment (Table I).

The effects of ketamine on the growth of C6 glioma and RNB cells. The effects of ketamine treatment significantly inhibited the proliferation of C6 cells at every concentration examined. However, there were no ketamine-induced effects on the proliferation of RNB cells at any given concentration (Fig. 1A). The proliferation of C6 cells was most significantly inhibited in response to ketamine at a dose of $30 \mu\text{M}$ ($n=7$, 66.4%, $P < 0.001$). The $30 \mu\text{M}$ dose of ketamine significantly inhibited C6 cell proliferation at 24-, 48-, and 72-h incubations compared with that of the control, however no notable effect was observed on RNB cell proliferation at any time point tested ($n=4$, Fig. 1B).

Detection of apoptotic cells following ketamine treatment. As presented in Fig. 2, the ratio of apoptotic cells following a 72-h incubation was significantly more pronounced in all ketamine-treated C6 cells compared with C6-control cells Control, ($7.4 \pm 2.8\%$; $3 \mu\text{M}$, $33.8 \pm 1.5\%$; $10 \mu\text{M}$, $34.1 \pm 1.7\%$; $30 \mu\text{M}$, $53.7 \pm 2.4\%$, and $100 \mu\text{M}$, $45.6 \pm 19.5\%$; $n=4$; Fig. 2A). In contrast, no significant differences in the proportion of apoptotic cells were observed between the RNB control cells and any of the ketamine-treated RNB cells ($n=6$; Fig. 2B).

Inhibition of apoptosis using DIDS. The increased proportion of apoptotic C6 cells following ketamine treatment were significantly reversed in the presence of DIDS, in a

dose-dependent manner (Fig. 3). The rate of apoptotic C6 cells observed in response to ketamine and $100 \mu\text{M}$ DIDS treatment was comparable to that observed with no treatment. DMSO alone had no effect on the percentage of apoptotic cells (data not shown).

Proliferation and apoptosis assay results following treatment with selective NMDAR antagonist D-AP5. Treatment with the selective NMDAR antagonist D-AP5 significantly inhibited the proliferation of C6 cells in the same manner as ketamine treatment ($n=4$, Fig. 4A) and induced apoptosis (Fig. 4B).

Discussion

In the present study, the gene expression profile screening of ketamine-treated C6 glioma cells using a cDNA microarray indicated that ketamine may suppress the growth of C6 cells via the regulation of growth factors and transcriptional regulators. The subsequent proliferation assays in ketamine-treated C6 cells confirmed these results. In addition, the results of the apoptotic assay demonstrated that ketamine-induced apoptotic cell death due to apoptosis in malignant astrocytes (C6 glioma cells), resulted in the suppression of C6 cell proliferation. In contrast, ketamine treatment had no effect on the growth of the non-malignant astrocytes (RNB cells). In the present study, it was observed that the effects of a selective blockade of NMDARs, by D-AP5, mimicked the ketamine-induced cell death apoptotic effect in C6 cells, indicating that a blockade of NMDARs is involved in the decreased proliferation of C6 glioma cells due to ketamine.

The results from the present study suggested that activation of NMDARs is associated with C6 glioma cell proliferation. Previous studies, which investigated the proliferation of C6 cells, are in accordance with the results from the present study, as it was reported that C6 cells release glutamate during proliferation, and glutamate promoted C6 proliferation via the activation of NMDARs (2,6). Normal glial cells uptake excitatory amino acids, ensuring the appropriate control of synaptic communication and preventing glutamate neurotoxicity (6). However, due to their lack of an efficient glutamate uptake system, C6 cells release excessive glutamate that facilitates their proliferation (2).

It was suggested that ketamine may increase neurodegeneration in neonatal animal brains (7-10). The continuous blockade of NMDARs due to ketamine upregulates NMDARs, and the upregulation of NMDARs results in an excessive excitatory response to glutamate, causing neuronal cell death (8,9). Despite these reports, in the present study ketamine did not affect the proliferation of RNB cells, even though RNB cells are neonatal. This result is probably due to the characteristics of the immortalized RNB cell line. RNB cells are non-malignant, and do not represent normal cells. To date, it is still unknown if RNB cells express NMDARs, and the results from the present study do not disprove a toxic effect of ketamine on astrocytes in the developing brain.

