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TGFβ1-Induced PAI-1 Contributes to a Pro-Fibrotic Network in Eosinophilic Esophagitis

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Abstract

Background: Eosinophilic esophagitis is an allergic disease of increasing worldwide incidence. Complications are due to tissue remodeling and involve transforming growth factor- β 1 (TGF β 1) mediated fibrosis. Plasminogen Activator Inhibitor-1 (PAI-1/serpinE1) can be induced by TGF β 1 but its role in EoE is not known.

Objective: To understand the expression and role of PAI-1 in EoE

Methods: We used esophageal biopsy specimens and plasma samples from control and EoE subjects, primary human esophageal epithelial cells, and EoE fibroblasts in immunohistochemistry, quantitative PCR, and immunoassay experiments to understand the induction of PAI-1 by TGF β 1, the relationship between PAI-1 and esophageal fibrosis, and the role of PAI-1 in fibrotic gene expression.

Results: PAI-1 expression was significantly elevated in epithelial cells of active EoE biopsies as compared with inactive EoE or control biopsies ($p < 0.001$). Treatment of primary esophageal epithelial cells with recombinant TGF β 1 increased PAI-1 transcription, intracellular protein expression, and secretion. Esophageal PAI-1 expression correlated with basal zone hyperplasia, a fibrosis, and markers of esophageal remodeling including vimentin, TGF β 1, collagen I, fibronectin, and matrix metalloproteases and plasma PAI-1 levels correlated with plasma TGF β 1. PAI-1 inhibition significantly decreased baseline and TGF β 1-induced fibrotic gene expression.

Conclusions: PAI-1 is significantly elevated in the EoE epithelium, reflects fibrosis, and its inhibition decreases TGF β 1-induced gene expression. Epithelial PAI-1 may serve as a marker of EoE severity and form part of a TGF β 1-induced pro-fibrotic network.

Clinical Implications

Since PAI-1 is induced by TGF β 1, expressed in EoE epithelial cells, correlates positively with fibrosis and its inhibition decreases TGF β 1-induced fibrotic gene expression, PAI-1 may be as an epithelial marker of esophageal remodeling and serve as a new therapeutic target.

Capsule Summary

PAI-1 has not been previously studied in EoE. This study shows that PAI-1 may be an epithelial marker of fibrotic severity, and could be a new therapeutic target in EoE.

Key Words

Eosinophil; Esophagitis; SerpinE1; Remodeling; Fibrosis; Transforming growth factor beta-1

Abbreviations

CPA-3: Carboxypeptidase A3

EoE: Eosinophilic esophagitis

PAI-1: Plasminogen activator inhibitor-1

TGF β 1: Transforming growth factor beta-1

BZH: Basal zone hyperplasia

LP: Lamina propria

IHC: Immunohistochemistry

IF: Immunofluorescence

HPF: high power field

Introduction

Eosinophilic esophagitis (EoE) is a chronic antigen driven allergic disease of increasing worldwide incidence and prevalence (1). One mechanism for disease complications is fibrosis which occurs largely in the subepithelium, begins early in life, and is progressive without intervention (2-8). EoE esophageal remodeling includes epithelial changes of basal zone hyperplasia (BZH) and dilated intercellular spaces and subepithelial fibrosis and angiogenesis in the lamina propria (LP). Fibrosis is variably responsive to anti-inflammatory interventions (8-14). Since the subepithelial tissue is not always present for histologic evaluation, the presence and response of remodeling changes to intervention can be difficult to gauge. Without treatment EoE patients suffer from vomiting, failure to thrive, dysphagia, food impactions and consistently progress to esophageal strictures (1, 3, 4).

Studies from our laboratory and others have demonstrated that TGF β 1 can be a major regulator of childhood EoE remodeling (5, 15-20). A number of genes that are involved in changes in cellular function and trafficking are induced by TGF β 1 including periostin which increases eosinophil trafficking (18) and phospholamban which induces TGF β 1-mediated esophageal smooth muscle cell contraction in culture (17). Among the genes that are induced by TGF β 1 is the serine protease inhibitor, plasminogen activator inhibitor-1 (PAI-1/serpinE1) which promotes tissue fibrosis in asthma (21-24). However, the role of PAI-1, its induction in EoE, or its role in esophageal fibrosis has not been previously studied.

PAI-1 is elevated in human asthma and promotes airway remodeling in murine asthma models (21, 23-28). PAI-1 overexpression, triggered by viruses and allergens, is associated with airway fibrosis while PAI-1 deficiency protects mice from fibrotic airway remodeling (21, 23-26, 29). Mast cell derived TGF β 1 increases PAI-1 expression from immortalized airway epithelial cells in culture (22). In addition, a single nucleotide polymorphism 4G in the PAI-1 gene associates with human asthma risk (27, 28). These results define a role for PAI-1 expression in

airway remodeling but the induction of PAI-1 in EoE and its role in esophageal fibrosis is not known.

Based on these findings and our prior work in EoE that shows that TGF β 1 promotes EoE associated remodeling, we explored the role of PAI-1 in EoE. Since EoE is associated with substantial tissue fibrosis that can lead to esophageal rigidity, food impactions, and strictures, determining the mechanisms and new potential targets for controlling remodeling is an issue of clinical interest. Herein we demonstrate that PAI-1 levels are increased in the active EoE esophagus as compared with inactive EoE, that TGF β 1 significantly induces PAI-1 in esophageal epithelial cells, and that pharmacologic inhibition of PAI-1 decreases TGF β 1-induced gene expression. Our discovery that PAI-1 is part of a fibrotic network induced by TGF β 1 indicates that PAI-1 could be a new target in the therapy of esophageal remodeling.

