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Smad3 deficient mice have reduced esophageal fibrosis and angiogenesis in a mouse model of egg induced eosinophilic esophagitis

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Abstract

Objectives—Eosinophilic esophagitis (EoE) is a food triggered disease associated with esophageal fibrosis and stricture formation in a subset of patients. In this study we utilized a murine model of egg (OVA) induced EoE to determine whether inhibiting TGF- β 1 signaling through the Smad3 pathway would inhibit features of esophageal remodeling including fibrosis, angiogenesis, and basal zone hyperplasia.

Methods—Wild type (WT) and Smad3 deficient (KO) mice were sensitized intraperitoneally and then challenged chronically with intra-esophageal OVA for one month. Levels of esophageal eosinophils, esophageal TGF- β 1+ and VEGF+ cells, as well as features of esophageal remodeling (fibrosis, angiogenesis, basal zone hyperplasia) were quantitated by immunohistochemistry and image analysis.

Results—OVA challenge induced a similar increase in the levels of esophageal MBP+ eosinophils and esophageal TGF- β 1+ cells in WT and Smad3 KO mice. However, Smad3 KO mice challenged with OVA had significantly less esophageal fibrosis and esophageal angiogenesis compared to OVA challenged WT mice. The reduced esophageal angiogenesis in Smad3 KO mice was associated with reduced numbers of VEGF+ cells in the esophagus. There was a trend for OVA challenged Smad3 KO to have reduced basal zone hyperplasia, but this was not statistically significant.

Conclusion—In a mouse model of egg induced EoE, Smad3 deficient mice have significantly less esophageal remodeling, especially fibrosis and angiogenesis which is associated with reduced

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DATA ANALYSIS

Results were compared by a Mann-Whitney test using a statistical software package (GraphPad Prism, San Diego, CA). P values <0.05 were considered statistically significant. Results are presented as the mean $\pm$ SEM.

STATEMENT OF CONTRIBUTION

Ja Youn Cho, Ashmi Doshi, Peter Rosenthal, Andrew Beppu, and Marina Miller, designed experiments, and performed experiments. Seema Aceves, and David Broide contributed to the design of the study, the interpretation of results, and the writing of the manuscript. All authors reviewed the final draft of the manuscript.

STATEMENT OF CONFLICTS OF INTEREST

None of the authors have a conflict of interest.

expression of VEGF. Targeting the TGF- β 1/Smad3 pathway may be a novel strategy to reduce esophageal fibrosis and its associated complications such as esophageal strictures in EoE.

Keywords

eosinophil; fibrosis; fibronectin

INTRODUCTION

Eosinophilic esophagitis (EoE) is characterized histologically by a dense esophageal eosinophilia (>15 eos/HPF), and clinically by the presence of symptoms related to esophageal dysfunction including dysphagia, chest pain, abdominal pain, and food impactions (1, 2). Food impactions are related to esophageal strictures which develop in a subset of subjects with EoE (3,4, 5). In this study we have investigated the role of TGF- β 1 and the Smad 3 pathway which mediates TGF- β 1 intracellular signal transduction (6) in contributing to esophageal fibrosis and remodeling using a mouse model of oral food induced EoE which is associated with esophageal fibrosis and remodeling including angiogenesis, and deposition of extracellular matrix components such as fibronectin (7). We have focused on the TGF- β 1/Smad3 pathway in this study as TGF- β 1 is a cytokine that is highly expressed in the esophagus in EoE (8,9,10,11,12), and the TGF- β 1/Smad3 pathway is known to be important in mediating fibrosis in many diseases associated with fibrosis (6).

In EoE, eosinophils (8) and mast cells (9) are a prominent source of TGF- β 1 in the esophagus. To contribute to esophageal fibrosis in EoE, TGF- β 1 released from eosinophils, mast cells, or other TGF- β 1 producing cells would need to bind to TGF- β 1 cell surface receptors (TGF- β RI and TGF- β RII) on fibroblasts which would subsequently activate intracellular Smads to translocate to the nucleus to active transcription of collagen genes which contribute to fibrosis (6). Smad proteins are thus a family of transcription factors that mediate TGF- β 1 signals. The binding of active TGF- β 1 to the TGF- β 1 cell surface receptor results in phosphorylation of receptor regulated Smad2/3 (6). Once phosphorylated, Smad2/3 forms a complex with Smad4 which then translocates from the cytoplasm to the nucleus where it regulates transcription of collagen genes. Studies of human fibroblasts have identified a number of collagen gene promoters that are induced by TGF- β 1 and dependent on Smad 3 (13). Activation of these collagen gene promoters by TGF- β 1 can be blocked by dominant-negative Smad3 expression vectors (13). Therefore, inhibition of TGF- β 1/Smad3 signaling may directly reduce the expression of collagen-producing genes and resultant fibrosis. The importance of this TGF- β 1 /Smad3 pathway to inhaled allergen induced airway fibrosis and remodeling of the airways in asthma has been demonstrated in mouse models of asthma using either Smad3 deficient mice (14) or anti-TGF- β 1 Abs (15). In this study we demonstrate that using a mouse model of egg induced EoE, Smad3 deficient mice have significantly less esophageal remodeling, especially fibrosis and angiogenesis. Targeting the TGF- β 1/Smad3 pathway in EoE may thus be a novel way to reduce esophageal fibrosis and its associated complications such as esophageal strictures.

