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CILIARY NEUROTROPHIC FACTOR INDUCED-INCREASE IN ß-AMYLOID PRECURSOR PROTEIN mRNA IN RAT C6 GLIOMA CELLS

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SUMMARY The effect of ciliary neurotrophic factor (CNTF) on ß-amyloid precursor protein (APP) gene expression was investigated in cultured rat C6 glioma cells and human SH-SY5Y neuroblastoma cells. CNTF increased APP mRNA abundance in C6 glioma cells in a dose-dependent manner, with an approximately 3-fold increase in maximum observed after 24 h with a concentration of 1 ng/ml. However, no significant differences in the splicing pattern of the three major isoforms of APP mRNA were apparent between control and CNTF-treated C6 glioma cells. CNTF had no effect on APP mRNA abundance in SH-SY5Y neuroblastoma cells. These findings suggest that CNTF can modulate APP mRNA expression and might affect amyloidogenesis in Alzheimer's disease.

The ß/A4 protein, the major component of the amyloid plaques that characterize Alzheimer's disease (AD) (1), is derived from the amyloid precursor protein (APP). APP is expressed in a variety of tissues, but is most abundant in brain (2). A secreted form of APP has a trophic effect on cortical neurons (3) and enhances neurite outgrowth (4). Although APP is thought to play an important role in physiological or pathological conditions, the mechanisms responsible for the regulation of APP expression have not been fully clarified.

Ciliary neurotrophic factor (CNTF) has originally been identified as a factor that promotes the survival of ciliary ganglionic neurons (5), but was subsequently shown also to regulate the survival of cultured parasympathetic, sympathetic, sensory, spinal motor, and hippocampal neurons (6) and oligodendrocyte (7). In mice, CNTF immunoreactivity is broadly distributed

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Abbreviations: AD, Alzheimer's disease; APP, ß-amyloid precursor protein; CNTF, ciliary neurotrophic factor; bFGF, basic fibroblast growth factor; TGF-ß1, transforming growth factor-ß1; IL-1, interleukin-1; DMEM, Dulbecco's modified Eagle's medium; SSC, saline-sodium citrate; SDS, sodium dodecyl sulfate; GAPDH, glyceroaldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction, KPI; Kunitz-type serine protease inhibitor.

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throughout neurons and glial cells of the nervous system (8). With respect to cellular differentiation, CNTF has been shown to play a role in the conversion of oligodendrocyte-type 2 astrocyte (O-2A) progenitors to the type 2 astroglial phenotype in vitro (9,10). CNTF also induces the expression of choline acetyltransferase (ChAT) (11) and the transcription of neuropeptide genes (12) in vitro. Moreover, disruption of the CNTF gene by homologous recombination results in motor neuron degeneration in mice (13). Recent reports showed a null mutation in the human CNTF gene that abolishes the expression of CNTF protein (14).

Recently, neurotrophic factors and cytokines, including basic fibroblast growth factor (bFGF) (15), transforming growth factor-ß1 (TGF-ß1) (16) and interleukin-1 (IL-1) (17, 18), have been shown to increase APP mRNA levels in cultured cells. To study the factors that regulate APP gene expressions, we have now investigated the effect of CNTF on the expression of APP mRNA in cultured glioma and neuroblastoma cells.

MATERIALS AND METHODS

Materials.

C6 glioma cells were obtained from the American Type Culture Collection. Human SH-SY5Y neuroblastoma cells were kindly provided by Dr. Sano (Kobe University, Kobe, Japan). Rat recombinant CNTF was purified by high-pressure liquid chromatography from lysates of Escherichia coli transfected with an expression vector (19) containing the complete coding sequence of rat CNTF. Fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe, and [α-^{32}P] dCTP (3000 Ci/nmol) were obtained from Commonwealth Serum Laboratories (Melbourne, Australia), Gibco (Grand Island, NY), Clontech Laboratories (Palo Alto, CA), NEN Research Products, respectively.

Cell culture.

C6 glioma cells and SH-SY5Y neuroblastoma cells were plated at a density of 8 x 10^5 per 100-mm dish and maintained for 24 h at 37°C under a 5% CO_2/ 95% air atmosphere in DMEM supplemented with penicillin G (100 U/ml), streptomycin (100 μg/ml), 25 mM HEPES and 10% heat-inactivated fetal bovine serum.

