Green Tea Polyphenols Protect Cochlear Hair Cells from Ototoxicity by Inhibiting Notch Signalling

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Abstract Notch signalling pathway plays an essential role in the development of cochlea, which inhibits the proliferation of hair cells. Epigallocatechin-3-gallate (EGCG) is the most abundant polyphenol in green tea, which presents strong antioxidant activation and has been applied for anti-cancer and anti-inflammatory. In this study, we treated the cochlear explant cultures with EGCG and found that EGCG can protect cochlear hair cells from ototoxic drug gentamicin. We demonstrated that EGCG could down-regulate the expression of Notch signalling pathway target genes, such as Hes1, Hes5, Hey1 and Hey5. However, the Notch pathway ligands such as Delta1, Jag1 and Jag2 were not affected by EGCG. To further illustrate the mechanism of recover cochlear hair cells, we demonstrated that EGCG inhibited the activity of \( \gamma \)-secrectase to suppress Notch signalling pathway and promoted the proliferation and regeneration of hair cells in the damaged cochlea. Our results suggest for the first time the role of EGCG as an inhibitor of the Notch signalling pathway, and support its potential value in hearing-impaired recovery in clinical therapy.

Keywords Cochlear hair cells · Notch signalling pathway · Polyphenol · EGCG · \( \gamma \)-Secretase

Abbreviations

IHCs Inner hair cells
EGCG Epigallocatechin-3-gallate
NICD Notch intracellular domain

Introduction

In all mammals, the sensory receptors of both the auditory system and the vestibular system are cochlear hair cells. Cochlear hair cells are arranged in four rows in the organ of corti along the entire length of the cochlear coil. The row of cells close to the centre of cochlea are called inner hair cells (IHCs), which provide the main neural output of the cochlea [1, 2]. The other three rows consist of outer hair cells and receive neural input from the brain, which detect movement through mechanotransduction as part of the cochlea’s mechanical pre-amplifier. Damage to these hair cells resulted in decreased hearing sensitivity and sensorineural hearing loss [3]. Because human hair cells are terminally differentiated and are incapable of regeneration, this damage could lead to permanent hearing loss. There are two avenues for the generation of new hair cells [4, 5]: one is the differentiation from inner ear stem cells; another is the trans-differentiation from supporting cells. A series of specific genes and signalling pathways control
these two avenues. In 1999, Bermingham et al. have reported that the gene Atoh1 (as also known as Math-1) serves to initiate inductive signals that regulate the development for hair cell in mice. In subsequent studies, the forced expression of Atoh1 in the mammalian adult inner ear has been reported to cause the trans-differentiation of supporting cells into hair cells [6–9]. Chen and Segil [10] have reported the cyclin-dependent kinase inhibitor p27 (Kip1) plays an essential role in the developmental control of progenitors of the hair cells and supporting cells. White et al. [11] have found the relationship between the expression of p27 (Kip1) and the proliferative capacity of supporting cells, which suggest p27 (Kip1) may be potential target for controlling the trans-differentiation of supporting cells into hair cell. Lanford et al. [12] have firstly demonstrated that the Notch pathway regulate the development of progenitor cells into hair cells, which support a role for the Notch pathway in the development of the cochlea.

