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CD34-Positive Fibroblasts in Reinke's Edema

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Objectives/Hypothesis: To elucidate whether and to what extent CD34+ fibroblasts (so-called CD34+ fibrocytes, CD34+ dendritic cells, and CD34+ stromal cells) occur in normal human vocal folds and in Reinke's edema.

Study Design: Histological study.

Methods: Conventional, immunohistochemical, and ultrastructural procedures were performed in histological blocks of 18 selected cases of Reinke's edema (with typical findings including acellular edematous spaces in the subepithelial connective tissue of vocal folds, and disarrangement of elastic, collagen, and reticular fibers). For control purposes, four normal vocal folds were analyzed.

Results: In normal vocal folds, most stromal cells were spindle-shaped CD34+ fibroblasts. In Reinke's edema, increased density and changes in the morphology and size of this subpopulation of fibroblasts were demonstrated in the connective tissue surrounding the edematous spaces, particularly in their borders, where together with some macrophages they formed boundaries, mimicking the walls of distended lymphatic vessels when conventional stains were used. These activated CD34+ fibroblasts acquired a dendritic morphology (with long, moniliform, often bifurcated, overlapping multipolar processes), and their cytoplasmic organelles were increased in number. In addition to CD34, they expressed vimentin, CD10 and CD99, but no α-smooth muscle actin (α-SMA), CD31, CD117, CD68, h-caldesmon, desmin, or S-100 protein.

Conclusions: CD34+ fibroblasts are a major cell component in the stroma of vocal folds in Reinke's edema, and their activation, with increased density and morphologic changes around the edematous spaces, occurs without immunophenotypic transformation toward myofibroblasts (no expression of α-SMA). The mechanisms by which these cells act in Reinke's edema require further study.

Key Words: CD34 fibroblasts, vocal folds, Reinke's edema.

Level of Evidence: NA

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INTRODUCTION

In Reinke's edema, the entire length of the membranous vocal fold is filled with fluid (swelling of Reinke's space). Several histologic modifications are involved in this lesion, including modified stromal cell activity, microvasculature alteration (with greater permeability, resulting in increased exudative fluid in the extracellular matrix), and disarrangement of collagenous and elastic fibers.[1–9] Risk factors in Reinke's edema include smoking, vocal abuse, and gastroesophageal reflux.[10,11] Of the stromal cells, fibroblasts are a major component of normal vocal folds.[12] Fibroblasts produce extracellular matrix components, including glycosaminoglycans (hyaluronic acid), proteoglycans, glycoproteins (fibronectin and laminin), collagen, and elastin. Vocal fold fibroblasts (VFFs) have been extensively studied, including methods for identification in culture,[13] senescence in primary culture,[14] paracrine potential and signaling,[15,16] immunoregulation,[16] mesenchymal potential,[17] interaction with adipose-derived stem/stromal cells,[18] transdifferentiation and deactivation,[19] influence of growth factors,[20–23] age-related changes,[24–26] changes after mechanical (vibration[27]) or chemical (smoking[15]) action, characterization of those immortalized,[23,28,29] or derived from chronic scars,[30,31] and their biological activity, such as maintaining the correct viscosity and elasticity of the vocal folds.[32] Furthermore, VFFs are considered as playing an important role in different lesions, including Reinke's edema and vocal fold wound healing and scarring.[12,23,26,30,31,33–36]

A subset of fibroblasts, CD34+ fibroblasts (so-called CD34+ fibrocytes, CD34+ dendritic cells, and CD34+ stromal cells), has been reported in normal tissue around different structures, such as vessels (the main cellular constituent of the vessel adventitia), nerves, glands, and skin annexes, and within capsules, septa, fibrous tracts, and interstitial reticular networks, including the upper respiratory tract (see Barth et al.[37,38]), although the vocal folds have not been specifically considered. CD34+ fibroblasts are also the major cell component of some benign and malignant tumors.[39] Furthermore, several groups of authors have reported

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the presence of CD34+ fibroblasts in other pathologic processes, such as repair and tumor stroma formation,[37,40,41] and some lesions with myxoid and edematous changes.[42–44] To our knowledge, CD34+ fibroblasts have not been investigated in Reinke's edema. The aim of this study therefore was to analyze CD34+ fibroblasts in normal human vocal folds and in Reinke's edema, especially around the edematous spaces in the subepithelial tissue.

