Changes in Gene Expression and Hearing Thresholds After Cochlear Implantation

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Hypothesis: Gene expression changes occur in conjunction with hearing threshold changes after cochlear implantation.

Background: Between 30 and 50% of individuals who receive electro-acoustic stimulation (EAS) cochlear implants lose residual hearing after cochlear implantation, reducing the benefits of EAS. The mechanism underlying this hearing loss is unknown; potential pathways include mechanical damage, inflammation, or tissue remodeling changes.

Methods: Guinea pigs were implanted in one ear with cochlear implant electrode arrays, with non-implanted ears serving as controls, and allowed to recover for 1, 3, 7, or 14 days. Hearing threshold changes were measured over time. Cochlear ribonucleic acid was analyzed using real-time quantitative reverse transcription-polymerase chain reaction from the following gene families: cytokines, tight junction claudins, ion and water (aquaporin) transport channels, gap junction connexins, and tissue remodeling genes.

Results: Significant increases in expression were observed for cochlear inflammatory genes (Cxc11, IL-1β, TNF-α, and Tnfrsf1a/b) and ion homeostasis genes (Senn1γ, Aqp3, and Gjb3). Upregulation of tissue remodeling genes (TGF-β, MMP2, MMP9) as well as a paracrine gene (CTGF) was also observed. Hearing loss occurred rapidly, peaking at 3 days with some recovery at 7 and 14 days after implantation. MM9 exhibited extreme up-regulation of expression and was qualitatively associated with changes in hearing thresholds.

Conclusion: Cochlear implantation induces similar changes as middle ear inflammation for genes involved in inflammation and ion and water transport function, whereas tissue remodeling changes differ markedly. The upregulation of MMP9 with hearing loss is consistent with previous findings linking stria vascularis vessel changes with cochlear implant-induced hearing loss. Key Words: Cochlear implants—Electro-acoustic stimulation—Gene expression—Guinea pig model—Hearing loss—Inflammation response—Ion homeostasis—Tissue remodeling.

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The hybrid or electro-acoustic stimulation (EAS) cochlear implant (CI) is shorter and thinner than a traditional CI and enables preservation of low-frequency residual hearing for combined acoustic and electric stimulation in the same ear (1,2). Compared to traditional, full-insertion CIs, EAS CIs dramatically improve speech perception in noise, voice recognition, and musical melody recognition (3–5). In addition, hearing preservation leads to superior speech perception outcomes even when the CI is used without the acoustic hearing, further indicating the importance of minimizing damage to neurosensory structures for effective electrical stimulation (6,7). However, between 30 and 55% of patients lose more than 30 dB of residual hearing within days to months after implantation, indicating a pressing need to improve hearing preservation rates and allow full usage of these benefits (8–10).

The exact mechanism of implantation-induced hearing loss is not yet clear. Proposed mechanisms include direct mechanical trauma to the basilar membrane or osseous spiral lamina (11–13), or an inflammatory or immune response leading to hair cell death (14). However, significant hearing loss can occur after cochlear implantation without evident mechanical trauma or hair cell loss (15,16). Fibrosis or osteogenesis after implantation can also theoretically cause hearing loss by attenuating the traveling wave (17); however, correlations between fibrosis/ossification and hearing loss are weak and require large numbers of animals to show significance (15,16). The lateral wall may also be vulnerable to damage because of its location in the path of the electrode insertion (18). Recent studies showed a correlation of reduced stria vascularis blood vessel density with hearing loss after...
cochlear implantation, suggesting that a reduced ability to maintain the endocochlear potential may cause hearing loss (15). Another possibility is that electrical stimulation and excessive current levels may also damage residual hearing via excitotoxic damage to afferent nerve terminals (19,20), especially with delayed hearing loss occurring months after implantation.

Gene expression changes have not been previously investigated in a cochlear implant animal model and may help to clarify whether the following factors and pathways—inflammation and hair cell loss, fibrosis and other tissue remodeling changes, or damage to ion homeostasis mechanisms—are responsible for the hearing loss.

