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Transcriptional Inhibition of the Beta-Amyloid Precursor Protein by Interferon-Gamma

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Attenuating beta-amyloid precursor protein (β-APP) gene expression may have relevance in diseases such as Alzheimer's disease, where β-APP has been implicated in neuropathological processes. We report here on the transcriptional down-regulation of β-APP by interferon-gamma (IFN-γ) in SKNMC human neuroblastoma cells. Treatment of the cells with IFN-γ resulted in a 85% dose-dependent inhibition of β-APP promoter activity after 24 h of exposure, with no changes observed at 5 h. For comparison, additional cytokines and signaling agents were also investigated for effects on β-APP promoter activity. Elevated levels of activity were observed after treatment with phorbol 12-myristate 13-acetate and basic fibroblast growth factor whereas no significant effects were seen after treatment with lipopolysaccharide or interleukin-1 beta. Thus, IFN-γ was shown here to be a suppressor of β-APP promoter activity and is the first cytokine reported to possess such down-regulating effects.

β-APP is a ubiquitously expressed glycoprotein whose proteolytic fragments have been implicated in the neuropathology of Alzheimer's disease (for review, see 1, 2). The protein consists of a small cytoplasmic domain, a single transmembrane-spanning region, and a large extracellular domain. Proteolytic processing of the protein releases bioactive polypeptides into the extracellular space. Peptides that consist of all or part of an internal 43 amino acid sequence, referred to as beta amyloid, have been shown to be neurotoxic (3), to induce proinflammatory cytokines (4), to destabilize neuronal calcium levels (5), and to increase neuronal vulnerability to excitatory amino acid neurotoxicity (5, 6).

The gene encoding β-APP is located on chromosome 21 (7, 8) and, once transcribed, gives rise to a variety of alternatively spliced mRNA's (9-11) leading to the observed heterogeneity of expressed protein isoforms. The promoter for the human APP has been cloned (12) and contains DNA consensus sequences for binding transcription factors such as USF, AP-1, AP-2, and NF-*k*b. In addition, the promoter has been shown to be up-regulated by a variety of effectors including: PMA; retinoic acid; IL-1β; nerve growth factor; and bFGF (13). We describe here the specific down-regulation of the β-APP promoter by IFN-γ in SKNMC human neuroblastoma cells stably expressing a luciferase reporter plasmid containing 3.8 kb of genomic DNA most proximal to the human β-APP gene.

MATERIALS AND METHODS

Materials. Human IFN-γ, IL-1β, and bFGF were purchased from Biosource International (Camarillo, CA, USA). The 12-well and 96-well Falcon culture plates were from Becton Dickinson & Co. (Lincoln Park, NJ, USA). LPS,

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Abbreviations: β-APP, beta amyloid precursor protein; IFN-γ, interferon-gamma; PMA, phorbol 12-myristate 13-acetate; IL-1β, interleukin-1β; bFGF, basic fibroblast growth factor; LPS, lipopolysaccharide.

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PMA, luciferin and all other unspecified chemicals used in the luciferase assay were from Sigma (St. Louis, Mo., USA). Geneticin and DMEM medium components used in the initial generation and maintenance of cells stably expressing the luciferase reporter plasmid were from GIBCO-BRL (Grand Island, NY, USA). Subsequent cell culturing for the studies presented here were in medium components from Mediatech, Inc. (Washington DC, USA). The CellTiter 96 AQueous proliferation assay measuring mitochondrial activity and the CytoTox 96 assay kit measuring lactate dehydrogenase activity were from Promega (Madison, WI, USA). The BioRad D_c protein assay kit was from BioRad Laboratories (Hercules, CA, USA).

Construction of the β-APP promoter-luciferase reporter plasmid. The human genomic DNA immediately upstream to the β-APP gene was isolated by oligonucleotide hybridization screening of a P1 phagemid genomic library from human placenta (Genome Systems, Inc., St. Louis, Missouri, USA). Based on information from the published sequence of the promoter (12), a *Bam*HI 3799 bp restriction endonuclease fragment, from position −3699 to position +100, was subcloned into a luciferase reporter vector at a *Bam*HI restriction site immediately proximal to a promoterless *Photinus pyralis* luciferase gene, which is flanked by the Simian virus 40 polyadenylation signal. The resulting β-APP promoter-luciferase plasmid is referred to as pAPP. The vector carries a HSV thymidine kinase promoter-driven neomycin resistance gene as a eukaryotic selection marker for stable subclone generation of transfected cells. Restriction map analysis and DNA sequencing of the promoter was performed to verify DNA sequence identity.

