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MOLECULAR PHARMACOLOGY, **53:**701–707 (1998).

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Nuclear Localization of Overexpressed Glyceraldehyde-3-Phosphate Dehydrogenase in Cultured Cerebellar Neurons Undergoing Apoptosis

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Received September 2, 1997; Accepted January 14, 1998

This paper is available online at http://www.molpharm.org

ABSTRACT

We recently reported that overexpression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) is directly involved in cytosine arabinonucleoside (ara-C)- and low K^+-induced neuronal death of cultured cerebellar granule cells. The former is entirely due to apoptosis, whereas the latter involves both apoptosis and necrosis. We examined the subcellular distribution of the overexpressed GAPDH occurring during apoptosis by using both subcellular fractionation and immunocytochemistry with a monoclonal antibody directed against this overexpressed protein. When immature cerebellar neurons were exposed to ara-C, an overexpression of GAPDH was observed, primarily in the nuclear fraction. In contrast, low K^+ exposure of mature cerebellar neurons induced the overexpression of GAPDH not only in the nuclear fraction but also in the mitochondrial fraction. In both paradigms, no significant change of GAPDH levels occurred in the microsomal and cytosolic fractions. Moreover, pretreatment with GAPDH antisense oligonucleotide or classic apoptotic inhibitors clearly suppressed the accumulation of GAPDH protein in these subcellular loci. This discrete nuclear localization of GAPDH during apoptosis was supported further by immunoelectron microscopy. Quantitative assessment of GAPDH immunogold labeling revealed that a ~5-fold increase in the intensity of gold particles was observed within the nucleus of apoptotic cells. Thus, the current results raise the possibility that neuronal apoptosis may be triggered by GAPDH accumulation in the nucleus, resulting in perturbation of nuclear function and ultimate cell death.

GAPDH (EC 1.2.1.12) is a highly conserved protein initially known to have a pivotal role in glycolysis, catalyzing reversible conversions between glyceraldehyde-3-phosphate and 1,3-diphosphoglycerate using NAD^+ as the cofactor. It is a tetramer composed of four subunits with a molecular mass of ~37 kDa. GAPDH has long been thought to be the product of a housekeeping gene whose transcript level does not vary in most biological conditions, and the protein is involved only in basic energy production. However, recent studies have indicated that there are >300 copies of GAPDH genes in the rat genome (Tso *et al*., 1985) and that GAPDH mRNA levels are highly regulated in certain malignant cells (Bhatia *et al*., 1994) and endothelial cells undergoing oxidative stress (Graven *et al*., 1994). Increasing evidence demonstrates that GAPDH is located in multiple cellular compartments, including the plasma membrane, mitochondria, cytoskeletons, and nuclei (Rogalski *et al*., 1989; Singh and Green, 1993), in addition to the cytosol, in which glycolysis occurs. GAPDH protein has been shown to be involved in a variety of nonglycolytic functions; these include interaction with microtubules (Huitorel and Pantaloni, 1985), tRNA (Singh and Green, 1993), and low-molecular-weight small G protein (Doucet and Tuana, 1991); involvement in nucleoside transport in synaptic vesicles (Schläfer *et al*., 1994); and regulation of protein phosphorylation (Kawamoto and Caswell, 1986). In addition, GAPDH is a target of covalent NAD^+ linkage mediated by nitric oxide (McDonald and Moss, 1993) and oxygen free radicals (Marin *et al*., 1995).

We reported previously that apoptosis of CGCs resulting from "aging" of the cultures is strikingly associated with overexpression of a particulate-bound 38-kDa protein, which has been identified as GAPDH (Ishitani *et al*., 1996a). Moreover, GAPDH antisense, but not sense, oligodeoxyribonucleotides specifically suppress the age-induced accumulation of GAPDH mRNA and protein before apoptotic neuronal death (Ishitani *et al*., 1996a, 1996b). By the same criteria, GAPDH overexpression in the particulate fraction has been implicated in ara-C- and low potassium chloride (5 mM; low K^+)-

ABBREVIATIONS: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ara-C, cytosine arabinonucleoside; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CGC, cerebellar granule cell; Act-D, actinomycin-D; CHX, cycloheximide.

