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MMPs-2 and -14 are Elevated in Eosinophilic Esophagitis and Reduced Following Topical Corticosteroid Therapy

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Abstract

Background—Eosinophilic esophagitis (EoE) is a chronic antigen mediated disease in children and adults associated with substantial esophageal remodeling and fibrosis. The expression of the remodeling-associated matrix metalloproteinases (MMPs) has not been previously detailed in EoE.

Methods—MMP-2 and -14 expression and cellular localization was assessed using qPCR and immunohistochemistry/immunofluorescence in EoE fibroblasts, active and inactive pediatric EoE biopsies, and non-diseased control biopsies. The effect of TGFβ1 treatment on MMP-2 expression in cultured esophageal epithelial (HET1A) cells was analyzed.

Results—MMP-2 and -14 mRNA were expressed in EoE fibroblasts and biopsies. Proliferating epithelial cells produced MMP-14 more abundantly in EoE than in controls ($p < 0.001$) and the degree of epithelial MMP-14 expression correlated positively with basal zone hyperplasia ($r = 0.65$, $p = 0.002$). EoE lamina propria had higher numbers of MMP-2 and -14 positive cells (906 ± 167 and 701 ± 93 cells/mm²) as compared with controls (258 ± 93 $p < 0.01$ and 232 ± 54 cells/mm² $p < 0.01$) and MMP-14 expression correlated with the severity of fibrosis. Following therapy with topical corticosteroids, MMP-14 and MMP-2 were significantly diminished ($p < 0.01$). TGFβ1 increased the expression and secretion of MMP-2 from esophageal epithelial HET1A cells.

Conclusions—MMP-2 and -14 are elevated in pediatric EoE subjects and significantly decrease following topical corticosteroid therapy. TGFβ1 increases MMP-2 in esophageal epithelial cells. This alludes to previously unappreciated role for MMPs in EoE associated esophageal remodeling and a potential positive feedback loop via TGFβ1.

Keywords

Eosinophilic esophagitis; Matrix metalloproteinase; Remodeling; Transforming Growth Factor-β1; Fibrosis

Introduction

Eosinophilic esophagitis (EoE) is a chronic, antigen driven allergic disease that is associated with substantial tissue remodeling.¹⁻⁵ Esophageal remodeling is the mechanism for EoE complications including fibrosis and smooth muscle dysfunction which ultimately cause esophageal rigidity, strictures, and dysmotility.^{1, 3, 6-10} Tissue remodeling involves collagen accumulation and leads to the entrapment of cells and growth factors in a fibrotic milieu. Angiogenesis and vascular activation provide increased numbers of blood vessels for inflammatory cell trafficking into the esophagus.^{3, 11}

Matrix metalloproteinases (MMPs) are a family of proteins that are integral to collagen breakdown, angiogenesis, and tissue remodeling. However, MMPs have not been previously studied systematically in EoE. MMPs function as gelatinases and collagenases that remodel extracellular matrix and activate other MMPs and growth factors such as TGF β 1.^{12, 13} TGF β 1 is over-expressed in EoE and has direct effects on esophageal fibroblasts, smooth muscle cells, and epithelium, causing increased fibrotic gene expression, smooth muscle cell contraction and epithelial mesenchymal transformation.^{3, 6, 7, 9, 14} In particular, MMP-14 and MMP-2 can act in sequence to activate one another and cause subsequent release of TGF β 1 from its latency complex.¹² Currently, a single preliminary report describes MMP-14 mRNA in EoE.¹⁵ In asthma, some of TGF β 1's pro-fibrotic effects can be mediated through MMP-2.¹⁶

In this study, we aimed to delineate the expression pattern and potential roles of MMP-2 and -14 in pediatric EoE. We report increased esophageal levels of MMP-2 and -14 in pediatric EoE and demonstrate that proliferating epithelial cells and EoE fibroblasts are cellular sources of both MMP-2 and -14. Cultured esophageal epithelial cells treated with TGF β 1 increased the production and release of MMP-2. As such, MMPs appear to play a previously unreported role in EoE.

