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Glyceraldehyde-3-phosphate dehydrogenase antisense oligodeoxynucleotides protect against cytosine arabinonucleoside-induced apoptosis in cultured cerebellar neurons

(over-expression/neuronal apoptosis/neuroprotection/cerebellar granule cells)

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ABSTRACT **Cytosine arabinonucleoside (AraC) is a pyrimidine antimetabolite that kills proliferating cells by inhibiting DNA synthesis and, importantly, is also an inducer of apoptosis. We recently reported that age-induced apoptotic cell death of cultured cerebellar neurons is directly associated with an over-expression of a particulate 38-kDa protein, identified by us as glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12). We now show that the AraC-induced neuronal death of immature cerebellar granule cells in culture is effectively delayed by actinomycin-D, cycloheximide, or aurintricarboxylic acid (a DNase inhibitor). Furthermore, two GAPDH antisense, but not their corresponding sense, oligodeoxyribonucleotides markedly arrested AraC-induced apoptosis. This protection was more effective than that induced by the above-mentioned classical inhibitors of apoptosis. Prior to AraC-induced neuronal death, GAPDH mRNA levels increased by ≈2.5-fold, and this mRNA accumulation was blocked by actinomycin-D and the GAPDH antisense (but not sense) oligonucleotide. Like actinomycin-D, a GAPDH antisense oligonucleotide also suppressed the AraC-induced over-expression of the 38-kDa particulate protein (i.e., GAPDH), while the corresponding sense oligonucleotide was totally ineffective. Thus, the present results show that GAPDH over-expression is involved in AraC-induced apoptosis of cultured cerebellar granule cells.**

Apoptosis, one form of programmed cell death, is a normal physiological process that occurs during development and prunes various cell populations to maintain a homeostasis (1, 2). Abnormal apoptosis has been linked to pathogenesis of a number of human diseases including cancer, viral infections, autoimmune diseases, and neurodegenerative disorders (3, 4). Apoptosis can be induced by various extrinsic and intrinsic factors such as activation of cell-surface receptors, growth factor withdrawal, oxidative stress, hormonal stimulation, and cell cycle perturbation (4). In this regard, it has been shown that cytosine arabinonucleoside (AraC) kills postmitotic rat sympathetic neurons in a manner similar to that caused by deprivation of nerve growth factor (5). Moreover, Dessi *et al.* (6) have reported that treatment of immature cerebellar granule cells (CGC) in culture with a high concentration of AraC induces a cascade of events including *de novo* synthesis of RNA and protein, leading to apoptosis of these neurons, as indicated by chromatin condensation and internucleosomal DNA cleavage. However, neither the underlying mechanism nor the protein(s) involved in AraC-induced apoptosis is clear.

CGC are the most abundant neurons in the cerebellum and mature postnatally, in contrast to other types of cerebellar neurons (7). Therefore, cultured CGC represent the most enriched neuronal model available for studying the molecular mechanisms by which apoptosis is involved. For example, it has been shown that apoptosis results when CGC are exposed to nondepolarizing concentrations of KCl (8–10) or β-amyloid peptide containing residues 25–35 (11). In a previous study (12), we found that CGC undergo age-induced apoptosis in culture, which is associated with the over-expression of a particulate 38-kDa protein that we identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12). Antisense oligodeoxyribonucleotides directed against GAPDH mRNA block this GAPDH over-expression and significantly delay age-induced apoptosis of cultured CGC. In an initial effort to explore the generality of the involvement of GAPDH in apoptosis, we undertook the present study. Here, we show that AraC-induced apoptotic death of immature CGC in culture is robustly inhibited by pretreatment with two antisense, but not sense, oligonucleotides to GAPDH. AraC-induced apoptosis is preceded by an increase in GAPDH mRNA which can be suppressed by these GAPDH antisense oligonucleotides, while the corresponding sense oligonucleotides are totally inactive in this regard.