The limitations of the present study are as follows; The cDNA microarray is a screening assay, and the results obtained from this assay may only be a rough estimate of gene expression changes. To confirm the results of the cDNA microarray,

Table I. Gene expression ratio of cell growth in C6 cells as demonstrated by cDNA microarray analysis.

A, Growth factors			
Gene name	Gene ID	Log expression ratio (n)	Regulation
Bone morphogenetic protein 6	25,644	-0.15±0.62 (8)	n.s.
Brain derived neurotrophic factor	24,225	-1.23±0.50 (4)	d
Connective tissue growth factor	64,032	0.10±0.09 (4)	n.s.
Early B-cell factor (olfactory neuronal transcription factor 1)	11,6543	-0.35±0.72 (4)	d
Early growth response 1	24,330	0.08±0.15 (2)	n.s.
Epidermal growth factor	25,313	-1.29±0.33 (2)	d
Growth arrest and DNA-damage-inducible-45 α	25,112	-0.11±0.76 (4)	n.s.
Growth factor receptor bound protein 2	81,504	-0.12±0.11 (4)	n.s.
Insulin-like growth factor 1	24,482	0.49±0.48 (2)	u
Insulin-like growth factor binding protein 1	25,685	-0.49±0.50 (4)	d
Insulin-like growth factor binding protein 2	25,662	-0.22±0.93 (4)	n.s.
Insulin-like growth factor binding protein 3	24,484	-0.16±0.54 (6)	n.s.
Insulin-like growth factor binding protein, acid labile subunit	79,438	-0.43±0.37 (2)	d
Insulin-like growth factor II (somatomedin A)	24,483	0.06±0.24 (2)	n.s.
Insulin-like growth factor-binding protein 5	25,285	-0.29±0.81 (6)	n.s.
Midkine	81,517	-1.07±0.30 (4)	d
Neural cell adhesion molecule	24,586	-0.32±1.00 (4)	d
Platelet-derived growth factor A chain	25,266	-0.01±0.13 (2)	n.s.
Platelet-derived growth factor- receptor α	25,267	-0.17±0.73 (4)	n.s.
Latent TGF β binding protein 1	59,107	0.32±0.28 (2)	u
TGF β inducible early growth response	81,813	-0.38±0.15 (2)	d
TGF β 1 induced transcript 1	84,574	-0.34±0.42 (2)	d
TGF β 3	25,717	-0.79±0.47 (4)	d
Vascular endothelial growth factor	83,785	0.16±0.06 (2)	n.s.
Vascular endothelial growth factor C	11,4111	0.44±0.37 (2)	u
B, Transcriptional regulators			
Gene name	Gene ID	Log expression ratio (n)	Regulation
Nuclear factor kappa B p105 subunit	81,736	0.57±0.63 (2)	u
V-myc avian myelocytomatosis viral-oncogene homolog	24,577	0.37±0.75 (4)	u
Wilms tumor 1	24,883	-0.72±1.37 (4)	d
PRKC, apoptosis, WT1, regulator	64,513	-0.88±0.32 (4)	d
Tumor protein p53 (Li-Fraumeni syndrome)	24,842	-1.34±1.00 (2)	d
Retinoblastoma 1 (including osteosarcoma)	24,708	-1.40±0.02 (2)	d
Fos like antigen 2	25,446	0.40±0.53 (4)	d
Retinoblastoma-binding protein 1-related protein	84,481	0.12±1.19 (4)	n.s.
Retinoblastoma-related gene	81,758	0.24±1.21 (2)	n.s.
Signal transducer and activator of transcription 1	25,124	-0.03±2.06 (4)	n.s.
Signal transducer and activator of transcription 3	25,125	-0.35±0.32 (4)	d
Suppression of tumorigenicity 13 (colon carcinoma)	81,800	-0.01±0.38 (4)	n.s.
Hsp70-interacting protein			

Data are expressed as the mean \pm standard deviation. Gene ID refers to the national center for biotechnology information gene ID. TGF, transforming growth factor; d, significant downregulation; u, significant upregulation, n.s., not significant.

further assays including reverse transcription quantitative polymerase chain reaction (RT-qPCR) may be required. However, for the purposes of the study, RT-qPCR was not conducted as the proliferation and apoptosis assays were the primary focus.

