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Age-Related Changes in the Vestibular Folds of the Human Larynx: A Histomorphometric Study

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Summary: Age-related changes were investigated in the human vestibular folds of the larynx. Twelve male Caucasian larynges were obtained from autopsy and studied via light microscopy. The donors were free from head-neck pathologies or laryngeal trauma. Specimens included a 6-week-old infant, and at least one each from the second through eighth decades. Each specimen was decalcified and prepared for paraffin embedding and subsequent sectioning in the coronal plane at 6 μm. Sections were stained alternately with safron-phloxine-hematoxylin and iron gallien elastic stains. Descriptive and quantitative data were obtained from light microscopy and planar morphometry using a computer image analysis system. Tissue changes included fatty infiltration of serous and mucous glands, fibrotic changes in connective tissue, and alterations in the distribution of mixed glands specific to serous/mucous ratios. Potential implications of findings to vocal function are discussed. **Key Words:** Larynx—Aging—Laryngeal glands.

Secretions from laryngeal glands have long been recognized as vital to the functional status of the larynx. As early as 1837, Hilton (1) noted the significance of glandular secretions in maintaining the vocal folds during phonation. The importance of hydration of the vocal folds has been emphasized for its role in sustaining the health of the epithelium as well as the structural integrity of the cover of the vocal fold and in maintaining the pliancy of the mucosal wave during phonation (2–5). The importance of secretions in the respiratory tract has been discussed by Sturgess (6), who pointed out that though other sources of secretion are present in the pharynx (goblet cells, intraepithelial cells), the major source of mucus in the respiratory tract is derived from submucosal glands. Specific to the vocal tract, topographical studies place a large percentage of these glands in the vestibular fold (7). Since the vestibular glands contain both mucous and serous acini, their functional contribution to the larynx cannot be underestimated. The secretory byproducts of mucous and serous glands (neutral and acidic glycoproteins) provide protection from effects of concentration gradients and abrasive and aeromechanical forces and immunologic protection through production of the enzyme lysozyme by serous acini (4). Each of these provisions is vital to maintenance of the cover or vibrating portion of the vocal fold.

A reduction in glandular secretions may alter the compliance characteristics of the cover of the vocal fold. This assumption is based on the apparent modification in vibratory characteristics of the true fold due to reduction in laryngeal secretions as demonstrated by Bless and Shaikh (8). Administration of atropine (DL-hyoscyamine) to human subjects and

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subsequent stroboscopic examination showed significant changes in amplitude, closed phase, duration, regularity, and phase symmetry of vocal fold vibration. Subjects also reported experiencing increased dryness of the vocal folds accompanied by increased effort during phonation and vocal fatigue. In a related study, Finkelhor et al. (9) studied the effects of changes in hydration on the internal tissue viscosity of the true vocal fold and on phonation. Using an in vitro model, they caused fluctuations in vocal fold oscillation threshold pressure by inducing fluid movement into and out of the vocal folds. A predicted shift in oscillation threshold occurred for each condition of hydration.

LARYNGEAL GLANDS

Anatomy of laryngeal glands

From the aforementioned, it is apparent that the laryngeal glands, particularly those of the vestibular folds, play a significant role in vocal fold functioning. A short description of relevant anatomical features of the vestibular glands is presented and serves as a backdrop against which to discuss the nature of reported age-related changes.

The vestibular folds of the larynx house the mixed seromucinous glands (Fig. 1), which are innervated by parasympathetic and sympathetic branches of the autonomic nervous system. Connective tissue surrounds these glands. Supportive functions of connective tissue depend largely on the properties of its extracellular matrix, which is composed of fibrous and aqueous portions. The fibers serve as a support structure on which fixed cells are suspended. The aqueous portion is a medium through which nutrients and waste products are exchanged. The consistency, composition, and state of hydration of the matrix exert an important influence upon this vital exchange (4).

Histologically, the vestibular glands are exocrine, compound tubuloalveolar glands (Fig. 2). They are located in the submucosa within the loose fibers of the quadrangular membrane. These mixed glands are separated from the more laterally placed saccular glands by areolar tissue and occasionally by the ventricularis portion of the vocalis muscle (2).