MATERIALS AND METHODS

Cell Culture. CGC were prepared from 8-day-old Sprague–Dawley rats and cultured as described (13). The dissociated cells were suspended in basal modified Eagle's medium containing 10% fetal calf serum, 2 mM glutamine, 50 μg of gentamicin per ml, and 25 mM (or 5 mM) KCl. The cells were seeded at a density of 1.7×10^6 cells/dish in 35-mm tissue culture dishes precoated with poly-L-lysine (50 μg/ml). AraC was added to the culture between 20 and 24 hr after seeding. All neuroprotective agents were added 1 hr before exposure to 300 μM AraC as reported (6).

Neuronal Survival. The culture medium was removed and neurons were lysed with 3% Triton X-100 in phosphate-buffered saline (PBS) as described (13). After staining with 0.15% trypan blue, intact nuclei with limiting membrane were counted in a hemocytometer. For an assessment of cells damaged by apoptosis, this method shows higher sensitivity than methods based on cytoplasmic enzymatic activity, as described (10, 14). Cell death was also quantified by fluorescein diacetate-propidium iodide double staining (15) for live and dead cells, respectively. The latter method produced

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Abbreviations: AraC, cytosine arabinonucleoside; CGC, cerebellar granule cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Act-D, actinomycin-D; CHX, cycloheximide; ATA, aurintricarboxylic acid.

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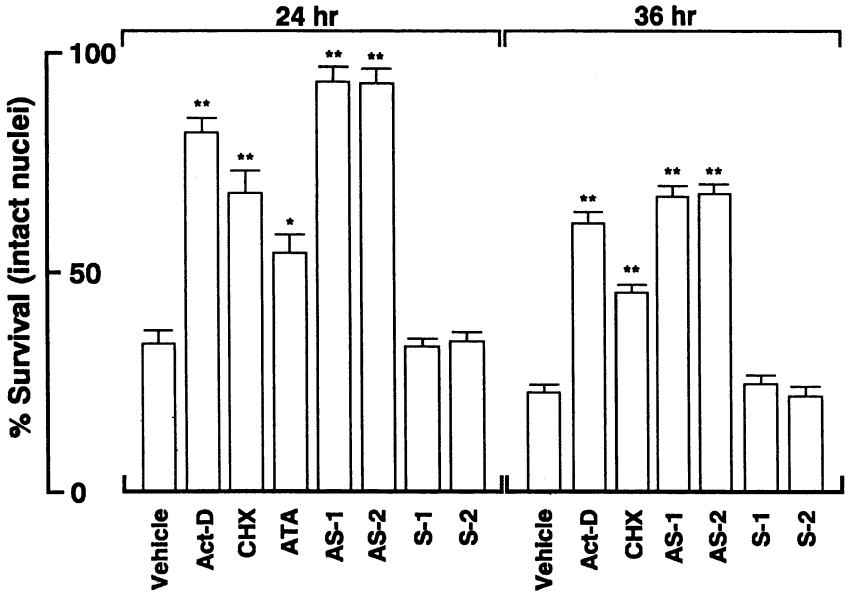

FIG. 1. Neuroprotective effects of various agents against AraC-induced neuronal death. CGC were pretreated with actinomycin-D (Act-D) (1 μg/ml), cycloheximide (CHX) (5 μg/ml), aurintricarboxylic acid (ATA) (10 μM), or GAPDH oligonucleotides (10 μM) and the number of intact nuclei was measured at 24 and 36 hr after AraC exposure as described. Data presented (percentage of unexposed control) are the mean ± SE of three independent experiments. *, $P < 0.02$; **, $P < 0.01$ compared with the corresponding untreated (vehicle) control at both time points, using the Student's t test. Bars: AS-1, plus GAPDH antisense-1 oligonucleotide; AS-2, plus GAPDH antisense-2 oligonucleotide; S-1, plus GAPDH sense-1 oligonucleotide; S-2, plus GAPDH sense-2 oligonucleotide.

comparable results and confirmed that cell death resulting from AraC exposure occurred almost exclusively on neurons.

