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Neurotoxicity of cytarabine (Ara-C) in dorsal root ganglion neurons originates from impediment of mtDNA synthesis and compromise of mitochondrial function

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ABSTRACT

Peripheral Nervous System (PNS) neurotoxicity caused by cancer drugs hinders attainment of chemotherapy goals. Due to leakiness of the blood nerve barrier, circulating chemotherapeutic drugs reach PNS neurons and adversely affect their function. Chemotherapeutic drugs are designed to target dividing cancer cells and mechanisms underlying their toxicity in postmitotic neurons remain to be fully clarified. The objective of this work was to elucidate progression of events triggered by antimitotic drugs in postmitotic neurons. For proof of mechanism study, we chose cytarabine (ara-C), an antimetabolite used in treatment of hematological cancers. Ara-C is a cytosine analog that terminates DNA synthesis. To investigate how ara-C affects postmitotic neurons, which replicate mitochondrial but not genomic DNA, we adapted a model of Dorsal Root Ganglion (DRG) neurons. We showed that DNA polymerase γ, which is responsible for mtDNA synthesis, is inhibited by ara-C and that sublethal ara-C exposure of DRG neurons leads to reduction in mtDNA content, ROS generation, oxidative mtDNA damage formation, compromised mitochondrial respiration and diminution of NADPH and GSH stores, as well as, activation of the DNA damage response. Hence, it is plausible that in ara-C exposed DRG neurons, ROS amplified by the high mitochondrial content shifts from physiologic to pathologic levels signaling stress to the nucleus. Combined, the findings suggest that ara-C neurotoxicity in DRG neurons originates in mitochondria and that continuous mtDNA synthesis and reliance on oxidative phosphorylation for energy needs sensitize the highly metabolic neurons to injury by mtDNA synthesis terminating cancer drugs.

1. Introduction

Peripheral nervous system neurotoxicities incidental to cancer therapies hinder effective drug dosing and compromise efficacy of treatments [1–3]. To address this issue, the mechanisms by which antimitotic drugs injure postmitotic neurons need to be clarified. To this end, we developed an in vitro model of anti cancer drug toxicity to the peripheral nervous system, using cultured dorsal root ganglion (DRG) neurons. Unlike the central nervous system neurons, DRG neurons are not protected by tight blood barrier and readily accessible to circulating compounds. For a proof of mechanism study, we chose the chemotherapeutic drug cytarabine (ara-C), a cytosine analog, which works as 'chain terminator' of DNA synthesis and leads to blockade of cancer cell proliferation [4–7]. Ara-C serves as first line chemotherapy in treatment of acute myeloid leukemia as well as other hematological cancers [6,8]. While ara-C mode of action in proliferating cells is well understood [9,10] and germane to other classes of antimitotic drugs which also hinder DNA synthesis [11,12], limited work was done to address mechanisms underlying adverse effects of ara-C in postmitotic cells, which do not replicate DNA, and particularly in the context of the peripheral nervous system [13–15]. To act as effective 'chain terminator' ara-C must first be incorporated into DNA. While postmitotic neurons do not replicate their genomic DNA, they continuously replicate mtDNA in support of vigorous mitochondrial biogenesis necessitated by high-energy demands in highly metabolic neurons.

Here we investigated whether features inherent to DRG neurons, namely their high mitochondrial content, reliance on mitochondria for energy needs and limited shielding from circulating compounds by the

Abbreviations: DRG, dorsal root ganglion; mtDNA, mitochondrial DNA; BER, base excision repair; NER, nucleotide excision repair; DDR, DNA damage response; OCR, oxygen consumption rate; PNS, peripheral nervous system; pol γ, DNA polymerase γ; ROS, reactive oxygen species; 8-oxoG, 8-oxoguanine; GSH, reduced glutathione; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; 6AN, 6-aminonicotinamide

