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Role of the PI3K/AKT pathway and PTEN in otitis media

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Abstract

Mucosal hyperplasia is common sequela of otitis media (OM), leading to the secretion of mucus and the recruitment of leukocytes. However, the pathogenic mechanisms underlying hyperplasia are not well defined. Here, we investigated the role of the AKT pathway in the development of middle mucosal hyperplasia using *in vitro* mucosal explants cultures and an *in vivo* rat model. The Akt inhibitor MK2206 treatment inhibited the growth of middle ear mucosal explants in a dose-dependent manner. *In vivo*, MK2206 also reduced mucosal hyperplasia. Unexpectedly, while PTEN is generally thought to act in opposition to AKT, the PTEN inhibitor BPV reduced mucosal explant growth *in vitro*. The results indicate that both AKT and PTEN are mediators of mucosal growth during OM, and could be potential therapeutic targets.

Keywords

Middle ear; nontypeable *Haemophilus influenza*; Mucosal growth regulation

1. Introduction

Otitis media (OM) is a common childhood disease. More than 90% of children under five years of age experience OM [1]. OM causes more pediatric physician visits, prescriptions and surgeries than any other condition [2]. Annually, it causes an estimated \$6 billion in health care and associated costs in the U.S [3].

While antibiotics are no longer recommended for uncomplicated acute OM (AOM) or OM with effusion in children over two [4], treatment patterns have in fact shown little change since this recommendation [5]. Antibiotic therapy is recommended for younger children [6].

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Author contributions statement

HHL preformed the experiments with assistance from AC, and KP. HHL and AFR did the literature review and wrote the original manuscript text. AK, KP, SIW and AFR contributed to data analysis and manuscript writing. KP did the *in vivo* assessment of inhibitors. HHL and AFR conceived the figures. All authors reviewed and approved the manuscript.

Declaration of competing interest

All authors declare that they have no competing financial interests. Dr. Ryan is a cofounder of Otonomy, Inc., serves as a consultant and member of the Scientific Advisory Board, and holds an equity position in the company. The UCSD and San Diego VA Committees on Conflict of Interest have approved this relationship. Otonomy, Inc. played no part in the research reported here.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yexcr.2019.111758>.

Streptococcal vaccines have reduced OM due to the covered strains, but increased incidence for other strains and microbes [7,8]. Ventilation tubes can be effective for recurrent or chronic OM [9] but there is controversy about long-term benefit and later TM abnormalities such as scarring or cholesteatoma [10]. Given these issues, alternative treatments for OM are needed.

A potential target for OM therapy is middle ear (ME) pathogenesis. A major feature of OM is hyperplasia of the mucosa that lines the ME. This converts the normal lining monolayer of simple squamous epithelium into a respiratory-type epithelium with goblet and ciliated cells as well as a robust stroma [11]. Hyperplasia contributes to the deleterious sequelae of OM, including mucous secretion into ME effusions [12]. Expansion of the mucosal vasculature also provides increased opportunity for leukocytic infiltration and tissue edema [13]. Increases in stromal cells, with their subsequent activation, can lead to the generation of matrix components and fibrosis that may permanently damage the sound conduction apparatus of the ME, resulting in persistent hearing loss [14]. A study of Danish teenagers found that 33% exhibited visible scarring of the tympanic membrane due to prior OM [15]. While mucosal hyperplasia is a major feature of OM, the intracellular pathways that mediate the growth of the ME mucosa in OM remain poorly understood. A better understanding of the pathophysiology of ME responses to infection may direct us toward potential new therapies for this disease.

The PI3K/AKT pathway is an important regulator of cell growth in many systems [16], which remains largely unexplored in the ME. The PI3K (phosphoinositide 3-kinase) family consists of Class 1, 2 and 3 kinases, which phosphorylate phosphatidylinositol biphosphate (PIP₂) to PIP₃. Class 1 PI3K kinases are involved in the activation of AKT (protein kinase B), whereas the Class 2 and 3 PI3Ks have other downstream targets [17]. AKT activation leads to stimulation of numerous downstream proteins, including mTOR (mammalian target of rapamycin), that affect cell growth, survival, and differentiation [18]. PTEN (phosphatase and tensin homolog deleted on chromosome ten) is well known as a negative regulator of PI3K, which operates by dephosphorylating PIP₃ to PIP₂ [19].