CNTF treatment.

After cells achieved subconfluence, CNTF dissolved in DMEM was added to culture medium. Control cells were received the same volume of DMEM. At indicated times after the addition, the cells were lysed with a solution containing 4M guanidinium thiocyanate. The cell lysates were frozen at -80°C until use.

RNA isolation and RNA blot hybridization.

Total cellular RNA was isolated according to the acid guanidinium-phenol-chloroform method (20). Equivalent amounts of total cellular RNA (10 μg) were electrophoresed on a 1.2% agarose-formaldehyde gel transferred to a nylon membrane. No RNA degradation of ribosomal RNA was detected by visual inspection of ethidium bromide stained-ribosomal RNA bands under ultraviolet illumination. The APP-specific cDNA probe was the 2.3 kb-fragment generated by the digestion of APP cDNA with *Kpn* I (nucleotide 203, Kang sequence) (21) and *Spe* I (nucleotide 2304). The probes were labeled with an Oligolabelling kit (Pharmacia LKB Biotechnology) to specific radioactivities of the probes were 3 x 10^8 to 6 x 10^8 cpm per microgram of DNA. The membranes were hybridized to the ^{32}P-labeled APP cDNA probe and washed in 0.1 x SSC (1 x SSC: 150 mM NaCl and 15 mM sodium citrate), 0.1% SDS at 55°C for 15 min. After washing, the membranes were exposed to X-ray film at -80°C with intensifying screens. Equal loading of total RNA was checked by rehybridizing with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (22). Quantitation of total APP mRNA was performed by measuring radioactivity on Northern blots with an image analyzer (FUJI BAS 2000). Experiments for quantitation of mRNA levels were performed at least triplicate.

PCR amplification of mRNA.

Alternatively spliced APP mRNA was analyzed by quantitative reverse transcription polymerase chain reaction (RT-PCR). First-strand cDNA was synthesized with random hexamer

(TOYOBO, Kyoto, Japan) and murine leukemia virus reverse transcriptase (BRL) from 1 μg of total RNA. After heating the RNA and primer to 70°C and cooling on ice, the RT reaction mixture was incubated at 25°C for 15 min, 42°C for 45 min, and terminated by heating at 95°C for 5 min. For PCR, the RT reaction products were added to 25 pmol of both forward primer labeled at the 5' end with ^{32}P and reverse primer and 2.5 U of Taq polymerase (Perkin Elmer Cetus), in 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 200 μM dNTPs. The sequence of the forward primer was 5'-GGCGGATCCGGGGGTCTCCAGGTACTTGT-3', which corresponds to nucleotides between 825 to 853 of the rat APP cDNA sequence (23). The sequence of the reverse primer was 5'-GGCGAATTCTACCACTGAGTCTGTGGAGG-3', which corresponds to nucleotides 902 to 930 of rat APP cDNA. PCR was performed for 18 cycles of denaturation for 1 min. at 95°C, annealing for 1 min. at 60°C, and primer extension for 2 min. at 72°C. PCR products were electrophoresed on 8% polyacrylamide gels. The radioactivity of each band was determined by measuring with an image analyzer.

RESULTS

Effect of CNTF on cell proliferation

Under control conditions, the number of C6 glioma cells increased from 1.31 x 10^4 cells/cm^2 at plating to 5.46 x 10^4 cells/cm^2 after 24 h. Cell number did not differ significantly among C6 glioma cells incubated for 24 h in the absence or presence of various concentrations (0.1 pg/ml to 1 ng/ml) of CNTF (Table 1). Similarly, CNTF had no significant effect on the number of SH-SY5Y neuroblastoma cells after 24 h. Furthermore, treatment of C6 glioma cells and SH-SY5Y neuroblastoma cells with CNTF had no obvious effect on cell morphology.

Time course of effect of CNTF on APP mRNA expressions

We examined whether CNTF brought about the change in APP mRNA levels. CNTF (1 ng/ml) increased the APP mRNA abundance in C6 glioma cells in a time-dependent manner. The increase was maximal (~ 2.6-fold) after 24 h (Fig. 1A and 1B). CNTF had no effect on APP mRNA abundance in SH-SY5Y neuroblastoma cells (Fig. 1B).

