

Title

Expression of reactive oxygen species during wound healing of vocal folds in a rat model

Running title

ROS in vocal fold healing

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Abstract

Objectives: Previous studies have indicated that while normal wound healing requires low levels of reactive oxygen species (ROS), excessive amounts of ROS impair wound healing. In injured vocal folds, this excess may result in dysphonia due to scarring which is difficult to treat. However, the expression of ROS during vocal fold wound healing has yet to be investigated. In this study we assessed the expression and localization of ROS in injured vocal folds by immunohistochemistry.

Methods: Vocal folds of Sprague-Dawley rats were unilaterally injured by stripping the mucosa under transoral endoscopy. Larynges were harvested at specific time points after injury and immunohistochemically examined for 4-hydroxy-2-nonenal (4-HNE), a ROS marker, and the presence of inflammatory cells.

Results: 4-HNE-immunopositive cells were significantly increased in the lamina propria of injured vocal folds compared to normal vocal folds on post-injury days 1 and 3. More than half of the 4-HNE-immunopositive cells were also immunopositive for a macrophage and granulocyte-specific antibody.

Conclusions: This study suggests that a large amount of ROS is produced during early-phase wound healing, until post-injury day 3, and this period may be crucial for regulating ROS levels. Results also suggest that inflammatory cells may contribute to

ROS generation.

Key words: vocal folds, wound healing, reactive oxygen species, 4-hydroxy-2-nonenal

Introduction

Reactive oxygen species (ROS) are traditionally considered to be toxic agents in various pathologies including aging, cancer, inflammation, and post-ischemic injury^{1, 2}. ROS are also considered deleterious to wound healing. Although recent studies describe some beneficial roles for ROS during the healing process, as a defense against invading pathogens and as mediators of cellular signaling³⁻⁵, overexposure to ROS, known as oxidative stress, leads to impaired wound healing^{3, 6, 7}.

Vocal fold scarring following injury disrupts the layered structure of the lamina propria, which is essential for optimal vibration, often resulting in severe dysphonia⁸. Therefore the prevention of scarring remains an important therapeutic challenge. Considering the deleterious effects of excessive ROS, regulating the amount of ROS during wound healing may lead to the prevention of vocal fold scarring. However the effect of ROS on injured vocal folds has yet to be fully characterized. In the current study, we assessed the expression and localization of ROS in injured vocal folds in a rat model using immunohistochemistry for 4-hydroxy-2-nonenal (4-HNE), which is an oxidative stress marker for lipid peroxidation. In addition, previous studies on the skin have reported that inflammatory cells produce a large amount of ROS during the early phase of skin wound healing^{9, 10}. We therefore hypothesized that inflammatory cells may also be a

source of ROS during vocal fold wound healing. To address this hypothesis, we also attempted to identify inflammatory cells by assaying for granulocytes and macrophages.

Materials and methods

Animals

Thirty-eight thirteen-week-old male Sprague-Dawley rats were used in this study.

Three rats (six vocal folds) were chosen randomly as the control group without vocal fold injury. All experimental protocols were approved by the Animal Research Committee of the Kyoto University Graduate School of Medicine. Animal care was provided by the Institute of Laboratory Animals of Kyoto University.

Surgical Procedure

The animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (45mg/kg) and xylazine hydrochloride (4.5mg/kg) after inhalative sedation with diethyl ether. Atropine sulfate (0.005mg/kg) was also injected intraperitoneally to reduce the secretion of saliva and sputum in the laryngeal lumen. Rats were positioned on a custom-made operation platform. Visualization of the larynx was achieved with a transoral endoscope connected to video equipment. Topical anesthesia (5% lidocaine) was applied to the vocal folds to reduce their movement.

Unilateral vocal fold stripping was performed with microscissors and microforceps.

The contralateral vocal fold was left intact. Tateya et al. had previously performed vocal fold stripping in rats with a 25-gauge needle and microforceps¹¹. We therefore conducted preliminary studies to confirm that our stripping method was easy to perform and resulted in scarring similar to that produced by Tateya's method.