Genes associated with inflammatory reactions in response to surgical trauma include pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β). When added to cochlear explants, TNF-α leads to hair cell death in vitro (21,22); its effects can be reversed by dexamethasone administration (23). Interestingly, TNF-α is localized not just in the organ of Corti but also to Type I fibrocytes and root cells in the spiral ligament where it mediates capillary constriction and reduction of cochlear blood flow (24,25). TNF-α and IL-1β are upregulated with middle ear inflammation (26,27); TNF-α is also upregulated after noise exposure (28). Other inflammation-related genes include chemokine (C-X-C motif) ligand 1 (CxCl1), tumor necrosis factor receptor superfamily member 1a and 1b (Tnfrf1a, Tnfrf1b), and TNF receptor-associated factor-2 and -4 (Traf2, Traf4). CxCl1 is upregulated with middle ear inflammation (26,27);

### TABLE 1. Primers used in the RT-PCR for guinea pigs

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<th>Target</th>
<th>Accession No.</th>
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Tnfr1a, Tnfr1b, and Traf4 are upregulated after noise exposure–induced apoptosis (28).

Genes associated with fibrosis and osteogenesis responses as part of the wound healing process include growth factors and tissue remodeling genes. Transforming growth factor β (TGF-β) is upregulated in fibrotic disease (29) and regulates fibroblast growth factors (FGFs) and fibroblast growth factor receptors (FGFRs). FGFs are involved in extracellular matrix remodeling, i.e., regulate bone resorption and formation by osteoblasts (30), and in turn stimulate interleukins (IL-6) and matrix metalloproteinases (MMPs; 31); FGF-2 has an additional role in modulating vascular tone (30). Connective tissue growth factor (CTGF) is an autocrine/paracrine growth factor localized to the Type IV fibrocytes of the spiral ligament (32). Vascular endothelial growth factor (VEGF) is upregulated with shear stress on vascular endothelial cells and may contribute to tissue remodeling and angiogenesis (33).

Tissue remodeling genes include bone morphogenetic proteins (BMPs) and the MMPs, zinc-dependent proteolytic enzymes that degrade extracellular matrix molecules and initiate the wound repair process. MMP2 and MMP9 are upregulated along with pro-inflammatory cytokines (TNF-β, IL-1β) in the heart during intrauterine hypoxia (hypoxic stress) (34,35) and have a role in blood vessel/basement membrane degradation/remodeling in the strial capillary basement membrane (36).

Genes associated with ion homeostasis include genes involved with ion channel regulation, water homeostasis, and gap junction proteins involved in potassium recycling and transport. Amiloride-sensitive sodium channel subunit alpha (Scnn1a) regulates sodium channel gene expression and is upregulated with inflammation in the middle ear (26). The aquaporin family of genes forms membrane pores involved in active water transport to maintain osmotic equilibrium in epithelial cells. Aquaporins 1 and 3 (aqp1, aqp3) are localized to the stria vascularis, spiral ligament, organ of Corti, and spiral ganglion (37), and aqp1 is expressed specifically by Type III fibrocytes (38). With middle ear inflammation, aqp1 and aqp5 are downregulated whereas aqp3 is upregulated (26). Claudins (e.g., cldn3) maintain tight junctions and prevent intercellular leakage of solutes and ions, and gap junction proteins are conduits for K+ movement in the recycling pathway (39). Gap junction protein Cx31 (Gjb3) is reduced with aging in the spiral ligament Type I and II fibrocytes and basal and intermediate cells in stria vascularis (40).

These genes are all part of an interconnected gene network, with expression in one part of the network potentially influencing the other parts. For instance, inflammatory cytokines also regulate the tissue remodeling and gap junction proteins (41). To clarify which pathways might be involved in hearing loss after cochlear implantation, we measured changes in gene expression of these various pathways in conjunction with hearing thresholds over time in a guinea pig cochlear implant model.

