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Application of stem cell derived neuronal cells to evaluate neurotoxic chemotherapy

Claudia Wing [a], Masaaki Komatsu [a,1], Shannon M. Delaney [a], Matthew Krause [b], Heather E. Wheeler [a,2], M. Eileen Dolan [a,*]

[a] Section of Hematology/Oncology, Department of Medicine, Chicago, IL, USA
[b] Committee of Molecular Pathogenesis and Molecular Medicine, The University of Chicago, Chicago, IL, USA

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ABSTRACT

The generation of induced pluripotent stem cells (iPSCs) and differentiation to cells composing major organs has opened up the possibility for a new model system to study adverse toxicities associated with chemotherapy. Therefore, we used human iPSC-derived neurons to study peripheral neuropathy, one of the most common adverse effects of chemotherapy and cause for dose reduction. To determine the utility of these neurons in investigating the effects of neurotoxic chemotherapy, we measured morphological differences in neurite outgrowth, cell viability as determined by ATP levels and apoptosis through measures of caspase 3/7 activation following treatment with clinically relevant concentrations of platinating agents (cisplatin, oxaliplatin and carboplatin), taxanes (paclitaxel, docetaxel and nab-paclitaxel), a targeted proteasome inhibitor (bortezomib), an antiangiogenic compound (thalidomide), and 5-fluorouracil, a chemotherapeutic that does not cause neuropathy. We demonstrate differential sensitivity of neurons to mechanistically distinct classes of chemotherapeutics. We also show a dose-dependent reduction of electrical activity as measured by mean firing rate of the neurons following treatment with paclitaxel. We compared neurite outgrowth and cell viability of iPSC-derived cortical (iCell® Neurons) and peripheral (Peri.4U) neurons to cisplatin, paclitaxel and vincristine. Goshajinkigan, a Japanese herbal neuroprotectant medicine, was protective against paclitaxel-induced neurotoxicity but not oxaliplatin as measured by morphological phenotypes. Thus, we have demonstrated the utility of human iPSC-derived neurons as a useful model to distinguish drug class differences and for studies of a potential neuroprotectant for the prevention of chemotherapy-induced peripheral neuropathy.

1. Introduction

With an estimate of >13.7 million cancer survivors in the United States (Siegel et al., 2012) there is concern regarding long-term effects of chemotherapy. Chemotherapy-induced peripheral neuropathy (CIPN) is one of the most common and potentially permanent side effects of modern chemotherapy that can result in dose reduction or cessation of therapy (Brewer et al., 2016). CIPN may develop in 20–40% of cancer patients as a consequence of treatment with platinum analogues (cisplatin, oxaliplatin, carboplatin), taxanes (paclitaxel, nab-paclitaxel, docetaxel), vinca alkaloids (vincristine), proteasome inhibitors (bortezomib), epothilones or other chemotherapeutics (Chu et al., 2015; Grisold et al., 2012). Differences in structural and mechanistic properties among various chemotherapeutic agents contribute to variations in clinical presentation including numbness, loss of proprioceptive sense, tingling, pins and needles sensations, hyperalgesia or allodynia in the hands or feet in a stocking-glove distribution (Brewer et al., 2016).

Mechanisms underlying CIPN include direct and indirect effects on sensory nerves such as damage to neuronal cell bodies in the dorsal root ganglion, alteration of the amplitude of the action potential or conduction velocity (Argyriou et al., 2012; Sisignano et al., 2014). Whereas CIPN may be reversible for some cytotoxic drugs (e.g. taxanes), for other agents (e.g. cisplatin), the persistence of CIPN is well documented (Argyriou et al., 2012; Avan et al., 2015). Wide ranges in incidence rates likely reflect not only differences in study populations, drug-related factors (e.g. dose-intensity) and potential confounders, but also genetic susceptibility (Argyriou et al., 2012; Bhatia, 2011). Patients at high risk could consider alternative chemotherapy regimens with similar efficacy or a treatment strategy that mitigates risk by limiting the cumulative dose of the neurotoxic drug.