Furthermore, a single TUNEL assay was conducted to verify that the cell death observed was due to apoptosis. Additional assays may prove helpful to examine apoptosis. However, the TUNEL method used herein

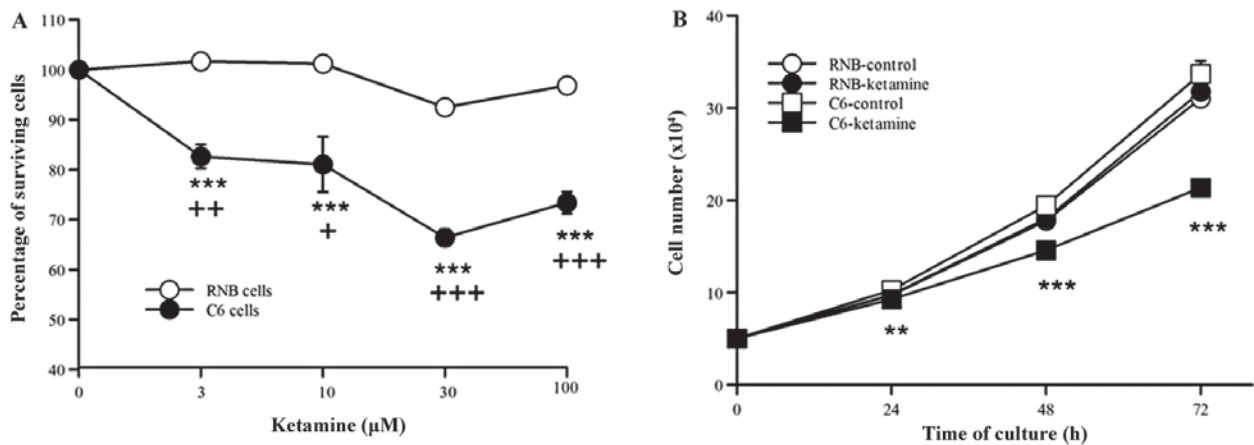


Figure 1. (A) Ketamine concentration-response curves and the percentage of survival of C6 and RNB cells. Cell proliferation was examined by cell counts following culture for 72 h (C6 control, $72.2 \pm 0.17 \times 10^4$ cells; RNB control, $68.8 \pm 0.13 \times 10^4$ cells). The results (mean \pm SEM) are expressed as percentages compared with control experiments (C6, n=7; RNB, n=3). (B) Time-response curves of ketamine for the number of C6 and RNB cells. Cell proliferation was examined in ketamine treated (30 μ M) C6 and RNB cells at 0-, 24-, 48-, and 72-h incubations. The results are expressed as the mean \pm standard error of the mean. **P<0.01, ***P<0.001 vs. C6 control; *P<0.05, **P<0.01 and ***P<0.001 vs. RNB cells. SEM, standard error of the mean.

