Tohoku J. Exp. Med., 2024, **264**, 93-99

Expression of Vascular and Tissue Repair Factors in Laryngeal Granulomas

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Voice abuse, chronic cough, laryngeal surgery, and tracheal intubation may lead to injury and subsequent repair/remodeling in the vocal fold mucosa. Periostin is known to be involved in airway remodeling and is also associated with various otolaryngological diseases. D-*β*-aspartic acid is the major isomer of D-aspartic acid found in tissues of elderly individuals. In ischemic heart disease, increased CD31 expression has been observed around cardiomyocytes during remodeling, and endothelial proliferation has been reported at these sites. In this study, we investigated the expression and role of CD31, CD34, D-*β*-aspartic acid, and periostin in the formation of laryngeal granulation tissue. This retrospective study involved five patients who underwent surgical treatment for laryngeal granulation tissue. The expressions of CD31, CD34, D-*β*-aspartic acid, and periostin in surgical samples were investigated by immunohistochemistry. Histologically, the specimens showed inflammatory cell infiltration, fibrin exudation, fibrosis, and neovascularization, but no tumor cells. No stratified squamous epithelial covering was observed. The expression of periostin and D-*β*-aspartic acid was also observed in the specimens. CD31-positive cells (endothelial cells) and CD34-positive cells (progenitors of endothelial cells) were observed in the specimens. Our results indicate that the overexpression of CD31, CD34, D-*β*-aspartic acid, and periostin may play a role in the pathogenesis of laryngeal granulation tissue, and we speculate that D-*β*-aspartic acid and periostin could serve as novel biomarkers and therapeutic targets for laryngeal granulation tissue.

Keywords: CD31; CD34; D-*β*-aspartic acid; laryngeal granulation tissue; periostin
Tohoku J. Exp. Med., 2024 October, **264** (2), 93-99.
doi: 10.1620/tjem.2024.J056

Introduction

Granulation tissue is a newly formed mass of connective tissue and capillaries that appear on a wound. Laryngeal granulation tissue is an inflammatory mass that tends to occur near the vocal fold process in the posterior part of the median portal, and is classified according to its cause into four categories: that resulting from laryngeal surgery, that occurring after endotracheal intubation, that caused by contact with the voice through overuse or force, and that of unknown cause (Kleinsasser 1969). The formation of this tissue can be attributed to voice overuse or forceful generation of voice, where the epithelium is damaged by friction caused by coughing or strenuous raising of the voice, resulting in an inflammatory mass at the site of the injury. Conditions occurring after endotracheal intuba-

Received March 26, 2024; revised and accepted June 18, 2024; J-STAGE Advance online publication June 27, 2024
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tion are similar, caused by irritation from the intubation. Recently, this condition has also been associated with gastric acid reflux into the pharynx. Pathologically, it is characterized by chronic inflammation and is not considered a tumor.

In the context of laryngeal diseases, the expressions of CD31, CD34, D-*β*-aspartic acid, and periostin have been reported in vocal polyps, which are non-neoplastic masses (Tateda et al. 2022, 2023a, b). Immunological studies on periostin and D-*β*-aspartic acid in laryngeal granulation tissue, including CD31 and CD34, which are thought to be involved in neovascularization in chronic inflammation, have not yet been performed. In the present study, we immunohistochemically examined the expression of periostin, CD31, and CD34 in seven samples from five patients undergoing surgical treatment for laryngeal granulation tissue, and D-*β*-aspartic acid in three samples.

Materials and Methods

Subjects

The patient group consisted of five individuals with laryngeal granulation tissue, aged 54–84 (mean age ± standard deviation (SD) 72.8 ± 11.2) years, who did not respond to at least three months of conservative therapy, necessitating surgical removal of the laryngeal granulation tissue by endolaryngeal microsurgery at the Division of Otolaryngology, Tohoku Medical and Pharmaceutical University Hospital, between January 2016 and November 2018. All patients had single lesions, and only cases with typical clinical and histological findings were included in this study.