We hypothesized that PI3K/AKT signaling, and its negative regulation by PTEN, would be active in OM, with AKT activation promoting mucosal hyperplasia, and PTEN limiting tissue growth. To address this hypothesis, we assessed the expression of genes encoding PI3K/AKT pathway members and PTEN during the complete course of an acute ME bacterial infection in mice. We also evaluated the effects of AKT or PTEN inhibition on ME mucosal growth *in vitro*, and AKT inhibition *in vivo*.

2. Materials and methods

Animals.

For gene expression studies, 60–90 days old wild-type (WT) C57/WB F1 hybrid mice, both male and female, were purchased from the Jackson Laboratory (Bar Harbor, ME). For the *in vitro* evaluation of ME mucosal growth, 250–330 g Sprague Dawley rats, both male and female, were obtained from Harlan, Inc. (Indianapolis, IN). All experiments were performed

humanely according to NIH guidelines, following approval by the Institutional Animal Care and Use Committee of the Veteran Affairs Medical Center, San Diego CA.

Bacteria.

Haemophilus influenzae strain 3655 (nontypeable, biotype II), originally isolated from the ME of an OM patient in St. Louis and provided by Dr. Asa Melhus, Lund University, Sweden, was used at a concentration of 5×10^3 – 10^4 bacteria/ml to induce infection of the mouse or rat ME.

Inoculation of the ME.

Mice or rats were anesthetized with rodent cocktail (2.0 mg/kg xylazine and 40.0 mg/kg ketamine i. m.). Both ME bullas were exposed in an aseptic surgery by cervical midline incision. A small hole was made in each bulla, so as not to perforate the tympanic membrane. A 30 g needle was then used to carefully inject 5 μ l (mouse) or 50 μ l (rat) of inoculum into the ME. The incision was then closed and the tympanic membranes visually confirmed to be intact. Animals were sacrificed at various intervals after inoculation for tissue harvest or histology. Uninoculated animals served as controls.

ME mucosal histology.

MEs were harvested at 3 h, 6 h, 1 day, 2 days, 3 days, 5 days and 7 days after NTHi inoculation. The samples were fixed in 4% paraformaldehyde (PFA) overnight. They were then decalcified (8% EDTA and 4% PFA) for 10 days, processed into paraffin and then sectioned at 9 μ m. H&E stained sections from standardized locations of the ME cavity were digitally recorded and mucosal thickness was quantified using NIH Image-Pro (14,25). Thickness was assessed at six standardized locations from each of 4 sections widely spaced through the ME, and averaged for each ear to obtain a measure of thickness. Data from six ears at each time point were averaged for each post-inoculation time point.

Whole-genome gene arrays.

Two groups of 20 C57BL/6:CB F1 hybrid mice per time point were inoculated in the ME as described above with *Haemophilus influenzae* strain 3655 (NTHi). ME tissues were harvested at 0 h (control), 3 h, 6 h, 1 d, 2 d, 3 d, 5 d and 7 d after inoculation, to obtain two independent biological replicates of the OM time course. The tissues samples were homogenized in TRIzol < SUP > TM < /SUP > (Invitrogen, Carlsbad, CA) and total RNA extracted. After confirmation of RNA quality, mRNA was reverse transcribed using a T7-oligodT primer then *in vitro* transcribed using T7 RNA polymerase to generate biotinylated cRNA probes that were hybridized to Affymetrix MU430 2.0 whole-genome microarrays. The raw hybridization intensity data were median-normalized and statistical differences in gene transcript expression levels were evaluated using variance-modeled posterior inference (VAMPIRE) [20]. Bonferroni multiple testing correction ($\alpha_{\text{Bonf}} < 0.05$) was applied to identify only those genes with the most robust changes. The expression of gene transcripts encoding Class 1 catalytic and regulatory PI3K subunits, AKT isoforms 1–3, PTEN, as well as growth factors and receptors likely acting upstream from PI3K/AKT, over the course of acute OM, was extracted from this dataset.