Methods

EoE subjects/biopsies

We utilized our UCSD/RCHSD EoE database to select a cohort of EoE subjects who had active and inactive EoE defined as ≥ 15 eosinophils and < 15 eosinophils per hpf, respectively. Inactive EoE was a consequence of standard of care EoE directed therapy of topical fluticasone or budesonide. Control subjects had no eosinophils in the esophagus and no endoscopic abnormalities. Archived biopsies were used for immunohistochemistry (IHC) and immunofluorescence (IF) and frozen biopsies were used for qPCR and were collected in RNAlater and kept at -80°C until use. Plasma samples were obtained at the time of EGD with biopsy. Written informed consent/assent was obtained for all samples under UCSD/RCHSD IRB approved protocol 091485. Procurement and use of epithelial cells from deceased organ donors was not considered human subjects research and approved under UCSD IRB protocol 130835. Clinical features of subjects whose biopsies were used in immunohistology experiments are shown Table E1.

Immunostaining and histologic assessment

H&E stained, formalin fixed, paraffin embedded specimens were scored by a single pathologist blinded to the diagnosis and treatment (R.N.) using our previously published standardized histology scoring tool (30). The numbers of epithelial and lamina propria (LP) eosinophils, the severity of basal zone hyperplasia (BZH), and the LP fibrosis score were quantified (30). Fibrosis severity was scored 0-3 based on the collagen bundle thickness, collagen accumulation, and number of fibroblasts. BZH was graded on the percent of the epithelial height that was comprised of basal cells from 0-3 (30). Subjects whose biopsies were studied were chosen from the database using a random number generator and used if there was well oriented epithelium and the presence of adequate LP for analysis (active EoE subjects).

Tissue sections (5 μ M) were deparaffanized and hydrated prior to immunostaining as previously described (5). Following antigen retrieval, slides were incubated with anti-PAI-1 (1:200 AbCam ab66705, Cambridge, MA) alone or in combination with anti-Ki67 (1:100, DAKO, Carpinteria, CA) or anti-cytokeratin 14 (1:400, AbCam ab7800, Cambridge MA). Samples were processed for IF or IHC using the appropriate species-specific secondary antibodies as previously described (5). Isotype control antibodies were used to ensure staining specificity. Epithelial PAI-1 was quantified using color detection and image analysis with Image J software for the height of positively staining cells relative to the total epithelial height (the same index utilized for calculating BZH). All images were quantified and analyzed under identical light or fluorescence microscopic conditions, including magnification, gain, camera position, and background illumination.

Cell culture and treatment

Human organ transplant donor esophagi provided by the National Disease Research Interchange and the Arkansas Regional Organ Recovery Agency were processed for epithelial cells using collagenase D to digest isolated mucosa and cultured initially in DMEM/F12 with 10% fetal bovine serum and later in complete EpiCM2 media (ScienCell, Carlsbad CA). Cells were placed in growth factor reduced media overnight and stimulated with recombinant human TGF β 1 (10ng/ml, R&D, Minneapolis MN) in basal media for 48 hours prior to collection of RNA, protein, and supernatants. EoE fibroblasts were isolated using collagenase I, cultured in SMCM (ScienCell, Carlsbad CA) pre-treated with 75 μ M TM5275 for 2 hours followed by 24 hours of TGF β 1 (5ng/ml, R&D, Minneapolis MN).

Quantitative PCR

RNA was isolated from human esophageal epithelial cells or frozen EoE/control biopsy specimens stored in RNAlater, converted to cDNA using the manufacturer's instructions, and subjected to quantitative real time (RT qPCR) using SYBR green and normalized to the

housekeeping gene RPL13A. All dissociation curves were single peak. Primer sequences are listed in supplemental Table E21.

ELISAs and Immunoblots

ELISAs for PAI-1, TGF β 1, and PF4 were completed using the manufacturer's instructions (DY1786, DY240, and DY795, R&D, Minneapolis MN) and cell supernatants or plasma samples. Plasma was collected in citrate plasma separator tubes and platelets were depleted using two centrifugation spins at 1800xg for 30 and 10 minutes. PAI-1 and TGF β 1 plasma levels were normalized for platelet activation using PF4 ELISA. For immunoblotting, cells were harvested on ice using RIPA lysis buffer (Teknova, Hollister, CA) with phosphatase and protease inhibitors (Roche Applied Science, Indianapolis, IN). Equivalent amounts of total protein was loaded into each well and electrophoresed on NuPAGE 4-12% Bis-Tris gels (Life Technologies, Grand Island, NY). Proteins were transferred to 0.2 μ m pore nitrocellulose membrane (Life Technologies, Grand Island, NY), blocked with 5% dry milk, incubated overnight with PAI-1 or Gap antibody and probed with the appropriate species specific HRP-linked secondary antibody. Immunoblots were developed with Immuno-Star WesternC Kit (BioRad, Hercules, CA), imaged, and analyzed using the GBOX XRQ and its accompanying software (Syngene, Frederick, MD).

Statistical analysis

All statistical analyses and graphing were done using GraphPad Prism (San Diego, CA). Comparisons between two groups with Gaussian distribution were done using a student's t-test for unpaired variables; non-Gaussian data/non-continuous variables were compared using a Mann-Whitney test. Pre-post comparisons were done using a t-test for paired variables for Gaussian data/continuous variables or Wilcoxon's test for non-parametric paired data. Spearman's coefficient was calculated for correlations. A two tailed p value <0.05 was considered statistically significant.

Results

PAI-1 expression in the esophagus of EoE subjects

We utilized 14 paired archived biopsies to analyze PAI-1 expression in the active versus inactive state. Twenty-seven additional active EoE biopsies and 11 control biopsies were used for comparison of active to non-diseased state. In the total group, the subjects had a mean age of 7 years (range 1-17 years), 83% were male, and 40% of subjects failed PPI monotherapy. Forty-eight percent had self-reported asthma, 69% had allergic rhinitis and 48% had food allergies. Sixty-three and 73% of subjects had IgE testing that was positive to foods and aeroallergens, respectively. Among the paired treatment group, 13 were treated with swallowed topical swallowed budesonide and 1 was treated with topical fluticasone.

