Published in final edited form as:

Acta Otolaryngol. 2013 July ; 133(7): 685–691. doi:10.3109/00016489.2013.771407.

Age-Related Cochlear Cytokine Gene Expression in the BALB/cJ Mouse with Systemic versus Intra-tympanic Dosing of Steroid Drugs

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Abstract

Conclusion—Age-related differences in the expression of inflammatory cytokines in the inner ear may contribute to the development of age-related hearing loss.

Objectives—Age-related hearing loss is characterized by tissue remodeling, ischemia, ion homeostasis, and inflammation. Steroid therapy is an otoprotective strategy that likely acts by reducing inflammation. We examined age-related changes in cytokine gene expression in the cochlea of the BALB/cJ mouse model of premature age-related hearing loss after systemic or intra-tympanic steroid delivery.

Methods—“Young” (2.5-3 months) and “Old” (5-9 months) mice were treated with dexamethasone or fludrocortisone administered either orally or intra-tympanically. Cytokine gene expression in cochlear RNA was analyzed using prefabricated cDNA arrays. Old groups were compared to Young to identify age-related changes.

Results—Down-regulation of a cytokine associated with bone remodeling (SPP1) was observed in the untreated Old group. Numerous genes were up- or down-regulated by more than two-fold by steroid treatment, including pro-inflammatory interleukins (IL-16) and anti-inflammatory cytokines.

Keywords

Aging; Animal model; Glucocorticoid; Inflammation; Inner Ear; Mineralocorticoid; Sensorineural hearing loss; SPP1

Introduction

Age-related hearing loss (ARHL) is a predominant neurodegenerative disease of aging. It is categorized into four groups according to the specific organ of degeneration: *sensory* (organ of Corti), *neural* (spiral ganglion cells), *strial* (stria vascularis), or mixed. Numerous biological factors contribute to ARHL, including altered vascular biology, hypoxia/ischemia, inflammation, bone remodeling, and perturbations in ion homeostasis, with many of these processes being inter-related [1]. For example, cytokines are responsible for initiating and regulating inflammation while also playing important roles in other cellular functions such as apoptosis, bone remodeling, and ion homeostasis [2, 3]. Our focus has been on the inflammation aspects of ARHL pathogenesis.

Inflammation has been linked to ARHL in mouse models, including the CD/1 mouse in which the HIF1- α marker for ischemia is associated with up-regulation of radical oxygen species leading to up-regulation of TNF- α , a strong promoter of inflammation [4]. ARHL is also observed in the SAMP8 mouse model with accelerated senescence that is used to study the effects of aging on biological processes [5]. ARHL in SAMP8 mice involves oxidative stress and altered levels of antioxidant enzymes which result in chronic inflammation and subsequent cellular apoptosis.

Age-related changes in response to glucocorticoid treatment in tissues other than the cochlea have been shown. Glucocorticoids and mineralocorticoids are a part of the hypothalamic-pituitary-adrenal axis where they act to provide negative feedback. This feedback inhibition is reduced with advancing age, suggesting that a similar mechanism may be involved in aging in other organ systems [6]. The cochlear hypothalamic-pituitary-adrenal axis (HPA) system functions to balance auditory sensitivity and susceptibility to noise-induced hearing loss, making this an important target for therapeutic interventions.

Corticosteroid therapy is commonly used to suppress inflammation and to protect the middle and inner ear from permanent damage. Steroids also have an impact on inner ear ion homeostasis and fluid transport [7] and have been suggested to prevent bone remodeling and labyrinthitis ossification after cochlear implant [8]. The various effects of corticosteroids likely result in part from a differential affinity to either the glucocorticoid receptor (GR) (anti-inflammation and immunosuppression) or the mineralocorticoid receptor (MR) (sodium transport and fluid reabsorption). Although glucocorticoid drugs are primarily used for immunosuppression, their affinity to other receptors like MRs may contribute to other functions and/or side-effects. As an example of the complexity of steroid therapy, MacArthur et al. [9] showed that pure glucocorticoids were more effective than pure mineralocorticoids at reducing hearing loss in the C3H/HeJ mouse model for spontaneous chronic otitis media, but treating with prednisolone which has both glucocorticoid and mineralocorticoid properties was a superior approach.

