CMLS, Cell. Mol. Life Sci. 58 (2001) 844–849
1420-682X/01/060844-06 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

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, two prominent abnormalities associated with AD. These results provide further evidence that aberrantly increased AD7c-NTP expression may have a role in AD-type neurodegeneration. In addition, we demonstrate that primary post-mitotic neurons can be efficiently transfected with conventional recombinant plasmid DNA to evaluate the effects of gene over-expression in relevant in vitro models.

Key words. Neuronal thread protein; neurodegeneration; primary neuron culture; DNA transfection.

Introduction

AD7c-NTP is a member of a novel family of genes termed 'neuronal thread proteins' (NTPs). The ~1.4-kb AD7c-NTP cDNA was isolated from an Alzheimer's disease (AD) brain library and encodes a ~41-kDa protein [1]. Analysis of post-mortem brains revealed significantly increased levels of AD7c-NTP mRNA and protein (~41 kDa) expression in AD relative to age-matched control cases [1, 2]. Further studies of Down syndrome brains demonstrated that aberrantly increased AD7c-NTP expression is detectable in the second decade of life and prior to the accumulation of characteristic AD-type lesions, including senile plaques, amyloid-β, and neurofibrillary tangles [2, 3]. Therefore, 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.

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

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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 (Panvera, 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 2×10^4 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 10^4 and 5×10^5 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 immunosorbant 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 Spectracount plate reader (Packard, Meriden, Conn.). Relative cell density was determined by subsequently 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 the ~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. 3A, B). However, at the 96-h time point, the levels of AD7c-NTP were relatively reduced (data not shown),

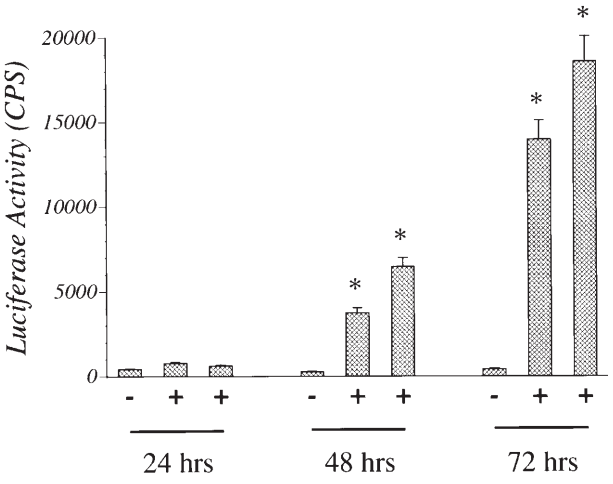

Figure 1. Detection of luciferase activity in rat cerebellar neuron cultures transfected with pcDNA3-Luc (+) or pcDNA3-AD7c (–) using the Mirus polyamine IT-100 reagent. The cultures were seeded into six-well plates and rendered post-mitotic by treatment with cytosine arabinoside. Five-day-old cultures were transfected, and luciferase activity was measured 24, 38, or 72 h later using the luciferase reporter assay. Values graphed represent the mean + SD of results obtained from four replicate culture wells per time point. Similar results were obtained using cells transfected with the Mirus polyamine LT-1 reagent (data not shown).

