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 neuropasm 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. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

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 (Honore 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-
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% CO2 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 mm² petri dishes in embryo media [1 mM MgSO4, 120 μM KH2PO4, 74 μM Na2HPO4, 1 mM CaCl2, 500 μM KCl, 15 μM NaCl, and 500 μM NaHCO3 in deionized H2O (dH2O)] 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 individual ventilation cages and were allowed free access to food and water. The temperature was maintained at 21 ± 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 and fluorescence microscopy. (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 mm² in two petri dishes with embryo medium (174 mM NaCl, 21 mM KCl, 12 mM MgSO4, 18 mM Ca(NO3)2, 4H2O, 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.
Scanning electron microscopy

For the scanning electron microscopy (SEM), zebrafish embryos (4 dpf) were fixed overnight in 2.5% glutaraldehyde in phosphate buffered saline (PBS) (pH 7.2) at 4°C. The embryos were washed three times (5 min per wash) in distilled water and dehydrated through a graded series of 25, 50, 70, 80, 95, and 100% ethanol solution for 10 min each. The samples were then desiccated through a graded series of 25, 50, 75, and 100% isoamyl acetate in ethanol solution for 10 min. They were then dried using a critical point dryer (CPD). The dried specimens were sputter-coated twice with carbon using an evaporator (MED010; Baltek, Hudson, NH, USA) and observed under a scanning electron microscope (JSM-6700F; JEOL, Tokyo, Japan) operating at 5 or 7 kV.

Transmission electron microscopy

For the transmission electron microscopy (TEM), the HEI-OC1 cells were fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) + 0.001% CaCl2 for 1 h on ice. The HEI-OC1 cells were washed in cacodylate solution, dehydrated through the graded ethanol series, and infiltrated and embedded in Spur’s epoxy resin via propylene oxide. The cells were oriented to obtain cross sections in a rostral to caudal manner. Semi-thin sections (2 μm) were collected and stained with 1% Toluidine Blue. Ultrathin sections (90 nm) were cut on an Ultracut S microtome (Leica, Wetzlar, Germany), mounted on 200-mesh Athene thin bar-grids, with uranyl acetate and lead citrate contrast. The grids were examined using a transmission electron microscope (EM 902A; Carl Zeiss, Oberkochen, Germany).

Cochlea immunohistochemical analysis

For immunohistochemistry study, DAKO immunohistochemistry kit (DAKO LSAB Universal K680, Carpenteria, CA, USA) was used according to the manufacturer’s instruction. Removable temporal bone was fixed in 4% paraformaldehyde for 16 h, and then decalcified with 10% EDTA in PBS for 2 weeks, dehydrated, and embedded in paraffin wax. Sections of 5 μm were deparaffinized in xylene and rehydrated through a gradual concentrations of ethanol. The endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min. In xylene and rehydrated through a gradual concentrations of 25, 50, 75, and 100% ethanol. The endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min. After three washes with PBS (pH 7.4), the sections were incubated with endogenous biotin by incubation with 0.1% biotin blocking solution in PBS. Nonspecific binding was blocked with 1% bovine serum albumin for 1 h. The sections were then incubated with biotinylated secondary rabbit antibody for 1 h and, then, covered with 30 min with a secondary antibody containing horseradish peroxidase. Finally, the sections were stained in a freshly prepared substrate solution (3 mg of 3-amino-9-ethylcarbazole in 10 ml of sodium acetate buffer pH 4.9, 500 μl of dimethylformamide, 0.03% hydrogen peroxide) for 10 min.

Cell viability assay

To determine cell viability, the HEI-OC1 and UB-OC1 cells were seeded in 96-well plates at densities of 5×103 cells/well in 1 ml complete medium after they were exposed to various doses of radiation (0–30 Gy), various concentrations of EC (0–200 μM), or radiation plus EC. At 72 h interval after irradiation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma Chemical Co.) was added to 40 μl of the cell suspension for 4 h. After three washes with PBS (pH 7.4), the insoluble formazan product was dissolved in dimethyl sulfoxide (DMSO). The optical density (OD) of each culture well was measured using a microplate reader (Bio-Tek, Winooski, VT, USA) at 540 nm.