Table 1. Effect of CNTF on C6 glioma cell proliferation

CNTF (ng/ml)	cell number (x10^4 cells/cm^2)
0	5.46 ± 0.29
1x10^{-4}	4.78 ± 0.20
1x10^{-3}	4.81 ± 0.10
1x10^{-2}	4.86 ± 0.14
1x10^{-1}	4.70 ± 0.16
1	4.83 ± 0.36

C6 glioma cells were cultured with various concentrations of CNTF. After 24 hour, cell number was counted. Values are expressed as mean ± S.E.M. Experiments were done in triplicate.

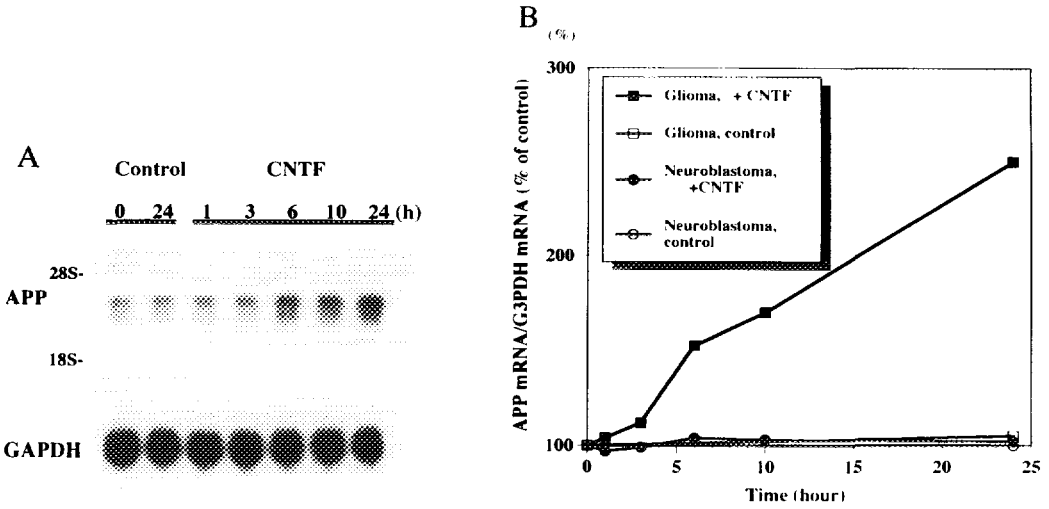

Figure 1. Time course of the effect of CNTF on APP mRNA abundance. Cells were incubated in the absence or presence of CNTF (1ng/ml) and harvested at the indicated time points. (A) Northern blot analysis in rat C6 glioma cells ; 10 μg of total RNA were loaded in each lane. (B) Quantitive analysis. APP mRNA and GAPDH mRNA abundance was quantified with an image analyzer and is expressed as a percentage of value at time 0.

Dose response of effect of CNTF on APP mRNA expressions

We next examined the level of APP mRNA after treatment with various concentrations of CNTF (0.1 pg/ml-1 ng/ml) to ascertain the concentration of CNTF necessary to elicit a change in APP mRNA transcripts in C6 glioma cells. The effect of CNTF on APP mRNA abundance was apparent at concentrations as low as 1pg/ml and was maximal at 1 ng/ml. These results indicate that APP mRNA expressions are increased in a dose-dependent manner (Fig. 2).

Analysis of alternatively splicing pattern of APP mRNA

We used RT-PCR to examine whether the CNTF-induced increase in APP mRNA was accompanied with a change in the relative proportion of transcripts encoding the three major isoforms of APP (APP695, APP751, APP770). The amplification efficiencies of the three transcripts isoforms remained constant up to 24 PCR cycles. Furthermore, we verified that the amounts of the three APP transcripts increased linealy with the amount of input RNA within the range of 0.05 to 3 μg (data not shown). On the basis of these results, we performed RT-PCR for 18 cycles to amplify the product from 0.1 μg of input RNA. As expected, three major bands were detected with the sizes of 105 bp (APP695), 273 bp (APP751), and 330 bp (APP770). The proportions (mean ± S.E.M.) of APP695, APP751, and APP770 mRNAs in control C6 glioma cells to CNTF were 14.5 ± 0.8%, 31.3 ± 0.5% and 55.7 ± 1.1% (n = 3), respectively. After exposure of C6 glioma cells to CNTF (1 ng/ml) for 24h, these values were 15.3 ± 0.7%, 33.6 ±

