

Cyclic Tensile Strain on Vocal Fold Fibroblasts Inhibits Cigarette Smoke-Induced Inflammation: Implications for Reinke Edema

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Summary: Objectives. To investigate whether patients with Reinke edema are more extroverted than patients with carcinoma and, whether cyclic tensile strain (CTS) attenuates cigarette smoke condensate (CSC)-induced inflammation in human vocal fold fibroblasts (HVFF).

Study Design. *In vitro* and *ex vivo* study.

Methods. Clinical investigation and Eysenck personality questionnaire were performed to evaluate the personality and smoking status in individuals among groups. 3-(4,5)-dimethylthiazolium (-z-y1)-3,5-di-phenyltetrazolium bromide (MTT) assay was applied to test the viabilities of cultured HVFF exposed to CSC and/or CTS. The messenger RNA (mRNA) and protein expressions of cluster of differentiation 44 (CD44), cyclooxygenase-2 (COX-2), and matrix metalloproteinase-9 (MMP-9) in tissues from Reinke edema, paracancerous site, normal vocal fold, and in HVFF with different treatments were measured by reverse transcriptase polymerase chain reaction or western blot, respectively.

Results. Patients with either Reinke edema or carcinoma were moderate/heavy smokers; the extraversion score, however, was higher in Reinke edema patients than that in carcinoma patients. CD44 mRNA and protein expressions were significantly higher, whereas, COX-2 and MMP-9 expressions were significantly lower in Reinke edema tissues than those in paracancerous tissues. Treatment of HVFF with CSC led to the decrease in cell viability, the reduction in CD44 expression, but, the increase in COX-2 and MMP-9 expressions and, moreover, administration of CTS inhibited such effects of CSC on HVFF.

Conclusions. Our results demonstrate that Reinke edema patients have more extrovert personality characteristics in comparison to carcinoma patients and, importantly, CTS attenuates CSC-induced inflammation in HVFF. Phonatory vibration may be a mechanism for lower expression of proinflammatory mediators in Reinke edema tissues in spite of cigarette smoke exposure.

Key Words: Vocal fold–Reinke edema–Inflammation–Fibroblasts–Cigarette smoke condensate–Cyclic tensile strain.

INTRODUCTION

Reinke edema, one of the cigarette smoke (CS)-associated laryngeal response diseases, characteristically presents with the edema of vocal folds due to the excessive production of extracellular matrix.¹ Although it has been reported that the fibroblasts in lamina propria are important for the production of extracellular matrix molecules,^{2,3} the exact mechanism underpinning Reinke edema remains unclear.

It has been documented that CS, one of the most detrimental factors to the human body, can elicit a significant inflammatory response in many tissues via regulation of certain key factors.⁴ Of these, cluster of differentiation 44 (CD44) is believed to be important in mediating many inflammatory reactions. As the receptor of hyaluronan, CD44 binding to hyaluronan can suppress proinflammatory cytokine-stimulated production of cyclooxygenase-2 (COX-2) and matrix metalloproteinase (MMP) in synovial fibroblasts.⁵⁻⁷ CD44^{-/-} animals showed enhanced and prolonged infiltration of neutrophil and macrophage.⁸

Through regulating these factors, CS is closely associated with numerous diseases, including vocal fold squamous cell carcinoma and Reinke edema.^{9,10} Recent studies showed that exposure to CS caused squamous metaplasia in the vocal folds of animals and the up-regulation of COX-2 and prostaglandin E2 (PGE2) expressions in human vocal fold fibroblasts, implying that CS may increase probability of neoplasia.^{11,12} On the contrary, one study demonstrated that, under CS-mediated oxidative stress, the fibroblasts in Reinke edema tissue exhibited a chemoprotective response, that is, the increased antioxidant gene activity in tissues, suggesting that Reinke edema may delay or even inhibit progression to malignancy.¹³ However, there have been no researches relevant to the etiology for eliciting this antioxidant mechanism. In this regard, it is important to know why the Reinke edema exhibits a chemoprotective reaction and why the two kinds of patients experience such different outcomes in spite of being exposed to similar condition of CS.

Besides smoking, another essential etiologic factor of Reinke edema is excessive speaking.^{10,14} It has been reported that to be talkative is a way of being extroverted, and extraversion tends to be manifested in outgoing, talkative, and energetic behavior.¹⁵ In our long-term clinical practice, we have observed that most Reinke edema patients are more extroverted with excessive speaking, whereas, vocal fold carcinoma patients, it seems, are more introverted with much less communication. In view of this, it is speculated that vibrational stimulation may be one major cause for the different conditions of vocal fold. Available data have manifested that, during phonation, vocal

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fold fibroblasts encounter various types of mechanical force, with tensile as the dominant form.¹⁶ A study has shown that tensile strain alters cell behavior and inhibits interleukin-1 β (IL-1 β)-induced inflammation in vocal fold fibroblasts.¹⁷ Moreover, recent studies have demonstrated that a proper physiological level of mechanical force benefits human fibroblasts of injured larynx and other tissues.^{18–20} We thus hypothesize that patients with Reinke edema have more extrovert personality characteristics in comparison to patients with carcinoma, that is, excessive phonation resulted from the extrovert personality involves in the pathogenesis of Reinke edema. In addition, like a double-edged sword, the same phonatory vibration may be the putative reason for protecting the vocal fold of Reinke edema patients from the CS-induced injuries via suppressing the inflammatory gene expression in fibroblasts.

