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VALPROIC ACID INDUCES POLARIZATION, NEURONAL-LIKE DIFFERENTIATION OF A SUBPOPULATION OF C6 GLIOMA CELLS AND SELECTIVELY REGULATES TRANSGENE EXPRESSION

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Abstract—Glioblastoma is the most frequent primary brain tumor, and for which standard therapies have not significantly increased the survival of patients. Recently, chromatin alterations have been linked to the pathogenesis of cancer, and drugs that modify chromatin structure, such as inhibitors of histone deacetylases (iHDAC), are now considered as a valuable strategy for the treatment of cancer. For instance, valproic acid (VPA), an iHDAC originally used for the treatment of bipolar disorders and epilepsy, is now being used in cancer therapy. In this work we show that VPA induces morphological changes in murine astrocytoma C6 cells, which are associated with inhibition of cell proliferation, growth arrest, decreased cell migration, cell death and histone 4 hyperacetylation. VPA-treated cells extended processes with characteristics similar to the structure of a growth cone, and we also observed both a down-regulation of glial protein markers and increased expression of a neuronal specific protein after VPA treatment. Finally, there is an increase in the expression of a reporter transgene driven by a neuronal-specific promoter and a decrease of gene expression using a glial specific promoter in VPA-treated cells. These results indicate that VPA induces a specific differentiation of C6 cells toward a neuronal-like phenotype. The present data highlight the importance of epigenetic phenomena in the development and differentiation of the nervous system.

Key words: histone deacetylases, epigenetic, brain tumors, gene therapy, oncology, GFAP.

The cellular origin of gliomas, the most frequent primary tumors of the CNS, and the process of gliomagenesis are not completely understood yet, but recent reports suggest that gliomas originate from neural stem cells. Moreover, it is known that many brain tumors share the same genetic alterations in signal-transduction and in cell-cycle arrest pathways, thus suggesting a common origin (Benitez et al., 2008). Within the tumor cells there is a wide genetic heterogeneity and conventional treatments such as chemotherapy and radiotherapy fail to increase the median survival of patients with gliomas; these results are associated with an elevated cell proliferation rate and high infiltration into the normal parenchyma.

In recent years, inhibition of histone deacetylases (HDAC), using epigenetic drugs, has emerged as a potential strategy to reverse aberrant chromatin changes associated with cancer and to induce tumor cell differentiation (Minucci and Pelicci, 2006; Yoo and Jones, 2006). HDACs are enzymes involved in the remodeling of chromatin, and have a key role in the regulation of gene expression (Jenuwein and Allis, 2001). Previous reports have indicated that sodium phenylbutyrate (SB), an inhibitor of histone deacetylases (iHDAC) classes I and II, reduces cell proliferation, inhibits cell migration and changes the pattern of expression of cell cycle proteins in glioma cells (Engelhard et al., 2001; Ito et al., 2001). However, very high doses of SB are required to obtain therapeutic effects (Gore et al., 2002).

Prior results indicated that valproic acid (VPA) induced the differentiation of transformed cells associated with the inhibition of HDACs (Gottlicher et al., 2001; Gurvich et al., 2004). VPA is a short-chain fatty acid employed in the treatment of epilepsy, bipolar disorders and migraine (Blaheta and Cinatl, 2002). This drug has been used to induce the differentiation of neural progenitor cells (Hsieh et al., 2004), to promote neurite growth and to increase the expression of neuronal markers in neuroblastoma cell lines (Stockhausen et al., 2005; Yuan et al., 2001); it also protects dopaminergic neurons from neurotoxicity (Peng et al., 2005) and, in some cases, it may stimulate neuronal cell death (Jin et al., 2005). The molecular mechanisms involved in these processes have not been elucidated yet. Nevertheless, VPA has been used to induce the expression of both the *gad67* (glutamic acid decarboxylase 67) and the *reelin* genes in GABAergic interneurons in a mouse model of schizophrenia, in which both genes had been silenced (Dong et al., 2005, 2007).

A clinical advantage of VPA with respect to SB is that the therapeutic level required to induce neuronal differentiation in neural progenitor cells and neuroblastoma cell lines, and to induce the expression of specific genes, is the same plasma concentration required for the treatment of epilepsy (Blaheta and Cinatl, 2002), thus the clinically effective concentrations of VPA, contrary to SB, are safe.

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Abbreviations: BDNF, brain-derived neurotrophic factor; BrdU, 5-bromo-2-deoxyuridine; CMV, cytomegalovirus; DAPI, 4,6-diamidino-2-phenylindole; DNMT, DNA methyltransferase; eGFP, enhanced green fluorescent protein; ERK, extracellular signal-regulated kinase; FSK, forskolin; *gad67*, glutamic acid decarboxylase 67; GDNF, glial derived neurotrophic factor; GFAP, glial fibrillary acidic protein; H3, histone 3; H4, histone 4; HDAC, histone deacetylases; iHDAC, inhibitor of histone deacetylase; LDH, lactate dehydrogenase; MTT, tetrazolium 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SB, sodium phenylbutyrate; VPA, valproic acid.

