Distribution and Appearance of Ki-67, IL-1 α , IL-10 and PGP 9.5 in Reinke's Oedema-affected Larynx Tissue Compared With Control Tissue

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Abstract

Background: Reinke's oedema (benign diffuse swelling of the vocal folds) induced various morphological changes occurs in the superficial lamina propria also known as the Reinke's space. Smoking, laryngopharyngeal reflux and vocal fold abuse can promote the development of this condition as well as lead to vocal fold dysfunction and injury. Patients with Reinke's oedema complain of vocal problems. 3,47/1000 is estimation of the general population prevalence of Reinke's oedema. Investigation of the distribution and appearance of Ki-67, IL-1 α , IL-10 and PGP 9.5 in Reinke's oedema-affected larynx tissue compared with control tissue was the aim of the work.

Methods: Routine histological and immunohistochemical analysis were applied for specimens with Reinke's oedema and the control group. Biotin-streptavidin biochemical method was used for detection of Ki-67, PGP 9,5, IL-10 and IL-1 α . Immunoreactive cells appearance local distribution were evaluated using semiquantitative grading method. Statistical analysis of the data was performed using nonparametric statistical methods (Mann Whitney U test and Spearman's rank coefficient).

Results: A high positive correlation was found between IL-1 α and PGP 9.5 epithelial immunoreactive cells and between epithelial and subepithelial IL-10 cells in the control group. Mann-Whitney U tests revealed significant differences in immunoreactive markers between the patients and the control group. With exception of PGP 9.5 positive subepithelial nerves, all other examined markers revealed higher number of immunoreactive positive structures in the patient tissue.

Conclusions: Intensive proliferation of the surface epithelium was observed in Reinke's oedema affected larynx tissue. Increased expression of IL-1 α structures is linked to tissue remodeling, inflammation and remodeling. Notable increase in IL-10 positive structures is indicating to the dominant anti-inflammatory tissue response. PGP 9.5 expression increase is involved in the morphopathogenesis of Reinke's oedema.

Trial registration: This study was approved by the Ethical Committee of Riga Stradins University, issued on 31-10-2019, No 6-2/2/25.

Background

Reinke's oedema (RE) is benign diffuse swelling of the vocal folds, also known as various polypoid degeneration and smoker's polyps. These lesions can be unilateral as well as bilateral [1]. The general population prevalence of Reinke's oedema is 3,47/1000 [2]. Changes appear in the superficial lamina propria (SLP), also called Reinke's space, which has an important functional role in vocal fold vibrations as well as voice production [3].

Reinke's space consists of regularly interrelated parallel connective tissue fibres. Accumulation of the oedematous transudate in Reinke's space can lead to varying levels of dysfunction of the vocal folds. Oedematous transudate is in motion during the phonation process. Overexpression of the glottic wave created by Reinke's oedema results in a deep unmodulated voice [3, 4]. Reinke's oedema causes the voice to become hoarse; thus, patients complain of vocal problems [2, 5].

Smoking is considered to be the main risk factor for Reinke's oedema; however, laryngopharyngeal reflux and vocal fold abuse also contribute to the development of this condition. Allergy is not considered a crucial factor in aetiology of Reinke's oedema [6-9]. The probability of suffering from Reinke's oedema is higher in older women (>39 years old), but a predilection in males has also been found by some authors [2, 10].

Various morphological changes can be found in Reinke's oedema, such as subepithelial vascularization, thickened basement membrane, dilated capillaries, dense reticular fibres around the vessels, inflammatory cell infiltration, elongated intracellular junctions and widening of intracellular spaces [11, 12].

Risk factors for Reinke's oedema can lead to vocal fold injury. The reaction of the vocal fold mucosa due to the damage may be excessive extracellular matrix production. The widely known proliferation marker Ki-67 indicated that epithelial cells above Reinke's oedema had higher proliferative activity than the epithelial cells without oedema [4]. A strict correlation between the nuclear protein Ki-67 and cell proliferation has been found [13]. Ki-67 expression is high in proliferating cells and absent in resting cells [14]. Thus, for determination of the cell growth fraction, Ki-67 is used, and Ki-67 staining intensity is increased from onset S phase until metaphase [15].