Annexin V-FITC/PI staining

Annexin V-FITC/Propidium iodide (PI) staining was processed to determine the percentage of apoptotic cells at 72 h after irradiation in a quantitative manner. The cells were stained with Annexin V-FITC apoptosis detection kit following the manufacturer’s protocols (BD Biosciences, Franklin Lakes, NJ, USA). For the assay, the HEI-OC1 cells were cultured overnight in 6-well plates and then treated with 20 Gy radiation in the presence or absence of EC (200 μM). Following the drug treatments, the cells were washed with PBS three times and resuspended in 1× binding buffer (10 mM HEPES/NaOH at pH 7.4, 140 mM NaCl, and 2.5 mM CaCl2). Afterward, the cells were incubated with Annexin V-FITC and PI for 15 min at room temperature and then analyzed by flow cytometry (BD Biosciences).

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay

Apoptosis in the HEI-OC1 cells was determined by the TUNEL method using an in situ cell detection kit (Roche Molecular Biochemicals, Mannheim, Germany). The HEI-OC1 cells were added to 24-well culture dishes containing growth medium and glass cover slips. After the monolayers achieved 60–70% confluence, the cells were exposed to medium with radiation (20 Gy) in the absence or presence of EC (200 μM). Thereafter, the cells were washed with PBS and fixed in 4% paraformaldehyde. The cells were then incubated with 50 μl of TUNEL reaction mixture (TdT and fluorescein-dUTP) at 37 °C for 60 min in a humid atmosphere. The cells were stained with Hoechst 33342 (5 μg/ml) for 5 min. The stained cells were analyzed under a fluorescence microscope (Carl Zeiss).

Mitochondrial membrane potential assay

The MMP of intact cells was measured by flow cytometry with the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetravinylbenzimidocarbocyanine iodide (JC-1; Molecular Probes). The culture medium was briefly removed from the adherent HEI-OC1 cells, and the cells were rinsed with PBS. Cell monolayers were incubated with DMEM and 5 μg/ml JC-1 at 33 °C for 20 min. The cells were subsequently washed twice with cold PBS and trypsinized. Cell pellets were then resuspended in 500 μl of PBS. The change in MMP was measured by flow cytometry (BD Biosciences) and fluorescence microscopy at 72 h after irradiation.

Measurement of intracellular ROS production

Intracellular generation of ROS was quantified using 5-(and 6)-carboxyl-2′,7′-dichlorodihydro fluorescein diacetate (DCFDA; Molecular Probes, Eugene, OR, USA). This esterified form is cell membrane-permeable and undergoes deacetylation by intracellular esterases. Upon oxidation, DCFDA is converted to highly fluorescent 2′,7′-dichlorofluorescein (DCF). For the assay, the HEI-OC1 cells were cultured overnight in 6-well plates and then treated with 20 Gy of radiation in the presence or absence of EC for 24 h. The cells were incubated in the dark with 10 μM DCFDA in serum-free medium for 10 min at 33 °C. An oxidative burst (hydrogen peroxide, H2O2) was detected using a FACScan flow cytometer (BD Biosciences) with excitation and emission settings at 488 and 530 nm, respectively.

Western blot assay

Total proteins were extracted using the ProteoExtract® Subcellular Proteome Extraction Kit (Calbiochem, La, Jolla, CA, USA) following the manufacturer’s instructions. Protein concentrations were measured using the BCA assay (Pierce, Rockford, IL, USA). The proteins were separated by electrophoresis on 12% and 10% sodium dodecyl sulfate polyacrylamide gels. An equal amount of protein (10 μg) was loaded in each lane. After electrophoresis, the proteins were transferred onto polyvinylidene difluoride (PVDF)
membranes. The membrane was blocked in Tris-Buffered Saline Tween-20 (TBST) containing 5% non-fat milk for 1 h, followed by overnight incubation at 4 °C with primary antibodies. After washing the membrane extensively, incubation with horseradish peroxidase-conjugated secondary antibody (1:1000, Cell Signaling Technology, Danvers, MA, USA) was performed for 1 h at room temperature. Protein bands on the blots were visualized by ECL Plus Western Blot detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistics
The Student t-test and one-way ANOVA were used for the statistical analyses of the data. All statistical analyses were conducted using SPSS 10.0 statistical software (SPSS, Chicago, IL, USA). Parameters of the data from three independent experiments are expressed as the mean±SD. A P<0.05 was considered statistically significant (* P<0.05; ** P<0.01; *** P<0.001).