Submucosal glands are considered to be a major source of mucus in the human respiratory tract, contributing 40 times more than the epithelial goblet cells (10). Mucous cells produce a range of neutral to acidic glycoprotein. Serous cells produce some neutral glycoprotein, lysozyme (an enzyme capable of breaking down bacterial cell walls), and possibly other serum-type glycoprotein (6).

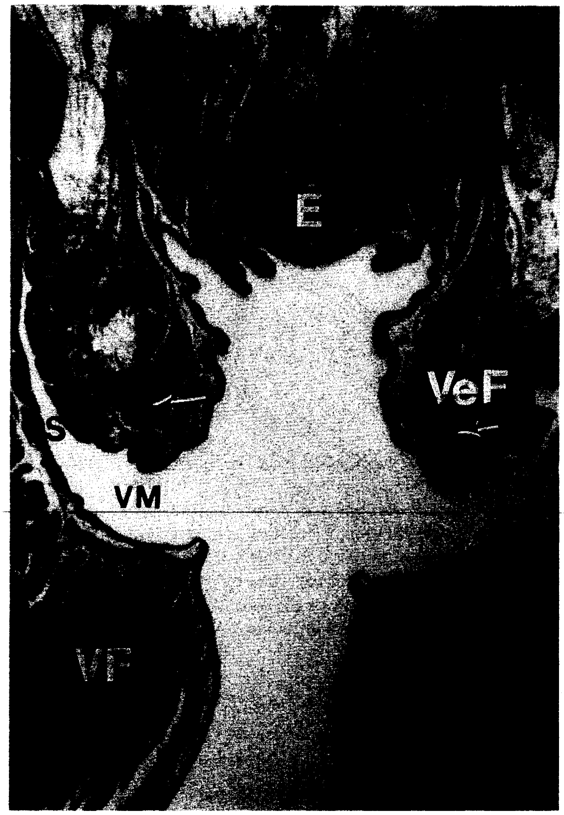

FIG. 1. Coronal section of the larynx through the anterior third of the vocal folds. Illustrated are the vocal folds (VF), ventricle of Morgagni (VM), sacculus (S), ventricular fold (VeF) with its seromucous glands (arrows), a portion of the epiglottis (E), and tissue from the preepiglottic space. Safron-phloxine-hematoxylin; Original magnification ×1.

Aging of laryngeal glands

Despite the potential functional roles of laryngeal glands, little has been written about age-related changes. While atrophic changes in mucous glands of the ventricular fold have been reported in subjects over age 70 (11), the research on other involutional age-related changes in laryngeal cartilage and connective tissue suggests that aging may be evidenced much earlier in life (12–15). Bak-Pedersen and Nielsen (16), however, found no statistically significant changes in distribution or composition in laryngeal glands based on age or sex. The reasons for these discrepancies are unclear, although Bak-Pedersen and Nielsen considered only subjects older than 56 years. Though aging of the laryngeal glands is not well documented, considerable research exists on aging of the salivary glands,

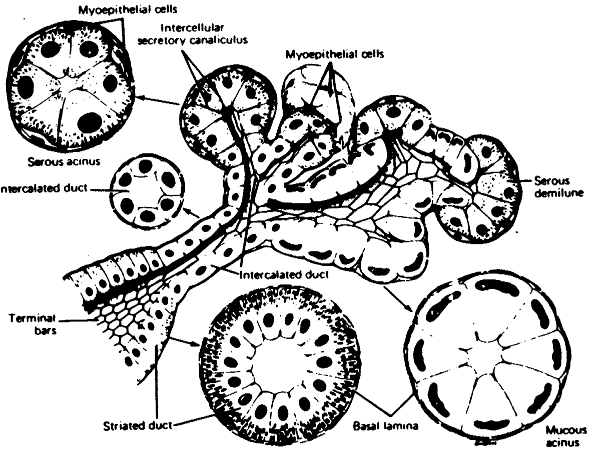

FIG. 2. Schematic representation of a mixed seromucinous gland. Serous and mucous acini comprise the secretory portion of the gland and are responsible for the manufacture of secretions. Note the difference in shape and location of serous and mucous cells. A cluster of such cells is termed an acinus. Nuclei of the serous acini are located centrally within the cells, while nuclei of the mucous acini are flattened and peripherally placed. [Reprinted with permission from Junquiera et al. (4).]

which are morphologically similar to glands in the larynx (17–19). Reports based on human and animal experiments suggest that degenerative changes include loss of acinar components, increases in fat, and vascular and connective tissue changes. However, these data may be difficult to generalize to laryngeal glands because of reported differences in type, rate, and extent of change specific to variation in location of salivary glands (20).