As compared with non-diseased control subjects, patients with active EoE had significantly more PAI-1 expression in the epithelium (79% versus 14% of epithelial height) (Figure 1a,b,d). In order to understand if esophageal PAI-1 expression was decreased following EoE-directed therapy, we utilized paired, well oriented biopsy specimens. Following therapy, PAI-1 expression was significantly decreased to 24% of the epithelial height ($p < 0.001$) (Figure 1b,c,e). Since epithelial height can vary between biopsies, we utilized a second methodology to confirm that PAI-1 expression was higher in active as compared with inactive or control biopsies. We assessed the numbers of highly positive PAI-1 epithelial cells per high power field and found that this method also demonstrated that active EoE biopsies had more PAI-1 than control or inactive biopsies (see Supplemental Material and Figure E1).

PAI-1 expression in the EoE esophagus and association with epithelial features

To assess if actively proliferating epithelial cells, expressed PAI-1, we performed double immunofluorescence confocal imaging studies using the basal zone marker, cytokeratin 14, and the proliferation marker, Ki67 (Figure 2a,b). Quantification of double positive cells demonstrated that both cytokeratin 14 positive basal cells and actively proliferating cells expressed PAI-1

(Figure 2c,d). Quantification demonstrated significantly more Ki67/PAI-1 and cytokeratin 14/PAI-1 double positive cells in the basal than the non-basal zone in the active disease state (Figure 2c,d) as well as more PAI-1/Ki67 double positive cells in the basal zone of active versus inactive subjects (Figure 2d).

We used our standardized histologic scoring tool in order to assess the relationship between the degree of PAI-1 expression and the severity of BZH. BZH is scored as 0, 1, 2, or 3 when <20%, 21-50%, 51-75%, or >75% of the epithelial height is encompassed by basal cells, respectively. The degree of PAI-1 expression correlated positively with the degree of basal zone hyperplasia on histologic scoring ($p<0.001$), consistent with the fact that PAI-1 was expressed in these cells (Figure 2e).

In order to understand if PAI-1 gene expression associated with the expression of epithelial proliferation markers, we ran a panel of epithelial genes including the proliferation marker, Ki67, and cytokeratin 5. These analyses demonstrated that gene expression of PAI-1 correlated positively with the expression levels of Ki67 and cytokeratin 5 (Figure 2f,g). Together these analyses show that proliferating cells express PAI-1 mRNA and protein.

PAI-1 expression relative to epithelial and fibrotic gene expression

Recent studies have demonstrated that mRNA panels can be used to gauge EoE disease activity (31). Indeed, these panels can be completed using formalin fixed paraffin embedded biopsy specimens, allowing post-biopsy analysis to occur even if a frozen/fresh sample was not procured specifically for RNA analysis at the time of EGD. We utilized frozen biopsy specimens from control (n=3), gastroesophageal reflux disease (n=1), active EoE (n=10), and inactive EoE (n=7) to determine if PAI-1 gene expression correlated with the expression of other pro-fibrotic genes and epithelial mesenchymal transformation genes that are induced by TGF β 1. Since TGF β 1 is a major regulator of EoE associated remodeling (5, 17, 32, 33), we hypothesized that PAI-1 would be elevated as part of a network of TGF β 1-induced

genes. Since PAI-1 causes fibrotic remodeling, we were curious to know if the degree of epithelial PAI-1 expression would correlate with the degree of subepithelial fibrosis. Using a histologic scoring system that we have previously published, we graded fibrosis from 0-3 based on the severity of collagen accumulation and collagen bundle thickness. PAI-1 expression level associated positively with subepithelial fibrosis score (Figure 3a**b**).

In terms of fibrotic gene expression, we found that the degree of PAI-1 gene expression corresponded with the expression of the fibrotic genes collagen I ($r=0.64$, $p<0.01$), MMP-2 ($r=0.63$, $p<0.01$), and MMP-14 ($r=0.74$, $p<0.001$) and the TGF β 1 induced marker of epithelial mesenchymal transformation vimentin (33) (Figure 3b-e). While we did not find that PAI-1 mRNA corresponded significantly with the gene expression of either of the inflammatory markers eotaxin-3 or CPA-3 (not shown), we did find that the numbers of tryptase positive cells and eosinophils correlated positively with the degree of PAI-1 expression by immunohistochemistry in the biopsies (Figure 3f, g).

TGF β 1 induces PAI-1 expression in human esophageal epithelial cells and EoE fibroblasts

In order to understand if epithelial PAI-1 expression and secretion are regulated by TGF β 1 in esophageal cells, we isolated primary human epithelial cells from organ transplant donors and treated them in culture with recombinant human TGF β 1 and assayed for PAI-1 gene expression by qPCR and protein expression and secretion using immunoblotting and ELISA. TGF β 1 induced PAI-1 gene expression 11-fold in primary human esophageal epithelial cells (Figure 4a). PAI-1 protein was induced 2-fold in esophageal epithelial cell extracts (Figure 4b,d) and there was an almost 6-fold increase in PAI-1 release into the supernatant following TGF β 1 treatment (Figure 4c).

Since TGF β 1 induced PAI-1 in human esophageal epithelial cells and PAI-1 was elevated in EoE, we determined whether PAI-1 and TGF β 1 expression correlated with one another. Immunohistochemistry for epithelial PAI-1 and LP TGF β in the same biopsy specimens

demonstrated that the esophageal expression of PAI-1 and TGF β 1 positively associated with one another (Figure 5a-c). In addition, TGF β 1 gene expression positively correlated with PAI-1 mRNA levels (Figure 5d).

Both PAI-1 and TGF β 1 have been reported to be detectable in the peripheral blood. We utilized stored, platelet depleted plasma samples from children with active and inactive EoE and from controls in order to explore if we could detect TGF β 1 or PAI-1 in the peripheral blood. Both PAI-1 and TGF β 1 were normalized to PF4 levels of both factors to control for platelet activation. Using 488 samples from 203 subjects, we found that, consistent with the esophageal data, the peripheral plasma levels of PAI-1 and TGF β 1 correlated with one another (Figure 5e) but the plasma levels of neither PAI-1 nor TGF β 1 correlated with esophageal disease activity or severity.

