의학박사 학위논문

청각유모세포에서 TNF-α에 의한 손상과 Dexamethasone에 의한 보호기전

Tumor Necrosis Factor-α (TNF-α)-induced Damage and Prevention Mechanism of Dexamethasone in Auditory Hair Cell

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이 논문을 의학박사 학위 논문으로 제출함

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국문요약

배경: 청각유모세포 손상은 감각신경성 난청의 가장 흔한 기전이다. 자가면역 질환으로 알려진 면역 관련 난청을 비롯해 돌발성 난청과 메니에르병 (Ménière's disease) 등 흔한 질환에서도 면역 기전이 병인의 일부로 제기되고 있다. 면역 관련 내이질환을 비롯해 여러 난청에서 와우 내에 tumor necrosis factor-α (TNF-α) 가 증가되어 있음이 알려져 있고, 스테로이드에 반응하여 치료를 위해 이용되나, 그 기전이 정확히 알려져 있지는 않다. 다양한 부작용을 나타낼 수 있는 스테로이드 치료는 면역 관련 난청의 잦은 재발과 만성 경과를 고려할 때 지속적으로 혹은 반복적으로 사용하기 어렵다. 따라서 난청 치료 시 전신적 스테로이드 치료를 줄이고자 여러 시도가 이루어지고 있으며, 난청의 여러 기전에 등장하는 TNF-α 역시 치료의 열쇠로 주목받고 있다. TNF-α의 역할과 스테로이드의 보호 기전에 대한 연구로 그 하위 신호전달체계를 탐색할 수 있으며, 이를 통해 스테로이드를 대체할 수 있는 예방 혹은 치료 물질을 발견할 수 있다면 면역 관련 난청을 비롯한 다양한 난청의 이해와 그 치료에 있어서 새로운 장을 열 수 있을 것으로 기대된다. 본 연구에서는 청각유모세포를 이용한 세포실험을 통해 TNF-α로 유도되는 청각유모세포의 손상과 스테로이드에 의한 보호 효과를 확인하고 그 기전을 탐색하고 관련 연구를 고찰하였다. 이를 통해 면역 관련 난청을 비롯한 여러 난청에서 TNF-α의 역할과 스테로이드에 의한 보호 효과를 더 깊이 이해하고, 최종적으로 난청 기전에 대한 이해를 넓히고 향후 난청 연구에 도움이 되고자 하였다.

방법: TNF-α로 인한 청각유모세포 손상과 보호기전을 세포실험 단계에서 확인하기 위해 와우의 감각 유모세포로 HEI-OC1 (House ear institute-organ of Corti 1)을 이용하였다. Dexamethasone과 TNF-α의 cell viability에 대한 효과를 확인하기 위해 다양한 농도로 처리 및 배양하여 CCK assay로 확인하였다. 세포주에 TNF-α와 dexamethasone을 처리하고 ROS (reactive oxygen species, 활성산소종)를 측정하였다. dexamethasone과 TNF-α 처리에 따른 apoptosis의 연관성은 Bcl-2, Bax, caspase 3, caspase 7, PARP, β-actin 등 antibody를 이용하여 Western blot으로 확인하였다. Dexamethasone의 cell viability, ROS generation, 그리고 apoptosis에 대한 보호효과를 확인하기 위하여 dexamethasone 6시간 전처리 후 TNF-α를 24시간 처리하여 control 및 TNF-α만 처리한 경우와 비교하였다. Apoptosis를 실제로 확인하기 위하여 control, TNF-α 처리, 그리고 dexamethasone 전처리 후 TNF-α 처리한 세포주를 by annexin V/propodium iodide (PI) 염색 후 형광현미경으로 관찰하였다.

결과: HEI-OC1 cell의 viability는 dexamethasone 처리 6시간 후 cell viability는 5 nM 이상의 농도부터 control에 비하여 증가하였고, TNF-α 처리 24시간 후 5 ng/ml 이상의 농도부터 control에 비해 유의하게 감소하였다. ROS level은 dexamethasone 처리 시에는 control과 차이가 없었으나 TNF-α 처리 시 유의하게 증가하였다. TNF-α 처리 시 Bcl-2의 감소와 Bax의 증가, cleaved caspase 3, cleaved caspase 7, cleaved PARP가 유의하게 증가하여 apoptosis pathway가 활성화됨을 확인하였고 dexamethasone 처리 시에는 control과 차이를 보이지 않았다. Dexamethasone 전처리 후 TNF-α를 처리한 실험에서는 control에 비해 TNF-α 처리 시 저하되었던 cell

viability의 저하 정도가 유의하게 감소하였고, 증가되었던 ROS generation의 증가 정도가 유의하게 감소하였으며, 활성화되었던 apoptosis 역시 변화 정도가 감소하였다. Annexin V/PI 염색 후 형광현미경으로 관찰하였을 때 control에 비해 TNF-α 처리 시 증가하였던 apoptotic cell은 dexamethasone 전처리 후 증가 정도가 감소하였다.

결론: TNF-α는 청각유모세포의 cell viability를 저해하고 ROS를 증가시키며 apoptosis를 활성화시킨다. Dexamethasone은 TNF-α에 의한 청각유모세포의 손상을 감소시키는 보호 효과를 보인다. 청각유모세포 손상에서 TNF-α의 역할과 dexamethasone에 의한 보호 효과, 그리고 그 기전에 대한 탐색은 향후 면역 관련 난청을 비롯한 다양한 난청 치료를 위한 연구에 중요한 바탕이 될 것이다.

목 차

표 및 그림 목차

Introduction

Auditory hair cell damage is the most common mechanism of sensorineural hearing loss. Various etiologies such as aging process, noise exposure, vascular disorders, viral infections, drug-induced ototoxicity, trauma, and immune-related mechanisms act as insults to the inner ear and cause sensorineural hearing loss [1-4]. Immune-related hearing loss is an autoimmune-mediated hearing loss characterized by frequent recurrence and chronic course, and it is known to occur in about 5 per 100,000 people every year, accounting for approximately 1% of the total hearing loss [5]. The hearing loss associated with autoimmune mechanism is thought to be higher than known, because immune-related response has been suggested as part of the etiology in more common diseases such as sudden sensorineural hearing loss and Meniere's disease [1,6,7].

Sudden sensorineural hearing loss is a rapid-onset hearing impairment without a definite cause. Clinically, it is defined as a case in which sensorineural hearing loss of 30dB or more at three or more consecutive frequencies in pure tone audiometry occurring in less than 72-hours [8]. Sudden sensorineural hearing loss affects 5 to 27 per 100,000 people annually in the United States according to recent reports [8,9]. Most common cause is idiopathic, but various etiologies including viral infection, vascular disorders, autoimmune diseases, and vestibular schwannomas are known as possible causes [5,6,8-11]. Since the cause is unknown and many possible causes are suggested, the response to treatment and prognostic factors have also been proposed in various ways. It was reported that changes of serum cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were correlated with

prognosis in patients with sudden sensorineural hearing loss [7]. There was a clinical study confirming that the posttreatment serum TNF-α level was more increased than pretreatment level in patients who did not respond to treatment of sudden sensorineural hearing loss [12]. These results suggest that TNF-α plays an important role in the pathophysiology of sudden sensorineural hearing loss. This is also expected to open a new field in the treatment of sudden sensorineural hearing loss through the blockade of TNF-α. The TNF-α inhibitor, or another better agent could block TNF-α-related mechanism in sudden sensorineural hearing loss.

Meniere's disease is a chronic inner ear disease characterized by fluctuating sensorineural hearing loss and recurrent vertigo [13-17]. It is also rare, but relatively more common than immune-related hearing loss, the prevalence rate is known to be about 0.5% according to the recently published meta-analysis [16,18]. Meniere's disease is known as a histopathology termed endolymphatic hydrops, but recent studies have shown that histopathological findings of endolymphatic hydrops do not cause symptoms of Meniere's disease, but coexist [17,19]. Recently, there are some studies of immunological mechanisms of Meniere’s disease such as inflammatory changes of endolymphatic sac and the association with arteritis [20-22].