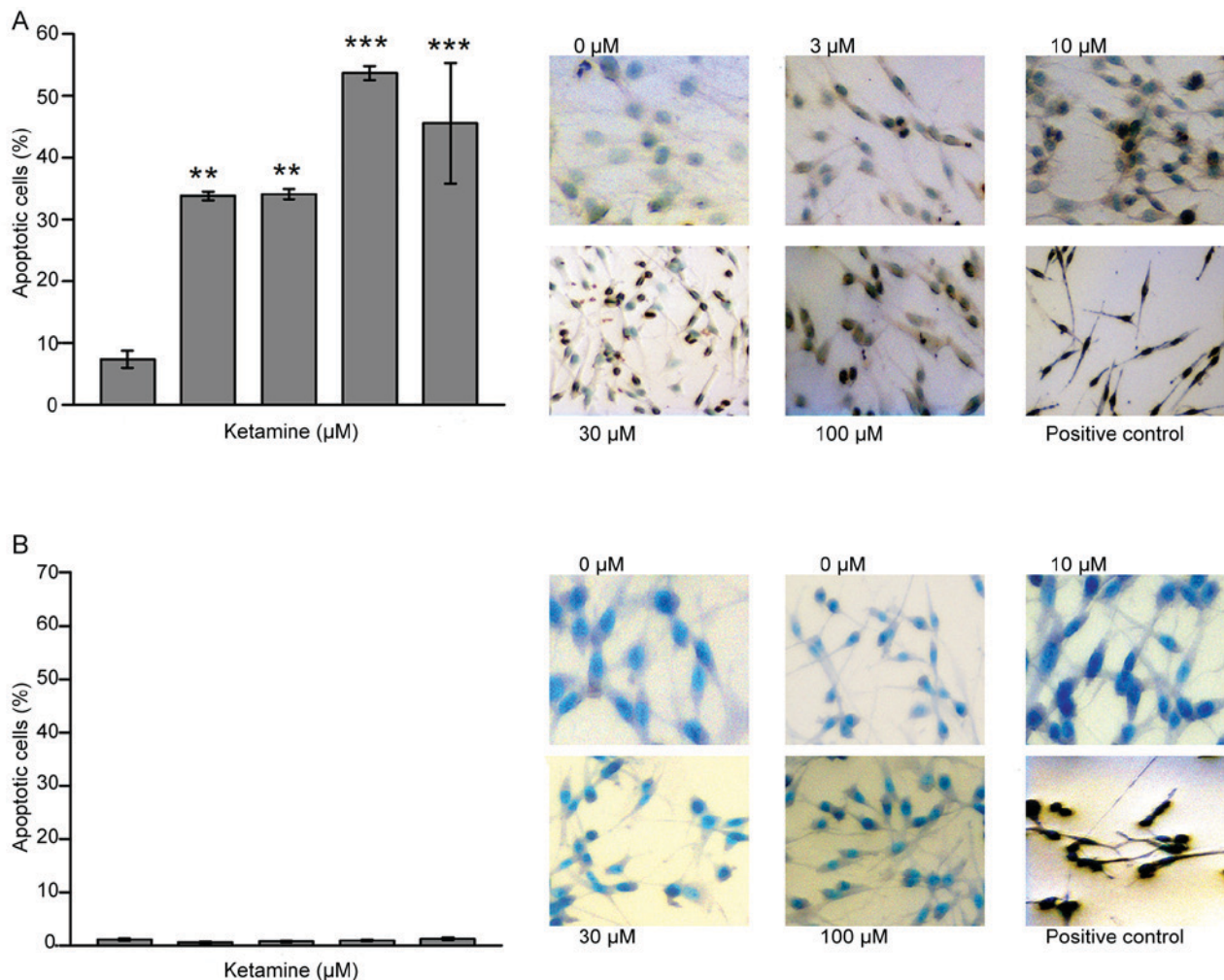


Figure 2. (A) Ketamine-induced apoptosis in C6 cells. The percentages of apoptotic cells were calculated relative to total cells. Values are expressed as the mean \pm SEM (n=4). (B) Ketamine-induced apoptosis in RNB cells. Values are expressed as the mean \pm SEM (n=7). **P<0.01, ***P<0.001 vs. control. Magnification, x400. SEM, standard error of the mean.

is recognized as one of the main methods for detecting apoptotic programmed cell death, as it identifies cells in

the last phase of apoptosis (11,12). In addition, the DIDS assay further confirmed the existence of apoptosis, since it

