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A fluorescence vital assay for the recognition and quantification of excitotoxic cell death by necrosis and apoptosis using confocal microscopy on neurons in culture

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Abstract

An automated fluorescence method for the detection of neuronal cell death by necrosis and apoptosis with sequential acridine orange (AO) and ethidium bromide (EB) staining using confocal microscopy is described. Since cell nuclei during apoptosis become acidic, AO staining was utilized to distinguish live neurons from neurons undergoing apoptosis, using the AO property to shift its fluorescence from green at normal pH toward brilliant orange-red in the process of acidification. Further EB application labels nuclei of necrotic neurons in red. Sequential treatment by AO and EB can be employed as an express vitality test to count fractions of live and dead cell via apoptosis and necrosis, respectively. An algorithm of automatic quantification of cell types is based on the image correlation analysis. Our conclusion is validated by experiments with the vital dye trypan blue and the pharmacological study of receptor subtypes involved in the excitotoxicity. The approach described here, therefore, offers an express, easy, sensitive and reproducible method by which necrosis and apoptosis can be recognized and quantified in a population of living neurons. Because this assay does not require any preliminary tissue treatment, fixation or dissociation in a cell suspension its utility is likely to be extended for measuring cell viability and cytotoxicity on a variety of living preparations (tissues, brain slices and cell cultures).

Keywords: Excitotoxicity; Cell culture; Cortical neurons; Acridine orange; Ethidium bromide; Confocal microscopy; Necrosis; Apoptosis

1. Introduction

Neurodegeneration or neuronal cell death is critically involved in both physiological processes of developing vertebrate central nervous system (CNS) and diseased states induced by many pathological factors such as hypoxia, hypoglycemia, energy deprivation, oxidative stress, or a combination of those mimicked by ischemia (Choi, 1988; Beal, 1992; Lipton, 1999; Khodorov, 2004). The most common feature of neuronal cell loss is an accumulation of glutamate (Glu), an excitatory neurotransmitter in the CNS, resulting in hyperactivation of glutamate receptors (GluR) and sustained cell depolarization (Antonov and Magazanik, 1988; Szatkowski et al., 1990; Rossi et al., 2000; Antonov, 2001). Glu excitotoxicity, which destroys neurons following excessive Glu exposure, can be revealed experimentally. When injected in the CNS, applied to brain slices or neuron cultures, GluR agonists trigger necrotic membrane injuries of neurons (Choi et al., 1987) that usually appear during hours of treatment and the delayed apoptotic death that begins from DNA digestion and nucleus fragmentation (Wyllie et al., 1980).

Primary cell cultures are routinely used to determine the mechanism by which GluR agonists mediate neuronal cell death. Quantification of neuronal cell death in experimental systems can be achieved using a variety of *in vitro* assays with different technical advantages (Bergmeyer and Bernt, 1974; Koh and Choi, 1987; Uliasz and Hewett, 2000). In many studies, the cellular uptake of the fluorescence dye propidium iodide (PI, or similar stain ethidium bromide, EB) has been used as a marker of dead or dying cells, since this dye is unable to penetrate the plasma membrane of live cells, and can enter cells exclusively through necrotic membrane damages (Monette et al., 1998; Noraberg et al., 1999). Apoptosis can be recognized by a set of morphological features (Wyllie et al., 1980). At the biochemical level apoptosis is characterized by DNA digestion (Compton, 1992) and is accompanied by expression of many specific proteins and molecules that can be visualized by

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immunostaining on fixed preparations (Nagata, 1997; Wolf and Green, 1999).

In principle, acridine orange (AO) staining without PI or EB may monitor apoptosis. In addition to the property to label double-stranded nucleic acids in green (Darzynkiewicz, 1990), AO in living cells serves as a pH indicator, being trapped by acidic compartments, which then fluoresce as brilliant orange-red (Zelenin, 1966). As nuclei during apoptosis undergo morphological changes and acidification, AO has been used to identify apoptotic cells in live *Drosophila* embryos (Abrams et al., 1993; White et al., 1994), *Tetrahymena* and chicken chondrocytes (Mpoke and Wolfe, 1997), and cell aggregates from human fetal brain (Pulliam et al., 1998). Based on these unique properties, we demonstrate that AO itself as a membrane permeable vital stain allows to discriminate live neurons from neurons undergoing apoptosis. Further, EB application labels nuclei of necrotic neurons in red. Sequential treatment by AO and EB, therefore, can be employed as an express test to count proportions of live cells and those dead by apoptosis and necrosis, respectively. Verification of our conclusion was accomplished by parallel experiments with the vital dye, trypan blue, and the pharmacological analysis of receptor subtypes that mediate Glu-induced neurodegeneration. Combined with confocal microscopy and an automatic quantification of neuron states, this method is fast and reliable and eliminated manual counting thus minimizing sample and systematic measurement errors.