MATERIALS AND METHODS

Tissue Samples

The archives of the Department of Anatomical Pathology of the University Hospital of the Canary Islands and of the Hospiten Hospitals of the Canary Islands were searched for cases that had been diagnosed as Reinke's edema. All protocols were performed in accordance with international ethical guidelines. Histological blocks of 18 cases, without other associated lesions, were selected (14 females and four males, aged 43–72 years, all smokers), and histological sections were again made. The controls consisted of four human vocal folds without microscopic lesions, which were obtained from two adult cadavers (one female and one male, aged 38 and 56 years, respectively. Both were nonsmokers and neither had undergone tracheal intubation).

LIGHT MICROSCOPY

Specimens were fixed in a buffered neutral 4% formaldehyde solution, embedded in paraffin, and cut into 4-μm-thick sections. Sections were stained with hematoxylin and Eosin (H&E), PAS-Alcian, Masson trichrome, Wilder's reticulin, orcein, and Verhoeff's resorcin-fuchsin stains.

Electron Microscopy

Three cases were also processed for electron microscopy study. Small pieces were fixed in 2% glutaraldehyde with sodium cacodylate buffer, postfixed in 1% osmium tetroxide in cacodylate buffer, dehydrated in a graded ethanol series, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate. The grids were examined at 60 kV with a JEOL 100B electron microscope (JEOL USA, Inc., Peabody, MA).

Immunohistochemistry

Sections that were 3-μm thick were cut and attached to silanized slides. After pretreatment for enhancement of labeling (antigen retrieval PT-Link [ref. 1012; Dako, Glostrup, Denmark]), the sections were blocked with 3% hydrogen peroxide and then incubated with primary antibodies (Dako) for 10 to 40 minutes. The primary antibodies used in this study were as follows: CD-34 (ready to use), code no. IR63261; CD-31 (ready to use), code no. IR61061; CD-10 (ready to use), code no. IR64861; CD-99 (ready to use), code no. IR05761; bcl2 (ready to use), code no. IR61461; CD117 (c-kit) (dilution 1:50), code no. A-4502; CD-68 (ready to use) code no. IR 60961; vimentin (ready to use), code no. IR63061; α-smooth muscle actin (α-SMA) (ready to use), code no. IR61161; h-caldesmon (ready to use), code no. IR05461; desmin (ready to use), code no. IR60661; cytokeratin AE1/AE3 (ready to use), code no. IR05361; S-100 protein (ready to use), code no. IR50461; epithelial membrane antigen (ready to use), code no. IR62961; collagen IV (dilution 1:50), code no. M-0785; laminin (dilution 1:20), code no. M-0638; Ki-67 (MIB1) (ready to use), code no. IR62661; and neurofilament protein (ready to use), code no. IR60761. The immunoreaction was developed in a solution of diaminobenzidine, and the sections were then briefly counterstained with hematoxylin, dehydrated in ethanol series, cleared in xylene, and mounted in Eukitt. Positive and negative controls were used. For the double immunostaining we used anti-CD34 antibody [diaminobenzidine (DAB) as chromogen] to highlight CD34+ fibroblasts and anti-CD68 [aminoethylcarbazole (AEC) substrate-chromogen] for macrophages.

RESULTS

General Histologic Characteristics of Reinke's Edema

In all the cases, the main distinguishing histologic alteration in H&E-stained sections was edema in the lamina propria of the vocal folds. The subepithelial connective tissue contained interconnected, acellular edematous spaces of varying size and morphology, ranging from thin fissures to broad lagoons (Fig. 1A). The connective tissue between the edematous spaces varied in thickness (becoming thin in large areas) and was lined by apparently flat cells (mimicking walls of lymphatic vessels/lymphatic-like distensions). Globular structures were present in the connective tissue and in the edematous spaces, either partially attached to the surface by thin or thick pedicles, or freely floating within (Fig 1A). In periodic acid-Schiff (PAS)-Alcian–stained sections, the extracellular matrix showed Alcian Blue positivity. The connective fibers lost their normal spatial arrangement. Orcein and Verhoeff's methods revealed abundant elastic fibers, which were fragmented, convoluted, focally condensed, and aggregated in skeins (Fig. 1B,C). These aggregates contributed greatly to the globular structures observed in the connective tissue and in the edematous spaces (Fig. 1B,C). Reticular fibers were demonstrated by Wilder method and were predominantly located in the vascular and subepithelial basement membranes, and frequently surrounded and inserted between the globular structures.

