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EXPOSURE TO DIPHTHERIA TOXIN DURING THE JUVENILE PERIOD IMPAIRS BOTH INNER AND OUTER HAIR CELLS IN C57BL/6 MICE

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Abstract—Diphtheria toxin (DT) administration into transgenic mice that express the DT receptor (DTR) under control of specific promoters is often used for cell ablation studies *in vivo*. Because DTR is not expressed in mice, DT injection has been assumed to be nontoxic to cells *in vivo*. In this study, we demonstrated that DT application during the juvenile stage leads to hearing loss in wild-type mice. Auditory brainstem response measurement showed severe hearing loss in C57BL/6 mice administered DT during the juvenile period, and the hearing loss persisted into adulthood. However, ototoxicity did not occur when DT was applied on postnatal day 28 or later. Histological studies demonstrated that hearing loss was accompanied by significant degeneration of inner and outer hair cells (HCs), as well as spiral ganglion neurons. Scanning electron microscopy showed quick degeneration of inner HCs within 3 days and gradual degeneration of outer HCs within 1 week. These results demonstrated that DT has ototoxic action on C57BL/6 mice during the juvenile period, but not thereafter, and the hearing loss was due to degeneration of inner and outer HCs by unknown DT-related mechanisms.

Key words: cochlea, degeneration, diphtheria toxin, hair cell, hearing loss, ototoxicity.

INTRODUCTION

Conditional ablation of specific cells is an indispensable technique for studying cellular functions *in vivo*. In recent studies, transgenic or knock-in mice, which are designed to express diphtheria toxin (DT) receptor (DTR) in a specific cell-type, have been used for conditional ablation studies (Saito et al., 2001). DT is an exotoxin secreted by *Corynebacterium diphtheriae*. After binding to DTR, which is identical to human heparin-binding epidermal growth factor-like growth factor (HB-EGF) (Naglich et al., 1992), DT is translocated to endosomes by endocytosis, and the A-fragment of DT is released into the cytoplasm (Collier and Kandel, 1971; Gill and Dinius, 1971; Dorland et al., 1979). The released A-fragment then catalyzes ADP-ribosylation of elongation factor-2 (EF-2) and inhibits protein synthesis, thereby inducing cell death (Honjo et al., 1968; Robinson et al., 1974). The DT-induced ablation system in mice is based on the fact that DT binds to human HB-EGF but not to mouse HB-EGF (Mitamura et al., 1995; Cha et al., 1998). Mice have been shown to be resistant to DT (Pappenheimer et al., 1982), and conditional ablation of specific cells, which exogenously express DTR, is achieved by systemic administration of DT (Saito et al., 2001).

Because DT itself is assumed to have no biological effect on wild-type (WT) mice, several studies failed to include a control group that treated WT mice with DT in their study (Kwon et al., 2014; Wang et al., 2014). Conversely, some studies have reported unexpected off-target effects of DT on WT mice, such as weight loss (Meyer Zu Horste et al., 2010; Goldwich et al., 2012; Christiaansen et al., 2014), proteinuria (Goldwich et al., 2012), and mucosal inflammation of the lung (Chapman

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Abbreviations: ABR, auditory brainstem response; DT, diphtheria toxin; DTR, DT receptor; EDTA, ethylenediaminetetraacetic acid; EF-2, elongation factor-2; H&E, hematoxylin and eosin; HB-EGF, heparin-binding epidermal growth factor-like growth factor; HC, hair cell; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IHC, inner hair cell; OHC, outer hair cell; P, postnatal; PB, phosphate buffer; PBS, phosphate-buffered saline; PFA, paraformaldehyde; SEM, scanning electron microscopy; SGN, spiral ganglion neuron; SPL, sound pressure level; SV, stria vascularis; TNBT, tetranitro blue tetrazolium; W, week old; WT, wildtype.

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and Georas, 2013). Furthermore, high-dose DT was shown to be lethal in mice (Bonventre et al., 1973; Goldwich et al., 2012; Christiaansen et al., 2014). Because these reports used purified DT with few contaminants, such as endotoxins, and demonstrated consistent results using DT supplied from different vendors (Meyer Zu Horste et al., 2010; Chapman and Georas, 2013; Christiaansen et al., 2014), these studies demonstrated that DT causes adverse effects on mice.