TGF β 1 induced PAI-1 is involved in TGF β 1-induced gene expression

To understand if induced PAI-1 was in its active or inactive conformation we treated EoE fibroblasts with TGF β 1 and assayed for PAI-1 activity. EoE fibroblasts released active PAI-1 at baseline and this was augmented in the presence of TGF β 1 (data not shown). We utilized the specific PAI-1 inhibitor TM5275 to assess if PAI-1 was involved in fibrotic gene expression. Treatment with TM5275 significantly decreased the baseline gene expression of collagen I and periostin in EoE fibroblasts (Figure 6a). In addition, PAI-1 inhibitor treatment significantly decreased TGF β 1-induced expression of α -smooth muscle actin (α SMA) and phospholamban (Figure 6b). It has been reported that PAI-1 can alter cellular function independently of its enzymatic activity via the low density lipoprotein receptor-related protein 1 (LRP1)/ α -2 macroglobulin receptor 2 (35-37). Using immunocytochemistry we found that LRP1 was expressed in the LP of active EoE biopsies and on esophageal fibroblasts (Figure 6c,d), supporting the ability of PAI-1 to mediate pro-fibrotic effects either dependently or independently of its enzymatic activity in EoE.

Discussion

In this manuscript we document a number of novel findings regarding the TGF β 1-induced serine protease inhibitor, PAI-1, in pediatric EoE. We show that PAI-1 is significantly elevated in the active EoE esophagus as compared with non-diseased esophagi and that EoE-directed therapy reduces the degree of PAI-1 expression. We further demonstrate that TGF β 1 increases PAI-1 in primary human esophageal epithelial cells and EoE fibroblasts. The levels of PAI-1 correlate with histologic features of epithelial and subepithelial esophageal remodeling and fibrotic gene expression. The induction of a subset of genes by TGF β 1 was significantly decreased by pharmacologic PAI-1 inhibition and genes involved in myofibroblast formation such as α SMA were decreased by PAI-1 inhibition even in the absence of TGF β 1. Together these data suggest that PAI-1 is part of a pathogenic fibrotic network that can be initiated by TGF β 1.

To our knowledge, this is the first report of the potential role of PAI-1 in EoE. In asthma, PAI-1's induction by allergen challenge and its role in fibrosis and airway remodeling have been studied for a number of years. In sensitized mice and humans, ovalbumin and house dust mite challenge, respectively, result in elevated pulmonary PAI-1 levels. Animals that are deficient in PAI-1 due to gene deletion or treatment with the PAI-1 inhibitor, tixplatinin, are protected from fibrin deposition and airway fibrosis and a murine model of TGF β 1-induced pulmonary fibrosis shows decreased myofibroblast formation and collagen deposition in the airways following treatment with oral TM5275 (23, 24, 38). In addition, rats treated with small interfering PAI-1 RNA have protection from bleomycin induced lung fibrosis and depletion of PAI-1 can improve hepatic fibrosis (39, 40). Our data suggests that PAI-1 is involved in EoE pathogenesis that also includes fibrosis. Since pharmacologic inhibition of PAI-1 decreased the baseline expression of the fibrosis genes collagen I and periostin, it is possible the use of PAI-1 blockade could be of utility in fibrotic EoE. Consistent with its role in TGF β 1-induced pulmonary fibrosis, PAI-1 was

part of the pathway for increases in α SMA expression. The ability of TM2575 to reduce TGF β 1-induction of both α SMA and phospholamban may reflect the role of PAI-1 in myofibroblast formation (38). Since LRP1 is expressed in the EoE esophagus, PAI-1 may act independently of its enzymatic activity to promote esophageal fibrosis (35-37).

While all esophageal biopsies contain adequate epithelial tissue for analysis, at our center, 50% and 15% of biopsies obtained during routine clinical care have LP and muscularis mucosa, respectively. For this reason, despite the hypothesis that substantial esophageal remodeling occurs beneath the superficial surface, this tissue is not always available for analysis of fibrotic severity. Currently, other than invasive procedures that can gauge transmural esophageal rigidity in adults, there is no manner in which to sample esophageal rigidity. Indeed, even the relationship between histologic fibrosis and progression to strictures is not yet clear, although a logical assumption is that increasing esophageal fibrosis is one of the main underlying mechanisms for esophageal rigidity and dysfunction. Since LP is not always available for assessment, an epithelial marker that accurately reflects the severity of LP fibrosis could be of substantial utility for evaluating biopsies. Since it is possible that LP fibrosis is the closest gauge to esophageal compliance that we have currently available in children, and since epithelium is always present in the biopsy specimens, an epithelial marker of fibrosis could potentially alter clinical interventional strategies. For example, in subjects with less, absent, or minimal fibrosis, less intervention may be required to prevent remodeling, strictures, and food impactions while in patients with high levels of fibrosis, more aggressive therapy may be warranted. In this context, epithelial PAI-1 staining, which is straightforward and easily performed, could potentially serve as an epithelial marker of fibrotic severity, at least in children, and may have the potential to change EoE management.

We have previously reported that MMP-14 is also increased in the epithelium of pediatric EoE subjects (16). Like PAI-1, MMP-14 expression and release is increased in epithelial cells by

TGF β 1 (16). Indeed, PAI-1 gene expression correlates positively with MMP-14 gene expression as well as with the expression of a number of other remodeling genes including collagen I, MMP-2, Ki67, vimentin, and TGF β 1. In addition PAI-1 levels correlated with the proliferation and epithelial genes Ki67 and cytokeratin 5. The fact that PAI-1 expression correlates with basal zone hyperplasia and fibrosis scores, both of which are histologic markers of remodeling, suggests that it is indeed a good epithelial marker of esophageal remodeling.