Given the paucity of information on age-related changes in the glands of the larynx, this research study examined age-related morphologic changes in the serous and mucous glands in the vestibular folds. The parenchyma of the glands (acini) and the stroma (connective tissues and other supporting elements) were studied using qualitative and quantitative analyses to characterize differences according to age, extent of change, and tissues affected.

METHODS

Specimens

Specimens for this study were selected from autopsies performed within the Department of Pathology at the University of Tennessee, Memphis. Ten male Caucasian subjects were selected for study, consisting of a 4-week infant and nine adult specimens, one from each decade from the second through ninth (aged 16–78 years; see Table 1). Specimens were free from laryngeal injury or systemic diseases that might adversely affect laryngeal functioning.

TABLE 1. *Age distribution of specimens in series (n = 10 male subjects)*

Subject no.	Age	Cause of death
1	4 wks	Sudden infant death syndrome
2	16 yrs	Multiple stab wounds
3	19 yrs	Gunshot wound to abdomen
4	28 yrs	Gunshot wound to head
5	32 yrs	Aortic and coronary atherosclerosis, acute alcoholism
6	40 yrs	Atherosclerotic cardiovascular disease, interstitial myocardial fibrosis, obesity, pulmonary edema, cerebral edema, visceral congestion
7	56 yrs	Arteriosclerotic cardiovascular disease
8	62 yrs	Carcinoma of lung
9	71 yrs	Arteriosclerotic cardiovascular disease
10	78 yrs	Arteriosclerotic cardiovascular disease

Fixation and specimen preparation

Larynges were fixed in 10% buffered formalin immediately following autopsy. They were decalcified in EDTA and the process was monitored chemically and via spot x-ray film; the end point of decalcification was determined radiographically. The specimens were prepared for paraffin embedding and sectioned in the coronal plane at 6-μm thickness taken at 125-μm intervals. Sections were stained alternately with safron-phloxine-hematoxylin stain to demonstrate general tissue features and iron gallein elastin stain for demonstration of elastic and collagenous fibers.

Instrumentation

Histologic examination of laryngeal sections was done using an Olympus BSH light microscope fitted with apochromatic objectives. Photodocumentation was done with an automatic photomicrographic system (Olympus PM 10AD) that was fitted to the trinocular head of the microscope.

Histologic detail was quantified using interactive planar morphometry software of a computer-based Microcomp PM2 image analysis system (Southern Micro Instruments, Atlanta, GA, U.S.A.). True color images were digitized directly from a video monitor using a digitizing tablet (Numonics model 2200) with programmable cursor accurate to 0.025 mm.

Definitions of anatomical boundaries

The midportion of each vestibular fold was selected for analysis. Selection of this area was based on topographical studies placing a large percentage of these mixed seromucinous glands in the vestibular fold over the vibrating portion of the true vocal fold. To standardize vestibular fold measurement areas across specimens, the following definitions, based on anatomical criteria, were used to establish the anteroposterior, midregion, and lateral boundaries of the vestibular folds.

All sections of the right and left vestibular folds of each specimen were examined under the microscope to establish the anterior and posterior boundaries of the folds. The following operational definitions were adopted:

The *anterior boundary* was defined as the fibroglandular area superior to the nodular portion of the vocal fold that also contained contiguous areas of thyroid cartilage and thyroarytenoid muscle. The *posterior boundary* was the posterior-most extent of the vestibular fold to attach to the arytenoid cartilage where the vocal process and posterior macula flava were well defined. The *superior boundary* extended from either the thyroartyenoid muscle or the lateral border of the fold near the corner of the saccule to the opposing corner formed by the medial fold margin and epiglottic intrusion. This boundary served as superior border for all measurements. The *lateral boundary* was defined relative to the anterior or posterior orientation of sections in a series. Anteriorly, the free margin of the lateral aspect of the vestibular fold (bordered by the saccular space) was the natural boundary. Posteriorly, the thyroarytenoid muscle (externus) served as the lateral boundary. The *medial boundary* was defined as the free margin of the vestibular fold up to and including the natural superior boundary where the intrusion of the epiglottis is apparent. The *midportion of the vestibular fold* was arithmetically determined by counting the total number of sections between the anterior and posterior boundaries. The middle 50% of sections were retained for analysis.