The response to steroid therapy is a common feature of sudden hearing sensorineural loss, Meniere's disease, and immune-related inner ear disease [5,8,16]. This can be a basis for speculating that autoimmune mechanism plays an important role in the pathogenesis of hearing loss. The cross-reactive circulating antibodies are found in patients with progressive sensorineural hearing loss including sudden sensorineural hearing loss, temporal arteritis, Wegener's granuloma, Cogan syndrome, polyarteritis nodosa, systemic lupus erythematosus [1,5-7,10,22,23]. It also supports that immune response is a possible common mechanism of

various disorders with hearing loss.

TNF-α is a well-established proinflammatory cytokine which can initiate apoptosis in various cells. It is originally produced in monocytes and macrophages. TNF-α activate extrinsic death signal pathway through tumor necrosis factor receptor-1 (TNFR1). It can induce activation of procaspase. And it can also involve intrinsic signal pathway through involving apoptosis-related proteins. These proteins include Bcl-2, an anti-apoptotic protein, and Bax families, a pro-apoptotic protein. TNF-α can initiate the intrinsic pathway and the extrinsic pathway, and these mechanisms act together in the downstream, and induce cell death by activating procaspase.

TNF-α plays a role in initiating inflammation against infectious stimulations [24]. This mechanism helps maintain homeostasis through death of damaged cells. However, long-standing increased level of TNF-α is associated with pathologic mechanism in autoimmune diseases such as Crohn disease, rheumatoid arthritis, psoriatic arthritis, and ankylosing spondylitis [25,26]. In inflammatory bowel diseases such as Crohn's disease and ulcerative colitis, TNF-α may be a therapeutic target [27].

The spiral ligament fibrocytes of cochlea also produce TNF-α. It plays a similar role, inducing cell death in the ear [28]. In addition to TNF-α, several cytokines are expressed in the cochlea and are known to be concerned in some diseases. Interleukin-1β (IL-1β), a proinflammatory cytokine, is expressed in fibrocytes of the spiral ligament and is known to increase after cochlear trauma. It was found that increased level of IL-1β is a common response to trauma, but increased level of TNF-α may induce cochlear disease as a response by immune-related cell infiltration [29]. Not only TNF-α, interleukin-6 (IL-6) also changes in sudden sensorineural hearing loss, the decreased level of TNF-α and increased level of IL-6

are found to be good prognostic factors for recovery [7].

These cytokines, of course, also appear in immune-related hearing loss. The role of cytokines in immune-related hearing loss has been investigated. It has been reported that Several cytokines including interleukin-1 (IL-1), interleukin-17 (IL-17), interferon-gamma (IFN-γ), and TNF-α are increased in immune-related hearing loss [5,6,30,31]. The level of TNF-α from peripheral circulation can be used as a diagnostic and prognostic factor for immune-related hearing loss [32].

Vestibular schwannoma is the most common neoplasm of internal auditory canal. Vestibular schwannoma most often occurs in the vestibular nerve, not the cochlear nerve. The mechanism of hearing loss is multifactorial, and there are many parts that cannot be explained by nerve compression, such as hearing loss even in small tumors that do not grow, and normal hearing in huge tumors [33]. Recently, it has been known that TNF-α secreted by vestibular schwannoma acts as an ototoxic molecule and causes hearing loss, and fibroblast growth factor 2 (FGF2) is an otoprotective molecule that acts as a protective role for hearing [11]. In addition to typical autoimmune diseases such as ankylosing spondylitis and Crohn's disease, TNF-α is emerging as a therapeutic target in vestibular schwannoma.

In an animal study, TNF-α was identified as a key cytokine of inner ear immune response [34]. It was found that increased TNF-α level during hearing loss and protected effects by TNF-α inhibitors in several animal studies, and the same is true in clinical studies [35-40]. It is questionable, however, whether TNF-α plays a role in mediating the process of hearing loss or can be called a direct etiology. There are some animal experiments that have suggested a correlation between the increase of TNF-α and hearing loss, but this was not a result obtained by direct TNF-α injection, but by other insults such as noise or ototoxic drugs. A study that

protected hearing loss from TNF-α by intratympanic injection of Etanercept, a TNF-α inhibitor, was an animal experiment conducted in rats, but it was a protective effect by Cisplatin-induced ototoxicity [35]. In animal experiments using rats, TNF-α-related hearing loss was blocked with Dexamethasone. [36]. But the otologic insult was Gentamicin, not TNF-α, in this study. There were animal experiments using Guinea pig that block TNF-α-related hearing loss with Etanercept [37,39]. In these studies, also, the otologic insult was cochlear implantation or noise, not TNF-α. There was also an animal experiment using Guinea pig that showed that Etanercept have a protective effect from TNF-α by preventing the reduction of cochlear blood flow [38]. It has suggested in many studies that TNF-α is associated with auditory hair cell damage or hearing loss and that blockade of TNF-α can prevent it. TNF-α increases in several systemic inflammations, however, not all of them lead to hearing loss. Namely, several ototoxic insults induce auditory hair cell loss through various cytokines and signal pathways, and the premise of current study is that TNF-α plays a mediatory role of auditory hair cell damage. It is not clear whether administration of TNF-α directly causes auditory hair cell damage and hearing loss. It is thought that the blockade of TNF-α-related mechanisms can prevent the result of hearing loss.

In immune-related hearing loss high dose steroids therapy have been used to regulate the process, but its mechanism is not accurately revealed. And it is burdensome to continue long term and repeated steroids pulse therapy, because of the frequent recurrence and chronic course of the disease and many side effects of steroids. Recently, there are some reports of using immunosuppressants for the treatment and prevention of hearing loss. The role of inflammatory process in hearing loss was reported by a study that the stress signaling molecules such as TRPV1 (transient receptor potential vanilloid 1), NOX3 (NADPH oxidase

3), COX2 (cyclooxygenase 2), and TNF-α cause chronic inflammation and damage to hair cells as a common mechanism of hearing loss, and TNF-α inhibitor prevents this process mechanism [41]. Several animal studies found that inner ear neuroinflammation, such as increased level of TNF-α, induces hearing loss and TNF-α inhibitor, protects this process [42,43]. It was found that this process is related to synaptic degeneration in experiments with guinea pig [42]. There was a clinical study that intratympanic infliximab, a TNF-α inhibitor, injection for refractory or recurrent patients with immune-related hearing loss [40].

In a study on ototoxicity using HEI-OC1 cells, changes in cytokines such as TNF-α, IL-1β and IL-6 were presented [44]. In this study, anti-TNF-α significantly increased HEI-OC1 viability when Cisplatin was treated, but anti-IL-1β and anti-IL-6 did not. The neutralization of TNF-α also inhibited the NF-κB in this experiment. This result indicated that among several cytokines which act in the downstream pathway related to the damage of auditory hair cells, TNF-α plays a key role.

As described above, it is known that TNF-α is increased in the cochlea in immune-related inner ear diseases and TNF-α is a pivotal cytokine that can induce inner ear inflammation [45]. Although it is impossible that block all related molecules, blocking of TNF-α is attractive attempt as a therapeutic target because it acts on several cytokines across the extrinsic death signal pathway and intrinsic signal pathway.

The corticosteroids have been used to treat a variety of inner ear diseases. The corticosteroids mean synthetic glucocorticoids. For sudden sensorineural hearing loss, both systemic use and local injection are effective [8]. In Meniere's disease or immune-related hearing loss, corticosteroids pulse therapy is used for acute hearing loss [1,5,15]. It is difficult to use corticosteroids for a long term due to systemic side effects such as acne, blurred vision,

glaucoma, insomnia, hypertension, lower resistance to infection, osteoporosis, and worsening of diabetes [46,47]. Therefore, many tries have been made to reduce systemic corticosteroids therapy such as local injection or alternative agents. Because intratympanic corticosteroid injection provides sufficient drug delivery to the inner ear through the round window, it is used as an initial or salvage treatment of sudden sensorineural hearing loss [8,48-51].

Published studies suggested that corticosteroids can protect the inner ear against cytokines such as IL-1β and TNF-α [52,53]. However, the detailed mechanisms or downstream pathways of this protective mechanism are not exactly known. Research on this mechanism cannot be able to suggest a prevention or treatment for hearing loss at once, however, it is expected that downstream molecules or alternative substances of corticosteroids can be developed through if continuous studies including current study. These studies could lead to a way to avoid the side effects of corticosteroids and to use safe substance as long-term maintenance therapy of hearing loss including immune-related hearing loss.