The laryngeal granulation tissues were histologically diagnosed at the Division of Pathology, Tohoku Medical and Pharmaceutical University Hospital. The study was approved by the ethics review committees of both Tohoku Medical University Hospital (Approval no. 2016-2-058) and Tohoku University Graduate School of Medicine (Approval no. 2017-1-321), and the requirement for informed consent was waived owing to the opt-out policy adopted in the study.

Immunohistochemistry analysis to detect CD31, CD34, and periostin

Sections (4 μm thick) were prepared from paraffin-embedded tissue blocks and deparaffinized, followed by rehydration in alcohols. Endogenous peroxidase activity was blocked using 3% H_2O_2 in absolute methanol at room temperature for 10 min. All sections were preincubated in Protein Block Serum-Free (Cat. No. X0909; Dako, Santa Clara, CA, United States) at room temperature for 20 min to block nonspecific background staining. The sections were then treated with monoclonal antibodies (diluted 1:100) against CD31 (Cat. No. M0823, Clone: JC70A; Dako, Santa Clara, CA, United States) and CD34 (Cat No. 413361, Clone: NU-4A1; Nichirei Biosciences Inc., Chuo, Tokyo, Japan), and with a polyclonal anti-periostin antibody (diluted 1:2730), and kept at 4°C overnight. The sections were then incubated with biotin-conjugated anti-mouse antibody (Cat. No. 42402, N-Histofine SABPO (M); Nichirei Biosciences Inc.) for 30 min and then with streptavidin-labeled peroxidase for another 30 min at room temperature. Finally, they were incubated with liquid diaminobenzidine (DAB) and counterstained with hematoxylin for CD31 and CD34 immunostaining. For periostin immunostaining, after the primary antibody incubation, sections were incubated with a secondary antibody, the EnVision+ Dual Link System-HRP (Cat. No. K4063; Dako) for 60 min at room temperature and finally with DAB + Substrate Chromogen System (Cat. No. K3468; Dako). They were counterstained with hematoxylin.

Immunohistochemistry analysis to detect Dβ-aspartic acid

About 4-μm thick sections were prepared from paraffin-embedded tissue blocks, deparaffinized, and rehydrated. Antigen retrieval was performed by heating the sections in antigen retrieval solution (pH 9.0) at 121°C in an autoclave for 5 min. Endogenous peroxidase activity was blocked by treating the sections with 3% H_2O_2 in absolute methanol at room temperature for 10 min. Before antibody incubation, all sections were preincubated with normal goat serum (Cat. No. MP-7444, ImmPRESS HRP REAGENT Kit Anti-Rat; Vector Laboratories, Newark, CA, United States) at room temperature for 20 min to block nonspecific background staining. The sections were then treated with a polyclonal antibody (diluted 1:800) against D-aspartic acid (Cat. No. AB-T047, Advanced Targeting Systems, Carlsbad, CA, United States) and kept at 4°C overnight. Thereafter, the sections were incubated with a secondary antibody (Cat. No. MP-7444, ImmPRESS HRP REAGENT Kit Anti-Rat; Vector Laboratories) for 30 min at room temperature and finally incubated with liquid DAB+ Substrate Chromogen System and counterstained with hematoxylin.

Assessment of slides

Immunostained sections were examined under a light microscope at × 20, × 40, and × 100 magnifications using an eyepiece reticle. Cell counts were expressed as means per high-power field, corresponding to an area of 0.202 mm^2. At least two sections were immunostained, and more than five areas per section were evaluated using the reticle. Results are expressed as the presence or absence of positive cells as follows: (–): Negative. (+): Positive.

Results

Histological subtypes of laryngeal granulation tissue

A characteristic feature of laryngeal granulation tissue is the absence of epithelium.

Fig. 1 shows control samples (A1–5), vocal fold polyps (B1–6), and laryngeal granulation tissue samples (C1–6, D1–6, E1–6).