Nuclear Staining of DNA. For microscopic nuclear DNA analysis, granule cells cultured on glass coverslips in 35-mm dishes were washed with PBS and fixed with 3% glutaraldehyde for 30 min at 4°C. After fixation, cells were washed two times with PBS and then stained with 0.5 μg/ml Hoechst 33258 in PBS for 15 min at 37°C. Stained cells were washed again with

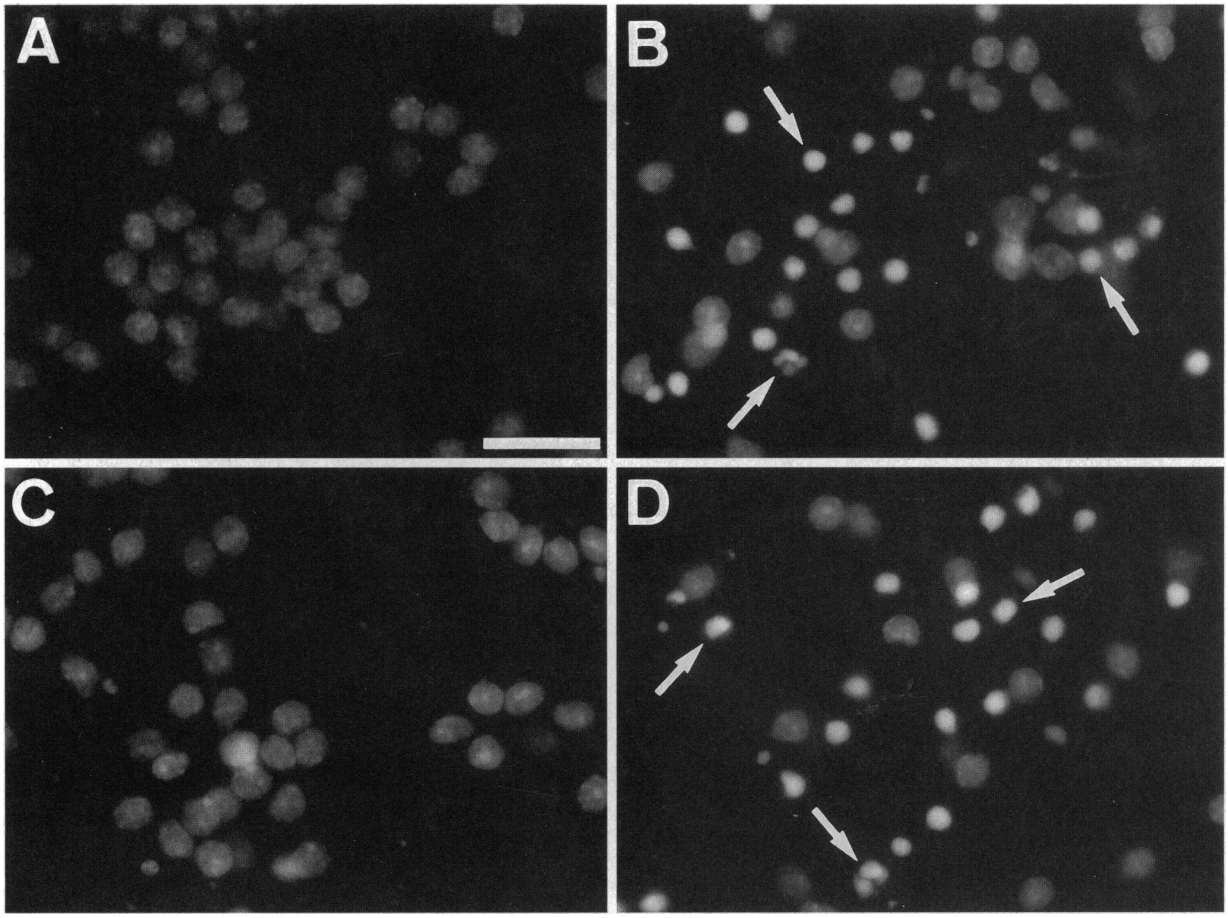

FIG. 2. Morphological features of AraC-induced apoptotic cell death of CGC: neuroprotection with GAPDH antisense oligonucleotide. Monolayered CGC cultures were pretreated with GAPDH oligonucleotides and maintained in the AraC-containing medium for 24 hr. Cells were fixed and stained with the DNA-specific fluorochrome Hoechst dye 33258 as described. (*A*) Without AraC exposure, (*B*) plus vehicle and AraC, (*C*) plus GAPDH antisense-1 oligonucleotide and AraC, and (*D*) plus GAPDH sense-1 oligonucleotide and AraC. Note that the number of apoptotic nuclei with condensed chromatin (arrows) can be seen in *B* and *D* and that the occurrence of the appearance of these apoptotic nuclei was almost completely blocked by GAPDH antisense-1 oligonucleotide (*C*), while its corresponding sense oligonucleotide was totally ineffective (*D*). Results shown are from a representative field of a typical experiment performed three times with similar results. (Bar = 20 μm.)