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induced neuronal death of CGC cultures (Ishitani and Chuang, 1996; Ishitani *et al*., 1997). Furthermore, we have demonstrated that the former is strictly due to apoptosis (Ishitani and Chuang, 1996), but the latter includes both apoptosis and necrosis and that only the apoptotic component involves overexpression of GAPDH (Ishitani *et al*., 1997). The current study was undertaken to determine the subcellular distribution of overexpressed GAPDH during neuronal death in the above-mentioned apoptotic paradigms as an initial step to exploration of the mechanism of action of GAPDH in neuronal apoptosis. We present the intriguing observation that overexpressed GAPDH in CGCs undergoing apoptosis is localized in the nuclear compartment, suggesting alteration of nuclear function may play a role in neuronal apoptosis. Some of these results have appeared in abstract form (Sunaga *et al*., 1997).

Materials and Methods

Cell culture. CGCs were prepared from 8-day-old Sprague-Dawley rats and cultured as described previously (Sunaga *et al*., 1993). The dissociated cells were suspended in basal modified Eagle's medium containing 10% fetal calf serum, 2 mM glutamine, 50 μg/ml gentamicin, and 25 mM KCl, which is required for the survival of CGCs. The cells were seeded at a density of 1.8×10^6 cells/dish in 35-mm tissue culture dishes precoated with poly-L-lysine (50 μg/ml). The ara-C at a final concentration of 300 μM was added to the culture at 20–24 hr after seeding. All neuroprotective agents were added 1 hr before ara-C exposure. In the case of low K^+-induced apoptosis of CGCs, mature cells at 7 days in cultures were washed twice with and maintained in serum-free basal modified Eagle's medium that contained 5 mM KCl and was supplemented with glutamine, gentamicin, and a low concentration (10 μM) of ara-C as described previously (Ishitani *et al*., 1997). Neuronal survival was assessed by fluorescein diacetate-propidium iodide double staining for live and dead cells, respectively, as described previously (Ishitani *et al*., 1996a).

Antibody production. A mouse monoclonal antibody specific for overexpressed GAPDH during apoptosis was raised against the increased 38-kDa protein in the particulate fraction from CGCs displaying age-induced apoptosis as described previously (Ishitani *et al*., 1996a). Briefly, the particulate fraction was prepared from CGCs after 17 days in culture and then subjected to SDS-PAGE. The target 38-kDa band was excised from the gel and used as an antigen for the production of monoclonal antibody. The immunized BALB/c mice were killed, and their spleen cells were fused to NE-1 myeloma cells. Hybridomas were used to generate ascites fluid according to the traditional method, and the resultant IgG was purified from its fluid by using a Protein A-DEAE column.

Subcellular fractionation and Western blotting. Subcellular fractions of CGCs were prepared essentially according to the method of Gray and Whittaker (1962). Scraped cells from each culture were ruptured by sonication at 4° in 4 mM HEPES, pH 7.4, containing 0.32 M sucrose. The homogenate was centrifuged for 10 min at $1,000 \times g$ to produce a pellet (P1), which was washed once by resuspension in an equal volume of homogenization buffer and recentrifuged at the same speed. The original supernatant was then centrifuged at $17,500 \times g$ for 20 min to produce a pellet (P2) and a supernatant. This supernatant fraction was centrifuged at $200,000 \times g$ for 30 min to produce a high speed pellet (P3) and a high speed supernatant (S). All steps were performed at 4°. Each fraction was dissolved in a small volume of SDS (2%)-containing sample buffer. An aliquot of the samples (3–6 μg of protein) was loaded onto each lane of the gel (8–16% linear gradient) for SDS-PAGE analysis, as described by Laemmli (1970). After electrophoresis, the protein was electroblotted onto a polyvinylidene difluoride membrane (Polyscreen; DuPont-New England Nuclear, Boston, MA) and probed with the GAPDH monoclonal antibody (diluted 1:400), and the specific antigen was visualized by enhanced chemiluminescence autography (Renaissance; DuPont-New England Nuclear) as described previously (Ishitani *et al*., 1997). Quantification of GAPDH protein band on the autogram was performed by using a charge-coupled device densitometric image analyzer. GAPDH protein levels in each of the fractions were normalized to an internal 56- or 43-kDa (or both) protein band in each sample because these proteins could be detected ubiquitously in all fractions (Fig. 1A, *lane 1*). In addition, electron microscopic examination revealed that P1, P2, and P3 fractions consisted mainly of nuclei, mitochondria, and microsomes, respectively. Fraction S contained no organized structures.

