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A novel model of spontaneous otitis media with effusion (OME) in the *oxgr1* knock-out mouse

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Abstract

Objective—A novel mouse model with a specific genetic mutation in a G protein coupled receptor (GPCR) encoded by the *oxgr1* gene results in a predisposition to spontaneous otitis media with effusion. As a primary component of interest in OME, mucin expression was examined in this model to assess expression as compared to wild type animals and suitability as a murine model of OME.

Method—Mutant (*oxgr1*^{−/−}) and wild-type (*oxgr1*^{+/+}) mice between ages of 2–5 months were examined by otoscopy and auditory brainstem response (ABR). Histology changes in the middle ear were evaluated. Expression of mucin genes in the middle ear epithelium was determined using RT-PCR and quantitative PCR.

Result—Otoscopy exam showed signs of inflammation in 82% of mutant mice. Significant elevated ABR thresholds were detected in mutant mice indicating hearing loss. Histology analysis of the middle ears demonstrated the presence of inflammatory cells, changes in the mucosal epithelium, and middle ear fluid. RT PCR using universal primers for bacterial 18s rRNA suggested the absence of bacteria in the middle ear. The knockout mice demonstrated expression of *muc1*, *muc2*, *muc3*, *muc4*, *muc5AC*, *muc5B*, *muc9*, *muc10*, *muc13*, *muc15*, *muc16*, *muc18*, *muc19* and *muc20*. There was a trend of increase in *muc5B* and *muc19* expression in the middle ear of the knockout mice compared to that of wild-type. There was no significant change in the level of *muc2*, and *muc5AC* was expressed at a level below the detection limit of quantification.

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Conflict of interest statement

None of the authors of this manuscript have any financial or non-financial competing interests to disclose.

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Conclusion—Development of a murine model with genetic defect has several attractive features. The rate of OME in these animals is high at 82%. It is clear that this OME is related to histopathologic changes in the middle ear epithelium of these knock-out mice. Induction of mucus effusion is evident though the violation in dysregulation of GFM does exist in this non-challenge study condition. The underlying cause of these differences between individual animal requires further investigation. Given this, the *oxgr1*^{-/-} model is likely to be an ideal model to examine mucin regulation in MEE and potentially develop novel GPCR-specific targeted interventions to regulate these processes.

Keywords

oxgr1; knockout mice; otitis media; mucin

Introduction

Otitis media (OM) continues to be a prevalent and challenging disease process for children. OM affects the majority of children early in life and is responsible for billions of dollars in health care expenditures world-wide. In severe cases, OM leads to hearing loss, resulting in the potential for developmental delays and the need for surgical intervention. Progress has been made in the molecular understanding of the pathophysiology that takes place in OM [1–5] and in developing vaccines to limit the impact of this disease. However, numerous questions and challenges remain in understanding OM pathogenesis and developing novel solutions. One area of particular difficulty involves otitis media with effusion (OME). In this subset of OM, the persisting middle ear effusion (MEE) that develops can lead to longer term hearing loss and difficulties for children. Mucins are the primary components in the MEE of OME, and these mucins are responsible for this fluid's viscosity and subsequent hearing loss [2]. In developing models for the study of acute otitis media (AOM) numerous animal models have proven efficacious. However, humans represent a unique species in which chronic OME develops in a subset of the population without obvious underlying genetic or anatomic deficits. Although studies have demonstrated genetic predisposition and genetic polymorphisms associated with OME; many patients with OME have no obvious genotypic defect [6]. In the primary animal models of OM; chinchilla, mouse and rat, OME does not develop in wild type animals. Thus, investigators have relied upon either creating anatomic changes, such as altering the Eustachian tube, or creating animals with a genetic defect predisposing them to OME [7–9]. Each of these approaches presents some difficulties in translating findings to human disease. However, they have provided robust systems for investigation and acceptable surrogates in studying the pathophysiology in OME.

This current investigation employed a novel mouse model with a specific genetic mutation in a G protein coupled receptor encoded by the *oxgr1* gene resulting in a predisposition to spontaneous OME. As a primary component of interest in OME, mucin expression in this model was examined to assess expression, comparison to wild type animals and suitability for future experimentation using this murine model of OME.