Cd34+ Fibroblasts in Normal Human Vocal Folds and in Reinke's Edema

Normal vocal folds. In normal vocal folds, the majority of stromal cells were CD34+ fibroblasts, which showed thin, mostly bipolar (Fig. 1D–F), and in a small subpopulation, multipolar cytoplasmic processes. High density of CD34+ fibroblasts was observed around vessels (Fig. 1E) (except in those under the epithelium [Fig. 1D]) and in the lamina propria of the mucosa adjacent to vocal folds (Fig. 1G). Fibroblasts also expressed vimentin, CD10, and CD99; bcl2 was scantily expressed.

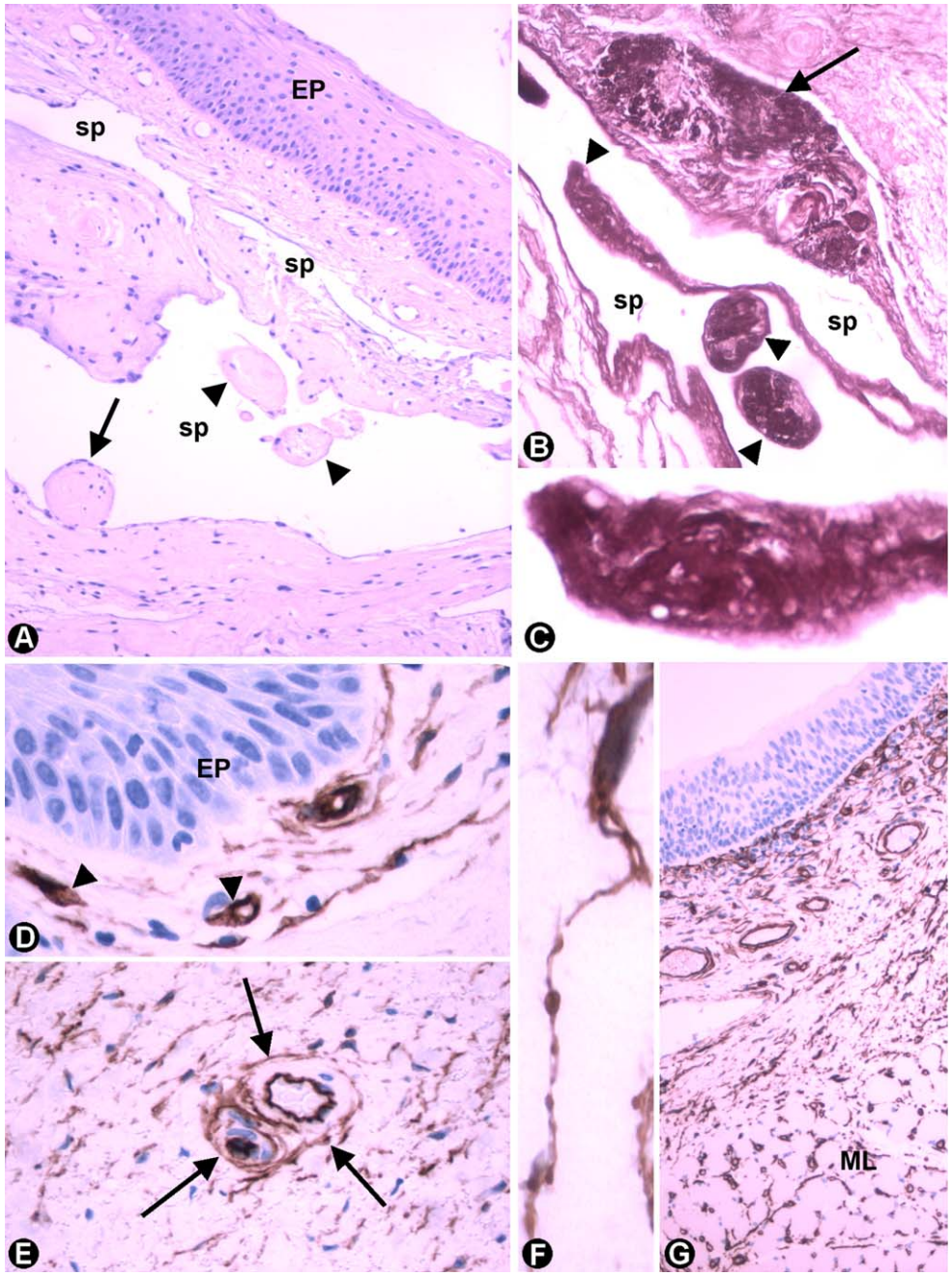

Fig. 1. (A–C) General histologic characteristics of Reinke's edema. (A) In an hematoxylin and eosin (H&E)-stained section, interconnected edematous spaces (sp), ranging from thin fissures to broad lagoons, are observed in the subepithelial connective tissue of a vocal fold. The connective tissue between the edematous spaces varies in thickness and with H&E is apparently lined by flat cells. Globular structures are present in the connective tissue and in the edematous spaces, either partially attached to the surface (arrow) or freely floating within (arrowheads). (B) Orcein-stained, fragmented, convoluted, focally condensed elastic fibers, originating aggregates in skeins (arrow) are observed. Note that the elastic fibers constitute an important part of the globular structures (arrowheads). (C) A detail of one orcein-stained globular structure. (D–F) CD34+ fibroblasts in normal vocal folds. (D and E) Scattered CD34+ fibroblasts are observed throughout the connective tissue. The vessels are surrounded by CD34+ fibroblasts (E, arrows), except for some located under the epithelium (D, arrowheads). (F) A CD34+ fibroblast, with thin, bipolar (spindle-shaped) cytoplasmic processes is shown. (G) Greater density of CD34+ fibroblasts in an area adjacent to vocal fold. (A and B) ×120. (C) ×340. (D and E) ×240. (F) ×680. (G) ×140. EP = epithelium; ML = muscle layer. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

These cells did not express α-SMA, CD31, CD117, CD68, h-caldesmon, desmin, cytokeratin, S100 protein, epithelial membrane antigen, or neurofilament protein. Vascular endothelium was heavily immunostained with anti-CD34 and anti-CD31, the latter only staining endothelial cells (CD34+ fibroblasts are CD31-negative, another feature that distinguishes them from endothelial cells). Positivity for anti–α-SMA was detected in