METHODS

Subjects

Twelve male albino Dunkin-Hartley guinea pigs (body weight 300–350 g) were divided into four groups (n = 3 per group), in which gene expression was examined at various times after surgery: 1, 3, 7, and 14 days. Baseline auditory brainstem response (ABR) measurements were conducted in all animals. At 5 weeks of age, all groups underwent cochlear implantation surgeries in the left ear. The 1-day and 3-day groups underwent final ABR testing at 1 and 3 days post-surgery, respectively, immediately before tissues were collected. The 7-day and 14-day groups underwent ABR testing at multiple time points: the 7-day group was tested at 1, 3, and 7 days post-surgery, and the 14-day group was tested at 1, 3, 7, and 14 days post-surgery. After final ABR testing, animals in each group were sacrificed and cochlear tissues harvested for gene expression analysis. All animal procedures in the study were approved by the Oregon Health and Science University Institutional Animal Care and Use Committee (Protocol MS15_IS00000672).

Auditory Brainstem Response (ABR) Testing

All ABR testing was performed in a soundproof booth. Before the testing, guinea pigs were anesthetized with ketamine (30 mg/kg) and xylazine (5 mg/kg) administered via intramuscular injection. Subcutaneous needle electrodes were placed at the vertex, below the ipsilateral pinna near the cheek, and below the contralateral pinna near cheek (ground). Test stimuli consisted of alternating phase tone bursts at frequencies of 1, 2, 4, 8, 16, and 32 kHz. Signals were presented using an RX6 D/A converter and PA5 programmable attenuator (Tucker-Davis Technologies, Alachua, FL, USA), amplifier (Crown D35, Elhart, IN, USA), and speaker (Vifa, Madisound, Middleton, WI, USA). The speaker was placed 10 cm from the ipsilateral external auditory meatus, and the contralateral ear was plugged with a silicon earplug. The animals’ evoked responses were amplified with a gain of 5,000 and bandpass filtered from 100 to 3,000 Hz using a Model 5113 preamplifier (Signal Recovery, Wokingham, UK). Responses to
300 sweeps were averaged at each stimulus level. Threshold was determined by decreasing the test signal level in 5-dB steps from 90 dB sound pressure level (SPL) to the lowest level that evoked a detectable and repeatable Wave III response.

**Surgical Procedures**

Each animal was anesthetized with ketamine (60 mg/kg) and xylazine (5 mg/kg) by intramuscular injection, with supplemental doses given as needed. Rectal temperature was monitored and controlled with a heating pad and feedback loop temperature controller. Electrocardiograms, breath rate, and SPO$_2$ were recorded by a veterinary monitor. The surgery area was shaved and aseptically prepared with povidone-iodine. Local anesthetics (lidocaine 2 mg/kg) were injected subcutaneously along the intended incisions.

Through a left post-auricular incision, the left bulla was exposed and opened to gain access to the round window niche. A cochleostomy was made just inferior to the round window by a 0.5-mm diamond burr at a slow rotation speed (800 rpm) to limit the acoustic trauma. An eight-electrode CI electrode array designed for guinea pigs (HL8; Cochlear Inc., Australia) was inserted into the scala tympani until the white mark reached the edge of the cochleostomy, corresponding to an insertion depth of 7 mm. The electrode materials are similar to those in human electrode arrays. The cochleostomy was sealed with a small piece of muscle fascia and the skin incision was closed with 5-0 sutures.

**Tissue Collection and RNA Extraction**

At 1, 3, 7, and 14 days after cochlear implantation surgery for the 1-day, 3-day, 7-day, and 14-day groups, respectively, animals were deeply anesthetized by excess xylazine (10 mg/kg) and decapitated. The cochleae were quickly removed and perfused through the round window with RNAlater (Qiagen, Valencia, CA, USA) to preserve RNA. The cochleae were carefully dissected in RNAlater. The cochlear sensory epithelium (organ of Corti, OC) and lateral wall (stria vascularis together with spiral ligament, SV + SL) were collected. Cochlear tissue from the surgery side (left) or control side (right) were put into liquid nitrogen separately as one sample and then stored at −80 °C until RNA was extracted.

Tissue total RNA was extracted using the RNeasy Mini Kit (Qiagen) after tissue homogenization as per manufacturer’s protocols. Total RNA of OC and SV + SL tissue from one cochlea was pooled for one sample each. RNase-Free DNase kit was used to remove DNA contamination. RNA quantity and quality were evaluated with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All samples demonstrated good quality RNA (A$_{260}$/A$_{280}$ > 1.8).