Fig. 1 A1–5 Images of a 73-year-old Japanese man with hypopharyngeal cancer are presented as the control. A

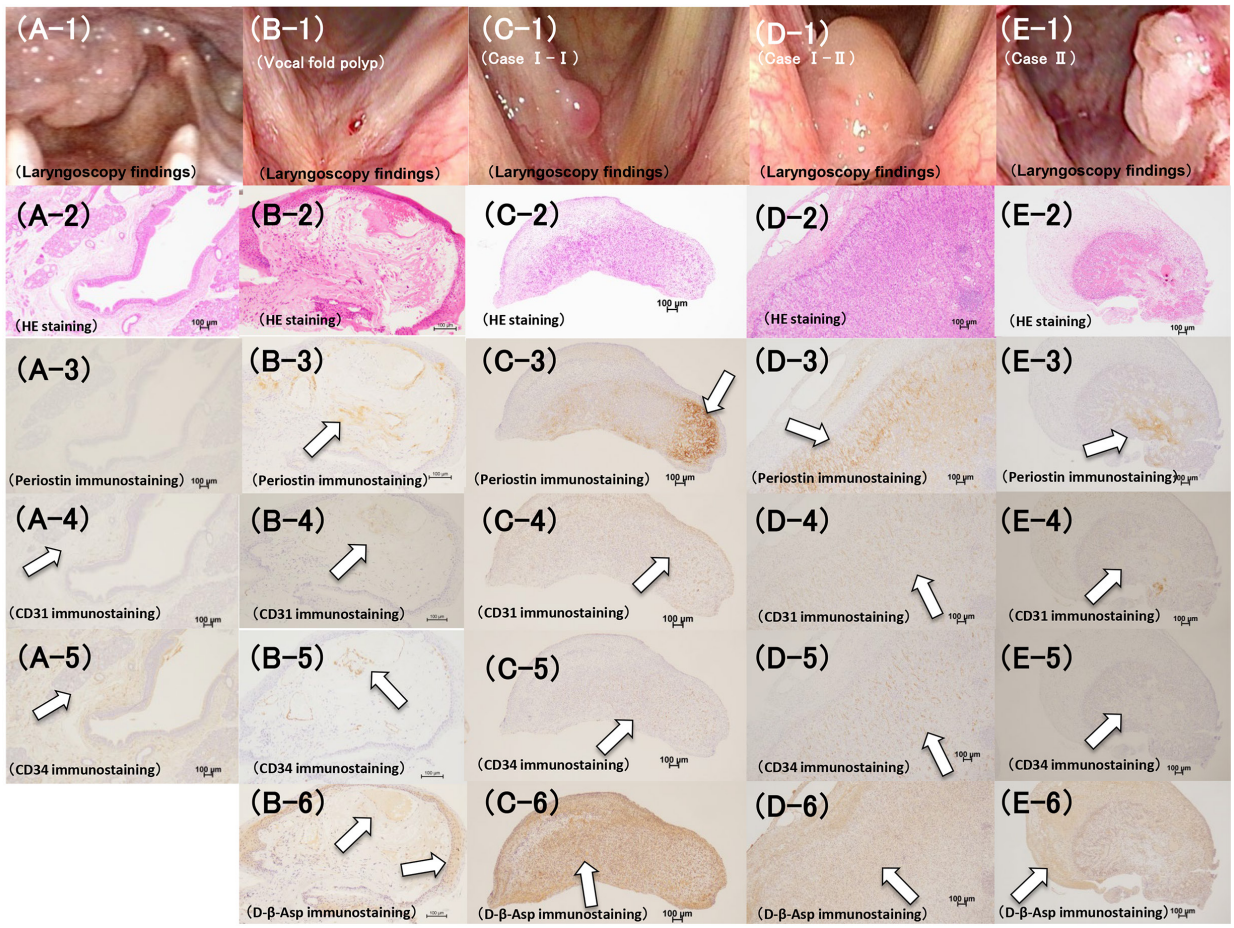

Fig. 1. Laryngeal findings, hematoxylin and eosin (HE) staining and immunohistochemical staining of periostin, CD31, and CD34 in control specimens, vocal fold polyps, and laryngeal granulation tissue (A-1) A 73-year-old Japanese man with hypopharyngeal cancer is presented as the control. A hypopharyngeal mass is observed in the right pyriform sinus. A normal vocal fold mucosa specimen was used. (A-2) Mucosal epithelium and lamina propria (HE staining, original magnification × 40).
(A-3) Expression of periostin was not detected (immunostaining, original magnification × 40).
(A-4) Minimal expression of CD31 and (A-5) CD34 was detected (corresponding arrows) (immunostaining, original magnification × 40).
(B-1) A 73-year-old Japanese man with red polyps on the left vocal fold. (B-2) This case is a typical vascular-hyaline type. Dense eosinophilic submucosal deposition of fibrin material closely apposed to a vascular space is observed (HE staining, original magnification × 100). (B-3) Periostin was observed from the basement membrane to the lamina propria in varying degrees (arrow) (immunostaining, original magnification × 100). (B-4) Noticeable expression of CD31 and (B-5) CD34 was detected (corresponding arrows) (immunostaining, original magnification × 100). (B-6) D-*β*-aspartic acid was detected in the epithelium and stroma (arrow) (immunostaining, original magnification × 100).
(C-1) An 84-year-old Japanese man with red laryngeal granulation tissue on the right vocal fold. (C-2) The specimens consisted of inflammatory cell infiltration, edema, fibrin exudation, fibrosis, and neovascularization, without epithelium and tumor cells. (HE staining, original magnification × 40). (C-3) Expression of periostin (C-4) CD31, (C-5) CD34, and (C-6) D-*β*-aspartic acid was detected (corresponding arrows) (immunostaining, original magnification × 40 for all).