Unlike the correlation of PAI-1 with other remodeling genes, its mRNA level did not correspond to gene expression of inflammatory markers such as eotaxin-3 and CPA-3. Given that we see PAI-1 expression largely in structural epithelial cells in EoE, it is possible that, in chronic EoE, PAI-1 may be part of a pro-fibrotic network that can function independently or in the presence of lower levels of inflammation. This is potentially important in the context of the inflammatory burn out and progressive fibrosis that is seen in EoE adults with long disease durations (7). In murine asthma, PAI-1 deficiency substantially reduces fibrosis but does not robustly decrease inflammation, consistent with the concept that PAI-1 has functions that are independent of inflammation (24). These observations have therapeutic implications in EoE since there are no agents that specifically target fibrosis and interventional strategies can have variable effects on remodeling. It is possible that the use of agents such as inhibitors of PAI-1 may be of utility in subjects with fibrotic EoE. In addition, since PAI-1 can be made by mast cells where it is induced by chymase-activated TGF β 1 (22), it will be of interest to see what role inflammation does play in PAI-1 expression in EoE.

PAI-1 can be detected in the peripheral blood of asthmatic subjects and can associate with asthma severity (21). In order to understand if we could detect PAI-1 in the periphery of EoE subjects, we utilized stored plasma samples. Since PAI-1 is released by platelets, it is important to control for the presence and degranulation of platelets. We found that plasma PAI-1

associated positively with peripheral TGF β 1 levels, supporting the concept that TGF β 1 is integral to PAI-1 expression.

In conclusion, we have demonstrated a novel potential role of PAI-1 in EoE as an epithelial marker of esophageal remodeling that is induced by TGF β 1. Since PAI-1 inhibition decreased the effects of TGF β 1 on gene expression, it is possible that PAI-1 could be a new therapeutic target in EoE. It will be of interest to validate and extend our findings in a larger EoE population in order to better understand the role of PAI-1 in EoE pathogenesis.

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Figure Legends

Figure 1. PAI-1 is expressed in the EoE esophageal epithelium. Representative image of PAI-1 immunohistochemistry on esophageal biopsy specimens from a non-diseased control (A), active EoE (B), and inactive EoE (C). Epithelial staining was quantified as a percent of the total epithelial height in control and active EoE (D) and in paired inactive and active EoE biopsies (E). Lines represent means.

Figure 2. Actively proliferating cells express PAI-1. Representative images of double immunofluorescence for PAI-1 (green, A, B) and cytokeratin 14 (Krt14) (red, A) or nuclear Ki67 (red, B) shows that PAI-1 co-localizes with cytokeratin 14 (yellow, A) and Ki67 positive cells (green cytoplasm, red nucleus, B). Quantification of cells that are KRT14-PAI-1 (C) and Ki67-PAI-1 (D) double positive in the basal (BZ) and non-basal (NBZ) zones of a subset of biopsies. Epithelial PAI-1 expression correlates with the severity of basal zone hyperplasia (E). PAI-1 mRNA expression correlates with Ki67 (F) and cytokeratin 5 (Krt5) (G) in esophageal biopsies from active, inactive, and control subjects.

Figure 3. PAI-1 expression associates with fibrosis and remodeling gene expression. Epithelial expression of PAI-1 correlates with the severity of LP fibrosis (A). PAI-1 mRNA expression correlates with mRNA expression of the pro-fibrotic genes collagen I (B), MMP-14 (C), and MMP-2 (D) and the epithelial mesenchymal transformation marker vimentin (VIM) (E). PAI-1 expression correlates with the numbers of tryptase positive cells (F) and eosinophils (G).

Figure 4. TGF β 1 induces PAI-1 expression in epithelial esophageal cells. Treatment of primary human esophageal epithelial cells with recombinant human TGF β 1 significantly induces PAI-1

gene expression (A), increases intracellular PAI-1 protein expression (B, D), and induces PAI-1 release into the supernatant (C).

Figure 5. PAI-1 expression associates with the degree of TGF β 1 expression. Representative images of epithelial PAI-1 expression (left) and LP TGF β 1 positive cells (right) in control (A) and EoE (B). Epithelial PAI-1 expression correlates with the numbers LP TGF β 1 positive cells (C). mRNA expression of PAI-1 correlates with TGF β 1 in esophageal biopsies (D). Plasma PAI-1 levels correlate with plasma TGF β 1 (E).

Figure 6. PAI-1 inhibition decreases gene expression in EoE fibroblasts. Treatment with the PAI-1 inhibitor TM5275 decreases baseline (A) and TGF β 1-induced (B) expression of pro-fibrotic and myofibroblast genes. Active EoE biopsy (C) and primary human esophageal fibroblasts (D) shows presence of low density lipoprotein like receptor-1.

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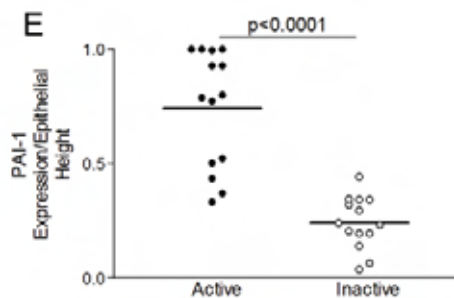
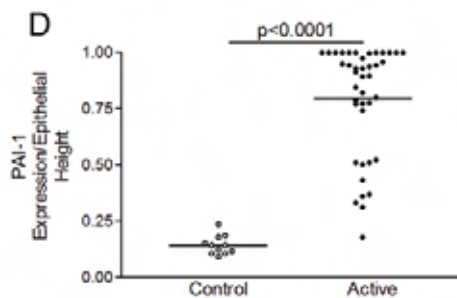
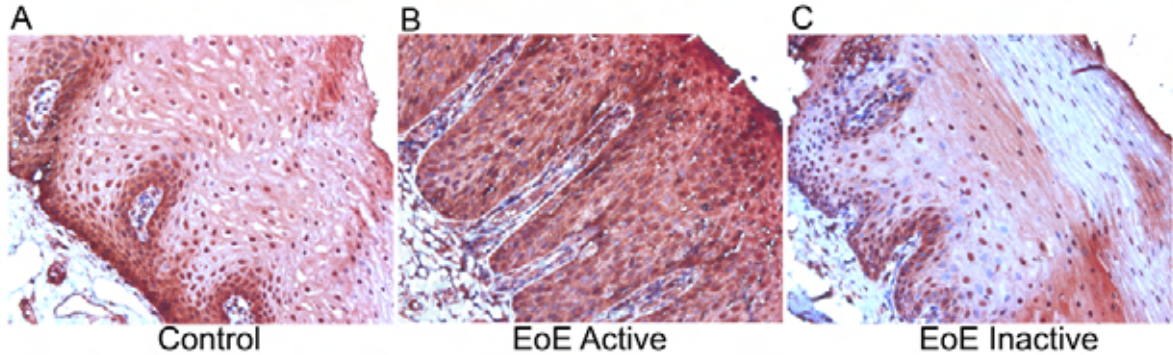