In an analysis using DTR knock-in mice from the C57BL/6 strain, results unexpectedly showed that DT-treated juvenile mice exhibited abnormal behaviors in response to auditory stimuli, and this abnormality was also observed in C57BL/6 WT mice. Therefore, we hypothesized that DT might induce side effects, such as hearing loss. In the present study, we investigated the effects of DT during postnatal development, and results revealed that inner hair cells (IHCs), outer hair cells (OHCs), and spiral ganglion neurons (SGNs) were impaired after DT treatment, and juvenile C57BL/6 mice were more susceptible to DT.

EXPERIMENTAL PROCEDURES

Animals

Male C57BL/6J and CBA/J mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). All experimental procedures were conducted in accordance with standard guidelines for animal experiments from the Nagoya University Graduate School of Medicine. This study was approved by the local animal ethics committee of Nagoya University (approval number: 26181, 27204 and 28303). All efforts were made to minimize the number of animals and their suffering.

Recording of auditory brainstem response (ABR)

Phosphate-buffered saline (PBS) or DT (50 μg/kg, Sigma, St Louis, MO, USA) was intraperitoneally injected into postnatal (P) 7, P14, P28, or 8-week-old (W) mice. Survival rates were almost 100%, and neither morphological nor behavioral abnormalities were observed after treatment with this DT dose. ABR was measured as described in our previous reports at 14 or 15 days after DT administration (AD Instruments Pty. Ltd., Castle Hill, Australia) (Ohgami et al., 2010). Tone-burst stimuli at 4, 12, 20, and 32 kHz were recorded in 10-dB increments from 0 to 90 or 100 dB sound pressure level (SPL). The threshold was determined by identifying the lowest level of wave I. When any wave was not observed at the highest stimulation level, the threshold was assigned to the highest presentation level plus 10 dB. The number of "scale-out" animals is indicated in each figure. In control mice, the thresholds were higher than previous reports, in particular at high frequency, which could be due to our ABR system setup.

Hematoxylin and eosin (H&E) staining and quantification of SGN numbers

Mice were intraperitoneally injected with PBS or DT (50 μg/kg) at P7. At P21 or P56, mice were anesthetized and perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Inner ears were dissected, post-fixed in the same fixative overnight at 4 °C, and decalcified in 0.1 M PB containing 10% EDTA for several days. The 5-μm-thick paraffin sections were cut on a microtome, deparaffinized with xylene, and then stained with H&E. After dehydration with an ascending ethanol series, sections were cleared with xylene and mounted. The number of SGNs in the apical, middle and basal turn were quantified and normalized to the spiral ganglion area. A total of 9 sections from 3 animals (3 sections per animal) were analyzed at each time point. The interval of each section was at least 30 μm, and there was no possibility of double-counting the same cells.

Whole-mount tetranitro blue tetrazolium (TNBT) staining of the cochlea

Mice were intraperitoneally injected with PBS or DT (50 μg/kg) at P7 and HC numbers were analyzed at P21. TNBT staining of cochlea was performed in accordance with a previous report (Wang et al., 2011). Briefly, cochleae were dissected and incubated in a staining solution containing sodium succinate and TNBT for 45 min at 37 °C. After fixation with 10% formalin, the bone was carefully removed from the apex using fine forceps, and images of the HC surface were taken by a stereoscopic microscope M60 (Leica Microsystems, Wetzlar, Germany) at the apical, middle, and basal turn. The number of IHCs and OHCs were quantified in each turn and normalized to the length of the basilar membrane. Five animals were analyzed in total.

Scanning electron microscopy (SEM)

Mice intraperitoneally injected with PBS or DT (50 μg/kg) at P7 were analyzed by SEM at P8, 10, 14, and 21. After decapitation, the inner ear was dissected and immediately immersed in a fixative (30 mM HEPES, pH 7.4, 5% glutaraldehyde, 2% PFA, 100 mM NaCl, 2 mM $CaCl_2$). The cochleae were perfused with the fixative, post-fixed in the same fixative for 1 week, and decalcified in 0.1 M PB (pH 6.6) containing 8% EDTA, 4% PFA, and 10% sucrose for at least 3 days. Then the cochleae were processed using the osmium tetroxide and thiocarbohydrazide method (Hunter-Duvar, 1978). After dehydration with a critical point dryer (EM CPD300, Leica Microsystems), images of the middle turn were taken by an electron microscope (JSM-7610F, JEOL Ltd, Tokyo, Japan) with a magnification of 2000× or 15,000×. At least three animals were analyzed and representative images are shown.

Statistical analyses

Values are expressed as mean ± S.E.M. Data were analyzed using Student's *t*-test, and $P < 0.05$ was considered statistically significant.