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Activation of The Transforming Growth Factor-Beta Pathway in Bacterial Otitis Media

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Abstract

Purpose—Granulation tissue is common in otitis media (OM), yet little is known about the signaling pathways in the formation of granulation tissue in response to infections. In this study, we sought to investigate the activation of the transforming growth factor-beta (TGF- β) signaling pathway in the formation of granulation tissue in response to middle ear pathogens.

Materials and Methods—Rat OM models were made by inoculating pneumococcus type 6A (Pn6A), non-typeable *H. influenzae* (NTHi) into the middle ear cavity, or by obstructing the eustachian tube (ETO). Various pathway activities in the middle ear mucosa were analyzed with microarrays.

Results—The TGF- β signaling pathway was highly regulated in the middle ear cleft with bacterial OM but not ETO. In bacterial OM, the TGF- β signaling pathway products were higher in NTHi-infected ears than in Pn6A-infected ears.

Conclusion—Bacterial OM triggers granulation tissue to thrive in the middle ear cleft of rats. NTHi is more potent than Pn6A in the formation of granulation tissue. ETO alone did not contribute to granulation tissue formation in the middle ear.

Keywords

bacterial otitis media; TGF- β pathway; granulation tissue formation; microarrays; rat

INTRODUCTION

OM is one of the most common upper respiratory tract infections and is frequently associated with granulation tissue forming in the middle ear. The formation of granulation tissue in the middle ear is considered destructive, irreversible, and intractable, which eventually results in the destruction of the ossicular chain and subsequent conductive hearing loss. The pathological characteristics of granulation tissue are the proliferation of new blood vessels in the submucosa and the thickening of the middle ear cleft with active production of extracellular matrix proteins (ECMs). However, little is known about the global gene expression profile of granulation tissue and the molecular signaling pathways involved in the process.

In our early studies, we demonstrated that a transcriptional factor family, inhibitor of differentiation (Id), was up-regulated in the middle ear of rats after bacterial challenges.¹ Id is a product of the TGF- β signaling pathway. The major role of the Id family is to form new

blood vessels²⁻⁵ and to metabolize ECMs,⁶ which suggests that TGF- β is involved in the formation of granulation tissue.

TGF- β is a pleiotropic cytokine, which is produced by many cell types including inflammatory cells.⁷ It is a key regulator of tissue remodeling.⁸⁻¹³ TGF- β exerts its effects *via* a signaling pathway involving receptors (serine/threonine kinases or activin-like receptor kinases, ALKs) at the cell surface and their substrates, the Smads.¹⁴ Upon phosphorylation and oligomerization, the Smads translocate into the nucleus to regulate the transcription of target genes,¹⁵ such as collagens (COL), fibronectins (FN), laminin (LAM), and tenascins (TN). Through the Smad¹⁶ and mitogen-activated protein kinase (MAPK) pathways,¹⁰ TGF- β regulates the expression of ECMs and early response oncogenes such as *c-fos* and *c-jun*.^{9, 17-19} as well as vascular wall proteins such as smooth muscle α -actin.²⁰ It is well known that ECM and blood vessel related proteins are involved in the formation of granulation tissue.

The expression of TGF- β isoforms (β 1, β 2, and β 3), TGF- β receptors (type I, II, and III), and TGF- β receptor signaling molecules (Smads and MAPK) under fibrotic disorders is highly regulated.^{7, 17, 21, 22} In addition, T-helper 2 (Th₂) cytokines IL-4, IL-5, IL-9, and IL-13 are also involved in submucosal fibrosis.²³⁻²⁶

TGF- β 1, β 2, and β 3 regulate the production of ECM and stimulate the proliferation of fibroblasts but inhibit the proliferation of epithelial cells.²⁷⁻²⁹ However, TGF- α exerts an opposite effect, stimulating the proliferation of epithelial cells but inhibiting the proliferation of fibroblasts. The effects of TGF- β and TGF- α on ECM profiles are distinct. This constitutes underlying pathologic changes in the mucosal and submucosal layers of the middle ear cleft. To understand the role of major middle ear pathogens in the formation of granulation tissue, we performed the global gene expression using microarrays and analyzed the effects of middle ear pathogens on the TGF- β signaling pathway products. The purpose of this study was to explore the disease mechanism through which granulation tissue is formed in OM.

