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Effect of low-dose thalidomide on dopaminergic neuronal differentiation of human neural progenitor cells: A combined study of metabolomics and morphological analysis

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ABSTRACT

Thalidomide is increasingly used in anticancer and anti-inflammation therapies. However, it is known for its teratogenicity and ability to induce peripheral neuropathy, although the mechanisms underlying its neurological effect in humans are unclear. In this study, we investigated the effect of thalidomide on the metabolism and neuronal differentiation of human neural progenitor cells. We found that levels of tyrosine, phenylalanine, methionine and glutathione, which are involved in dopamine and methionine metabolism, were decreased following thalidomide treatment. Morphological analysis revealed that treatment with 100 nM thalidomide, which is much lower than clinical doses, significantly decreased the number of dopaminergic (tyrosine hydroxylase-positive) neurons, compared with control cells. Our results suggest that these adverse neurological effects of thalidomide should be taken into consideration prior to its use for the treatment of neurodegenerative and other diseases.

1. Introduction

A number of neurodegenerative diseases, including Parkinson's disease (PD), are characterized by the progressive loss of dopaminergic neurons in the substantia nigra and their projections to the striatum, which leads to various motor deficits (Ebert et al., 2008; Jeon et al., 2010). Human neural progenitor cells (hNPCs), which are capable of dividing and differentiating into many cells types of the nervous system including neurons, astrocytes and oligodendrocytes, can respond to environmental demands by increasing their proliferation and differentiation (Ryu et al., 2009). Transplantation of genetically modified hNPCs to replace and protect dopaminergic neurons, as a potential therapeutic strategy for the treatment of PD, has become increasingly popular among researchers (Kitiyanant et al., 2011; Lunn et al., 2011; Ryu et al., 2009). Furthermore, hNPCs have recently been proposed as a powerful model system for developmental neurotoxicity testing, as they have the capacity to differentiate into any cell type in the nervous system. The use of hNPCs also affords better predictive power, as there is no need to extrapolate results obtained with non-human species (Breier et al., 2008, 2010).

Thalidomide was used as a sedative drug to treat morning sickness in pregnant women in the 1950s, but was subsequently withdrawn from the market in 1961 because of severe teratogenicity and neurotoxicity (Franks et al., 2004; Lenz, 1988; Matthews and McCoy, 2003). Interestingly, subsequent studies on the mechanisms of thalidomide teratogenicity revealed that the compound was an effective anticancer and anti-inflammatory agent. The US Food and Drug Administration approved thalidomide for the treatment of lepromatous leprosy and multiple myeloma in 1998 and 2006, respectively (Kim and Scialli, 2011; Teo et al., 2005; Uhl et al., 2006). However, major obstacles to the use of thalidomide are its diverse neurological side effects.

It has been hypothesized that impairment of energy metabolism might contribute to nerve cell death in neurodegenerative diseases (Beal et al., 1993). Dysregulation of metabolites in the methionine (Met) transmethylation and transsulfuration pathways has been implicated in several neurodevelopmental diseases,

Abbreviations: CE-TOFMS, capillary electrophoresis time-of-flight mass spectrometry; DoD, day of differentiation; GSH, glutathione; HCA, hierarchical clustering analysis; hNPCs, human neural progenitor cells; MAP2, microtubule-associated protein 2; Met, methionine; PCA, principal component analysis; PD, Parkinson's disease; Phe, phenylalanine; TH, tyrosine hydroxylase; Tyr, tyrosine.

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such as PD and autism (Glaser et al., 2005; James et al., 2004). However, little is known of the effect of thalidomide on the pathogenesis of neurodegenerative diseases. It is likely that further investigation of thalidomide's mechanism of action will provide a rational basis for the development of effective and safe thalidomide-based therapies for the treatment of neurodegenerative and other diseases.

In the present study, we investigated the effect of thalidomide on dopaminergic neuronal differentiation of hNPCs using a combination of metabolomics and morphological analysis. Metabolomics of hNPCs has recently been described as a novel approach for biomarker discovery for human neurological diseases (Maletic-Savatic et al., 2008). Here, we report for the first time that thalidomide at concentrations much lower than clinical doses might impair dopamine and Met metabolic pathways and inhibit dopaminergic neuronal differentiation of hNPCs.

2. Materials and methods

2.1. Chemicals

Dimethyl sulfoxide (DMSO) was obtained from Sigma–Aldrich Co. (St. Louis, MO). Thalidomide was obtained from Wako Pure Chemicals (Osaka, Japan). DMSO was used as the primary solvent for all chemicals, and the DMSO solutions were further diluted in cell culture media for treatments. The final concentrations of DMSO in the media did not exceed 0.1% (v/v).

