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Ototoxicity of paclitaxel in rat cochlear organotypic cultures

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ABSTRACT

Paclitaxel (taxol) is a widely used antineoplastic drug employed alone or in combination to treat many forms of cancer. Paclitaxel blocks microtubule depolymerization thereby stabilizing microtubules and suppressing cell proliferation and other cellular processes. Previous reports indicate that paclitaxel can cause mild to moderate sensorineural hearing loss and some histopathologic changes in the mouse cochlea; however, damage to the neurons and the underlying cell death mechanisms are poorly understood. To evaluate the ototoxicity of paclitaxel in more detail, cochlear organotypic cultures from postnatal day 3 rats were treated with paclitaxel for 24 or 48 h with doses ranging from 1 to 30 μM. No obvious histopathologies were observed after 24 h treatment with any of the paclitaxel doses employed, but with 48 h treatment, paclitaxel damaged cochlear hair cells in a dose-dependent manner and also damaged auditory nerve fibers and spiral ganglion neurons (SGN) near the base of the cochlea. TUNEL labeling was negative in the organ of Corti, but positive in SGN with karyorrhexis 48 h after 30 μM paclitaxel treatment. In addition, caspase-6, caspase-8 and caspase-9 labeling was present in SGN treated with 30 μM paclitaxel for 48 h. These results suggest that caspase-dependent apoptotic pathways are involved in paclitaxel-induced damage of SGN, but not hair cells in cochlea.

Introduction

Paclitaxel is an antineoplastic drug widely used for the treatment of ovarian, breast, lung and, cervical cancers as well as many head and neck neoplasms (Forastiere et al., 1993; Hunter et al., 2011; Jeansonne et al., 2011; Bicaku et al., 2012). As a tubulin stabilizer, paclitaxel inhibits proliferation of tumor cells by inducing tubulin polymerization, hindering the formation of the mitotic spindle causing the arrest of proliferating cells in the G2/M-phase of the cell cycle which ultimately leads to tumor cell death by either apoptosis or phagocytosis (Tishler et al., 1992; Foland et al., 2005). Critical side-effects of paclitaxel include neurotoxicity, hypersensitivity reactions, hematologic toxicity, cardiac disturbances and gastrointestinal tract symptoms (Arbuck et al., 1993; Laskin et al., 1993; Rowinsky et al., 1993) limiting its clinical use. Peripheral neuropathy is the most common side-effect of paclitaxel resulting in a variety of sensory symptoms such as numbness and paresthesia in a glove-and-stocking distribution (du Bois et al., 1999; Pace et al., 2007). In neurons, paclitaxel-induced tubulin polymerization interferes with axonal transport which presumably is responsible for the peripheral neuropathy. The neurotoxic effects of paclitaxel on the dorsal root ganglia (DRG) are dose and time dependent and often result in sensory disorders such as allodynia and hyperalgesia and a reduction of sensory nerve conduction velocity (Cavaletti et al., 2000). The damaging effects of paclitaxel on peripheral axons are thought to be due to it neurotoxic effects on satellite cells, peripheral glial cells that form the protective and supporting envelope of myelin surrounding peripheral axons (Kiya et al., 2011).

Since paclitaxel preferentially damages sensory neurons in the dorsal root ganglion, it may be predicted to similarly disrupt neurons within the auditory periphery. Although several clinical reports suggest that paclitaxel can cause mild to moderate sensorineural hearing loss (Gogas et al., 1996; Tibaldi et al., 1998), direct evidence elucidating the nature and locations of its toxic effects on sensory hair cells, cochlear support cells and neurons is lacking. One reason factor that clouds our understanding of the ototoxic of paclitaxel is that it is often co-administered with a variety of other antineoplastic agents, such as cisplatin (Ding et al., 2011a; Jamesdaniel et al., 2012), that are known to cause hearing loss (Bellmunt et al., 2012). Consequently, functional deficits to the auditory system occurring during or after antitumor treatment were almost always attributed to other ototoxic drugs (Cavaletti et al., 1997; Wandt et al., 1999). Another possible reason why paclitaxel by itself may not be considered ototoxic is that it may cause a "hidden hearing loss" by primarily damaging the auditory nerve fibers rather than the outer hair cells (OHC). Damage to auditory nerve fibers or inner hair cells (IHC) has little effect on hearing thresholds when assessed with the "gold standard" audiogram (Lobarinas et al., 2013).