MATERIALS AND METHODS

Eighty-four Sprague Dawley rats (both sexes) were purchased from Charles River. The Institutional Animal Care and Use Committee approved the use of animals in this study. Young and healthy rats, weighing 200-225 g, were randomly divided into 12 groups with their bullae challenged with middle ear pathogens (Pn6A and NTHi, respectively) or ETO, as previously described.³⁰ Each group had seven animals and each animal was inoculated with Pn6A (10^6 cfu, 50 μ L/bulla) and NTHi (10^9 cfu, 50 μ L/bulla), which is equivalent to 10^6 cfu of Pn6A in the induction of middle ear inflammation according to our previous study in the bullae of rats.³¹ Bullae with the same amount of phosphate-buffered saline (PBS, 50 μ L/bulla) inoculation served as controls. Animals were killed on days 3 and 7, respectively. On day 3, middle ear effusion peaked, and on day 7, effusion was mainly resolved. ETO was performed in two groups of rats, as previously described.³¹ The rats were killed on days 7 and 30, respectively. Sham ETO was performed in two additional groups of rats as ETO controls (i.e., performance of the ETO surgery without mechanical obstruction of the Eustachian tube). After anesthesia with ketamine hydrochloride (80 mg/kg) and xylazine (16 mg/kg), intramuscularly, both bullae were precisely dissected out at scheduled time points for total RNA extraction and subsequent Affymetrix microarray analysis. At the same time, middle ear tissue sections were made from the above animal models (3 bullae each group) to examine histopathologic changes, as previously described.³¹

Rat middle ear epithelial cells (MEEC),³² immortalized with temperature-sensitive simian vacuolating virus 40 (SV40), were used to identify genes expressed in the epithelial cells. Rat MEEC cultures were maintained in Ham's F-12K media supplied with 1.5 g/L sodium bicarbonate, 2.0 mmol/L L-glutamine, 10 ng/mL epidermal growth factor, 10 mg/mL insulin-transferrin-sodium selenite, 2.7 g/L glucose, 500 ng/mL hydrocortisone, 0.1 mmol/L nonessential amino acids, and 4% fetal bovine serum. The media were changed every 3–4 days. Cells were cultured at 33°C (permissive temperature, allowing cells to grow) or 39°C (non-permissive temperature, allowing cells to differentiate) in a 5% carbon dioxide cell culture incubator for the proliferation of MEEC (pMEEC) or the differentiation of MEEC (dMEEC).

Approximately 9–11 bullae from each group of rats were used for RNA isolation using the StrataPrep[®] Total RNA Miniprep Kit (Stratagene). Each bullae yielded approximately 3–4 µg of total RNA. In each group, approximately 30–45 µg of total RNA was used. Affymetrix microarrays were performed, as previously described.³³ Briefly, cDNA was prepared from 20 µg total RNA using a T7-dT₂₄ primer. cRNA was synthesized from cDNA and biotinylated using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY) and hybridized with the Rat U34 arrays (Affymetrix). The intensity of each gene expression in the samples pooled from 9–11 bullae is presented as relative light units (RLU).³⁴ Data presented in this study represent multiple signal molecules related to the TGF-β signaling pathway.