Larynges were harvested on post-injury days 1, 3, 5, 7, 14, 28, and 56 (five animals per time point). The rats were humanely euthanized with an intracardiac injection of Pentobarbital sodium (200mg/kg) after inhalative sedation with diethyl ether. The harvested whole larynges were immersed in 4% paraformaldehyde for 24 hours then in 30% sucrose for 24 hours. They were then soaked in embedding medium (Optimum Cutting Temperature Compound; Tissue-Tek Inc., Kyoto, Japan) and frozen quickly with liquid nitrogen. Ten-micrometer-thick cryostat sections (Leica, CM 1850 Kryostat; Leica Instruments GmbH, Nussloch, Germany) of whole larynges were made vertical to the glottis (coronal sections), air dried, and stored at -80°C until used.

Immunohistochemistry

After blocking, specimens were incubated overnight at 4°C with a rabbit anti-4-HNE antibody (Alpha Diagnostic International Inc., San Antonio, Texas, USA, 1:500) and OX41^{12, 13}, a mouse anti-granulocyte + macrophage antibody (Abcam Inc., Cambridge,

Massachusetts, USA, 1:400). Samples were washed then incubated for 1 hour at room temperature with Alexa-Fluor 488 goat anti-rabbit IgG (Invitrogen, France, 1:500) and Alexa-Fluor 555 donkey anti-mouse IgG (Invitrogen, France, 1:500) as secondary antibodies. 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI) was used to stain cell nuclei in each specimen. Lastly, samples were mounted on coverslips for observation under a fluorescent microscope (Biorevo, BZ 9000; Keyence Corp., Osaka, Japan). Omission of the primary antibody served as a negative control.

After the images were randomized, the numbers of 4-HNE-immunopositive cells across the entire lamina propria were counted manually under 20X magnifying power at three different anatomic sites per rat larynx: the anterior, middle, and posterior regions of the vocal fold; the data from each region were averaged. A small amount of 4-HNE is present within all animal tissue, therefore cells in which the intensity of immunofluorescence was greater than that of normal muscle cells were designated as immunopositive. Additionally, to confirm our hypothesis that inflammatory cells are involved in ROS generation, we counted the number of cells from each immunophenotype identified by double-immunostaining for 4-HNE and OX41 on post-injury days 1 and 3. Cells were counted manually under 40X magnifying power at five different regions from the middle portion of the vocal fold: the center of the lamina

propria, the anterior region, the posterior region, as well as the medial and lateral portions flanking the center of the lamina propria. The total number of cells per specimen was used to determine the ratio of each immunophenotype.

Intrajudge and interjudge reliability were separately assessed using Spearman's correlation. Ten percent of the specimens were selected at random and re-measured.

Initial measurements significantly correlated with repeated measurements (intrajudge reliability, $r=0.96$; interjudge reliability, $r=0.89$).

Histological examination

Elastica van Gieson staining was performed to identify collagen and elastin.

Statistical Analysis

The Kruskal-Wallis test was performed to evaluate the difference in the number of 4-HNE-immunopositive cells at each time point. On the basis of the Kruskal-Wallis test, the Steel's test was performed to compare the difference between the control and each time point. Differences of $p < 0.05$ were regarded as statistically significant.

Results

EVG staining showed severe fibrotic changes in the lamina propria of the injured vocal folds at day 56, demonstrating considerable tissue contraction and collagen deposition (Fig. 1).

Immunohistochemistry for 4-HNE

As a small amount of 4-HNE is present within all animal tissue, even uninjured control vocal folds had several 4-HNE-immunopositive cells. However, the number of 4-HNE-immunopositive cells in the injured vocal folds was significantly increased in the epithelium and the lamina propria on post-injury days 1 and 3, compared to control (Fig. 1, 2). On post-injury day 5, 4-HNE-immunopositive cells were substantially reduced and after day 7, few 4-HNE-immunopositive cells were detected (Fig. 1, 2).

Double immunostaining for 4-HNE and OX41 (granulocytes + macrophages)

OX41-immunopositive cells were never detected in controls, but appeared in the injured folds on post-injury days 1 and 3 (Fig. 3). After day 5, the number of OX41-immunopositive cells was reduced in the injured vocal folds. Of the total number of 4-HNE-immunopositive cells, 68.5% (SD=11.2) were OX41-immunopositive on post-injury day 1, and 53.0% (SD=5.1) on day 3. A few OX41-immunopositive and 4-HNE-immunonegative cells were also observed on day 7, which might mean that ROS production was almost stopped from OX41-immunopositive cells at this time point (Fig. 3).