RESULTS
Epicatechin increased HEI-OC1 cell viability after radiation treatment

As shown in Fig. 1A, C, radiation decreased the viability of the HEI-OC1 and UB-OC1 cells in a dose-dependent manner. We then examined the effect of various concentrations of EC on the radiation-treated cells and discovered that EC significantly protected the HEI-OC1 and UB-OC1 cells from radiation induced cytotoxicity in a dose-dependent manner (Fig. 1B, D).

Epicatechin inhibited radiation-induced apoptosis in HEI-OC1 cells

To quantify and verify the number of apoptotic cells induced by radiation treatment of the HEI-OC1 cells, we used flow cytometry. Annexin V-FITC and Propidium Iodide staining were used to analyze the percentage of apoptotic cells treated with radiation in the absence or presence of EC (Fig. 2A). We found that radiation plus EC treatment (mean 2.27%) significantly decreased the number of early apoptotic cells compared to the cells treated with radiation only (mean 23.02%) (P<0.05). When HEI-OC1 cells were treated with EC alone, no significant change in the Annexin V-FITC/PI positive cells compared to the control was observed. These results indicate that radiation promotes apoptotic cell death and radiation-induced apoptosis can be inhibited by EC pre-treatment.

The TUNEL reaction and DAPI staining were performed to determine if radiation-induced apoptosis of the HEI-OC1 cells occurred by apoptosis and if this can be prevented by EC treatment. As shown in Fig. 2B, EC treatment decreased the number of TUNEL-positive cells. Moreover, EC treatment decreased the radiation-induced cytotoxicity.

Fig. 1. Effect of EC on viability of the HEI-OC1 and UB-OC1 cell lines after treatment with radiation: HEI-OC1 (A, B) and UB-OC1 (C, D) were exposed to various doses of radiation (0–30 Gy), various concentrations of EC (0–200 μM) or radiation plus EC. At 72 h interval after irradiation, cell viability was measured by MTT assay. The data represent the mean±SD of three independent experiments. * P<0.05, ** P<0.01, *** P<0.001, compared to medium alone (A, C) or radiation alone (B, D).
nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies in the HEI-OC1 cells as detected by DAPI staining. These results indicate that radiation promotes apoptotic cell death and that apoptosis was inhibited by EC pre-treatment.

The MMP can be used as an index of mitochondrial pore opening, which is an indicator of mitochondrial dysfunction. Quantitative analysis of red and green fluorescent signals from intracellular JC-1 dye reflects the degree of mitochondrial damage. Therefore, we examined the effect of radiation on MMP in the HEI-OC1 cells to determine whether the loss of MMP could play a role in radiation-induced apoptosis. As shown in Fig. 2C, in the control cells, a high MMP was maintained, as indicated by the pre-dominantly red fluorescence of the JC-1 dye. However, radiation treatment increased the green cell fluorescence indicating a loss of MMP and mitochondrial damage. Treatment with EC inhibited radiation-induced changes in the MMP and cell morphology (Fig. 2C).