MATERIALS AND METHODS

Oral OVA allergen induced esophageal eosinophilic inflammation

Eight week old female Smad3 deficient on a BALB/c background (kindly provided by Dr. Stuelten, NCI, with the permission of Dr Lise Wogensen, Aarhus, Denmark who generated the mice) and control BALB/c mice (12 mice/group; The Charles River Laboratory, Wilmington, MA) were sensitized intraperitoneally on day 0 and 14 (50 µg of OVA adsorbed to 1 mg of aluminum hydroxide adjuvant in phosphate buffered saline (PBS), Sigma-Aldrich, St. Louis, MO) and challenged intra-esophageally three times/week for four weeks with 10 mg OVA suspended in 100 µl PBS on days 28, 30, 32, 35, 37, 39, 42, 44, 46, 49, 51, 53 as previously described (7). OVA was administered through an intragastric feeding needle (20-gauge, 1.5-inch; Pepper and Sons, Inc, New Hyde Park, NY). Mice were sacrificed 24 hrs after last administration of intraesophageal OVA (day 54). Control BALB/c mice were neither sensitized nor challenged. The esophagus was removed in its entirety and fixed with 4% paraformaldehyde solution (Electron Microscopy Sciences, Hatfield, PA) for 24 hrs., oriented and embedded in 1% agarose (Invitrogen, Carlsbad, CA), and then sectioned (upper, middle and lower). Five-micron esophageal sections were then prepared from each layer and equivalent numbers of sections from each layer were included in every experiment for analysis. Results in each group are presented as a combined score of the 3 layers analyzed (upper, middle, lower) as previously described (7). For immunohistochemistry experiments, the total area of lamina propria in each slide was counted and results expressed per mm² of lamina propria.

MBP+ esophageal eosinophils

Eosinophils were detected in esophageal tissue by immunohistochemistry using an anti-mouse Major Basic Protein (MBP) antibody (kindly provided by James Lee PhD, Mayo Clinic, Scottsdale, AZ) as previously described (7). Quantitation of the number of eosinophils was performed using a light microscope attached to an image-analysis system with the entire cross section of the esophagus visualized. The area of the esophageal lamina propria analysis was outlined and this area determined by the image analysis software (Image-Pro Plus; Media Cybernetics). Results are expressed as the number of eosinophils per mm² of lamina propria.

Esophageal IL-5, eotaxin-1, and TGF-β1+ cells

Esophageal tissue sections were processed for immunohistochemistry using a primary mAb directed against either TGF-β1 (Santa Cruz Biotechnology Inc, Santa Cruz, CA), IL-5 (R&D), or eotaxin-1 (R&D), as described (16). Results are expressed as the number of IL-5, eotaxin-1, or TGF-β1 positive cells per mm² of lamina propria.

Esophageal TGF-β1 mRNA

The esophagus derived from WT and Smad3 deficient mice were processed for qPCR to detect TGF-β1 mRNA as previously described in this laboratory (17). In brief, total RNA was extracted with RNA-STAT-60 (Tel-Test) and reverse transcribed with Oligo-dT and SuperScript II kit (Life Technologies). qPCR was performed with TaqMan PCR Master Mix

and TGF- β 1, primers (Applied Biosystems). The relative amounts of transcripts were normalized to those of housekeeping gene (GAPDH) mRNA and compared by the Ct method as previously described in this laboratory (17).

Esophageal fibrosis

The area of trichrome staining in paraffin embedded esophagus was outlined and quantified using a light microscope attached to an image analysis system as previously described (7). Results are expressed as the area of trichrome staining per μ m length of basement membrane.