Gene expression intensity that was higher than the background (100 RLU) was included in this study. TGF-β pathway genes were selected from the Affymetrix array data. In consideration of the effector molecules being controlled by a battery of genes, instead of by a single gene, all of the pathway genes were combined and analyzed by analysis of variance (ANOVA). In consideration of possible positive and negative responses from the genes to bacterial challenges, genes up-regulated and down-regulated in the same pathway were grouped together, respectively, and evaluated separately. Differences between controls and experiments were determined by using a single factor, ANOVA. Transcripts of the genes with significant changes (up- and down-regulated) in the pathway were determined. Next, genes being up-regulated and down-regulated were evaluated respectively. Expression changes of multiple genes in the same TGF-β pathway were evaluated with two-factor ANOVA with their up-regulated and down-regulated genes being evaluated separately. *P* values less than 0.05 were considered significant when weighing differences between experiments and controls. In a similar way, microarrays were performed on the rat temperature-sensitive MEEC³² under proliferating and differentiating conditions (pMEEC and dMEEC) to evaluate gene expression changes in the epithelial cells.

Reverse transcription polymerase chain reaction (RT-PCR) was performed on total RNA isolated from the middle ear tissue of rats as prepared above. Specific primers for TGF family genes were designed with Oligo 4.0 and shown in Table I. Sequence information of the TGF-β signaling pathway genes was available at the website of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). RT-PCR was performed as described previously³⁵ using 1 µg of RNA, 0.2 µM up- and down-primer pairs, and 30 cycles. Negative controls for RT-PCR were performed to exclude the possibility of contamination. PCR products were analyzed on a 2% agarose gel.

Bullae were fixed in 10% paraformaldehyde solution overnight, dehydrated in graded alcohol, immersed in xylene, embedded in paraffin, and cut at a thickness of 4 microns. Sections were deparaffinized in xylene, dehydrated in graded alcohol and then water, and stained with hematoxylin and eosin (H&E) for histology, as previously described.³¹

Differences of data between the groups were analyzed by non-paired Student's *t*-test, ANOVA, or both, with $p < 0.05$ considered as statistically significant.

RESULTS

Along the TGF- β signaling pathway, there are many genes including TGF- β ligands, TGF receptors, Smads, and effectors (Fig. 1). Approximately 26 TGF- β pathway genes altered their expressions (up- or down-regulated) and 57 ECM genes (e.g., TGF- β pathway effectors) altered following bacterial challenge in the middle ear of rats. The range of regulation for the TGF- β pathway genes in experimental groups was between 0.048–12.7 times compared with their respective controls (Table II). Since the TGF signaling pathway and its effectors are highly affected by bacterial OM, we focused on the expression profile of the TGF- β signaling pathway in this study.

The Affymetrix® microarrays demonstrated that TGF- α was mainly expressed in MEEC whereas TGF- β 1 was mainly in the fibroblasts. Note that β 2 and β 3 were mainly expressed in dMEEC (Fig. 2A). ETO slightly increased the expression of TGF- α but not Pn6A and NTHi (Fig. 2A, 5th ~9th, 1st column). It suggests that effusion in the middle ear cavity stimulates the expression of TGF- α . To our surprise, TGF- β 1 (mainly expressed in the fibroblasts) was down-regulated in Pn6A and ETO but not responsive to NTHi compared with their controls at the time points studied (Fig. 2A, 2nd column). TGF- β 2 was moderately expressed in MEEC but weakly expressed in the submucosal layer and not responsive to Pn6A, NTHi, and ETO (Fig. 2A, 3rd column). TGF- β 3 was mainly expressed in pMEEC, slightly responsive to Pn6A, NTHi, and ETO (Fig. 2A, 4th column). Genes up-regulated in the TGF- β signaling pathway in the middle ear cleft with OM are presented in heat map (Fig. 2B).