In the case of vocal fold trauma, the inflammatory response has demonstrated a major role in the removal of injured tissue and in reactions with invading organisms. Wound healing initially starts with the inflammatory stage, where recruitment of inflammatory cell infiltration, synthesis of growth factors, cytokines and blood flow changes occur; thus, inflammatory inhibitors, including cytokines, are important. The interleukin-10 (IL-10) gene has anti-inflammatory properties and is produced by B cells, mast cells, eosinophils, macrophages and many subsets of T cells [16, 17].

Damage caused to tissue can initiate cellular and tissue signaling pathways, increasing mediator (for example, cytokine) release. IL-10 has an essential role in the regulation of immune responses, limiting the host immune response, preventing damage and maintaining homeostasis [18]. It has been reported that tissue motion can trigger an increase in IL-10 concentration [19]. Interestingly, average normalized IL-10 concentrations have been detected to be highest 24 h after resonant voice exercises and lowest after vocal rest [16].

An increased intensity of phonation causes increased expression of inflammatory mRNA in normal rabbit vocal folds. One of the major proinflammatory interleukin-1 (IL-1) family members is IL-1 α . Two main proinflammatory forms of IL-1 are IL-1 α and IL-1 β , whose effects are mediated through the IL-1R1 receptor. Healthy individual mesenchymal-originated cells constitutively contain the IL-1 α precursor [20-23]. The IL-1 α precursor is localized in the cytosol and the nucleus, where it is bound to chromatin [24]. IL-1 α is released from cells due to cell death or injury. IL-1 α activity may be increased by proteolysis [25]. The release of IL-1 α from macrophages is triggered by necrotic cells in vitro [26]. Blockage of IL-1 α influences the acute neutrophilic response but has barely any effect on monocytes. IL-1 α necrotizing cells stimulate neutrophil infiltration [27].

Another important immunohistochemical marker is protein gene peptide 9.5 (PGP 9.5), which is also known as ubiquitin C-terminal hydrolase (UCHL-1). Ubiquitin carboxy-terminal ethyl esterase activity has been demonstrated by PGP 9.5. PGP 9.5 is widely used as a neural and neuroendocrine cell marker [28-31].

The aim of this study was to investigate the distribution and appearance of Ki-67, IL-1 α , IL-10 and PGP 9.5 in Reinke's oedema-affected larynx tissue compared with control tissue.

Methods

2.1. Characteristics of the subjects

This study was approved by the Ethical Committee of Riga Stradins University, issued on 31-10-2019, No 6-2/2/25. We examined five vocal fold specimens from patients with Reinke's oedema (1 male and 4 females) aged 58 to 71

years (all were irregular smokers), whereas for the control group, we evaluated seven larynx tissue samples obtained during a postmortem autopsy. The control specimens used for this study were the property of the Institute for Anatomy and Anthropology of Riga Stradins University.

2.2. Routine histological analysis

Biopsy samples were fixed for 24 h with 2% formaldehyde and 0.2% picric acid in 0.1 M phosphate (pH=7.2). After fixation, Tyrode's buffer was applied to biopsy tissue for 12 h and then embedded into paraffin. Four-micrometre-thick sections were cut and stained with haematoxylin and eosin. This staining was used to evaluate tissue morphological structure.

2.3. Immunohistochemical analysis

For detection of Ki-67, PGP 9.5, IL-10, and IL-1 α biotin-streptavidin biochemical method was used. Rabbit antibodies were used for the detection of Ki-67 (CMC27531040, diluted 1:100, Sigma-Aldrich Company, CA, USA), IL-10 (orb100193, diluted 1:600, Biorbyt, Ltd., Cambridge, UK), PGP 9.5 (439273A, diluted 1:100, Zymed Laboratories, Invitrogen Corporation, Carlsbad, CA, USA), and IL-1 α (orb308737, diluted 1:100, Biorbyt Ltd.).

Immunoreactive cells were evaluated in larynx tissue in three random visual fields at X400 (ocular X10, objective X40). Ki-67 immunoreactive cells were counted in three randomly selected visual fields at X400. For further data analysis, the average number of structures was chosen. Slides were examined under a light microscope (Leica DC 300F, Leica Biosystems Richmond, Richmond, VA, USA). Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) was used for image analysis and editing.