Methods

EoE subjects/biopsies

The UCSD/RCHSD EoE database was screened to randomly select a cohort of EoE subjects who met the following criteria: 1) EoE defined as histologic criteria (≥ 15 eosinophils per high power field (hpf) on hematoxylin/eosin (H&E) stain at 400x magnification on light microscopy); 2) the presence of typical symptom and endoscopic features; 3) successful treatment (defined as <15 eosinophils per hpf; subjects also had symptomatic and endoscopic improvement following therapy) with topical esophageal corticosteroids; 4) adequate and well oriented epithelial tissue in paired archived paraffin specimens blocks; 5) adequate LP for analysis (defined as at least 3 hpf) in pre- and post-therapy specimens in the same subject. A total of 18 subjects were analyzed and 78% failed PPI monotherapy. Of those not treated with PPI, 2 (of 3) were diagnosed prior to the consensus recommendations. An additional 10 EoE biopsies were utilized for qPCR studies and/or EoE esophageal fibroblast isolation. Subject characteristics are listed in supplemental Table 1. Controls had normal appearing esophagi with no eosinophilia. All studies were approved under UCSD/

RCHSD IRB number 091485. Procurement of cells from organ donors was not considered human subjects research and approved under UCSD IRB protocol 130835.

Immunostaining and histologic assessment

H&E stained, formalin fixed, paraffin embedded specimens were evaluated by a single pathologist blinded to the diagnosis and treatment (RN). The numbers of epithelial and lamina propria (LP) eosinophils, the severity of basal zone hyperplasia, and the LP fibrosis score were quantified using our previously published pathology scoring tool.¹⁷ Specifically, fibrosis severity was scored 0-3 by a single pathologist (RN) based on the collagen bundle thickness, collagen accumulation, and number of fibroblasts.

Tissue sections (5µM) were deparaffinized and hydrated prior to immunostaining as previously described.³ Primary antibodies used are listed in supplemental Table 2. Samples were processed for fluorescence or immunohistochemistry using the appropriate species-specific secondary antibodies as previously described.³ The mean of the peak numbers of MMP-2 or -14 positive cells from 3-5 hpf were quantified and calculated as cells per mm² using ImagePro (ImagePro, Media Cybernetics, Bethesda MD). MMP-14 was quantified in the epithelium using color detection and image analysis for the height of positively staining cells relative to the total epithelial height which is the same index utilized for calculating basal zone hyperplasia (BZH) (BZH is calculated as the height of proliferating epithelial cells to the total epithelial height). All images were quantified and analyzed under identical light or fluorescence microscopic conditions, including magnification, gain, camera position, and background illumination.

Cell culture

EoE fibroblasts—fibroblasts were isolated from pediatric EoE esophageal biopsies by dispersion with collagenase VIII (Sigma-Aldrich, St. Louis MO) and cultured in smooth muscle cell media (ScienCell, Carlsbad CA). Fibroblast phenotype was confirmed by the production of collagen I, fibronectin, and appropriate morphology. *Esophageal longitudinal smooth muscle cells*: Human esophagi were obtained from the Arkansas Regional Organ Procurement Agency and from the National Disease Research Interchange from organ transplant donors. Longitudinal smooth muscle bundles were dissected and isolated smooth muscle cells were cultured in smooth muscle cell media (ScienCell, Carlsbad CA). *Epithelial cells*: HET1A cells were purchased from ATCC and cultured in EpiCM2 media (ScienCell, Carlsbad CA).

Quantitative PCR

RNA was isolated from EoE fibroblasts or frozen EoE/control biopsy specimens stored in RNA later, 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 GAPDH or RPL13A. All dissociation curves were single peak. Primer sequences are listed in supplemental Table 2.

MMP-2 Assay

MMP2 levels were measured in cultured esophageal cells using the Biotrak MMP2 Activity Assay (GE Healthcare Life Sciences, Pittsburgh, PA). Cells were incubated in serum free media overnight followed by treatment with recombinant human TGF β 1 (10ng/ml, R&D) for 72 hours. Supernatants were collected and cells were washed, lysed, and subjected to analysis according to the manufacturer's instructions.

Statistical analysis

All statistical analyses and graphing were done using GraphPad Prism (San Diego, CA). Comparisons between two groups were done using a student's t-test for unpaired variables. Pre-post comparisons were done using a t-test for paired variables. A two tailed p value <0.05 was considered significant.

Results

Subject Characteristics

EoE subject characteristics are listed in supplemental Table 1. Among the subjects whose samples were used, 22 (73%) patients were male, mean age was 7.3 years, all were atopic, defined as positive IgE to aeroallergens or foods on skin or serum testing, and 97% had typical endoscopic features with mean peak eosinophil counts of 86 per hpf.