This study was initially designed to further explore the role of TNF-α and protective effects of corticosteroid in immune-related hearing loss. There are many studies about noise-induced hearing loss using DBA mice, age-related hearing loss studies using rats, and acoustic trauma using guinea pigs, but there is no adequate animal model for immune-related hearing loss [54-56]. Therefore, this study was conducted to investigate the relationship between immune-related hearing loss and TNF-α at the cellular level first. In this study, the HEI-OC1 (House ear institute-organ of Corti 1) cell line was used for experiments.

The HEI-OC1 cells were isolated from the auditory organ of the transgenic mouse. The HEI-OC1 cells express prestin and myosin 7a, which are specific markers of auditory hair cells. They also express connexin 26, which is specific to supporting cells [57]. Therefore,

HEI-OC1 can be seen to represent the general characteristics of the organ of Corti. The HEI-OC1 is widely used in various researches including apoptosis, autophagy and senescence pathways, ototoxicity and its protective mechanism [58]. Several laboratories have tried to establish an inner ear cell line suitable for the purpose of their research [59]. Among them, the OC-k3 cell line, the adult organ of Corti cell line, is also used as the inner ear research. The OC-k3 cells were derived from organ of Corti of 2-week-old H-2Kb-tsA58 (Immortomouse) transgenic mice [60]. The OC-k3 can be used to experiment about cell differentiation of the inner ear. But, the HEI-OC1 cell line is the most actively used in research, and therefore has a wealth of scientific evidence. There are many previous studies comparing the results as an inner ear sensory cell study. Thus, it is reasonable to use this cell line for this study. In this study, the HEI-OC1 cell was also used.

In this study, TNF-α was treated to inner ear hair cell line and the changes of cytokines and signals related to cell viability, reactive oxygen species (ROS), and apoptotic pathway observed to investigate the mechanisms of cell damage and proliferation. In addition, it was evaluated whether TNF-α-induced damage of inner ear cells could be prevented by pretreatment with Dexamethasone, a commonly used steroid for inner ear diseases. Through this study, the roles of TNF-α and Dexamethasone in various hearing loss including immune-related hearing loss were explored to make the basis for the development of a potential therapeutic agents to replace steroids. This study was finally intended to broaden the understanding of the mechanism of hearing loss and to be helpful in future studies on the treatment of hearing loss.

Materials and Methods

Cell culture and reagents

The cochlear auditory cell line HEI-OC1 was kindly provided by Dr. Federico Kalinec (Department of Cell and Molecular Biology, House Ear Institute, Los Angeles, CA, USA). The HEI-OC1 cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA). The cells were cultured in DMEM supplemented with 10% FBS at 33 °C in a humidified chamber containing 10% CO_2 for the experiments. Dexamethasone (BML-EI126) and mouse recombinant TNF-α (893964) were purchased from Enzo Life Sciences (Farmingdale, NY, USA) and R&D Systems (Minneapolis, MN, USA), respectively.

Cell viability assay

A colorimetric D-Plus CCK cell viability assay kit (Dongin LS, Seoul, South Korea) was used to estimate cell viability. The method of evaluation was performed according to the manufacturer's instructions. The cells were seeded in 96-well plates at a density of 4×10^3 cells/well. Next, the cells were grown for 24 hours under standard conditions. These cells were exposed to different concentrations of dexamethasone (0, 5, 10, 15, and 20 nM) or TNF-α (0, 1, 5, 10, 15, 20 ng/ml). After incubation for 3 hours at 37 °C, the optical density (OD) at 450 nm was measured using a microplate spectrophotometer (Molecular Devices, San Jose, CA, USA). The absorbance values were converted to percentages relative to the untreated

control. The experiments were repeated at least three times to confirm that consistent results were obtained.

Measurement of intracellular ROS generation

The generation of intracellular ROS was measured using 5-(and-6)-chloromethyl-2′-7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA; Molecular Probes, Eugene, OR, USA,) following the manufacturer's instructions. The cells were seeded in 96-well plates, and, after 24 hours, incubated with dexamethasone, TNF-α, or pretreated with dexamethasone prior to treatment with TNF-α. The cells were washed with Hank's balanced salt solution (HBSS) and supplied with phenol red-free medium containing 5 μM CM-H_2DCFDA. Then, the cells were incubated for 20 min at 37 °C in the dark. For quantification of generated ROS levels, fluorescence was measured at 485 nm (excitation) and at 535 nm (emission) using the VICTOR 3 (Perkin-Elmer, Waltham, MA, USA) fluorescence plate reader.

Western blot analysis

Total protein was extracted using the radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma-Aldrich, St. Louis, MO, USA). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Waltham, MA, USA). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted into nitrocellulose transfer membranes (GE Healthcare, Chicago, IL, USA). This was followed by incubation with primary antibodies against Bcl-2 (3498), caspase-3 (9662),

cleaved caspase-3 (9661), poly (ADP-ribose) polymerase (PARP)/cleaved PARP (9542), and β-actin (A5441), which were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibody against Bax (556467) was purchased from BD (Franklin Lakes, NJ, USA), against caspase-7/cleaved caspase-7 (sc-81654) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and against β-actin from Sigma-Aldrich. The antibodies were diluted in 5% bovine serum albumin in Tris-buffered saline (TBS). Next, the membranes were washed with Tween 20-containing TBS (TBST) and were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Bethyl Laboratories, Montgomery, TX, USA) in TBST for 1 hour. The immunoreactive bands were detected using an enhanced chemiluminescence assay kit (ECL; Dongin LS) and quantified using the ImageQuant LAS 500 biomolecular imager (GE Healthcare).

Apoptosis by annexin V/propidium iodide (PI) analysis

The annexin V/PI assay was performed following the manufacturer's protocols (Annexin V-FITC kit, Bender Med Systems GmbH, Vienna, Austria). The cells grown on glass coverslips in 6-well culture dishes pretreated with 0 or 10 nM dexamethasone for 6 hours and further incubated with 10 ng/ml of TNF-α for 24 hours. After washing, the cells were mounted on glass slides and examined using the appropriate filter of an Olympus IX71 fluorescence microscope: green fluorescence (excitation/emission: 495/515 nm) for early apoptotic cells, and red fluorescence (excitation/emission: 561/615 nm) for late apoptotic and necrotic cells.

Statistical analysis

Data are expressed as means ± the standard error (SE) of three or four independent experiments. Statistical analyses were conducted using one-way or two-way analysis of variance (ANOVA) to identify statistically significant differences. To determine statistically significant relationships between the distribution of categorical values, the Chi-squared test was performed. Differences with p-values < 0.05 were considered statistically significant, and the p-values of the tests are provided in the figure legends.

Results

TNF-α-induced auditory hair cell damage

HEI-OC1 cells were treated with different concentrations of dexamethasone (0, 5, 10, 15, and 20 nM) for 6 and 24 hours or with TNF-α (0, 1, 5, 10, 15, 20 ng/ml) for 24 hours. Cell viability was evaluated using a CCK-8 kit. After 6 hours of treatment with dexamethasone, cell viability increased with increasing concentration starting from 5 nM (129.6%) and reached a peak at 15 nM (142.7%) (**Figure 1A**). However, treatment with dexamethasone for 24 hours resulted in decreased cell viability (83.5%) at concentrations >15 nM. Thus, cell proliferation was observed after 6 hours of treatment with dexamethasone, but cell damage occurred after 24 hours of treatment at high concentrations of dexamethasone. Because treatment with 10 nM dexamethasone led to cell proliferation after 6 hours, with no cell damage observed after 24 hours, a 6-hour pretreatment period with 10 nM dexamethasone was used for subsequent experiments.

When the cells were treated with TNF-α for 24 hours, cell viability decreased in a dose-dependent manner starting from 5 ng/ml (**Figure 1B**); treatment with 5 ng/ml and 10 ng/ml TNF-α led to 70.6% and 50.0% cell viability, respectively, relative to the untreated control. We performed treatment with 10 ng/ml TNF-α for 24 hours in subsequent experiments because this concentration was the estimated half-maximal cytotoxic dose.

