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The critical chemical and mechanical regulation of folic acid on neural engineering

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ABSTRACT

The mandate of folic acid supplementation in grained products has reduced the occurrence of neural tube defects by one third in the U.S since its introduction by the Food and Drug Administration in 1998. However, the advantages and possible mechanisms of action of using folic acid for peripheral nerve engineering and neurological diseases still remain largely elusive. Herein, folic acid is described as an inexpensive and multifunctional niche component that modulates behaviors in different cells in the nervous system. The multiple benefits of modulation include: 1) generating chemotactic responses on glial cells, 2) inducing neurotrophin release, and 3) stimulating neuronal differentiation of a PC-12 cell system. For the first time, folic acid is also shown to enhance cellular force generation and global methylation in the PC-12 cells, thereby enabling both biomechanical and biochemical pathways to regulate neuron differentiation. These findings are evaluated *in vivo* for clinical translation. Our results suggest that folic acid-nerve guidance conduits may offer significant benefits as a low-cost, off-the-shelf product for reaching the functional recovery seen with autografts in large sciatic nerve defects. Consequently, folic acid holds great potential as a critical and convenient therapeutic intervention for neural engineering, regenerative medicine, medical prosthetics, and drug delivery.

1. Introduction

Folic acid (a synthetic form of folate) is an essential source of the single carbon group used in DNA methylation and plays a pivotal role in the development, function, regeneration, and repair of the central nervous system (CNS) [1–4]. Folic acid deficiency has been associated with a variety of CNS disorders [5–8], such as neural tube defects (NTDs) [9–11], developmental delays, dementia, and seizures [12,13]. Supplementing the diet with folic acid can prevent some of these clinical problems, provided the supplementation occurs sufficiently early in embryonic development. For instance, periconceptional folic acid intake was shown to considerably reduce the occurrence of NTDs such as spina bifida and anencephaly in newborn babies [14]: the recommended daily dose of folic acid of 4 mg/day resulted in a remarkable 72% reduction in NTD recurrence [15,16].

Notably, the systemic benefits of folic acid are not just limited to stimulating growth and differentiation during embryonic brain and spinal cord development. Iskandar et al. have shown that parenteral folic acid consumption produces up to 10-fold, dose-dependent improvement in axonal regrowth and functional recovery after injury to the adult CNS, an effect well in excess of other interventions [7]. Despite clinical efficacy of folic acid supplementation for preventing NTDs and other neurological disorders, and the potential benefits of folic acid on CNS nerve repairs reported in animal models [17], the exact pro-regenerative effects and underlying mechanisms of folic acid on the peripheral nervous system (PNS) at the cellular and subcellular levels have not been revealed.

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This paper aims to show how folic acid plays a role as a multifunctional, pro-regenerative niche component by affecting different cells of the PNS, their behavior (proliferation and migration of glial cells, neurotrophin release of Schwann cells, and cell survival and differentiation of a model neuronal PC-12 cell system), and their network with each other, ultimately leading to improved peripheral regeneration. The new findings will elaborate how certain doses of exogenous folic acid affect the PNS axon regeneration by exerting both chemical and mechanical stimuli to the neurons. These outcomes will serve as important benchmarks for designing innovative and advanced biomaterials that can provide microenvironments aimed at inducing specific cell behaviors in glial cells and neurons.

Furthermore, distinguishing it from all of the previous work involving systemic injections of folic acid into animals to aid in CNS regeneration, we designed nerve guidance conduits out of biodegradable crosslinked urethane-doped polyester (CUPE) that locally deliver folic acid at the injury site for the repair of critical-sized peripheral nerve gaps (>20 mm) in Wistar rats. CUPE was used here to fabricate NGC s due to its reported merits that include excellent biocompatibility, hemocompatibility, and soft and highly elastic property [18–20], making it suitable for nerve tissue engineering and other vascular applications. A folic acid-incorporated nerve guidance conduit (Fig. 1a) is considered to create a chemical gradient in the surrounding tissue as a guide for Schwann cells to migrate into the device, and then serve as a suitable microenvironment that provides both chemical and mechanical cues to facilitate 1) Schwann cell proliferation, 2) the release of neurotrophins from the Schwann cells, and 3) axonal regeneration (Fig. 1b). In conclusion, we propose that folic acid may offer multiple benefits to orchestrate the regeneration of peripheral nerves as a cost-effective regenerative niche component, and can be distinguished from other biological agents such as nerve growth factors or transplanted cells due to its known structure and chemical stability. The outcomes generate great insights into unveiling the regulatory effects of folic acid effects and establishing a folic acid niche model for future neuroregenerative medicine.

