Cytokine 61 (2013) 228–236

Contents lists available at SciVerse ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Vocal fold fibroblasts immunoregulate activated macrophage phenotype ☆,☆☆

Suzanne N. King [a], Fei Chen [b], Marie E. Jetté [a], Susan L. Thibeault [a,c,*]

[a] Division of Otolaryngology – Head and Neck Surgery, University of Wisconsin–Madison, United States
[b] Division of Speech and Hearing Sciences, The University of Hong Kong, Hong Kong
[c] University of Wisconsin Carbone Cancer Center, Madison, Wisconsin, United States

ARTICLE INFO

Article history:
Received 1 September 2012
Accepted 25 September 2012
Available online 2 November 2012

Keywords:
Macrophage
Vocal fold
Fibroblasts
Scar
Polyp

ABSTRACT

Recent evidence suggests that fibroblasts play a critical role in regulating inflammation during wound healing because they express several inflammatory mediators in response to bacteria. The objective of this study was to analyze the effects of lipopolysaccharide (LPS) on the immunomodulatory properties of vocal fold fibroblasts (VFFs) derived from polyps, scar and normal tissue co-cultured with macrophages, to provide insight into their interactions during the inflammatory process. Fibroblasts were co-cultured with CD14+ monocytes and after 7 days, wells were treated with LPS for 24 and 72 h. Culture supernatants were collected and concentrations of TNF-α, IL-6, IL-8, IL-10, IL-12, IL-1β and MCP-1 were quantified by ELISA. Normal VFF and CD14+ monocultures were used as controls. Twenty-four hours after LPS activation, macrophages co-cultured with polyp VFF had significantly increased expression of TNF-α, IL-1β, IL-12 and IL-10 compared to controls ($p < 0.0001$). In contrast, macrophages co-cultured with scar VFF had significantly lower expression of TNF-α, IL-1β and IL-12 with significantly higher IL-10 compared to control ($p < 0.0001$). After 72 h, macrophages co-cultured with polyp VFF increased expression of TNF-α, IL-1β, IL-10, IL-6, IL-8, MCP-1 and TGF-β ($p < 0.01$) and macrophages co-cultured with scar VFF significantly decreased their expression of IL-1β and IL-12 compared to control ($p < 0.0001$). Scar VFF at both time points produced significantly lower levels of IL-8, MCP-1, IL-6 and TGF-β compared to controls ($p < 0.05$). Based on our findings, VFF and macrophages secrete several inflammatory mediators that modify their diverse functions. Polyp and scar VFF may play a role in regulating abnormal inflammatory responses, which could result in excessive ECM deposition that disrupts the function of the vocal folds.

1. Introduction

In the vocal fold lamina propria, fibroblasts and macrophages are the two most prominent cells orchestrating the complex inflammatory events involved in wound healing [1]. Prolonged inflammation (i.e. infection, vocal abuse) can lead to excess deposition of extracellular matrix (ECM) resulting in benign vocal fold lesions [2] and dysphonia; however, the contribution of fibroblasts to the resolution of inflammation remains unclear. Recent evidence has suggested that fibroblasts contribute to fighting infection by synthesizing danger signals that can modify macrophage immunophenotype [3,4]. Such interactions may contribute to the formation of inflammatory lesions in the vocal fold and impair its normal biomechanical function which is vital for normal voice production.

Vocal fold fibroblasts (VFFs) are essential to tissue homeostasis, because they provide a rich source of glycosaminogylcans, proteoglycans, elastin and collagen molecules that influence the migration, growth, differentiation and activity of neighboring cells [1,5,6]. Damage to the vocal folds due to external challenges (i.e. tobacco, reflux, bacteria), abusive behaviors (e.g., excessive voice use) or infection can alter fibroblast function. Beyond their structural function, very little is known about the role of fibroblasts as inflammatory mediators within the vocal folds. They are known to express a rich source of inflammatory cytokines, chemokines and lipid mediators, including IL-6, IL-8, IFN-γ, prostaglandin E2 (PGE2) and cycloxygenase (COX-2) [7–11]. Fibroblasts from various other tissues have also been shown to produce pro-inflammatory cytokines that are involved in recruiting and activating macrophages, such as monocyte chemotactic protein (MCP)-1 or macrophage inflammatory protein (MIP)-1β [12,13] and produce several Toll like receptors (TLRs) in response to microbial products [14]. Overt secretion of cytokines and chemokines are known to cause chronic inflammation in the vocal fold [15], how-