2.4. Quantification of immunoreactive cells

For evaluation of the appearance and local distribution of IL-1 α , IL-10 and PGP 9.5 immunoreactive cells, a semiquantitative grading method was used [31-33]. The semiquantitative scoring system was as follows: 0 - no positive structures; 0/+ - occasional positive structures; + - few positive structures; +/++ - few to a moderate number of positive structures; ++ - moderate number of positive structures; ++/+++ - moderate to numerous positive structures; +++ - numerous positive structures; +++/++++ - numerous to abundant positive structures; ++++ - an abundance of positive structures in the visual field.

2.5. Statistical analysis

For statistical analysis of the data, nonparametric statistical methods were used. Almost all data were ranked as ordinal values. Only Ki-67 was ranked as a scale value. For analysis of the IL-1 α , IL-10, PGP 9.5- and Ki-67- positive structures within the larynx samples from patients with Reinke's oedema and controls, the Mann-Whitney U test was used [33, 34].

The collected data were transformed in SPSS as follows: no stained cells, 0; occasional stained cells, 0.5; few stained cells, 1.0; few to moderate number of stained cells, 1.5; moderate number of stained cells, 2.0; moderate to numerous stained cells, 2.5; numerous stained cells, 3.0; numerous to abundant stained cells, 3.5; and abundant stained cells, 4.0.

Spearman's rank correlation coefficient r_s (Spearman's rho) was calculated for evaluation of the cross-compliance of two variables [35]. The acquired correlation coefficient (r_s) results were interpreted as follows: 0.80-1.00 very

high, 0.60-0.80 high, 0.40-0.60 moderate, 0.20-0.40 low and <0.2 very low correlation. Statistically significant values were considered p values of ≤ 0.05 . Statistical analysis of the data was performed using the statistical programme SPSS v22.0 (IBM Co., Chicago, IL).

Results

3.1. Findings of routine histological analysis

Various degrees of basal cell hyperplasia and thickening of the basement membrane were found in the larynx tissue from the patients with Reinke’s oedema. Intraepithelial infiltration was detected in three patient samples, while in the control group, infiltration was not predominant (Figures A1 and A2).

3.2. Immunohistochemistry findings

IL-10- and IL-1 α -positive epithelial and subepithelial cells and PGP 9.5-positive subepithelial nerves were observed in all tissue samples (Table 1). There were no differences in IL-10 expression between the epithelium and subepithelium in the patient group, being expressed from moderate to numerous positive structures. IL-10 fluctuated from a few to a moderate number of immunoreactive cells in the epithelium, while only a few subepithelial positive structures were observed in the controls (Table 1, Figures B1 and B2). Ki-67 expression was observed only in the patient samples (Table 1, Figures C1 and C2) and displayed 174.4 $\pm$ 23.81 positive epithelial cells. The number of PGP 9.5-positive epithelial neuroendocrine cells (NECs) and PGP 9.5-positive subepithelial nerves varied from few to moderate in the patients. The controls showed a stable number of a few PGP 9.5-positive subepithelial nerves. No PGP 9.5 epithelial NEC expression was observed in this group (Table 1, Figures D1 and D2). The controls demonstrated stable expression of IL-1 α both in the epithelium and subepithelium, staining occasional structures. Patient tissue revealed numerous IL-1 α epitheliocytes and moderate to numerous positive structures in the subepithelium (Table 1, Figures E1 and E2).

Table 1. Relative number of immunoreactive structures in the larynx samples from patients and the controls

Factors Groups	PGP 9.5 epithelial NEC	PGP 9.5 subepithelial nerves	IL-1 α epithelial	IL-1 α subepithelial cells	IL-10 epithelial	IL-10 subepithelial cells	Ki-67 $\pm$ SD
Patients	+ / ++	+ / ++	+++	++ / +++	++ / +++	++ / +++	174.4 $\pm$ 23.81
Controls	0	+	0 / +	0 / +	+ / ++	+	0

Abbreviations: Ki-67 – proliferation marker; IL-1 α – interleukin 1 *alpha*; IL-10 interleukin 10; PGP 9.5 - protein gene peptide 9.5, NEC – neuroendocrine cells, SD – standard deviation