![FIG. 2. Changes in target gene expression after cochlear implantation as compared to non-surgery control ears. Error bars depict SEM. Asterisks indicate statistically significant values as compared to control ears. The gene expression changes are ordered as follows: housekeeping gene (column 1), ion homeostasis genes (columns 2–8), tissue remodeling genes (columns 9–16), and inflammation-related genes (columns 17–23).](http://example.com/2.png)
Quantitative RT-PCR Analyses

After total RNA extraction, first-strand cDNA was synthesized using 500 ng total RNA from each sample and Oligo-dT primers synthesized by reverse transcription (RT² First Strand Kit; SA Biosciences Corp., Frederick, MD, USA). Then, cDNA product was prepared for real-time PCR using the RT² SYBR Green/ROX qPCR Master Mix (Qiagen). Primers were designed using OLG OIGO Primer Analysis Software, version 7 (42; Molecular Biology Insights, Inc., Cascade, CO, USA; Table 1). The gene sequences of each target were downloaded from GenBank. Primers were then tested by reverse-transcription PCR and electrophoresis. PCR primers were custom-synthesized by Integrated DNA Technologies (Coralville, IA, USA).

A StepOnePlus Real-Time PCR System (SA Biosciences Corp.) was used to run each sample in triplicate. Negative controls without a template were included in every PCR run. Thermal cycle conditions were set as follows: 95 °C 10 minutes, then 40 cycles of 95 °C 15 seconds, 60 °C 1 min followed by a melt curve. The amplification efficiency was estimated. Two housekeeping genes, β-actin and glyceraldehyde-3-phosphate dehydrogenase, were chosen as the endogenous reference set for all samples. The relative gene expression of every target gene was calculated using the ΔΔCt method (43,44). The results were expressed as a fold change in gene expression in the surgery side relative to the control side.

Statistical Analysis

A two-way analysis of variance was employed to assess differences in ABR thresholds (group × frequency) using SPSS software (version 19.0; IBM). Relative expression software, REST 2009 (Qiagen) was used to calculate the relative expression ratios on the basis of group means for target genes versus the housekeeping gene set (45). REST software also tested the group ratio results for significance using the randomization test (46). Statistical significance was based on p values less than 0.05.

RESULTS

ABR Threshold Shifts Over Time After Cochlear Implantation Surgery

Average post-surgery ABR threshold shifts relative to baseline ABRs are shown for each frequency and group in Figure 1. All groups experienced significant increases

FIG. 3. Changes in target gene expression (A, C) and ABR threshold shifts (B, D) in the implanted ear after cochlear implantation for each animal from the 1-day and 3-day groups. The gene expression changes are again grouped in each subplot from left to right as follows: housekeeping gene (column 1), ion homeostasis genes (columns 2–6), tissue remodeling genes (columns 7–11), and inflammation-related genes (columns 12–16).
in hearing threshold at high frequencies in the implanted ear after surgery. Smaller threshold shifts occurred at low frequencies. The threshold changes between high frequencies and low frequencies were statistically significant at 1, 3, and 7 days. Also note that there was a peak threshold shift at 3 days post-surgery with a slight recovery at 7 and 14 days post-surgery. No threshold shifts were observed for the control/non-implanted ear.

RT-PCR Expression Changes Over Time After Surgery

Implantation led to significant upregulation of genes involved in all three pathways: ion homeostasis, tissue remodeling, and inflammation, as shown in Figure 2.

Statistically significant upregulation of several ion homeostasis function genes was seen at multiple time points. Scnn1γ was upregulated 2.8-fold in the cochlea 1 day after electrode array insertion relative to control non-implanted contralateral cochleae (Fig. 2A). Scnn1γ was also upregulated to 3- and 1.7-fold at 3 days (Fig. 2B) and 7 days (Fig. 2C) post-surgery, respectively, but the small increase in mRNA expression in the 14 days post-surgery group implanted cochleae (Fig. 2D) was not significant. Aqp3 was upregulated to 2.3-, 1.7-, and 2.7-fold at 1, 3, and 7 days (Fig. 2, A–C), but not at 14 days post-surgery (Fig. 2D). Gjb3 was upregulated to 1.47- and 1.41-fold at 3 and 14 days (Fig. 2, B–D).