Tissue culture.

Rat MEs were harvested 48 h after inoculation with NTHi. The ME mucosa was micro-dissected and carefully removed from the hypotympanum as an intact sheet. The mucosa was placed in PBS into a Petri dish with a thin layer black Sylgard 184 silicone elastomer (Dow Corning). A diamond scalpel (Fine Science Tools) was used to divide the mucosa into square explants approximately 0.5 mm on a side. Explants were cultured in 24-well plates (Falcon), epithelium uppermost, for 24 h in Dulbecco's modified Eagle's medium and Ham's F12 medium (3:1) supplemented with fetal calf serum (5%), hydrocortisone (0.4 mg/mL), isoproterenol (10 μ M), penicillin (100 U/ mL), and streptomycin (100 mg/mL), using a volume just sufficient to cover the tissue, so that attachment to the dish was encouraged. Explants from uninfected rat MEs were identically treated. The explants were incubated in a 5% CO₂ humidified atmosphere at 37 °C. Culture media was replaced every second day. Each explant was photographed daily with RT-SPOT color digital camera to document the extent of primary culture growth.

In vitro inhibition of AKT or PTEN.

On day 1, all wells with healthy, attached, explants were randomly divided into 4 groups. The pan-AKT inhibitor MK2206 (IC₅₀ 8–12 nM for AKT1 and 2; Selleckchem, Houston, TX) was added at 0, 1, 10 or 100 nM. The PTEN inhibitor bpV(HOpic) (IC₅₀ 14 nM, Calbiochem, San Diego, CA) was added, at 0, 5, 50 or 500 nM. The 0 nM groups served as negative controls, with the media receiving a supplement of 1 μ l/ml of DMSO alone, the same concentration of DMSO used for all concentrations of MK2206 and bpV. All explants were maintained in culture for 10 days. For each individual inhibitor concentration, 8–12 explants were imaged daily for 10 days and their surface areas were calculated using SPOT computer software.

In vivo inhibition of AKT.

To determine whether or not inhibition of the PI3K/AKT/PTEN pathway would decrease mucosal hyperplasia *in vivo*, rats were inoculated with NTHi as above, in combination with 500 μ M of MK2206 in DMSO. The animals were allowed to survive for 48 h, after which the MEs were harvested and fixed in 4% PFA. The growth response of the mucosa was assessed by measuring its thickness at standardized locations in the hypotympanum, attic, and promontory of the cochlea, for six MEs per condition. The results were averaged within each ME and treatments data analyzed by Student's T-test. To control for possible antibiotic effects of MK2206, we cultured NTHi in the presence of DMSO or DMSO with MK2206. Bacterial growth and titers were similar to those seen in bacterial media alone.

Data analysis.

Analysis of gene array data was described above. GraphPad prism 5.0 was used for statistical analysis of *in vitro* explant growth and *in vivo* mucosal thickness data. Explant data were compared using two-way analysis of variance, followed by Tukey-Kramer's *t*-test to determine statistical differences. A *P* value of < 0.05, with Bonferonni correction for multiple tests, was considered significant. *In vivo* mucosal thickness data were analyzed by Student's *t*-test.

3. Results

The ME mucosa exhibits robust but reversible hyperplasia during OM.

Changes in the morphology of the mucosa after ME inoculation with NTHi are illustrated in Fig. 1. Before infection, the mucosa throughout the majority of the ME consists of a monolayer of simple squamous epithelium with a minimal stroma. Modest changes in mucosal thickness due to edema were apparent by 6 h. Proliferation of epithelial and stromal cells was noted at 24 h, and edema remained apparent. By 2 days, the mucosal epithelium had differentiated into a multilayer, pseudostratified epithelium with a thickened and complex stromal layer with neovascularization. At 3 days the mucosa had begun to recover toward its normal thickness, a process that continued at 5 days. By 7 days the mucosa had largely recovered its pre-infection appearance. Mucosal hyperplasia occurred similarly in all regions of the ME, including the hypotympanum, on the promontory of the cochlea and in the attic. A quantitative representation of mucosal thickness over the course of OM is presented in Fig. 2A.