(D-1) An 84-year-old Japanese man with white laryngeal granulation tissue on the right vocal fold. (D-2) The specimens consisted of inflammatory cell infiltration, edema, fibrin exudation, fibrosis, and neovascularization, without epithelium and tumor cells. (HE staining, original magnification × 40).
(D-3) Expression of periostin, (C-4) CD31, (C-5) CD34, and (C-6) D-*β*-aspartic acid was detected (corresponding arrows) (immunostaining, original magnification × 40 for all).
(E-1) A 54-year-old Japanese man with white laryngeal granulation tissue on the left vocal cord cartilage area. (E-2) The specimens consisted of inflammatory cell infiltration, edema, fibrin exudation, fibrosis, and neovascularization, without epithelium and tumor cells. (HE staining, original magnification × 40). (E-3) Expression of periostin, (E-4) CD31, (E-5) CD34, and (E-6) D-*β*-aspartic acid was detected (corresponding arrows) (immunostaining, original magnification × 40 for all).

Table 1. Patient demographics, Clinical features and Pathological findings.

Case	age/gender	Chief complaint	Side	Period illness (month)	Tobacco	Alchohol	Reflex esophagitis	Periostin	CD31	CD34	D-β-Asp
I-I	84/M	Hoarseness	R	12M	(+)	(+)	(–)	(+)	(+)	(+)	(+)
I-II	84/M	Hoarseness	R	1M	(+)	(+)	(–)	(+)	(+)	(+)	(+)
II	54/M	Laryngeal discomfort	L	7M	(+)	(+)	(–)	(+)	(+)	(+)	(+)
III	77/M	Hoarseness	L	12M	(–)	(+)	(–)	(+)	(+)	(+)	ND
IV-I	74/F	Hoarseness	L	7M	(–)	(–)	(–)	(+)	(+)	(+)	ND
IV-II	74/F	Hoarseness	L	2M	(–)	(–)	(–)	(+)	(+)	(+)	ND
V	75/F	Hoarseness	L	4M	(–)	(–)	(–)	(+)	(+)	(+)	ND

M: male; F: female; R: right; L: left, ND: not done.

hypopharyngeal mass is observed in the right pyriform sinus, and a normal vocal fold mucosa specimen was used for comparison. Fig. 1A-2 shows the mucosal epithelium and lamina propria.

Vocal fold polyps were categorized into four histological types (edematous type, vascular-hyaline type, fibrous type, and myxoid type). Fig. 1B1–6 Images of a 73-year-old Japanese man with red polyps on the left vocal fold, which is a case of the vascular-hyaline type. Dense eosinophilic submucosal deposition of fibrin material closely adjacent to a vascular space is observed.