The levels of intracellular ROS were measured through reactive fluorescence after dexamethasone or TNF-α treatment. ROS generation in dexamethasone-treated cells was not different from that in the untreated control. ROS generation significantly increased by 1.40-

fold after TNF-α treatment, relative to the untreated control and to treatment with dexamethasone alone (**Figure 2**). This suggests that the generation of ROS was induced by TNF-α treatment of auditory hair cells but was not affected by dexamethasone treatment.

Dexamethasone treatment did not lead to significant changes to the apoptotic pathway when compared to the untreated control. By contrast, the levels of the anti-apoptotic protein Bcl-2 decreased 0.52-fold and the levels of the pro-apoptotic protein Bax increased 1.52-fold after TNF-α treatment. We further confirmed that the levels of cleaved caspase-3 and -7, which are catalytically active forms of caspases, also increased in TNF-α-treated cells by 2.31-fold and 1.81-fold, respectively. The levels of cleaved PARP fragments, the inactivated form of PARP, increased 1.60-fold. (**Figure 3**). These suggest that the apoptotic pathways were activated by TNF-α treatment in auditory hair cells but was not affected by dexamethasone treatment.

Protective effects of dexamethasone pretreatment against TNF-α-induced auditory hair cell damage

We next investigated whether the decrease in cell viability, increase in ROS levels, and the activation of apoptotic pathways induced by TNF-α could be prevented by dexamethasone pretreatment. The damage to the cells after treatment with TNF-α alone (49.8% cell viability) was significantly reduced by dexamethasone pretreatment (68.2% cell viability) (**Figure 4A**). The improvement in cell viability suggests that dexamethasone is protective against TNF-α-induced auditory hair cell damage.

Similarly, the increase in ROS levels triggered by TNF-α was significantly reduced by

dexamethasone pretreatment. ROS generation increased 1.42-fold in cells treated with TNF-α alone; meanwhile, ROS generation increased 1.15-fold when the cells were treated with dexamethasone prior to treatment with TNF-α (**Figure 4B**). These findings show that dexamethasone pretreatment is protective against TNF-α-induced generation of ROS.

The activation of the apoptosis pathway induced by TNF-α was also significantly reduced in cells pretreated with dexamethasone (**Figure 4C**). Bcl-2 was reduced 0.40-fold when the cells were treated with only TNF-α, but it was reduced 0.91-fold in cells pretreated with dexamethasone prior to treatment with TNF-α, showing no significant difference from the control. The increase in Bax also showed the same trend. When the cells were treated with only TNF-α, Bax levels increased 1.72-fold, whereas in cells treated with dexamethasone prior to TNF-α treatment, Bax levels increased 0.92-fold, which was not significantly different from the control. The changes in apoptosis-related proteins also showed similar results. The levels of cleaved caspase-3, caspase-7, and PARP increased 8.00-, 2.28-, and 3.68-fold, respectively, after treatment with TNF-α alone. However, the levels of cleaved caspase-3, caspase-7, and PARP increased only 4.00-, 1.58-, and 2.10-fold, respectively, in cells pretreated with dexamethasone. These findings show that dexamethasone pretreatment protects auditory hair cells from TNF-α-induced apoptosis.

The apoptotic effects of TNF-α and the protective effects of dexamethasone pretreatment were further observed when the cells were subjected to annexin V/PI staining (**Figure 5**). When observed under the fluorescence microscope, the percentages of early apoptotic cells as determined by annexin V staining were 1.80% in the control cells, 3.97% in cells treated with TNF-α alone, and 3.50% in cells treated with dexamethasone prior to TNF-α treatment. It appears that dexamethasone could not protect auditory hair cells from the apoptotic

pathway induced by TNF-α; however, the results from staining of late apoptotic cells suggest otherwise. The percentages of late apoptotic cells as stained with PI were 1.26% in the control cells, 29.82% in cells treated with TNF-α alone, and 6.53% in cells treated with dexamethasone prior to TNF-α treatment. Thus, the level of apoptosis induced by TNF-α was reduced by dexamethasone pretreatment. These findings further confirm that TNF-α induces apoptosis and dexamethasone protects the cells from TNF-α-induced apoptosis.

Discussion

In this study, the mechanisms by which TNF-α induces damage in HEI-OC1 cells were investigated through the evaluation of cell viability, ROS generation, apoptosis-related proteins, and apoptotic rates. We have confirmed that dexamethasone protects HEI-OC1 cells against these TNF-α-induced mechanisms. Several previous studies on TNF-α and dexamethasone in various cells including auditory hair cells have been reported, and the results of the current study were consistent with those of the previous studies.

The effects of dexamethasone on various cells, including auditory hair cells, are diverse. Dexamethasone has been reported to induce apoptosis in leukemia cells in a concentration-dependent manner [61]. A study has evaluated the effects of various chemicals, including cisplatin and dexamethasone, on HEI-OC1 cells, human embryonic kidney cells (HEK-293), and HeLa cells [62]. Dexamethasone showed cytotoxicity in HEK-293 and HeLa cells, but not in HEI-OC1 cells. Dexamethasone decreased cell viability without significant cell death in HEI-OC1 cells after treatment for 24 and 48 hours. In the current study, cell viability also decreased when HEI-OC1 cells were treated with dexamethasone for 24 hours. Similar to previous studies, cytotoxicity can be observed when HEI-OC1 cells are treated with high concentrations of dexamethasone for a long time. Additionally, in this study, 6 hours of dexamethasone treatment promoted cell proliferation; therefore, 6 hours was selected for dexamethasone pretreatment of HEI-OC1 cells. The reported changes caused by dexamethasone in HEI-OC1 cells are decreased senescence and increased autophagy [62]. These mechanisms are likely related with the protective effects of dexamethasone against

stimulation. The current study also showed that a 6-hour pretreatment with dexamethasone protects HEI-OC1 cells from the effects of TNF-α, which are consistent with the results of previous studies. This is also consistent with the general knowledge on the effects of dexamethasone, which is widely used clinically for the treatment of various sudden hearing impairments.

The protective effects of dexamethasone in relation to senescence and autophagy still require further understanding. Senescence is the process by which normal somatic cells cease to divide as a part of cellular aging. Senescence is associated with telomere shortening but is sometimes induced by various causes, including oxidative stress and chemical agents, which is called stress-induced premature senescence (SIPS). In contrast to apoptotic cells, senescent cells can survive stably [63]. Autophagy is a catabolic process used for cellular adaptation to diminish nutrient resources. Autophagy usually blocks the induction of apoptosis, and caspase activation blocks the autophagic process [64]. Thus, autophagy is considered a protective response mechanism. A recent study has suggested that autophagy plays a cytoprotective role against oxidative stress-induced necrosis in HEI-OC1 cells [65].

Apoptosis, autophagy, and senescence are mediated by several cytokines such as IL-1β, IL-1, IL-6, IL-17, IFN-γ, and TNF-α. As a result, the downstream signaling pathways, such as the mitogen-activated protein kinase (MAPK) and transcription factor NF-κB pathways, are involved. Various studies have been conducted to further understand these processes. Although recent studies have bridged gaps in knowledge regarding these mechanisms, several questions remain unanswered especially in their involvement in sudden sensorineural loss and potential treatments. Various treatment attempts have been made, but treatments other than systemic or intratympanic steroids cannot yet be applied clinically for sudden

sensorineural hearing loss. TNF-α inhibitors and other immunosuppressive treatments are considered potential therapeutics, but high-dose systemic steroids are still more commonly used in immune-related hearing loss. Furthermore, the specific mechanisms underlying the protective effects for steroids are still not understood. Therefore, studies on the mechanisms of hearing loss are important for understanding the condition and the potential treatments.