Postembedding EM immunocytochemistry. Neurons grown in 35-mm plastic dishes were prefixed and postfixed in 3% glutaraldehyde and 1% OsO_4, respectively, dehydrated in ethanol, and *in situ* embedded as described previously (Ishitani *et al*., 1996a). Ultrathin sections were mounted on nickel grids and processed for immunogold cytochemistry, essentially according to the method of Tanaka *et al*. (1997). Briefly, the sections were pretreated with 5% H_2O_2 for 10 min (i.e., etching), washed in phosphate-buffered saline, and incubated at room temperature for 1 hr sequentially with (1) 10% goat serum in phosphate-buffer saline, (2) the first antibody to GAPDH (1:400 dilution), and (3) the second antibody (10 nm gold-labeled goat anti-mouse IgG, 1:50 dilution; British Biocell International, Cardiff, UK). Finally, the sections were stained with uranyl acetate and examined in a JEM-1210 electron microscope (JEOL, Tokyo, Japan). Control experiments included omission of the primary antibody to GAPDH and preabsorption of the primary antibody with purified GAPDH. Quantitative analysis of subcellular distribution of the immunogold particles was performed on a series of electron micrographs (total of 60) by using an image analyzer (C-Imaging 1280; Compix, Mars, PA). To ensure the validity of data, the immunolabeling density was

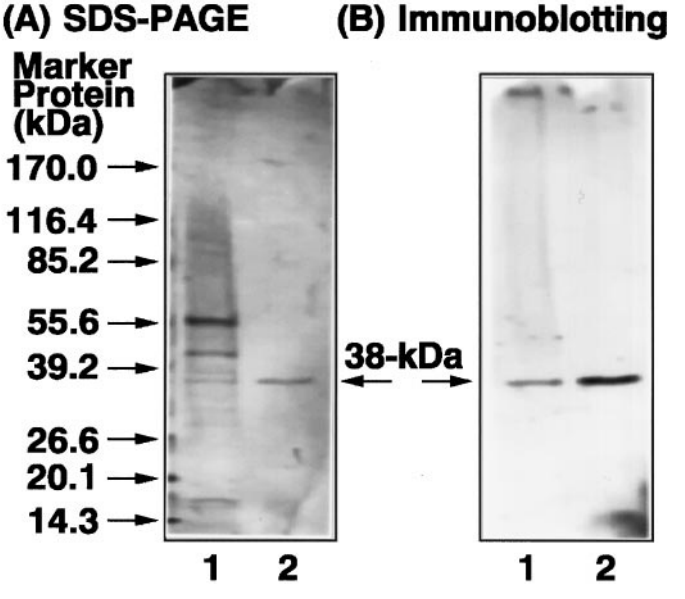

Fig. 1. Specificity of a monoclonal antibody directed against overexpressed GAPDH. Mature CGCs were exposed to low K^+ in serum-free medium for 12 hr (with a viability of 75%), and the particulate fraction was prepared and subjected to SDS-PAGE for immunoblotting as described in the text. A, SDS-PAGE analysis of the proteins in the particulate fraction (*lane 1*) and the purified GAPDH preparation (*lane 2*). Shown are the protein bands on polyvinylidene difluoride membrane, which were transferred from the gel. *Left*, molecular mass standards (in kDa). B, Immunoblotting pattern of the particulate proteins (*lane 1*) and GAPDH (*lane 2*). After protein transfer, blots were incubated with this monoclonal antibody as described in Materials and Methods. *Lane 1*, 6 μg of particulate protein. *Lane 2*, 0.1 μg of purified GAPDH preparation. No significant cross-reaction with any peptide fragments, except for the 38-kDa band, is demonstrated in *lane 1* (B).

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expressed as the number of gold particles per μm^2 of only the nucleus and cytoplasm at an early stage of apoptosis in the cells because of the difficulty in tracing the exact area of other intracellular compartments that are usually not well defined in the micrographs of electron microscopic immunohistochemical studies.

Synthesis of oligonucleotide. The phosphorothioated antisense oligodeoxyribonucleotide against rat GAPDH gene was prepared as described previously (Ishitani *et al*., 1996a). The sequence was 5′-GACCTTCACCATCTTGTCTA-3′, corresponding to a sequence flanking the ATG initiation codon (the phosphorothioated nucleotide is underlined).

Results

Characterization of a monoclonal antibody raised against overexpressed GAPDH during apoptosis. To study the intracellular distribution of overexpressed GAPDH, primarily we prepared a monoclonal antibody against this overexpressed protein. Our initial immunodot blot analysis indicated that this monoclonal antibody reacted in a concentration-dependent manner with purified chicken muscle GAPDH obtained commercially (data not shown). We then examined its reactivity with overexpressed GAPDH in CGCs. The high speed particulate fraction (pellet; 200,000 × *g* for 30 min) was derived from CGCs treated with 5 mM KCl in the absence of serum (termed low K^+) for 12 hr and the amount of 38-kDa band was estimated with SDS-PAGE. The results confirmed previous observations (Ishitani *et al*., 1997) that the level of the 38-kDa protein band was increased by ~2-fold at this stage (data not shown). To determine the specificity of this monoclonal antibody, Western blots were made using the above-mentioned particulate sample derived from low K^+-exposed CGCs as shown in Fig. 1. The blot revealed only a single band at a molecular mass of 38 kDa, comigrating with the purified GAPDH. In addition, the monoclonal antibody preabsorbed with purified GAPDH preparation (>10-fold) failed to recognize this 38-kDa protein (data not shown).

