Research Article

Neurodegeneration changes in primary central nervous system neurons transfected with the Alzheimer-associated neuronal thread protein gene

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Received 31 January 2001; received after revision 31 March 2001; accepted 4 April 2001

Abstract. The AD7c-NTP gene is over-expressed in brains with Alzheimer’s disease (AD), and increased levels of the corresponding protein are detectable in cortical neurons, brain tissue extracts, cerebrospinal fluid, and urine beginning early in the course of AD neurodegeneration. In the present study, we utilized a novel method to transfect post-mitotic primary neuronal cell cultures, and demonstrated that over-expression of the AD7c-NTP gene causes cell death and neuritic sprouting, AD7c-NTP gene is associated with AD, and accumulation of AD7c-NTP protein in the brain begins early in the course of disease. Moreover, the prominent localization of AD7c-NTP immunoreactivity in degenerating neurons, neuropil threads, and dystrophic neurites (swollen cell processes) [2] suggests a relationship between AD7c-NTP protein accumulation in the brain and the distribution of AD-type cellular degeneration.

In preliminary studies, we observed a dimorphic phenotype characterized by reduced viability and increased neuritic sprouting in PNET2 neuronal cells transfected with the AD7c-NTP cDNA [1]. Importantly, apoptotic cell loss and neuritic sprouting are major correlates of dementia in AD. This study demonstrates that efficient gene transfer of recombinant plasmid DNA can be achieved in primary neuronal cultures using a Mirus transfection reagent, and that over-expression of the AD7c-NTP gene is associated with AD, and accumulation of AD7c-NTP protein in the brain begins early in the course of disease. Moreover, the prominent localization of AD7c-NTP immunoreactivity in degenerating neurons, neuropil threads, and dystrophic neurites (swollen cell processes) [2] suggests a relationship between AD7c-NTP protein accumulation in the brain and the distribution of AD-type cellular degeneration.

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Key words. Neuronal thread protein; neurodegeneration; primary neuron culture; DNA transfection.
gene in post-mitotic neurons results in increased cell death and neuritic sprouting, as seen in AD.

Materials and methods

In vitro model

Post-mitotic primary rat cerebellar neuron (rCBN) cultures were generated with brain tissue derived from postnatal day 6 pups as described previously [4–6]. Five-day-old cultures were transfected with the full-length AD7c-NTP cDNA (pcDNA3-AD7c) or the luciferase (pcDNA3-Luc) or LacZ (pcDNA3-βGal) reporter gene ligated into the pcDNA3.1 vector (Invitrogen, Carlsbad, Calif.) in which gene expression was regulated by a CMV promoter. Cells seeded into 6- or 96-well plates were transfected using the Mirus IT-100 or LT-1 reagent (Pansera, Madison, Wis.) following the manufacturer’s protocol. Transfection efficiency ranged from 10 to 25% as demonstrated by co-transfection with recombinant plasmid DNA expressing green fluorescent protein (pcDNA3-GFP) and visualizing the percentage of labeled cells by fluorescence microscopy. The cells were analyzed for gene expression, viability, and morphology 24, 48, or 72 h after transfection.

Viability assays

Viability was measured using the crystal violet assay [7] since crystal violet dye labels only live cells. The assays were performed with cells seeded into 96-well plates at a density of 2 × 10⁴ cells/well. The absorbances (540 nm) were measured using a Spectracount plate reader (Packard, Meriden, Conn.). The crystal violet absorbances increased linearly with cell density between 10⁴ and 5 × 10⁵ cells/well.

Protein expression

Western blot analysis, the microtiter immunocytochemical ELISA (MICE) assay, and immunocytochemical staining were used to detect and measure protein expression. For Western blot analysis, the cells were lysed in radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors [8]. Protein concentrations were determined using the BCA assay (Pierce, Rockford, Ill.). Samples containing 60 μg of protein were analyzed by Western immunoblotting as described previously [6, 9, 10]. The MICE assay is a rapid and sensitive method for quantifying immunoreactivity in 96-well microcultures and combines the advantages of the enzyme-linked immunosorbent assay with immunocytochemical staining to permit sensitive in-situ quantification of protein expression with values normalized to cell density [11]. Briefly, the cells were fixed overnight in Histochoice (Amresco, Solon, Ohio), permeabilized with 0.05% saponin in Tris-buffered saline (TBS; 50 mM Tris, pH 7.5, 0.9% NaCl), and blocked with Superblock-TBS (Pierce). The cells were then incubated overnight at 4°C with primary antibody diluted in TBS containing 0.05% Tween-20 and 0.5% bovine serum albumin (TBST-BSA). Immunoreactivity was detected using horseradish peroxidase conjugated secondary antibody (Pierce) and the TMB soluble peroxidase-substrate (Pierce). Absorbances were measured at 450 nm using a Spectracoat plate reader (Packard, Meriden, Conn.). Relative cell density was determined by subsequent staining the cells with 0.1% Coomassie blue dye, lysing the labeled cells with 1% SDS, and measuring the absorbances at 540 nm [11]. The MICE index was calculated from the ratio of the absorbances measured for immunoreactivity and cell density, multiplied by 100. Eight or 16 replicate culture wells were analyzed in each experiment. All experiments were repeated at least three times.

For immunocytochemical staining, adjacent culture wells were pretreated and incubated with primary antibody as described above for the MICE assay. Immunoreactivity was revealed with biotinylated secondary antibody and avidin-biotin horseradish peroxidase reagents (Vector Laboratories, Burlingame, Calif.), and with diaminobenzidine used as the chromogen [1]. All studies included negative controls in which the primary or secondary antibody was omitted.

Results

Effective gene transfer into rCBN cultures

Figure 1 demonstrates luciferase activity in rCBN cultures transfected with pcDNA3-Luc. Increased luciferase activity was detected 48 h after transfection, and at the 72-h time point, the levels were further increased. However, after 96 h, the levels of luciferase activity declined (data not shown), indicating that peak gene expression occurred 72 h after transfection. Western blot analysis using the N3I4 monoclonal antibody generated to recombinant AD7c-NTP protein [2] demonstrated increased levels of about 41-kDa AD7c-NTP protein in cells transfected with pcDNA3-AD7c relative to cells transfected with pcDNA3-Luc (fig. 2). Densitometric analysis of the autoradiographs showed that transfection with pcDNA3-AD7c resulted in three- to fivefold higher levels of AD7c-NTP protein relative to the pcDNA3-Luc control transfected cells at the 72-h time point (fig. 2). Using the MICE assay and the N3I4 or N2J1 monoclonal antibody to quantify AD7c-NTP immunoreactivity, substantially increased levels of AD7c-NTP expression were measured in rCBN cultures 48 and 72 h after transfection with pcDNA3-AD7c relative to control transfected cultures (figs. 3 A, B). However, at the 96-h time point, the levels of AD7c-NTP were relatively reduced (data not shown).