The Notch signalling pathway is a highly conserved cell signalling system in multicellular organisms and controls numerous cellular processes, such as cell differentiation, proliferation and apoptosis. As a transmembrane receptor, Notch plays an important role in mediating critically cellular functions by cell to cell communications [13]. Activation of Notch upon DSL (Delta/Serrate/Lag-2) ligands induces proteolytic processing of Notch receptor, within its extracellular domain (NECD) via a disintegrin and metalloprotease (ADAM), which facilitates γ-secretase proteolysis to release the Notch intracellular domain (NICD) from the plasma membrane [14, 15]. The NICD then translocates to the nucleus and interacts with the DNA-binding protein CSL (CBF1, SuH, LAG-1), and participates in the transcriptional regulation of target genes, such as Hes1, Hes2, Hey1 and Hey2 [16, 17]. With the further research of the development of cochlear hair cell, various studies have demonstrated Notch signalling pathway plays essential roles in the regulation of the cochlear hair cell. In 1998, both of Adam et al. [18] and Haddon et al. [19] have reported the lateral inhibition mediated by Notch signalling pathway in the control of hair cell differentiation, which plays an essential role in the development of vertebrate inner ear. In 1999, Lanford et al. have demonstrated the same inhibition in the differentiation of mammalian hair cell by Notch signalling pathway. In their studies, Notch signalling pathway exerts lateral inhibition in the development of cochlear mosaic, which plays an important role in the determination of various cell fates. According to the recent studies, inactivation of the Notch signalling pathway can promote the proliferation of hair cells [7, 20–22]. Therefore, the inhibitors of γ-secretase might promote the proliferation and regeneration of hair cells in the damaged cochlea by targeting the Notch receptors to suppress the Notch activity [22].

In recent years, tea has attracted increasing attention for health and delaying aging because of their powerful antioxidant properties. Tea polyphenols (include catechins, theaflavins, tannins and flavonoids) extracted from tea leaves have been extensively used for the prevention and treatment of human cancers [23]. Among the various polyphenols in tea, the most significant phytochemical is Epigallocatechin gallate (EGCG). EGCG is a type of catechin, which is the most abundant, and active compound and presents much stronger antioxidant activation than Vitamin C [24]. In biochemical research fields, the benefits of EGCG have begun to emerge [25]. In 1998, Pietta et al. [26] have found that EGCG has the ability to inhibit lipid peroxidation, which may decrease the risk of heart disease. In 2000, Fujiki et al. [27] have suggested EGCG can inhibit the release of tumor necrosis factor-alpha causing the suppression of tumor promotion. Besides cancer and heart disease, lots of studies have reported EGCG can confer protection against other diseases, such as osteoporosis [28], liver and kidney damage [29], bacterial infection [30] and viral infection [31].

In the present work, we demonstrated EGCG can promote the regeneration of cochlear hair cells damaged by ototoxic drug gentamicin. From immunostaining assay, we found the number of cochlear hair cells remained the same by EGCG treatment after gentamicin insult. Mechanotransduction currents recordings from cochlear hair cells indicate that EGCG not only protects the number of cochlear hair cells but also their functions. Furthermore, we provide evidence that the protective effect of EGCG on cochlear hair cells was due to its inhibition of Notch pathway. These findings offer insights into the function of EGCG in regeneration of cochlear hair cells and support the medical value of tea polyphenols on the hearing-impaired recovery.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Qianfo Shan Hospital Affiliated to Shandong University. The IACUC committee members at Qianfo Shan Hospital Affiliated to Shandong University approved this study. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.
**Experimental Animals**

Transgenic mice carrying GFP under the control of an enhancer from the Atoh1 gene (Atoh1/GFP) were purchased from Cyagen, Shanghai. The promoter of Atoh1/GFP transgene contains about 1.4 kb sequence from the Math1 enhancer which is fused to the reporter gene BGnEGFP. The reporter gene BGnEGFP contains the positive autoregulatory elements, the h-actin promoter, and a nuclear localization signal for GFP [32, 33]. Transgenic mice were identified by direct observation of GFP-mediated fluorescence. The following primers were used for PCR based genotyping: 5’ CGAAGGCTACGTCCAGGAGCGCAC CAT 3’ and 5’ GCACGGGGCCGCGATGGGG TGTTCTGC 3’.