Discussion

Wound healing is regulated by numerous factors including cells, growth factors,

cytokines, and hormones^{14, 15}. In addition to these, recent studies have demonstrated that the appropriate amount of ROS is another essential factor in the wound healing process as they provide defense against invading microorganisms and aid in cellular signaling^{3, 5, 16}. However, excessive amounts of ROS lead to impaired wound healing due to their high reactivity^{3, 6}.

ROS are normal by-products of cellular metabolism. Under basal conditions, 3-5% of the total oxygen consumed by mitochondria is converted to ROS^{5, 17}. In addition, inflammatory cells such as neutrophils and macrophages produce ROS as a defense against invading pathogens. This phenomenon is described as a “respiratory burst” and creates large amounts of superoxide radical anions via NADPH oxidase⁹. Superoxide radical anions are rapidly converted into hydrogen peroxide (H₂O₂) by superoxide dismutases (SODs). Although H₂O₂ itself is nonradical, it can easily cross cellular membranes and decompose into highly reactive hydroxyl radicals through the Fenton reaction in the presence of iron or copper ions^{6, 18}. Hydroxyl radicals are highly reactive causing significant oxidative stress in cells, including DNA modification, lipid peroxidation, and protein modification. Moreover, superoxide radical anions, in conjunction with nitric oxide, form toxic peroxynitrite which is itself not a free radical but is an unstable oxidant.

Physiologic defenses have evolved to control possible damage from the generation of ROS; these include ROS-detoxifying enzymes and low-molecular-weight antioxidants. The former includes SODs, glutathione peroxidases, catalase, and peroxiredoxins. While SODs catalyze the reaction from superoxide radical anion to H₂O₂, the others detoxify H₂O₂ before it can be converted to a hydroxyl radical. The low-molecular-weight antioxidants include not only endogenous molecules, such as glutathione, uric acid, and lipoic acid, but also exogenous molecules such as vitamins E and C, and carotenoids⁹. These antioxidants “sacrifice” themselves to be oxidized becoming less active radicals and therefore less damaging than the radicals they scavenge¹⁶. Although cells have multiple systems to protect themselves from oxidative stress, they can suffer severe damage due to oxidative stress if excessive amounts of ROS are produced or if the ROS-detoxifying system is insufficient.

The participation of ROS during wound healing has been well studied in the skin^{3, 5-7, 19}. ROS are involved in all stages of the wound healing process, including migration, adhesion, proliferation, neovascularization, remodeling, and apoptosis. Given these various roles, some amount of ROS is likely needed for wound healing. For example, Roy et al. reported the significance of H₂O₂ in regulating wound healing in vivo. The decomposition of endogenous H₂O₂ at the wound site by adenoviral catalase gene

transfer impaired wound angiogenesis and closure³. Likewise, Sen et al. reported that dysfunctional NADPH oxidase impaired the ability to produce superoxide radical anions, resulting in compromised wound healing in mice⁵. Conversely, excessive amounts of ROS lead to impaired wound healing due to their high reactivity. For example, it was shown that high doses of H₂O₂ impaired wound closure, while low doses moderately facilitated closure³. Kümin et al. reported the beneficial role of peroxiredoxin 6 during wound healing which is a member of the peroxiredoxin family that catalyzes the reduction of a broad spectrum of peroxides including H₂O₂. Moreover, the overexpression of peroxiredoxin 6 was shown to result in enhanced wound closure in aged mice⁷. Taken together, these results suggest that regulating the amounts of ROS may aid in the prevention of scar formation.

Recent studies have examined the contribution of ROS to vocal fold pathology. Branski et al. reported the upregulation of Heme oxygenase (HO)-1, an antioxidant enzyme, in Reinke's edema in smokers, and identified an increase in intra-cellular ROS in human vocal fold fibroblasts induced by cigarette smoke condensate²⁰. Similarly, Alper et al. reported about acute stress to excised vocal fold epithelium from ROS²¹. The present study aimed to further clarify the role of ROS on vocal fold wound healing using an in vivo set up. The in vivo concentration of ROS is difficult to determine directly because

of the short half-life and high reactivity of ROS⁹. Previous studies have indirectly determined ROS levels in vivo by determining the oxidation by-products of lipids, proteins, or DNA. 4-HNE is a major by-product of lipid peroxidation^{7, 22-27}. While it is found within all animal tissue at some level, oxidative stress increases the amount of 4-HNE, therefore this study employed 4-HNE as a marker of oxidative stress.