A study has shown that TNF-α, IL-1β, and IL-6 are related to cisplatin-induced ototoxicity in HEI-OC1 cells [44]. The downstream signaling pathways implicated in this previous study were the MAPK and NF-κB pathways. The MAPK family includes extracellular signal-regulated kinases (ERK), and c-Jun N-terminal kinases (JNK), and p38-MAPKs [66]. Generally, ERK pathways are related with cell survival, while the JNK pathways and p38-MAPK pathways are involved in apoptosis. In a previous cisplatin-induced ototoxicity study, the pathways were blocked via pharmacological inhibition; namely, PD98059 and U0126 were used as MEK1/ERK inhibitors, SB203580 as a p-38 inhibitor, and SP600125 as a JNK inhibitor. Pharmacological inhibition of MEK1/ERK was found to markedly reduce the transcription of genes encoding TNF-α, IL-1β, and IL-6 in cisplatin-treated cells [44]. Anti-TNF-α, anti-IL-1β, and anti-IL-6 antibodies were used for cytokine neutralization. The antibody against TNF-α improved cell viability, while those against IL-1β and IL-6 did not. The anti-TNF-α antibody also showed inhibitory effects on NF-κB. These results indicate that TNF-α plays a pivotal role in cisplatin-induced ototoxicity. In the current study, we instead used dexamethasone, which is widely used clinically, instead of an antibody that neutralizes TNF-α. Although our experimental design cannot directly evaluate the mechanisms for the inhibition of a signaling pathway, it allows us to evaluate the protective effects of a clinically used treatment.

A study using HEI-OC1 cells and dexamethasone has been previously conducted. The previous study has suggested that IL-1β could induce matrix metallopeptidase 9 (MMP-9), a type IV collagen matrix metalloproteinase, and this mechanism was inhibited by dexamethasone [53]. In addition, RU486, the glucocorticoid receptor (GR) antagonist, reverses the inhibitory effects of dexamethasone. However, ROS was observed to be related to the protective mechanism of dexamethasone. The results of this previous study suggest that the action of dexamethasone is mediated by receptor activation. By evaluating the mRNA levels through reverse transcription-polymerase chain reaction (RT-PCR) of genes encoding key proteins, the results of the previous study showed that the activation of the p38 MAPK signaling pathway was important in IL-1β-induced MMP-9 expression, while JNKs and ERKs were not. Our study has confirmed that the effects of TNF-α in HEI-OC1 cells were also inhibited by dexamethasone. However, because we did not determine the mRNA levels of key players in the putative pathways involved, further studies about transcriptional downregulation are needed.

In this study, three or more independent experiments were performed for each experiment and then averaged to perform statistical analysis. In the evaluation of cell viability after TNF-α and dexamethasone treatment, the same tendency was confirmed by four repeated independent experiments. Thus, the mean value was indicated with the standard error in **Figure 1**. In subsequent experiments, after confirming the same trends in three repeated experiments, **Figures 2** to **Figure 5** are similarly displayed with standard errors. There are no established specific guidelines for the number of repetitions and evidence of statistical significance for cellular experimental design as in the current study. In recent similar cellular experiments related to cytotoxicity and apoptosis using HEI-OC1 cells, the values were

derived from three experiments for analyzing cell viability, ROS generation, and apoptosis [44,53,62,65,67]. As in previous studies, when the same tendency was shown in three repeated experiments, the mean values were presented with standard errors. Therefore, scientifically, this method of presentation can be considered valid. Because the values for the control were calculated as one, the standard error is not indicated in the control.

Through this study, we have confirmed that TNF-α treatment of auditory hair cells decreases cell viability, promotes ROS generation, and induces apoptosis, and that dexamethasone protects the auditory hair cells from the effects of TNF-α. However, this study only confirmed these effects in a previously established cell line. Thus, *ex vivo* experiments through cochlea primary cultures, especially of the organ of Corti, are needed to verify our *in vitro* results. Further, results of clinical significance can be assessed *in vivo* through animal experiments.

Ultimately, studies on the effects of dexamethasone and TNF-α inhibitors, or their synergistic effects should be performed. Research about this subject will have important clinical implications for broadening the understanding of hearing loss and the potential range of treatment. Although our study did not completely reveal the mechanisms underlying the effects of TNF-α and dexamethasone in hearing loss, our findings are fundamental to the ongoing research in this field.

Conclusions

In summary, TNF-α inhibits cell viability, increases ROS production, and activates apoptosis in auditory hair cells, while dexamethasone pretreatment attenuated these effects. Thus, dexamethasone exhibits protective effects against TNF-α-induced auditory hair cell damage. These findings about the role of TNF-α in immune-related hearing loss, the protective effects of dexamethasone, and the exploration of the corresponding mechanisms are important bases for future studies for the treatment of hearing loss.

References

1. Dayal VS, Ellman M, Sweiss N. Autoimmune inner ear disease: clinical and laboratory findings and treatment outcome. J Otolaryngol Head Neck Surg. 2008 Aug;37(4):591-6.
2. Kujawa SG, Liberman MC. Adding insult to injury: cochlear nerve degeneration after "temporary" noise-induced hearing loss. J Neurosci. 2009 Nov 11;29(45):14077-85.
3. Kuhn M, Heman-Ackah SE, Shaikh JA, Roehm PC. Sudden sensorineural hearing loss: a review of diagnosis, treatment, and prognosis. Trends Amplif. 2011 Sep;15(3):91-105.
4. Wang X, Jiang R, Su J. Sudden sensorineural hearing loss with end-stage renal disease: a report of 32 cases. Acta Otolaryngol. 2019 Nov;139(11):1004-7.
5. Vambutas A, Pathak S. AAO: Autoimmune and Autoinflammatory (Disease) in Otology: What is New in Immune-Mediated Hearing Loss. Laryngoscope Investig Otolaryngol. 2016 Oct;1(5):110-5.
6. Bovo R, Aimoni C, Martini A. Immune-mediated inner ear disease. Acta Otolaryngol. 2006 Oct;126(10):1012-21.
7. Tsinaslanidou Z, Tsaligopoulos M, Angouridakis N, Vital V, Kekes G, Constantinidis J. The Expression of TNFα, IL-6, IL-2 and IL-8 in the Serum of Patients with Idiopathic Sudden Sensorineural Hearing Loss: Possible Prognostic Factors of Response to Corticosteroid Treatment. Audiology and Neurotology Extra. 2016;6(1):9-19.
8. Chandrasekhar SS, Tsai Do BS, Schwartz SR, Bontempo LJ, Faucett EA, Finestone

SA, et al. Clinical Practice Guideline: Sudden Hearing Loss (Update). Otolaryngol Head Neck Surg. 2019 Aug;161(1_suppl):S1-S45.

9. Alexander TH, Harris JP. Incidence of sudden sensorineural hearing loss. Otol Neurotol. 2013 Dec;34(9):1586-9.

10. Zadeh MH, Storper IS, Spitzer JB. Diagnosis and treatment of sudden-onset sensorineural hearing loss: a study of 51 patients. Otolaryngol Head Neck Surg. 2003 Jan;128(1):92-8.

11. Dilwali S, Landegger LD, Soares VY, Deschler DG, Stankovic KM. Secreted Factors from Human Vestibular Schwannomas Can Cause Cochlear Damage. Sci Rep. 2015 Dec 22;5(18599.

12. Demirhan E, Eskut NP, Zorlu Y, Cukurova I, Tuna G, Kirkali FG. Blood levels of TNF-alpha, IL-10, and IL-12 in idiopathic sudden sensorineural hearing loss. Laryngoscope. 2013 Jul;123(7):1778-81.

13. Derebery MJ, Berliner KI. Allergy and its relation to Meniere's disease. Otolaryngol Clin North Am. 2010 Oct;43(5):1047-58.

14. Gazquez I, Requena T, Espinosa JM, Batuecas A, Lopez-Escamez JA. Genetic and clinical heterogeneity in Meniere's disease. Autoimmun Rev. 2012 Oct;11(12):925-6.

15. Nakashima T, Pyykko I, Arroll MA, Casselbrant ML, Foster CA, Manzoor NF, et al. Meniere's disease. Nat Rev Dis Primers. 2016 May 12;2(16028.

16. Lopez-Escamez JA, Carey J, Chung WH, Goebel JA, Magnusson M, Mandala M, et al. Diagnostic criteria for Meniere's disease. J Vestib Res. 2015;25(1):1-7.

17. Committee on Hearing and Equilibrium guidelines for the diagnosis and evaluation of therapy in Meniere's disease. American Academy of Otolaryngology-Head and Neck Foundation, Inc. Otolaryngol Head Neck Surg. 1995 Sep;113(3):181-5.

18. Murdin L, Schilder AG. Epidemiology of balance symptoms and disorders in the community: a systematic review. Otol Neurotol. 2015 Mar;36(3):387-92.