Expression of genes encoding the PI3K/AKT pathway, PTEN and upstream growth factors increases during OM.

Genes encoding the Class 1 PI3K alpha, beta, delta and gamma catalytic subunits, the PI3K regulatory subunits 1–4, the three isoforms of AKT, and PTEN were included in the gene arrays employed. The resulting data are presented in Fig. 2B. Of the PI3K catalytic subunit genes, the *Pik3a* subunit was slightly upregulated (1.5-fold), while *Pik3g* and *Pik3d* subunit genes were significantly upregulated 8.5- and 3.5-fold, respectively, peaking at 24 h after ME inoculation with NTHi. Meanwhile, *Pik3b* was not regulated. None of the regulatory subunit genes *Pik3r1–4* were increased by NTHi infection. There was a tendency for all of the regulatory subunits to be down-regulated 3–6 h after inoculation, but this was statistically significant only for *pik3r1* at 6 h. The genes encoding AKT1, AKT2 and AKT3 were significantly upregulated 9.0-, 3.0- and 2.6-fold, respectively, also peaking at 24 h after NTHi inoculation. The *Pten* gene was significantly upregulated 6.1-fold at the same time. The peak of expression occurred 1 day prior to the peak of mucosal growth. Interestingly, expression of the gene encoding mTOR, a frequent target of AKT signaling and a known growth stimulatory protein, was not affected by NTHi infection.

We also assessed the expression of growth factor genes that regulate the proliferation of epithelial and stromal cells, the main constituents of the ME mucosa. As can be seen in Fig. 2C, expression of the epithelial growth factors HB-EGF, amphiregulin and epiregulin was sharply upregulated within hours of bacterial inoculation. mRNA encoding the stromal growth factors FGF and PDGF also rapidly increased, peaking at 3–6 h after NTHi inoculation, although not to the same high fold changes as epithelial factors. Expression of TGFB mRNA, in contrast, peaked at 24 h.

In addition to growth factors, some cytokines and chemokines can induce cell proliferation. This includes the CXCL chemokines. Interestingly, the *Cxcr2* gene was upregulated 35-fold at 1 day after inoculation of the mouse ME with NTHi. Detailed information on the

expression of AKT signaling, growth factor and growth factor receptor genes, including fold change range and significance levels, are presented in the Supplementary Table.

AKT inhibition reduces ME mucosal growth *in vitro*.

To assess the role of AKT in mucosal hyperplasia, ME mucosal explants were grown *in vitro* and treated with MK2206, an inhibitor active against all three AKT isoforms. When explants from uninfected rats were grown for 10 days in culture, a steady increase in the size of the explants was observed. As can be seen in Fig. 3, treatment with the pan-AKT inhibitor MK2206 had no effect on this growth at 1 or 10 nM. However, 100 nM of the inhibitor significantly inhibited the expansion of explant area.

When explants of mucosa from MEs that had been infected with NTHi 48 h previously were grown in culture, similar growth was similar to that observed for uninfected tissue. Again, no growth inhibition by MK2206 was observed with MK2206 at 1 nM and 10 nM, but significant growth reduction was seen with 100 nM (Fig. 4).

4. PTEN inhibition also reduces ME mucosal growth *in vitro*

When ME mucosal explants from uninfected rats were treated with the PTEN inhibitor bpV, significantly reduced growth was observed at 50 and 500 nM, but not at 5 nM (Fig. 5). In contrast, BPV significantly inhibited explant outgrowth at all concentrations for explants from previously infected MEs (Fig. 6).

5. AKT inhibition reduces mucosal hyperplasia *in vivo*

When the MEs of rats were infected by intrabullar inoculation with NTHi plus DMSO, robust mucosal hyperplasia was observed 48 h later (Figs. 6 and 7). However, when 100 μ M of the AKT inhibitor MK2206 was injected along with the bacteria, significantly less mucosa thickness resulted ($p < 0.01$) (see Fig. 8).