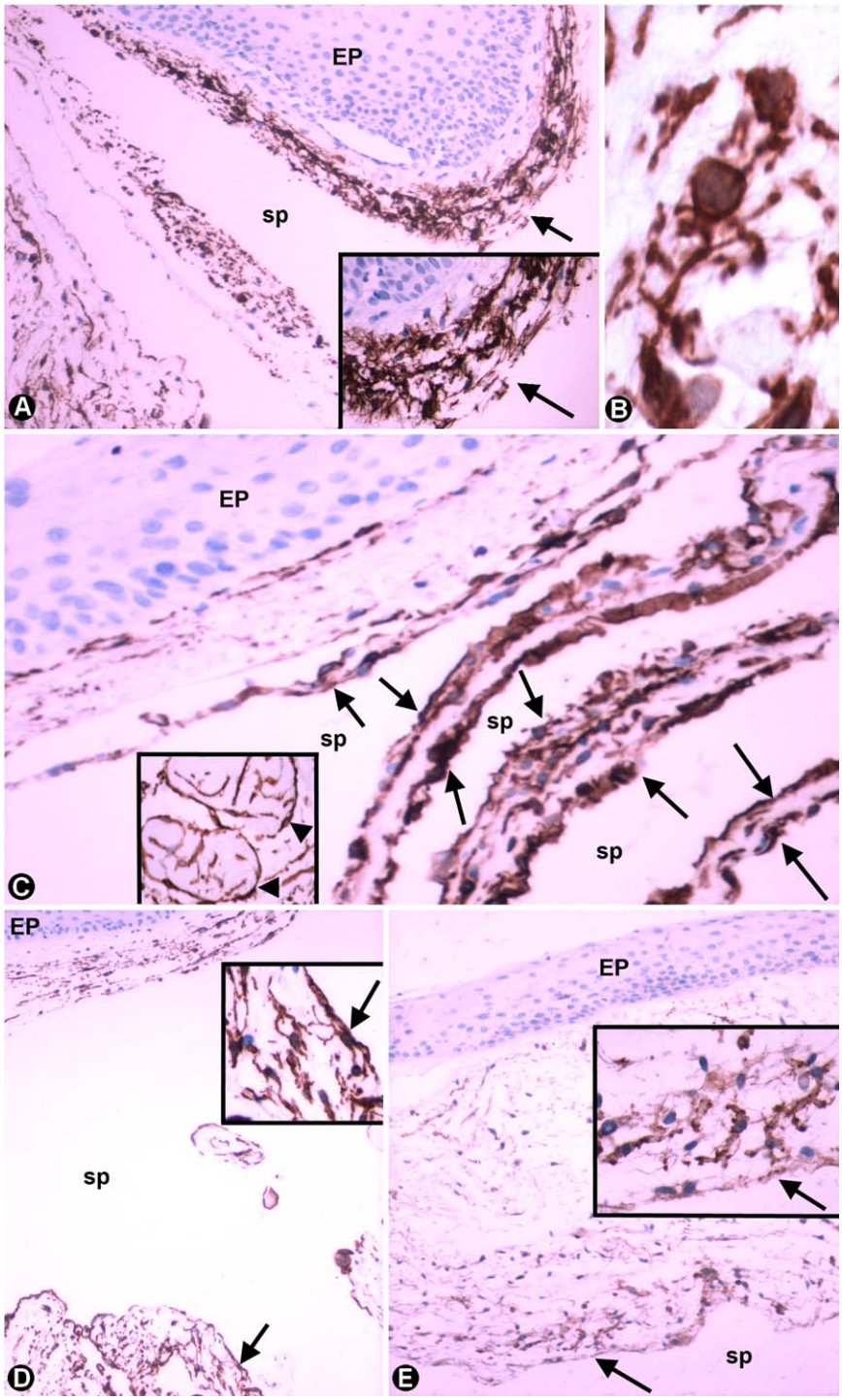

Fig. 2. (A–C) CD34+ fibroblasts in Reinke's edema. (A) Note the increase in number and size of CD34+ fibroblasts, above all on the border of the edematous spaces, forming boundary layers. Insert shows a detail of a boundary of CD34+ fibroblasts. (B) The dendritic morphology of a CD34+ fibroblast is shown by high magnification. (C) CD34 expression demonstrating that the limiting boundaries converge where the connective tissue between the contiguous edematous spaces is rather thin (arrows). Insert shows globular structures surrounded by CD34+ fibroblasts. (D and E) Expression of CD10 (D) and CD99 (E) is also observed. Inserts show details of CD10 and CD99 expression. (A, D and E) ×120. (A, D, and E inserts) ×260. (B) ×680. (C) ×280. (Insert) ×120. EP = epithelium; sp = edematous spaces. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

pericytes and vascular smooth muscle cells. A dense reticular network of CD34+ fibroblasts was observed around striated muscle cells in the muscle layer.

Reinke's edema. In Reinke's edema, the immunohistochemical characteristics of the CD34+ fibroblasts were unchanged in relation to the normal vocal folds, with expression of CD34 (Fig. 2A–C), CD10 (Fig. 2D), and CD99 (Fig. 2E). However, CD34+ fibroblasts increased their number and showed changes in morphology and size, above all on the border of the edematous spaces, forming boundary layers (Fig. 2A,C). When the connective tissue between the contiguous edematous spaces was rather thin, the opposite boundary layers converged (Fig. 2C). The globules within the edematous spaces were also partially or totally covered by CD34+ fibroblasts (Fig. 2C insert).

CD34+ fibroblasts had a larger cellular body (nuclear or somatic region) than in normal conditions, and their cytoplasmic processes were more often multipolar (dendritic-like) (Fig. 2B). The cellular body

