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Upregulation of death pathway molecules in rat cerebellar granule neurons undergoing apoptosis

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Abstract

Cerebellar granule neurons can be maintained in culture in a medium containing high serum and depolarising levels of KCl. When serum is removed and the KCl levels lowered from 25 to 5 mM, the cells undergo apoptosis. Apoptosis can be prevented by inhibitors of transcription or translation, suggesting a need for macromolecular synthesis in the apoptotic process. Using quantitative reverse transcription-polymerase chain reaction the levels of mRNA for a range of genes postulated to be important in apoptosis have been examined. Elevated levels of caspase 3, c-Jun, and Fas ligand were found, in addition to a corresponding increase in c-Jun protein and activation of caspase-3. These results suggest that cerebellar granule neurons upregulate components of both death receptor-mediated and the mitochondrial-mediated death pathways.

Keywords: Apoptosis; mRNA; Caspase; Fas ligand; c-Jun; Cerebellar; Reverse transcription-polymerase chain reaction

Programmed cell death plays an important role in shaping the developing nervous system, removing unwanted neurons, and in establishing correct synaptic connections with the targets they innervate [12]. Many neurons are dependent upon neurotrophic factors for survival and, during development, a limiting supply of trophic factors is thought to be a signal which initiates the apoptotic process [13]. In the adult, inappropriate neuronal apoptosis has been suggested to be involved in a number of neurodegenerative diseases [8], including Alzheimer's disease, Parkinson's disease and stroke. A greater understanding of the mechanisms which govern neuronal death could lead to more effective therapies for these disorders [2].

Neuronal survival in vitro can be supported by a number of non-protein agents in the absence of neurotrophic factors. For example, several neuronal populations can be maintained by depolarising levels of KCl [4]. One of these, cerebellar granule neurons, can be cultured in serum and high KCl conditions [1]. Granule neurons cultured from early postnatal rat cerebellum differentiate and acquire several morphological, biochemical and electrophysiological characteristics of mature neurons. Since they are the most abundant neuronal population in the brain they can be grown in sufficient quantities to allow biochemical experiments to be performed. Removal of serum and lowering the KCl to the more physiological level of 5 mM triggers apoptosis in these cells, presumably mimicking the cell death which occurs naturally between 3 and 5 weeks postnatally [1].

In many cases of neuronal apoptosis, including cerebellar granule neurons, the process is dependent upon transcription and translation. This suggests that the synthesis of new proteins, or maintenance of existing labile ones might be central to the process [10]. A number of genes are upregulated in the granule neuron model of apoptosis. One such gene is caspase 3, a member of the cysteine protease family related to interleukin-1β converting enzyme (ICE) [3]. Genetic studies in *Caenorhabditis elegans* identified the ced3 gene, a gene homologous to ICE, as crucial for developmental cell death [18]. Subsequent experiments have shown that this family of proteases plays a key role in apoptosis [15]. Another gene which has been strongly implicated in neuronal death is the transcription factor c-Jun. In cerebellar granule neurons the level of c-Jun is upregulated during apoptosis [11] and inhibition of c-Jun function using dominant negative constructs or the use of chemical inhibitors of the JNK/p38 pathways protected granule neurons

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from apoptosis [5,17]. These data suggest that genes which are upregulated by c-Jun may be important in neuronal apoptosis. In this study we have examined the expression of a range of genes during apoptosis of cerebellar granule neurons using quantitative reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting.

Cerebellar granule neurons were obtained from 8-day-old Sprague–Dawley rat pups as described previously [14]. Briefly, cells were dissociated from dissected cerebella by mechanical disruption in the presence of trypsin and DNase and plated in poly-L-lysine coated culture dishes. Cells were seeded at 3×10^5 cells/cm^2 in basal modified Eagle's medium, 2 mM glutamine, 25 mM KCl, 50 μg/ml gentamicin and 10% heat-inactivated fetal bovine serum. Cytosine-β-D-arabinofuranoside (10 μM) was added after 20–22 h to prevent the growth of non-neuronal cells. Apoptosis was induced by shifting cultures at 6–8 days in vitro to serum-free basal modified Eagle's medium containing 5 mM KCl; cells were harvested at 0, 2, 4, 8 or 18 h later for RNA analysis (one time course did not contain an 18 h timepoint).