Esophageal angiogenesis and VEGF + cells

Blood vessels in esophageal tissue were identified by immunohistochemistry using a rat anti-mouse PECAM monoclonal antibody (BD Bioscience, San Jose, CA) which detects the blood vessel adhesion molecule PECAM as previously described in this laboratory (7). To enhance the ability to detect new vessels, only those small blood vessels $\leq$ 5 microns were counted as previously described in this laboratory (7). Results are expressed as the number of PECAM-1 positive vessels per mm^2 of lamina propria.

In addition we quantitated the number of VEGF positive cells using an anti-VEGF primary Ab (Santa Cruz Biotechnology, Santa Cruz, CA). Results are expressed as the number of VEGF positive cells per mm^2 of lamina propria.

Esophageal Basal Zone Thickness

The epithelial basal zone thickness was assessed in esophageal sections stained with hematoxylin and eosin using a light microscope attached to an image-analysis system (7). The maximal thickness of the basal layer in each slide was recorded in μ m.

Effect of TGF- β 1 on Smad3 deficient versus WT mast cell proliferation

To determine whether Smad3 deficient cells have a reduced capacity to respond to TGF- β 1, purified populations of mouse bone marrow derived mast cells (MBMMC) ($> 99\%$ pure) from WT and Smad3 deficient mice were cultured in triplicate in the presence or absence of TGF- β 1 (10 ng/ml) for 48 hrs as previously described in this laboratory (18). The number of MBMMC in WT and Smad3 deficient cultures were quantitated using the CyQUANT cell proliferation assay kit (Invitrogen). We have previously demonstrated that TGF- β inhibits WT MBMMC proliferation (18). Results are expressed as the % inhibition of MBMMC proliferation quantitated as (Baseline number of MBMMC-48 hour number of MBMMC) divided by 100.

RESULTS

MBP+ esophageal eosinophils

The number of esophageal eosinophils increased significantly in WT mice challenged with OVA compared to non-OVA challenged mice (122.9 ± 24.8 vs. 12.2 ± 2.2 eosinophils/ mm^2 ; $p < 0.0001$) (Figure 1). OVA challenge in Smad3 deficient mice induced a similar increase in

the number of esophageal eosinophils (OVA Smad3 KO vs no OVA Smad3 KO; $p < 0.0001$) as noted in OVA challenged WT mice (Figure 1).

Esophageal IL-5+ cells and eotaxin+ cells

There was no difference in the number of IL-5 positive cells in OVA challenged WT mice compared to OVA challenged Smad3 KO mice (0.72 ± 0.33 vs 0.85 ± 0.45 IL-5 positive cells/mm²; $p = \text{ns}$). Eotaxin-1 positive cells were not detected in either WT or Smad3 KO mice.

Esophageal TGF- β 1+ cells and esophageal TGF- β 1 mRNA

The number of esophageal TGF- β 1+ cells increased significantly in WT mice challenged with OVA compared to non-OVA challenged mice ($p < 0.01$) (Figure 2A). There was no significant difference in the number of esophageal TGF- β 1+ cells in OVA challenged Smad3 deficient mice compared to OVA challenged WT mice ($p = \text{ns}$) (Figure 2A).

Levels of esophageal TGF- β 1 mRNA increased significantly in WT mice challenged with OVA compared to non-OVA challenged mice ($p < 0.01$) (Figure 2B). There was no significant difference in levels of esophageal TGF- β 1 mRNA in OVA challenged Smad3 deficient mice compared to OVA challenged WT mice ($p = \text{ns}$) (Figure 2B).

Esophageal Fibrosis

The area of esophageal trichrome staining (which detects collagen) increased significantly in WT mice challenged with OVA compared to non-OVA challenged mice (1.52 ± 0.10 vs. 0.54 ± 0.05 $\mu\text{m}^2/\mu\text{m}^2$ area of trichrome staining) ($p < 0.0001$) (Figure 3). Smad3 deficient mice challenged with OVA had a significant reduction in the area of esophageal trichrome staining compared to OVA challenged WT mice ($p < 0.0001$) (Figure 3).

Esophageal angiogenesis

WT mice challenged with OVA had a significant increase in the number of esophageal small blood vessels as quantitated by PECAM staining ($p < 0.0001$ vs no OVA) (Figure 4). In contrast, OVA challenged Smad3 deficient mice had a significant reduction in the number of esophageal small blood vessels compared to OVA challenged WT mice ($p < 0.0001$) (Figure 4).