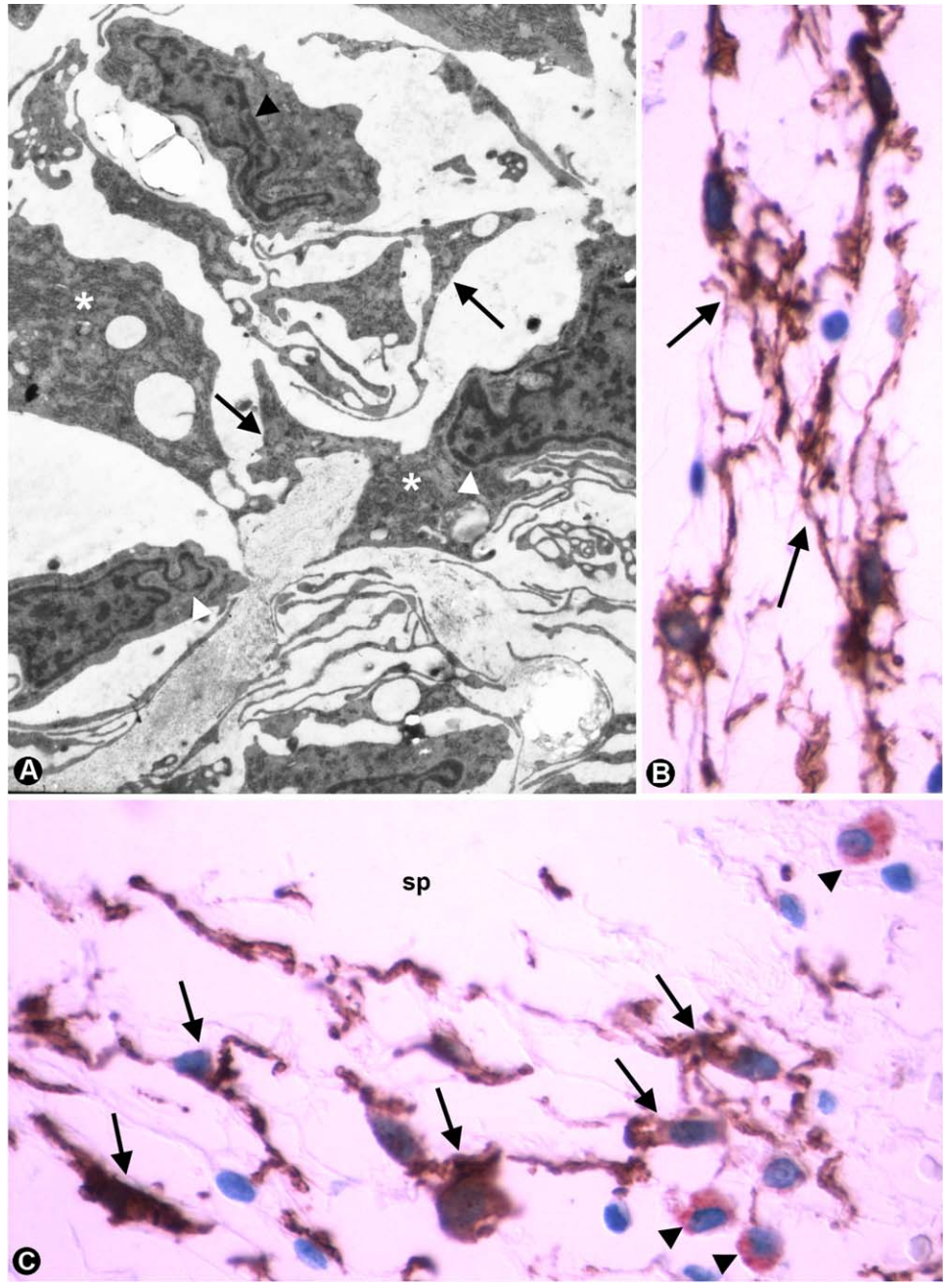

Fig. 3. (A) Ultrastructural characteristics of fibroblasts in Reinke's edema. Overlapping multipolar, thin, moniliform and bifurcated cell processes (arrows), folded and creased nuclei (arrowheads), rough endoplasmic reticulum (asterisks) and edematous intercellular matrix are observed. (B) A similar area of anti–CD34-stained fibroblasts by light microscopy. (C) Distribution of CD34+ fibroblasts and macrophages on the border of the edematous spaces. In a double-stained section CD34+ and CD68-negative (arrows) fibroblasts, CD34 negative and CD68+ (arrowheads) macrophages are observed forming a boundary layer. (A) ×10,000. (B and C) ×280. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

presented either a spindle-like or triangular morphology. The cytoplasmic processes had a moniliform bead-like aspect and were long, initially thin, and often bifurcated (Fig. 2B). Frequently, the processes around the edematous spaces overlapped and formed a dense network with those of neighboring CD34+ fibroblasts (Fig. 3A,B). Ultrastructurally, the nucleus (Fig. 3A) was folded and creased, and relatively large in relation to the content of cytoplasm in the cell body. Patches of heterochromatin were observed, particularly near the nuclear membrane (Fig. 3A). The nucleolus was inconspicuous. The somatic cytoplasm contained polysomes, cisternae of smooth and rough endoplasmic reticulum (Fig. 3A), few mitochondria, a small Golgi apparatus, centrioles, and some lipidic droplets. In the processes, the cytoplasm had few organelles, which increased in number in the dilated portions, where mitochondria, endoplasmic reticulum, and some caveolae could be observed. The cellular surfaces had no basement membrane (Fig. 3A). The extracellular matrix in the connective boundary that lined the edematous spaces was also edematous.

Cells Other Than Cd34+ Fibroblasts in the Stroma of Vocal Folds Affected by Reinke's Edema

Macrophages, fewer in number than the CD34+ fibroblasts, not stained with anti-CD34, and expressing

CD68 (Fig. 3C), appeared scattered throughout the connective tissue and contributed to form the boundaries on the border of the edematous spaces. Mastocytes were also scattered in the connective tissue and focally increased in number. Few lymphocytes were observed in all the selected cases.