Abbreviations: taxol, Paclitaxel; SGN, spiral ganglion neurons; OHC, outer hair cells; IHC, inner hair cells; ABR, auditory brainstem response; ANF, auditory nerve fibers.

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However, if paclitaxel were to selectively damage auditory nerve fibers within the inner ear its toxic effects might only be detected by measuring the compound action potential (Ding et al., 1999; Salvi et al., 1999) or the auditory brainstem response (ABR), in particular wave I (Starr et al., 2001; Ye et al., 2012). This is consistent with previous animal studies in which auditory brainstem response (ABR) threshold elevations were observed in mice and guinea pigs with intact sensory hair cell suggesting that selective nerve damage, characteristic of peripheral neuropathy, is responsible for the normal cochlear potentials (Atas et al., 2006). Thus, the histopathological consequences of paclitaxel treatment may be interesting to investigate under well controlled conditions to determine if paclitaxel damages the sensory hair cells or if it preferentially damages the nerve fibers of the spiral ganglion neurons (SGN) similar to "auditory neuropathy," a condition in which the hair cells are functional normal, but the auditory nerve is damaged thereby preventing sound transmission to the central nervous system (Starr et al., 2003; Berlin et al., 2005; Zeng et al., 2005; Berlin et al., 2010). Accordingly, studies were performed to determine whether paclitaxel by itself is damaging to the sensory hair cells, supporting cells and neurons in the inner ear. To accomplish this, we applied various doses of paclitaxel to cochlear organotypic cultures from postnatal day three (P3) rats and identified the histopathological changes occurring in the hair cells, support cells, SGN and their nerve fibers.

Materials and methods

Cochlear organotypic cultures and paclitaxel treatment. Cochlear organotypic cultures were prepared from postnatal day 3 SASCO Sprague-Dawley rats following procedures similar to those described previously (Wei et al., 2010; Ding et al., 2011b). The cochlea was removed and the entire organ of Corti and SGN transferred on to rat tail type I collagen gel in basal Eagle medium containing 2% sodium carbonate. A 15-μl drop of the collagen solution was placed on the surface of a 35 mm culture dish and allowed to gel for approximately 30 min. Afterwards, 1.3 ml of culture medium (0.01 g/ml bovine serum albumin, 1% Serum-Free Supplement (Sigma I-1884), 2.4% of 20% glucose, 0.2% penicillin G, 1% BSA, 2 mM glutamine, 95.4% of 1 BME) was added to the dish. The cultures were placed in an incubator at 37 °C in 5% CO_2 overnight. On the following day, fresh medium was added alone or in the presence of various concentrations of paclitaxel. Paclitaxel stock solution was made at a concentration of 30 mM in 1 μl DMSO and diluted in culture medium to a final paclitaxel concentration of 0 μM, 1 μM, 10 μM or 30 μM. Cochlear explants were treated with culture medium containing 1 μM, 10 μM or 30 μM paclitaxel or control medium (0 μM paclitaxel) containing an equivalent concentration of DMSO (0.001 μl) and cultured in 5% CO_2 at 37 °C in a humidified atmosphere. These experiments were approved by the University at Buffalo's Animal Care and Use Committee.