Several chemokine and tissue remodeling genes exhibited significant upregulation at various time points. TGF-β1 was upregulated 1.38- to 1.92-fold at 1, 3, 7, and 14 days (Fig. 2, A–D). CTGF was significantly upregulated 1.63-fold at 3 days (Fig. 2B). MMP2 was upregulated 1.53- and 1.44-fold at 7 and 14 days (Fig. 2, C–D). MMP9 was upregulated by a factor of 12 at 1 day, 9.5 at 3 days, 19 at 7 days, and 12.3 at 14 days (Fig. 2, A–D). FGF-2 was downregulated, most notably at 7 days, but this was not statistically significant.

Several inflammation-related genes showed upregulation at all time points. Cxcl1 was upregulated 6.04-, 6.4-, 4.15-, and 2.37-fold at 1, 3, 7, and 14 days (Fig. 2, A–D). IL-1β was upregulated 4.16-, 17.1-, 6.7-, and 3.7-fold at 1, 3, 7, and 14 days (Fig. 2, A–D). TNF-α was upregulated 6.4-, 7.2-, 6.6-, and 4.4-fold at 1, 3, 7, and 14 days (Fig. 2, A–D). Tnfrsf1a was upregulated 1.65-fold...
at 3 days (Fig. 2B). Tnfrsf1b was upregulated 3.1-, 2.5-, 1.8-, and 1.6-fold at 1, 3, 7, and 14 days (Fig. 2, A–D).

**Individual ABR Threshold Shift and Gene Expression Results Within Groups**

ABR threshold shifts post-surgery at different time points and associated gene expression changes are shown for individual animals in the 1-day and 3-day groups in Figure 3, the 7-day group in Figure 4, and the 14-day group in Figure 5. Only genes with significant expression changes are shown.

Within the 1-day group, GE2 had the most hearing loss (shaded circles in Fig. 3B), and gene expression changes of ion homeostasis genes (Aqp3, Gja1, Gja3) and tissue remodeling genes (BMP3, MMP9) were correspondingly largest for GE2 (gray shaded bars in Fig. 3A). Inflammation response genes, on the other hand, showed the largest increase in expression for GE3 (open bars in last five columns of Fig. 3A).

Within the 3-day group, there was a mix of hearing loss patterns versus frequency. GE6 had more upregulated genes, especially inflammation-related genes (open bars in last five columns in Fig. 3C), than the other two animals in the group, but did not show more overall hearing loss than the other animals (open triangles compared to other symbols in Fig. 3D).

Within the 7-day group, ABR thresholds over multiple time points indicate some recovery of thresholds over time after surgery. GE13 had a peak hearing loss of 45 dB threshold shift at 8 kHz at 1-day post-surgery, which recovered over time to a 15-dB threshold shift by 7 days post-surgery (Fig. 4E). GE9 also showed a recovery after 1 day post-surgery, ultimately ending with the lowest threshold shifts at 7 days post-surgery (Fig. 4D; gray shaded circles in Fig. 4B). GE8 showed the most post-surgery hearing loss at 7 days post-surgery (Fig. 4C; black squares in Fig. 4B) as well as the greatest upregulation of cochlear mRNA for MMP9, MMP2, Cxcl1, TNF-α, IL-1β, TGF-β1, Aqp3, and Gjb3 (black bars in Fig. 4A). GE13, which had the second highest amount of threshold shift at 7 days post-surgery (open triangles in Fig. 4B), also showed more upregulation of these genes (open bars compared to other bars in Fig. 4A) compared to GE9 which had the smallest threshold shift, but generally less than GE8 which had the largest threshold shift.

Within the 14-day group, more hearing loss was observed for GE10 and GE12 than for GE11 (black squares and open triangles compared to gray shaded circles in...
Tissue Remodeling Post-surgery

Cochleae of all animals were dissected under a light microscope and the microstructure checked before cochlear tissue harvesting. No fracture of osseous spiral lamina or tear of basilar membrane was observed in any of the implanted cochleae. Osteogenesis in the cochleostomy area was seen in GE13, but no evidence of fibroplasia or osteogenesis was observed for any other cochlea. Note that GE13 did not have the largest threshold shift in the 7-day group.