DISCUSSION

CD34 is a 110 kDa transmembrane cell surface glycoprotein, whose expression was first reported in hematopoietic progenitor cells and vascular endothelium,[45,46] and more recently in a subset of fibroblasts (see Barth and Westhoff[38]). CD34+ fibroblasts have been described in numerous tissues and organs, including the normal upper respiratory tract.[37] However, the presence of CD34+ fibroblasts in vocal folds has not been specifically considered. Our findings show that most stromal cells in the lamina propria of the normal human vocal folds are CD34+ fibroblasts, whose density increases in neighboring areas.

In this work, we report morphologic changes and increased density of CD34+ fibroblasts in the stroma of vocal folds affected by Reinke's edema, particularly in the borders of the edematous spaces, where this subpopulation of fibroblasts formed boundaries, along with some macrophages, mimicking the wall of distended lymphatic vessels when H&E staining is used (pseudolymphatic condition[2]). Reinke's edema should therefore be added to those processes in other sites where CD34+ fibroblasts have been described as playing an important role, including lesions with myxoid and edematous changes.[42–44]

CD34+ fibroblasts involved in Reinke's edema may originate from two sources: endogenous and exogenous. On the one hand, the presence of CD34+ fibroblasts in the normal vocal folds suggests their contribution to the hyperplasic CD34+ fibroblasts that delimit the edematous spaces. This contribution could be predominantly from macula flava, which is considered an important structure in the growth and development of the human vocal mucosa and whose vocal stellate cells (morphologically similar to CD34+ fibroblasts on the border of the edematous spaces) constantly synthesize extracellular matrix,[47] and/or from Reinke's space, whose fibroblasts may change their cell shape (toward stellate) and organelle development (e.g., in vitro effect of HGF[23]). On the other hand, the participation of intrinsic CD34+ fibroblasts does not exclude an exogenous contribution, as described for tissue repair (when resident CD34+ fibroblasts are insufficient to meet the need for stromal repair, CD34+ circulating fibrocytes[48] enter the injured tissue[38,44,49]).These possibilities require further studies.

Recently, considerable attention has been given to the characteristics and properties of VFFs. The contributions of these studies facilitate understanding of the possible significance and molecular mechanisms of CD34+ fibroblasts in Reinke's edema, which is discussed below.

Hanson et al.[17] demonstrated that human VFFs isolated from the lamina propria meet the criteria established to define multipotent mesenchymal stem cells. VFFs showed similar cell surface markers, immunophenotypic properties, and differentiation potential as bone marrow and adipose tissue-derived mesenchymal stem cells. These criteria include the nonexpression of CD34.[50] However, the phenotype and abilities of mesenchymal stromal cells, including CD34 expression, may vary between in vitro and in vivo settings.[51,52] Thus, by day 4 of culture, downregulation of CD34 expression was demonstrated during the mesenchymal stromal cell derivation phase of adipose tissue (CD34+, CD146−, CD31−, CD271± stromal cell subset, obtained from adipose stromal fraction, downregulated CD34, and upregulated CD105, CD146, and CD271[51]), a fact that could also occur with CD34+ fibroblasts obtained from vocal folds. CD34+ VFFs could therefore be resident mesenchymal cells in the vocal fold lamina propria.

VFFs produce several inflammatory mediators[16,21,53] and play a role in regulating abnormal inflammatory responses by suppressing proinflammatory cytokines and neutrophil recruitment, and interfering with macrophage activation.[16,54] As mentioned above, VFFs meet the criteria of mesenchymal stem cells, which have immunosuppressive properties, including inhibition of monocyte differentiation, T and B lymphocyte proliferation, and NK cell function. Thus, it has been demonstrated that VFFs may regulate the immunophenotype of macrophages, which acquire an anti-inflammatory profile based on low CD16, high CD206, and low human leukocyte antigen-DR expression.[55] The characteristics of the cases selected for our study suggest that CD34+ fibroblasts may also have this role in Reinke's edema.