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blood nerve barrier [16], make DRGs particularly sensitive targets for chemotherapeutic drugs. We show in vitro that the mitochondrial DNA polymerase γ, which is responsible for mtDNA synthesis [17], is strongly inhibited by incorporated ara-C suggesting that in vivo induced ara-C DRG toxicity might be initiated by impediment of mtDNA synthesis [18] and resultant impairments of mitochondrial function. We found that in DRG neurons, sublethal ara-C exposure leads to increased production of reactive oxygen species (ROS), formation of oxidative adducts in mtDNA, reduction in mtDNA copy number and mitochondrial content, as well as, compromised mitochondrial respiration. Recent evidence supports the notion of retrograde cross talk from mitochondria to nucleus that involves stress signaling and activation of the DNA damage response [19,20]. Elevated mitochondrial ROS has been previously implicated in induction of oxidative DNA damage and genomic instability [21,22]. Here, consistently with this scenario, we observe chromatin modifications detectable by immunoreactivity of phosphorylated forms of the histone variant H2AX (γH2AX) and of ataxia-telangiectasia mutated (ATM), a PI3-family kinase [23,24]. Phosphorylated γH2AX and γATM proteins are involved in oxidative stress and damage signaling and nuclear foci containing the phosphorylated forms of these proteins are considered biomarkers of modifications that facilitate chromatin decondensation and activation of the DNA damage response (DDR). Considered together, our findings support causal link between impediment of mtDNA synthesis, resultant respiratory compromise and subsequent activation of the DNA damage response in DRG neurons exposed to sublethal ara-C treatment.

2. Methods

2.1. Culture and treatment of mouse dorsal root ganglion (DRG) neurons

The University of Texas Medical Branch Institutional Animal Care and Use Committee approved all mouse-handling procedures. Dorsal root ganglion neurons were isolated from 3 to 4 months old C57BL/6 male mice (Envigo Laboratories, USA) according to established protocols [25–27] and as we described [28–30]. Briefly, ganglia were collected from all spinal levels, placed in cold dissecting solution (130 mM NaCl, 5 mM KCl, 2 mM KH2PO4, 1.5 mM CaCl2, 6 mM MgCl2, 10 mM glucose and 10 mM Hepes, pH 7.2), incubated with collagenase A (Roche) and trypsin (1 h/37 °C) followed by DNase I (Roche), dissociated by 20 gentle triturations and spun (175 g/3 min). Pellets were passed through 70-μm strainer and re-suspended in DMEM/F12 (Sigma) with 10% FBS, 10 ng/ml nerve growth factor (Sigma) and penicillin/streptomycin. Cells were seeded on pre-coated (10 μg/ml laminin and 100 μg/ml poly-L-ornithine, Sigma) glass coverslips or wells at $(4-5) \times 10^3$ and $3 \times 10^4/cm^2$, respectively. Treatments were initiated 24 h post seeding when neurite network has been established. Cytarabine (ara-C) purchased from MP Biomedicals (Solon, OH) was dissolved in DMSO and stored as 10 mM stock solution at −20 °C. Stock was diluted 1:10 with complete culture medium prior to supplementation at 50 μM for indicated incubation times.

2.2. Immunofluorescent staining

DRG neurons seeded on coverslips were treated as indicated and fixed in 4% paraformaldehyde as we described [28–30]. Cells were permeabilized with 0.1% Triton X-100/0.1% sodium citrate in PBS and incubated with 3% BSA (w/v)/1% Donkey serum (v/v) in PBS. Primary antibodies were: rabbit anti-Neurofilament 200 (1:20,000, Sigma-N4142); mouse anti-COX1 (1:1500, Millipore-459600); mouse anti-8-oxoguanine (1:200, Millipore #MAB3560); rabbit anti-TFAM (1:2000, Genetex #GTX103231); mouse anti γH_2AX (1:4000, Millipore #05–636) or rabbit anti γH_2AX (1:1000, Santa Cruz SC-101696) and mouse anti-ATM phosphorylated at serine 1981 (1:1500, Cell Signaling #4526 L). For detection of 8-oxoG immunoreactivity, a published protocol [31] was used as follows: Coverslips were fixed in 100% methanol (−20 °C), pretreated for one hour with RNase A (100 μg/ml), denatured with 50 mM NaOH (10 min) followed by 3 × 3 min washes in PBS and incubation with primary antibody. Subsequently, coverslips were washed 3× with 1% BSA and incubated 45 min with goat-anti mouse 488 and goat anti-rabbit 594 AlexaFluor secondary antibodies, mounted with Prolong® Gold Anti-fade with DAPI and viewed with 40× objective on Olympus IX71 with QIC-F-M-12-C cooled camera (QImaging, Surrey, BC) and QCapture Pro (QImaging) software.