Methods

Animals

Heterozygous (*Oxgr1*^{+/-}) mice with mixed (C57BL/6J-Tyr^{c-Brd} × 129Sv/Ev^{Brd}) genetic background generated by homologous recombination were developed by Lexicon Pharmaceuticals Incorporated (The Woodlands, TX). The homologous recombination removed the full length of *Oxgr1* gene. The *oxgr1* (NM_001001490) targeting vector was constructed from lambda POS system. The PCR generated selection cassette including flanking homology sequences of the specific gene was introduced into the mouse genomic

clone by yeast recombination resulting in the full length removal of *Oxgr1* gene. The linearized targeting vector was electroporated into 129Sv/Ev^{Brd} embryonic stem (ES) cells. To confirm *Oxgr1* deletion, the select clones were analyzed by southern hybridization using specific external probes on both arms of homology. The targeted ES clones were injected into C57BL/6J-Tyr^{c-Brd} blastocysts. The resulting chimeras were back crossed to C57BL/6J-Tyr^{c-Brd} to generate F1 heterozygous offsprings. Heterozygous cross set up generated wild-type control (*Oxgr1*^{+/+}), homozygous null (*Oxgr1*^{-/-}) and heterozygous litter mates for the study population. Genotype was verified by PCR reaction of tail genomic DNA. Primer pairs UTT069-21 (5'-GAGCCATGATTGAGCCACTG-3') and UTT069-25 (5'-CACCCTGGCATAGTAATGG-3') generated a 294 bp *Oxgr1* specific fragment which present in wild type but absent in mutant allele and UTT069-3 (5'-CAGAGCCATGCCTACGAG-3') and GT (5'-CCCTAGGAATGCTCGTCAAGA-3') amplified a 378 bp fragment specific to the selection cassette in mutant allele. The PCR amplicon from each allele was subjected to restriction analysis to confirm sequence specificity. In conjunction with the scientific team at Lexicon Pharmaceuticals the predictability of OME formation and suitability of this construct for the proposed experiments was determined and found acceptable. All animals were handled in accordance with the specific guidelines of the Institutional Animal Care and Use Committee at the Medical College of Wisconsin.

Auditory Brainstem Response and Otoscopy

Animals were housed in a pathogen-free environment and colonies were generated. Mixed gender adult animals were culled at 8–20 weeks for experimentation. A total of 12 wild type and 14 mutant mice were subjected to ABR threshold measurement performing at 4, 8, 12, 16, 24 and 32 kHz according to standard protocol [10]. All ears were examined by otoscopy following ABR for presence of middle ear fluid, inflammation, or infection.

Middle ear histopathology and OME pathogen detection

Five animals from each group were utilized for histology analysis of the middle ears. Animals were euthanized with an overdose of pentobarbital and intracardiac perfused with 10% buffered formalin. The intact bullas were removed bilaterally and immediately immersed in 10% buffered formalin. The resulting fixed bullas were decalcified, dehydrated and embedded in paraffin. Paraffin embedded bullas were sectioned horizontally at 5 microns and serially collected at every 50um. The sections were mounted on glass slides and stained with hematoxylin and eosin for histologic evaluation, and with the Periodic acid-Schiff (PAS) reaction to highlight mucus glycoprotein.

Despite housing in a pathogen-free environment, several steps were employed to ensure that the OME identified was related to spontaneous development of OME rather than pathogen-associated OME. Bulla sections were stained for bacterial contamination using Gram staining. Furthermore, polymerase chain reaction was utilized to amplify ribosomal RNA of both nonspecific bacteria and those prominent pathogens causing OME such as *H influenzae*, *S pneumoniae* and *M catarrhalis*. The specific primers and PCR conditions were as previously described [11].

Mucin gene expression in MEM

Following euthanasia, the temporal bone was harvested and homogenized in TriZol reagent (Invitrogen). The homogenate was prepared from each ear of the wild type and mutant mice. Total RNA was extracted following the manufacturer's instructions. Genomic DNA was removed using RQ1 RNase-Free DNase (Promega). Two micrograms of purified total RNA was reverse transcribed using Superscript III First Strand Synthesis System (Invitrogen), following the manufacturer's instructions. Specific mucin genes (*muc1*, *muc2*, *muc3*, *muc4*,

muc5AC, *muc5B*, *muc6*, *muc9*, *muc10*, *muc13*, *muc15*, *muc16*, *muc18*, *muc19* and *muc20*) were amplified with Platinum Blue PCR master mix (Invitrogen), each in 20ul reactions containing 0.2uM of each primer, 1.5mM MgCl₂ and 1ul of cDNA template using MiniAmp (Biorad). Specific primers and PCR conditions utilized for mouse mucin genes were as previously described [12].