TGF family members are known to act through type I and type II serine/threonine kinase receptors. There are seven type I receptors (e.g., ALK1-7) and five type II receptors (ActRII, ActRIIB, T β RII, BMPRII, and AMHR) in tissues.¹⁶ The TGF receptors expressed in the middle ear cleft were ALK5 (S81584), ALK7 (U69702), ActRII, and ActRIIB. T β RII (M87067) and T β RIII (M80784) were highly expressed, especially in pMEEC but weakly expressed in dMEEC. T β RII was slightly up-regulated in NTHi but less responsive to NTHi and ETO. In contrast, T β RIII was moderately expressed in pMEEC, slightly down-regulated in dMEEC, slightly up-regulated in Pn6A at day 3, and down-regulated in ETO (Fig. 3A). The expression of the receptors was confirmed by RT-PCR (Table III).

It is known that Smad2 is a downstream molecule for ActRII, ActRIIB, ALK5, and ALK7.^{16, 36} The expression of Smad2 (AB017912) was high in pMEEC but low in dMEEC. Smad2 was up-regulated in Pn6A infection and ETO but not changed in NTHi infection. In contrast, the expression of inhibitory Smad7 (AF042499) was relatively low. It was down-regulated in pMEEC but up-regulated in dMEEC. It was down-regulated in ETO and not much changed in Pn6A and NTHi infected ears. Smad8 (AF012347) was expressed in the MEEC and tissues at a very low level. The expression of Smads was confirmed by RT-PCR (Table III).

There are 57 genes that were recognized as effectors of the TGF- β signaling pathway in this study. These genes include ECMs (COL, TN, FN, and LAM), early responding genes (*c-fos* and *c-jun*), vascular α -actin (essential for angiogenesis), heat shock proteins (chaperones of collagen synthesis such as HSP47),³⁷ and Th₂ interleukins (IL-4, IL-5, and IL-13), which are actively involved in submucosal remodeling and fibrotic disorders.²⁶ Generally speaking, there were both up- and down-regulated expressions of these genes in response to the challenge of Pn6A, NTHi, and ETO in a time-dependent manner. The majority of the

genes responded to the bacterial OM and ETO with an up- or down-regulated expression persistently. There were several genes that responded to bacterial OM with up- and down-regulated expressions. However, ETO generally caused the down-regulated expression of many genes (COL1A1, COL3A1, and FN) whereas NTHi caused the up-regulated expression of these genes (Fig. 4A). On day 3, heat shock proteins (HSP27 and 70) were down-regulated in the middle ear cleft following Pn6A and NTHi infections (Fig. 3B). On day 7, FN3M1 was up-regulated in NTHi infection but not in Pn6A infection (Fig. 3C). In ETO, FN γ was up-regulated on day 7 whereas FN and COL4A1 were up-regulated in ETO on day 30 (Fig. 3D). The expression of the major effectors was confirmed by RT-PCR (Table III).

The changes of the ECM gene expression were evident. First, fibronectins were highly up-regulated in Pn6A and NTHi infected ears on days 3 and 7 as well as in the ears with ETO, either on days 7 and 30. Examination of fibronectins in the middle ear epithelial cells and entire cleft demonstrated that fibronectins were expressed in both the mucosal and submucosal layers. The major ECMs in response to NTHi infection on day 3 were COL1A1 and COL3A1 (Fig. 5A), COL3A1 and FN3M1 on day 7 (Fig. 5B). The expression levels of FN-1, TN-C, TN-X, COL8A1 in dMEEC were higher than those in pMEEC and the middle ear cleft (Fig. 5C-D). Interestingly, COL8A1, TN-C, and laminins were mainly expressed in dMEEC (Fig. 5C-D).

Secondly, collagens were highly expressed in the middle ear cleft including types I, II, III, IV, V, VIII, IX, X, XI, and XII (all alpha 1 subunit except alpha 3 for type IV). Major collagens in the submucosa (fibroblasts) were types I, III, V, XII, and II whereas major collagens in MEEC were VIII, V, XI, and X in the orders of mRNA abundance. There were 4 genes encoding type 1 alpha 1 (U75405, M27207, Z78279, and AF050214, counting for approximately 76% of all collagens in terms of mRNA transcripts) that were significantly up-regulated on day 3 with NTHi infection ($115,483 \pm 38,115$ RLU) compared with day 3 treated with PBS ($77,573 \pm 25,656$). In contrast, Pn6A on day 3 increased but did not significantly increase the expression of type 1 alpha 1 collagens. Collagen type III alpha 1, which is the second highest collagen expressed in the submucosa (accounting for approximately 16% of all collagens in terms of mRNA transcripts), had the same response patterns as collagen type I alpha 1.