19. Merchant SN, Adams JC, Nadol JB, Jr. Pathophysiology of Meniere's syndrome: are symptoms caused by endolymphatic hydrops? Otol Neurotol. 2005 Jan;26(1):74-81.

20. Berlinger NT. Meniere's disease: new concepts, new treatments. Minn Med. 2011 Nov;94(11):33-6.

21. Moller MN, Brandt C, Ostergaard C, Caye-Thomasen P. Endolymphatic sac involvement in bacterial meningitis. Eur Arch Otorhinolaryngol. 2015 Apr;272(4):843-51.

22. Caulley L, Quimby A, Karsh J, Ahrari A, Tse D, Kontorinis G. Autoimmune arthritis in Meniere's disease: A systematic review of the literature. Semin Arthritis Rheum. 2018 Aug;48(1):141-7.

23. Tayer-Shifman OE, Ilan O, Tovi H, Tal Y. Cogan's syndrome--clinical guidelines and novel therapeutic approaches. Clin Rev Allergy Immunol. 2014 Aug;47(1):65-72.

24. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell. 2001 Feb 23;104(4):487-501.

25. Akazawa A, Sakaida I, Higaki S, Kubo Y, Uchida K, Okita K. Increased expression of tumor necrosis factor-alpha messenger RNA in the intestinal mucosa of inflammatory bowel disease, particularly in patients with disease in the inactive phase. J Gastroenterol. 2002;37(5):345-53.

26. Shanahan JC, St Clair W. Tumor necrosis factor-alpha blockade: a novel therapy for rheumatic disease. Clin Immunol. 2002 Jun;103(3 Pt 1):231-42.

27. Abraham BP, Ahmed T, Ali T. Inflammatory Bowel Disease: Pathophysiology and Current Therapeutic Approaches. Handb Exp Pharmacol. 2017;239(115-46.

28. Ichimiya I, Yoshida K, Hirano T, Suzuki M, Mogi G. Significance of spiral ligament fibrocytes with cochlear inflammation. Int J Pediatr Otorhinolaryngol. 2000 Nov 30;56(1):45-51.

29. Satoh H, Firestein GS, Billings PB, Harris JP, Keithley EM. Tumor necrosis factor-alpha, an initiator, and etanercept, an inhibitor of cochlear inflammation. Laryngoscope. 2002 Sep;112(9):1627-34.

30. Lorenz RR, Solares CA, Williams P, Sikora J, Pelfrey CM, Hughes GB, et al. Interferon-gamma production to inner ear antigens by T cells from patients with autoimmune sensorineural hearing loss. J Neuroimmunol. 2002 Sep;130(1-2):173-8.

31. Pathak S, Hatam LJ, Bonagura V, Vambutas A. Innate immune recognition of molds and homology to the inner ear protein, cochlin, in patients with autoimmune inner ear disease. J Clin Immunol. 2013 Oct;33(7):1204-15.

32. Svrakic M, Pathak S, Goldofsky E, Hoffman R, Chandrasekhar SS, Sperling N, et al. Diagnostic and prognostic utility of measuring tumor necrosis factor in the peripheral circulation of patients with immune-mediated sensorineural hearing loss. Arch Otolaryngol Head Neck Surg. 2012 Nov;138(11):1052-8.

33. Ren Y, Stankovic KM. The Role of Tumor Necrosis Factor Alpha (TNFalpha)in Hearing Loss and Vestibular Schwannomas. Curr Otorhinolaryngol Rep. 2018 Mar;6(1):15-23.

34. Satoh H, Firestein GS, Billings PB, Harris JP, Keithley EM. Proinflammatory cytokine expression in the endolymphatic sac during inner ear inflammation. J Assoc Res Otolaryngol. 2003 Jun;4(2):139-47.

35. Kaur T, Mukherjea D, Sheehan K, Jajoo S, Rybak LP, Ramkumar V. Short interfering RNA against STAT1 attenuates cisplatin-induced ototoxicity in the rat by suppressing

inflammation. Cell Death Dis. 2011 Jul 21;2(e180.

36. Bas E, Van De Water TR, Gupta C, Dinh J, Vu L, Martinez-Soriano F, et al. Efficacy of three drugs for protecting against gentamicin-induced hair cell and hearing losses. Br J Pharmacol. 2012 Jul;166(6):1888-904.

37. Arpornchayanon W, Canis M, Ihler F, Settevendemie C, Strieth S. TNF-alpha inhibition using etanercept prevents noise-induced hearing loss by improvement of cochlear blood flow in vivo. Int J Audiol. 2013 Aug;52(8):545-52.

38. Ihler F, Sharaf K, Bertlich M, Strieth S, Reichel CA, Berghaus A, et al. Etanercept prevents decrease of cochlear blood flow dose-dependently caused by tumor necrosis factor alpha. Ann Otol Rhinol Laryngol. 2013 Jul;122(7):468-73.

39. Ihler F, Pelz S, Coors M, Matthias C, Canis M. Application of a TNF-alpha-inhibitor into the scala tympany after cochlear electrode insertion trauma in guinea pigs: preliminary audiologic results. Int J Audiol. 2014 Nov;53(11):810-6.

40. Mata-Castro N, Sanz-Lopez L, Varillas-Delgado D, Garcia-Fernandez A. Intratympanic infliximab is a safe and effective rescue therapy for refractory immune-mediated hearing loss. Eur Arch Otorhinolaryngol. 2020 Feb;277(2):393-400.

41. Dhukhwa A, Bhatta P, Sheth S, Korrapati K, Tieu C, Mamillapalli C, et al. Targeting Inflammatory Processes Mediated by TRPVI and TNF-alpha for Treating Noise-Induced Hearing Loss. Front Cell Neurosci. 2019;13(444.

42. Katsumi S, Sahin MI, Lewis RM, Iyer JS, Landegger LD, Stankovic KM. Intracochlear Perfusion of Tumor Necrosis Factor-Alpha Induces Sensorineural Hearing Loss and Synaptic Degeneration in Guinea Pigs. Front Neurol. 2019;10(1353.

43. Wang W, Zhang LS, Zinsmaier AK, Patterson G, Leptich EJ, Shoemaker SL, et al. Neuroinflammation mediates noise-induced synaptic imbalance and tinnitus in rodent

models. PLoS Biol. 2019 Jun;17(6):e3000307.

44. So H, Kim H, Lee JH, Park C, Kim Y, Kim E, et al. Cisplatin cytotoxicity of auditory cells requires secretions of proinflammatory cytokines via activation of ERK and NF-kappaB. J Assoc Res Otolaryngol. 2007 Sep;8(3):338-55.

45. Keithley EM, Wang X, Barkdull GC. Tumor necrosis factor alpha can induce recruitment of inflammatory cells to the cochlea. Otol Neurotol. 2008 Sep;29(6):854-9.

46. McDonough AK, Curtis JR, Saag KG. The epidemiology of glucocorticoid-associated adverse events. Curr Opin Rheumatol. 2008 Mar;20(2):131-7.

47. Nash JJ, Nash AG, Leach ME, Poetker DM. Medical malpractice and corticosteroid use. Otolaryngol Head Neck Surg. 2011 Jan;144(1):10-5.

48. Ahn JH, Han MW, Kim JH, Chung JW, Yoon TH. Therapeutic effectiveness over time of intratympanic dexamethasone as salvage treatment of sudden deafness. Acta Otolaryngol. 2008 Feb;128(2):128-31.

49. Ahn JH, Yoo MH, Yoon TH, Chung JW. Can intratympanic dexamethasone added to systemic steroids improve hearing outcome in patients with sudden deafness? Laryngoscope. 2008 Feb;118(2):279-82.

50. McCall AA, Swan EE, Borenstein JT, Sewell WF, Kujawa SG, McKenna MJ. Drug delivery for treatment of inner ear disease: current state of knowledge. Ear Hear. 2010 Apr;31(2):156-65.

51. Garavello W, Galluzzi F, Gaini RM, Zanetti D. Intratympanic steroid treatment for sudden deafness: a meta-analysis of randomized controlled trials. Otol Neurotol. 2012 Jul;33(5):724-9.