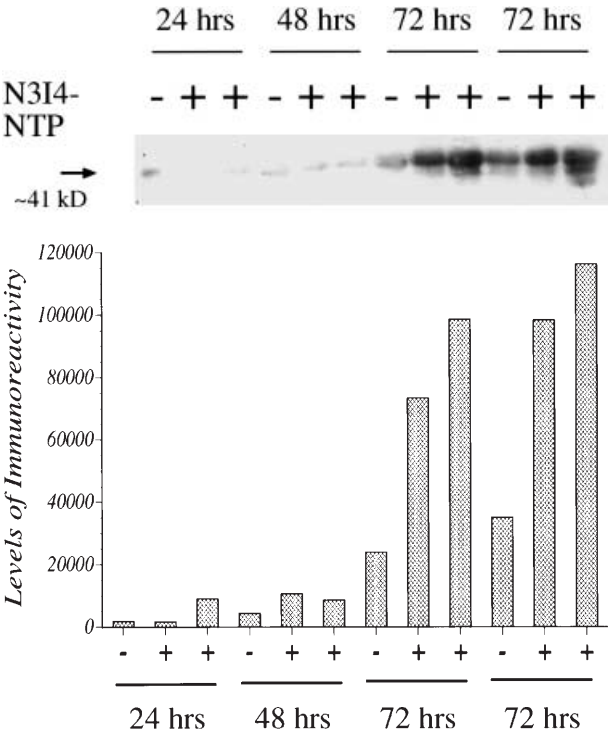

Figure 2. Detection of N3I4-immunoreactive AD7c-NTP expression in rat cerebellar neuron cultures transfected with pcDNA3-Luc (–) or pcDNA3-AD7c (+) using the Mirus polyamine IT-100 reagent. The cultures were seeded into six-well plates and rendered post-mitotic by treatment with cytosine arabinoside. Five-day-old cultures were transfected and AD7c-NTP expression was detected by Western blot analysis (above) and quantified by densitometry (below). The arrow indicates the position of the ~41 kDa N3I4-immunoreactive AD7c-NTP protein. Similar results were obtained in repeated experiments.

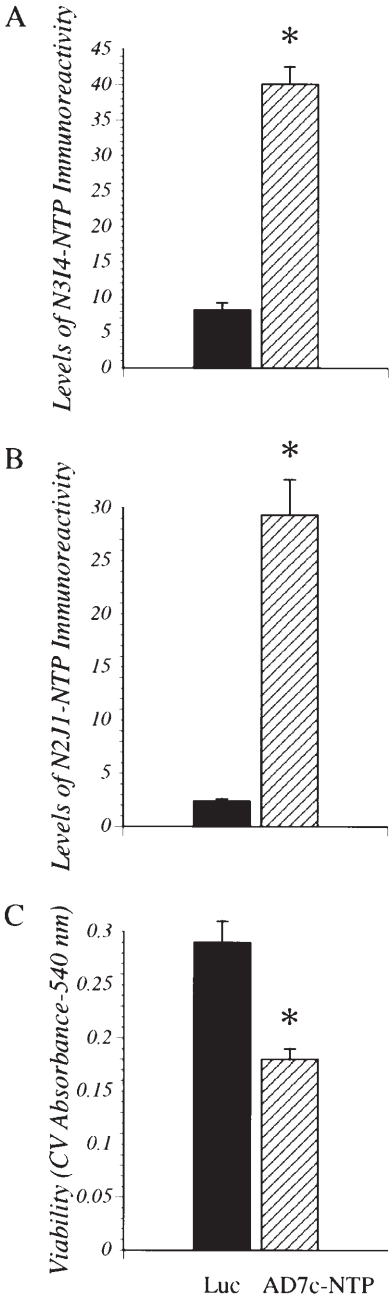

Figure 3. Increased AD7c-NTP expression associated with reduced viability of post-mitotic rat cerebellar neuron cultures demonstrated 72 h after transfection with the AD7c-NTP cDNA. (*A, B*) The graphed data reflect the mean + SD of results obtained from 16 replicate culture wells. Asterisks indicate significant differences from control as demonstrated by Student t-test analysis ($P < 0.001$). N3I4 and N2J1 are monoclonal antibodies generated against recombinant AD7c-NTP protein. AD7c-NTP expression was measured using the microtiter immunocytochemical ELISA (MICE) assay in which the levels of immunoreactivity were adjusted for differences in culture cell densities (see Materials and methods). (*C*) Viability was measured using the crystal violet assay.

indicating that peak levels of AD7c-NTP gene expression occurred 72 h after transfection, corresponding with results obtained in pcDNA3-Luc transfected cells. Similar results were obtained in at least four separate experiments.

Effects of AD7c-NTP over-expression on neuronal viability and morphology

Viability was measured using the crystal violet assay. Over-expression of AD7c-NTP cDNA resulted in significant neuronal cell loss relative to the control cultures. After 72 h, the cultures that had been transfected with pcDNA3-AD7c exhibited approximately 36% lower mean cell densities relative to pcDNA3-Luc-transfected control cultures (fig. 3C). Phase contrast microscopy demonstrated progressive depletion of the granule cell neurons in cultures transfected with pcDNA3-AD7c (figs. 4A, B). In addition, the remaining granule cell neurons in the pcDNA3-AD7c-transfected cultures exhibited prominent neuritic sprouting manifested by the presence of long thin interconnecting processes emanating from nearly all cells (fig. 4A), compared with the short processes associated with the control cells (fig. 4B). Immunocytochemical staining studies revealed abundant AD7c-NTP immunoreactivity 48 and 72 h after transfection with pcDNA3-AD7c, and relatively low levels of AD7c-NTP immunoreactivity in corresponding control (pcDNA3-LacZ or pcDNA3-Luc) transfected cultures (fig. 4C–F).