PBS and mounted with 50% glycerol in PBS. Fluorescence microscopy was performed using an Olympus IMT-2 microscope (filter: UG1, L420).

Northern Blot Analysis. Total RNA isolation and Northern blotting were performed essentially as described (16), except that the human GAPDH cDNA probe was 1.1-kb in length (Clontech) and high-stringency washing of the hybridized blots was performed three times in 0.1× standard saline citrate (SSC) containing 0.1% SDS at 60°C for 10 min. An amount of ≈9 μg of total RNA from each sample was separated by electrophoresis through a 1.2% agarose-formaldehyde gel. Specific hybridization bands were quantified by charge-coupled device (CCD) densitometry of the autoradiograms and then normalized to total cellular RNA in each sample, as described (16, 17).

SDS/PAGE Analysis. Scraped cells were ruptured by sonication in 50 mM Tris·HCl (pH 7.4), and the homogenates were centrifuged at 200,000 × *g* for 30 min. The particulate (pellet) fraction was redissolved in a small volume of the sample buffer. An aliquot of these preparations was loaded onto each lane of the gel (8–16% linear gradient) for SDS/PAGE analysis, as described by Laemmli (18). After electrophoresis, protein bands were visualized by staining with 0.1% Coomassie brilliant blue and then quantified by using a CCD densitometric image analyzer.

Synthesis of Oligonucleotides. Two preparations of phosphorothioated antisense and sense oligodeoxyribonucleotide against rat GAPDH gene (19) were synthesized according to the method of Iyer *et al.* (20). Briefly, the GAPDH antisense-1 oligonucleotide sequence was 5′-GACCTTCACCATCTT-GTCTA-3′, corresponding to a sequence flanking the ATG initiation codon. The GAPDH antisense-2 oligonucleotide sequence was 5′-GTGGATGCAGGGATGATGTT-3′, which was against the theoretical single-stranded region of GAPDH mRNA between residues 637 and 656. The sequences of the sense-1 and -2 oligonucleotides were the exact inverse of those of the respective antisense oligonucleotide with phosphorothioate bonds in the corresponding positions. A concentration of 10 μM of the GAPDH antisense oligonucleotides was found to be optimal and this dose was used in the present knockdown study.

RESULTS

AraC-Induced Apoptosis of CGC: Effects of GAPDH Antisense Oligonucleotides and Classical Neuroprotective Agents. Exposure of immature CGC (20–24 hr after plating) to AraC (300 μM) resulted in apoptotic cell death (Fig. 1), confirming a previous report (6). At 24 hr after AraC exposure, the cell viability was decreased to 33.6 ± 2.7% of the unexposed control and this cell loss was attenuated by Act-D, a RNA synthesis inhibitor; CHX, a protein synthesis inhibitor; and ATA, an endogenous DNase inhibitor. Pretreatment with GAPDH antisense-1 or antisense-2 oligonucleotides completely prevented AraC-induced apoptosis. In contrast, their corresponding GAPDH sense oligonucleotides were ineffective. At 36 hr, the cell viability continued to decline and the apoptotic death was still robustly inhibited by these two GAPDH antisense oligonucleotides and, to a slightly lesser extent, by Act-D and CHX. Additionally, we also found that both the AraC-induced neuronal death and the neuroprotective effects of Act-D, CHX, and the GAPDH antisense oligonucleotides were observed when cells were cultured under a more physiological potassium concentration—i.e., 5 mM KCl (data not shown).

