TGF-β1–induced PAI-1 contributes to a profibrotic network in patients with eosinophilic esophagitis

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Background: Eosinophilic esophagitis (EoE) is an allergic disease of increasing worldwide incidence. Complications are due to tissue remodeling and involve TGF-β1–mediated fibrosis. Plasminogen activator inhibitor 1 (PAI-1/serpinE1) can be induced by TGF-β1, but its role in EoE is not known.
Objective: We sought to understand the expression and role of PAI-1 in patients with EoE.
Methods: We used esophageal biopsy specimens and plasma samples from control subjects and patients with EoE, primary human esophageal epithelial cells, and fibroblasts from patients with EoE in immunohistochemistry, quantitative PCR, and immunoassay experiments to understand the induction of PAI-1 by TGF-β1, the relationship between PAI-1 and esophageal fibrosis, and the role of PAI-1 in fibrotic gene expression.
Results: PAI-1 expression was significantly increased in epithelial cells of biopsy specimens from patients with active EoE compared with that seen in biopsy specimens from patients with inactive EoE or control subjects ($P < .001$). Treatment of primary esophageal epithelial cells with recombinant TGF-β1 increased PAI-1 transcription, intracellular protein expression, and secretion. Esophageal PAI-1 expression correlated with basal zone hyperplasia, fibrosis, and markers of esophageal remodeling, including vimentin, TGF-β1, collagen I, fibronectin, and matrix metalloproteases, and plasma PAI-1 levels correlated with plasma TGF-β1 levels. PAI-1 inhibition significantly decreased baseline and TGF-β1–induced fibrotic gene expression.
Conclusions: PAI-1 expression is significantly increased in the epithelium in patients with EoE and reflects fibrosis, and its inhibition decreases TGF-β1–induced gene expression. Epithelial PAI-1 might serve as a marker of EoE severity and form part of a TGF-β1–induced profibrotic network. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: *Eosinophil, esophagitis, SerpinE1, remodeling, fibrosis, TGF-β1*

Eosinophilic esophagitis (EoE) is a chronic antigen-driven allergic disease of increasing worldwide incidence and prevalence.[1] One mechanism for disease complication is fibrosis, which occurs largely in the subepithelium, begins early in life, and is progressive without intervention.[2-8] Esophageal remodeling in patients with EoE 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] Because 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, patients with EoE experience vomiting, failure to thrive, dysphagia, and food impactions and consistently progress to esophageal strictures.[1,3,4]

Studies from our laboratory and others have demonstrated that TGF-β1 can be a major regulator of childhood EoE remodeling.[5,15-20] A number of genes involved in changes in cellular function and trafficking are induced by TGF-β1, including periostin, which increases eosinophil trafficking,[18] and phospholamban, which induces TGF-β1–mediated esophageal smooth muscle cell contraction in culture.[17] Among the genes that are induced by TGF-β1 is the serine protease inhibitor plasminogen activator

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Abbreviations used
BZH: Basal zone hyperplasia
EoE: Eosinophilic esophagitis
LP: Lamina propria
LRP1: Lipoprotein receptor–related protein 1
MMP: Matrix metalloproteinases
PAI-1: Plasminogen activator inhibitor 1
PF4: Platelet factor 4
αSMA: α-Smooth muscle actin

inhibitor 1 (PAI-1/serpinE1), which promotes tissue fibrosis in asthmatic patients.[21-24] However, the role of PAI-1, its induction in patients with EoE, and its role in esophageal fibrosis have not been previously studied.

PAI-1 expression is increased in human subjects with asthma and promotes airway remodeling in murine asthma models.[21,23-28] PAI-1 overexpression, which is triggered by viruses and allergens, is associated with airway fibrosis, whereas PAI-1 deficiency protects mice from fibrotic airway remodeling.[21,23-26,29] Mast cell–derived TGF-β1 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 patients with EoE and its role in esophageal fibrosis is not known.

On the basis of these findings and our prior work in EoE that shows that TGF-β1 promotes EoE-associated remodeling, we explored the role of PAI-1 in patients with EoE. Because 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 esophagus in patients with EoE compared with those in patients with inactive EoE, that TGF-β1 significantly induces PAI-1 in esophageal epithelial cells, and that pharmacologic inhibition of PAI-1 decreases TGF-β1–induced gene expression. Our discovery that PAI-1 is part of a fibrotic network induced by TGF-β1 indicates that PAI-1 could be a new target in the therapy of esophageal remodeling.