Histological evaluation. At the end of each experiment, the cochlear explants were fixed with 10% formalin for 2 h and subsequently washed with 0.1 M phosphate buffered saline (PBS). Auditory nerve fibers and SGN were labeled and evaluated along the entire length of the cochlea (Ding et al., 2011b). Specimens were immunolabeled with primary monoclonal antibody against neuronal class III β-tubulin (Covance, TUJ1, # MMS-435P; 1:100 dilution) overnight at 4 °C. After washing with PBS three times, specimens were labeled with a secondary antibody conjugated with Cy3 (goat anti-mouse IgG, Jackson ImmunoResearch; #115-165-205, lot 80054; 1:200 dilution) for 60 min at room temperature. To visualize F-actin that is heavily expressed in the stereocilia bundles and cuticular plate of the hair cells specimens were labeled with phalloidin conjugated to Alexa Fluor 488 (Life Technologies, A12379; lot 1488579; 1:100 dilution); labeling was carried out after completing the secondary antibody labeling. To assess paclitaxel-induced pathological changes to the nucleus, specimens were stained with To-Pro-3 (Life Technologies; #53605; Lot 1351934; 1 mM stock solution diluted 1:1000) for 20 min following the manufacturer's protocol. After rinsing with PBS, specimens were mounted on glass slides in glycerin, coverslipped and examined using a confocal microscope (Zeiss LSM-510 meta, step size 0.5 mm per slice) using appropriate filters to detect the red fluorescence of Cy3 labeling in auditory nerve fibers and SGN (excitation 550 nm, emission 570 nm), green fluorescence of Alexa Fluor 488 in the stereocilia bundles and cuticular plate of hair cells (excitation 488 nm, emission 520 nm) and far red fluorescence of To-Pro-3 in the nucleus (excitation 642 nm, emission 661 nm). Confocal images were stored on disk and processed using Confocal Assistant and Adobe Photoshop 5.5 software.

Hair cells counts. To quantify hair cell loss produced by paclitaxel, cochlear cultures were examined under a fluorescence microscope (Zeiss Axioskop 400×) equipped with appropriate filters for detection of Alexa Fluor 488 fluorescence. The numbers of missing IHC and OHC were counted in consecutive 0.24 mm segments along the entire length of the cochlea (Ding et al., 2011b). Hair cells were classified as missing if the stereocilia and the cuticular plate were absent. Using hair cell count data from normal SASCO Sprague-Dawley rats as a reference (i.e., lab norms), a cochleogram was constructed showing the percent IHC and OHC loss as a function of the percent distance from the apex of the cochlea. Hair cell losses from individual cochleograms were averaged to generate a mean cochleogram for each condition using custom software.

TUNEL and caspase staining. Cochlear explants were treated with 30 μM paclitaxel in culture medium or control medium for 48 h to evaluate the extent of TUNEL labeling using the APO-BrdU TUNEL Assay Kit (Life Technologies, #A23210) according to the manufacturer's protocol. To determine if cell death was caspase-mediated, cochlear explants were also evaluated for the expression of activated caspases-6, -8 or -9 using fluorescently-labeled caspase-6 inhibitor (CaspaTag 6, FAM-VEID-FMK, # 654, 1:30 in solvent, Intergen), caspase-8 inhibitor (CaspaTag 8, # 8004, 1:30 in solvent, Intergen) or caspase-9 inhibitor (CaspaTag 9, # 8003, 1:30 in solvent, Intergen) following the manufacturer's instructions. The caspase inhibitors enter living cells and irreversibly bind to the specific activated caspase present in cells undergoing apoptosis (Amstad et al., 2001). Cochlear specimens were incubated for 1 h at 37 °C with a caspase-6, -8 or -9 probe ($n = 6$/caspase probe). Samples were subsequently fixed with 10% formalin in PBS for 20 min and then labeled with To-Pro-3 as described above. Cochlear cultures were mounted in glycerin on glass slides and examined with a confocal microscope using appropriate filters to detect the carboxyfluorescein activated caspases (excitation 488 nm, emission 529 nm) and with To-Pro-3 which labels the nuclei. Images were processed with Advanced Imaging Microscopy (version 4.0, Carl Zeiss) and Adobe Photoshop as described previously (Qi et al., 2008).

Results

Paclitaxel damaged hair cells, auditory nerve fibers and SGN

After 24 h treatment, no obvious histopathologies were observed in hair cells, auditory nerve fibers or SGN of organotypic cultures ($n = 5$/condition 1 μM, 10 μM and 30 μM) with any of the doses of paclitaxel employed (data not shown). However, after 48 h exposure, paclitaxel produced significant damage to cochlear hair cells which increased in a dose-dependent manner. Fig. 1A shows an example of a normal control cochlea maintained in culture for 48 h ($n = 5$). Staining with Alexa Fluor 488 revealed bright green staining of the stereocilia and to a lesser extent the cuticular plate of the three orderly rows of OHC and one row of IHC (Fig. 1A). Cell nuclei were labeled with To-Pro-3;