Morphological examination of cells stained with Hoechst dye 33258 revealed a large number of nuclei showing condensed chromatin at 24 hr after AraC exposure (Fig. 2*B*), as compared with the unexposed control (Fig. 2*A*). This AraC-induced chromatin condensation was prevented by GAPDH antisense-1 oligonucleotide (Fig. 2*C*), while the sense-1 oligo-

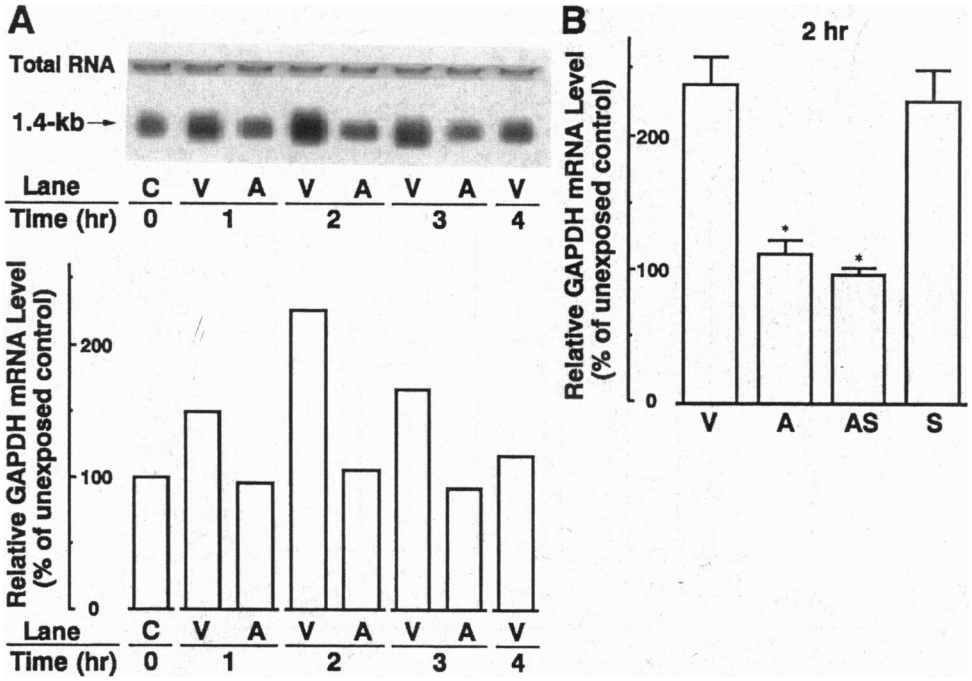

FIG. 3. Northern blot analysis of GAPDH mRNA levels in the CGC exposed to AraC. Experimental conditions for Northern blotting are as described. (*A*) AraC-induced increase of GAPDH mRNA levels in CGC. Levels of GAPDH mRNA in bar graphs are expressed as values relative to the unexposed control (i.e., 0 hr). Results shown are from a typical experiment that has been repeated three times with similar results. (*B*) Suppressive effects of the GAPDH oligonucleotides and Act-D on the over-expressed GAPDH mRNA levels in CGC at 2 hr after AraC exposure. Cells were pretreated with indicated agents as described in the legend to Fig. 1. Values are the mean ± SE of three independent experiments. *, $P < 0.01$ compared with the untreated control (V), using the Student's *t* test. C, unexposed control; V, plus vehicle and AraC; A, plus Act-D and AraC; AS, plus GAPDH antisense-2 oligonucleotide and AraC; S, plus GAPDH sense-2 oligonucleotide and AraC.

nucleotide was without effect (Fig. 2*D*). Electron microscopic examination revealed that CGC exposed to AraC showed the characteristic morphological hallmarks of apoptosis—i.e., membrane blebbing, perinuclear heterochromatic patches, dilatation of endoplasmic reticulum, and condensed chromatin (data not shown). Moreover, no obvious swelling of CGC was noted after AraC exposure. These morphological features and the necessity of macromolecular synthesis further support the notion that AraC-induced cell death involves an apoptotic process.