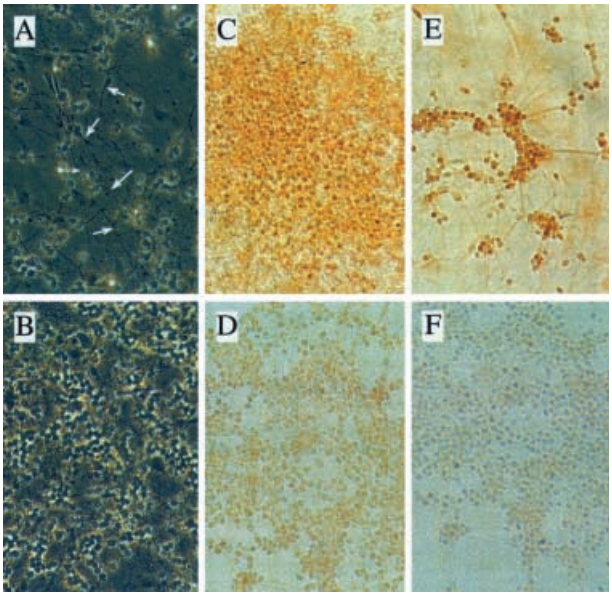

Figure 4. Reduced viability and increased N3I4-immunoreactive AD7c-NTP expression in post-mitotic rat cerebellar neuron cultures transfected with the AD7c-NTP cDNA (*A, C, E*) relative to LacZ control transfected cultures (*B, D, F*). (*A, B*) Phase contrast images of cells in culture, 72 h after transfection. Note the conspicuous loss of small granule cell neurons, and the prominence of long thin cell processes on most of the remaining neurons (arrows). (*C–F*) Transfected cultures were immunostained with the N3I4 monoclonal antibody to detect AD7c-NTP protein. Immunoreactivity was revealed by the avidin-biotin horseradish peroxidase complex method using diaminobenzidine as the chromogen (brown precipitate). Note the abundant AD7c-NTP immunoreactivity present in cultures that had been transfected 48 (*C*) or 72 (*E*) h earlier with the AD7c-NTP cDNA relative to cells that had been transfected 48 (*D*) or 72 (*F*) earlier with the LacZ gene.

Discussion

This study demonstrated that efficient gene transfer in post-mitotic neurons can be achieved using the Mirus polyamine transfection reagent, and that over-expression of the AD7c-NTP gene causes neuronal cell death and neuritic sprouting in post-mitotic neurons, as described previously in the PNET2 neuronal cell line [1, 12]. In the rCBN cultures, optimum gene expression was detected 72 h rather than 24 or 48 h after transfection, as generally occurs in cell lines that are transiently transfected with conventional reagents. Most likely, the delayed time course of optimum gene expression was related to the efficiency or mechanism of gene delivery effected by the polyamine carrier, since SH-Sy5y neuroblastoma cells transfected with pcDNA3-Luc or pcDNA3-AD7c using the Mirus LT-1 or IT-100 reagent exhibited the same time course of peak-level gene expression (data not shown). The advantage of using the Mirus polyamine transfection reagent is that studies can now be done with primary neuronal cultures rather than transformed cell lines, and without the need to employ viral vectors. Moreover, the effects of gene expression can be determined using conventional blotting assays rather than single-cell analyses since sufficiently high percentages (10–25%) of the cells will express the gene of interest. Finally, the use of primary neuronal cultures provides a more relevant model for studying the effects of aberrant gene expression in relation to neurodegeneration. Although cerebellar granule neurons are generally not targets of AD neurodegeneration, they were used in these studies because: (i) the culture model is well-established and provides a relatively uniform population of post-mitotic neurons and (ii) we have found that the adverse effects of AD7c-NTP over-expression on neuronal viability are similar for different subtypes of cultured neuronal cells. In this regard, our most recent studies have demonstrated that cultured fetal cortical neuron transfected with pcDNA3-AD7c using Mirus polyamine reagents also exhibit reduced viability and increased neuritic sprouting as reported here.