RNA was extracted from four time courses by homogenising pelleted cells in TRIzol reagent (Life Technologies Inc., Gaithersburg, MD, USA). The RNA was resuspended in PCR grade water, the concentration calculated by A_{260} measurement and assessed by gel electrophoresis. First strand cDNA was synthesized from 1 μg of each RNA sample in a reaction mixture containing: 0.01 M dithiothreitool (DTT), 0.5 mM each dNTP, 0.5 μg oligo(dT) primer, 40 U RNAseOUT ribonuclease inhibitor (Life Technologies Inc.), and 200 U SuperscriptII reverse transcriptase (Life Technologies Inc.). Triplicate reverse transcription reactions were performed, with an additional reaction omitting the reverse transcriptase to allow for assessment of genomic DNA contamination. The resulting cDNA products were divided into 20 aliquots using a Hydra 96 robot (Robbins Scientific, Sunnyvale, CA, USA) for parallel Taqman PCR reactions, using different primer and probe sets. Taqman PCR was carried out using an ABI prism 7700 sequence detector (Perkin–Elmer Applied Biosystems, Foster City, CA, USA) on the cDNA samples in a mixture containing: 2.5 mM $MgCl_2$, 0.2 mM dATP, dCTP, dGTP and dUTP, 0.1 μM each primer, 0.05 μM Taqman probe, 0.01 U AmpErase uracil-N-glycosylase, 0.0125 U Amplitaq Gold DNA polymerase (all reagents from Perkin–Elmer) 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. Additional reactions were performed on each 96 well plate using rat genomic DNA (Clontech Laboratories Inc., Palo Alto, CA, USA) to produce a standard curve relating threshold cycle to template copy number [6]. Primer and probe sets were designed from sequences in the Genebank database using Primer Express software (Perkin–Elmer).

Primers, GAPDH forward (F) 5′-GAACATCATCCCTGCATCCA-3′, reverse (R) 5′-CCAGTGAGCTTCCCGTTCA-3′, probe (P) 5′-CTTGCC CACAGCCTTGGCAGC-3′. β-actin F 5′-TCTGTGTGGATTGGTGGC TCTA-3′, R 5′-CTGCTTGCTGATCCACATCTG-3′, P 5′-CCTGGCCTC ACTGTCCACCTTCC-3′. Caspase 3 F 5′-AATTCAAGGGACGGGTCA TG-3′, R 5′-GCTTGTGCCGTACAGTTTC-3′, P 5′-TTCATCCAGTCA CTTTGCGCCATG-3′. c-Jun F 5′-TCCACCGCCAACATGCT-3′, R 5′-CCACTGTTAACGTGGTTCATGAC-3′ P 5′-TCTGTTTAAGCTGTG CCACCTGTTCCCT-3′. Fas Ligand F 5′-AAAAGCCAGTGTCCCATT GG-3′, R 5′-CCCCTCTTATTTCTCTGTTAGCATCA-3′ P 5′-CATCTT TATTTTTAACTGATGTTTTCTGAGCCCACC T-3′.

For protein analysis, samples were homogenized in lysis buffer (25 mM Tris–HCl (pH 7.5), 3 mM EDTA, 3 mM EGTA, 50 mM NaF, 2 mM orthovanadate, 0.27 M sucrose, 10 mM glycerophosphate, 2 μM microcystin, 1 mM mercaptoethanol/DTT, 0.5% Triton X-100, complete protease tablets (Boehringer)). Twenty microgrammes of protein was run on 4–20% SDS-polyacrylamide gels and transferred to nylon membranes. Blots were blocked for 2 h in buffer A (phosphate-buffered saline (PBS)/0.1% Tween 20 and 10% (w/v) non-fat dry milk). Primary antibodies were diluted in buffer A, incubated at room temperature for 1–2 h, washed 3×15 min in PBS/0.1% Tween-20 and incubated a further hour in horseradish peroxidase-conjugated secondary antibody. Blots were washed 3×15 min in PBS/0.1%Tween-20 and 2×10 min in PBS before incubation in ECL (Amersham) and exposure to X-ray film. Caspase-3 and -8 antibodies were raised in-house to recombinant human protein, while c-Jun and FasL antibodies were purchased from Transduction Laboratories. Blots shown are representative of three experiments.

RNA quality was assessed by examining the expression of a number of 'housekeeper' or control genes commonly used in Northern blotting and PCR experiments. These include cyclophilin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin; all three genes were found to follow identical patterns within an RNA time-course. RNA changes were measured in four independent experiments. To control for variations in RNA quality the results were first expressed as a percentage of the level of β-actin expression. To compare between the time-courses the value at 0 time was taken as 1 and the results expressed as fold-increase over this value. As previous studies have indicated a gradual loss of mRNA during apoptosis, these results, normalized to the level of β-actin mRNA, will show expression changes relative to this gene.

c-Jun is known to be upregulated in a number of neuronal cells following an apoptotic stimulus. An increase in the level of c-Jun was seen by 2 h (Fig. 1), peaking by 4 h and this level was maintained. Previous experiments suggested a decrease in the level of c-Jun mRNA by 4 h [17]. Caspase-3 has also been described as increasing at the transcriptional level consequent to apoptosis. When expression of this transcript was examined an increase in level was evident by 4 h and peaked at 8 h; caspase-3 was still significantly increased at 18 h. The Fas death receptor pathway is thought to play a role in some forms of apoptosis, and the