To test our hypothesis, we conducted both clinical and basic research. Specifically, smoking status of Reinke edema patients or vocal fold carcinoma patients was evaluated. In addition, Eysenck personality questionnaire (EPQ), an objective measure, was used to determine the personality of patients, which, in turn, may indirectly reflect the amount of vocal fold vibration. It has been shown that the differentiation of epithelial cell after injury is greatly influenced by the metabolism of fibroblasts in Reinke space and,²¹ also, the fibroblasts in paracancerous tissue may drive malignant tumorigenesis by providing the inflammatory microenvironment for tumor development.²² Accordingly, Reinke space tissues from Reinke edema and paracancerous site were collected for this study, then, the primary cultured human vocal fold fibroblasts (HVFF) were treated with cigarette smoke condensate (CSC) in the presence or absence of cyclic tensile strain (CTS) and, finally, the gene and protein expressions of CD44, COX-2, and MMP-9 were detected in tissues or cells. Through the aforementioned experiments, we sought to provide preliminary data regarding the effect of vibration on CS-induced responses in vocal folds. The investigation is a first step toward addressing the large question of why Reinke edema exhibits chemoprotective reaction.

MATERIALS AND METHODS

Participants

Written informed consents were obtained from all subjects before their inclusion in the study, in accordance with the ethical standards of the Local Ethics Committee of Shandong Provincial Hospital. All subjects gave their written informed consent. In total, 55 patients (54 male, aged 42–71) with vocal fold squamous cell carcinoma, 67 patients (58 male, aged 38–69) with Reinke edema and 60 physically and mentally healthy control adults (52 male, aged 40–70) were included for the smoking status investigation and EPQ survey. All the participants possessed good understanding of the EPQ. The patients were hospitalized for the surgery in the Department of Otorhinolaryngology-Head and Neck Surgery of Shandong Provincial Hospital from September 2009 to December 2011. The diagnosis of the lesions was determined by at least two specialists on stroboscopic inspection and confirmed by the pathologist.

For *in vitro* and *ex vivo* study, paracancerous and Reinke edema tissues were collected from 20 of the 55 vocal fold carcinoma patients and 20 of the 67 Reinke edema patients during routine surgical resection. Meanwhile, normal human vocal folds were obtained from 14 hypopharyngeal and six esophageal carcinoma patients, without smoking history or phonotrauma, who had undergone total laryngectomy as initial treatment. The vocal folds were judged to be without any evidence of disease by at least two specialists. Patients with neither systemic inflammatory disease nor current anti-inflammatory medications were selected for the study.

Assessment of smoking status

The association between cancer occurrence and cigarette smoking status was assessed via the classical method based on the duration of smoking and the number of cigarettes smoked per day.²³ Both Reinke edema and carcinoma patients had prolonged duration of exposure to CS. Assessment of smoking status was based on the total smoke exposure (pack-years, PKY), which was calculated by multiplying the number of packs (1 pack = 20 cigarettes) of cigarettes smoked per day by the number of years the person has smoked. The obtained PKY was then compared between Reinke edema and carcinoma patients.

EPQ survey

A Chinese version of EPQ modified by Gong et al²⁴ was used in this study. It comprised 88 items to be answered in a binary mode of “yes/no.” The responses to these items define four independent personality dimensions: extraversion (E), neuroticism (N), psychoticism (P), and lie (L), among which E dimension, characterized by enjoyment of social interactions, talkative personality, novel activities, and a tendency toward impulsive behavior, and higher score on E dimensionality represents extroversion personality.²⁵

The collection of vocal fold tissues

Reinke space tissues were collected from vocal fold carcinoma patients and Reinke edema patients during routine surgical resection, and normal Reinke space tissues were obtained from hypopharyngeal or esophageal carcinoma patients who had undergone total laryngectomy. In detail, phonomicrosurgery via the direct endoscopic approach under general anesthesia was performed for Reinke edema, and the Reinke edema contents in Reinke space were removed using cup forceps by microflap approach. CO₂ laser microlaryngoscopy was applied for vocal fold carcinoma, and Reinke space tissues of paracancerous site (5 mm away from cancer) were collected before the laser excision of lesions. Reinke space of normal vocal fold was dissected under microlaryngoscopy after total laryngectomy. Specifically, the epithelial layer was dissected using sickle knife and a small curved elevator, and then, Reinke space was directly visualized and collected using cup forceps under microlaryngoscope. All the tissues were used for reverse transcriptase polymerase chain reaction (RT-PCR) and western blot analyses of CD44, COX-2, and MMP-9 expressions and, in addition, the normal vocal fold tissues were also used for primary culture of vocal fold fibroblasts.

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