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EPICATECHIN INHIBITS RADIATION-INDUCED AUDITORY CELL DEATH BY SUPPRESSION OF REACTIVE OXYGEN SPECIES GENERATION

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Abstract—Radiation-induced toxicity limits the delivery of high-dose radiation to head and neck lesions. The aim of this study was to investigate the effectiveness of epicatechin (EC), a minor component of green tea extract, on radiation-induced ototoxicity *in vitro* and *in vivo*. The effect of EC on radiation-induced cytotoxicity was analyzed in the organ of Corti-derived cell lines, HEI-OC1 and UB-OC1. The cell viability, apoptosis, reactive oxygen species generation, and mitochondrial membrane potential as well as changes in the signal pathway related to apoptosis were investigated. Then, the therapeutic effects of hearing protection and drug toxicity of EC were explored in a zebrafish and rat model. Radiation-induced apoptosis and altered mitochondrial membrane potential in HEI-OC1 and UB-OC1 were observed. EC inhibited radiation-induced apoptosis and intracellular reactive oxygen species generation. EC markedly attenuated the radiation-induced embryotoxicity and protected against radiation-induced loss and changes of auditory neuromast in the zebrafish. In addition, intratympanic administration of EC was protective against radiation-induced hearing loss in the rat model, as determined by click-evoked auditory brainstem ($P<0.01$). EC significantly reduced the expression of p-JNK, p-ERK cleaved caspase-3, and cleaved PARP compared to their significant increase after radiation treatment. The results of this study suggest that EC significantly inhibited radiation-induced apoptosis in auditory hair cells and may be a safe and effective candidate treatment for the prevention of radiation-induced ototoxicity.

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Abbreviations: ABR, auditory brainstem response testing; DCFDA, 5-(and 6)-carboxyl-2′,7′-dichlorodihydro fluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; dpf, days post-fertilization; EC, Epicatechin; FBS, fetal bovine serum; IHC, inner hair cell; IT, intratympanic; MMP, mitochondrial membrane potential; NAC, *N*-Acetylcysteine; OHC, outer hair cell; PBS, phosphate buffered saline; PI, Propidium Iodide; ROS, reactive oxygen species; SEM, scanning electron microscopy; SNHL, sensorineural hearing loss; TEM, transmission electron microscopy; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling.

Key words: radiation, ototoxicity, apoptosis, epicatechin, ROS.

The use of radiation therapy to treat cancer inevitably involves exposure of normal tissues to toxic treatment. As a result, patients may experience symptoms associated with damage to normal tissue during the course of therapy for a few weeks after therapy or for months or years after treatment. Radiotherapy has become increasingly important for the treatment of head and neck cancer. However, radiotherapy for head and neck cancers can potentially cause hearing loss because the cochlea and auditory pathways are often included in the radiation fields (Low et al., 2008). Radiation-induced sensorineural hearing loss (SNHL) has been observed in 49% of patients, immediately after treatment, and has a frequency of 55% at 2 to 8 years after therapy, among patients treated with cranial irradiation that had exposure to the inner ear (Ho et al., 1999; Kwong et al., 1996). In studies on chemoradiation treatment, a 53% frequency SNHL and 14% of ototoxicity have been reported (Pearson et al., 2006; Langenberg et al., 2004). Among patients who received radiotherapy for nasopharyngeal carcinoma, at least one-third developed significant SNHL after treatment (Ho et al., 1999; Kwong et al., 1996).

Irreversible hearing loss as a consequence of cisplatin administration has been studied, with numerous causative factors and mechanisms identified. Whereas studies on radiation-induced hearing loss are limited, the precise mechanism of radiation-induced hearing loss remains unknown. Radiation can cause two types of hearing impairment, conductive hearing loss originating in the outer and middle ear, and SNHL caused by damage to the cochlea and auditory nerve (Honoré et al., 2002). Radiation-induced changes that could indirectly lead to cochlear hair cell death have been well documented in animals. Radiation primarily damages the cochlear duct, as well as the Organ of Corti and its surrounding elements (Keleman et al., 1963). In addition, it might cause degeneration of the stria vascularis (Gamble et al., 1968). In humans, Schuknecht and Karmody reported atrophy of the basilar membrane, spiral ligament, and stria vascularis in a deafened man who had complete loss of hearing after receiving 5,220 rads (Schuknecht and Karmody, 1966). Recently, Low et al. reported dose-dependent cochlear cell apoptosis and associated reactive oxygen species (ROS) generation after radiation, with p53 possibly playing a key role (Low et al., 2006). They demonstrated that L-*N*-Acetylcysteine (L-NAC) significantly re-

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duced ROS generation and cochlear cell apoptosis in the cochlear cell line after irradiation (Low et al., 2008).

Green tea, consumed in a balanced and controlled diet was reported to improve the overall anti-oxidative status and protect humans against oxidative damage (Erba et al., 2005). In our previous study, we investigated epicatechin (EC), a minor component of green tea, as a factor that prevented cisplatin-induced ototoxicity caused by ROS generation as well as changes in the mitochondrial membrane potential (MMP) (Kim et al., 2008). However, the function and mechanism of EC as a radioprotective agent against radiation-induced ototoxicity has not yet been investigated. The goal of the present study was to investigate the *in vitro* effects of EC on radiation-induced hair cell death in the cochlear organ of Corti-derived cell lines, HEI-OC1 and UB-OC1, and in addition, the *in vivo* effects of EC in zebrafish and rats. Moreover, the associated signaling mechanisms, specifically those involving p53, MAPK, and caspase-3 were also studied.