Figure 1

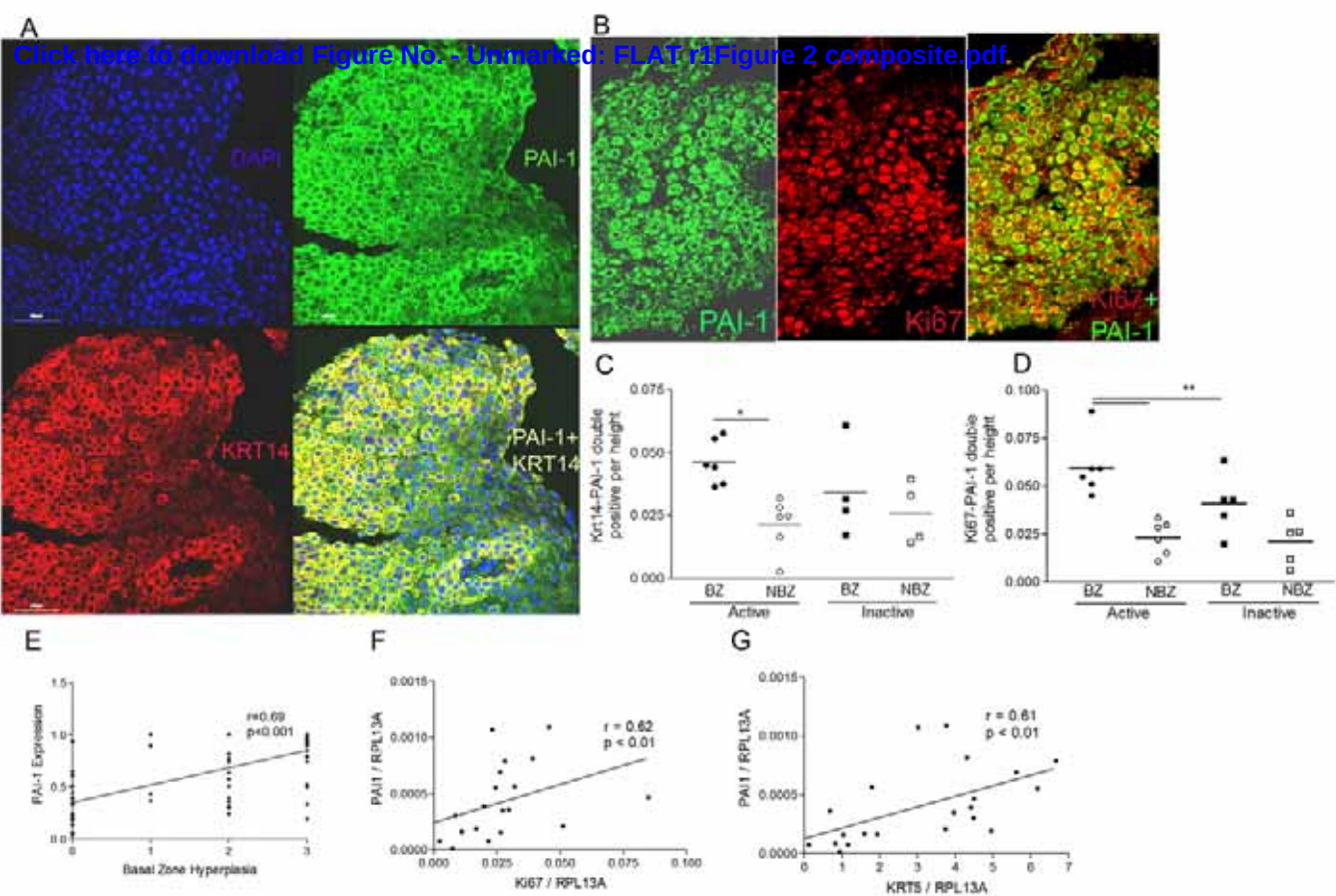


Figure 2

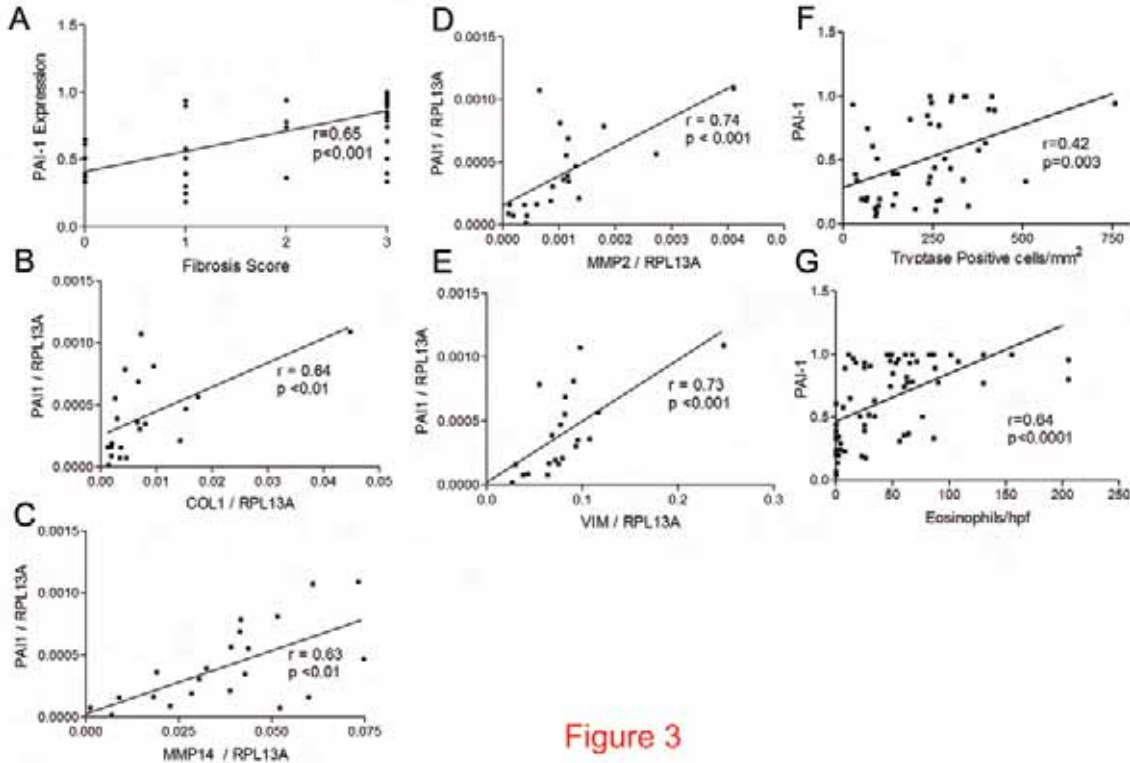


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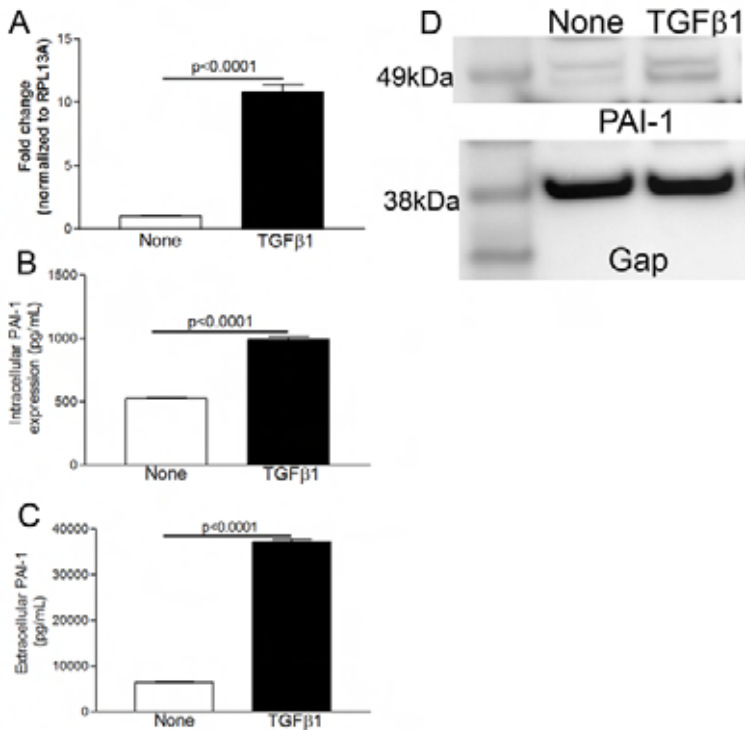


Figure 4

A

Control

PAI-1

TGF β 1

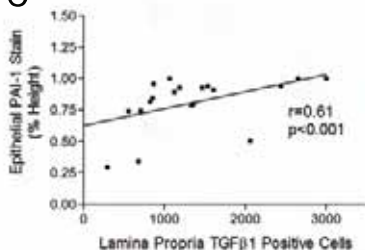
B

EoE

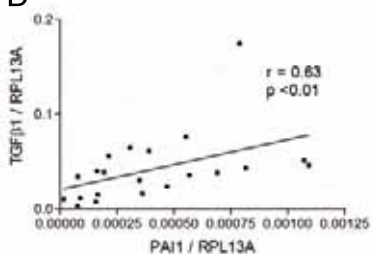
PAI-1

TGF β 1

C



D



E

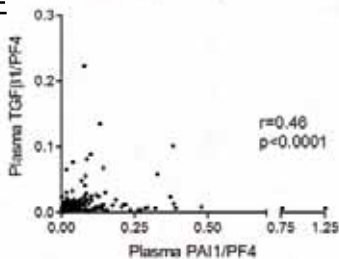


Figure 5

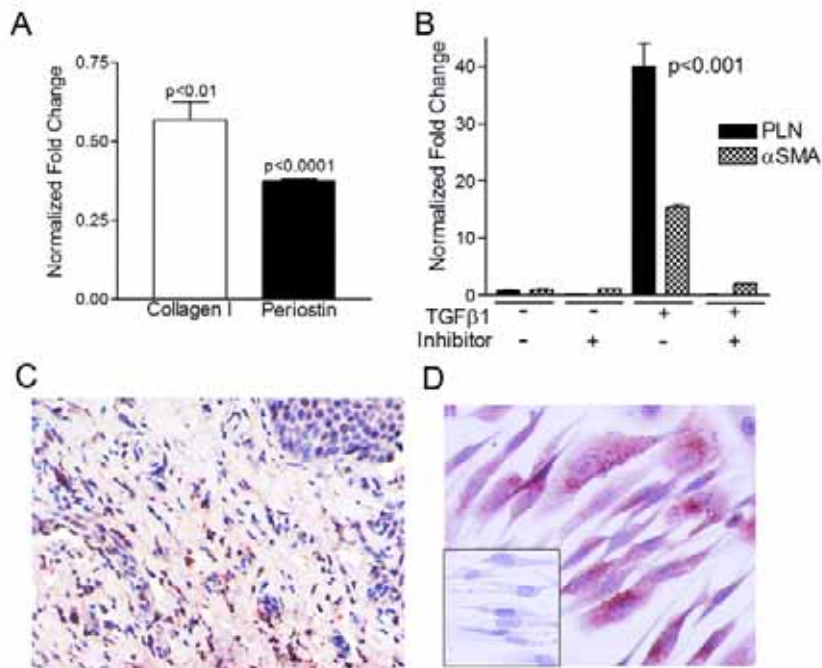


Figure 6

Rawson et al. Supplemental Material**Supplemental Methods**

A subset of biopsies were re-stained for PAI-1 and quantified using digital image analysis and the ImageJ plug-in (IHC Profileron) to identify the numbers of “high positive” cells. All images were quantified and analyzed under identical light or fluorescence microscopic conditions, including magnification, gain, camera position, and background illumination.