☆ *Source of funding:* NIDCD – NIH Grants R01 DC4336, DC9600, T32 DC009401.
☆☆ *Author contributions:* Conceived and designed the experiments: SK ST. Performed the experiments: SK FC MJ. Analyzed the data: SK FC. Wrote the paper: SK MJ ST. Edited manuscript: SK FC MJ ST.

* Corresponding author. Address: Otolaryngology Office, WIMR 5107, 1111 Highland Avenue, Madison, WI 53705-2725, United States. Tel.: +1 608 263 6751; fax: +1 608 263 6199.

E-mail addresses: kings@surgery.wisc.edu (S.N. King), carolfchen@gmail.com (F. Chen), jette@surgery.wisc.edu (M.E. Jetté), thibeaul@surgery.wisc.edu (S.L. Thibeault).

1043-4666/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.cyto.2012.09.023

ever not all inflammatory events result in the formation of benign lesions [16]. Therefore, fibroblasts from normal and disease states may play a diverse role in their microenvironment that could influence the function of surrounding immune cells.

Macrophages exhibit functionally distinct phenotypes during wound healing that can both exacerbate the injury or initiate wound repair. These polarizations are broadly characterized into two subpopulations; classically activated macrophages (M1) exhibit pro-inflammatory responses (i.e. interleukin [IL]-12, tumor necrosis factor [TNF]-α, inferon [IFN]-γ) and alternative activated macrophages (M2) exhibit anti-inflammatory responses (i.e. IL-10, IL-4) [17]. Classically activated macrophages are produced during cell-mediated immune responses to resist against infection by bacteria or other microorganisms [18]. Alternatively activated macrophages include several subtypes involved in wound healing or tissue regulation, which have suppressed inflammatory functions thereby inhibiting their host defense [18]. Our research group has previously demonstrated that normal VFF can modulate macrophage expression of HLA-DR and CD206 to a more anti-inflammatory phenotype [19]. However, after infection, endogenous danger signals produced in the microenvironment by fibroblasts can potentially alter macrophages phenotype and influence the resolution of inflammation [20].

The objective of this study was to analyze the complex inflammatory signaling between macrophages and VFF derived from polyps, scar or normal tissue. We hypothesized that the interaction between these cells plays an important role in the pathogenesis of chronic inflammation in the vocal fold as evidenced by differences in macrophages cytokine expression with normal and pathologic VFF. In this study, we used an *in vitro* inflammatory co-culture model of macrophages and fibroblasts to analyze their paracrine signaling. We co-cultured fibroblasts with CD14+ cells for 7 days and then activated macrophages with LPS for 24 and 72 h. Lipopolysaccharide (LPS), the outer membrane of a gram-negative bacterium causes activation and secretion of pro-inflammatory cytokines from macrophages [4]. Results suggest that functionally distinct subpopulations of macrophages exist *in vitro* and that our various VFF play a critical role in regulating macrophages inflammatory response.

2. Materials and methods

2.1. Human vocal fold fibroblasts

Primary fibroblast cell lines were harvested from normal, polyp and scar human vocal fold biopsies derived from four adult donors based on protocols approved by the University of Wisconsin Health Sciences Institutional Review Board (IRB) as previously described [21,22]. Polypoid tissue was obtained from a 24-year-old female undergoing microlaryngoscopy with bilateral excision of vocal fold polyps. Polyp tissue was carefully removed with cold instruments, making sure to preserve the overlying epithelium and with maximal preservation of the surrounding native lamina propria. The donor's past medical and surgical histories were unremarkable.