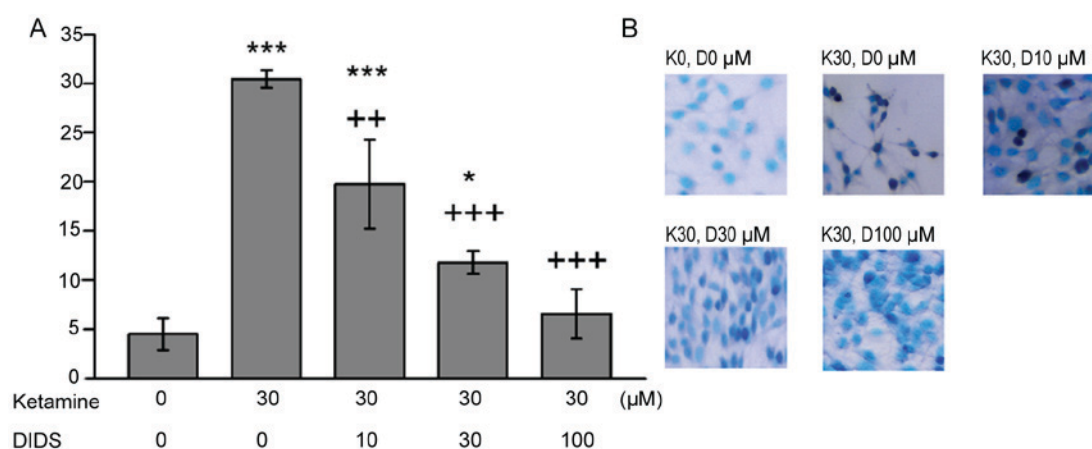


Figure 3. Effects of DIDS on ketamine-induced apoptosis in C6 cells. (A) The percentages of apoptotic cells were calculated relative to the total cells. The ketamine-induced apoptosis in C6 cells was reversed in the presence of DIDS. Data are expressed as the mean \pm standard error of the mean ($n=4$). * $P<0.05$, *** $P<0.001$ vs. the control; ++ $P<0.01$, +++ $P<0.001$ vs. 30 μ M ketamine. (B) Representative images of TUNEL-stained C6 cells. K, Ketamine; D, DIDS. Magnification, $\times 400$. DIDS, 4,4'-diisothiocyanate-2,2'-disulfonic acid stilbene.

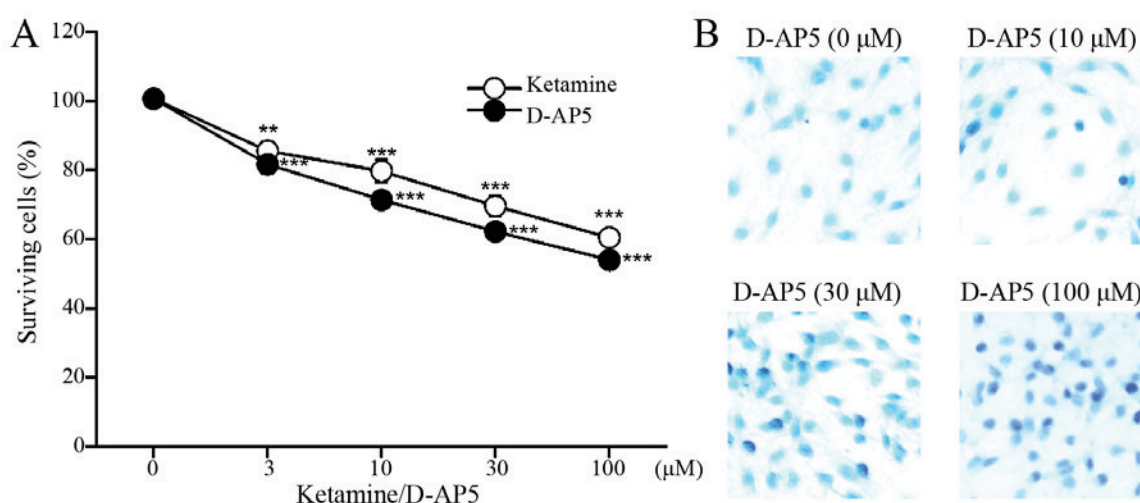


Figure 4. (A) Ketamine and D-AP5 concentration-response curves and percentage of survival of C6 cells. The data are expressed as the mean \pm standard error of the mean (C6 cells, $n=4$; RNB cells, $n=4$). ** $P<0.01$, *** $P<0.001$ vs. each control. (B) TUNEL staining of C6 cells treated with D-AP5. Magnification, $\times 400$. D-AP5, D(-)-2-amino-5-phosphonopentanoic acid.

is widely accepted that DIDS prevents apoptotic cell death. The increase in the level of ketamine-induced cell death demonstrated by TUNEL-positive signals was significantly inhibited by DIDS, and it was therefore concluded that the present protocol was sufficient to detect apoptosis. Finally, a cell viability assay may have been required. Nevertheless, it is worth noting that the cell counting assay performed in our study is effective and sufficient to assess cell proliferation, since the cell viability was confirmed using an apoptosis assay.

In conclusion, the present study confirmed that ketamine induced glioma cell death and suppressed C6 cell proliferation. Gliomas are the most common malignant tumors of the central nervous system. The majority of brain tumors are gliomas (~80%), and gliomas represent a leading cause of mortality among young children and adults with brain tumors, due to intensive growth and invasion ability (13). Although the glioma cell lines investigated in the present

study were rodent rather than human cell lines; and it would be premature to apply the results of this basic study to human cases, these results contribute to the knowledge regarding ketamine-induced effects. In current clinical practice, ketamine is the only viable anesthetic option among NMDAR antagonists, and it is widely used in neurosurgery. The results from the present study suggest that ketamine is an anesthetic candidate providing potential benefit for glioma resection. We thus hope that these results will be useful to oncologists, including anesthesiologists.

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