There were remarkably up- and down-regulated genes in bacterial OM or ETO (more than 5 times compared with their controls). These genes included TGF- β 1, receptors, p38MAPK, collagens, and heat shock proteins (HSP), which are chaperones of collagen synthesis, tenascins, and fibronectins (Fig. 5). In Pn6A infection, days 3 and 7, some collagens and fibronectins were down-regulated whereas in NTHi infection, days 3 and 7, collagens and fibronectins were up-regulated. In ETO-treated ears, genes in the TGF- β pathway and effectors have an inclination to be down-regulated.

It is noted that genes up- or down-regulated in ETO are different from those up- and down-regulated in bacterial OM. For example, TGF- α was up-regulated in ETO but down-regulated in bacterial OM whereas TGF- β 1 was down-regulated in ETO but up-regulated in bacterial OM. Smad2, a common mediator for TGF- β 1 and TGF- α , was up-regulated in both bacterial OM and ETO. In terms of effectors, NTHi increased the mRNA transcripts (approximately 12% on day 3 and 4% on day 7) for collagens whereas ETO reduced the mRNA transcripts (approximately 5% on day 7 and 17% on day 30, respectively) for collagens. However, with ETO, the expression of COL1A2, 3A1, 5A1, 10A1, 11A1 and 12A1 was reduced but COL2A1 and 4A1 increased whereas NTHi increased COL1A1, 3A1, 8A1, and 12A1 but reduced COL9A1. Compared with Pn6A, NTHi significantly increased the expression of collagens on day 3.

To verify the formation of granulation tissue, rat bullae were treated with PBS, ETO, Pn6A, and NTHi, respectively, for 14 days and then harvested for histological studies. It was found that the PBS-treated middle ear had limited proliferation of fibroblasts in the submucosal layer (Fig. 6A). Like PBS, ETO had no or little submucosal response (Fig. 6B) while NTHi had a remarkable submucosal response (Fig. 6C). Compared with PBS or ETO, Pn6A had a moderate response in the submucosal layer of the middle ear cleft (Fig. 6D).

DISCUSSION

Granulation tissue is frequently formed during the process of OM. TGF- β plays an important role in the formation of granulation tissue. Recent mouse models for chronic OM support the notion that the TGF- β signaling pathway is involved in this process³⁸ although the detailed mechanism is not well understood at the present time.

Granulation tissue formation begins at the acute stage of OM, persists, and accelerates in the chronic stage of OM. It results in the proliferation of fibroblasts in the submucosa and the thickening of the middle ear cleft. In the chronic stage of OM, proliferation of fibroblasts persists and ECM proteins continuously deposit to the middle ear submucosa layer, finally leading to fibrosis. In our previous study, we have shown that Pn6A induced mucous cell metaplasia of the middle ear mucosa of rats more than NTHi,³¹ suggesting that pneumococcus is a potent pathogen that causes OM with mucoid effusion. In this study, we demonstrated that NTHi significantly induced the expression of collagens (1A1 and 3A1) during the acute phase (day 3) in comparison with the controls. In contrast, Pn6A induced the expression of collagens to a lesser degree. It has been known in an *in vitro* study that NTHi activates T β R-Smad3/4 signaling.³⁹ It suggests that NTHi is a more potent pathogen implicated in granulation tissue and subsequent fibrotic disorders than Pn6A.