Epicatechin inhibited intracellular ROS generated by radiation

We next investigated the effect of radiation on intracellular ROS generation. Cells were treated with 20 Gy of radia-

**Fig. 2.** Effect of EC on radiation-induced apoptosis and mitochondrial membrane potential in HEI-OC1 cells: (A) To quantify the effects of EC on radiation-induced apoptosis, we used flow cytometry; Annexin V-FITC and propidium iodide staining were used to analyze the percentage of apoptotic cells treated with radiation (20 Gy) in the absence or presence of EC (200 µM). The data represent the mean±SD of three independent experiments. * P<0.05, ** P<0.01. (B) TUNEL assay. Apoptosis in HEI-OC1 cells was determined by the TUNEL method using an in situ cell detection kit. After monolayers achieved 60–70% confluence, the cells were exposed to medium with radiation (20 Gy) in the absence or presence of EC (200 µM). The cells were then incubated with 50 µl of TUNEL reaction mixture (TdT and fluorescein—dUTP) and stained with Hoechst 33258 (5 µg/ml). The stained cells (arrow) were observed under a fluorescence microscope. Scale bar=50 µm. (C) Inhibition of MMP in the radiation-treated HEI-OC1 by EC. The cells were treated with radiation (20 Gy) and EC (200 µM) and stained with JC-1 for visualization under the fluorescent microscope. The MMP change was objectively measured using the flow cytometry FACScan. The data represent the mean±SD of three independent experiments. * P<0.05, ** P<0.01. Scale bar=50 µm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
tion, and the level of intracellular ROS was monitored by FACScan flow cytometry with the peroxide sensitive fluorescent probe, DCFDA. Treatment with radiation significantly increased the generation of intracellular ROS. We further examined the effects of EC on radiation-induced ROS generation. As expected, EC significantly inhibited radiation-induced intracellular ROS generation (Fig. 4A, B), suggesting that intracellular ROS may be directly involved in radiation-induced hearing loss. To demonstrate the effects of EC on radiation-induced ROS generation in vivo, we performed an immunohistochemistry study for NOX. In immunohistochemistry study, radiation markedly induced the expression of NOX-3 protein in the cochlea of rat model. However, NOX-3 protein was significantly decreased in the spiral ligament, stria vascularis, spiral limbus, and the hair cells of the organ of Corti in rat treated with both EC plus radiation compared to rat treated with radiation alone (Fig. 4C).

**Inhibition of MAPK activity by epicatechin rescued HEI-OC1 cells from radiation-induced cytotoxicity**

To elucidate the mechanism underlying the activity of radiation and EC, we evaluated the radiation and EC-induced changes on gene expression. We evaluated gene related apoptosis of p53, p38, JNK, ERK, cleaved caspase-3 and cleaved PARP. A representative Western blot is shown in Fig. 5A, B; the results obtained confirmed increased expression of p-JNK, p-ERK, p-p53 (Ser6), p38, cleaved PARP, and cleaved caspase-3 after radiation treatment. Next, the effects of EC treatment on the expression of p53, p38, JNK, ERK, cleaved caspase-3, and cleaved PARP were investigated by Western blot analysis to determine whether EC blocked the MAPK pathway and the apoptotic signal pathway mediating the observed apoptotic responses. EC significantly reduced expression of p-JNK, p-ERK, cleaved caspase-3, and cleaved PARP that were significantly increased after radiation treatment (Fig. 5). These results suggest that EC blocked radiation-induced apoptosis via down-regulation of JNK and ERK.