METHODS

Patients with EoE and biopsy specimens

We used our University of California, San Diego and Rady Children's Hospital–San Diego (UCSD/RCHSD) EoE database to select a cohort of patients with active and inactive EoE, which were defined as 15 or more eosinophils and less than 15 eosinophils per high-power field, 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 biopsy specimens were used for immunohistochemistry and immunofluorescence, and frozen biopsy specimens were used for quantitative PCR, collected in RNAlater, and kept at −80°C until use. Plasma samples were obtained at the time of esophagogastroduodenoscopy with biopsy. Written informed consent/assent was obtained for all samples under a UCSD/RCHSD institutional review board–approved protocol 091485. Procurement and use of epithelial cells from deceased organ donors was not considered human subjects research and was approved under UCSD institutional review board protocol 130835. Clinical features of subjects whose biopsy specimens were used in immunohistology experiments are shown Table E1 in this article's Online Repository at www.jacionline.org.

Immunostaining and histologic assessment

Hematoxylin and eosin–stained, formalin-fixed, paraffin-embedded specimens were scored by a single pathologist blind to the diagnosis and treatment (RN) by using our previously published standardized histology scoring tool.[30] The numbers of epithelial and LP eosinophils, the severity of BZH, and the LP fibrosis score were quantified.[30] Fibrosis severity was scored as 0 to 3 based on the collagen bundle thickness, collagen accumulation, and number of fibroblasts. BZH was graded on the percentage of epithelial height comprised of basal cells from 0 to 3.[30] Subjects whose biopsy specimens were studied were chosen from the database by using a random-number generator and used if there was well-oriented epithelium and the presence of adequate LP for analysis (patients with active EoE).

Tissue sections (5 microns) were deparaffinized and hydrated before immunostaining, as previously described.[5] After antigen retrieval, slides were incubated with anti–PAI-1 (1:200 ab66705; Abcam, Cambridge, Mass) alone or in combination with anti-Ki67 (1:100; DAKO, Carpinteria, Calif) or anti–cytokeratin 14 (1:400, ab7800; Abcam). Samples were processed for immunofluorescence or immunohistochemistry by using appropriate species-specific secondary antibodies, as previously described.[5] Isotype control antibodies were used to ensure staining specificity. Epithelial PAI-1 expression was quantified by using color detection and image analysis with ImageJ software (National Institutes of Health, Bethesda, Md) for the height of positively staining cells relative to total epithelial height (the same index used 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 by using collagenase D to digest isolated mucosa and cultured initially in Dulbecco modified Eagle medium/F12 with 10% FBS and later in complete EpiCM2 medium (ScienCell, Carlsbad, Calif). Cells were placed in growth factor reduced medium overnight and stimulated with recombinant human TGF-β1 (10 ng/mL; R&D Systems, Minneapolis, Minn) in basal medium for 48 hours before collection of RNA, protein, and supernatants. Fibroblasts from patients with EoE were isolated with collagenase I and cultured in SMCM (ScienCell) pretreated with 75 μmol/L TM5275 for 2 hours, followed by 24 hours of TGF-β1 (5 ng/mL, R&D Systems).

Quantitative PCR

RNA was isolated from human esophageal epithelial cells or frozen biopsy specimens from patients with EoE/control subjects stored in RNAlater, converted to cDNA by using the manufacturer's instructions, subjected to quantitative real-time PCR with SYBR Green, and normalized to the housekeeping gene ribosomal protein L13a *(RPL13A)*. All dissociation curves were a single peak. Primer sequences are listed in Table E2 in this article's Online Repository at www.jacionline.org.

ELISAs and immunoblots

ELISAs for PAI-1, TGF-β1, and platelet factor 4 (PF4) were completed according to the manufacturer's instructions (DY1786, DY240, and DY795; R&D Systems) and cell supernatants or plasma samples. Plasma was collected in citrate plasma separator tubes, and platelets were depleted by means of 2 centrifugation spins at 1800*g* for 30 and 10 minutes, respectively. PAI-1 and TGF-β1 plasma levels were normalized for platelet activation by using PF4 ELISA. For immunoblotting, cells were harvested on ice with RIPA lysis buffer (Teknova, Hollister, Calif) with phosphatase and protease inhibitors (Roche Applied Science, Indianapolis, Ind). Equivalent amounts of total protein were loaded into each well and electrophoresed on NuPAGE 4-12% Bis-Tris gels (Life Technologies, Grand Island, NY). Proteins were transferred to 0.2-μm-pore nitrocellulose membrane (Life Technologies), blocked with 5% dry milk, incubated overnight with PAI-1 or Gap antibody, and probed with the appropriate species-specific horseradish peroxidase–linked