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Research report

The gas7 protein potentiates NGF-mediated differentiation of PC12 cells

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Abstract

The growth-arrest-specific protein gas7 is required for morphological differentiation of cultured mouse cerebellar neurons and PC12 cells. Moreover, its overexpression in various cell types induces neurite-like outgrowth. The role of gas7 in neuronal differentiation was further characterized by adenovirus-mediated overexpression in PC12 cells and quantification of the expression of various neuronal markers, in the absence and presence of different concentrations of nerve growth factor (NGF). The potential neuroprotective activity of gas7 against various neurotoxic insults was also assessed. In addition to promoting the formation of neurite-like extensions, overexpression of gas7 potentiated NGF-mediated neuronal differentiation of PC12 cells, as shown by the enhanced expression of the neuronal proteins βIII-tubulin, synaptotagmin, α7 subunit of the acetylcholine receptor, and dihydropyrimidinase related protein-3. This effect was exerted independently of cell cycle progression, as gas7 did not affect proliferation of PC12 cells. While some differentiation enhancers protect PC12 cells against lethal insults, gas7 overexpression in PC12 cells did not protect against oxygen–glucose deprivation, the calcium ionophore A23187, or the nitric oxide donor sodium nitroprusside, suggesting that gas7 is not neuroprotective. The ability of gas7 to potentiate neuronal differentiation makes it a potential therapeutic target to promote re-establishment of neuronal connections in the injured or diseased brain, such as following stroke.

Theme: Development and regeneration
Topic: Cell differentiation and migration

Keywords: gas7; Growth-arrest-specific protein; Neuronal differentiation; Nerve growth factor; Cell cycle; PC12 cells

1. Introduction

The mature nervous system coordinates the functions of an organism through a complex arrangement of neurons, which transport information to the various organ systems. Numerous neurodegenerative conditions such as stroke, Alzheimer's, and Parkinson's are accompanied by damaged neuronal circuitry and often result in permanent disabilities. Although the regenerative capacities of the brain are far from understood, recent data raise the possibility that amplification of brain intrinsic repair mechanisms including neuronal plasticity and de novo neuronal differentiation might be of therapeutic value [1,21,29,34]. The creation of alternate communication pathways may be an important part of the brain recovery process [1,21,34]. The roles of various molecules in proper organization of the nervous system, such as neurotrophins, Rho GTPases, and the actin cytoskeleton, and their potential clinical application against neurodegeneration are being explored, but there are still

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many unanswered questions [14,20]. Improved comprehension of the pathways and mechanisms involved in neuronal development is thus of major importance for brain repair.

The growth-arrest-specific (gas) protein gas7 was first identified as being preferentially expressed in growth-arrested NIH3T3 fibroblasts [6,16]. Messenger RNAs of the gas genes accumulate when cells reach density inhibition or are induced to exit the cell cycle [8,26]. The expression of gas7 in mouse primary tissues is enriched in the brain, selectively in neuronal cells [16]. Antisense-mediated downregulation of gas7 in maturing mouse Purkinje cultures was shown to prevent the formation of neurites and transfection of mouse Neuro 2A and rat pheochromocytoma (PC12) cells with gas7-expressing plasmids induced the extension of neurite-like processes [7,16]. gas7 is thus believed to be involved in neurite outgrowth. Moreover, gas7 was suggested to be essential for nerve growth factor (NGF)-mediated differentiation of PC12 cells [7]. Treatment of PC12 cells with NGF rapidly and transiently increased the expression of a 60-kDa gas7 isoform and antisense-inhibited expression of this protein prevented NGF-induced differentiation of the cells [7]. Better understanding the functions of the gas7 protein in neuronal differentiation might be valuable in the treatment of neurodegenerative conditions. The gas7 gene itself may be a direct therapeutic target or alternatively studying the actions of this gene may lead to a better comprehension of neurodegeneration and repair mechanisms.

As was shown for gas7, NGF also transiently induces the expression of the antiproliferative protein PC3, in PC12 cells [4]. PC3 is thought to be involved in neuronal differentiation through its inhibitory effect on cell cycle progression [4,9]. Overexpression of PC3 in PC12 cells, by transient transfection, potentiated the differentiation elicited by NGF, as was shown by the enhanced expression of the neuronal markers tyrosine hydroxylase and neurofilament 160 kDa [9]. Expression of PC3 also protected PC12 cells from apoptosis triggered by NGF deprivation [9]. The similar kinetics of expression of gas7 and PC3, following treatment of PC12 cells with NGF, suggest that gas7 could be endowed with roles comparable to those of PC3.

The goal of this study was to further characterize the effects of gas7 expression on neuronal differentiation and to determine its protective effects against neurotoxic insults in PC12 cells.

2. Materials and methods

2.1. Cell culture

PC12 cells were grown on poly-L-lysine-coated dishes, in DMEM supplemented with 10% horse serum (HS) and 5% fetal bovine serum (FBS). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. For neuronal differentiation, DMEM was supplemented with 2% HS, 1% FBS, and 50 ng/mL of nerve growth factor (NGF; Alomone Laboratories, Israel). The sub-optimal differentiation media was made of DMEM, 2% HS, 1% FBS, and 10 ng/mL NGF.

2.2. Construction and production of replication-defective recombinant gas7 adenovirus

A sequence linker for additional cloning sites (*Hin*dIII-*Eco*RV-*Eco*RI-*Pst*I-*Bam*HI-*Xba*I-*Not*I) was first inserted into the adenovirus transfer vector pQBI-AdCMV5-GFP, containing two cytomegalovirus (CMV) IE-driven transcription units (Q BIOgene, Carlsbad, CA, USA). The murine full-length gas7 cDNA (accession number U19860) was obtained by double *Hin*dIII/*Not*I digestion of a previously cloned plasmid (pcDNA3gas7) [16] and subcloned into the *Hin*dIII/*Not*I sites of the modified pQBI-AdCMV5-GFP vector. The pQBI-AdCMV5-GFP/gas7 construct was linearized with FseI and cotransfected into HEK293 cells, together with adenoviral large-arm fragment DNA (Q BIOgene), using the Lipofectamine 2000 reagent (Invitrogen, Bethesda, MD, USA) according to the manufacturer's instructions. Six hours after transfection, cells were overlaid with agarose for viral plaque formation. After 7 to 10 days, viral plaques expressing GFP were picked under a fluorescent microscope and further amplified in HEK293 cells. The expression of the gas7 transgene was confirmed by PCR and Western blot analysis. The recombinant adenoviruses were purified by cesium chloride-gradient centrifugation and titrated by a plaque assay, as previously described [15].

2.3. gas7 overexpression in PC12 cells

PC12 cells were plated at 10^5 cells/cm^2 and allowed to adhere to culture dishes for 24 h before infection. For experiments with differentiated PC12 cells, cells were first incubated for 5 days in differentiation media and replated 24 h before infection, at the same density as the undifferentiated cells. For all of the following steps, differentiated cells were kept in differentiation media, whereas growth media was used for undifferentiated cells. The gas7 adenovirus was added at a multiplicity of infection (MOI) of 1 plaque-forming unit (pfu) per cell, and the media was changed 16 h later to remove extra viral particles. To assess non-specific effects resulting from the infection process itself, a similar adenovirus construct without the gas7 cDNA sequence (referred to as the GFP adenovirus) was also used, at 3 MOIs. The conditions of infection resulted in 37.4 ± 1.6% and 39.9 ± 1.7% of infected cells with the GFP and gas7 adenoviruses, respectively. With higher MOIs, cells tended to detach from dishes 3 to 4 days after infection with either adenovirus (data not shown).