Cell culture and selection of stably transfected cells. All cells were grown at 37°C in a 5% CO_2 humidified incubator. SKNMC cells used for transfection were from American Type Culture Collection (Rockville, MD, USA) and maintained in DMEM medium (DMEM containing 10% fetal bovine serum, DMEM nonessential amino acids, 20 mM HEPES, 50 U/ml each of penicillin and streptomycin). Cells were harvested by trypsinization, centrifuged at 1000 × *g* for 5 min, washed once in PBS and centrifuged again, followed by resuspension in 200 μl PBS containing 10 μg of *Xmn*I restriction cut linearized plasmid. Cells were electroporated at a setting of 220 V and 800 μFd, and seeded into a 75 cm^2 flask with 10 ml culture medium. After 24 h, the culture medium was replaced with selection medium (culture medium plus 0.5 mg/ml geneticin) and grown one week to select for stable plasmid incorporation. A clonal cell line (referred to as Sma7) generated by dilution subcloning that expresses luciferase activity from a single plasmid copy as assessed by Southern analysis was used in the present studies.

Cell culture and extract preparations. All subsequent studies of the Sma7 cells were obtained from cells grown in DMEM/F12 culture medium (DMEM/F12 containing 10% fetal bovine serum, 15 mM HEPES, pH 7.4, 4 mM glutamine, 50 U/ml each of penicillin and streptomycin, 0.25 mg/ml geneticin). Cells were seeded at 2.5×10^4 cells/well/100 μl culture medium in 96-well Falcon culture plates for mitochondrial and lactate dehydrogenase assays and at 5×10^5 cells/well/ml culture medium in 12-well Falcon plates for luciferase and protein content assays. After 24 h, the medium was replaced with the same volume of fresh culture medium in the absence or presence of LPS, PMA or cytokines and incubated for the indicated times.

Cell extracts for luciferase and protein assays were prepared by aspirating off the culture medium and washing the cells two times at room temperature for 1 min with 1 ml of phosphate buffered saline, pH 7.4. After the last wash, the plates were put on a bed of ice and the cells lysed by the addition of 300 μl of lysis buffer (1% triton X-100, 100 mM Tris-Cl, pH 7.9, 20 mM magnesium acetate, 2 mM EGTA, 10 μg/ml leupeptin, 25 μM AEBSF), freeze-thawing the plates once, and transferring the contents to a microfuge tube for a further 1 h incubation on ice. Insoluble debris was removed by centrifugation for 1 min at 12,000 × *g*. Aliquots of the supernatent were assayed for either total protein content or luciferase activity.

Luciferase activity measurements. Luciferase assays were carried out by adding 50 μl of cell extracts to individual wells of a white, 96-well microlite plate from Dynatech Laboratories (Chantilly, VA) followed by rapid addition of 50 μl of luciferase reaction mix (100 mM Tris-acetate, pH 7.9, 20 mM magnesium acetate, 2 mM EGTA, 2 mM ATP, 2 mM mercaptoethanol, 600 μM luciferin) to all the samples and reading on a Dynatech ML1000 luminometer. All values were normalized to total protein content for comparison purposes. Linearity of the reaction was verified by serial dilutions of purified luciferase from R&D Systems (Minneapolis, MN).

Cell viability. Cytotoxicity and relative cell number values were obtained by measuring the amount of lactate dehydrogenase activity in 50 μl of culture medium or cell lysate, respectively, using the CytoTox 96 assay kit according to the manufacturer's suggestions. Cell number quantitation was also assessed by measuring mitochondrial activity in the cell cultures with the CellTiter 96 AQueous proliferation kit according to the manufacturer's suggestions.