BALB/cJ mice are one of the best-characterized mouse models for ARHL. Hearing loss in the BALB/cJ strain as measured by auditory brainstem response (ABR) thresholds is seen at 2-4 months of age, with severe hearing loss seen by 23 months of life [10]. Hearing loss in the mouse is accompanied by a progressive loss of hair and spiral ganglion cells (SGC) in association with cochlear atrophy, which are also common findings in ARHL [11].

The focus of this article is to examine the effects of age and pharmacological intervention with steroids on cytokine gene expression in the cochlea in order to inform future therapeutic approaches for ARHL.

Materials and Methods

Mouse Strains

All experimental animal procedures were reviewed and approved by the ----- Institutional Animal Care and Use Committee. BALB/cJ mice were obtained from the Jackson Laboratories (Bar Harbor, ME). The animals were studied at two different ages: 'Young' animals were 2.5-3 months old, whereas 'Old' animals were 5-9 months of age. The animals were sacrificed by an overdose of anesthetic (ketamine and xylazine). The distribution of animals into the various study groups is shown in Table 1.

Systemic Steroid Drugs

The study drugs were administered according to equivalent therapeutic doses as defined by their relative glucocorticoid effect [12]. The drug preparations used were the standard hospital pharmacy formulation given to patients. Systemic steroid treatments were given either in the drinking water (fludrocortisone 10 µg/kg/d), or subcutaneously (dexamethasone 0.75 mg/kg/d) [9]. Oral steroid doses were ingested over a 24-hour period and the animals were sacrificed at the end of the 24 hours. Subcutaneous dexamethasone was administered once and animals sacrificed 24 hours after dosing. Age-matched untreated control animals were also studied.

Intratympanic (IT) Steroid Drugs

For IT steroid treatment, either 5µl of dexamethasone sodium phosphate (4 mg/ml) (Dex-21P; American Regent Laboratories, Shirley, NY, U.S.A.), or 5 µl of fludrocortisone acetate (0.12 mg/ml) was injected into the middle ear [13]. Animals were given one injection and sacrificed 24 hours later. Age-matched control animals were injected with saline, which was the vehicle used for administration of drug in the study animals.

Gene Expression Analysis

Cochleas were isolated and stored in RNAlater (Ambion, Inc., Austin, TX) at -70°C until RNA extraction at which time they were transferred to sterile microcentrifuge tubes. Cochleas from each experimental group were pooled, mechanically homogenized in lysis buffer, and the RNA-isolated (SV Total RNA Isolation Kit, Promega Corp., Madison, WI). To increase RNA purity, the lysate was passed through a membrane homogenizer (Qiashrepper; Qiagen Corp., Valencia, CA) prior to RNA isolation. RNA was concentrated by sodium acetate/ethanol precipitation, rehydrated in 10 µl RNAase-free water, quantified using a spectrophotometer, and stored at -70°C.

A commercially available prefabricated gene membrane array was used to evaluate the expression of 96 common murine cytokine genes (Mouse Common Cytokine cDNA Array, #MM-003; SuperArray Bioscience Corp., Frederick, MD). Inner ear RNA underwent reverse transcription with ³²P-dCTP, and the radiolabeled cDNA was used to probe the gene expression array. The membranes were prehybridized with sheared salmon sperm DNA before hybridization with the cDNA overnight. The membranes were washed, exposed to radiographic film, and scanned with an HP Precision Scan 2.0 Pro scanner. The experiments were performed in triplicate from the pooled cochleas of each experimental group.

Data Analysis

The image data extraction and data analyses were performed using the web-based GEM Express Expression Analysis Suite (SuperArray Bioscience Corp.). Gene expression signal data were exported into Microsoft Excel and analyzed to determine cytokine gene expression relative to embedded standards on the arrays. Mean signal intensity was adjusted for local

background by subtracting the minimum background intensity. Data were normalized against GAPDH expression. Signal AP threshold was set to 2.0 and boundary was set to 1.8. Using the three data points from each membrane, each gene was assessed for fold-change and p-value. A two-fold increase or decrease and a two-sided p-value of less than .05 was considered to be significant.

Results

Control Baseline Comparisons

Systemic control animals were untreated and also served as the baseline controls for comparing IT delivery of vehicle (saline) to steroid. When IT saline groups were compared to untreated animals, a mild change in cytokine expression was seen in Old groups as a result of the local inflammation caused by the injection (Table 2). However, no significant changes were observed in saline-IT treated cochleas in Young animals.