Specimens of laryngeal granulation tissue consisted of inflammatory cell infiltrate, edema, fibrous exudation, fibrosis, and neovascularization, with no epithelial or tumor cells (Fig. 1C-2, D-2, E-2).

Expression of periostin in laryngeal granulation tissue

The expression of periostin was investigated in seven samples of laryngeal granulation tissue from five patients. It was detected in all 7 (100%) samples obtained from patients with laryngeal granulation tissue (Fig. 1C-3, D-3, E-3) (Table 1). In the laryngeal granulation tissue, periostin was found to be expressed internally, near the surface, or in a mixed distribution of internal and near-surface areas.

Expression of D-β-aspartic acid in laryngeal granulation tissue

The expression of D-β-aspartic acid was investigated in the same three samples. All three cases showed diffuse staining, ranging from the interior to near the surface areas (Fig. 1 C-6, D-6, E-6) (Table 1).

Expression of CD31 in laryngeal granulation tissue

The expression of CD31 was investigated in seven samples of laryngeal granulation tissue from five patients. It was detected in all 7 (100%) samples obtained from patients with laryngeal granulation tissue (Fig. 1C-4, D-4, E-4) (Table 1). The CD31-positive structures showed a vascular appearance and were distant from each other, with some having point-positive structures.

Expression of CD34 in laryngeal granulation tissue

The expression of CD34 was investigated in seven samples of laryngeal granulation tissue from five patients. It was detected in all 7 (100%) samples obtained from patients with laryngeal granulation tissue (Fig. 1C-5, D-5, E-5) (Table 1). The CD34-positive structures had a vascular appearance and were distant from each other, with some having point-positive structures.

Patient characteristics and the clinical features of laryngeal granulation tissue

Patient characteristics and the clinical features of the five cases are summarized in Table 1. The age of the patients ranged from 54 to 84 (mean age 72.8) years. The distribution of lesions was predominantly unilateral, with four cases presenting on the left side and one case on the right side; no cases involved both sides. The majority of cases occurred on one side, more frequently on the left side. Three of the patients were male, and two were female. Two patients had a history of smoking, and three had a history of alcohol consumption. No patient had a history of reflux esophagitis. The clinical features resembled those of vocal fold polyps. The most commonly observed primary symptom was recurrent hoarseness, although a wide variety of clinical features were observed, including laryngeal discomfort, pharyngodynia, and chronic cough.

In all cases, the laryngeal granulation tissue eventually resolved.

Discussion

Laryngeal granulation tissues are elevated lesions caused by nonspecific inflammation that may occur unilaterally or bilaterally. These lesions are particularly important in the diagnosis of laryngeal granulomatosis, typically localized to the vocal fold process or cartilage areas. Morphologically, granulation tissues appear smooth, spherical, hemispherical, or lobulated, and vary in size and shape. Their color ranges from white to red, depending on the degree of inflammation. Historically, lesion etiology has been classified into four categories: those resulting from laryngeal surgery, those occurring after endotracheal intu-

bation, those caused by voice overuse or excessive force generation, and those with no apparent cause (Kleinsasser 1969). Recently, these lesions have also been associated with gastric acid reflux into the pharynx, i.e., pharyngolaryngeal gastric acid reflux disease. Despite their infrequent occurrence, some cases of laryngeal granulation tissue recur and are refractory to treatment. The primary clinical manifestations are hoarseness and an abnormal sensation in the pharynx, although pharyngotracheal irritation and chronic cough may also be present. In some instances, the condition remains asymptomatic and may be detected incidentally during upper gastrointestinal endoscopy. During patient interview, in addition to smoking, alcohol consumption, and voice use, the patient's coughing habits, conditions that result in coughing, history of tracheal intubation, and symptoms of gastric acid reflux (e.g., heartburn) should be assessed. It is also important to check for the presence of drug-induced cough, such as that associated with the use of angiotensin-converting enzyme inhibitors.