52. Dinh CT, Haake S, Chen S, Hoang K, Nong E, Eshraghi AA, et al. Dexamethasone

protects organ of corti explants against tumor necrosis factor-alpha-induced loss of auditory hair cells and alters the expression levels of apoptosis-related genes. Neuroscience. 2008 Nov 19;157(2):405-13.

53. Nam SI, Kwon TK. Dexamethasone inhibits interleukin-1beta-induced matrix metalloproteinase-9 expression in cochlear cells. Clin Exp Otorhinolaryngol. 2014 Sep;7(3):175-80.

54. Diao MF, Liu HY, Zhang YM, Gao WY. [Changes in antioxidant capacity of the guinea pig exposed to noise and the protective effect of alpha-lipoic acid against acoustic trauma]. Sheng Li Xue Bao. 2003 Dec 25;55(6):672-6.

55. Ahn JH, Kang HH, Kim TY, Shin JE, Chung JW. Lipoic acid rescues DBA mice from early-onset age-related hearing impairment. Neuroreport. 2008 Aug 27;19(13):1265-9.

56. Tavanai E, Mohammadkhani G, Farahani S, Jalaie S. Protective Effects of Silymarin Against Age-Related Hearing Loss in an Aging Rat Model. Indian J Otolaryngol Head Neck Surg. 2019 Nov;71(Suppl 2):1248-57.

57. Kalinec GM, Webster P, Lim DJ, Kalinec F. A cochlear cell line as an in vitro system for drug ototoxicity screening. Audiol Neurootol. 2003 Jul-Aug;8(4):177-89.

58. Kalinec GM, Park C, Thein P, Kalinec F. Working with Auditory HEI-OC1 Cells. J Vis Exp. 2016 Sep 3115).

59. Rivolta MN, Holley MC. Cell lines in inner ear research. J Neurobiol. 2002 Nov 5;53(2):306-18.

60. Kalinec F, Kalinec G, Boukhvalova M, Kachar B. Establishment and characterization of conditionally immortalized organ of corti cell lines. Cell Biol Int. 1999;23(3):175-84.

61. Kizawa Y, Furuya M, Saito K, Masuko T, Kusama T. Effects of dexamethasone and

aminophylline on survival of Jurkat and HL-60 cells. Biol Pharm Bull. 2006 Feb;29(2):281-5.

62. Kalinec G, Thein P, Park C, Kalinec F. HEI-OC1 cells as a model for investigating drug cytotoxicity. Hear Res. 2016 May;335(105-17.

63. Campisi J. Aging, cellular senescence, and cancer. Annu Rev Physiol. 2013;75(685-705.

64. Marino G, Niso-Santano M, Baehrecke EH, Kroemer G. Self-consumption: the interplay of autophagy and apoptosis. Nat Rev Mol Cell Biol. 2014 Feb;15(2):81-94.

65. Hayashi K, Dan K, Goto F, Tshuchihashi N, Nomura Y, Fujioka M, et al. The autophagy pathway maintained signaling crosstalk with the Keap1-Nrf2 system through p62 in auditory cells under oxidative stress. Cell Signal. 2015 Feb;27(2):382-93.

66. Wada T, Penninger JM. Mitogen-activated protein kinases in apoptosis regulation. Oncogene. 2004 Apr 12;23(16):2838-49.

67. Yi J, Kim TS, Pak JH, Chung JW. Protective Effects of Glucose-Related Protein 78 and 94 on Cisplatin-Mediated Ototoxicity. Antioxidants (Basel). 2020 Aug 2;9(8).

English abstract

Background: Auditory hair cell damage is the most common mechanism for sensorineural hearing loss. Immune-related hearing loss is autoimmune-mediated and is characterized by frequent recurrence and a chronic course. It is rare, but it may be underreported or underdiagnosed as it has been suggested to be part of the etiology of more common diseases such as sudden sensorineural hearing loss and Ménière's disease. The role of cytokines in immune-related hearing loss has been investigated. Increased levels of tumor necrosis factor-α (TNF-α) in the cochlea have been found in various types of hearing loss, including immune-related inner ear disease. Further, TNF-α has been identified as a key cytokine of the immune response in the inner ear by activating a number of signaling pathways. Corticosteroids have been used to treat a variety of inner ear diseases, but its mechanism are not completely understood. However, due to the side effects of corticosteroids, attempts have been made to reduce the requirement for systemic corticosteroid therapy especially in immune-related hearing loss, which may require long term and repeated steroid pulse therapy. Therefore, TNF-α is in the spotlight as a potential therapeutic target. By exploring the role of TNF-α and the protective effects of corticosteroids, downstream signaling pathways may be identified, which may open new avenues in the understanding and treatment of various types of hearing loss, including immune-related hearing loss. Additionally, prophylactic and therapeutic substances that can replace corticosteroids may be identified by understanding these mechanisms. Therefore, this study was performed to evaluate TNF-α-induced damage and the protective effects of corticosteroids in hearing loss using an auditory hair cell line. This study was intended to broaden the understanding of the mechanisms underlying hearing

loss and to contribute to ongoing efforts to identify treatments for the condition.

Materials and Methods: House Ear Institute-organ of Corti 1 (HEI-OC1) cells were used as an auditory sensory hair cell model. HEI-OC1 cells were treated with various concentrations of dexamethasone and TNF-α to first identify their effects on HEI-OC1 cell viability, which was determined using a Cell Counting Kit-8 (CCK-8) assay. Oxidative stress was assessed via the production of reactive oxygen species (ROS) through reactive fluorescence after treatment with TNF-α and dexamethasone. The effects of dexamethasone and TNF-α treatment on the apoptosis pathway were confirmed by western blotting using antibodies against as B-cell lymphoma 2 (Bcl-2), Bcl-2-associate X protein (Bax), caspase-3, caspase-7, poly (ADP-ribose) polymerase (PARP), and β-actin. To evaluate the protective effects of dexamethasone on TNF-α-induced effects on cell viability, ROS generation, and apoptosis, the cells were treated with dexamethasone for 6 hours before a 24-hour treatment with TNF-α, and then compared with the control (no treatment) and the treatment with TNF-α alone. To further confirm the protective effects of dexamethasone against apoptosis, the control, TNF-α-only treated, and dexamethasone-pretreated TNF-α-treated cells were evaluated by annexin V/propodium iodide (PI) staining.

Results: The cell viability of HEI-OC1 cells increased relative to the control after 6 hours of treatment with dexamethasone at a concentration of 5 nM or higher. Meanwhile, cell viability significantly decreased after 24 hours of treatment with TNF-α at a concentration of 5 ng/ml or higher. TNF-α treatment significantly increased ROS production, compared with the control and dexamethasone treatment. Levels of proteins in the apoptosis pathway did not differ between the untreated control and the cells treated with dexamethasone. However, treatment with TNF-α activated apoptosis, as shown by decreased levels of Bcl-2 and

increased levels of Bax, cleaved caspase-3, cleaved caspase-7, and cleaved PARP. Pretreatment with dexamethasone significantly reduced the TNF-α induced decrease in cell viability and increase in ROS generation and apoptosis activation. Fluorescence staining showed that the percentage of apoptotic cells increased with TNF-α and decreased with dexamethasone pretreatment.

Conclusions: TNF-α reduces cell viability, increases ROS generation, and activates the apoptosis in auditory hair cells. Dexamethasone can protect auditory hair cell against the TNF-α-induced damages. The findings we present here can be used as basis for future studies on the treatment of hearing loss.

Figure 1. Effects of Dexamethasone (Dex) and tumor necrosis factor-α (TNF-α) on the viability of HEI-OC1 cells.

(A, B) Values are expressed as the means ± SE (standard error) for four independent experiments, expressed as a percentage of untreated control value. HEI-OC1 cells were incubated for 24h in the presence of TNF-α

* $P < 0.05$, compared with untreated control.