Increased Levels of GAPDH mRNA and Protein in AraC-Exposed CGC. The neuroprotective effects of GAPDH antisense oligonucleotides suggested that AraC-induced apoptosis involved an increased expression of GAPDH. To examine this possibility, the levels of GAPDH mRNA and protein were measured by Northern blot hybridization and SDS/PAGE analysis, respectively. The GAPDH mRNA level increased in a time-dependent manner after AraC exposure (Fig. 3*A*). Pretreatment with Act-D effectively suppressed this mRNA increase throughout the time course examined. The increase was detected between 1 and 3 hr with a maximal effect at 2 hr, which was approximately 2.5-fold of the unexposed control. Moreover, AraC-induced increase in GAPDH mRNA was completely blocked by Act-D and GAPDH antisense-2, but not by its corresponding sense oligonucleotide (Fig. 3*B*). Consistent with the time-dependent accumulation of GAPDH mRNA, it was observed that only 50% of the dead cells could be rescued by the addition of Act-D at 3.5 hr after AraC exposure (data not shown). SDS/PAGE analysis revealed that the level of the 38-kDa protein in the particulate fraction was not significantly increased at 6 hr after exposure to AraC and at this time no significant loss of cell viability was detected (Fig. 4). The 38-kDa protein level was significantly increased at 12 hr and further increased at 24 hr to about 2-fold of the control level. The increased level at 24 hr was blocked by Act-D and GAPDH antisense-1, but not sense-1 oligonucleotide. In all cases, the levels of the 38-kDa proteins were inversely correlated with the degree of cell survival. Our microsequencing