The current study demonstrated that the number of 4-HNE-positive cells increased significantly in the lamina propria from post-injury days 1 to 3, and suggested that cells such as fibroblasts might suffer from oxidative stress during this period in the wound healing process. Double immunostaining for 4-HNE and OX41 (granulocytes + macrophages) showed that control vocal folds contained a few 4-HNE-immunopositive and OX41-immunonegative cells with a spindle-shaped morphology, which suggested that a few fibroblast-like cells are involved in ROS under normal conditions. On post-injury days 1 and 3, many 4-HNE-immunopositive cells were detected and more than half of the 4-HNE-positive cells were OX41-immunopositive. This suggested that inflammatory cells contribute to the generation of ROS during the early phase of vocal fold wound healing. It was assumed that the 4-HNE-positive and OX41-negative cells are fibroblasts suffering oxidative stress, although the current study did not directly address this issue. After post-injury day 5, both 4-HNE-immunopositive cells and

OX41-immunopositive cells were decreased.

Histological analysis showed severe scarring in these injured vocal folds, suggesting that the early phase of wound healing, up to post-injury day 3, may be a crucial period for regulating oxidative stress.

Future studies will be needed to further confirm the effects of ROS on vocal fold scarring.

Conclusions

The current in vivo study examined the presence and localization of ROS in injured vocal folds. Immunohistochemistry showed that 4-HNE-immunopositive cells were present until post-injury day 3 in the lamina propria, and more than half of the 4-HNE-immunopositive cells were also OX41-immunopositive, suggesting ROS are produced during the early phase of vocal fold wound healing and inflammatory cells contribute to the generation of ROS. These data provide a basic understanding of the expression of ROS during vocal fold wound healing.

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Figure legends

Figure 1

Representative examples of immunohistochemistry for 4-hydroxy-2-nonenal (4-HNE) and Elastica van Gieson (EVG) staining at each time point in coronal sections of normal and injured vocal folds. 4-HNE appears green, while nuclei appear blue.

4-HNE-immunopositive cells are increased in the lamina propria and are most evident on post-injury days 1 and 3. EVG staining reveals severe scarring with tissue contraction and collagen deposition (brown in the EVG staining) on day 56. Scale bar: 100µm.

Figure 2

Number of 4-HNE-immunopositive cells in the lamina propria.

4-HNE-immunopositive cells in the lamina propria were significantly increased on post-injury days 1 and 3 compared to control. *: significant difference ($p < 0.05$) compared to control (Kruskal-Wallis test, post hoc Steel test).

Figure 3

Double immunostaining for 4-HNE (green) and OX41 (granulocytes + macrophages, red).

OX41-immunopositive cells were never detected in controls, but appeared in the injured

folds on post-injury days 1 and 3. After day 5, the number of OX41-immunopositive cells was reduced in the injured vocal folds. Of the total number of 4-HNE-immunopositive cells, 68.5% (SD=11.2) were OX41-immunopositive on post-injury day 1, and 53.0% (SD=5.1) on day 3. A few OX41-immunopositive and 4-HNE-immunonegative cells were also observed on day 7. White arrows: 4-HNE- immunopositive and OX41-immunonegative cells; yellow arrows: 4-HNE-immunonegative and OX41-immunopositive cells; scale bar: 50 μ m.