Data analysis

Qualitative and quantitative analyses were made from histologic sections. Qualitative analyses consisted of systematic observations of tissue samples using low (×2, ×4), medium (×10, ×20), and high (×40, ×60) power objectives with ×2.5 photoeyepieces. The effective magnifications used were determined by multiplying the power of the objective by the power of the photoeyepiece. Specific observations included descriptions of adipose tissue, condition of dense regular connective tissue, general appearance of acinar cells, and distribution of serous and mucous acini within glands. A four-point equal-appearing interval scale was used to assign ratings for each category of descriptor.

Intrajudge and interjudge reliability was determined from 10% agreement of repeated measurements from 10% of all measurements made. A high degree of reproducibility was found for qualitative measurements as reflected in 95% agreement of intrajudge and 90% of interjudge ratings. A 98% level of agreement was found for quantitative measurements.

Quantitative analyses were made using a Microcomp image analysis system programmed for planar morphometry. The microscopic field was displayed as a true color video image on a Sony RGB monitor and was digitized from the monitor. Measurements were made of vestibular fold area and frequency distribution of mucous, serous, and intralobular fat cells within a given section of vestibular fold. Cell distribution values were related to the area measurements, expressed as number of cell types to total tissue sample per section. The distribution of serous, mucous, and fat cells per area represented the concentration of cell types per section in the described area of vestibular fold.

RESULTS

Bilateral vestibular fold relationships

Results of this study indicate that the distribution of age-related changes was similar bilaterally. Comparison of right and left sides of vestibular folds for each subject was accomplished to establish homogeneity of the samples. A one-way analysis of variance, repeated measures design, was applied for each set of observations quantified. The statistical comparisons were made for area measurements as well as cell counts to include serous, mucous, and fat cells. Results of the analysis demonstrated no significant differences between right and left sides for cell distribution or area measurements (area, $F = 0.81$, df 1,208; serous, $F = 0.19$, df 1,208; mucous, $F = 0.11$, df 1,208; fat, $F = 1.06$, df 1,208).

Mucous and serous acini

Mucous glands appeared to be less prominent than serous glands in aged specimens. Younger specimens showed well-defined serous and mucous components (Fig. 3). However, both serous and mucous glands appeared to exhibit slight and inconsistent amounts of atrophy, as early as age 28 (Fig.

FIG. 3. Well-defined mucous and serous acini in a region of the ventricular fold of a 4-week-old male infant. (S,s) serous acini; (M,m) mucous acinus; (CT) parenchymal connective tissue; Safron-phloxine-hematoxylin; ×10 original magnification.

4A). Qualitative analysis revealed that "parenchymal status" declined with age as reflected in atrophy of acini and accumulations of intra- and extraglandular fatty tissue. In older specimens (above age 50) morphologic changes were typically found and were more extensive (Fig. 4B). Some atrophy of both serous and mucous cells was also observed in early adulthood (age 32); however, atrophy of serous acini alone was never encountered.

In specimens aged 56 and older, it was often the case that serous and mucous atrophy was present (Fig. 5). Overall, it was noted that cells in the center of the glands (typical location of mucous cells) displayed the most consistent degeneration (Fig. 6). Often the centrally located cells were not intact (mucous cells), while cells located in the periphery were well preserved (serous cells) (Fig. 6). From these data, it appears that central portions of the vestibular glands, which are composed of mucous acini, are most vulnerable to the effects of aging.