2.2. Cell culture and differentiation

The hNPCs, derived from H9 human embryonic stem cells, were obtained from Chemicon-Millipore (ENStem Human Neural Progenitor Expansion Kit; Norcross, GA). This cell line forms an adherent cell monolayer and can readily differentiate into different neuronal subtypes (Shin et al., 2005). On the first day of differentiation (DoD0), cells were cultured on a coating of 0.54 μg/cm^2 Laminin-511 (BioLaminaAB, Stockholm, Sweden) in 90-mm dishes (1.2×10^6 cells/dish) or 24-well plates (5×10^4 cells/well). The cells were cultured in neural differentiation medium containing phenol red-free neurobasal medium, 1× B27, 1× N2, 10 ng/ml BDNF (Invitrogen, Carlsbad, CA), and 2 mM glutamine (Sigma–Aldrich). The medium was replaced every 3 days. The cells were exposed to 0.1% DMSO or thalidomide (100 nM) during DoD3–5 and isolated for morphological analysis or metabolite analysis on DoD12 (Fig. 1).

2.3. Sample preparation for metabolite analysis

On DoD12, differentiated cells (approximately 1×10^7 cells/dish in triplicate) were washed twice with 5% mannitol solution to remove residual medium. Excess mannitol solution was removed via two rounds of centrifugation at maximum speed, and then 1.3 ml methanol solution containing 10 μM internal standards was added. Cells were collected using a cell scraper and the cell pellet was stored at −80 °C for subsequent extraction. Metabolome extraction was then performed as previously described (Ohashi et al., 2008). Metabolic profiling was initially performed using a single sample, and then the effect of thalidomide on target metabolites involved in dopamine and Met metabolic pathways was verified using four independent culture experiments.

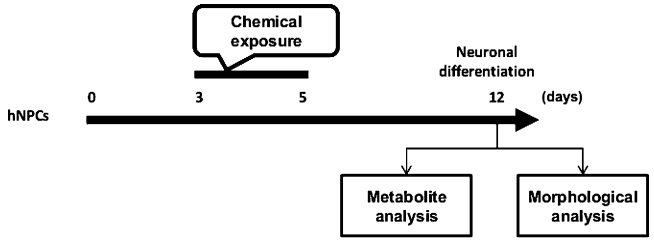

Fig. 1. Schematic overview of experimental procedures. Cells were exposed to thalidomide at a concentration of 100 nM or 10 μM during DoD3–5, and the subsequent effects on cellular metabolism and morphology were examined on DoD12.

2.4. Capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS)

Metabolic changes in response to thalidomide were examined during the neural differentiation of hNPCs using a CE-TOFMS-based metabolomics technique. This approach has been described as a sensitive, selective and rapid analytical method for anionic species, suitable for single-cell metabolomics (Lapainis et al., 2009; Ohashi et al., 2008; Soga et al., 2002). CE-TOFMS was performed using an Agilent CE Capillary Electrophoresis System equipped with an Agilent 6210 Time-of-Flight mass spectrometer, Agilent 1100 isocratic HPLC pump, Agilent G1603A CE-MS adapter kit and Agilent G1607A CE-ESI-MS sprayer kit (Agilent Technologies, Waldbronn, Germany). Detailed methods were as previously described (Ohashi et al., 2008; Qin et al., 2011).

2.5. Data analysis for CE-TOFMS

Raw data obtained by CE-TOFMS were analyzed with KEIO Master Hands software (Sugimoto et al., 2010a). Data analysis was performed using the raw data and included noise filtering, baseline correction, peak detection, deconvolution and integration of the peak area from sliced electropherograms with a 0.02 mass-to-charge ratio (m/z). Detailed methods were as previously described (Sugimoto et al., 2010a). To quantify the major metabolites, the injected volume for CE and the sensitivity of MS were corrected using internal standards, and then all annotated metabolites were further corrected with the same chemicals in a standard mixture to overcome the effect of different ionization patterns.

2.6. Identification of metabolites

Detailed methods are as previously described (Sugimoto et al., 2010b). Briefly, the peaks were identified based on the matched m/z values and normalized migration times of the corresponding standard compounds.

2.7. Immunofluorescence

On DoD12, differentiated cells were immunolabeled with human anti-microtubule-associated protein 2 (MAP2; M4403, 1:200 dilution; Sigma–Aldrich) or anti-tyrosine hydroxylase (TH; AB152, 1:200 dilution; Millipore) antibodies and Hoechst 33342 solution (Dojindo, Tokyo, Japan). Cells were fixed with 4% paraformaldehyde for 15 min and then blocked for 30 min in PBT buffer (phosphate-buffered saline with 5% goat serum and 0.1% Triton). Cells with primary antibodies were incubated overnight at 4 °C. On the next day, cells were washed and blocked in BBT-BSA (bicine-buffered saline with 1 mM $CaCl_2$, 0.1% Triton and 0.5% bovine serum albumin) and then incubated with Alexa-conjugated secondary antibodies (1:1000 dilution; Alexa Fluor 546, Invitrogen).

2.8. Morphological analysis

The immunofluorescence images were acquired using the IN Cell Analyzer 6000 (GE Healthcare, Buckinghamshire, UK) with 6