Developmental shift in the apostat: Comparison of neurones and astrocytes

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Abstract The intrinsic pathway of apoptosis was investigated in cell-free extracts of neurones and astrocytes at various stages of maturation. Neuronal extracts were activated 65-fold after 3 days, 9-fold after 7 days, and were not activated after 10 days in culture. In contrast, astrocyte extracts were activated to a similar extent at all stages, up to 60 days in culture. The co-incubation of neuronal and astrocyte extracts followed by addition of cytochrome *c*/2′-deoxyadenosine 5′-triphosphate led to a 40-fold activation, suggesting that the development-associated neuronal shift does not involve the appearance of a dominant inhibitor, but rather downregulation of some key component(s) involved in caspase activation.

Keywords: Brain ageing; Neuronal apoptosis; Caspase activation; Intrinsic pathway; Astrocytes; Neurones

1. Introduction

Neuronal cell death occurs in many different physiological and pathological states, from development to ischaemia to neurodegeneration. The biochemical pathways underlying neuronal cell death in these paradigms are of interest from the standpoints of pathogenesis and potential therapeutics. One of these pathways is the intrinsic pathway of apoptosis which is triggered by several stimuli that target mitochondria and induce cytochrome *c* (cyt *c*) release from the mitochondrial inter-membrane space to the cytosol thus activating the apoptotic cascade [1,2].

Over the past 10 years, it has been shown that the expression of mutant proteins associated with neurodegeneration (e.g., mutants of copper/zinc superoxide dismutase that are associated with familial amyotrophic lateral sclerosis) leads to apoptosis in cultured neuronal cells [3–5]. However, in vivo, the cell loss associated with neurodegenerative diseases is, in at least some cases, non-apoptotic [6,7]. Among the many possible explanations for this combination of observations are two for which there is experimental support: first, alternative, non-apoptotic cell death programs have been shown to exist [8,9], and it is possible that some of the neuronal cell death in neurodegenerative diseases may proceed via these alternative pathways [10]; second, a developmental shift has been shown to occur in the apostat, such that the intrinsic pathway is progressively inactivated in association with downregulation of the apoptotic protease activation factor-1 (Apaf-1) and caspase-3 [11]. The apostat was first described as a hypothetical entity that refers to the set point of the cell with respect to apoptosis [12,13] and later was ascribed to mitochondria [14]. Previous studies showed that an apostat shift could be reproduced in cultured neurones [11], however, the caspase activation pattern of a glial population such as astrocytes remains unknown. In this work, we investigated the activation of the intrinsic pathway of apoptosis using cell-free extracts from primary cultures of neurones and astrocytes at various stages of maturation. The ability of astrocytes and neurones to demonstrate such a shift was compared. Furthermore, when we found that astrocytes did not display the shift, we used a combination of astrocyte and neurone extracts to determine the nature of the shift displayed by neurones. Our results support the proposal that the development-associated neuronal apostat shift is not associated with the expression of a dominant inhibitor of apoptosis, but rather is associated with the downregulation of a crucial component or components required for apoptosis activation such as Apaf-1 and caspase-3 [11]. In contrast, astrocytes may not undergo the maturation-associated shift seen in neurones.

2. Materials and methods

2.1. Materials

The trinucleotide 2′-deoxyadenosine 5′-triphosphate (dATP), phosphate-buffered saline, pH 7.4 (PBS), HEPES buffer (1 M), heat-inactivated fetal bovine serum, and the cell culture reagents MEM media, neurobasal media, GlutaMAX-I, and B-27 supplement were from Invitrogen. Cytosine arabinoside (Ara-C), the antibiotic solution penicillin/streptomycin and horse heart cyt *c* were from Sigma–Aldrich. The caspase substrates acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC) and acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (LEHD-AMC) were obtained from Enzyme Systems Products. 10 mM stock solutions of the substrates was prepared in dimethyl sulfoxide (DMSO). All other reagents were purchased from Sigma–Aldrich unless otherwise stated.