**Cochlear Tissue Isolation and In Vitro Culture**

According to the procedure from published reports [33, 34], the timed mated pregnant mice at E13 to E14.5 were sacrificed and the embryos were isolated and placed in PBS (GIBCO). Then unfixed bulla from embryos were dissected and incubated in calcium-magnesium-free PBS (Invitrogen) containing dispase (1 mg/ml; Invitrogen) and collagenase (1 mg/ml; Worthington) to free the cochlear duct from the surrounding condensed mesenchyme for 10–15 min. After that, the enzyme solution was replaced with Hank’s balanced salt solution (HBSS, Life Technologies) and the spiral ganglia, Reissner’s membrane, and the most basal cochlear segment should be removed to obtain a flat cochlear surface preparation. For RT-PCR experiments, both the most cochlear base and the cochlear apex were removed, only the cochlear mid-turn was used. The remaining cochlear tissue was cultured on SPI filter membranes (Spi Supplies) in DMEM-F12 (Invitrogen) with B27 supplement (Invitrogen), 2.5 ng/ml FGF2 (NIH), 5 ng/ml EGF (Sigma) and 100 U/ml Penicillin (Sigma). All cultures were maintained in a 5 % CO2/20 % O2-humified incubator.

**Drug Treatments**

After 24 h cultured in the incubator, cochlear tissues (3 in a group) were randomly divided into 3 groups of 3–5 cochlear tissues per treatment group. These 3 groups were separately treated with PBS (as control), 40 μg/ml gentamicin or 40 μg/ml gentamicin + 40 μg/ml EGCG for 24 h.

**EdU Incorporation Assay**

EdU (5-ethynyl-29-deoxyuridine, Life Technologies) was prepared in DMSO as 100 mM stock solution and used at a final concentration of 3 mM. Click-iT Alexa Fluor 555 Kit (Life Technologies) was used to detect incorporated EdU. The assay was performed according to manufacturer’s specifications.

**Hair Cell Electrophysiology**

The hair cell electrophysiology procedures were modified from published reports [35, 36] as follows: after cochlear tissues treated separately with PBS, gentamicin or gentamicin + EGCG for 24 h, utricles and cochleae were excised, mounted on glass coverslips, and viewed on an Axioskop FS upright microscope (Zeiss) equipped with a 63x water-immersion objective and differential interference contrast optics. Electrophysiological recordings were performed at room temperature in solutions containing (in mM): 137 NaCl, 5.8 KCl, 10 HEPES, 0.7 NaH2PO4, 1.3 CaCl2, 0.9 MgCl2, and 5.6 d-glucose, vitamins (1:100) and amino acids (1:50) as in MEM (Invitrogen), pH 7.4 (311 mOsm/kg). Recording electrodes (2–4 MΩ) were pulled from R-6 glass (King Precision Glass) and filled with (in mM): 135 KCl, 5 EGTA-KOH, 5 HEPES, 2.5 Na2ATP, 2.5 MgCl2, and 0.1 CaCl2, pH 7.4 (284 mOsm/kg). The whole-cell, tight-seal technique was used to record mechanotransduction currents using an Axopatch 200B (Molecular Devices) for utricles and a Multiclamp 700A amplifier (Molecular Devices) for cochleae. Cells were held at 84 mV, a physiologically relevant holding potential. Currents were filtered at 2–5 kHz with a low-pass Bessel filter, digitized at at least 20 kHz with a 12-bit acquisition board (Digidata 1322A or 1440A), and recorded using pClamp 8.2 software (Molecular Devices). Inner hair bundles were deflected using a stiff glass probe mounted on a PICMA chip piezo actuator (Physik Instruments) driven by a 400-mA ENV400 amplifier (Piezosystem Jena) filtered with an 8-pole Bessel filter at 10–40 kHz to eliminate residual pipette resonance.

**Western Blotting Assay**

For western blotting analysis, mouse fibroblasts cells were seeded in 6-well plates and treated for 24 h with PBS, EGCG or L685458 (Sigma). L685458 is a potent, selective, structurally novel γ-secretase inhibitor [37]. To isolate protein, cells were washed with PBS, and harvested using the lysis buffer (50 mM tris-Cl pH = 6.8, 2 % SDS, 6 % glycerol, 1 % β-mercaptoethanol, 0.004 % bromophenol blue). Protein bands in the gel were then transferred to Nitrocellulose Blotting membranes and incubated with the appropriate primary antibody. The antibody dilutions were as follows: 1:1000 for Notch (ab27526) and 1:1000 for actin (ab1801). Membranes were incubated overnight at 4 °C and washed the next day with buffer (1 × PBS, 0.05 %...
Two-tailed Student’s t tests were used for secondary incubation for 1 h at room temperature. Proteins were then visualized with chemiluminescent substrates.