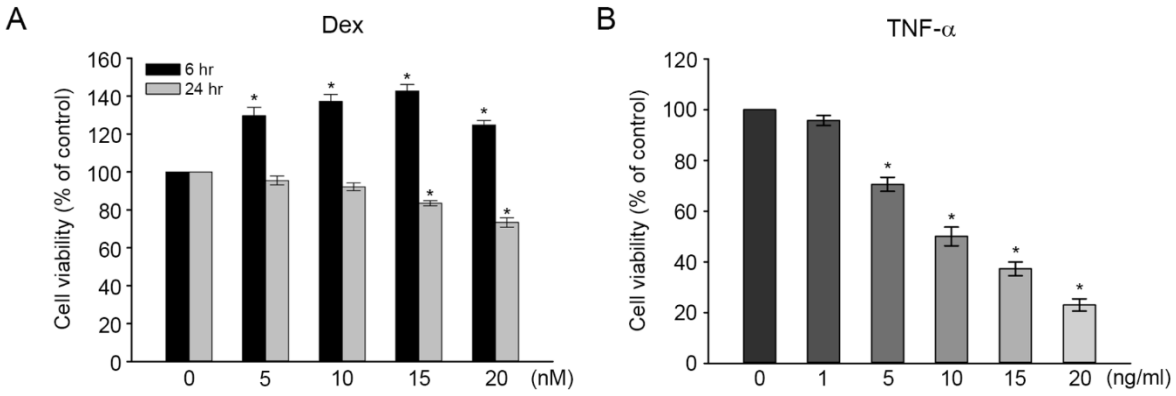

Figure 2. Effects of Dexamethasone (Dex) and tumor necrosis factor-α (TNF-α) on the ROS generation of HEI-OC1 cells.

Values are expressed as means ± SE (standard error) for three independent experiments.

*P < 0.05, compared with the untreated control (Con).

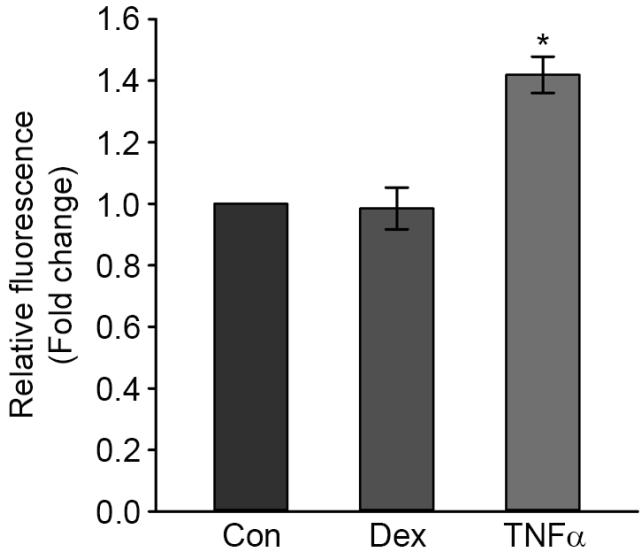

Figure 3. Effects of Dexamethasone (Dex) and tumor necrosis factor-α (TNF-α) on the expression of apoptosis-related proteins.

Individual bands were normalized to β-actin. The values in a graph are represented as fold changes relative to the untreated control, expressed as means ± SE (standard error) of three independent experiments.

*P < 0.05; compared with untreated control (Con).

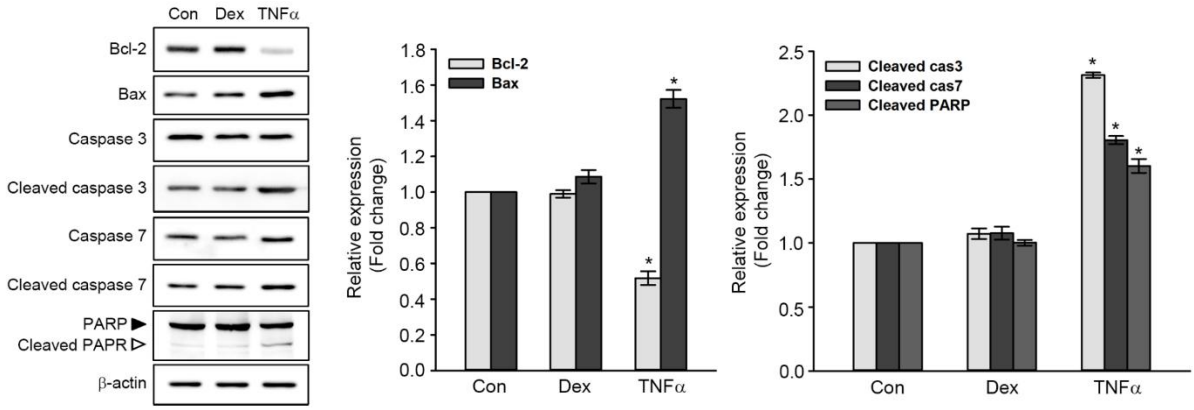

Figure 4. Protective effect of Dexamethasone (Dex) on the cell viability, ROS generation, and expression of apoptosis-related proteins.

(A) Values are expressed as the means ± SE (standard error) for four independent experiments, expressed as a percentage of untreated control (Con) value.

(B) Values are expressed as means ± SE for three independent experiments.

(C) Individual bands were normalized to β-actin. The values in a graph are represented as fold changes relative to the untreated control, expressed as means ± SE of three independent experiments.

*,# $P < 0.05$, *; compared with untreated control, #; TNF-α only versus Dex plus TNF-α.

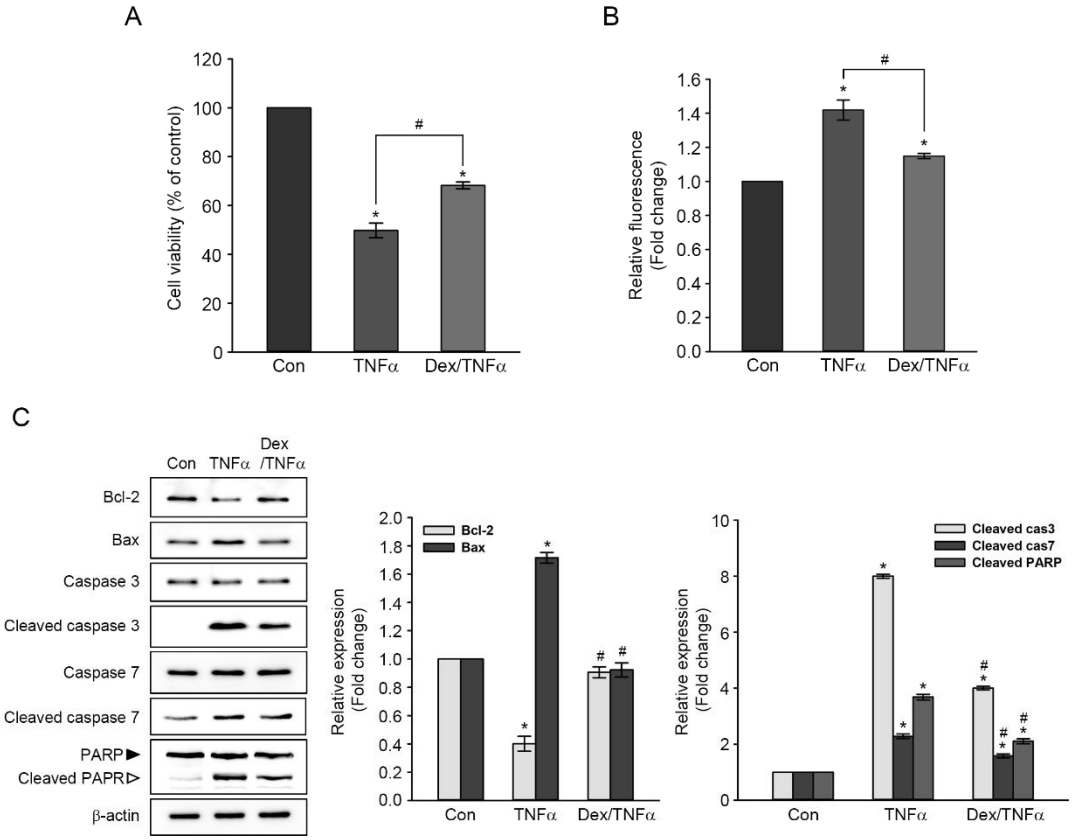

Figure 5. Staining of apoptotic cell death

Values are expressed as means ± SE (standard error) for three independent experiments.

Scale bar = 100 μm, Original magnification, 100×

*,# P < 0.05, ** P < 0.01, *** P < 0.001

*,**,***; compared with untreated control

#; TNF-α only versus Dex plus TNF-α.