Age-related changes in cytokine expression in untreated BALB/cJ mice

Comparison of cytokine gene expression in the untreated Old group compared to the untreated Young group showed a significant change in Secreted Phosphoprotein 1 (SPP1) which was down-regulated 2.5 fold ($p=.029$) in the cochlea of older as compared to young animals (data not shown). SPP1 acts as a pro-inflammatory cytokine and is present in the extra-cellular matrix of bone [14, 15].

Age-related cytokine expression in response to Systemic Steroid Treatment

We looked at cytokine expression in both Old, and Young groups treated systemically with either dexamethasone (GC) or fludrocortisone (GC/MC). In the dexamethasone treatment group, we found changes in the expression of several cytokines. The proinflammatory cytokine IL-16 was up-regulated nearly 5-fold in the Old as compared to the Young group (Table 3-Systemic). In addition, we found a slight down-regulation of a subset of cytokines including lymphotoxin A (LTA), IL-9, and IL-1 subfamily 5 (IL-1F5). Overall, however, the data suggest that the cellular response to dexamethasone/ glucocorticoid in the older versus younger animals does not differ markedly. In contrast, when treated with fludrocortisone, there was down-regulation of 9 cytokines in the older mice, including the pro-inflammatory cytokines SPP1, IL-5, and IL-6, as well as the anti-inflammatory cytokine IL-21 (Table 4-Systemic).

In a less stringent analysis using 1.5-fold change as our cut off (data not shown), other cytokines showed changes in expression in response to systemic Fludrocortisone steroid treatment, including up-regulation of BMP5 and Fibrosin 1 and down-regulation of IL-12A, IL-22, IFNG, NODAL, Granulin, and Oncostatin M.

Age-related cytokine expression in response to IT Steroid Treatment

Glucocorticoid and mineralocorticoid effects in Old versus Young groups were also compared when the drug was delivered intra-tympanically (IT), and a very different cytokine pattern was observed than was noted with systemic treatment. There was significant down-regulation of a subset of common pro-inflammatory cytokines, including Inhibin- β A (INHBA), IL-12A, IL-25, as well as several Interferon-alpha family members in both the dexamethasone and the fludrocortisone in Old groups (Tables 3 and 4, IT). In addition, both treatment groups showed a 2.2 to 5 fold increase of the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1Ra) in the older population. IL-11 was also shown to be down-regulated by nearly 2-fold in Old animals in the IT dexamethasone treatment group. Finally, with fludrocortisone treatment in the Old groups as compared to similarly

treated Young animals, two bone morphogenic proteins (BMP2 and BMP8b) were downregulated by 2.3 and 4.4 fold, respectively.

When including genes that exhibited a 1.5-fold change, we showed that there was downregulation in IL-5, Granulin, BMP7, FGF10, and GDF15 in mice treated with IT fludrocortisone, and when using IT dexamethasone, we also showed that there was downregulation in IL-17B and Granulin.

Cytokine expression in Systemic versus IT delivery of steroids

IT delivery of dexamethasone resulted in a more dramatic change in cochlear cytokine expression than did systemic treatment, and the pattern of cytokine expression was different between the two delivery methods (Table 3). When using fludrocortisone, the delivery method showed no effect on the number of cytokines that were affected (Table 4). There was overlap in the changes seen between the two IT treated groups (dexamethasone and fludrocortisone), with 9 cytokines affected in common.

Discussion

We were surprised to discover that there is little difference in the overall pattern of cytokine expression when comparing untreated BALB/cJ Old to Young mice (data not shown). There was, however, a significant down-regulation of SPP1 cytokine expression in the Old compared to Young untreated groups. SPP1 was further reduced in the older experimental group treated with systemic fludrocortisone. We had anticipated that there would be a more dramatic change because hearing loss in the BALB/cJ mouse is progressive with increasing age [16]. This could be explained by the age of the animals used. While hearing loss is first exhibited in the BALB/cJ mouse at 2-4 months, significant hearing loss is seen closer to 23 months. We studied animals at 5-9 months of age to explore the early events of an active disease process leading to hearing loss which may no longer be evident at end-stage disease due to permanent tissue alterations, such as bone loss or scarring.