DISCUSSION

Consistent with previous studies, significantly greater hearing loss was induced at high frequencies than at low frequencies after implantation, as expected because of the shallow electrode insertion and limitation of surgical trauma to the basal, high-frequency region. Hearing loss peaked at 3 days post-surgery, with thresholds showing some recovery at 7 and 14 days post-surgery. The temporarily higher threshold shifts may reflect temporary fluid accumulation or threshold recovery resulting from upregulated genes involved in inflammation and ion homeostasis.

There was a statistically significant upregulation of nearly all inflammatory cytokines studied (TNF-α, CxCl1, IL-1β, Tnf1r1a, and Tnf1r1b). Some ion homeostasis genes were also upregulated (Scnn1a, Aqp3, and Gjb3/Cx31), whereas others were downregulated (Aqp1). The upregulation of inflammation genes TNF-α, CxCl1, and IL-1β, upregulation of the ion homeostasis genes Scnn1a, Aqp3, and Gjb3/Cx31, and downregulation of Aqp1 qualitatively resemble the trends seen with middle ear inflammation (26,27), suggesting generic gene expression changes associated with an inflammatory response. The upregulation of apoptosis-related genes Tnf1r1a and Tnf1r1b is consistent with upregulation seen after noise exposure; however, Traf4 was downregulated instead of upregulated, suggesting activation of slightly different pathways (28).

Tissue remodeling genes, on the other hand, showed different gene expression patterns after cochlear implantation, compared to those seen with middle ear inflammation. MMP2 and MMP9 were significantly upregulated, and FGF2 was downregulated slightly, whereas middle ear inflammation does not lead to changes in expression of these genes (27). These genes have specific roles related to blood flow; FGF-2 regulates vascular tone (29), whereas MMP2 and MMP9 are involved in stria vasularis capillary basement membrane remodeling (36). In addition, when the hearing loss of individual animals was compared with gene expression, MMP9 was the single factor most associated with degree of high-frequency hearing loss. The upregulation of genes involved in blood flow is consistent with previous findings linking reduced stria vasularis blood vessel density with increased hearing loss after cochlear implantation (15). It is not clear, though, whether the MMP9 expression changes directly cause the hearing loss or are a downstream result of electrode insertion trauma to blood vessels in the stria vasularis which could affect the ability to maintain the endocochlear potential. The continued elevated expression of MMP9 at 14 days post-implantation, long after the hearing loss has stabilized, suggests the latter interpretation.

There was little upregulation of bone morphogenesis-related genes such as BMP3, consistent with the lack of ossification observed around the electrode array in all but one animal. TGF-β and CTGF, which were not studied in (26), were also upregulated. TGF-β is involved with the fibrotic tissue response (29), so upregulation of these genes is consistent with the typically observed fibrotic tissue (wound healing) response to surgical trauma; however, the downregulation of FGF2 implies opposite or differing effects on multiple fibrosis-related pathways. In addition, the time frame of the hearing loss, within 1 to 3 days after cochlear implantation, was too rapid to be explained by bone or fibrotic tissue growth effects on cochlear mechanics. Little is known about the role of CTGF, a growth factor expressed in the Type IV fibrocytes of the spiral ligament (32); it may be that direct damage to the spiral ligament region triggers a response in these fibrocytes.

In summary, genes affected by cochlear implantation include those involved in inflammation, ion homeostasis, and tissue remodeling, with tissue remodeling changes differing most from those seen with inflammation of the middle ear. Genes involved in tissue remodeling, particularly MMP2 and MMP9, were significantly upregulated and qualitatively associated with the degree of hearing loss. In this study, only a subset of genes has been ruled out as potential players in implantation-induced hearing loss. Further study with more animals and more genes, as well as examining the effects of electrical stimulation, is needed to clarify the mechanisms of implantation-induced hearing loss. Gene expression changes may also be used to measure the effects of clinically applicable interventions including various drugs (steroids, antioxidants) or new electrode designs in the future.

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