MMP-14 and -2 expression in EoE

Preliminary screening data using cultured fibroblasts and esophageal biopsies showed the presence of MMP-14 and -2 in EoE specimens (data not shown). This observation combined with the prior literature showing that MMP-14 and -2 can function in tandem for TGF β 1 activation and that MMP-14 is elevated in EoE¹⁵ led us to further assess their expression in EoE¹².

Quantitative RT-PCR studies demonstrated that EoE fibroblasts and biopsies expressed detectable MMP-14 and MMP-2 mRNA (Figure 1a,b). While MMP-14 was more abundant in EoE biopsies than fibroblasts, MMP-2 was more abundant in fibroblasts than in the biopsy specimens (Figure 1). Since esophageal biopsies are largely comprised of epithelium, this data aligns with our finding that MMP-14 expression was consistently elevated in epithelial cells but that MMP-2 demonstrated more variability in its epithelial expression (see below).

We utilized immunohistochemistry followed by image analysis with quantification in order to assess the degree of expression and cellular localization of MMP-2 and -14 in EoE and control esophageal biopsies. MMP-14 was strongly expressed in the expanded epithelial basal zone of EoE subjects (Figure 2a). MMP-14 expression encompassed a larger portion of the epithelial height in EoE as compared with control subjects (Figure 2c) and correlated with the degree of basal zone hyperplasia ($r=0.65$, $p=0.002$). Active EoE biopsies had a mean of 56% (95% CI 43-70%) of the total epithelial height expressing MMP-14 while control biopsies had a mean of 4.5% (95% CI 0.1-9%) ($p<0.0001$) (Figure 2c).

MMP-2 stain was present in the epithelium of EoE subjects but had variable expression and could not be reliably quantified (n=23 subjects evaluated). However, MMP-2 expression in the LP was isolated to distinct populations of cells (Figure 3a). Quantitation showed significantly more MMP-2 positive cells in the LP of EoE subjects ($m=906 \pm 167$ cells/mm²) than control subjects (232 ± 54 cells/mm²) ($p=0.003$) (Figure 3b). The LP in EoE subjects also had more MMP-14 positive cells (mean= 701 ± 93 cells/mm²) than control LP (mean= 263 ± 93 cells/mm²) ($p=.003$) (Figure 2b, d). Interestingly, the degree of epithelial MMP-14 expression correlated with the severity of LP fibrosis ($r=0.70$, $p=0.02$).

MMP Expression is reduced following topical corticosteroid therapy

In order to assess if topical corticosteroid therapy would decrease MMP expression, we evaluated MMP-2 and -14 expression in the epithelium and LP following therapy with esophageal topical corticosteroids. All patients studied were considered responders; 14 subjects were treated with oral viscous budesonide and 5 were treated with fluticasone propionate.

Epithelial MMP-14 expression was significantly reduced following therapy with a mean of 19% of the epithelial height showing MMP-14 expression following therapy ($p<0.0001$) (Figure 2a, c). Topical steroids also reduced the levels of MMP-14 positive cells in the LP (264 ± 50 cells/mm²) ($p<0.0001$, Figure 2b, d). The numbers of MMP-14 positive cells in the LP following therapy was not significantly different from the numbers seen in control subjects (Figure 2b, d). Similarly, LP MMP-2 expressing cells were significantly decreased following topical corticosteroid therapy (239 ± 67 cells per mm²) ($p=0.002$). The numbers of MMP-2 positive cells in the LP post therapy was not significantly different from the numbers of cells seen in non-diseased control subjects (Figure 3a, b). As such, topical esophageal corticosteroids are capable of reducing MMP-2 and -14 expression in EoE and, in the LP, the numbers of MMP-2 and -14 expressing cells are reduced to normal levels following therapy.

Proliferating epithelial cells express MMP-2 and -14 and increase MMP-2 in response to TGFβ1

Since MMP-14 was expressed in the basal zone, since the degree of epithelial MMP-14 expression correlated with BZH severity, and since the basal zone comprises the renewal source for the epithelium, we hypothesized that actively proliferating epithelial cells would be a source of MMP-14. Using the epithelial cell proliferation marker Ki67, we found that proliferating epithelial cells had cytoplasmic MMP-14 expression (Figure 4a). In addition, double immunofluorescence using a subject biopsy that had robust epithelial MMP-2 demonstrated that MMP-2 also was expressed in proliferating epithelial cells (Figure 4b).