It is well known that the TGF- β plays a critical role in fibrotic disorders.^{10, 11, 13, 21, 40} In this study, we demonstrated that the TGF pathway activity in bacterial OM was significantly higher than in controls, suggesting that the pathway was active although the majority of the genes in this pathway were not dramatically up-regulated at the time studied. To our knowledge, individual genes in this pathway synergistically contribute to the activity of the pathway and lead to fibrotic disorders. Interestingly, the activity of the same pathway was not significantly regulated in ETO. It suggests that ETO is not related to the formation of granulation tissue in the middle ear cleft.

Indeed, histology of the middle ear mucosa with bacterial OM revealed that submucosal responses are obvious whereas ETO revealed little or no submucosal thickening. It should be noted that when a single gene was selected for analysis, it was difficult to reflect the entire pathway activity: individual genes may be down-regulated while the entire pathway activity is actually up-regulated. For example, TGF- β 1 is an important member of the TGF family involved in fibrotic disorders, but it was not up-regulated in Pn6A-infected middle ears. Instead, TGF- β 1 was up-regulated in NTHi-infected middle ears on day 3. However, when TGF- β 2, β 3, and their receptors and subsequent molecules, such as Smads, were combined together, their changes were significant. TGF family genes are in part up-regulated and in part down-regulated. By means of microarray data analysis, it is becoming clear that the TGF- β signaling pathway, as a whole, is up-regulated—although the regulation of individual genes at the ligand or receptor levels may not be so at the time studied. Interestingly, TGF- β (especially β 2 and β 3) and its receptors (T β RII) are highly expressed in MEEC, which suggests that the TGF signaling pathway begins at the epithelial layer then disseminates to the submucosal layer.

In addition to TGF- β , T-helper cells that produce cytokines, such as IL-4, IL-5, and IL-13, may be involved in the process of fibrotic disorders because those cytokines are highly up-regulated, which then results in the hyperproduction of collagens I and III alpha 1 subunit. We know that TGF- β is involved in tissue remodeling of the middle ear mucosa, but we do not know which TGF- β members are involved in this process and how it occurs. In this study, we demonstrate that TGF- β 1, mainly expressed in fibroblasts, and TGF- β 2 and β 3, mainly expressed in the middle ear epithelial cells, are all involved in the inflammatory response of the middle ear cleft.

As shown in our previous studies, bacterial OM leads to up-regulation of the Id family, Id1, and Id3,1 which are potential triggers for new blood vessels developing. In this study, bacterial OM increases the production of ECMs. This constitutes a pathological basis for the formation of granulation tissue in the middle ear cleft.

To summarize, the expression of ECM mRNA transcripts (COL1A1, 3A1, 5A1, etc.) and major collagens in the middle ear cleft was reduced in ETO. The expression of those ECM mRNA transcripts increased in bacterial OM, especially in NTHi. Since 1A1, 3A1, and 5A1 collagens count for approximately 90% of collagen mRNA transcripts, it is plausible to assume that bacteria, especially NTHi, are potent triggers for the formation granulation tissue.

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ABBREVIATIONS

OM	otitis media
TGF-β	transforming growth factor-beta
ETO	obstructing the eustachian tube
Pn6A	pneumococcus type 6A
NTHi	non-typeable <i>H. influenza</i>
ECMs	extracellular matrix proteins
Id	inhibitor of differentiation
ALKs	activin-like receptor kinases
COL	collagen
FN	fibronectin
LAM	laminin
TN	tenascin
IL	interleukin
MAPK	mitogen-activated protein kinase
MEEC	middle ear epithelial cells
ANOVA	analysis of variance

ActR	activin receptor
TβR	TGFβ receptor
BMPR	bone morphogenic protein receptor
HSP27	heat shock protein 27
TF	transcriptional factor
MEKK1	mitogen-activated protein kinase kinase kinase

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Fig. 1.