Electronic Figures

Figure E1. PAI-1 is increased in EoE. Quantification of the % high positive cells in control, active EoE (A) and active and inactive EoE (B) shows higher numbers of PAI-1 positive cells. Bars represent means.

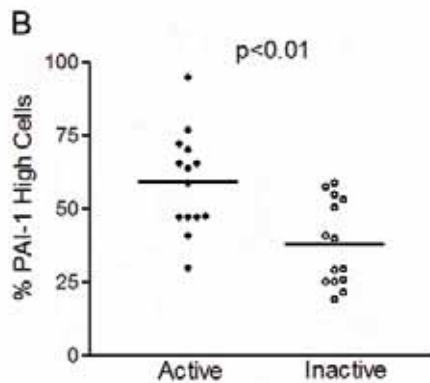
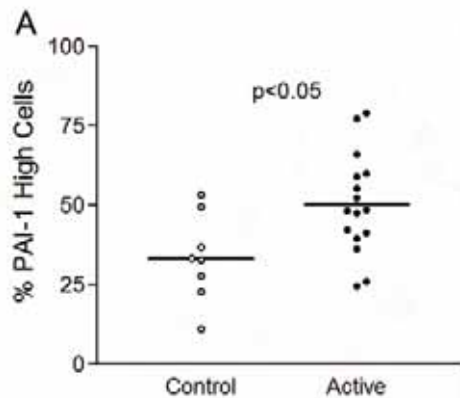


Figure E1

Table E1. Clinical Features

Subject	Age	Sex	Atopic Disease	Skin Test Positive	TCS	Eos/hpf (Pre/Post)
1	13	M	AR	No		48
2	4	M	AR, Eczema, Asthma, Food allergies	F, A		108
3	17	M	AR, Eczema, Asthma, Food allergies	F		50
4	3	M	Food allergies	F, A		87
5	3	M	AR, Asthma, Food allergies	NA		71
6	1	M	AR, Asthma, Food Allergies	F, A		55
7	2	M	AR, Eczema, Asthma, Food Allergies	F, A		155
8	5	M	AR, Asthma, Food Allergies	F		130
9	9	M	Food allergies	F		62
10	3	M	AR, Eczema	F, A		48
11	3	M	Eczema, Asthma, Food allergies	F, A		90
12	3	M	None	No		34
13	17	M	Asthma	F, A		50
14	2	M	NA	F, A		21
15	9	M	None	A		45
16	15	F	AR	A		130
17	3	M	NA	NA		17
18	5	M	None	NA		60
19	2	M	None	NA		84
20	17	M	NA	NA		26
21	7	M	AR	F, A		56
22	7	M	AR, Asthma, Food allergies	F, A		46
23	5	F	AR, Eczema, Food allergies	F, A		15
24	15	M	AR, Asthma, Food allergies	F, A		86
25	16	M	AR	F, A		30
26	7	M	AR, Asthma, Food allergies	F, A		67
27	2	M	AR, Eczema, Asthma, Food allergies	F		25
28	4	F	AR, Asthma, Food allergies	F	Budesonide	101/4
29	16	M	AR	A	Fluticasone	29/0
30	3	F	Asthma	NA	Budesonide	76/2
31	10	M	AR, Eczema, Asthma, Food allergies	A	Budesonide	25/8
32	7	F	NA	No	Budesonide	63/0
33	1	F	Eczema, Asthma, Food Allergies	F	Budesonide	61/2
34	2	F	None	F	Budesonide	64/0
35	2	M	AR, Food allergies	F, A	Budesonide	67/0
36	5	M	None	F, A	Budesonide	65/0
37	12	M	AR	F, A	Budesonide	82/0
38	4	M	Asthma	A	Budesonide	53/0
39	1	M	AR, Eczema, Food allergies	No	Budesonide	86/0
40	10	M	AR, Eczema	No	Budesonide	130/1
41	14	M	AR, Eczema, Asthma	F, A	Budesonide	205/0

*Relevant for paired analysis only

M: Male, AR: Allergic rhinitis, F: Food, A: Aeroallergen, hpf: high power field

Table E2

Gene	Primer Sequence
MMP-14	F-TTGTCTCCTGCTCCCCCTGCT
	R-CGCCTCATGGCCTTCATGGTGTCT
MMP-2	F- AGCTCTATGGGGCCTCTCCTGA
	R- TCACCACGGATCTGAGCGATGC
GAPDH	F- TGGTATCGTGGAA-GGGACTCAT
	R- ATGCCAGTGAGCTTCCCGTTC
RPL13A	F- AAACCTCCCCAGCTAAACACC
	R- GAACTTCAGAGAAGCATCACGATGATA
PAI-1	F- TGG TTC TGC CCA AGT TCT CCC TG
	R- TGC CAC TCT CGT TCA CCT CG
Fibronectin	F- TCA ACA GTG GGA GCG GAC CT
	R- TGG CAG CGG TTT GCG ATG GT
Collagen I	F- CAG CCG CTT CAC CTA CAG C
	R- TTT TGT ATT CAA TCA CTG TCT T
VIM:	F- GAG TCC ACT GAG TAC CGG AGA C
	R- TGT AGG TGG CAA TCT CAA TGT C
Ki67	F- CCA CAC TGT GTC GTC GTT TG
	R- CCG TGC GCT TAT CCA TTC A
KRT5	F- TGG AGG GCG AGG AAT GCA GAC TC
	R- ACT GCT ACC TCC GGC AAG ACC