Cultures transfected with pcDNA3-AD7c exhibited increased levels of AD7c-NTP gene expression as demonstrated by Western blot analysis, the MICE assay, and immunocytochemical staining using monoclonal antibodies generated to the human recombinant protein [1, 2]. In contrast, cells transfected with non-relevant genes exhibi-

ted much lower levels of AD7c-NTP expression. However, as illustrated by the Western blot autoradiograph and densitometry results shown in figure 2, control cultures exhibited modest increases in the levels of AD7c-NTP protein 72 h after transfection and 8 days after seeding. Further studies demonstrated that transfection of rCBNs with another non-relevant cDNA that encodes human aspartyl-asparaginyl-β-hydroxylase (AAH) [13], in which gene expression was also under the control of a CMV promoter, was associated with increased levels of AAH and very low levels of AD7c-NTP, except after 72 h when modest elevations in AD7c-NTP immunoreactivity were detected by Western blot analysis (data not shown). However, it is noteworthy that the modest increases in AD7c-NTP expression observed in control cultures were not accompanied by neuronal cell loss relative to younger cultures or non-transfected cultures of the same age. Since NTP expression can increase with neurite outgrowth associated with differentiation or repair/regeneration following oxidative or ischemic injury [6, 14, 15], one possible interpretation of the findings in control cultures is that aging or maturation of the post-mitotic neuron cultures may itself cause modest elevations in the levels of AD7c-NTP expression.

Although high levels of AD7c-NTP protein were detected by the MICE assay or immunocytochemical staining 48 h after transfection with pcDNA3-AD7c, increased levels of AD7c-NTP protein were not detected by Western blot analysis until 72 h after transfection. This discrepancy was probably due to the higher sensitivities of the MICE assay and immunocytochemical staining compared with immunoblotting methods. Increased levels of luciferase or β-galactosidase activity were also detected at the 48-h time point, although peak activity levels also occurred 72 h after transfection. Both the luciferase and β-galactosidase assays are luminescence based and therefore highly sensitive relative to Western blot analysis.

The phase contrast microscopy studies demonstrated progressive depletion of granule cells, and prominent growth of long, thin interconnecting processes from the remaining viable neurons in the pcDNA3-AD7c-transfected cultures. Therefore, over-expression of AD7c-NTP in post-mitotic neurons causes both increased neuronal cell death and prominent neuritic sprouting. This same dual phenotype was observed in PNET2 neuronal cells that over-expressed AD7c-NTP following either transient transfection [1], or stable transfection with a vector system in which gene expression was regulated by an inducible promoter [12]. However, since PNET2 cells are immature, proliferative, and transformed, and AD neurodegeneration exclusively affects post-mitotic neurons, it was necessary to show that over-expression of the AD7c-NTP gene has the same effect in post-mitotic neurons.

The mechanism by which the dual phenotype occurs in AD7c NTP-transfected cultures is not known. One possible explanation is that subpopulations of neurons may exhibit different responses to AD7c-NTP over-expression. Alternatively, secreted AD7c-NTP protein may have either cytotoxic or growth-stimulatory effects on neighboring neurons. Analysis of the translated AD7c-NTP protein predicts the presence of a hydrophobic leader sequence, a myristoylation motif, and a potential cleavage site, suggesting that the protein can be secreted [1]. The finding of elevated levels of AD7c-NTP protein in cerebrospinal fluid and urine of patients with AD [1, 16–18] also supports the hypothesis that over-expression of the AD7c-NTP gene results in increased secretion or release of the protein by degenerating neurons. The higher degrees of cell loss (36%) relative to transfection efficiency (10–25%) may have been due to the adverse effects of secreted AD7c-NTP protein on neighboring cells. Although we have not been able to accurately measure AD7c-NTP protein secreted into the culture medium due to the very low concentrations of intact protein, recent preliminary studies suggest that recombinant AD7c-NTP protein can have cytotoxic effects on some cultured neurons. The mechanism by which over-expression of the AD7c-NTP gene causes neuronal death resembles the effects of oxidative injury [12], but is distinguished by the fact that the neuronal loss due to oxidative stress is accompanied by neurite retraction rather than sprouting [6, 10]. Future investigations will determine how over-expression of the AD7c-NTP gene mediates both cell death and increased neuritic growth in differentiated neurons.

Acknowledgements. This work was supported by grants AA-02666 and AA-10102 from the National Institute of Health.

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