2.2. Primary cultures

Experimental animals used in these studies fulfil all the requirements stated by NIH and IACUC guidelines. Primary cultures of cortical neurones and astrocytes were prepared as described [15]. Briefly, primary astrocyte cultures were derived from one day old rat pups

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Abbreviations: cyt *c*, cytochrome *c*; dATP, 2′-deoxyadenosine 5′-triphosphate; DEVD-AMC, Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; LEHD-AMC, Acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin

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and cultured in MEM media containing HEPES, 10% fetal bovine serum and 1% penicillin/streptomycin for 7–60 days at 37 °C and in 5% carbon dioxide atmosphere. Primary cortical neurones were established from day 17 Sprague–Dawley rat embryos. Dissociated cells were seeded onto 10-cm poly-D-lysine coated plastic dishes and incubated in neurobasal medium containing 1 mM GlutaMAX-I and B-27 supplement. The cells were seeded at a density of 3×10^5 cells and cultured at 37 °C in a 5% carbon dioxide atmosphere for 3–10 days. Glial growth was prevented by adding Ara-C to the culture media to 25 μM.

2.3. Preparation of cell-free extracts

All steps were carried out at 4 °C unless otherwise stated. Cell-free extracts from neuronal and astrocytes were prepared according to a modified procedure in which the addition of protease inhibitors was omitted [15]. Cells were washed twice with PBS solution, scrapped and centrifuged at $1000 \times g$ for 5 min. The pellet was resuspended in hypotonic buffer at 1:1 ratio (w/v) and kept on ice for 30 min to allow swelling. The cells in suspension were then disrupted by passage through a 27-gauge needle (Becton–Dickinson) to shear DNA and centrifuged at $16000 \times g$ for 30 min in a refrigerated Eppendorf 5417R centrifuge. The pellet was discarded and the supernatant consisting of a cytosolic extract and light membrane structures (S16) was aliquoted and stored at −80 °C until used. The hypotonic buffer consisted of 20 mM piperazine-1,4-bis(2-ethane)sulfonic acid (Pipes) buffer, pH 7.4, containing 10 mM potassium chloride, 5 mM sodium EGTA, 2 mM magnesium chloride and 1 mM dithiothreitol.

2.4. Kinetics of activation

Caspase activity was measured according to a procedure modified from that described [15].

Prior to the kinetic measurements the substrates DEVD-AMC and LEHD-AMC were dissolved in assay buffer consisting of 50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer, pH 7.4, containing 1 mM EDTA, 100 mM sodium chloride, 10% sucrose (w/v), 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps) (w/v), and 10 mM dithiothreitol.

30 μg of cell-free extracts of astrocytes or cortical neurones at different stages of maturation was activated with 10 μM horse heart cyt *c* and 1 mM dATP at 37 °C during 60 min. A control experiment was carried out simultaneously in the absence of cyt *c*/dATP and gives the background activity of the sample. The samples were loaded in a 96-well microplate (Costar) and the reaction started by addition of the substrate (50 μM final concentration). The reaction was monitored continuously for 30 min at 37 °C in a thermostated Molecular Devices SpectraMax Gemini spectrofluorimeter, at excitation and emission wavelengths of 370 and 460 nm, respectively. The steady-state rates of substrate hydrolysis were obtained from the linear parts of the curves. Results were expressed in arbitrary units of fluorescence (AU). The data are reported as means ± S.D. of three independent experiments.