Localization of overexpressed GAPDH during apoptosis: subcellular fractionation and EM immunocytochemistry. Subcellular fractionation using a differential centrifugation was conducted to determine whether the overexpressed GAPDH during apoptosis was associated with certain intracellular organelles. We adopted two types of apoptotic inducers (i.e., ara-C and low K^+) because the former results in only apoptotic death, whereas the latter induces both apoptosis and necrosis (Ishitani and Chuang, 1996; Ishitani *et al*., 1997). Western blot analysis of the subcellular fractions derived from immature CGCs (20–24 hr after plating) exposed to ara-C clearly revealed that a majority of the overexpressed GAPDH was present in the nuclear (P1) fraction, with a small but statistically insignificant increase in the mitochondrial (P2) fraction and no change in the microsomal (P3) and cytosolic (S) fractions (Fig. 2A). Furthermore, an accumulation of this protein in the nucleus (P1 fraction) was prevented by pretreatment of its antisense oligonucleotide or Act-D; these pretreatments also resulted in rescue of neuronal death. In addition, all these observations were confirmed using the age-induced apoptotic paradigm of CGCs (data not shown). On the other hand, when mature cells were exposed to low K^+, overexpressed GAPDH was found in both the P1 and P2 fractions but not in the P3 and S fractions (Fig. 2B). Pretreatment with the GAPDH antisense oligonucleotide or CHX also effectively blocked the increase of this protein in both fractions, accompanying the rescue of neuronal death. In addition, in both models, pretreatment with GAPDH sense oligonucleotide did not show any suppressive effect on the overexpression of this protein, confirming our previous reports (Ishitani and Chuang, 1996; Ishitani *et al*., 1997). Moreover, another antisense oligonucleotide directed against the coding region of GAPDH (Ishitani *et al*., 1996a) also showed a similar inhibitory effect on the translocation of GAPDH to these subcellular fractions (data not shown).

We compared the subcellular localization of overexpressed GAPDH during apoptosis by using a postembedding immunogold electron microscopy. Electron microscopic examination detected the perinuclear heterochromatic patches and membrane blebbing on the early stage of apoptotic cells (data not shown). This procedure clearly confirmed the nuclear localization of the GAPDH, which was detected by our biochemical studies. Within cells undergoing ara-C-induced apoptosis, immunoparticles were located mainly on the nuclear compartment and associations with cytoplasmic organelles were rare (Fig. 3B). In contrast, low K^+-induced apoptotic cells showed that immunoparticles were also associated with the cytoplasmic elements, including the mitochondria, as well as the nucleus (Fig. 3C). In addition, Fig. 3A shows that the gold labeling in the nucleus of the unexposed healthy cell is very scarce. Furthermore, quantitative assessment of this GAPDH immunogold labeling strongly supported the morphological observations. As shown in Fig. 4, a ~5-fold increase over the untreated control in the density of gold particles was observed in the nucleus of cells treated with ara-C (1.41 ± 0.20 particles/μm^2) or low K^+ (1.36 ± 0.22 particles/μm^2). On the other hand, within the cytoplasmic region, a small but statistically insignificant increase of immunoparticles were observed in the ara-C-induced apoptotic cells, whereas a considerable degree of enhancement (~3.5-fold) was detected in the low K^+-exposed CGCs. When the mouse primary antibody was omitted in the immunohistochemical experiments, only background level of immunoparticles (0.149 ± 0.025 particles/μm^2, 12 assessments) was observed throughout the cells.