Esophageal VEGF positive cells

To determine whether the Smad3 pathway influenced levels of angiogenic cytokines, we quantitated the number of VEGF positive cells within the lamina propria. WT mice challenged with OVA had a significant increase in the number of VEGF positive cells in the LP ($p < 0.0001$; vs WT no OVA) (Figure 5). Smad3 deficient mice challenged with OVA had significantly reduced numbers of VEGF positive cells in the LP ($p < 0.0001$; vs WT OVA) (Figure 5).

Esophageal Basal Zone Thickness

OVA challenged WT mice had a slight increase in basal zone thickness compared to non-OVA challenged WT mice which was not statistically significant ($p=0.12$). OVA challenged Smad3 deficient mice had a trend for reduction in basal layer thickness compared to OVA challenged WT mice which was not statistically significant ($p=0.40$).

Effect of TGF- β 1 on Smad3 deficient versus WT mast cell proliferation

WT MBMMC cultured in the presence of TGF- β 1 for 48 hours had a significantly greater inhibition of MBMMC proliferation (48% inhibition) compared to Smad3 deficient MBMMC cultured in the presence of TGF- β 1 (18 % inhibition) ($p < 0.001$)(Figure 6).

DISCUSSION

In this study we have used a mouse model of egg induced EoE and esophageal remodeling to demonstrate the importance of the Smad3 signaling pathway to esophageal fibrosis and angiogenesis which are features of esophageal remodeling in EoE. As esophageal strictures and food impactions are an important complication of EoE (3,4,5), understanding the mechanism by which esophageal fibrosis and strictures are induced in pre-clinical mouse models of EoE provides insight into potential novel targets such as the TGF- β 1/Smad3 signaling pathway in the subset of EoE subjects with esophageal strictures. The potential relevance to human EoE of our findings related to the Smad3 pathway in the mouse model of EoE is supported by studies of esophageal biopsies in EoE demonstrating increased numbers of esophageal TGF- β 1+ cells (including eosinophils and mast cells expressing TGF- β 1)(8,9,10,11,12), activation of the Smad3 signaling pathway (8), increased fibrosis (8,9,10), and increased angiogenesis (8). While the pre-clinical model of EoE we have used in this study provides important insight into many features of OVA induced esophageal remodeling associated with EoE, we are not able to model esophageal strictures and food impactions with this mouse model.

Our studies in Smad3 deficient mice demonstrated that they have similar levels of esophageal eosinophilic inflammation and esophageal TGF- β 1+ cells as WT mice when challenged with OVA. Thus, targeting the Smad3 pathway does not inhibit the ability of eosinophils to traffic from the bone marrow to the esophagus, or inhibit the ability of cells in the esophagus such as eosinophils and mast cells to express TGF- β 1. However, targeting the Smad3 pathway does inhibit the ability of cells which express TGF- β receptors coupled to intracellular Smad3 signal transduction pathways to respond to TGF- β 1 released by eosinophils and mast cells. Thus, in Smad3 deficient mice esophageal fibroblasts (which highly express TGF- β receptors that are coupled to intracellular Smad3 signal transduction pathways), are unable to respond to TGF- β 1 to express collagen which results in less esophageal fibrosis. Our in vitro studies with Smad3 deficient mast cells confirm that Smad3 plays a significant role in mediating the function of TGF- β 1, although a minor inhibitory effect of TGF- β 1 that was Smad3 independent was also noted.

Our studies also demonstrated that Smad3 deficient mice have reduced esophageal angiogenesis another feature of esophageal remodeling noted in esophageal biopsies from

subjects with EoE (8). Angiogenic vessels are known to exhibit increased expression of adhesion molecules (19) and may contribute to increased inflammation through increased recruitment of eosinophils into the esophagus. Studies in EoE have demonstrated increased angiogenic blood vessels with increased levels of expression of the adhesion molecule VCAM-1 (8) which binds VLA-4 expressed by eosinophils. As VEGF is a key mediator of angiogenesis (20), we quantitated levels of VEGF positive cells in the esophagus in WT and Smad3 deficient mice. These studies demonstrated that OVA challenge induced increased numbers of VEGF + cells and angiogenesis in the esophagus in WT mice, while levels of VEGF+ cells and angiogenesis were significantly reduced in Smad3 KO mice. The mechanism by which Smad3 influences levels of VEGF is likely to be indirect as the Smad3 transcription factor is not a key regulator of VEGF transcription. However, there is considerable evidence that TGF- β 1 (which we have detected at increased levels in EoE and which signals through Smad3) can mediate angiogenesis *in vivo* (21,22,23,24). Because of TGF- β 1's inhibitory effects on endothelial cells *in vitro* (25), TGF- β 1 is hypothesized to induce angiogenesis *in vivo* through an indirect mechanism, by inducing expression of VEGF and/or other angiogenic factors (25). For example TGF- β 1 deficient mice die *in utero* and show defective vasculogenesis (21). Similarly mice deficient in the TGF- β 1 receptor I (ALK1 deficient mice) die from defects in angiogenesis (21,22,23). In addition, *in vivo* administration of TGF- β 1 subcutaneously in newborn mice induces angiogenesis (24). As levels of VEGF were reduced in the OVA challenged Smad3 deficient (who had reduced angiogenesis), it suggests that TGF- β 1 is indirectly inducing angiogenesis in this model most likely mediated by angiogenic cytokines such as VEGF. However, further studies will need to be performed using inhibitors of VEGF and/or other angiogenic cytokines to determine their individual roles in contributing to angiogenesis in EoE.