Quantification of gel forming mucin genes

Following demonstration of mucin gene expression as described above, quantitative PCR was performed to assess the expression of the gel-forming mucin genes: *muc2*, *muc5AC*, *muc5B* and *muc19*. TaqMan primer/probe sets were obtained commercially (Applied Biosystems) for mouse *muc2* (P/N: Mm00458299_m1), *muc5AC* (P/N: 01276735_m1), *muc5B* (P/N: Mm00466407_m1), *muc19* (P/N: Mm01306462_m1) and *hprt* (P/N: Mm03024075_m1). Two micrograms of total RNA from mouse middle ear was random primed and reverse transcribed using Superscript III First Strand Synthesis System (Invitrogen). Quantitative PCR reaction was performed using one-tenth of the cDNA reaction on a ViiA7 (Applied Biosystems). Each cDNA sample was analyzed in triplicates. The mRNA level of each target gene was normalized to *Hprt* gene. The relative fold change was then calculated using $2^{-\Delta\Delta C_t}$ method [13].

Results

Genotypic screening of *Oxgr1* knockout mice

Cross set up between heterozygous (*Oxgr1*^{+/-}) mice generated wild-type control, homozygous null (*Oxgr1*^{-/-}) and heterozygous population for the study consistent with Mendelian ratios. The genotyping was confirmed by PCR amplification of the tail genomic DNA using specific primers for either the wild type or mutant allele. The specific primers 21 and 25 amplified 294 bp fragment of *Oxgr1* coding region in the wild type but not mutant allele. The specific primers for mutant allele amplified a 378 bp fragment of the selection cassette that replaced the gene by homologous recombination (Figure 1).

Oxgr1 knockout mice spontaneously develop otitis media with effusion

Otoscope examination of 8–20 week old mutant mice revealed 25 of 34 ears (74%) were affected with middle ear fluid, middle ear air-fluid levels or bulging tympanic membranes. About 82% of animals had at least one affected ear. ABR thresholds measured on affected ears of these animals showed 20 to 30dB elevation across all frequencies (Figure 2). A Two Way Repeated Measures ANOVA showed a significant difference in hearing thresholds between the mutant and wild type mice ($p < 0.001$).

Histology analysis of middle ears from 5/5 mutant mice compared to wild type mice demonstrated chronic tissue injury with host response as evidenced by relatively abundant, densely eosinophilic/PAS-positive luminal fluid adherent to middle ear epithelial surfaces, entrapment of large foamy macrophages within the adherent fluid, focal deposition of hemosiderin within the macrophages and subepithelial stroma, and submucosal fibrosis (Figure 3). Acute inflammation changes were not present, and no statistically significant increase in middle ear goblet cell density in mutants was observed. Ossicles of the mutant animals were much more heavily mineralized than those of control wild types (Figure 3 E–F). These histopathological changes observed within the middle ears of the mutant mice would be predicted to negatively impact hearing. Attempts to amplify 16s ribosomal RNA of *H influenza*, *S pneumoniae* and *M catarrhalis* revealed negative results indicating the absence of these prominent OME causing pathogens. The negative result obtained from 16s ribosomal RNA universal primers with the detection limit of 12pg of bacterial genomic DNA, in addition, indicates the absence of other nonspecific bacteria. This result correlated

with Gram staining of ME sections showing absence of both gram positive or gram negative bacteria. The *oxgr1*^{-/-} mice universally develop spontaneous OME at a very high rate (82%) based on histologic evaluation and conductive hearing loss documented on ABR.