RNA Isolation and Quantitative RT-PCR Statistical Analysis

Twenty-four hours after cochlear tissues treated separately with PBS, gentamicin or gentamicin + EGCG, cochlear epithelia were isolated from these treated cochlear tissues using dispase (1 mg/ml; Invitrogen) and collagenase (1 mg/ml; Worthington) [34]. The total RNA was extracted from cochlear epithelia using TRIzol reagent (Invitrogen) and the amount of RNA was quantitated by a spectrophotometer (Nano-Drop ND-2000). Total RNA (2 µg) was reverse transcribed to cDNA using the reverse transcriptase kit from Bio-Rad according to the manufacturer’s instructions. The mRNA levels of the target genes were quantified by real time PCR using SYBR green (Life Technologies) in an ABI Prism 7500 real-time PCR system (Applied Biosystems). Each PCR reaction was carried out in triplicate.

Cell Counts

Hair cells were quantified after the cochlear tissues treated with PBS, gentamicin or gentamicin + EGCG and identified by Atoh1/GFP expression. High-power images of cochlear explants were assembled and analysed in Adobe PhotoShop CS3. ImageJ software (NIH) was used to measure the length of analysed cochlear segments and hair cell density (cells per 100 micron) was then calculated for each segment. The total number of inner hair cells or outer hair cells was counted in each of the four cochlear segments of 1200–1400 mm (apical, mid-apical, mid-basal, and basal). Values are presented as mean ± standard error (SEM) (n = 3, cochlear explants per condition were analyzed from two independent experiments). Two-tailed unpaired Student’s t tests were used to determine confidence interval. p ≤ 0.05 was considered as significant differences.

Statistical Analysis

Two-tailed Student’s t tests were used to determine confidence interval. The statistical differences between the experimental and control groups were analyzed by one-way analysis of variance (ANOVA). Values are presented as mean ± standard error (SEM). Statistical significance is indicated as *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. *p ≤ 0.05 was considered as significant difference, **p ≤ 0.01 was considered as very significant difference, ***p ≤ 0.001 was considered as extremely significant difference.

Results and Discussion

Chemical Structure of EGCG and DIC Image of Stage P2 Isolated Mouse Cochlear Explant

Tea is a popular worldwide beverage with numerous potential beneficial effects such as refreshing and antioxidative properties. The main active compounds in green tea are polyphenols. In this study, we aimed to study the role of EGCG (Fig. 1a), which is the most abundant and powerful antioxidant in polyphenols in green tea leaves, in regeneration of cochlear hair cells. According to the procedure from published reports [33, 34], we isolated the cochlear tissues from the embryos of timed mated pregnant Atoh1/GFP transgenic mice at E13 to E14.5 and cultured these tissues in vitro. To further observe the cochlear tissues carefully, the specimens were mounted in Slow Fade and were captured the differential interference contrast (DIC) and fluorescent images by an inverted microscope and a cooled CCD camera. In Fig. 1b, the structure of the cochlear tissues was shown by the DIC image, including apex (AP), mid apex (MA), mid base (MB) and base (BA) parts. Then we tested the expression of Atoh1/GFP in the isolated cochlear tissues. The Atoh1/GFP was expressed in inner and outer hair cell nuclei clearly (Fig. 1c).