For the treatment of painful neuropathies, most drugs fall short of providing adequate relief (Sisignano et al., 2014). A systematic evaluation of 48 randomized controlled trials concluded that there are no

* Corresponding author at: University of Chicago, 900 E. 57th St, KCBD 7100, Chicago, IL 60637, United States.
E-mail address: edolan@medicine.bsd.uchicago.edu (M.E. Dolan).
[1] Current address: Department of OBGYN, JR Hiroshima Hospital, 3-1-36 Futabanosato, Higashi-ku, Hiroshima, Japan, 732–0057.
[2] Current address: Loyola University Chicago, Departments of Biology & Computer Science, LSB 324, 1050 W Sheridan Rd, Chicago, IL 60660.

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agents that can be recommended for the prevention of CIPN (Hershman, et al., 2014). With regard to the treatment of existing CIPN, the best available data support a moderate recommendation for treatment with duloxetine, a selective serotonin and norepinephrine reuptake inhibitor (Smith, et al., 2013). Goshajinkigan (GJG), a traditional Japanese herbal medicine, has been shown to inhibit the progression of neuropathy or alleviate symptoms of nerve pain resulting from chemotherapy treatment with paclitaxel/carboplatin for ovarian and endometrial cancer patients (Kaku et al., 2012), docetaxel in breast cancer patients (Abe et al., 2013), nab-paclitaxel for breast cancer patients (Ohno et al., 2014) and oxaliplatin in colorectal cancer patients (Nishioka et al., 2011; Hosokawa et al., 2012; Yoshida et al., 2013). In animal models, GJG has been shown to suppress various transient receptor potential channels that may mitigate the pain responses in the patient (Mizuno et al., 2014; Kato et al., 2014; Matsumura et al., 2014).

Given the paucity of available treatments and increasing number of cancer survivors living with CIPN, there is an urgent need to identify a reasonable model system to identify more effective compounds supporting multiple targets and providing relief to patients undergoing treatment. Previously, we have demonstrated that induced-pluripotent stem cell (iPSC) derived neurons can be used as a preclinical model system to study CIPN (Wheeler et al., 2015). In the present manuscript, we extend these studies by: 1) evaluating additional platinating agents (oxaliplatin, carboplatin) and taxane analogs (docetaxel, nab-paclitaxel), a proteasome inhibitor (bortezomib), an antiangiogenic (thalidomide), and a chemotherapeutic that does not cause neuropathy (5-fluorouracil); 2) determining the effect of paclitaxel treatment on electrical activity of neurons; 3) comparing drug sensitivity in iPSC- derived cortical versus peripheral neurons; 4) and testing GJG, as a potential neuroprotectant to counteract the effects of paclitaxel, cisplatin and oxaliplatin by evaluating in neurons and cancer cell lines.

2. Methods

2.1. iCell® Neurons

Commercial human iPSC-derived neurons (iCell® Neurons) were purchased from Cellular Dynamics International (CDI, Madison, WI). The cells have been characterized by CDI to represent a pure neuronal population with >95% pan-neuronal population of GABAergic and to a lesser degree glutamatergic subtype expressing ßIII-Tubulin, MAP-2, peripherin having <1% dopaminergic neurons. iCell® Neurons were determined to express multiple ligand gated and voltage gated ion channels and be characteristically similar to neurons from the neonatal forebrain (Dage et al., 2014).

2.2. Peri.4U neurons

Commercial human iPSC-derived neurons (Peri.4U) were purchased from Axiogenesis (Cologne, Germany) with >90% purity and expressing ßIII-Tubulin, MAP-2, peripherin and vGLUT2. These peripheral-like neurons are not DRG nociceptive neurons. All batches of iPSC-derived neurons were tested for sterility, viability, purity, and morphology. Neurons were maintained according to the manufacturers' protocol.

2.3. Cancer lines

Ovarian adenocarcinoma, SKOV3 (HTB-77) and non-small cell lung cancer, A549 (CCL-185) were obtained from ATCC (Manassas, VA). Authentication of the cancer cell lines were performed by IDEXX BioResearch (Columbia, MO) for interspecies contamination and misidentification, Case # 10952–2014. This authentication was conducted by measuring short tandem repeat (STR) using the Promega CELL ID System (Madison, WI) (8 STR markers (CSF1PO, D13S317, D16S539, D5S818, D7S820, TH01, TPOX, vWA)) and amelogenin (for gender).