Discussion

In the current study, we developed a monoclonal antibody against overexpressed GAPDH in rat CGCs undergoing apoptosis for the biochemical and electron microscopic immunocytochemical studies on the subcellular localization of this overexpressed protein during apoptotic neuronal death. The major finding is the discrete nuclear localization of this protein within cerebellar neurons undergoing apoptosis induced by ara-C and low K^+. This is the first biochemical and morphological evidence that the putative "killing protein or proteins" in the apoptotic pathway of neuronal death primarily are present in the nuclear compartment. Moreover, in the low K^+-induced neuronal death, which includes both apoptosis and necrosis (Ishitani *et al*., 1997), an overexpressed GAPDH also is present in the mitochondrial fraction, in addition to its nuclear fraction. It is unclear whether translocation of GAPDH to the mitochondrial fraction contributes to the occurrence of necrosis during low K^+-induced death of CGCs. However, this possibility is consistent with the proposal that secondary necrosis is the result of progressive apoptosis and

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related to perturbation of mitochondrial function (Ankarcrona *et al.*, 1995). A role of GAPDH translocation to the nucleus, mitochondria, or both in neuronal apoptosis is supported further by our observations that neuroprotective agents such as GAPDH antisense oligonucleotides, Act-D, and CHX are all effective in preventing the accumulation of GAPDH protein in these organelles. In addition, in our preliminary studies on subcellular distribution of GAPDH during low K^+- and ara-C-induced apoptosis of CGCs, an overexpression of GAPDH protein in the particulate fraction (a >2-fold increase) and its partial nuclear localization were demonstrated by Western blotting using a commercially available anti-rabbit muscle GAPDH monoclonal antibody (Saunders *et al.*, l996; Ishitani *et al.*, 1997).

A majority of GAPDH protein is expected to be found in the cytoplasm where glycolysis occurs. Surprisingly, our electron microscopic immunohistochemical studies show that immunoparticles in the cytoplasm are scarce. Because the monoclonal antibody used in our study was raised against overexpressed GAPDH excised from SDS-PAGE, it is possible that its determinant group is mainly against nuclear GAPDH, which might be in a monomeric, denatured form. Conversely, the monoclonal antibody may show relatively weak affinity for cytoplasmic GAPDH, which is likely to be a tetrameric protein catalyzing glycolysis. Mounting evidence suggests that GAPDH has multiple isoforms endowed with diverse cellular functions. In addition to its prominent role in glycolysis in the cytosol, it can be bound to membranes to regulate endocytosis, perhaps through perturbation of cytoskeletal structures (Caswell and Corbett, 1985; Robbins *et al.*, 1995). Some isoform or isoforms of GAPDH in the brain have membrane fusion activity independent of glycolysis (Glaser and Gross, 1995). In the nucleus, GAPDH participates in tRNA transport (Singh and Green, 1993) and controls DNA replication and DNA repair (Meyer-Siegler *et al.*, 1991; Baxi and Vishwanatha, 1995). In addition, GAPDH has been suggested to regulate transcriptional activity in neurons (Morgenegg *et al.*, 1986). Thus, it is conceivable that accumulation of overexpressed GAPDH in the nucleus can result in alteration of nuclear functions and ultimate neuronal death. GAPDH is a target protein of nitric oxide-catalyzed nonenzymatic covalent modification by NAD^+ (McDonald and Moss, 1993). It is conceivable that GAPDH protein accumulated in the nucleus might be covalently modified or represent a specific isoform or isoforms of this protein. On the other hand, the differential effects of antisense oligonucleotides on overexpressed rather than basal GAPDH protein may reflect distinct properties of these two classes of