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In the present work we studied the effect of VPA in the C6 astrocytoma cell line. We observed that there is a correlation between the inhibition of cell proliferation and the increase of cell death, in a time- and dose-dependent manner after the administration of VPA. We also show that VPA induces the differentiation of C6 cells, an effect associated with decreased cell proliferation, growth arrest, and diminished cell migration. Interestingly, in C6 cells treated with VPA for 72 h we found extended processes, which had characteristics similar to the structure of a growth cone. Protracted treatment of C6 cells for 6 days with 3 mM VPA induced morphological changes associated with the down-regulation of glial fibrillary acidic protein (GFAP), glial derived neurotrophic factor (GDNF), and brain-derived neurotrophic factor (BDNF) and increased the expression of βIII-tubulin, a specific neuronal marker. These results were specific for VPA treatment, because we did not observe expression of neuronal markers or neurite-like growth using the activator of adenylyl cyclase, forskolin (FSK), a drug known to induce astrocyte differentiation (Segovia et al., 1994, 1998; Salero-Coca et al., 1995). We also observed that VPA differentially regulates the expression of transgenes in C6 cells, depending on the cell-type specific promoter. In agreement with our data, the expression of a transgene driven by a neuron specific promoter was augmented by VPA, whereas the expression of a transgene driven by a glial promoter was decreased by the drug. These results show that the differentiation-promoting activity of VPA toward a neuron-like phenotype in C6 cells is based on the regulation of gene expression. Taking these results together, we propose that VPA induces the morphological differentiation of C6 cells toward a neuronal-like phenotype, and that this process is related with the down-regulation of glial markers and expression of neuronal genes. The present data also highlight the importance of epigenetic phenomena in the development and differentiation of the nervous system.

EXPERIMENTAL PROCEDURES

Cell culture

C6 and U373 cells were grown in Dulbecco's modified Eagle's F12 medium (DMEM-F12, Invitrogen, Carlsbad, CA, USA) with 10% fetal calf serum (Invitrogen) at 37 °C in a 5% CO_2–95% air humidified atmosphere. Drugs were applied to cultures, and for long-term cultures the medium was changed after 72 h of treatment, with fresh medium and drugs.

Cell proliferation and cell death assays

To determine the effect of VPA on cell proliferation and cell death we used the 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays, respectively. C6 cells were seeded in 96-well plates at a density of 12,500 cells/cm^2. After 24 h, cells were treated with different concentrations of VPA (Sigma, St. Louis, MO, USA). Twenty-four, 48 and 72 h after treatment, cell proliferation and cell death were determined using the MTT assay kit (Promega, Madison, WI, USA) and the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega), respectively, as described by the manufacturers. Absorbance was read at 492 nm using an automated microplate reader (Bio-Rad, Hercules, CA, USA). Assays were performed in quadruplicate.

Proliferation index

Cells were incubated in the presence of 50 μM of 5-bromo-2-deoxyuridine (BrdU) for 6 h before completing the 72 h of treatment. Cells were fixed with 4% *p*-formaldehyde and BrdU positive cells were revealed using a BrdU staining kit (Zymed, South San Francisco, California, USA), according to the manufacturer's instructions. Two hundred to 300 cells were examined per coverslip to determine the percentage of cells that incorporated BrdU. We performed three independent experiments.

Cell migration assay

To determine the effect of VPA on cell migration we performed *in vitro* "scratch wounds" by scraping confluent cell monolayers seeded in Petri dishes (35-mm diameter) with a 1 ml sterile pipette tip. After 72 h of treatment, migration was determined by measuring the width of the scratch from the edge of the wound (Khwaja et al., 2006). The width of the scratch was measured using the Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD, USA). We measured the width of the scratch in three independent experiments for each treatment.

Morphological analysis of C6 cells by immunofluorescence

Cells were seeded on 1 cm^2 slide-covers in 24 well microplates and 24 h later cells were treated with VPA or FSK (Sigma) or received no treatment, as control. After 72 h, cells were fixed with 4% *p*-formaldehyde. Morphological analysis of C6 cells was performed using an anti-β-tubulin antibody 1:300 (Chemicon, Temecula, CA, USA) or anti-GFAP antibody 1:450 (DAKO, Carpinteria, CA, USA). The primary antibodies were developed using a fluorescein streptavidin-conjugated secondary antibody (1:70 Vector Laboratories, Burlingame, CA, USA). Cells were observed using a fluorescence microscope (Olympus BX51, Tokyo, Japan).

Western blot analysis

Total protein was isolated using the TriPure reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Twenty micrograms of total protein were separated on 8% SDS-PAGE gels and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked for 1 h at room temperature in 5% nonfat milk/TBST (0.05% Tween-20, TBS) and incubated with the primary antibodies overnight at 4 °C. Blots were then incubated with a peroxidase-coupled secondary antibody followed by enhanced chemiluminescence detection (Perkin Elmer, Boston, MA, USA), according to the manufacturer's instructions. The primary antibodies were: anti-acetylhistone 4 (H4) 1:1000 (Upstate Cell Signaling Solution, Charlottesville, VA, USA); anti-GFAP 1:1000 (Chemicon); anti-GDNF 1:50 (Santa Cruz Biotechnology); anti-BDNF 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-βIII-tubulin 1:100 (Covance, Princeton, NJ, USA); and anti β-actin 1:400 (Garcia-Tovar et al., 2001). Images from Figs. 1 and 5A and B are each from the same gels, but since lanes were not contiguous, lanes between the experimental ones were cut from the image.

Cell transfection with the Thy1.2eGFP plasmid

C6 cells were seeded on slide-covers as previously described. Seventy-two hours after treatment with the different agents, cells were transfected with the Thy1.2eGFP plasmid (generously provided by Dr. Rusty Lansford, California Institute of Technology, USA) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The Thy1.2eGFP plasmid uses the *thy* promoter to drive the expression of enhanced green fluorescent protein (eGFP) in neuronal cells (Dittgen et al., 2004). Finally, 24 h after transfection cells were fixed with 4% *p*-formaldehyde,