Since MMP-2 can activate TGFβ1 and since TGFβ1 has direct effects on esophageal epithelial cell function^{14, 18}, we were curious to know if there was a potential positive feedback loop for TGFβ1 via MMP-2. TGFβ1 treatment of the esophageal epithelial cell line, HET1A, increased MMP-2 transcription as well as intracellular and extracellular MMP-2 (Figure 4c). In addition, both EoE fibroblasts and esophageal longitudinal smooth muscle cells produced MMP-2 (data not shown). In order to understand the effects of

TGFβ1 on MMP-14 expression, we treated HET1A cells with TGFβ1 and assessed MMP-14 mRNA and protein. We found that TGFβ1 increased MMP-14 expression (Figure 4d).

Discussion

In this study, we demonstrate the presence and potential functions of MMP-14 and -2 in pediatric EoE. Our current study includes a number of novel observations. We demonstrate that MMP-14 and -2 are elevated in the esophagus of pediatric EoE subjects and are expressed in proliferating epithelial cells as well as LP cells. In addition, we show that MMP-14 and -2 expression is reduced following topical corticosteroid therapy, consistent with the concept that proteins promoting esophageal remodeling can decrease with successful topical corticosteroid therapy. Since TGFβ1 can increase MMP-2 and MMP-14 in cultured epithelial cells, our data suggest a potential positive feedback loop for TGFβ1 activation.

The role of MMPs has not been systematically addressed in EoE despite multiple studies demonstrating esophageal tissue remodeling.³ We chose to focus on MMP-14 and -2 since they can act in concert for TGFβ1 activation and since screening data demonstrated their presence in EoE.^{1, 15} Once released, TGFβ1 activates both the canonical phosphorylated Smad and non-canonical signaling pathways to initiate fibrosis, epithelial mesenchymal transformation, smooth muscle contraction, and changes in calcium flux via the induction and phosphorylation of calcium channel regulating proteins such as phospholamban.^{6, 7, 14, 18} Since we show that TGFβ1 itself induces the expression of MMP-2 and -14 and the release of MMP-2 from esophageal epithelial cells, our data suggest a feedback loop for TGFβ1 activation such that TGFβ1 increases MMP-2 and -14 expression and the presence of MMP-2 and -14 in turn activates latent TGFβ1 to its active form to cause tissue fibrosis. Since the Smad3 pathway is important for esophageal EoE fibrosis and angiogenesis¹⁹, since TGFβ1-induced airway fibrosis can occur via MMP-2,²⁰ and since both MMP-14 and -2 are known to be essential for proper angiogenesis,²¹⁻²³ it will be interesting to determine if any of these Smad3/TGFβ1 proangiogenic effects are MMP-dependent in EoE.

Short term topical corticosteroid treatment reduces esophageal subepithelial fibrosis and TGFβ1 positive cells in EoE children and adults.²⁴⁻²⁶ Here we show that topical corticosteroids also reduce MMP-14 and -2 in the epithelium and LP of pediatric EoE subjects. Our finding that MMP-14 expression correlates with the remodeling features of basal zone hyperplasia and fibrosis is consistent with the theory that MMPs promote remodeling in EoE and that in children some features of remodeling are reversible.²⁵⁻²⁷

In conclusion, we provide the first data for the expression and cellular location of MMPs as well as their potential regulation by topical corticosteroids in EoE. These MMPs could affect not only fibrosis but also EoE associated angiogenesis since angiogenic remodeling is a known function of both MMP-2 and -14. As such, it will be of interest to understand which other MMP family members are elevated in adult and pediatric EoE, whether their expression correlates with tissue fibrosis, and whether they serve as additional therapeutic targets in EoE.

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Figure 1.

MMP-14 and -2 mRNA are present in EoE cells and biopsies. MMP-14 (A) and MMP-2 (B) message is present in both EoE biopsies and fibroblasts. Data is shown as ΔCt normalized to housekeeping gene.

Figure 2.

MMP-14 expression in EoE. Representative images of control, active EoE (pre) and inactive EoE (post) subject biopsies showing MMP-14 (red) in the epithelium (A). Lines depict the portion of the epithelium that is MMP-14 positive. Representative images of LP MMP-14 positive cells (arrows) in control, active EoE (pre), and inactive EoE (post) subject biopsies (B). Original magnification 200x. Quantification demonstrates that MMP-14 is more abundant in active EoE (pre) subject epithelium (C) and LP (D) than in controls or inactive EoE (post). Bars represent means. Isotype control image for MMP-14 (E).

