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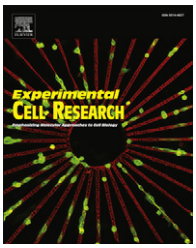

Research Article

Esophageal epithelial and mesenchymal cross-talk leads to features of epithelial to mesenchymal transition *in vitro*

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ABSTRACT

Background: Esophageal fibrosis is a complication of eosinophilic esophagitis (EoE) which has been attributed to both subepithelial fibrosis and to epithelial to mesenchymal transition (EMT), a process by which epithelial cells acquire mesenchymal features. Common to both causes of EoE-fibrosis is the notion that granulocyte-derived TGF-β, induces myofibroblast differentiation of the target cell. To date, the role of esophageal epithelial cells as effector cells in esophageal fibrosis has never been explored. Herein, we investigated consequences of cross-talk between esophageal epithelial cells and fibroblasts, and identified profibrotic cytokines which influence the development of EMT *in vitro*.

Methods and results: Stimulation of primary fetal esophageal fibroblasts (FEF3) with conditioned media (CEM) from esophageal epithelial cells (EPC2-hTERT), primed FEF3 cells to secrete IL-1β and TNFα, but not TGFβ. To determine whether these cytokines signaled in a paracrine fashion to esophageal epithelial cells, FEF3 cells were stimulated with CEM, followed by transfer of this fibroblast conditioned media (FCM) to EPC2-hTERT cells. Epithelial FCM stimulation increased expression of mesenchymal markers and reduced E-cadherin expression, features of EMT which were TNFα and IL-1β-dependent. Using organotypic culture models, primary EoE epithelial cells exhibited features of EMT compared to non-EoE cells, corresponding to patterns of EMT in native biopsies.

Conclusions: Esophageal epithelial cell and fibroblast cross-talk contributes to esophageal fibrosis. Our results suggest that features of EMT can develop independent of TGF-β and granulocytes, which may have important implications in treatment of EoE.

Abbreviations: EoE, eosinophilic esophagitis; EMT, epithelial to mesenchymal transition; IL-1β, interleukin 1-beta; TNFα, tumor necrosis factor alpha; TGFβ, transforming growth factor beta; αSMA, alpha smooth muscle actin; CEM, conditioned epithelial media; FCM, fibroblast conditioned media; OTC, organotypic cell culture

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Introduction

Eosinophilic esophagitis (EoE) is a chronic allergic disease affecting 4 in 10,000 children [1] and adults, characterized by eosinophilic infiltrates of the esophageal mucosa. In older children and adults, the most problematic complication of EoE is the development of esophageal fibrosis leading to dysphagia and esophageal food bolus impactions. The precise etiology of EoE-associated fibrosis remains unknown.

Fibrosis is defined as the inappropriate deposition of extracellular matrix (ECM), leading to deformation of the parenchyma. It is widely believed that stimulation with pro-fibrotic cytokines activates fibroblasts to acquire the activated phenotype of myofibroblasts, morphologic intermediates between fibroblasts and smooth muscle cells which synthesize ECM components including collagen, α-smooth muscle actin (αSMA), fibronectin, and proteoglycans. Although local fibroblasts are considered to be the most common myofibroblast progenitors, myofibroblasts have also been shown to originate from bone marrow-derived fibrocytes [2–4] and smooth muscle cells [5]. In addition, epithelial cells can acquire a myofibroblast characteristics and lose epithelial cell features [6] via epithelial to mesenchymal transition (EMT) [7]. In EMT, epithelial cells gain contractile and cytoskeleton proteins found in myofibroblasts while losing their characteristic tight junction and adhesion proteins.

Others have recently shown that EoE-associated fibrosis occurs through several mechanisms, including EMT. Aceves et al. showed that esophageal biopsies from EoE patients exhibit increased subepithelial collagen deposition compared to biopsies from control patients and patients with gastroesophageal reflux disease [8] suggesting that activation of fibroblasts within the subepithelium contributes to EoE fibrosis. In contrast, Kagalwalla et al. recently demonstrated that esophageal biopsies from pediatric EoE subjects exhibit features of EMT, characterized by increased expression of the mesenchymal marker vimentin and decreased expression of the epithelial marker cytokeratin within the epithelial compartment [9]. Interestingly, Kagalwalla et al. also observed a correlation between EMT scores and subepithelial fibrosis in pediatric EoE biopsies, indicating that the two processes are not mutually exclusive. In addition, these investigators also showed that features of EMT could be induced *in vitro*, through stimulation of the HET-1A esophageal epithelial cell line with the profibrotic cytokine, transforming growth factor-β (TGF-β), consistent with findings of Ohashi et al., who also showed that TGF-β stimulation induced EMT in the EPC2-hTERT esophageal epithelial cell line [10].