Mucin gene expression in MEM of *oxgr1* knock-out mice

To investigate the impact of *oxgr1* deficiency on expression of middle ear mucin gene expression, conventional RT PCR was used to verify this expression in mouse middle ear mucosa. From previous experiments, full characterization of mouse middle ear epithelial mucin gene expression has been determined in an immortalized cell-culture model [12]. These experiments demonstrated expression of *muc1*, *muc2*, *muc3*, *muc4*, *muc5AC*, *muc5B*, *muc9*, *muc10*, *muc13*, *muc15*, *muc16*, *muc18*, *muc19* and *muc20*. Results from this current investigation demonstrated expression of each of these mucin genes (Figure 4) representing 100% correlation of the *oxgr1*^{-/-} model to our previous *in vitro* mouse middle ear cell-culture model as well as correlation to human *in vivo* and *in vitro* models developed in our laboratory [2,12]

The relative expression of mRNA levels of each of the 4 gel-forming mucin genes in MEM of mutant mice was assessed using quantitative PCR (Figure 5). The mRNA level of *muc2* was comparable between the mutant samples and wild type samples (t-test p-value = 0.920). The expression level of *muc5AC* mRNA in both groups of animals was below the detection limit of quantification, with the threshold cycles higher than 40, most likely due to the low number of goblet cells present in mouse middle ear. A trend of increase in *muc5B* level was detected in mutant animals though not significant (Mann-Whitney Rank Sum p-value = 0.381). A similar trend was observed in *muc19* mRNA level (t-test p-value = 0.368).

Discussion

Mucins continue to be an important area of focus in research directed at the physiology and pathophysiology of respiratory epithelium, and particularly this epithelium in the middle ear. Mucins are necessary to mechanically protect the underlying middle ear epithelium (MEE), to assist with the processing of antigens, and to provide trapping and clearance of invading pathogens. However, in too much abundance or in the incorrect composition of mucins there exists stasis of secretions and eventual hearing loss. Given their secretory nature, high viscosity and up-regulation during periods of inflammation such as OM, the gel-forming mucins MUC2, MUC5AC, MUC5B and MUC19 have been a primary area of interest [14–16]. However, we have previously demonstrated that a wide variety of mucins are produced in the MEE [2] and that experimental models should recognize these other mucins as possibly important in the overall regulation of MEE mucosal immunity, protection and function. Given this, the initial experiments utilizing the *oxgr1* knock-out mouse (*oxgr1*^{-/-}) required validation of an appropriate expression of mucin genes in the middle ear as compared to other mouse and human models employed in our laboratory. As demonstrated in Figure 4, the expression of each of the gel-forming mucins *muc2*, *muc5AC*, *muc5B* and *muc19* in the *oxgr1*^{-/-} model correlates with the other models for OM which have been investigated in our laboratory. This includes human *in vivo* and *in vitro* models, mouse *in vitro* models and chinchilla *in vivo* and *in vitro* models. [2,3,12]. In addition, the profile of non-gel-forming mucins expression in the *oxgr1*^{-/-} model correlates well with previously described human, murine and chinchilla models [2,3,12,17]. Given these findings, the *oxgr1*^{-/-} model appears to be an appropriate murine model of spontaneous OME development for future investigation of MEE mucin physiology and pathophysiology.

The development of the *oxgr1*^{-/-} model for the study of OM has a number of critically important aspects. First, efforts to identify or create an animal model of chronic OME have primarily relied on creating anatomic changes to the ET [7, 18] or murine models with

evident craniofacial deficits [9,19]. Although these methods have merit and have proven as viable models for investigation, permanently altering the ET presents some limitations in experimental design. An additional model of chronic OME has been developed and relies on an abnormality in the toll-like 4 receptor [8]. However, this abnormality creates a significant systemic abnormality for these mice with a complete inability to clear Gram-negative bacteria, again presenting some limitations in experimental design. The *oxgr1*^{-/-} model avoids each of these difficulties by focusing on a novel knock-out model in the G protein-coupled receptor (GPCR) family.