EGCG Rescues Cochlear Hair Cell Number from Gentamicin Damage

Gentamicin is an aminoglycoside antibiotic to treat many types of bacterial infections, which is composed of a mixture of related gentamicin components and fractions. However, gentamicin can cause severe hearing and kidney problems. Therefore gentamicin is frequently used as the agent to induce damage in studies on cochlear hair cell protection [38, 39]. Prior to our major experiments, we examined the dosage effects of gentamicin and EGCG in the culture, and found their respectively optimal concentration both to be 40 µg/ml (supplementary Fig. S1), therefore these dosages have been used throughout the study. Therefore to examine whether EGCG could protect the hair cells from damage caused by gentamicin, we treated cochlear explant cultures with PBS as control, gentamicin or gentamicin + EGCG respectively (Fig. 2). In the PBS treated group, the highly organised structure composed by neat and orderly cells of cochlea could be clearly seen, with three regular rows of outer hair cells (OHC) and one regular row of inner hair cells (IHC). Following the treatment with gentamicin, the hair cells
showed serious signs of damage and the number of hair cells were significantly decreased, causing obvious structural lesions in cochlea. In the third group treated with the combination of gentamicin and EGCG, the structure lesion was rescued (Fig. 2a). Quantitative analysis in Fig. 2b directly indicated an 80% reduction of hair cells cause by gentamicin compared to the control. EGCG exhibited excellent protection against the damage caused by gentamicin, the number of hair cells has remained almost the same as the control. In order to rule out the possibility that EGCG only abolishes the adverse effect of gentamicin rather than promoting hair cell proliferation originated from dividing cells, EdU incorporation assay was performed, where EdU can be actively incorporated into newly synthesized DNA of dividing cells. No EdU labeled hair cells were observed in control or hair cell-ablated culture treated gentamicin, whereas a number of hair cells treated with the combination of gentamicin and EGCG are positively stained for EdU (Fig. 2c). These results indicated EGCG regenerates cochlear hair cells from damages induced by ototoxic drug gentamicin.

**Rescue of the Gentamicin Damaged Hair Cell Mechanotransduction by EGCG**

According to the fluorescent images and cell counting experiments, EGCG exhibit powerful ability to protect cochlear hair cells from the damage caused by gentamicin, evident by the number of hair cells as well as cochlea structure. We further examined whether the cochlear hair cells rescued by EGCG retain their mechanosensory functions. We recorded mechanotransduction currents from the cochlear hair cells in the same three experimental groups (Fig. 3). To avoid using hair bundles damaged during dissection, we selected the hair cells from the mid apex cochlea for analysis. The three groups of hair cells were bathed in 1.3 mM Ca$^{2+}$ with no other permeant cations. As expected, the hair cells treated with gentamicin had significantly attenuated transduction currents, reduced to only one third of the control group. However, the hair cells treated with gentamicin + EGCG had normal transduction currents as control. These data provided strong evidence that, apart from rescuing cochlea structural...
lesions damaged by gentamicin, EGCG also recovers the mechanosensory function of the hair cells.

**EGCG Efficiently Inhibits the Activity of Notch Signalling Pathway**

The differentiation and proliferation of hair cells have been shown to be controlled by Notch signalling pathway. The inactivation of the Notch signalling pathway can promote the proliferation of hair cells. To further study the mechanisms of EGCG protection of hair cells, we focused on four Notch pathway target genes (Hes1, Hes5, Hey1 and Hey5), which are also negative regulators of neurogenesis [40–42]. In Fig. 4, we analysed the expression of Hes1, Hes5, Hey1 and Hey5 in these 3 groups (the hair cells are separately treated with PBS, gentamicin or gentamicin + EGCG) by RT-PCR assay. EGCG treatment alone didn’t have any effect on the expression levels of these Notch effector genes (data not shown). As shown in Fig. 4, compared to the control, we found these four genes had no obvious changes at mRNA level when the hair cells were treated with gentamicin. Whereas in the gentamicin + EGCG group of hair cells, the mRNA levels of all these four genes have been significantly down-regulated. Therefore, EGCG can negatively regulate the expression of Hes1, Hes5, Hey1 and Hey5. These results suggest that EGCG may inhibit the expression of the target genes of Notch signalling to cause the inactivation of Notch signalling.