SPP1 acts as a pro-inflammatory cytokine by enhancing production of interferon-gamma and IL-12, which in turn leads to a reduction in the production of IL-10, an essential member of the pathway that leads to type I immunity [14]. Interestingly, SPP1 is abundant in bone extracellular matrix and is produced by osteoblasts involved in bone formation [15]. In experimental models of acute otitis media, inoculation with *Streptococcus pneumoniae* leads to an initial phase of bone loss from osteoresorption, followed by bone growth due to osteoneogenesis, with subsequent normalization of bone homeostasis [3]. We speculate that SPP1 may be involved in a similar mechanism contributing to ARHL.

When treatment was compared in Young vs. Old animals, systemic treatment with steroids revealed cytokine changes. The cytokines involved in these changes differed between glucocorticoid and mineralocorticoid treatment groups (Table 3 and 4-systemic). In the case of dexamethasone, there was a 5-fold increase in the cytokine IL-16 in the Old group (Table 3). IL-16 is a pro-inflammatory cytokine that is a potent chemotactic stimulus for CD4+ T-lymphocytes and eosinophils. Dexamethasone also reduced expression of Lymphotoxin alpha (LTA). Polymorphisms in LTA that result in decreased activity of LTA have been implicated in chronic inflammation disorders, including psoriatic arthritis [17, 18], and we speculate that chronic inflammation may also play a role in aging.

Systemic treatment with fludrocortisone resulted in the down-regulation of several cytokines, including pro-inflammatory SPP1, IL-5, and IL-6, as well as anti-inflammatory IL-21, and Cytokine K (IK) in the Old group (Table 4). IL-6, in addition to being a pro-inflammatory cytokine, is a potent stimulator of osteoclast-induced bone resorption in the

context of chronic inflammation [19]. There was also a trend toward up-regulation in expression of the cartilage-inducing protein BMP5 (1.5-fold). Up-regulation of BMP5 in tandem with reduced Spp1 levels suggests that there may be altered bone homeostasis in the older animals.

Control Young saline-IT animals showed no significant changes in cytokine expression, but Old saline-IT animals showed a change in the expression of some cytokines (Table 2). The change in the Old group suggests that the IT injection may cause a localized inflammation response in this age group, whereas the lack of change in the young group suggests that this localized response is either reduced or may be resolved prior to the 24hr study time-point.

IT treatment with steroids revealed slight differences in the response of the Old as compared to the Young mice, with many similarities in the pattern of response in both Old and Young animals when exposed to glucocorticoid or mineralocorticoid treatment (Tables 3 and 4). Pro-inflammatory cytokines such as Inhibin- β A (INHBA), IL-12A, IL-25, and several Interferon-alpha family members were down-regulated. Inhibins are involved in regulating a number of diverse functions such as hypothalamic and pituitary hormone secretion. Glucocorticoids and mineralocorticoids have been shown to reduce hypothalamic-pituitary-adrenal (HPA) axis signaling which has a protective effect against noise-related hearing loss. The increased down-regulation of INHBA in IT versus systemic groups suggests that the IT delivery may enhance the effect of glucocorticoid on the HPA system.

There was an up-regulation of the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1Ra) in the older population. In addition, IL-11, which promotes osteoclast formation and bone resorption [20], was shown to be down-regulated in older animals in the IT dexamethasone treatment group. IL-11 is known to down-regulate GRs [20] which suggests that there may be a positive feed-back loop in which dexamethasone lowers IL-11 thereby increasing the expression of GRs, resulting in increased dexamethasone efficacy. In the case of fludrocortisone treatment, two bone morphogenic proteins, BMP2 and BMP8b, were down-regulated in the Old group. BMPs are involved in bone remodeling, a process implicated in hearing loss. An ability to down-regulate BMPs could be an asset in developing a therapy to prevent ARHL.

When comparing the effect of systemic versus IT delivery of dexamethasone, similar patterns of gene expression were found with each treatment approach. Most notably, in all groups except dexamethasone in Old mice, Inhibin- β A (INHBA) was markedly up-regulated (11- to 20-fold increase).