2. Materials and methods

2.1. Materials

Folic acid (purity ≥ 97%), 1,6-hexamethyl diisocyanate (purity ≥ 99%), and 1,4-dioxane (purity 99.8%, anhydrous) were purchased from Sigma-Aldrich. Citric acid (purity ≥ 99.5%, anhydrous, ACS) and 1,8-octanediol (purity ≥ 98%) were purchased from Alfa Aesar.

2.2. Cell culture

Rat Schwann cells (ATCC, Manassas, VA, USA; catalog #: CRL-2768™) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic antimycotic 100× solution. The cells were maintained in a 37 °C incubator with 5% CO2 and a relative humidity of 95%. The medium was changed every two or three days. Rat PC-12 Adh cells (ATCC, Catalog #: CRL-1721.1™) were seeded in 25 cm^2 CellBIND® flasks (Corning, Corning, NY, USA) and maintained in complete F-12K medium (Kaighn's Modification of Ham's F-12 Medium) (ATCC) supplemented with 15% horse serum (ATCC), 2.5% fetal bovine serum (ATLANTA biologics, Flowery Branch, GA, USA), and 1% antibiotic antimycotic 100× solution (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C in a humidified incubator with 5% CO_2. The medium was changed every two days. To induce differentiation, PC-12 cells were treated with 50 ng/mL of nerve growth factor (NGF)-β from rat (Sigma-Aldrich) dissolved in differentiation media (F-12K medium containing 2.5% horse serum and 1% antibiotic antimycotic 100× solution) for three consecutive days.

Human astrocytes or HAs (HA; ScienCell Research Laboratories, Corte Del Cedro, Carlsbad, CA, USA; catalog #: 1800) were received as a gift from Dr. Gong Chen (Pennsylvania State University, University Park, PA, USA) and maintained in astrocyte medium (ScienCell Research Laboratories) at 37 °C in a humidified atmosphere containing 5% CO_2. These HAs were isolated from human cerebral cortex.

2.3. Cell cytotoxicity and proliferation assays

Both Schwann and PC-12 cells were seeded in 96-well plates (Corning) at a cell density of 10,000 cells/well for cytotoxicity assays and 500 cells/well for a proliferation assays, respectively. The cells were incubated in 100 μL of their complete growth media containing different concentrations of folic acid (Sigma-Aldrich) at 37 °C and 5% CO_2. After the incubation for predetermined times (24 h for cytotoxicity assays and one, three, five, and seven days for proliferation assays, respectively), colorimetric cell counting kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was performed according to the manufacturer's specifications using a plate reader (Tecan, Charlotte, NC, USA) in order to determine the number of viable cells in each sample. The experiment was repeated three times, independently.

2.4. Cell migration assay

Chemotactic property of folic acid on promoting the migration of Schwann cells and astrocytes was studied in the cell migration experiments. Migration assay was performed on Schwann cells and astrocytes using the transwells (Corning; 6.5 mm Diameter polycarbonate membrane inserts with 8 μm pore size). Before the migration assay, the cells were starved overnight in serum-free media and the bottom of each transwell insert was coated with fibronectin (Corning) and incubated overnight at 4 °C in order to allow cells to adhere to the bottom side of the membrane during migration assays. A 100-μL serum-free medium containing resuspended Schwann cells or astrocytes (10^6 cells/mL) was transferred to the top chambers of each transwell and allowed to adhere for 1 h at 37 °C in 5% CO_2. After the cells adhered, 600 μL of serum-free medium was added into the lower chambers and the cells were allowed to migrate for 4 h. After the cells migrated, the upper surface of each membrane was cleaned with a cotton swab and the cells adhering to the bottom surface of each membrane were stained with 0.1% crystal violet and rinsed with DI water until it ran clear, imaged using a light microscope (Nikon USA, Melville, NY, USA), and counted using ImageJ. Assays were performed three times using triplicate wells.

2.5. Immunostaining for neurite outgrowth assay

Undifferentiated PC-12 cells were plated in a density of 5×10^4 cells/well in their growth medium in 6-well plate and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After cell attachment overnight, each well was treated with different concentrations of folic acid and with or without 50 ng/mL NGF. After certain days of incubation, the cells in each well were fixed in 4.0% paraformaldehyde and stained with 4′,6-Diamidino-2-Phenylindole or DAPI (Sigma Aldrich; catalog #: D9542) and Alexa Fluor® 555 Phalloidin (Thermo Fisher Scientific, Waltham, MA, USA; catalog #: A34055). Fluorescent images of stained PC-12 cells were taken with a fluorescence microscope (Nikon). Five random fields (200–300 cells/well) were examined in each well. The

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