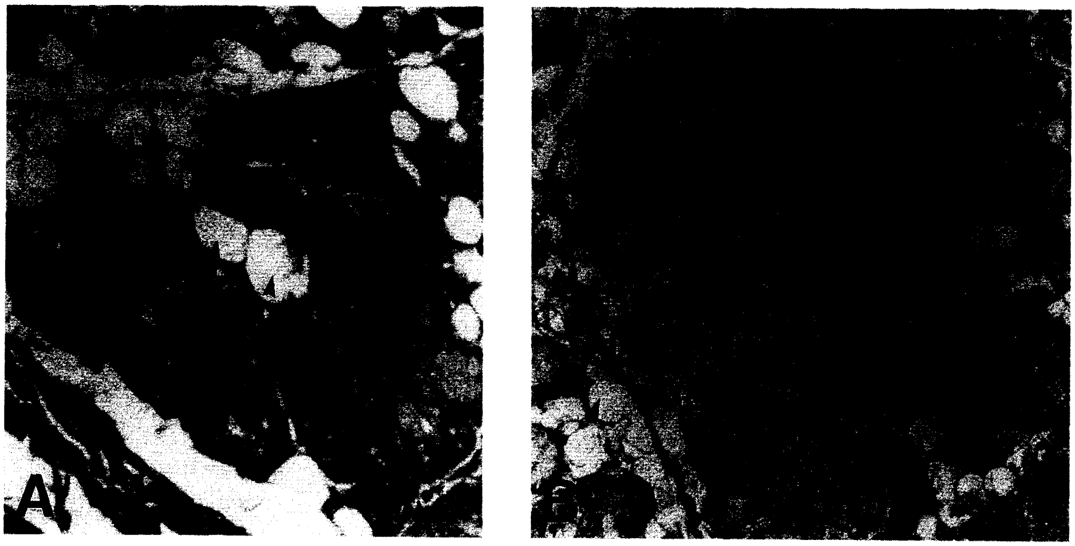

FIG. 4. Micrographs of coronal sections of the ventricular folds from a 28-year-old (A) and 71-year-old (B) man illustrating the replacement and displacement of gland acini with fat cells. In A there are slight fatty accumulation and mild fragmentation of connective tissue fibers. ×20 original magnification. In B note moderate fatty infiltration and replacement of gland acini. ×10 original magnification. White spherical bodies are fat cells (arrowheads). Safron-phloxine-hematoxylin.

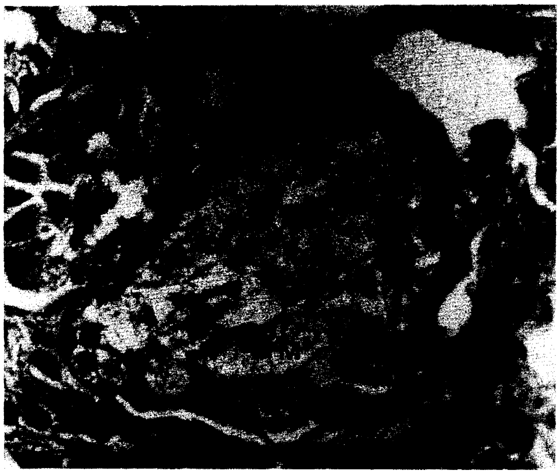

FIG. 5. Severe disorganization of serous (S) and mucous (M) acini in a 56-year-old man. Safron-phloxine-hematoxylin; ×50 original magnification.

Older subjects (56 years and older) appeared generally to have less intact mucous components than younger subjects, while subjects in the seventh and eighth decades tended to show evidence of degeneration in both types of acinar cells. The potential influence of these changes is particularly important, since the quality and quantity of secretions are likely to be affected by the loss of mucous components in the secretory product.

Connective tissues

The connective tissues investing the vestibular glands were found to change with increasing age. Moderate fragmentation of connective tissue fibers was observed in subjects as young as age 28. Severe fragmentation was consistently observed in older subjects, beyond the age of 60 years. There appeared to be a close relationship between connective tissue changes (stroma) and those in and of the acini (parenchyma) of the glands. This is well demonstrated in Fig. 7, which compares the histologic characteristics of the stroma of young (16-year) and old (71-year) subjects. In young specimens, the connective tissues were arranged in dense aggregates supporting well-defined lobules of the gland (Fig. 7A), while in the older specimens the connective tissues exhibited increased fragmentation and reduced density characterized by separation and fragmentation of the fibers (Fig. 7B). The intimate interdigitation of connective tissue with the glandular acinar cells observed in younger specimens (19 years and younger) was obviously disturbed in specimens of older age categories. The most severe cases were older than 60, with some suggestion of thickening of connective tissue fibers as early as age 32.