Periostin is a type of extracellular matrix protein that has been reported to be strongly expressed in lesions of airway inflammatory diseases such as bronchial asthma, allergic rhinitis, and chronic sinusitis. Recent studies have shown that periostin may be differentially involved in various otolaryngological diseases such as vocal cord polyps, allergic rhinitis, chronic rhinosinusitis with nasal polyps, IgG4-related diseases, tissue hematomas, eosinophilic otitis media, and eosinophilic otitis media (Ishida et al. 2012; Ohta et al. 2013a, 2014; Shiono et al. 2015; Tateda et al. 2022). However, the role of periostin in laryngeal granulation tissue has not been previously reported. Periostin is characterized not only by its role as an extracellular matrix structural protein that regulates fibrosis and collagen deposition but also as a matrix protein that regulates the Th2-mediated inflammatory cascade (Ishida et al. 2012; Ohta et al. 2013a; Shiono et al. 2015). In vocal polyps, periostin is expressed in the stroma but not in the epithelium. Four patterns of periostin expression have been reported: negative, superficial, infiltrate, and diffuse. In the present cases of laryngeal granulation tissue, periostin expression varied; in some cases, it was localized around the base of the mass, while in other cases, it was centered on the superficial layer of the mass. Notably, TGF-β, but not IL-13, expression was found in the same region as periostin expression, suggesting that TGF-β may be involved in the induction of periostin expression in vocal polyps (Tateda et al. 2022). This observation raises the prospect of further investigating the inducer of periostin expression in laryngeal granulation tissue.

Contrary to popular belief that all proteins that make up living organisms are composed of L-amino acids, recent findings highlight the accumulation of D-aspartic acid, which should not be present in the body, in various tissues such as the ligaments (Ritz-Timme et al. 2003), brain (Shapira and Chou 1987), aorta (Powell et al. 1992), cataracts (Masters et al. 1977), lens (Fujii et al. 1999; Kaji et al. 2007), skin (Fujii et al. 2002), and teeth (Helfman and Bada 1976) due to aging and UV light exposure. The presence of D-β-aspartic acid is thought to be the result of the racemization of L-β-aspartic acid in proteins (Kaji et al. 2007), making it a novel indicator of aging-related damage (Fujii et al. 1999; Kaji et al. 2007). UV irradiation of the skin is closely associated with the formation of D-β-aspartic acid in elastic fibers of the skin (Miura et al. 2004). In vocal polyps, D-β-aspartic acid was reported to be upregulated in the epithelium and stroma. It has also been reported that increased D-β-aspartic acid expression in the vocal polyp stroma was associated with the expression pattern of periostin (Tateda et al. 2023a). In the present study, D-β-aspartic acid expression was detected in laryngeal granulation tissue, even though the laryngeal cavity was not exposed to UV irradiation. These results indicate that racemization of L-amino acids may occur due to reasons other than aging or UV irradiation.

CD31 plays important roles in leukocyte trafficking, mechanotransduction, angiogenesis, vascular permeability, and regulation of cellular responsiveness. In previous reports, CD31 expression was found to be upregulated during myocardial remodeling following ischemic injury; however, this upregulation disappeared once the remodeling process was complete (Kondo et al. 2022). Furthermore, CD31 expression in human ischemic heart disease is not upregulated in complete fibrotic foci in the myocardium (Kondo et al. 2022). CD34+ cells, which are multipotent hematopoietic stem cells also known as endothelial progenitor cells, migrate to target sites, where they enhance angiogenesis, neovascularization, and tissue regeneration. Increased expression of CD31 and CD34 was observed in the stroma of vocal fold polyps, with three different expression patterns reported (negative, vascular, and diffuse). Three histological subtypes of vocal polyps were observed: edematous, vascular-hyaline, and fibrous, highlighting a potential correlation between CD31 expression patterns and the histological subtypes of the polyps (Tateda et al. 2023b).

CD31 is a marker of endothelial cells, with CD31 revealing that these cells maintain their structural integrity and a dilated pattern in limited areas (Ohta et al. 2013b). CD31 immunostaining showed that these endothelial cells were present in laryngeal granulation tissue. Interestingly, this study also highlighted the absence of a correlation between increased CD31 expression and areas of periostin expression in laryngeal granulation tissue.