3.3. Statistical analyses

Statistically significant data were found between the patient and control groups in PGP 9.5-positive epithelial NECs (Mann-Whitney U: 1.0; Z-score: -2.81: p-value: 0.005), IL-1 α epitheliocytes (Mann-Whitney U: 0; Z-score: -2.90: p-value: 0.04), IL-1 α -positive subepithelial cells (Mann-Whitney U: 0; Z-score: -2.98: p-value: 0.03), IL-10 epitheliocytes (Mann-Whitney U: 4.5; Z-score: -2.14: p-value: 0.033), IL-10 subepithelial cells (Mann-Whitney U: 0; Z-score: -2.94: p-value: 0.03), and Ki-67 epitheliocytes (Mann-Whitney U: 0; Z-score: -3.17: p-value: 0.002) (Table 2).

Table 2. Results of the Mann-Whitney U test between patients and the control group

Immunoreactive marker	Location	Mann-Whitney U	Z-score	p-value
PGP 9.5	epithelial NEC	1.0	-2.81	0.005
IL-1 α	epithelial	0	-2.90	0.04
IL-1 α	subepithelial	0	-2.98	0.03
IL-10	epithelial	4.5	-2.14	0.033
IL-10	subepithelial	0	-2.94	0.03
Ki-67	epithelial	0	-3.17	0.002

Abbreviations: Ki-67 – proliferation marker; IL-1 α – interleukin 1 *alpha*; IL-10 interleukin 10; PGP 9.5 - protein gene peptide 9.5, NEC – neuroendocrine cells

Spearman's rank coefficient revealed a high positive correlation between IL-1 α and PGP 9.5 in control epithelial immunoreactive cells [$r_s=0.794$; $p=0.033$; $N=7$] and between epithelial IL-10 and IL-10 subepithelial cells in the control group [$r_s=0.759$; $p=0.048$; $N=7$].

Discussion

In this study, almost all examined markers in Reinke's oedema-affected larynx tissue revealed significant ($p < 0.05$) changes between the patients and the controls, with a higher number of immunoreactive positive structures in the patient tissue, with the exception of PGP 9.5-positive subepithelial nerves.

Ki-67 showed significant changes in the epithelium with higher expression in the patients than the controls. Only the proliferation marker Ki-67 showed no expression in the epithelium of the controls from all examined markers. We assume that differences between the patients and controls may be explained by proliferative changes and that highly increased Ki-67 expression findings in patients reflect an active proliferative process. These changes could be caused by continuous inflammation in larynx tissue due to persistent risk factors (smoking, inflammation). Notably, the control samples used in this research were obtained during a postmortem autopsy. However, Ki-67 requires an active cell division cycle for its expression, explaining the absence of Ki-67 in the controls. Reduction and lack of Ki-67 expression have been found in postmortem material, as proliferation does not occur [13-15, 36].

Significant changes were also found in IL-1 α expression. Reinke's oedema affected the larynx epithelium, and the subepithelium revealed higher expression of IL-1 α than that of the controls. We suggest that proinflammatory cytokine IL-1 α expression in patients could be higher due to cellular stress-associated factors and cell damage, and a broad spectrum of stimuli can cause these expression changes. For example, a direct effect of air flow on the larynx as well as possible microbial contamination, inhaled particles and extensive vocal exercise may constitutively increase IL-1 α expression by promoting inflammation, and intraepithelial infiltration was detected in routine histological examination in our patients. Thus, this observation supports the concept that IL-1 α release might be related to cell injury. In accordance with other authors, theoretically, this cytokine may be used as an indicator for tissue health. Notably, elevated levels of IL-1 α expression are also related to epithelial proliferation, loss of cell attachment and cellular apical migration and could modulate larynx tissue proliferation and

remodelling processes. We hypothesized that increased IL-1 α in Reinke's oedema is connected to persistent inflammation, proliferation and tissue remodelling [20, 36, 37].