2. Materials and method

2.1. Cell culture

Experiments were performed at room temperature (20–22 °C) on primary cultures of neurons from embryonic rat brain cortex. All procedures using animals were in accordance with the European Communities Council Directive (24th November 1986; 86/609/EEC) and were approved by the local Institutional Animal Care and Use Committee. Cultures were prepared using a modification of the method as previously described (Dichter, 1978; Antonov et al., 1998). Pregnant Wistar rats were sacrificed by CO_2 inhalation at 16 days after conception. Cortices were obtained by aseptic dissection, incubated for 30–40 min at 37 °C in growth medium contained 83% minimum essential medium (MEM), 25 mM Hepes, 2 mM L-glutamine, 10% D-glucose plus 0.03% trypsin, and then dispersed mechanically by trituration with a fire-polished glass Pasteur pipettes. Cells were pelleted by light centrifugation ($410 \times g$, 5 min at 25 °C), the supernatant discarded and the cell pellet resuspended in pre-warmed Hanks solution. For better washout of reagents this procedure was repeated. A plating suspension at a density of 130,000 cells per ml was prepared in growth medium. This density was optimal for the neuronal survival, the neuronal network formation and further experimental manipulations with the cultures. The suspension of cells was plated onto 15 mm diameter glass coverslips that had been coated with poly-D-lysine in 35 mm plastic Petri dishes. Cultures were kept at 37 °C in a humidified 5% CO_2—containing atmosphere. The growth medium was refreshed twice a week. Cells were used for experiments after 7 and 14–17 days in culture. MEM, DMEM, calf serum and trypan blue (TB) were purchased from Biolot (St.Petersburg, Russia). All other reagents for culturing were from Sigma Chemical Co. (St. Louis, MO).

2.2. Neurotoxic insults by glutamate receptor agonist exposure

Directly before the experiments, coverslips with neuronal cultures were placed in the bathing salt solution consisted of 140 mM NaCl, 2.8 mM KCl, 1.0 mM $CaCl_2$, 10 mM Hepes; pH was adjusted to 7.2–7.4 with NaOH. In order to trigger the necrotic cell injury and apoptosis the indicated concentrations of glutamate receptor agonists were added to the bathing salt solution. When effects of antagonists and modulators were studied the compounds were coapplied with the glutamate receptor agonists. Measurements of the proportion of dead cells among whole cell population were performed every 60 min during 360 min of recording in the continuous presence of the compounds. Glutamate (Glu), *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainic acid (KA), DL-2-amino-5-phosphono-pentanoic acid (AP5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), cyclothiazide (CTZ), acridine orange and ethidium bromide (EB) were purchased from Sigma Chemical Co. (St. Louis, MO). All drugs were kept frozen in stock solutions at concentrations of 1–100 mM and were diluted to the required concentrations before use.

2.3. Imaging and image processing

Light microscopy images were captured by CCD camera Mintron (Alfa Telecom, St. Petersburg, Russia). Fluorescence images were captured using a Leica (Leica Microsystems, Heidelberg, Germany) TCS SL scanning laser confocal microscope (upright) equipped with argon laser of 50 mW (excitation wavelengths 458, 476, 488 and 514 nm, approximately 10 mW each). Cultures were viewed with a 40× water objective (HCX APO L 40×/0.80, Leica Microsystems, Heidelberg, Germany). To resolve fine details an additional electronic zoon with a factor of 1.5–3.5 was used. Since EB has a second peak of excitation, which is consistent with the excitation of AO, both AO and EB could be visualized using the same laser line. For two-channel imaging of AO and EB, neuronal cultures were excited with 488 nm laser line, which could be varied between 0.1 and 10 mW by means of a neutral density filter. The emitted fluorescence was acquired at 500–560 nm (green region of spectrum, for AO) and >600 nm (red region of spectrum, for EB) and collected simultaneously using separate photo multiplier tubes. Microscope settings were adjusted so that imaging conditions for both channels were kept equal and constant. When cultures were labeled by AO alone (without EB treatment) the same confocal imaging procedure was utilized. To improve signal-to-noise ratio 6 scans (512×512 pixel array) were averaged at each optical section. The confocal images from both channels were merged using standard Leica software and program ImageJ