IL1-Ra is a strong anti-inflammatory gene which was up-regulated 5-fold in the Old group after IT dexamethasone, and 2.5-fold after IT fludrocortisone. In designing an optimal treatment for ARHL, it may be more prudent to explore the direct anti-inflammatory gene up-regulation activity shown by glucocorticoids rather than its trans-repression of pro-inflammatory genes. The up-regulation of IL-1Ra in each of the IT treated groups suggests that this may be the more effective delivery route.

We have shown that there are different biochemical responses to steroids in relation to age and delivery system. Elucidating how age may change the response to the different steroidal treatments may contribute to an improved understanding of the complex etiology of ARHL, and may thereby ultimately lead to better therapies for this important and debilitating aspect of the aging process.

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Abbreviations

ABR	auditory brainstem response
ARHL	age-related hearing loss
GR	glucocorticoid receptor
MR	mineralocorticoid receptor
IT	Intra-tympanic

Table 1

Distribution of Mice into Treatment Groups

		Dexamethasone (n=18)		Fludrocortisone (n=35)	
		Systemic	IT	Control	IT
Young	5	5	4	10	5
Old	5	5	4	5	5

Table 2

Relative Cytokine Expression in Saline IT to Untreated

Gene	IT (Intra-Tympanic)	
	Young*	Old
BMP2		4.6 (p=.001)
BMP3		5.0 (p=.015)
BMP4		2.0 (p=.006)
BMP6		2.6 (p=.001)
BMP10		5.3 (p=.027)
CSF1		2.6 (p=.039)
CSF2		2.1 (p=.015)
CSF3		2.2 (p=.020)
Fibrosin 1		3.2 (p=.049)
GDF2		2.9 (p=.001)
GDF9		2.2 (p=.001)
IL-1α		-6.3 (p=.027)
IL-21		3.1 (p=.002)
TNFSF4		2.9 (p=.003)
TNFSF8		2.1 (p=.021)
TNFSF10		2.5 (p=.023)

* No significant changes noted.

Table 3

Relative Cytokine Expression after Dexamethasone Treatment in “Old” BALB/c Cochleas when Compared with Cochleas from “Young” BALB/c*

Gene	Systemic		IT (Intra-Tympanic)	
	Up-Regulated	Down-Regulated	Up-Regulated	Down-Regulated
ARTS-1	2.4 (p=.022)			
IL1F5		-2.1 (p=.010)		
IL1-Ra			5.1 (p=.003)	
IL-9		-2.7 (p=.025)		
IL-11				-2.0 (p=.019)
IL-12A				-3.5 (p=.0004)
IL-16	4.8 (p=.021)			
IL-17C				-2.8 (p=.002)
IL-25				-3.5 (p=.001)
INHBA				-14.3 (p=.001)
INFA4				-2.1 (p=.006)
INFA5				-2.7 (p=.001)
INFA6				-3.1 (p=.003)
LTA		-2.0 (p=.019)		
S100A11				-2.0 (p=.001)
TNFSF4			4.5 (p=.025)	
GDF15				-2.4 (p=.001)

* All fold changes presented in the table have a p-value < 0.05

Table 4

Relative Cytokine Expression after Fludrocortisone Treatment in “Old” BALB/c Cochleas when Compared with Cochleas from “Young” BALB/c*

Gene	Systemic		IT (Intra-Tympanic)	
	Up-Regulated	Down-Regulated	Up-Regulated	Down-Regulated
CD40-Ig		-2.8 (p=.017)		
IK		-3.2 (p=.001)		
IL-5		-2.1 (p=.010)		
IL-6		-3.5 (p=.001)		
IL-21		-2.5 (p=.024)		
SPP1		-3.0 (p=.003)		
THPO		-2.5 (p=.005)		
TNFSL-11b		-2.9 (p=.003)		
TNFSL 13b		-2.6 (p=.004)		
BMP2				-2.3 (p=.007)
BMP8b				-4.4 (p=.009)
IL1-Ra			2.2 (p=.015)	
IL-12A				-2.5 (p=.003)
IL-17C				-2.0 (p=.012)
IL-25				-2.3 (p=.003)
IL-27				-2.0 (p=.012)
INFA2				-2.0 (p=.022)
INFA5				-2.2 (p=.015)
INFA6				-2.3 (p=.013)
INHBA				-11.1 (p=.0004)

* All fold changes presented in the table have a p-value < 0.05