For primary cell culture, true vocal fold tissue was cut into small pieces and suspended in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 0.01 mg/mL streptomycin sulfate and 1× non-essential amino acid (NEAA) (all from Sigma Inc., St. Louis). Cells were expanded out on uncoated plastic tissue culture dishes (Focal) at 37 °C in 5% CO2-humidified atmosphere. Once a confluent monolayer of cells was achieved, adherent cells were then harvested and passaged. Vocal fold fibroblast categorization and identification has previously been reported with this culture methodology [21]. Polyp, scar, T21 and T59 vocal fold fibroblasts (VFFs) were expanded until passages six to eight for use in this study.

2.2. Human monocyte isolation

Human peripheral blood mononuclear cells (PBMCs) were collected from buffy coat samples obtained from normal healthy donors using density grade centrifugation (Interstate Blood Bank, TN). Pure populations of CD14+ cells were isolated by magnetic bead separation methods as previously described [19,23]. Briefly, cell suspension was incubated with anti-human CD14 microbeads (Miltenyi Biotech, Auburn, CA, USA) for 15 min at 4 °C degrees and cells were separated using an AutoMACS Pro Separator (Milenyi Biotech) according to manufacturer's instructions. Human monocytes separated from buffy coats were then frozen at −80 °C in freezing media (70% phenol red free RPMI-1640 supplemented with 20% FBS, 2 mM L-glutamine-alanine, 1% sodium pyruvate, 1% NEAA and 10% DMSO) and stored at −180 °C until used. This method has previously been shown to yield >95% cell viability and approximately 35% of CD14+ monocytes at a 1×10^6 starting concentration will differentiate into CD14+/CD206+ macrophages after 7 days culture as previously described [19,23].

2.3. Cell culture

To induce monocyte differentiation into macrophages in the presence of VFF, we co-cultured fibroblasts with CD14+ cells for 7 days (Fig. 1). VFF (20×10^5) suspended in 200 μL R10 media (phenol red free RPMI-1640 supplemented with 10% FBS, 2 mM L-glutamine–alanine, 1% sodium pyruvate and 1% NEAA) supplemented with an extra 10% FBS to facilitate cell adhesion were seeded on the basolateral underside of a tissue culture treated transwell (0.4 μm pore; Millicell). After incubation at 37 °C 5% CO_2 for 4 h, transwells were gently turned over and 1.5 mL of R10 media was added to the top and bottom of each transwell. Fibroblasts were incubated for 48 h to allow cells to initiate growth on the transwell. Purified CD14+ monocytes were thawed and washed, then seeded onto the apical side of the transwell at a concentration of 1×10^6 per well and cultured for 7 days at 37 °C in 5% CO_2 humidified atmosphere. For control conditions, fibroblasts (i.e. T21, T59, polyp, scar) and CD14 + cells were seeded alone on the transwell. R10 media was changed on day 4 as previously described [19]. Briefly, media was removed and centrifuged at 1200 RPM for 10 min to collect any non-adherent CD14+ cells. These cells were then suspended in fresh media and added back to the appropriate well on the apical side.

Following the 7th day of co-culture, media including non-adherent CD14+ cells was aspirated from each well. Fresh R10 media was added to the bottom of each well and R10 culture medium containing 10 μg/mL LPS from *Escherichia coli* O26:B6 (Sigma–Aldrich, USA) was added to the apical side of each well where macrophages were culturing. Non-stimulated cells were used as controls.

Our experimental protocol was based upon work previously published, demonstrating significant increases in fibroblast proliferation during co-culture with macrophages on polystyrene [19]. By day 7, VFF were confluent on plates and after harvesting co-culture conditions CD14+ macrophages were undetectable using flow cytometry techniques [19]. Transwell co-culture methods were used to allow for long-term co-culture and they prevent direct physical contact between fibroblasts and macrophages, keeping them 10 μm apart.

2.4. Fibroblast proliferation assay

To compare the growth rate of fibroblasts derived from polyp and scar vocal fold tissues to normal T21 and T59 VFF, cells were harvested and stained with trypan blue, then manually counted using a hemocytomer. VFF were seeded in triplicate on a 12-well plate at 50×10^4 cells/well and untreated conditions were