TGF-β is known as a prototypical profibrotic cytokine in many models of fibrosis [11–13]. Consistent with this notion, both Aceves et al. and Kagalwalla et al. have suggested that TGF-β is necessary for myofibroblast activation in the context of EoE-associated fibrosis. This assumption is supported by the work of others, who have previously shown that TGF-β is produced and released by circulating immune effector cells known to infiltrate the esophageal epithelium in EoE, including mast cells [14] and eosinophils [15].

While TGF-β plays an established role in tissue remodeling, other profibrotic cytokines and soluble mediators can activate fibroblasts and induce ECM production [16]. IL-1β, for example, enhances the effects of TGF-β in the acquisition of the mesenchymal phenotype in human bronchial epithelial cells *in vitro* [17]. TNF-α has been implicated in the development of EMT in retinal pigment epithelial cells [18], and enhances TGF-β-induced EMT in human alveolar epithelial cells [19]. To date, the potential role for IL-1β and TNF-α in EoE-associated tissue remodeling has not been investigated.

Others have shown that cross-talk between epithelial and mesenchymal cells contributes to remodeling in other model systems [20–22]. Building upon our previous reports that human esophageal epithelial cells function as effector cells in the pathogenesis of esophageal inflammation [23,24], we hypothesized that esophageal epithelial and mesenchymal cross-talk plays a role in EoE-associated fibrosis. In this study, we show for the first time that esophageal epithelial cells prime esophageal fibroblasts to secrete fibrogenic cytokines IL-1β and TNF-α. Surprisingly, we demonstrate that these cytokines play a role in the development of EMT *in vitro*, and this can occur in a TGF-β-independent fashion. Using a primary EoE cell line grown in organotypic culture with primary fibroblasts, we further demonstrate that esophageal epithelial cells can function as innate immune effector cells in the context of EoE.

Materials and methods

Cell lines: Three human esophageal epithelial cell lines, EPC2-hTERT, EPC394, and EPC425, were grown at 37 °C in a humidified 5% CO_2 incubator, and maintained keratinocyte serum free medium (KSFM, Invitrogen, Grand Island, NY) containing human epidermal growth factor (1 ng/mL), bovine pituitary extract (50 μg/mL), and penicillin (100 units/mL) and streptomycin (100 μg/mL). The EPC2-hTERT cell line is a telomerase-immortalized and nontransformed cell line, whereas the EPC394 and EPC425 cells lines are primary cell lines obtained from an EoE (EPC394) and a non-EoE control (EPC425) patient. Fetal esophageal fibroblasts (FEF3 cells, gift of Hiroshi Nakagawa MD, PhD) and a primary fibroblast cell line (PEF429) from an adolescent patient with EoE, were maintained in Dulbecco's Minimum Essential Media (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO), and grown at 37 °C in a humidified 5% CO_2 incubator.

Primary esophageal cell lines: Esophageal biopsies were placed in Hanks BSS buffer, transferred to dispase (BD Biosciences, 50 U/mL) for 20 min at 37 °C, then trypsinized (trypsin-EDTA, GIBCO) at 37 °C. Trypsin was inactivated using soybean trypsin inhibitor (SIGMA) and biopsies were gently manually shaken. Samples were poured through a cell strainer and cells were collected in a conical tube. Cells were pelleted by centrifugation at 4 °C for 5 min. For epithelial cell isolation, pellets were resuspended in KSFM containing antibiotics and fungizone (1:500) (GIBCO). For fibroblast isolation, pellets were resuspended in DMEM with antibiotics and fungizone (1:500) (GIBCO). Cell suspensions were then seeded in tissue culture plates. Cells were used at passage 2–3.

Conditioned epithelial media (CEM) stimulation: Conditioned epithelial media (CEM) was collected from confluent EPC2-hTERT cells grown in complete KSFM, and used to stimulate fibroblast monolayers for 3 and 6 h. Prior to stimulation of fibroblasts, CEM was supplemented with 10% FBS. Fig. 1A shows the schematic of the experimental design. For control conditions,