In summary, we have demonstrated that in a mouse model of OVA food induced EoE that Smad3 deficient mice have significantly less esophageal remodeling, especially fibrosis and angiogenesis. In addition, reduced angiogenesis in Smad3 deficient mice was associated with reduced levels of expression of VEGF. Targeting the TGF- β 1/Smad3 pathway may thus be a novel strategy to reduce esophageal fibrosis and its associated complications such as esophageal strictures in EoE.

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ABBREVIATIONS

EoE	Eosinophilic esophagitis
MBMMC	Mouse bone marrow derived mast cell
MBP	Major Basic Protein

OVA	Ovalbumin
PECAM	Platelet endothelial cell adhesion molecule
VEGF	Vascular endothelial growth factor

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Figure 1. Eosinophils in the Esophagus

Eosinophils in the esophagus were detected using immunohistochemistry staining with an anti-Major Basic Protein antibody. A) WT No OVA. B) WT OVA. C) Smad3 KO no OVA. D) Smad3 KO OVA. E) OVA challenge induced a significant increase in esophageal eosinophils in WT mice (OVA vs no OVA; $p<0.0001$), as well as in Smad3 KO mice (OVA vs no OVA; $p<0.0001$).

A **B**

Figure 2. Esophageal TGFβ1+ cells and Esophageal TGFβ1 mRNA

(A). TGFβ1 positive cells in the esophagus were detected using immunohistochemistry staining with an anti-TGFβ1 antibody. OVA challenge induced a significant increase in esophageal TGFβ1 positive cells in WT mice (OVA vs no OVA; p=0.01). There was no difference in numbers of TGFβ1 positive cells in OVA challenged Smad3 KO mice compared to OVA challenged WT mice (p=NS).

(B). The esophagus derived from WT and Smad3 deficient mice were processed for qPCR to detect TGF- β 1 mRNA. The relative amounts of transcripts were normalized to those of housekeeping gene (GAPDH) mRNA.

Figure 3. Esophageal fibrosis

The area of esophageal trichrome staining was quantitated using light microscopy and image analysis. OVA challenge induced a significant increase in the area of esophageal trichrome staining in WT mice (OVA vs no OVA; $p < 0.0001$). There was a significant reduction in the area of esophageal trichrome staining in OVA challenged Smad3 KO mice compared to OVA challenged WT mice ($p < 0.0001$).

Figure 4. Esophageal angiogenesis

Blood vessels <5 μm in diameter in the esophagus were detected using immunohistochemistry staining for PECAM. OVA challenge induced a significant increase in esophageal small blood vessels in WT mice (OVA vs no OVA; $p<0.0001$). There was a significant reduction in the number of esophageal small blood vessels in OVA challenged Smad3 KO mice compared to OVA challenged WT mice ($p<0.0001$).

Figure 5. Esophageal VEGF expression

VEGF positive cells in the esophagus were detected using immunohistochemistry staining with an anti- VEGF antibody. OVA challenge induced a significant increase in the number of esophageal VEGF+ cells in WT mice (OVA vs no OVA; $p < 0.0001$). There was a significant reduction in the number of esophageal VEGF+ cells in OVA challenged Smad3 KO mice (OVA WT vs OVA Smad3 KO; $p < 0.0001$).

Figure 6. Effect of TGF- β 1 on Smad3 deficient versus WT mast cell proliferation

Mouse bone marrow derived mast cells (MBMMC) (> 99% pure) from WT and Smad3 deficient mice were cultured in triplicate in the presence or absence of TGF- β 1 (10 ng/ml) for 48 hrs. The number of MBMMC in WT and Smad3 cultures were quantitated using a CyQUANT cell proliferation assay kit. Results are expressed as the % inhibition of MBMMC proliferation.