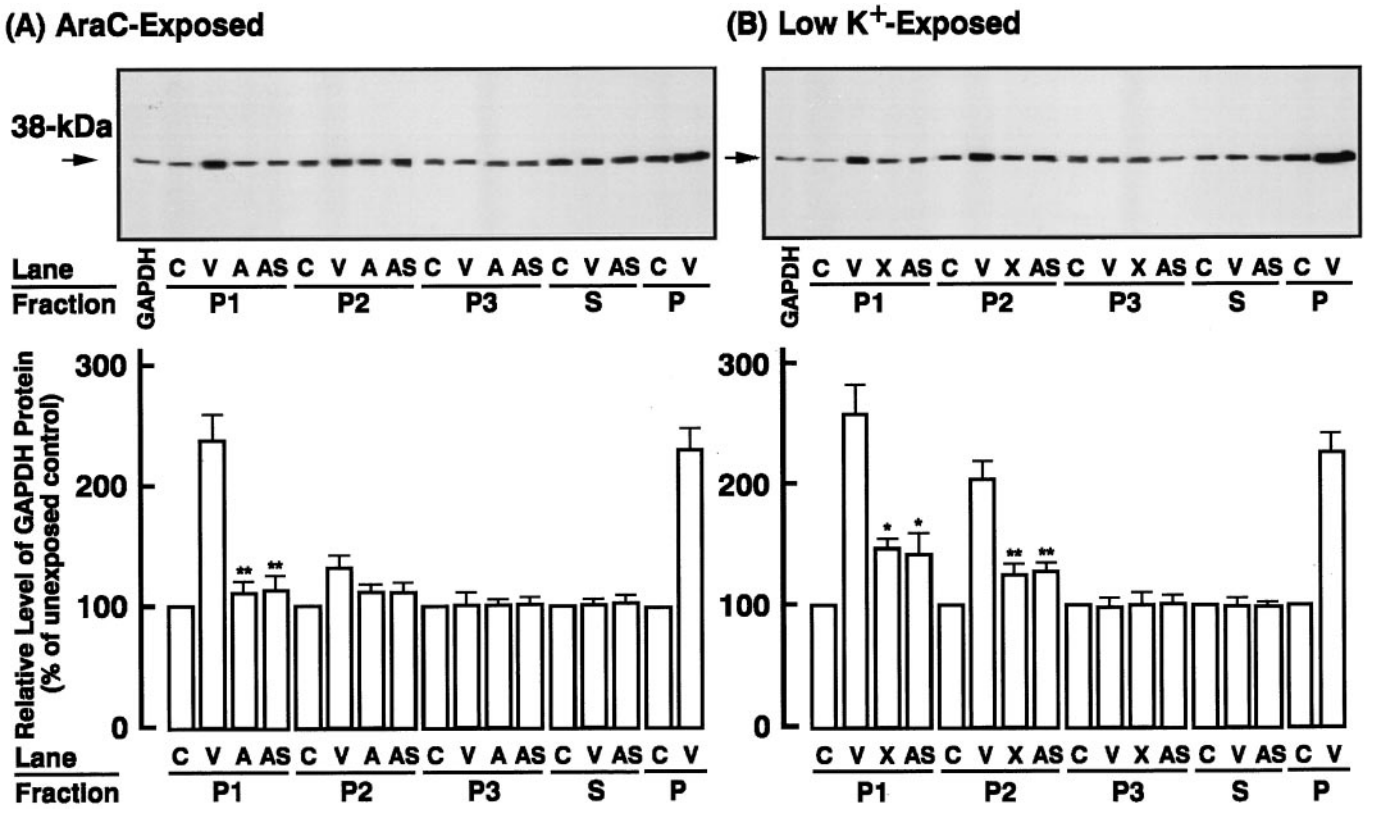

Fig. 2. Localization of overexpressed GAPDH in apoptotic CGCs detected by using subcellular fractionation and Western blotting. A, Subcellular fractionation of immature CGCs (20–24 hr after plating) exposed to ara-C for 24 hr. B, Subcellular fractionation of mature CGCs exposed to low K^+ for 12 hr. Subcellular fractions from various CGC samples were prepared, resolved by SDS-PAGE, electroblotted onto polyvinylidene difluoride membranes, and reacted with a monoclonal antibody for the overexpressed GAPDH as described in Materials and Methods. Measurement of level of GAPDH protein on the autogram also is described in the text. *Bar graphs*, levels of quantified GAPDH proteins expressed as relative values compared with the unexposed control (*C*). Values are mean ± standard error of three independent experiments. *, $p < 0.05$; **, $p < 0.01$ compared with the corresponding untreated (vehicle) control in each fraction using Student's *t* test. *V*, Plus vehicle (i.e., H_2O). *A*, Plus Act-D (1 μg/ml). *X*, Plus CHX (5 μg/ml). *AS*, Plus GAPDH antisense oligonucleotide (10 μM). *GAPDH*, purified GAPDH preparation. Note that GAPDH overexpression using these two apoptotic paradigms was detected in the nuclear (*P1*) or mitochondrial (*P2*) fraction but not in the microsomal (*P3*) and cytosolic (*S*) fractions. *P*, Particulate fraction, including P1, P2, and P3. Viability (%) of CGC (A): *C*, 95.0 ± 0.8; *V*, 40.2 ± 3.5; *A*, 82.6 ± 3.1; *AS*, 89.9 ± 3.1, and (B) *C*, 95.7 ± 0.6; *V*, 77.0 ± 2.0; *X*, 95.1 ± 0.6; *AS*, 90.4 ± 2.1.

