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Research Article

Esophageal epithelial cells acquire functional characteristics of activated myofibroblasts after undergoing an epithelial to mesenchymal transition

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ABSTRACT

Background and Aims: Eosinophilic esophagitis (EoE) is an allergic inflammatory disease that leads to esophageal fibrosis and stricture. We have recently shown that in EoE, esophageal epithelial cells undergo an epithelial to mesenchymal transition (EMT), characterized by gain of mesenchymal markers and loss of epithelial gene expression. Whether epithelial cells exposed to profibrotic cytokines can also acquire the functional characteristics of activated myofibroblasts, including migration, contraction, and extracellular matrix deposition, is relevant to our understanding and treatment of EoE-associated fibrogenesis. In the current study, we characterize cell migration, contraction, and collagen production by esophageal epithelial cells that have undergone cytokine-induced EMT *in vitro*.

Methods and Results: Stimulation of human non-transformed immortalized esophageal epithelial cells (EPC2-hTERT) with profibrotic cytokines TNFα, TGFβ, and IL1β for three weeks led to acquisition of mesenchymal αSMA and vimentin, and loss of epithelial E-cadherin expression. Upon removal of the profibrotic stimulus, epithelial characteristics were partially rescued. TGFβ stimulation had a robust effect upon epithelial collagen production. Surprisingly, TNFα stimulation had the most potent effect upon cell migration and contraction, exceeding the effects of the prototypical profibrotic cytokine TGFβ. IL1β stimulation alone had minimal effect upon esophageal epithelial migration, contraction, and collagen production.

Conclusions: Esophageal epithelial cells that have undergone EMT acquire functional characteristics of activated myofibroblasts *in vitro*. Profibrotic cytokines exert differential effects upon esophageal

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epithelial cells, underscoring complexities of fibrogenesis in EoE, and implicating esophageal epithelial cells as effector cells in EoE-associated fibrogenesis.

Introduction

Eosinophilic esophagitis (EoE) is a chronic allergic inflammatory disease characterized by eosinophil infiltration into the esophageal epithelium. The most important clinical complication of EoE is esophageal fibrosis, resulting in progressive dysphagia and recurrent esophageal food impactions requiring urgent endoscopic removal. Unfortunately, very little is currently known about the mechanisms by which fibrosis develops in EoE.

Activated myofibroblasts are the key effector cells in all models of fibrosis [1,2]. In wound healing, tissue strain and cytokine release activate myofibroblasts to begin migration, extracellular matrix (ECM) deposition, and tissue contraction, thus maintaining tissue homeostasis [3]. However, in fibrosis, exaggerated myofibroblast responses result in inappropriate ECM deposition, increased tissue stiffness, and organ dysfunction.

The cellular origin of the myofibroblast is controversial. Lineage–tracing studies in the kidney, lung, and liver have provided conflicting results about the existence of epithelial to mesenchymal transition (EMT), although the most recent evidence suggests that epithelial cells are not a significant source of myofibroblasts in these tissues [4–8]. In EoE, it is presumed that activated myofibroblasts originate from resident fibroblasts in the lamina propria. This is supported by the findings of Aceves et al. who demonstrated marked increases in collagen deposition in the lamina propria of biopsies from patients with EoE compared to normal and GERD controls [9]. Alternatively, we and others have shown epithelial cells from patients with EoE have increased markers of activated myofibroblasts (αSMA and vimentin) and decreased epithelial markers (cytokeratin and E-cadherin) suggesting that one source of the activated myofibroblasts may be epithelial cells [10,11]. We have also shown that epithelial cells in organotypic culture acquire markers of activated myofibroblasts suggesting that EMT may play a role in EoE-associated fibrogenesis [10,11].

Though best known for its roles in development and malignancy [12], EMT is also required for normal tissue homeostasis. During normal embryological development, epithelial cells lose cell-cell connections, become migratory, and eventually form the mesenchyme [13]. Because epithelial cells are capable of transdifferentiation in these settings, it has been postulated that during chronic inflammation, epithelial cells contribute to fibrosis via EMT. As the epithelium is often the site of primary injury and inflammation, it stands to reason that epithelial cells may also function as effector cells in fibrogenesis.

While EMT has been implicated in fibrosis of other organ systems [14–19], definitive evidence for EMT requires *in vivo* lineage tracing studies. In a bleomycin-induced mouse model of lung fibrosis, Tanjore et al. found that while myofibroblasts of epithelial origin were rare, they did contribute to overall lung fibrosis [20]. However, Chu et al. refuted the notion of EMT in a murine model of hepatic fibrosis by showing a lack of colocalization of epithelial markers with α-smooth muscle actin (α-SMA), a prototypical marker of activated myofibroblasts [21]. Unfortunately, similar *in vivo* lineage tracing studies to refute or support EMT in EoE are lacking, as there have been no published animal models of EoE-associated fibrogenesis. Furthermore, others have suggested that EMT cannot be defined by marker analysis alone, and must demonstrate acquisition of functional characteristics of activated myofibroblasts, including migration, contraction, and collagen deposition [8,21].

Our previous work, along with that of Kagalwalla et al., has shown that markers of EMT are expressed in esophageal biopsies from subjects with EoE [10,11]. In the current study, we build upon our previous findings and demonstrate that the acquisition of EMT markers by cultured epithelial cells is partially reversible, and that esophageal epithelial cells can acquire functional properties of activated myofibroblasts including migration, contraction, and expression of type I collagen following acquisition of EMT markers.

Materials and methods

Esophageal epithelial cells

EPC2-hTERT{Harada, 2003 #1166}, an extensively characterized telomerase-immortalized, non-transformed human esophageal epithelial cell line, was used in all experiments. In addition, primary human esophageal epithelial cells were isolated as described [11] from IRB-approved six pediatric esophageal biopsies. Cells were grown at 37 °C in a humidified 5% CO_2 incubator and maintained in keratinocyte serum free media (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). For all experiments, EPC2-hTERT cells were used between passages 36 and 39. Primary cells were used between passages 3–5.

Cytokine stimulation

Cells were seeded in 6-well plates and stimulated in triplicate with one of the following human recombinant cytokines: TNFα (40 ng/mL), TGFβ (10 ng/mL), or IL1β (10 ng/mL). All recombinant cytokines were purchased from R&D Systems (Minneapolis, MN). Media containing individual cytokines was refreshed weekly for 3 weeks. For cytokine rescue experiments, cells were either cultured for an additional week in the presence of individual cytokine, or were cultured in standard media for one week.

Quantitative RT-PCR

RNA was purified using an RNeasy Kit (Qiagen, Valencia, CA) according to manufacturer's recommendations. RNA samples were reverse transcribed using a high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, CA). Pre-formulated TaqMan Gene Expression Assays were purchased from Applied Biosystems for