CD34 is a crucial marker of endothelial cell progenitors and neovascularization. In the field of otorhinolaryngology, CD34-positive connective tissue is presumed to appear at sites of recovery or regeneration after pathology or injury. The regular vascular arrangement of CD34-positive structures along the epithelium underscores the role of the epithelium in differentiation, suggesting a sequential recovery where blood vessel restoration precedes or accompanies the regeneration of the epithelial tissue in the otorhi-

nolaryngological region (Katori et al. 2011). CD34 is expressed on the progenitors of endothelial cells and might play an important role in neovascularization and wound healing (Dreger et al. 2002; Pleșea et al. 2005; Oe et al. 2007). Immunostaining for CD34 has shown that these endothelial cell progenitors were present in laryngeal granulation tissues.

Periostin is a regulator of fibrosis and collagen deposition, marking its significance in the domain of myocardial repair/remodeling following myocardial infarction (Kondo et al. 2022). Also, evidence suggests that its overexpression in the nasal mucosa and vocal fold polyps contributes to tissue repair and fibrosis. These findings may support the negative spiral theory immunohistopathologically.

Laryngeal granulation tissues are thought to begin forming at the epithelial defect site after epithelial damage. Laryngeal granulation tissues are formed by direct stimulation of the epithelial defect site, but the characteristic feature of laryngeal granulation tissue is the absence of epithelium. Vocal fold polyps are thought to cause edema, hematoma, neovascularization, and fibrosis due to local interstitial circulation disorder after irritation is applied to the vocal folds due to vocal abuse. In vocal fold polyps, periostin was expressed only in the stroma. It has been reported that there are four periostin staining patterns in vocal fold polyps: negative type, superficial type, infiltrative type, and diffuse type (Tateda et al. 2022, 2023a).

Periostin expression was observed in all cases of laryngeal granulation tissues. However, the site of high expression varies depending on the case, such as on the surface layer or near the base, and the mode of expression was considered to be a subject for future investigation.

In vocal fold polyps, CD31 and CD34 were expressed only in the stroma. It has been reported that there are three staining patterns for CD31 and CD34 in vocal fold polyps: negative type, vascular type, and diffuse type (Tateda et al. 2023b). In laryngeal granulation tissues, expression of CD31 and CD34 was observed in all cases. However, the site of high expression varies depending on the case, such as on the surface layer or near the base, and the mode of expression was considered to be a subject for future investigation.

In vocal fold polyps, D-β-aspartic acid was expressed in the epithelium and stroma.

In vocal fold polyp epithelium, the expression rate of D-β-aspartic acid is reported to be 100% (Tateda et al. 2023a). It has been reported that there are two D-β-aspartic acid staining patterns in vocal fold polyps stroma: negative and positive (Tateda et al. 2023a).

In laryngeal granulation tissues, D-β-aspartic acid was expressed in all cases. High expression of D-β-aspartic acid in laryngeal granulation tissues was observed throughout the granulation tissues, from the superficial layer to the base. Due to the small number of cases, the mode of expression was considered to be a subject for future investigation.

Laryngeal granulation tissue presents a significant clinical challenge due to its high recurrence rate, rendering it a difficult condition to treat. It is important to accurately determine the cause of the disease and provide appropriate treatment by combining drug therapy, voice therapy, lifestyle modifications, and surgical interventions. It is believed that a deeper understanding of its pathology is crucial for developing strategies to reduce the recurrence rate.

Conclusion

Our results indicate that overexpression of CD31, CD34, D-β-aspartic acid, and periostin is likely involved in the pathogenesis of laryngeal granulation tissue. The presence of D-β-aspartic acid and periostin, in particular, presents a promising avenue for research, potentially serving as novel biomarkers for diagnosis and therapeutic targets of laryngeal granulation tissue.

Acknowledgment

This work was supported by a Grant-in-Aid for Scientific Research (C) (17K11363) from the Ministry of Education, Science, Sports and Culture of Japan. We express our sincere gratitude to Mr. Junya Ono for his assistance.

Funding

This work was supported by a Grant-in-Aid for Scientific Research (C) (17K11363) from the Ministry of Education, Science, Sports and Culture of Japan.

Conflict of Interest

The authors declare no conflict of interest.

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