6. Discussion

The results support the hypothesis that the PI3K/AKT/PTEN pathway is involved in hyperplasia of the ME mucosa during OM. Many of the genes encoding elements of the PI3K/AKT pathway and its inhibitor PTEN were strongly upregulated during the course of OM, with mRNA levels peaking 1 day prior to the maximum observed mucosal growth. Inhibition of AKT was found to reduce the growth of both uninfected and previously infected ME mucosal explants *in vitro*. AKT inhibition also reduced mucosal hyperplasia during OM *in vivo*. Unexpectedly, inhibition of PTEN also reduced ME mucosal growth *in vitro*.

The observation that *Pik3* and *Akt* genes were upregulated during OM, and that AKT inhibition reduced ME mucosal hyperplasia, were not surprising. In other systems, PI3K/AKT signaling has a well-documented role in tissue proliferation and differentiation. Constitutively activated AKT isoforms have been associated with a number of cancers [21]. AKT inhibitors can reduce cell replication and tissue growth in cancer [22], and in a variety of normal cell types (e.g. Refs. [23,24]). It is also not surprising that the *Pten* gene was

similarly upregulated, since PTEN often acts as major negative regulator of PI3K/AKT [19]. Given the potent growth promoting capacity of AKT activation, enhanced negative regulation to prevent excessive ME tissue growth would not be unexpected.

The PI3K/AKT pathway is typically activated by the binding of growth factors to their cognate receptors. Since FBS was included in our culture media, it is highly likely that the many growth factors present in FBS activated the pathway. However, in the *in vivo* ME intrinsic factors are likely to be involved. As can be seen in Fig. 2C and the Supplementary Table, growth factors known to promote the proliferation of epithelial and stromal cells, and their receptors, are rapidly expressed in the ME during OM. Moreover, we previously found that HB-EGF strongly promotes the growth of ME mucosal explants *in vitro* [25]. It thus seems likely that AKT acts downstream from HB-EGF and other growth factors during OM.

Another potential mechanism of AKT activation is inflammation. Li et al. [24], and Shang et al. [26] found that cell growth that is promoted by the activation of the chemokine receptors CXCR1 and CXCR2 is mediated by AKT. as noted above, the *Cxcr2* gene is strongly upregulated in the ME 1 day after inoculation of the mouse ME with NTHi. This provides another potential mechanism of AKT activation during OM.

It has been noted that the epithelium of the ME has two embryonic origins. The mucosa in the hypotympanum originates from endodermal cells, while those in the upper ME are derived from the neural crest [27]. Cells in both regions can be ciliated [28]. During OM, we observed mucosal hyperplasia to be similar in both regions. This suggests that the ME mucosa responds as a unit during infection, regardless of the embryonic origin of the epithelium. Given the importance of epithelial responses to bacterial clearance, this is perhaps not surprising.

It should be noted that the inhibition of AKT reduced, but did not eliminate, hyperplasia of the ME mucosa during OM (Figs. 5, 6 and 9). This presumably reflects the activity of other growth signaling pathways that can operate downstream from growth factor receptor activation. We have previously shown that an inhibitor of JNK MAP kinase can reduce ME mucosal growth both *in vitro* and *in vivo* [29]. Thus there is redundant growth signaling within the ME mucosa during OM.

Our most surprising finding was the potent decrease in ME mucosal hyperplasia *in vitro* by PTEN inhibition, since we had predicted that PTEN inhibition would promote growth through increased PIP3 and AKT activation. While PTEN is best known for its inhibition of PIP3 phosphorylation and downstream AKT activation, it is a multifunctional phosphatase with many other known targets, as well as other functions related to tissue proliferation. While loss of function mutations of PTEN cause several cancers [30], gain of function mutations can also cause oncogenic transformation and growth, especially in B-cells [31]. In addition, PTEN cooperates with Fanconi anemia proteins in DNA crosslink repair, a process important to cell replication and survival [32]. Interestingly, mucosal explants from infected MEs were more sensitive to PTEN inhibition than explants from uninfected MEs, a phenomenon not observed for AKT inhibition. This suggests that infection alters growth regulation in favor of PTEN signaling.