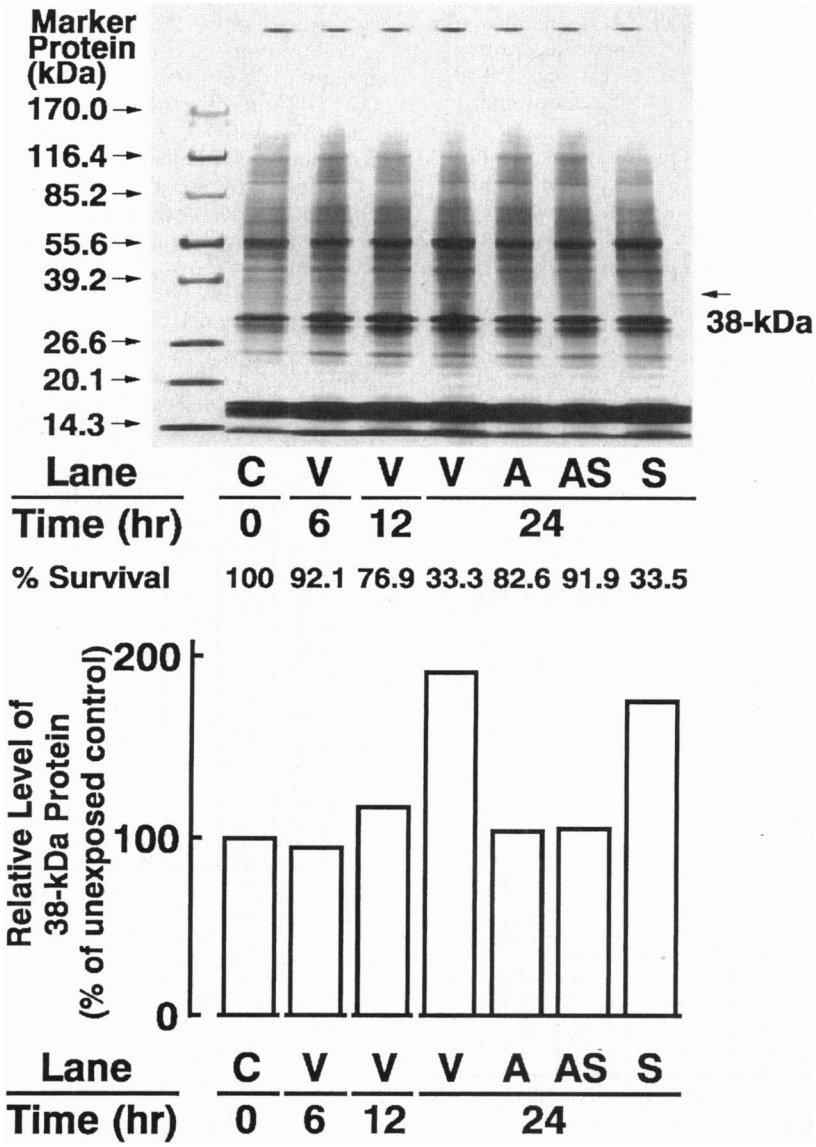

FIG. 4. SDS/PAGE analysis of particulate proteins of CGC: effects of GAPDH oligonucleotides and Act-D. Granule neurons were pretreated with indicated agents as described in the legend to Fig. 1. The assessment of percent survival and experimental conditions for SDS/PAGE analysis are as described. After exposing to AraC, cells were scraped at indicated times and the particulate fractions were prepared. An aliquot of samples (14 μg particulate protein) was loaded onto each lane of the gel. Molecular masses of marker proteins (kDa) are shown in the left lane. Lanes: C, unexposed control; V, plus vehicle and AraC; A, plus Act-D and AraC; AS, plus GAPDH antisense-1 oligonucleotide and AraC; S, plus GAPDH sense-1 oligonucleotide and AraC. Comparable results were obtained in two other independent experiments. Bars shown at the bottom of the figure are levels of 38-kDa proteins and the data are expressed as relative values compared with the unexposed control.

results revealed that the bulk of protein present in the over-expressed 38-kDa band on SDS/PAGE represented GAPDH (data not shown).

DISCUSSION

In an attempt to search for putative "killing protein(s)" involved in AraC-induced apoptosis, Dessi *et al.* (6) performed two-dimensional SDS/PAGE analysis of CGC extracts and observed two groups of proteins induced by AraC exposure. One group had molecular masses of 30–36 kDa and the other had molecular masses of 25–28 kDa. The nature of these AraC-induced proteins has not been identified, but it has been speculated that they may be endonucleases or proteases reported to be involved in the apoptotic process (6). The similarity in masses seen by Dessi *et al.* and those seen in our present study strongly suggest that their 36-kDa protein corresponds to our 38-kDa protein—i.e., GAPDH.

AraC-induced GAPDH mRNA accumulation precedes the over-expression of GAPDH protein whose level is inversely correlated with the cell viability after AraC exposure. To explore whether over-expression of GAPDH is an epiphenomenon or directly involved in cell death, we employed the antisense knockdown strategy. We found that GAPDH antisense oligonucleotides not only block the GAPDH mRNA and protein increase but effectively arrest the AraC-induced apoptotic death (Figs. 1, 3, and 4). These results strongly suggest that the neuroprotective effects of these antisense oligonucleotides are due to GAPDH knockdown and provide compelling support that GAPDH is an important factor involved in AraC-induced apoptosis. At present it is unclear as to whether GAPDH *per se* is an apoptotic protein or GAPDH requires concerted actions of other cellular protein(s) to elicit its apoptotic effect. Future experiment using GAPDH gene transfection into host neurons is essential to address this issue. In any event, it is noteworthy that GAPDH over-expression has been implicated in age-induced apoptosis in cultured CGC (12) and cultured cerebro-cortical neurons (21) as well as low KCl-induced death of CGC cultures (R.I., K. Sunaga, M. Tanaka, H. Aishita, and D.-M.C., unpublished data). These results suggest that GAPDH may have a profound role in apoptosis in neurons and possibly other cell types.

GAPDH is an enzyme with multiple functions and is present not only in the cytosol but also in many structural elements and organelles in the cell (22). In addition to its prominent role in glycolysis, catalyzing the NAD-dependent conversion of glyceraldehyde-3-phosphate to form 1,3-bisphosphoglycerate, GAPDH has been shown to have many nonglycolytic activities. For example, it regulates the structures of microtubules and microfilaments (23, 24) and is a major component in synaptic vesicles, possibly being involved in neurotransmitter release (25). In rabbit brain, as many as 16 isoforms of GAPDH have been detected, and one of these isoforms catalyzes the fusion of membrane bilayers *in vitro* (26). Further studies are necessary to determine which of these activities and which isoform(s) are involved in the apoptotic mechanism. Interestingly, GAPDH was found to bind β-amyloid precursor protein (27) and to interact with a monoclonal antibody to amyloid plaques from Alzheimer's disease brain (28). Recently, Burke *et al.* (29) reported that Huntingtin, the gene product of Huntington disease, and proteins of dentatorubral-pallidoluysian atrophy selectively interact with GAPDH via their expanded polyglutamine regions. These observations together with our results raise the interesting possibility that GAPDH over-expression may be involved in certain forms of neurodegenerative disorders.

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