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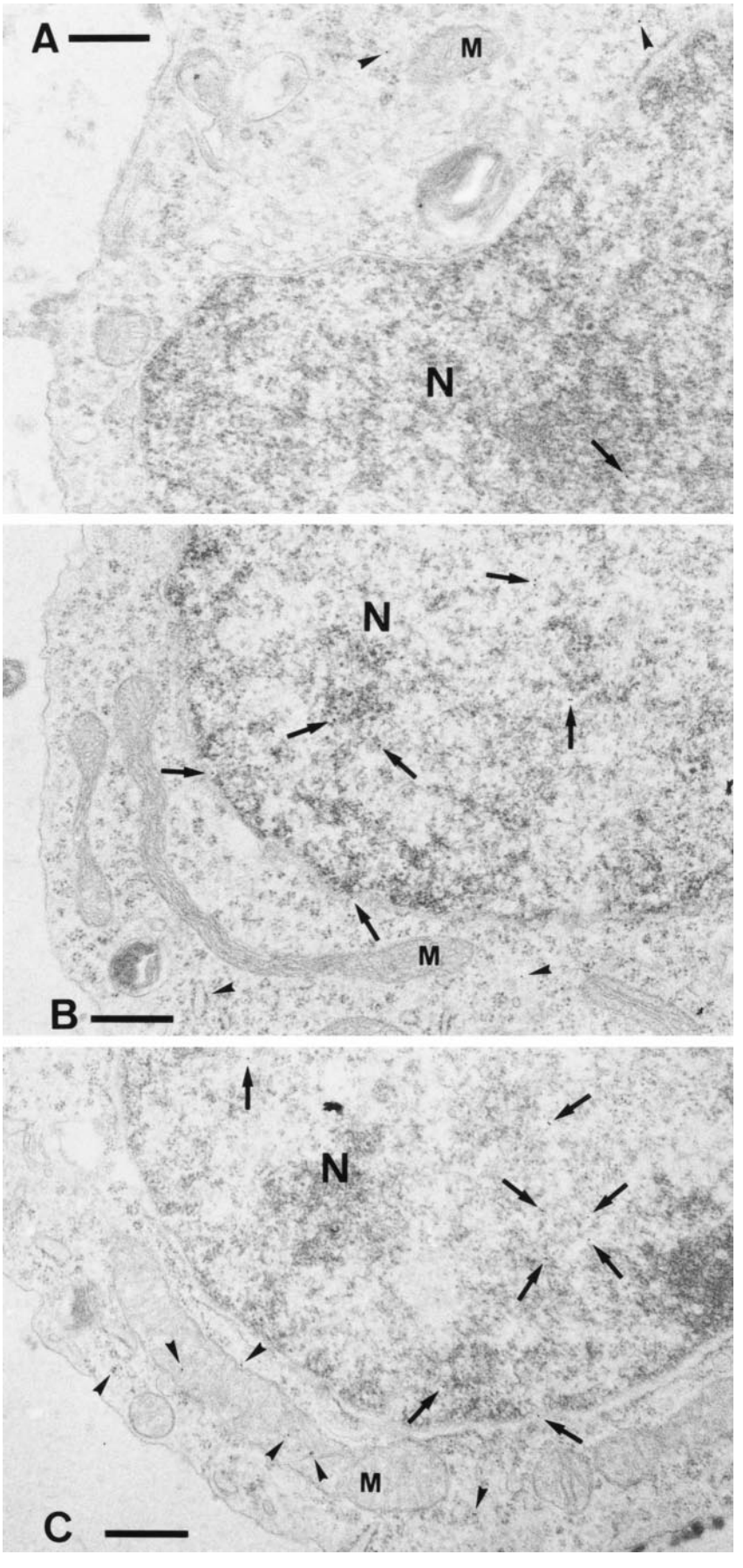

Fig. 3. Subcellular localization of overexpressed GAPDH in apoptotic CGCs detected by using the postembedding immunogold method. The immunoelectron microscopic examination was performed at an early stage of neuronal apoptosis as described in Materials and Methods. Immunoelectron micrographs: A, unexposed healthy CGCs; B, ara-C-induced apoptosis of CGCs at 24 hr; and C, apoptotic CGCs exposed to low K^+ for 12 hr. As the earliest apoptotic alteration, a diffuse increase in the density in the nucleoplasm of cells in B and C was noted compared with normal healthy cell in A. The immunoparticles (*arrows*) were located mainly on the nuclear compartment (*N*) in B. In contrast, low K^+-induced apoptotic cell C shows that immunoparticles (*arrowheads*) also were associated with the cytoplasmic elements, including mitochondria (*M*), besides the nucleus (*N*). Results shown are from a representative field of the typical experiment. *Scale bars*, 0.5 μm.

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GAPDH. It is imaginable that overexpressed GAPDH has a faster protein turnover rate than basal GAPDH and therefore is more sensitive to antisense knockdown. It also is possible that these two pools of GAPDH consist of different isoforms and are products of different genes, with the mRNA of induced GAPDH being more vulnerable to the attack by antisense oligonucleotides.