7. Conclusions

We have identified two potential pathways that mediate mucosal hyperplasia in OM. The PI3K/AKT pathway is a classical stimulant of tissue growth and cell survival. Normally inhibitory PTEN appears to mediate ME cell growth during OM paradoxically, independent of its negative regulation of PI3K, by an as yet unidentified process. Inhibition of AKT and possibly PTEN may have the potential to reduce the pathophysiological sequelae of this common childhood disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A. Representative sections illustrating hyperplasia of the mouse middle ear (ME) mucosa at various times after inoculation of the ME with nontypeable *Haemophilus influenzae* (NTHi). Mucosal hyperplasia peaked at 2 days after inoculation, and subsequently recovered to its normal thickness.

Fig. 2.

A. Kinetics of mean mucosal thickness during experimental OM in mice. **B.** Change of PI3K/AKT/PTEN pathway gene expression during OM. Each data point represents the mean from four arrays, two from each biological replicate. Relative gene expression (fold change compared to uninfected ME tissue) was assessed at intervals that captured the entire course of an acute OM episode. Several genes encoding elements of the PI3K/AKT/PTEN pathway were upregulated, peaking at 24 h. **C.** Change in the expression of epithelial and stromal growth regulatory genes during OM. Genes encoding growth factors are expressed earlier than those for AKT and PI3K. Variability and P values are presented in Supplementary Table 1.

Fig. 3.

Representative images of *in vitro* outgrowth of ME mucosal explants from rat MEs on day 10 of culture. **A.** Uninfected explant treated with 10 nM MK2206, not significantly different from control explants. Growth of the remaining explants was significantly reduced by inhibitor treatment. **B.** Infected explant treated with 100 nM MX2206. **C.** Uninfected explant treated with 5 nM Bpv. **D.** Infected explant treated with 5 nM Bpv. For each image, the circumference of the explant is indicated by the white line, and the explant area is provided.

Uninfected Mucosal Explants (MK2206)

Fig. 4.

Surface area of mucosal explants from uninfected rat MEs treated with MK2206 (0, 1, 10 and 100 nM), over culture time from 2 to 10 days. Only 100 nM MK2206 had a significant impact on mucosal growth ($P < 0.05$, ANOVA). Values are means $\pm$ one standard error; $n = 8-12$ explants at each concentration.

Infected Mucosal Explants (MK2206)

Fig. 5. Surface area of mucosal outgrowth of mucosal explants from infected rat MEs (all harvested 48 h after bacterial inoculation) treated with MK2206 (0, 1, 10 and 100 nM). Again, only 100 nM MK2206 significantly reduced the growth of explants ($P < 0.05$, ANOVA).

Uninfected Mucosal Explants (Bpv)

Fig. 6.

Surface area of mucosal explants from uninfected rat MEs treated with. BPV (0, 5, 50 and 500 nM). Significant growth inhibition was observed 50 and 500 nM BPV ($P < 0.05$, ANOVA).

Infected Mucosal Explants (Bpv)

Fig. 7.

Surface area of mucosal outgrowth of mucosal explants from infected rat MEs (all harvested 48 h after bacterial inoculation) treated with BPV (0, 5, 50 and 500 nM). Significant growth inhibition was observed at all BPV concentrations ($P < 0.05$, ANOVA).

Fig. 8.

A. Representative sections illustrating thickness of the mouse ME mucosa 48 h after inoculation of the ME with nontypeable *Haemophilus influenzae* (NTHi) plus either DMSO alone (control) or DMSO plus 5 μM MK2206.

Fig. 9.

Average thickness of the rat ME mucosa prior to infection (Cont) 48 h after inoculation of the ME with NTHi plus DMSO alone (NTHi) or NTHi and DMSO plus 5 μM of AKT inhibitor (NTHi + MK2206). Error bars represent $\pm$ one standard deviation. P values from Student's T-test.