A representative cascade of the TGF-β pathway. TGF-β₁₋₃, TGF-β receptors (I & II), Smads, and transcriptional factor (TF) constitute the cascade. TGF-β ligands (including TGF-β₁₋₃, activins, BMPs, etc.) with the assistance of accessory receptor (betaglycan) activate the formation of heterotetrameric complexes of type I and II serine/threonine kinase receptors which in turn activate the intracellular effectors of TGF-β signaling, Smad2/3 by phosphorylation. The action recruits Smad4 to the complex and translocate into the cellular nucleus where Smad2/3/4, working together with a cellular context-specific transcriptional factor (TF), bind to DNA and trigger the expression of a battery of genes including collagens, fibronectins, laminins, etc. Eventually, the TGF-β signaling pathway leads to fibrotic responses.

Fig. 2.

Effects of bacterial OM and ETO on the expression of the TGF- α and - β genes (A). TGF- α was mainly expressed in MEEC whereas TGF- β 1 was not. Unlike TGF- β 1, the expression of TGF- β 2 and TGF- β 3 was mainly in dMEEC. The TGF- β 1 gene expression was down-regulated by Pn6A at the early stage (day 3~7) compared with their controls. The TGF- β 2 gene was not responsive to bacterial OM and ETO. The TGF- β 3 gene expression was slightly responsive to Pn6A, NTHi, and ETO. Pn6A-3D, day 3 following pneumococcal infection in the middle ear cavity. The genes in the TGF- β signaling pathway, regulated by bacterial OM and ETO, are presented in heat map (B). Note that many genes in the TGF- β signaling pathway are up-regulated in bacterial OM, especially NTHi day 7, but only few genes are slightly up-regulated in ETO.

Fig. 3.

Altered expression of the TGF- β receptors following the challenge of Pn6A, NTHi, and ETO. The TGF receptors expressed in MEEC were ActR, ALK7, T β RII (II2), and T β RIII (M80784) whereas those expressed in fibroblasts were III and II2. T β RII (II2) was highly expressed in pMEEC but not responsive to Pn6A, NTHi, and ETO. In contrast, T β RIII was moderately expressed in pMEEC, responding to Pn6A, NTHi, and ETO.

Fig. 4.

Altered expression of the effectors in the TGF- β signaling pathways following the challenge of Pn6A, NTHi, and ETO. Collagens type I alpha 1 (COL1A1), type III alpha 1 (COL3A1), and fibronectins (FN) were low in MEEC but highly expressed in the middle ear cleft (panel A) following NTHi 3~7D but not Pn6A 3~7D or ETO 7~30D. Specifically, on day 3, FN3M1, IL-13, FN1, HSP70, and TGF- β 1 were down-regulated in Pn6A or NTHi infected ears (panel B). On day 7, there were 2 genes including FN3M1 and IL-13 were up-regulated where there were three genes including FN1, HSP70, and TGF- β 1 were down-regulated (panel C). In ETO from 7 to 30 days, there were 7 genes including COL4A1, COL5A1, FN, FN-1, TGF- β 1, and HSP70 were regulated (panel D).

Fig. 5.

Highly regulated effectors of the TGF- β signaling pathway following Pn6A, NTHi, and ETO. Effector genes up-regulated or down-regulated more than 5 times or higher in comparison with their controls were selected and demonstrated. These genes included COL, FN, TN, and LAM. COL (1A1, 3A1), highly responding to NTHi 3D, were listed in (panel A). COL (3A1) and FN (3M1), highly responding to NTHi 3~7 D, were listed in (panel B). FN (FN1), highly expressed in dMEEC, was listed in (panel C). COL (8A1) and TN (TN-C, TN-X), highly expressed in both pMEEC and dMEEC, were listed in (panel D). Note that smooth muscle protein, SMA, responded to NTHi (panel D).