Figure 1

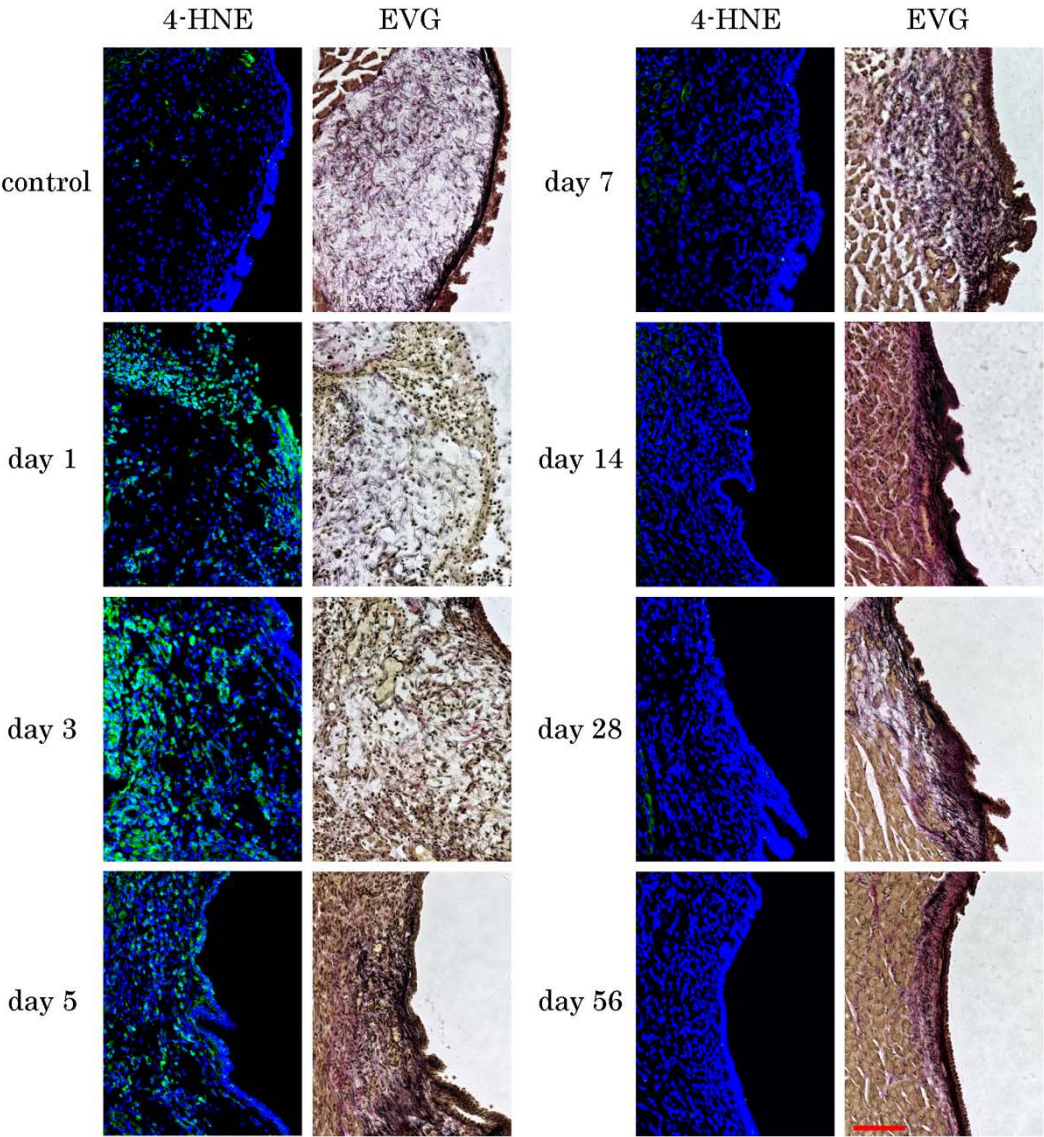


Figure 2

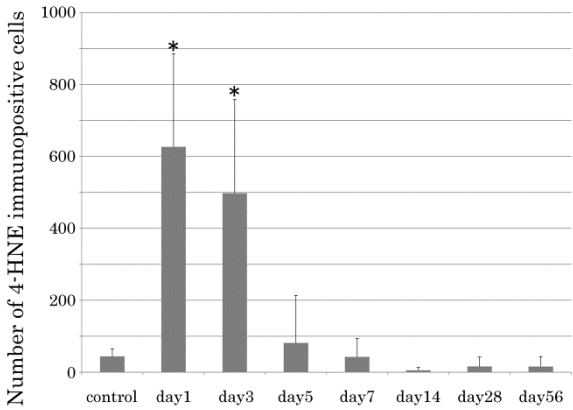


Figure 3

