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Original article

Use of human induced pluripotent stem cell-derived neurons as a model for Cerebral Toxoplasmosis

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Abstract

Toxoplasma gondii is a ubiquitous protozoan parasite with approximately one-third of the worlds' population chronically infected. In chronically infected individuals, the parasite resides primarily in cysts within neurons in the central nervous system. The chronic infection in immunocompetent individuals has been considered to be asymptomatic but increasing evidence indicates the chronic infection can lead to neuropsychiatric disorders such as Schizophrenia, prenatal depression and suicidal thoughts. A better understanding of the mechanism(s) by which the parasite exerts effects on human behavior is limited due to lack of suitable human neuronal models. In this paper, we report the use of human neurons derived from normal cord blood CD4+ cells generated via genetic reprogramming, as an *in vitro* model for the study *T. gondii* in neurons. This culture method resulted in a relatively pure monolayer of induced human neuronal-like cells that stained positive for neuronal markers, MAP2, NFL, NFH and NeuN. These induced human neuronal-like cells (iHNs) were efficiently infected by the Prugniad strain of the parasite and supported replication of the tachyzoite stage and development of the cyst stage. Infected iHNs could be maintained through 5 days of infection, allowing for formation of large cysts. This induced human neuronal model represents a novel culture method to study both tachyzoite and bradyzoite stages of *T. gondii* in human neurons.

Keywords: Latent toxoplasmosis; Induced neuronal cells; Cysts

1. Introduction

Toxoplasma gondii is a ubiquitous intracellular protozoan parasite with approximately one-third of the worlds' population chronically infected [1]. In chronically infected individuals, the parasite resides primarily in cysts in muscular and neural tissues [2,3]. In immunocompromised individuals, such as AIDS patients, individuals undergoing chemotherapy or transplantation recipients, the parasite can reactivate in the brain causing a severe to potentially fatal encephalitis [4]. The chronic infection in immunocompetent individuals has traditionally been considered to be asymptomatic but serological evidence indicates the chronic infection may be an etiological factor for development of neuropsychiatric disorders such as Schizophrenia, prenatal depression and suicidal behavior [5–11]. Furthermore, chronic Toxoplasmosis has also been associated with cryptogenic epilepsy, migraine headaches and mild cognitive effects in elderly individuals, further indicating the chronic infection exerts significant effects on neuronal activity in the central nervous system [12–16]. The mechanisms by which the parasite exerts affects on behavioral, cognitive and other neuronal functions are not well understood.

A better understanding of the mechanism(s) by which the parasite exerts effects on human brain and neurological functions is limited due in part to lack of suitable human neuronal models. In the human host the parasite consists of two phases, the rapidly replicating tachyzoite stage and the slower replicating bradyzoite stage. The tachyzoite stage

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replicates within vacuoles leading to host cell lysis while the more slowly replicating bradyzoite stage leads to formation of cysts that persist in muscle and neural tissue for the lifetime of the host [17,18]. *In vivo* studies have established that in the brain, neurons are the predominant host cell in which the cysts persist in the chronically infected host [19,20]. Previous *in vitro* studies of *Toxoplasma*-infected neurons have consisted primarily of mice or rat neuronal cultures composed of glial cells and neurons, and focused on differential development of tachyzoite and bradyzoite stages in glial cells vs. neurons but were not able to study development of the parasite in pure neuronal cells [21–26]. A recent study using human neurons derived from patients with brain disorders and healthy controls, reported these neurons could support growth of tachyzoite stage and cyst development and offers a potentially better *in vitro* model to study the effects of the parasite on human neuronal functions [27].

In this paper, we report on the use of human neurons derived from NCRM-1 cells, a neural stem cell (NSC) line derived from normal cord blood CD4+ cells generated via genetic reprogramming, as another *in vitro* model for the study of *T. gondii* in neurons. The phenotype of the NSC-derived human neurons was validated using neuronal markers, MAP-2, the neurofilament heavy and light chains, NFH and NFL respectively, and NeuN. This culture method resulted in a relatively pure, high-density monolayer of neuronal-like cells. These induced human neuronal-like cells (iHNs) were efficiently infected by the Prugniad (type II) strain of the parasite and supported replication of the tachyzoite stage and cyst development. Infected iHNs could be maintained through 5 days of infection, allowing for formation of large cysts. This induced human neuronal model represents a novel and effective culture method to study effects of *Toxoplasma* tachyzoite and bradyzoite stages and cyst development in human neurons.