To further study how EGCG down-regulated Notch signalling pathway, we analysed the expression of the Notch pathway ligands Delta1, Jag1 and Jag2 in the 3 groups (the hair cells are separately treated with PBS, gentamicin or gentamicin + EGCG) by RT-PCR. In Fig. 5a, compared to the control, the mRNA level of Delta1, Jag1 and Jag2 had been down-regulated by Gentamicin. However, we found that this down-regulation was due to the reduced number of hair cells rather than low expression of genes in intracellular level, since the protein levels of Delta1 in individual hair cells were not affected (data not shown). It was worth noting that, in the gentamicin + EGCG treated group, there was no obvious change of the mRNA levels of these 3 genes compared to the control. According to the results of Figs. 4 and 5a, we hypothesized that the inhibitory effect of EGCG on Notch signalling was not conferred by interfering with either its upstream ligands or downstream effectors, but rather on the Notch receptor itself. One of the top candidates of EGCG target we examined was γ-secretase. To test our hypothesis, we employed L685458, known inhibitor of γ-secretase [37], and compared its effect with EGCG in mouse fibroblasts. The cells were separately treated with PBS (as control), EGCG or L685458 for 24 h, and the release of NICD was analysed by Western Blot assay. In Fig. 5b, there was clear NICD bands (indicated by the arrow) in the control group, whereas in the EGCG or L685458 treated groups there were no NICD bands. This
finding suggests that EGCG acts as an inhibitor of γ-secretase, same as L685458, to suppress the proteolysis of Notch receptor, and in turn down-regulates Notch signalling pathway.

Mammalian cochlea is composed of highly differentiated and stabilized cells. Under normal circumstances, these cells are incapable of regeneration when damaged by internal or external factors. Therefore, this damage could lead to permanent hearing loss. To date, ototoxicity has been reported of some anti-cancer drugs (CDDP etc.) and anti-bacterial drugs (gentamicin etc.). However, the protection and curing of hearing loss are still challenging. Notch signalling pathway is a highly conserved cell signalling system and plays an essential role in controlling the development of cochlea. It has been shown that inactivation of the Notch signalling pathway can promote the proliferation of hair cells [7, 20–22]. In the recent studies, more and more scientists devote to explore the targets in Notch signalling pathway to recover the regeneration of damaged hair cells.

In this study, we found that EGCG has the ability to protect cochlear hair cells from ototoxic drug gentamicin. Both number (Fig. 2) and function (Fig. 3) of cochlear hair cells can be recovered by EGCG and the Notch signalling target genes has been suppressed under the treatment of EGCG (Fig. 4). However, the Notch pathway ligands Delta1, Jag1 and Jag2 have no obvious change at mRNA level with the treatment by EGCG and gentamicin (Fig. 5a). We speculated that the inactivation of Notch signalling pathway by EGCG was caused by the Notch receptor itself, rather than interfering with either Notch upstream ligands or downstream effectors. To further illustrate this essential mechanism, we examined the top candidate of EGCG target γ-secretase. Our studies revealed that the suppression of Notch signalling pathway by EGCG depends on the inactivation of γ-secretase (Fig. 5).

EGCG is the most abundant, and active compound in tea polyphenols, which presents strong antioxidant activation and has been reported to exhibit anti-cancer [43] and anti-inflammatory [44] activities in biochemical research. According to abundant studies, EGCG could play different roles in various pathways and targets to participate the therapies of diseases. In 2008, Shimizu et al. [45] have reported EGCG decreased the mRNA and protein levels of IGF-1 and IGF-2, which might be an inhibitor of critical RTKs involved in hepatocellular carcinoma proliferation. In our research, compared with L685458, EGCG may become a potential inhibitor of γ-secretase to suppress the Notch signalling pathway and be useful for hearing-impaired recovery in clinical therapy. Further studies can use the drug-tagging system with EGCG into the needed portion of the inner ear [46], thereby making EGCG to repair the hair cell efficiently and selectively.

Conclusions

In summary, we demonstrated EGCG could protect cochlear hair cells against the damage from gentamicin in the cochlear explant cultures. The mechanism is based on the inactivation of γ-secretase by EGCG and eventually causes suppression of Notch signalling pathway. In the near future, we envision that the drug-tagging system with EGCG into the targeted portion of the inner ear could be useful for clinical research and bring emerging prospects for hearing-impaired recovery.

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Conflict of interest The authors declare that they have no competing interests.

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