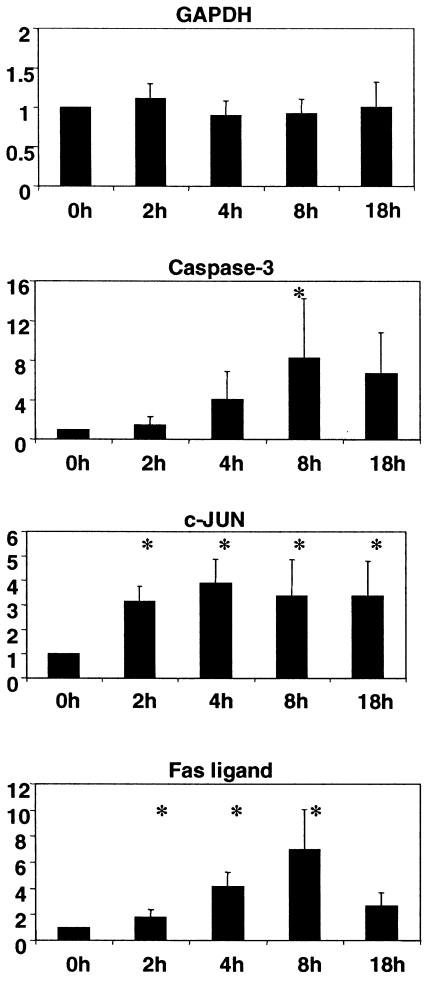

Fig. 1. Quantitative analysis of mRNA expression in apoptosising cerebellar granule neurons. Taqman PCR was carried out on RNA extracted from four timecourses of neurons induced to die by serum withdrawal and lowering K^+ to 5 mM. Data is expressed as fold induction over the level of mRNA detected at 0 h. * indicates that this is significant to $P < 0.05$.

level of Fas ligand mRNA has been reported to increase in a permanent middle cerebral occlusion model of stroke [7]. Fas ligand was investigated in this model of rat central nervous system apoptosis and was found to be upregulated, starting around 2 h and increasing to a maximum of 7–8 fold by 8 h. By 18 h the increase in Fas ligand had decreased to 2 fold. In contrast, the levels of caspase-8, a downstream effector of Fas receptor, did not significantly increase although there was a trend upwards in level within individual experiments (data not shown).

While RNA changes are suggestive of a role in the apoptotic process it was also of interest to determine whether similar changes occurred at the protein level. A marked increase in the level of c-Jun protein was observed by 4 h and this was maintained at 8 h but had decreased in level by 24 h (Fig. 2), consistent with previous reports [17]. This suggests that there is a mismatch between the level of mRNA present and protein production, which might arise

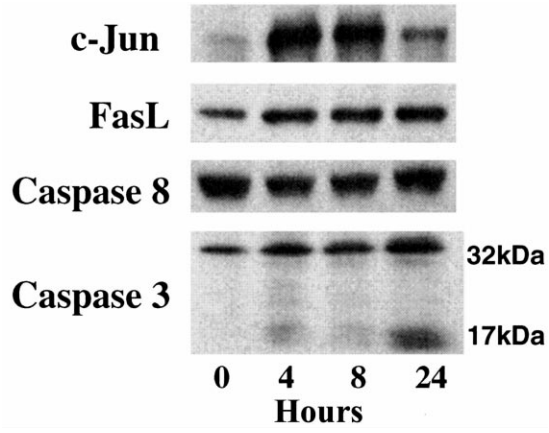

Fig. 2. Western blot analysis of apoptosing cerebellar granule neurons. Neurons were induced to die by serum withdrawal and lowering K^+ to 5 mM. The positions of pro-caspase-3 and the active 17 kDa product are indicated.

due to reductions in energy available during apoptosis. Activation of caspase-3 was seen as early as 4 h following the apoptotic insult, as indicated by the an increase in the 17 kDa large subunit cleavage product (Fig. 2). Increases in the level of FasL were not consistently seen but since FasL can be cleaved from the surface of cells it is possible that increased levels of this protein were not detected. Interestingly, it has been shown that in mice which have a non-functional FasL (gld mice), cerebellar granule neurons were less sensitive to apoptosis following KCl reduction and that a Fas-Fc decoy molecule could also reduce apoptosis [9].

We did not find any change in the level of uncleaved caspase 8, using an antibody reported to recognize the prodomain (SK441), and did not detect a cleaved caspase 8 product with this antibody [16]. It is possible that caspase 8 is being activated in this system but that the turnover of the cleaved product is too rapid for us to detect.

In this paper we have looked at the temporal expression of c-Jun, FasL and caspase 3 mRNA over an 18 h period. Our results are consistent with a model in which activation of a transcription factor, c-Jun, leads to the transcription of FasL, which binds to the death receptor Fas and downstream signalling culminates in the activation of caspase-3 and apoptosis.

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