Figure 3.

Representative image of LP MMP-2 positive cells (inset with arrows) in control, active EoE (pre), and inactive EoE (post) subject biopsies (A). Quantification demonstrates that MMP-2 is more abundant in active EoE (pre) subject epithelium and LP than in controls or inactive EoE (post) (B). Original magnification 200x. Bars represent means. Isotype control for MMP-2 (C)

Figure 4.

MMP-14 and -2 are expressed in actively proliferating esophageal epithelial cells and MMP-2 is increased by TGFβ1. Representative image of active EoE biopsy shows nuclear Ki67 (red) and cytoplasmic MMP-14 (green) (A) as well as nuclear Ki67 and cytoplasmic MMP-2 (green) (B). Original magnification 200x. Treatment of HET1A esophageal epithelial cells with recombinant TGFβ1 causes increased MMP-2 mRNA and cellular and secreted (C) MMP-2. MMP-14 mRNA and protein are increased by TGFβ1 (D). Graphs show mean with standard errors.

Table 1

EoE Patient Characteristics

Patient	Age (years)	Gender (male)	Atopic Status	Eosinophils per hpf (D/M/P)	Endoscopic Features
Subjects for fibroblast isolation and/or quantitative PCR					
1	16	M	AR, FS	29/46/0	P, F, Fr, Thick
2	1.5	F	AR, FS	70/50/30	P, Pl, F
3	6	M	Asthma, AR, FA	78/168/68	P, F, Pl
4	16	M	AR, FA	99/200/129	P, F, Pl
5	5	F	Asthma, AR, FA	200/60/6	P, Pl
6	3	M	Asthma, AR	0/20/ND	Normal
7	17	M	Asthma, AR, FS	50/150/ND	R, F, Pl
8	14	M	Asthma, AR	4/75/0	P, R, F
9	1	F	AR	30/32/0	P, F
10	3	M	AR, FA	0/0/0 (on topical corticosteroids)	Normal
Subjects for immunostaining					
1	10	M	Asthma, AR	30/25/26	P, F
2	7	F	Asthma, AR	42/56/56	F
3	3	F	Asthma, AR, FA	58/28/44	Pl
4	1	M	Asthma, AR	100/ND/80	Edema
5	5	M	Asthma, AR, FA	60/75/0	P, F, Pl
6	4	F	AR	80/75/40	F
7	5	M	Asthma, FA	40/40/22	F, P, Pl
8	16	M	AR	22/30/28	P, F, Pl
9	3	M	Asthma, AR, FA	150/160/15	P, F, Pl
10	18	M	Asthma, AR, FA	18/24/19	F
11	13	M	Asthma, AR, FA	215/142/134	F, P
12	3	M	AR, FA	66/41/66	F
13	13	F	Asthma, AR	98/26/72	LF, R
14	5	M	AR, FS	21/32/17	P
15	5	M	FA, AR	40/40/0	F
16	6	M	Asthma, AR, FA	60/110/15	P, Pl
17	2	M	Asthma, AR, FA	50/150/120	R, Edema
18	1	M	FA	100/85/30	Pl, F

ND=not done

Table 2

Antibodies and Primer Sequences Utilized in the Study

Antigen	Concentration	Manufacturer
MMP-14	1:100	ab3644, Abcam, Cambridge MA
MMP-2	1:200	ab37150, Abcam, Cambridge MA
Macrophages (Mac387)	1:300	ab22506 Abcam, Cambridge MA
Epithelial cells (Ki67)	1:100	M724029-2, Dako, Carpinteria, CA
Eosinophils (MPB)	1:50	K54914, Meridian Life Sciences, Memphis, TN
aSMA	1:400	A2547, Sigma, St Louis, MO
MMP-14	F-TTGTCTCCTGCTCCCCCTGCT R-CGCCTCATGGCCTTCATGGTGTCT	
MMP-2	F- AGCTCTATGGGGCCTCTCCTGA R- TCACCACGGATCTGAGCGATGC	
GAPDH	F- TGGTATCGTGGAA-GGGACTCAT R- ATGCCAGTGAGCTTCCCGTTC	
RPL13A	F- AAACCTCCCCAGCTAAACACC R-GAACTTCAGAGAAGCATCACGATGATA	

F- forward; R- reverse