2.3. In situ imaging of superoxide mediated dihydroethidium oxidation

Changes in superoxide levels were assessed in situ, in live DRG neurons by imaging superoxide-mediated oxidation of dihydroethidium (#D23107, Invitrogen) to 2-hydroxyethidium [32,33] and as we previously described [28,29,34]. Briefly, upon termination of ara-C exposure, dihydroethidium was added at final concentration of 100 nM for 20 min in the dark. Incubation was terminated by quick washes with PBS and coverslips were mounted with DAPI containing mounting medium. Coverslips were observed with Olympus IX71 fluorescence microscope and images were captured sequentially with QIC-F-M-12-C cooled camera fitted with the QCapture Pro software. Fluorescence intensity of individual DRG neurons demarcated by circular boundaries was scored with ImageJ software (NIH) and exported to Excel for determination of average intensity and further analysis. At least 30 DRG neurons obtained in 3 independent experimental sets were scored for each condition.

2.4. mtDNA copy number determination by real-time quantitative PCR

Total DNA was isolated from $\sim 3 \times 10^4$ DRG neurons using easy DNA isolation kit (Life Technologies) and RNA was digested with RNase A (40 μg/ml/30 min/37 °C). Real-time qPCR reactions were assembled in duplicates with 10 ng total DNA and SSO FAST Evagreen supermix (Biorad, Hercules, CA). The single-copy nuclear gene beta-2-microglobulin (B2M) was used as reference for determination of mtDNA copy number. A 180-nucleotide fragment of mitochondrial gene encoding cytochrome oxidase subunit 3 (COX3) was amplified after validation of qPCR data by targeting two additional mitochondria encoded genes, COX1 and ND1 that yielded similar results. mtDNA copy number was calculated using the formula: mtDNA copy number = 2 × 2 (CTC°xIII - CTB2M) [28,35,36]. Primers for COX3 (ID17710) were F-caattacatgagctcatcatagc and R-ccatggaatccagtagcca and for B2M (NM_009735) F-atccaaatgctgaagaacgg and R-atcagtctcagtgggggtga. Three sets of independent experiments were carried out and mean ± SEM values for mtDNA copy number were calculated.

2.5. Measurement of mitochondrial gene expression by real-time quantitative PCR

Total cellular RNA was isolated from cultured DRG neurons (4×10^4/3.5 cm dish) using RNeasy plus mini kit (Qiagen) and reverse transcribed with iScript RT supermix (Biorad), which contains random as well as oligo dT primers. Real-time PCR was done with CFX96 Real-Time System (Biorad) as we described [28–30]. B2M and 18 S gene transcripts were used as internal controls. PCR reactions were assembled in duplicates with SSO FAST Evagreen supermix (Biorad). PCR program was: 95 °C 2 min, 40 cycles of 95 °C 5 s, 55 °C 15 s. Data represent averages of at least 3 sets of independent biological experiments. The relative amount of target gene RNA was calculated as described [37] using the formula: -ΔΔCt = [(CT gene of interest - CT internal control) sample - (CT gene of interest - CT internal control) control]. Primer sequences are listed in Table 1.

2.6. Measurement of oxygen consumption rates (OCR)

Oxygen consumption rates (OCR) were measured using XF24