RESULTS

Regulation of β-APP promoter activity by LPS, IL-1β, PMA, and bFGF. Generation of a human SKNMC neuroblastoma cell line (Sma7) that stably expresses a single copy of the β-APP promoter plasmid was described above. The β-APP luciferase reporter plasmid used to generate this cell line is depicted in Figure 1. Treatment of the cells with PMA and bFGF resulted in increases of 130% and 116% at 5 h, respectively, and 170% and 169% at 24 h,

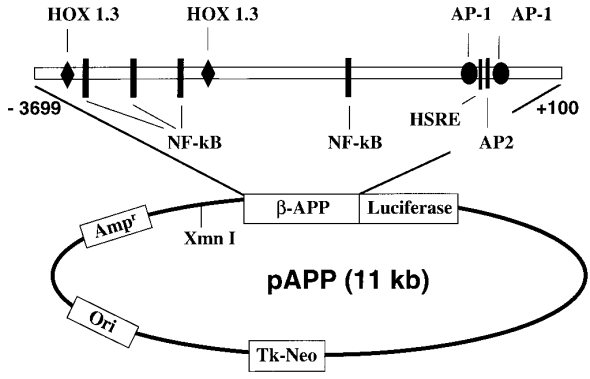

FIG. 1. The pAPP reporter gene plasmid construct. A 3.8 kb genomic DNA fragment flanked by Bam HI restriction sites was cloned into the luciferase reporter-gene plasmid. The cloned β-APP promoter contains 100 bp of the 5′ untranslated region of β-APP and 3.7 kb of DNA proximal to the transcription start site. The indicated transcription factor elements (HOX 1.3, NF-*k*B, AP-1, AP-2, HSRE) were deduced from the published promoter sequence (12). Abbreviations used are: Amp^r, ampicillin resistance gene; Tk, thymidine kinase promoter; neo, neomycin resistance gene; ori, origin of replication from the pBR322 plasmid.

respectively (Fig. 2). Treatment with LPS and IL-1β did not significantly differ from control cells at 5 or 24 h.

Inhibition of β-APP transcription by IFN-γ. Treatment of the Sma7 cells with IFN-γ resulted in a dose-dependent decrease in β-APP promoter activity with a maximal inhibition of 85%

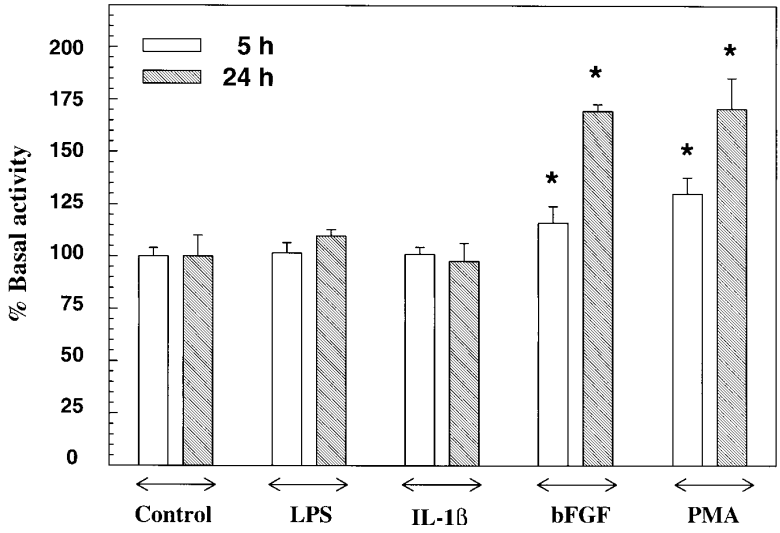

FIG. 2. Characterization of β-APP promoter activity in response to LPS, PMA and cytokines. Cells were seeded into 12-well plates and incubated for 24 h as described in Materials and Methods. Medium was then replaced with 1 ml of fresh medium containing LPS (1 μg/ml), IL-1β (1 nM), PMA (5 nM), or bFGF (100 pM) and the cells incubated an additional 5 or 24 h before assaying for protein content and luciferase activity. The data depicted is from a single representative experiment performed three independent times. Sample calculations were performed by normalizing measured light emissions to total protein content and the values expressed as the mean of quadruplicate values $\pm$ SD. Statistical significance was based on one way repeated measure ANOVA followed by Dunnett's test. *$p<0.05$ vs untreated control cells.