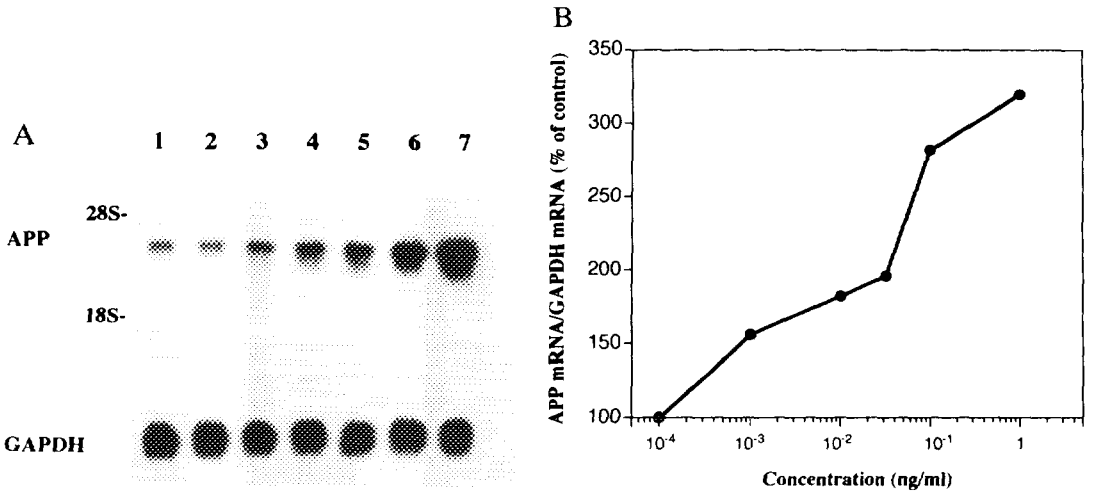

Figure 2. Dose-response relation for the effect of CNTF on APP mRNA abundance in rat C6 glioma cells. Cells were incubated with various concentrations of CNTF for 24 h. (A) Northern blot analysis; 10 μg of total RNA were loaded in each lane. Lane 1, no addition (control) ; lane 2, 0.1 pg/ml; lane 3, 1 pg/ml; lane 4, 10 pg/ml; lane 5, 33 pg/ml; lane 6, 100 pg/ml; lane 7, 1 ng/ml. (B) Quantitative analysis of the data shown in (A). APP and GAPDH mRNA abundance was quantified with an image analyzer and is as expressed as a percentage of control.

0.8%, and 50.8 ± 0.5% (n = 3), respectively. Thus, the splicing pattern of the three major isoforms of APP mRNA did not deffer significantly between control and CNTF-treated cells (Fig. 3).

DISCUSSION

We have shown that CNTF increased the APP mRNA levels in rat C6 glioma cells in a dose-dependent (1pg/ml to 1ng/ml) manner. The survival effect of CNTF was shown to be elicited with the median effective concentration (EC_{50}) of 23 pg/ml in embryonic chick motoneurons (24) and of 1 ng/ml in oligodendrocyte (7). Thus, the concentration of CNTF required for APP mRNA induction is within the same range as that required for promotion of the survival of neurons and glial cells. However, we did not detect an effect of CNTF on APP mRNA in SH-SY5Y neuroblastoma cells, even though these cells possess CNTF receptors (25). These findings suggest that the actions of CNTF might differ in intracellular signal transduction between glioma and neuroblastoma cell lines.