**Epicatechin decreased radiation induced embryotoxicity and ototoxicity in the zebrafish model**

To determine EC associated toxicity, we exposed zebrafish embryos at 1 dpf to various concentrations of EC and investigated the effects of this treatment on their morphologic appearance and survival up to 6 dpf of development. EC administration was not toxic to the zebrafish embryos; neither viability nor gross morphology were adversely affected (Fig. 6A). Next, we focused on the radioprotective effects of EC as determined by viability assessment of irradiated zebrafish embryos pre-treated by EC. The embryos were exposed at 1 dpf to 10 Gy, whereas 20 Gy caused death in 100% of irradiated embryos within 5 days after radiation exposure. In addition, organogenesis was commenced by 1 dpf; thus, this period of time is the preferred developmental stage to assess radiation-induced effects on most major organs. EC that was given before radiation had a significant effect on the survival advantage of the zebrafish embryos before exposure to 10 Gy ($P<0.05$). This effect was maximal at a concentration of 200 $\mu$M (Fig. 6B). Fig. 6C, D shows the distribution of neuromasts in live 4 dpf zebrafish as detected by staining with YO-PRO1. No adverse effect of the number or the gross morphology of the hair cells in the neuromasts proved the non-toxicity of EC to the neuromasts of zebrafish (Fig. 6C). Radiation exposure resulted in a loss of YO-PRO1 staining of the neuromasts. Treatment of the zebrafish with 10 Gy of radiation decreased the neuro-
masts, and 20 Gy of radiation resulted in additional decrease of the neuromast immunofluorescence. Co-treatment of the cells with radiation plus 200 μM H9262 EC resulted in increased fluorescence intensities compared to cells treated with radiation alone; indicating that EC protected the organ against radiation-induced ototoxicity in a dose-dependent manner (Fig. 6D). SEM was performed to determine hair cell damage by change of YO-PRO1 labeling was indicative of hair cell damage. Fig. 6E shows scanning electron micrographs of stereocilia and kinocilium from radiation and EC-treated zebrafish. Compared to control radiation treated zebrafish showed marked damage to the hair cells in the neuromasts. Exposure to radiation (20 Gy) resulted in severe morphological damage such as loss or cut of the kinocilium. Co-treatment with EC reduced radiation-induced hair cell loss compared to treatment with radiation alone.

Epicatechin inhibited the hearing threshold shift that occurred with radiation in the rat model

As shown in Fig. 7, the ear treated with radiation had an average click-evoked ABR threshold shift that was found to be 68.8 dB ± 7.1 (56–79), whereas the ear treated with intratympanic EC had a minimal average click-evoked ABR threshold shift of 42.5 dB ± 8.3 (29–48). The ABR threshold in the EC treatment group was significantly lower than the radiation only group (P < 0.01), suggesting that EC had an otoprotective effect in the rats subjects given radiation (Fig. 7).

DISCUSSION

Ionizing radiation damages DNA by direct and/or indirect effects on the irradiated cells (Shinomiya, 2001). The frequency of cell cycle disturbance, aberrant mitosis or cell death may increase as the dose of irradiation increases (Stone et al., 2003). Although there is a great deal of information on radiation-associated cell death, there is limited information on the effects of radiation on the auditory hair cells of the cochlea. The aim of the present study was to determine the effects of radiation-induced apoptotic cell death in cochlear cells in vitro and in vivo as well as the protective effects of EC on radiation-induced apoptosis.

In this study, we showed that radiation-induced apoptosis was demonstrated by increased TUNEL-positive cells and by changes in the MMP and EC dose-depend-
ently prevented radiation-induced apoptosis of HEI-OC1 and UB-OC1 cells. EC decreased radiation-induced nuclear condensation, DNA fragmentation, and perinuclear apoptotic body formation in HEI-OC1 cells. Moreover, EC decreased ROS that were generated in response to radiation. These findings suggest that radiation-induced ototoxicity is mediated by ROS and that EC may prevent toxicity by decreasing oxidant-mediated cell damage. Moreover, EC prevented changes in the MMP that were caused by radiation, suggesting that inhibition of ROS generation and preservation of mitochondrial integrity may be critical for the protection of auditory cells from the toxic effects of radiation.

ROS is thought to play a key role in the promotion of apoptosis by affecting mitochondrial permeability, release of cytochrome c, activation of p53 and caspases (Devarajan et al., 2002). Low et al. demonstrated a dose-dependent intracellular generation of ROS at 1 h post-irradiation; this was thought to be an important triggering factor in the apoptotic process (Low et al., 2006). They demonstrated that α-NAC significantly reduced ROS generation and cochlear cell apoptosis after irradiation (Low et al., 2008). Therefore, oxidative stress may play a direct role in radiation-induced apoptosis.