EXPERIMENTAL PROCEDURES

Cell culture

The establishment and characterization of the conditionally immortalized HEI-OC1 cell line was described by Kalinec et al. (Kalinec et al., 2003). HEI-OC1 and UB-OC1 were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco) and 50 U/ml interferon-γ (Genzyme, Cambridge, MA, USA). For the experiments described later in the text, HEI-OC1 and UB-OC1 were cultured under permissive conditions: 33 °C, 5% CO_2 in DMEM supplemented with 10% FBS.

Animals

Zebrafish (*Danio rerio*) embryos of the AB wild-type strain were produced by paired mating of adult fish at 28.5 °C. The embryos were maintained in 100 mm^2 petri dishes in embryo media [1 mM $MgSO_4$, 120 μM KH_2PO_4, 74 μM Na_2HPO_4, 1 mM $CaCl_2$, 500 μM KCl, 15 μM NaCl, and 500 μM $NaHCO_3$ in deionized H_2O (dH_2O)] at a density of about 50 embryos per dish. Beginning 4 days post-fertilization (dpf), larvae were fed dehydrated paramecia.

Twelve female Sprague–Dawley rats from Samtaco (Samtaco, Osan, Korea) weighing between 180 and 220 g were used for the present study. After transportation, the animals were maintained in the central animal laboratory for at least 1 week. The animals were housed in independent ventilation cages and were allowed free access to water and food. The temperature was maintained at 21 °C±1 °C, and lights were turned on from 8:00 AM to 8:00 PM. Rats with an inner ear infection were not used.

This study was approved by the Committee for Ethics in Animal Experiments of the Ajou University School of Medicine.

Radiation exposure and drug treatments of the rats

Animals were sedated using an i.p. injection of 3.125 mg/kg tiletamine, 3.125 mg/kg zolazepam, and 11.5 mg/kg xylazine hydrochloride. Then, the rat was placed and restrained in the prone position on an acryl plate. The radiation was restricted to the head centered across the cochlea to spare the rest of the body. A single dose of the radiation, 20 Gy was delivered by opposed photon beams at a rate of 2 Gy per min bilaterally with a distance of 100 cm from the source to the axis using the LINAC, 6MV (21EX, Varian Co., Palo Alto, CA, USA). The 12 rats were divided into three groups (Control group, Radiation only group, and Radiation plus EC group). The treatment group (eight rats, 16 ears) received 2 mM intratympanic (IT) EC (Sigma Chemical Company, St. Louis, MO, USA) in the right ear (Radiation plus EC group) and saline in the left ear (Radiation only group), before radiation treatment. The control group (four rats, eight ears) received intratympanic saline in both ears.

For animals receiving EC or saline, an intratympanic injection was given slowly through a myringotomy in the anterosuperior quadrant with an operating microscope before the radiation treatment. Immediately after irradiation, the animals were removed from the Lucite jig and housed (five animals/cage) in a climate and a light/dark-controlled environment and allowed free access to food and water.

Auditory brainstem response testing

Pre-treatment ABR thresholds were obtained in all experimental mice and the post-treatment ABR was obtained on day 7. Preyer's reflex and otoscopy were used to confirm that the middle ears were normal. The rats were anesthetized using an i.p. injection of 3.125 mg/kg zolazepam and 11.5 mg/kg xylazine. Subdermal sterile stainless steel electrode needles were attached, with the active lead at the vertex and referred to a second electrode located at the tip of the nose. The ground electrode was placed on the arm muscles. ABR stimuli were generated using a DT Auditory Evoked Potential Workstation (Tucker-Davis Technologies, Alachua, FL, USA); 10-ms tone burst stimuli (8 kHz) were delivered monaurally through a hollow rat ear bar. Tone bursts (rise–fall time 1 ms, duration 10 ms) were delivered at the rate of 20 s^{-1}, with increasing intensity from 10 to 80 dB sound–pressure levels in 5-dB steps; 1500 trials were averaged to assure an adequate brain response. The lowest response that clearly demonstrated a reproducible waveform was interpreted as the threshold response.

Analysis of treatment effects on zebrafish morphology and survival

Dechorionated embryos at 4 dpf were anesthetized with 8 μg/ml 3-aminobenzoic acid ethyl ester methanesulfonate salt (MS-222; Sigma Chemical Co.) and immobilized by placing them on 3% methylcellulose on a glass depression slide. The morphology was assessed visually using light transmission microscope (AXIO vert 200, Carl zeiss, Göttingen, Germany) at ×60 to ×100 magnification, and representative images were recorded using Axiovision. Similarly, survival of embryos was assessed visually at 24-h intervals up to 7 dpf by light microscopy. The criterion used for embryonic survival was the presence of cardiac contractility.

Examination of neuromasts in zebrafish

Wild-type zebrafish (*D. rerio*) were maintained at 28.5 °C on a 14 h light/10 h dark cycle. At 4 dpf, larvae were maintained at a density of 50 per 100 mm in two petri dishes with embryo medium (174 mM NaCl, 21 mM KCl, 12 mM $MgSO_4$, 18 mM $Ca(NO_3)_2$ $4H_2O$, 15 mM HEPES) and placed in a tissue incubator at 28.5 °C. The EC was prepared by adding the pure powder to the embryo medium. The EC was diluted in embryo medium to a final concentration of 200 μM in a 6-well plate. Then 4 dpf zebrafish larvae were exposed to 10 Gy, 15 Gy, and 20 Gy of radiation. The hair cell lateral line neuromasts were labeled using 2 μM YO-PRO1 (Molecular Probes, Eugene, OR, USA) for 1 h followed by three rinses. The zebrafish were then rinsed three times (5 min per wash) in embryo medium and anesthetized with 8 μg/ml MS-222 (Sigma Chemical Co.). The zebrafish were mounted with methylcellulose in a depression slide for observation under a fluorescence microscope.