The splicing patterns of the KPI insert domain of APP mRNA were not affected by CNTF. Thus, CNTF increased the three major isoforms of APP mRNA in proportion to their initial abundance. TGF-ß also increased the APP transcript isoforms without affecting their relative abundance (16). In contrast, bFGF increases the amount of KPI-containing APP transcript

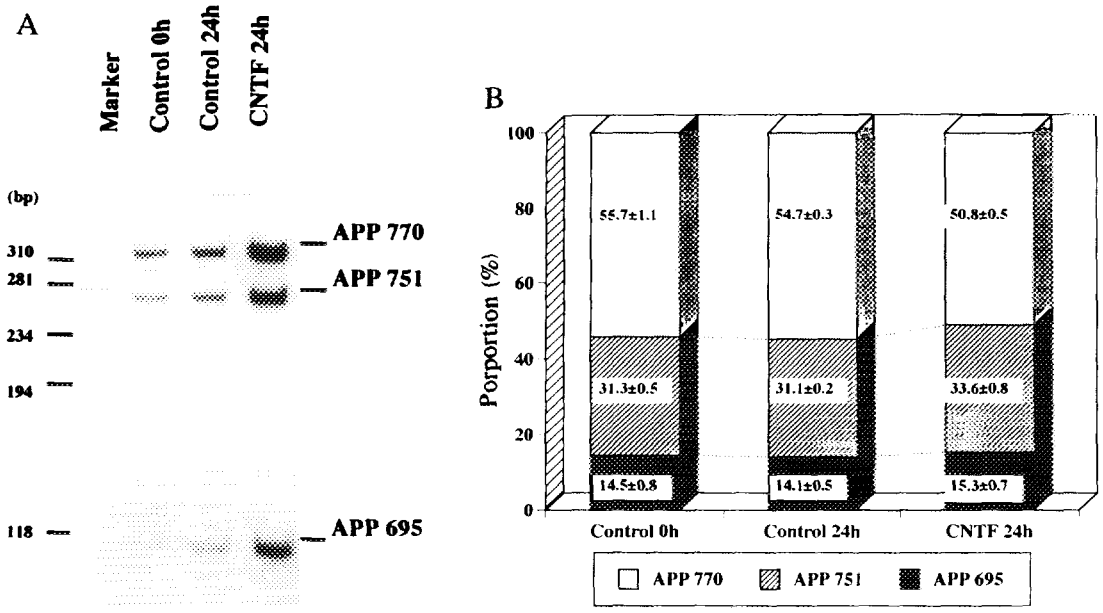

Figure 3. RT-PCR analysis of the three major APP mRNA isoforms in C6 glioma cells. Equal amounts of total RNA were reverse-transcribed and amplified with rat APP-specific primers. (A) ^{32}P-labelled PCR products were separated by electrophoresis on polyacrylamide gels and visualized by autoradiography.
(B) APP transcripts were quantified with an image analyzer and the amount of each isoform (APP 695, APP 751, APP 770) Autoradiographic bands were quantified by image analyzer and the amounts of each APP isoform (695, 751, 770) was calculated as a percentage of total APP mRNA. Values are means ± S.E.M. of three experiments.

isoforms more than it increases APP695 mRNA (26). Thus, the difference in the effects of neurotrophic factors on the splicing pattern of APP mRNA might be associated with variation in the effect of these factors on cell functions.

In the AD brain, the mechanism leading to ß/A4 amyloid deposition is yet unknown. Injury to the brain has been found to trigger ß/A4 deposition (27) and APP immunoreactivity was increased in the perikarya of neurons in the brain after head injury (28). In addition, a number of cytokines and growth factors play crucial roles in the processes associated with brain injury. The increase of IL-1 is correlated with the increase of APP in the early reactive phase following head injury in humans (29). Similarly, CNTF mRNA is dramatically up-regulated after aspiration induced injury in rat cortex and hippocampus (30) and CNTF can act as a survival factor for preventing the lesion-induced degeneration in neurons (31). Together with our results, these findings suggest that increased levels of CNTF in pathological conditions such as brain injury can increase APP gene expression. APP is cleaved within the ß/A4 protein sequence and secreted into the extracellular matrix (32). The secreted form of APP promotes neuronal survival in vitro (3). Therefore, elevated levels of APP by CNTF may play an important role in the protection of

injured neurons. On the other hand, 4 kDa full-length Aß peptide, which can produce an amyloidogenic source is secreted as part of normal APP metabolism (33, 34). Although the generation of Aß peptide from APP is not well understood, elevated production of APP might result in an increase in production of the Aß peptide. Thus, elevated expression of APP by CNTF might lead to increased ß-amyloid deposition. Additional studies will be needed to determine whether CNTF is implicate in the pathogenesis in AD.

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