The GPCR superfamily is comprised of cell surface proteins containing seven transmembrane domains and the receptor ability to interact with G protein. The family was classified based on phylogenetic analysis of their sequence structure into 5 groups; Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2, and Secretin [20]. *Oxgr1* encodes oxoglutarate (alpha-ketoglutarate) receptor 1, a GPCR in Rhodopsin group. Very little is known about its biological functions. The physiological ligand for OXGR1, alpha ketoglutarate (2-oxoglutarate), was reported to involve in regulation of VEGF, an important inducer of angiogenesis and vascular permeability [21]. Increased production of VEGF in response to bacterial components and hypoxia resulted in increase of vascular permeability of middle ear mucosa and production of middle ear effusion. These contributed significantly to pathogenesis of the disease both in experimental animals and patients with OME [22–23]. This should warrant an investigation on whether OME associated VEGF induction is regulated through *oxgr1*. In addition, members of GPCR family have been linked to numerous biologic signaling pathways; a number of which involve important inflammatory events [24–25]. Some preliminary work would suggest that these GPCRs have an important regulatory function in epithelial mucosal integrity, mucin regulation and inflammatory pathways [26].

Development of a murine model with this particular genetic defect has several attractive features. First, the rate of development of OME in these animals is high at 82%. Additionally, it is clear from the initial work presented here that this OME is related to histopathologic changes in the middle ear of these mice. Induction of mucus effusion is evident though the variation in dysregulation of GFM does exist in this non-challenge study condition. The underlying cause of these differences between individual animal requires further investigation. Given this, the *oxgr1*^{-/-} model is likely to be an ideal model to examine mucin regulation in MEE and potentially develop novel GPCR-specific targeted interventions to regulate these processes.

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Figure 1.

Genotype verification in litter mates by PCR amplification of tail genomic DNA. (A) Specific wild type allele primers generated a 294bp fragment in the homozygote wild type (*oxgr1^{+/+}*) and heterozygotes (*oxgr1^{+/-}*) animals. Specific mutant allele primers amplified a 378bp fragment of the selection cassette that generated homozygotes null (*oxgr1^{-/-}*) via homologous recombination. (B) Restriction analysis of the amplified fragments from wild type and mutant alleles. The amplified wild type fragment yielded a 152 and 142bp in *Sma*I digestion, and a 150 and 144bp in *Xma*I digestion. The amplified mutant allele subjected to *Bam*HI digestion resulted in 177 and 201bp, while *Hind*III reduced the fragment to 168 and 210bp. M; 50–1000bp DNA ladder, U; uncut, S; *Sma*I, X; *Xma*I, B; *Bam*HI, and H; *Hind*III.

Figure 2.

Comparison of auditory brainstem thresholds in affected ears of *oxgr1^{-/-}*, as indicated by otoscopic exam, and wild type mice. Error bar indicated standard error of mean. Two Way Repeated Measures Analysis of Variance showed a significant hearing loss ($p < 0.001$).

Figure 3.

Histology of the middle ear from *oxgr1*^{-/-} mice compared to wild type mice. (A,D) H&E staining of middle ears from wild type (WT) mice revealed no significant inflammatory or chronic reactive changes. In A, notice the intact ciliated epithelium overlaying delicate lamina propria abutting bone. In D, notice the normally scant, wispy eosinophilic luminal material (open white arrow) that has survived histology processing and the lightly mineralized ossicular bone (black asterisk). The tympanic membrane (TM) is thin and delicate. (B, C, E) H&E staining of middle ear from mutant mice demonstrate evidence of chronic tissue injury and host response. In B and C, notice clusters of large foamy macrophages (long black arrows) adherent to injured ciliated epithelium and set within abundant densely eosinophilic fluid (C and E, open white arrow). Focal deposits of golden brown hemosiderin are present within the macrophages and subepithelial stroma (B, see small green arrows in enlarged inset). The submucosa (B) is fibrotic. Ossicular bone (black asterisks) with the middle ear cavity (MEC) is much more heavily mineralized in the mutant mice (E) than in the wild type control (D).

Figure 4.

Conventional RT PCR identified mucin genes expressed in middle ear mucosa of wild type (w) and mutant (m) mice. This expression pattern was 100% correlated to the mouse and human middle ear epithelium culture models.

Figure 5. Relative quantification of gel forming mucin genes in the temporal bone of *oxgr1*^{-/-} mutant demonstrated comparable level of *muc2*. The mRNA of *muc5AC* was observed at lower level than the detection limit with cyclo thresholds higher than 40 on both wild type and mutant. A trend of increasing in *muc5B* and *muc19* level was observed in mutant mice.