Fig. 6. Remarkable proliferation of the middle ear submucosal layer in response to NTHi. In histological examination, PBS-treated middle ear for 14 days had limited proliferation of fibroblasts in the submucosal layer (Fig. 6A). Like PBS, ETO had no or little submucosal response (Fig. 6B). In contrast, NTHi had a remarkable submucosal response compared with Pn6A (Fig. 6C). Pn6A had a moderate submucosal response compared with PBS or ETO (Fig. 6D). Original amplifications, 40x in panels a, b, and d; 10x in c.

Table I

Primer pairs used in RT-PCR and oligo inhibition studies

Gene (GenBank #)	Upper/down primer sequence for RT-PCR	Size (bp)
TGF- β 1(X52498)	5'-aagaccatgcacatggagc -3'/5'-gttcatgtcatggatgggtgc-3'	663
T β RI (NM_012775)	5'-cgtctgcattgcact-3'/5'-agcagtggtaaacctgatcc-3'	180
ActRIIB (L10640)	5'-gctcacgggtgctggccta -3'/5'-ccagcaccgcggtaggtc-3'	608
Smad2 (NM_019191)	5'-ccaggctcttggatggtcgt-3'/5'-ggcggcagttctgttagaat-3'	253
COL3A1 (NM_032085)	5'-gatcaggccaatggcaatgt-3'/5'-aaaagcaaacaggccaatg-3'	259
COL1A1 (NM_053304)	5'-ttccctggacctaagggtact-3'/5'-ttgagctccagcttcgcc-3'	114
FN-1(NM_019143)	5'-gagagatctggaggtcat-3'/5'-gggtgacacctgagttaa-3'	854
Tenascin-C (U15550)	5'-ttcaatctctggacagctaccg-3'/5'-ataagtaatcggaaactctccactg-3'	593
MEKK1(U48596)	5'-agcggaggaatcaggaaa-3'/5'-tataagacagaggcgaccag-3'	694
c-jun (X17163)	5'-gtaagaacacaagcaggag-3'/5'-aggagtcgcagaatccaag-3'	756

Table II

Altered expression of the pathway genes and their effectors following the challenge with Pn6A, NTHi, and ETO in comparison with their controls analyzed by two factor ANOVA

	pMEEC vs. dMEEC	PBS vs Pn6A (3d)	PBS vs Pn6A (7d)	PBS vs NTHi (3d)	PBS vs NTHi (7d)	Ctrl vs. ETO (7d)	Ctrl vs. ETO (7d)
TGF- β	12 $\uparrow$	8 $\uparrow$	.0027	16 $\uparrow$	.0067	14 $\uparrow$	.0621
	.0249			.0499	.0024	13 $\uparrow$	.0524
	14 $\downarrow$	18 $\downarrow$	.0957	10 $\downarrow$	.1927	14 $\downarrow$	.0018
	.0219			.0281	.0711	21 $\uparrow$	.0138
Effectors	38 $\uparrow$	19 $\uparrow$	.0066	26 $\uparrow$	.0724	36 $\downarrow$	.0842
	.0184			.0953	.0294	36 $\downarrow$	.0256
	19 $\downarrow$	38 $\downarrow$	.0064	31 $\downarrow$	.0019		
	.0037						

Table III

Verification of the gene expression by RT-PCR*

Sample	dMEEC	pMEEC	PBS		Pn6A		NTHi		ETO	
			3D	7D	3D	7D	3D	7D	3D	7D
TGF-β1	-	±	±	±	+	+	+	+	±	±
TβR1	+	+	+	+	+	+	+	+	+	+
ActRIIB	+	+	+	+	+	+	+	+	+	+
Snad2	+	+	+	+	+	+	+	+	+	+
Tenascin-C	+	+	±	±	-	-	±	±	±	±
COL1A1	+	+	+	+	+	+	+	+	+	+
COL3A1	±	±	+	+	+	+	+	+	+	+
Fibronectin-1	+	±	+	+	±	±	+	+	±	±
MEKK1	+	+	+	+	+	+	+	+	+	+
c-jun	+	+	+	+	+	+	+	+	+	+