3. Results and discussion

Apoptosis is one of the cell death programs actively involved during neurodevelopment and neurodegeneration. In order to understand the possible links between neuronal cell death and ageing we compared cyt *c*/dATP induction of the caspase cascade in cell-free extracts of primary cultures of neurones and astrocytes as a function of time. Cortical neurones were cultured for 3, 7 and 10 days, and astrocytes for 14–60 days. At defined times, the cells were harvested, cell-free extracts produced and activated by cyt *c*/dATP [1,15]. Caspase activity was monitored using the executioner caspase substrate DEVD-AMC. Astrocyte extracts were activated by cyt *c*/dATP to a similar extent at all stages evaluated (14–60 days) (Fig. 1A). In contrast, neurone extracts were activated 65-fold after 3-days, only 9-fold after 7-days in culture, and were not activated by cyt *c*/dATP after being in culture for 10 days (Fig. 1B), in

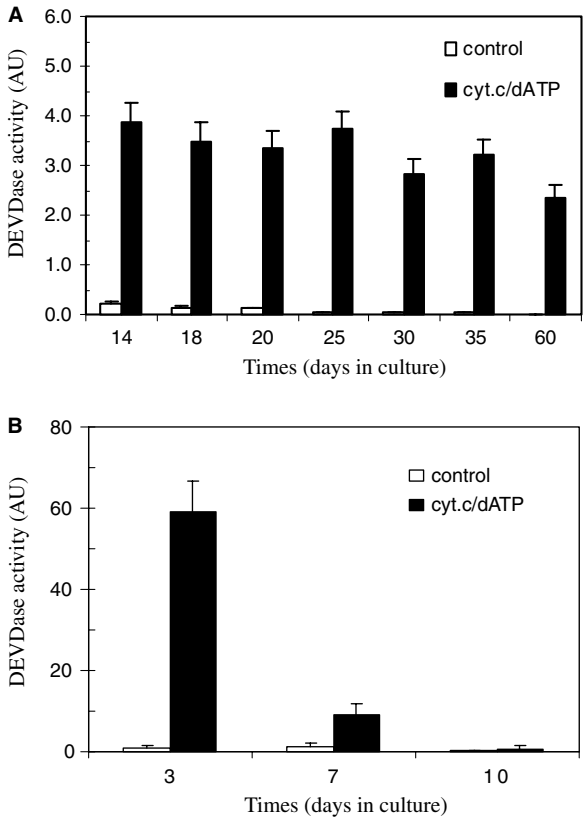

Fig. 1. Time dependence of the cyt *c*/dATP activation of neuronal and astrocyte extracts. 30 μg of cell-free extracts of primary cultures of astrocytes (A) or cortical neurones (B) at different stages of maturation were activated with cyt *c*/dATP. The reaction was followed fluorometrically after addition of DEVD-AMC substrate. The bars represent the steady-state rates of substrate hydrolysis. Control and cyt *c*/dATP treated samples are indicated with white and black squares, respectively. The details of the experiments are described in Section 2.

agreement with a previous study [11]. Given that neurone development events that occur in vivo have been shown in a number of studies to occur at a similar time in primary cell cultures [16], this finding indicates that the activation of the intrinsic pathway of apoptosis is age-dependent, and neuronal cultures can recapitulate this developmental apostat shift.

The difference between the findings for neurones and astrocytes could be the result of either of two general scenarios. Neuronal development could be associated with the appearance of a dominant inhibitor of caspase activation (e.g., XIAP or a functional analogue); alternatively, it is possible that neuronal development is associated with the downregulation of a critical component or components of the intrinsic apoptotic pathway. A previous study provided support for the latter possibility, but did not exclude the coexistence of the former [11]. Therefore, cell-free extracts of 7-day primary cultures of cortical neurones and astrocytes were co-incubated for 1 h at 37 °C, followed by 30 min cyt *c*/dATP activation. The course of the reaction was monitored fluorometrically by release of aminomethylcoumarin from the substrates LEHD-AMC and DEVD-AMC, which were the targets of the initiator caspase 9 and the executioner caspases-3 and -7, respectively [13]. As assayed on LEHD-AMC substrate, neuronal and astrocyte extracts were both activated by cyt *c*/dATP 8-fold and 13-fold, respectively (Fig. 2A). Co-incubation of neuronal and astrocyte