2.4. Compound preparations

Drug stocks were prepared and filtered using a 0.22 μM solvent resistant filter (EMD Millipore, Billerica, MA, USA) for sterility. Paclitaxel (Sigma-Aldrich, St. Louis, MO) and docetaxel (LKT Laboratories Inc., St. Paul, MN) were dissolved in DMSO to obtain a stock solution of 58.4 mM and 60 mM, respectively. Cisplatin and carboplatin (Sigma-Aldrich) were dissolved in DMSO and water, respectively, at a stock solution of 20 mM. Oxaliplatin and 5-fluorouracil (both Sigma-Aldrich), bortezomib and thalidomide (both LKT Laboratories Inc.) were dissolved in DMSO at a stock solution of 100 mM. Abraxane (nab-paclitaxel; 1 part paclitaxel/9 parts human albumin; Celegene, Summit, NJ) was purchased from University of Chicago pharmacy and dissolved in PBS to obtain a stock solution of 1 mM. Nab-paclitaxel could not be tested at 100 μM due to its insolubility at this dose. Vincristine (Sigma-Aldrich) was prepared on ice and in the dark (biological safety and room lights off, samples under cover) with PBS at a stock solution of 100 mM. Hydroxyurea (Sigma-Aldrich) was prepared by dissolving powder in PBS and filtered to obtain a stock solution of 1 M. All stock drugs were serially diluted in media for final concentrations from 1 nM to 100 μM for treatment of iCell® Neurons, 0.01 nM to 100 μM for Peri.4U neurons or 1.56 to 100 nM for treatment of the cancer lines. Vehicle controls for each drug were used at corresponding dilutions of final drug solution (0.1–0.2% DMSO).

Goshajinkigan (GJG), supplied by Tsumura & Co. (Tokyo, Japan), was stored desiccated at −20 °C. Prior to treatment, GJG was dissolved at 10 mg/mL in PBS, sonicated for 10 min and diluted to obtain a 50 to 200 μg/mL GJG solution in specific media per cell line.

2.5. Drug treatment of iCell® Neurons

iCell® Neurons were mixed with 3.3 μg/mL laminin (Sigma-Aldrich) in maintenance media containing 0.025 g/L albumin (final concentration) prior to seeding on Poly-D-Lysine coated 96-well Greiner Bio-One plates (Monroe, NC, USA) in 100 μL for a density of 1.33×10^4 cells/well. Four hours following plating, iCell® Neurons were treated with chemotherapeutic drug (1 nM to 100 μM) for 48 and 72 h and evaluated for morphological changes. For experiments with neuroprotectants, GJG was added at the same time as the chemotherapeutic agent.

2.6. Drug treatment of peripheral neurons

Peri.4U were thawed using Axiogenesis thawing media and suspended in 100 μL complete Peri.4U media containing 0.025 g/L albumin (final concentration) prior to seeding (final density 1.0×10^4 cells/well) onto Gel-Trex, reduced-growth factor basement membrane matrix (Life Technologies Inc., Carlsbad, CA) coated Poly-D-Lysine 96-well Greiner Bio-One plates as described above. Four hours following plating, Peri.4U cells were treated with chemotherapeutic drug (0.01 nM to 100 μM) for 48 and 72 h and evaluated for morphological changes.

2.7. High content imaging of neuronal morphological characteristics

After drug treatments (48 or 72 h), neurons were stained for 15 min at 37 °C with 1 μg/mL Hoechst 33342 (Sigma-Aldrich) and 2 μg/mL Calcein AM (Molecular Probes, Life Technologies) then washed twice using dPBS without calcium or magnesium. Imaging was performed at 10× magnification using an ImageXpress Micro imaging device (Molecular Devices, LLC, Sunnyvale, CA) at the University of Chicago Cellular Screening Center. Supplemental Fig. 1 illustrates the processing of a representative image used to quantitate individual cell measurements of mean/median/maximum process length, total neurite outgrowth (the sum of the length of all processes), number of processes, number of branches, cell body area, mean outgrowth intensity, straightness and cell numbers using the MetaXpress software Neurite Outgrowth