2. Materials and methods

2.1. Culture of neural stem cells (NSCs)

Induced human neuronal-like cells (iHNs) were derived from the cell line, NCRM-1, obtained from NIH Center for Regenerative Medicine (http://commonfund.nih.gov/stemcells). NCRM-1 is an induced pluripotent stem cell (ihPSC) line that was derived using the episomal vectors (Oct4, Sox2, c-Myc, Klf4, Lin28 2, and SV40 Large T antigen) as a reprogramming method. The somatic starting material was cord blood $CD34^+$ cells. Upon this reprogramming, the NCRM-1 cells are a neural stem cell phenotype that can be passaged numerous times. In this study, NCRM-1 neural stem cells, were used to differentiate human neurons. NCRM-1 cells, hereafter referred to as Neural Stem Cells (NSCs), were passaged weekly, and retained the ability to differentiate into human neurons through 20 passages. NSCs were cultured in StemPro NSC-SFM (GIBCO; Cat. No. A10509-01) consisting of KnockOUT™ DMEM/F12 media supplemented with recombinant human FGF-basic, recombinant human EGF, and StemPro Neural Supplement. The NSC-SFM media was supplemented with GlutaMAX and Antibiotic-Antimycotic solution (GIBCO Cat. No.15240). This complete media called NSC-expansion media (NSC-EM) was used to expand and passage the NSC cells. NSC cells were plated onto CELLstart (GIBCO Cat no. A10142) coated 6 well plates, according to the manufacturers directions, at a cell density of 5×10^4 cells/cm^2. NSCs were then cultured at 37 °C in a humidified atmosphere of 5% CO_2. NSC cells were fed with fresh NSC-EM every two days and passaged, using Accutase to dissociate, when cells were 95% confluent. NSCs maintained in culture up to 20 passages, were used in these experiments.

2.2. Differentiation of induced human neuronal-like cells (iHNs) from NSCs

Neuronal differentiation was adapted from established protocols by Yan et al. [28]. Briefly, neuronal differentiation was initiated by dissociating NSCs followed by plating onto poly-L-ornithine and laminin coated dishes at a density of 4×10^5 cells/cm^2 in NSC-EM. The next day, the spent medium was replaced with a neuronal induction medium (NIM) consisting of Neurobasal medium, MEM nonessential amino acids, GlutaMAX, B27 supplement (17504-044; GIBCO), 200 ng/ml Sonic Hedge Hog (SHH) (SRP 3156, Sigma), and 100 ng/ml recombinant human Fibroblast Growth Factor 8 (FGF8) (PHG0274; GIBCO) for 10 days, with medium changes every alternative day for 10 days. On day 10, cells were dissociated with Accutase and plated onto laminin-coated four-well LabTek II chamber slides or laminin coated coverslips at a density of 2×10^4 cells/cm^2 in a neuronal differentiation medium (NDM) consisting of Neurobasal medium, MEM nonessential amino acids, GlutaMAX, B27 supplement, 200 μm ascorbic acid, 20 ng/ml human Brain-Derived Neurotrophic Factor (BDNF) (PHC7074; GIBCO), and 20 ng/ml Glial-Derived Neurotrophic Factor (GDNF) (PHC7045; GIBCO) for another 10 days, with a medium change every other day, to allow maturation of neurons. At the end of this induction/differentiation period, these cells achieved a neuronal morphology, exhibiting processes and a small cell body. These cells were characterized for neuronal markers (see Sec. 2.3 below), and found to stain positive for neuronal markers and were subsequently referred to as induced human neuronal-like cells (iHNs).

2.3. Characterization of iHNs using neuronal markers and immunofluorescence

Immunocytochemical staining was used to determine the phenotype of differentiated iHNs. Differentiated iHNs were fixed with 4% paraformaldehyde at room temperature for 15 min, washed 3× with PBS and then permeabilized and blocked in a solution containing 1% Bovine Serum Albumin (BSA) and 0.05% Saponin for 30 min. Following this blocking/permeabilization step cells were incubated in primary antibodies at 4 °C overnight in blocking buffer consisting of DPBS + 1% BSA, followed by 3 washes in DPBS,

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