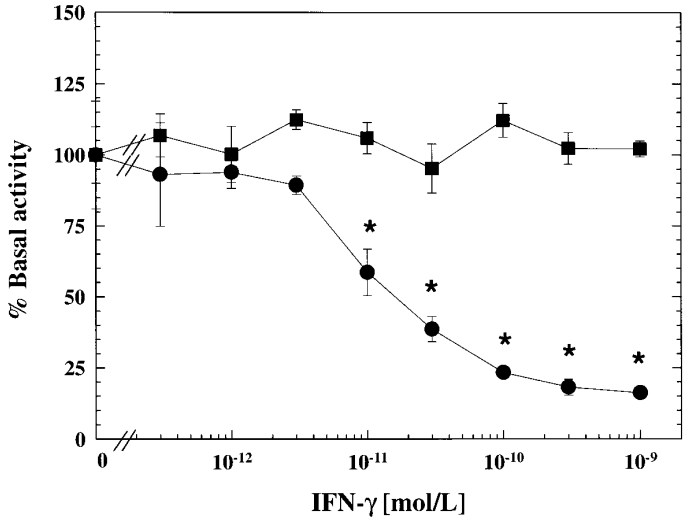

FIG. 3. Time course and dose-response of IFN-γ-induced suppression of β-APP promoter activity. Sma7 cells were seeded into 12-well plates at 5×10^5 cells/well in 1 ml of medium and incubated for 24 h. Medium was then replaced with 1 ml of fresh medium containing the indicated concentrations of IFN-γ. Luciferase activity was assessed after 5 h (■) and 24 h (●) of incubation. The data are from a single representative experiment performed on three different occasions and are presented as the mean of quadruplicate values +/− SD of the light emission values normalized to total protein content. Statistical significance was based on one way repeated measure ANOVA followed by Dunnett's test. *$p<0.05$ vs untreated control cells.

and an ED_{50} of 10 pM observed at 24 h (Fig. 3). No decline at 5 h was evident. No significant change in cell number was evident at 5 h or 24 h as assessed by measuring intracellular mitochondrial activity, lactate dehydrogenase activity, or total cellular protein nor was there evidence of cytotoxicity as measured by cellular lactate dehydrogenase activity released into the medium (data not shown).

DISCUSSION

We report in this study that β-APP transcription in SKNMC human neuroblastoma cells is up-regulated by PMA and bFGF (Fig. 2) and is down-regulated by IFN-γ (Fig. 3). The observation that IFN-γ lowers basal promoter activity of β-APP is significant in that it indicates the existence of a dynamic balance in cells between positive and negative regulators of β-APP transcription. To date, only cytokines that induce β-APP transcription have been reported. Our observations that PMA and bFGF induced transcription in SKNMC cells are similar to previous results obtained from PC12 cells (13). In contrast to results from that same study on PC12 cells, however, we did not observe β-APP promoter induction by IL-1β.

Evidence that overexpression of the β-APP gene may be one factor among several contributing to the progression of the neuropathology observed in Down syndrome and Alzheimer's disease can be found from several sources. In Down syndrome, where the chromosome 21 trisomy condition provides an extra copy of the β-APP gene, an increase in β-APP mRNA occurs that may account for the development of beta amyloid plaques in the brains of these patients by the age of 40 (14). In Alzheimer's disease, elevations of β-APP mRNA have been reported to occur in specific localized regions of the brain (15-17). In addition, one particular form of familial Alzheimer's disease linked to chromosome 14 has been shown to be associated with an increase in β-APP transcription (18). Lastly, overexpression of β-APP in transgenic

mice has been shown to exhibit some of the features observed in Alzheimer's disease including beta amyloid deposits and synaptic loss (19).

β-APP mRNA expression has been shown to be regulated both at the level of transcription and degradation. For example, specific stimuli such as PMA, IL-1β, and bFGF, have been shown to enhance β-APP promoter activity (13, 20). Interestingly, IL-1β and bFGF are also elevated in the brains of Alzheimer's disease patients (21, 22), suggesting a possible connection between these two cytokines and β-APP expression. Degradation of β-APP mRNA also appears to be a factor in determining net expression levels and is regulated by a sequence of 29 nucleotides in the 3′ untranslated region of the transcript (23). What effects IFN-γ has on β-APP transcript stability is unknown, and future studies will address this as well as possible protein processing effects.

Physiological processes down-regulating β-APP transcription have not been previously described, but may be an additional mechanism by which cells regulate β-APP mRNA levels. The identification of IFN-γ as a factor that reduces β-APP promoter activity raises the question as to whether there are additional factors negatively regulating β-APP transcription. The involvement of such factors in controlling β-APP expression may be of critical importance in determining temporal and spatial elevations of β-APP during stress, injury, or disease. Further characterization of the mechanism(s) by which IFN-γ achieves its effects should lead to a better understanding of β-APP gene regulation and thus, to our understanding of diseases involving beta amyloid.

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