Radiation induces apoptotic death of various cells through the activation of various signaling pathways. Important signaling events that regulate the cell death of radiation-damaged cells include: p53-dependent pathways (Miyashita et al., 1994; Bertout et al., 2009), MAPK protein (Westwick et al., 1995; Verheij et al., 1996), caspases (Tsuji moto, 1998), and necrotic cell death (Shinomiya et al., 2000). Although p53-independent mechanisms have been described (Wichmann et al., 2006), radiation-induced p53 is believed to play an important role; it is associated with cell cycle delay in order to facilitate DNA repair and to trigger apoptosis if irreversible damage has occurred (Verheij and Bartelink, 2000).

Other molecular mechanisms of radiation, include, interaction of ionizing radiation with cellular membranes that induces rapid sphingomyelin hydrolysis to ceramide (Haimovitz-Friedman et al., 1994), and activation of the pro-apoptotic SAPK/JNK pathway that may activate downstream of membrane-derived ceramide signals (Westwick et al., 2006; Verheij et al., 1996). The subsequent phosphorylation of c-Jun upregulates the transcription of apoptosis-regulating genes. Cross talk between the SAPK/JNK pathway and caspase cascade also plays a role in radiation-induced apoptosis. In addition, MAPK and Akt activated by PI3 kinase also play an important role in the mitochondrial path-
Since pre-mitotic apoptosis is a rapid mode of cell death, a prompt activation of pre-existing cytoplasmic caspase-3 may be involved in this process.

The data from this study demonstrate that JNK, ERK, and p38 in MAPKs were activated in the radiation-treated cells. Pharmacological inhibition of JNK and p38 activity was much more effective in the prevention of cell death in radiation-treated HEI-OC1 auditory cells than inhibition of pERK (data not shown). We investigated the effects of EC treatment on the expression of MAPKs in order to deter-

Fig. 6. Effect of EC on the protection of auditory hair cells in vivo: (A) The toxicity profile of EC as assessed by the survival of zebrafish embryos. The time-dependent survival of embryos exposed to EC at 1 dpf at concentrations ranging from 0 to 200 μM. (B) Dose-dependent effects on survival (at day 4 of development) of zebrafish embryos exposed to 10 Gy of ionizing radiation at 1 dpf in the presence of increasing concentrations of EC (0, 50, 100, 200 μM). EC was given 3 h before irradiation. (C) The toxicity profile of EC as assessed by hair cells of the neuromast in the zebrafish. The 4 dpf zebrafish were treated with various concentrations of EC for 24 h. The zebrafish were then stained with YO-PRO-1. Neuromasts that were located on the body were stained as white dots. Scale bar=50 μm. (D) Embryos were exposed to ionizing radiation (20 Gy) at 4 dpf in the absence or presence of 200 μM EC given 4 h before exposure to ionizing radiation and evaluated at 6 dpf. EC treatment preserved hair cell content as assessed by YO–PRO 1 staining of the irradiated zebrafish embryos. Scale bar=50 μm. (E) Scanning electron microscopy micrographs of the control, radiation alone, EC alone and radiation plus EC treated hair cells. * P<0.05, ** P<0.01, *** P<0.001. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
In vivo conservation between the two species (Zon and Peterson, 2008; Daldal et al., 2007). The intratympanic administration of EC significantly decreased the ABR threshold shift. Several studies have demonstrated that intratympanic injection of EC as a drug delivery method in the rat model. Our results showed that administration of these antioxidants reduced deleterious effects, caused by radiation exposure, in normal tissues of tumor bearing mice (Lawenda et al., 2007).

In conclusion, we demonstrated that radiation-induced apoptosis may be mediated by ROS production in auditory hair cell lines. EC, one of the tea catechins, protected auditory cells in vitro and in vivo from radiation by directly blocking ROS generation, inhibiting changes in the MMP, and inhibition of MAPKs. These findings suggest that EC can potentially be used to reduce radiation-induced SNHL, which is a common complication of radiotherapy for head and neck tumors. In addition, EC can be administrated topically through the middle ear, thereby minimizing systemic side effects.

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