Recently, GAPDH has been implicated in certain forms of neurodegenerative diseases. To date, five neurodegenerative disorders have been shown to be caused by expansions of CAG repeats that lie within the coding regions of their respective genes; these include spinobulbar muscular atrophy, Huntington's disease, spinocerebellar ataxia type 1, dentatorubropallidoluysian atrophy, and Machado-Joseph disease (Koshy *et al*., 1996). It has been reported that the gene products from these neurodegenerative disorders selectively interact with GAPDH via the expanded polyglutamine stretches encoded by their CAG repeats (Burke *et al*., 1996; Koshy *et al*., 1996). Although the etiology of these neurodegenerative disorders remain enigmatic, these findings suggest that GAPDH protein may contribute to a common modality of pathogenesis. Recent reports have provided compelling evidence for apoptotic cell death in Huntington's disease (Portera-Cailliau *et al*., 1995) and Alzheimer's disease (Su *et al*., 1994). Moreover, it has been shown that β-amyloid precursor protein binds GAPDH to its carboxyl terminal (Schulze *et al*., 1993). In this context, we found that GAPDH cross-interacts with a monoclonal antibody raised against amyloid plaques from the brains of patients with Alzheimer's disease (Sunaga *et al*., 1995) and that tetrahydroaminoacridine, an antidementia drug (Summers *et al*., 1986), effectively suppresses the overproduction of GAPDH mRNA and protein (Sunaga *et al*., 1995; Ishitani *et al*., 1996b). It has been proposed that binding of GAPDH to the gene products of these neurodegenerative diseases results in loss of energy production due to inactivation of the glycolytic activity, and this in turn contributes to cell death (Burke *et al*., 1996). Although this hypothesis is intriguing, the results of the current study raise an alternative possibility that the pathogenesis of certain forms of neurodegenerative diseases could be primarily due to overexpression and subsequent accumulation of GAPDH protein in the nucleus to trigger apoptotic events. It is interesting to note that NH_2-terminal fragments of mutant Huntington's disease protein (i.e., huntingtin) and Machado-Joseph disease protein (i.e., ataxin-3) are located next to intranuclear inclusions in the neurons of affected brain regions (Davies *et al*., 1997; DiFiglia *et al*., 1997; Paulson *et al*., 1997). Thus, it seems conceivable that GAPDH may function as a chaperon, being involved in the transport of these mutant protein complexes to the nucleus. Clearly, detailed future investigations are needed to substantiate these possibilities.

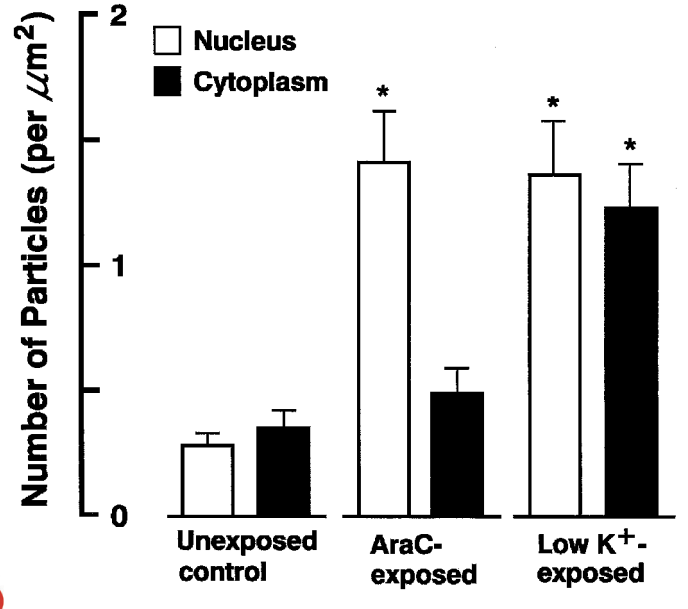

Fig. 4. Quantitative analysis of the immunoreactive product, gold particle, occurring within the ara-C- and low K^+-induced apoptotic CGCs. Ultrathin sections from several CGC monolayers were immunostained with a monoclonal antibody to the overexpressed GAPDH by the indirect postembedding electron microscopic immunocytochemical procedure, using immunogold as described in Materials and Methods. The immunoparticles on the micrographs were analyzed quantitatively by using an image analyzer as described in the text, and the results are mean ± standard error of 9–19 assessments from a typical experiment to ensure identical GAPDH overexpression and labeling conditions. *, $p < 0.01$ compared with the respective unexposed control, using Student's t test.

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