A high relative number of stained cells and a significantly higher number of epithelial and subepithelial IL-10 expression in patients than controls suggests that IL-10 plays an important role in Reinke's oedema. Not only IL-1 α but also IL-10 is involved in the regulation of neutrophil infiltration during the inflammatory process, while elevated IL-1 α expression indicates inflammation in patients. We assume that IL-10 expression is related to the limitation of tissue damage due to anti-inflammatory properties by suppressing proinflammatory cytokines. Additionally, tissue motion triggers IL-10 expression, thereby demonstrating that IL-10 expression is related to the phonation process. These aspects may explain the higher IL-10 expression in our patients. Prolonged phonation could damage the vocal folds, stimulating inflammation and IL-1 α expression, thus indirectly repeatedly stimulating IL-10 expression [16, 18].

Reinke's oedema-affected larynx tissue revealed significant changes in PGP 9.5 expression, where patient epithelial NECs had higher expression than control epithelial NECs. Studies have shown that excess neuropeptides lead to an increase in immune infiltrates, which can promote the remodelling process. Thus, we suggest that increased PGP 9.5 expression in epithelial NECs of patients could be a result of persistent inflammation and chronic irritation of environmental factors, which was also observed by other scientists [38].

Finally, we presume that KI-67, IL-1 α , IL-10 and PGP 9.5-positive epithelial NECs have a role in the developmental process of the morphopathogenesis in Reinke's oedema. These immunoreactive markers could be involved in inflammation, proliferation and remodelling of the larynx.

Conclusion

Reinke's oedema-affected larynx tissue shows intensive proliferation of the surface epithelium, probably due to persistent environmental factors (smoking) and inflammation.

An increased number of IL-1 α structures in the larynx epithelium and subepithelium of patients with Reinke's oedema is linked to inflammation, proliferation and tissue remodelling.

Notably, a significant increase in IL-10-positive cells indicates the dominant anti-inflammatory tissue response.

Notable increases in PGP-9.5-containing cells in Reinke's oedema affected the larynx epithelium, indicating neuropeptide involvement in the morphopathogenesis of this disease.

Abbreviations

RE – Reinke's oedema

Ki-67 – proliferation marker

IL-1 α – interleukin 1 *alpha*

IL-10 – interleukin 10

PGP 9.5 – protein gene peptide 9.5

NEC – neuroendocrine cells

SD – standard deviation

Declarations

Ethics approval and consent to participate

This study was approved by the Ethical Committee of Riga Stradins University, issued on 31-10-2019, No 6-2/2/25.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

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Authors' contributions

Conceptualization, M.P., D.S.; methodology, M.P.; software, V.K.; validation, M.P.; formal analysis, V.K. and G.S.; investigation, V.K. and D.S.; resources, M.P. and G.S.; data curation, M.P.; writing—original draft preparation, V.K.; writing—review and editing, M.P., D.S. and G.S.; visualization, V.K. and M.P.; supervision, M.P.; project administration, M.P.; funding acquisition, M.P. All authors have read and agreed to the published version of the manuscript.

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Figures

Figure 1

Representative microphotographs for routine morphological evaluation of slides stained by haematoxylin and eosin (A1-A2). Microphotographs stained by immunohistochemistry (B1-E2). Figure A1. Image of intact larynx mucosa of the control group. X250. Figure A2. Basal cell hyperplasia and thickening of the basement membrane in the patient. X200. Figure B1. Note a few to a moderate number of IL-10-positive structures in the epithelium and a few in the subepithelium in the control. IL-10, X200. Figure B2. IL-10 is present in a moderate number to numerous epithelial and subepithelial cells in the patient. IL-10, X250. Figure C1. Almost no positive Ki-67 structures were observed in the control group. Ki-67, X200. Figure C2. Numerous Ki-67-positive cells in the patient. Ki-67, X250. Figure D1. No PGP 9.5-positive epithelial NECs and few positive subepithelial nerves were observed in

the control group. PGP 9.5, X200. Figure D2. Few to moderate numbers of PGP 9.5-positive epithelial NECs and subepithelial nerves were observed in the patient group. PGP 9.5, X250. Figure E1. Occasional IL-1 α epithelial and subepithelial expression in the control. IL-1 α , X200. Figure E2. Numerous positive IL-1 α epitheliocytes and moderate to numerous positive structures in the subepithelium of the patient. IL-1 α , X250.