Different mechanisms may contribute to VFF behavior, including the regulation of vocal fold fibroblast/myofibroblast differentiation and expression of α-SMA (e.g., cultured VFFs derived from vocal fold scar reveal increased expression of α-SMA, when compared with cultured normal VFFs[31]). This VFF differentiation mainly depends on molecular mechanisms associated with transforming growth factor $\beta 1$ (TGF-$\beta 1$).[20] For instance, isolated human VFFs can differentiate into a myofibroblast phenotype (expressing α-SMA) by means of TGF-$\beta 1$ (dose dependent), whose effect is attenuated by hepatocyte growth factor (HGF) and interleukin-6.[20] Likewise, TGF-β stimulates collagen secretion and migration of immortalized human VFFs,[56] and TGF-$\beta 1$ action on VFFs is blunted in a dose-dependent manner in response to prostaglandin E_2,[57] which also regulates fibroblast cytoskeletal dynamics, inducing a disruption of actin filaments.[58] Moreover, interaction of VFFs with extracellular matrix is also essential to fibroblast proliferation, migration, and cell differentiation.[21] Thus, fibromodulin (increased in Reinke's edema[59,60]) binds to and potentially inhibits TGF-β.[61] HGF has intense antifibrotic activity and stimulates the production of hyaluronic acid,[23,62] which interacts with VFFs and mesenchymal stem cells through various cell surface receptors[17,63] and inhibits myofibroblast differentiation without decreasing TGF-$\beta 1$ receptor expression.[22] Therefore, depending on several factors, vocal fold repair after injury may occur with myofibroblast differentiation (e.g., during vocal fold

wound healing and scarring) or without immunophenotypic transformation to myofibroblasts, which concurs with observations on the origin of myofibroblasts[64–66] and behavior of CD34+ fibroblasts[37,38,40,41] in other locations. In our study of Reinke's edema, the activated fibroblasts increased in number and remained positive for CD34, without α-SMA expression. The stromal cell behavior in Reinke's edema may therefore be included within the tissue response in which the CD34 immunohistochemical staining pattern of fibroblasts is conserved.

Differential gene expression has been observed in benign vocal fold lesions. For example, Reinke's edema, vocal fold polyps, and scarred vocal folds have different levels of gene activity for interstitial protein fibronectin (involved in the development of fibrosis), fibromodulin (see above), and decorin (binds to fibronectin and promotes lateral association of collagen fibrils).[59,60,67] Thus, in Reinke's edema, messenger RNA levels were reported as decreased for fibronectin, and increased for fibromodulin and decorin, whereas the opposite characterized polyps and scarred vocal fold tissue.[59,67] Likewise, in another study on gene expression profiling of vocal polyps and Reinke's edema, 65 genes were found to differentiate both lesions.[60] All these findings concur with the different vocal fold responses to injury outlined above. Genes involved in protection against oxidative stress (mitogen-activated protein kinase 3, superoxide dismutase 1 [SOD1], glutatione peroxidase 2, and glutatione S-transferase A2) and apoptosis (caspase 9) showed increased expression in Reinke's edema, suggesting a defense mechanism that protects cells from oxidative stress, such as smoking or reflux.[60] Thus, it has also been observed that cigarette smoke condensate increases intracellular reactive oxygen species, as well as heme oxigenase 1 and SOD1 gene expression in VFFs.[68] Furthermore, VFFs are capable of participating in paracrine regulation of pathological blood vessel formation in cigarette smoking-related Reinke's edema (cigarette smoke extract increases vascular endothelial growth factor).[15]

The significance of increased CD34+ fibroblasts in Reinke's edema could therefore be related to the roles discussed above for VFFs. Thus, CD34+ VFFs may behave as resident cells with mesenchymal stem cell properties, participating in tissue homeostasis, and in immunomodulatory and anti-inflammatory mechanisms. CD34+ VFFs may display differences in α-SMA expression (myofibroblast differentiation), and Reinke's edema is a type of lesion in which myofibroblast differentiation does not occur. The interrelation of CD34+ fibroblasts with the modified extracellular matrix (e.g., decreased fibronectin and increased fibromodulin and decorin) may contribute to the behavior of these cells. Furthermore, gene expression in Reinke's edema may be related to the mechanism that protects cells from oxidative stress, such as smoking or reflux. Additional studies are needed to clarify the significance and role of CD34+ fibroblasts in Reinke's edema.

CONCLUSION

We report for the first time that VFFs in Reinke's edema are CD34+ fibroblasts that show increased density and changes in their morphology, structure, and size when compared with CD34+ VFFs in normal vocal folds. In Reinke's edema, this subset of fibroblasts, with no differentiation to myofibroblasts, was mainly distributed in the border of edematous spaces, forming delimiting boundaries, together with some macrophages. Further studies are needed to clarify the origin, significance, and role of CD34+ VFFs in Reinke's edema, including mesenchymal properties, immunomodulatory and anti-inflammatory effects, gene expression, and protection from oxidative stress.

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