DISCUSSION

Although the importance of glandular secretions to vocal fold function has been suggested in the literature (1–3), it was not until recently that research demonstrated the functional importance of these early claims (8,9). Results from the present study provide anatomical evidence that the vestibular glands in the male larynx undergo involution. These structural changes appear to provide the mechanical bases for changes in laryngeal function that have been recently reported. More important, however, is the fact that these data call for more detailed study of the mechanisms that contribute to involutional change in the larynx.

Changes in gland acini

A principal finding of the present investigation is that with increasing age, there is atrophy of the acini (secretory units) of the vestibular glands. These changes were characterized by loss of parenchymal tissue and accumulation of intra- and extraglandular fatty tissue. These findings are consistent with those of Ruckes and Hohmann (11), but are not supported by Bak-Pedersen and Nielsen (16), who studied the status of laryngeal glands in persons aged 56 and older. They found no significant age-related differences. However, these data may be affected by the skewed age distribution of the sample.

Another interesting aspect of the data from the present study concerns the replacement of glandular tissue by fat. Increased accumulation of fat in glands is the most telling indication of degenerative change (17) and has been associated with decline of function in the thymus gland (4,21) and in the salivary glands (18,19,22). Thus, it can be inferred that the structural changes in glands have some associated decrement in function. Specifically, the loss of the product of mucous and/or serous components may precede loss of the ability to produce secretions in quantity. The quality of these secretions may vary as well depending on the specific components lost. This aspect needs to be empirically tested in future research.

Unlike Ferrerri (2) who reported that the vestibular folds increased in size with advancing age, data from the present study did not reveal significant

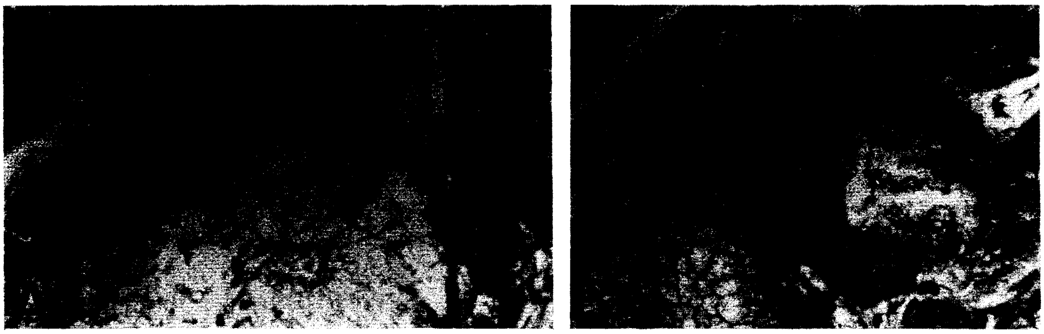

FIG. 6. A: Micrograph of a portion of ventricular gland from a 71-year-old man showing the greater tendency toward breakdown of the centrally located mucous acini compared with serous acini located on the periphery. ×50 original magnification. Compare this morphology with glands from a 4-week-old boy (B) illustrating prototypical intactness and spatial orientation of serous and mucous acini. ×25 original magnification. Safron-phloxine-hematoxylin.

bilateral or systematic increase with age. Ferrerri maintained that these increases in size were due to accumulations of fat in the vestibular fold. In the present study, increased fat was most prominent in the replacement of the acini of the glands. However, Ferrerri's suggestion raises the possibility that extralobular fat may form an effective barrier to diffusible transport routes from the connective tissues to the acini or blood vessels that feed them. This may contribute to decreased viability of the glands.

Observations from the present study suggest that mucous acini were more often affected than serous acini in the glands sampled. The center of glands, where the mucous acini are located, exhibited the most frequent evidence of degeneration or atrophy compared to the periphery of the glands, where the serous acini for these particular glands are found. Thus, although the relative distribution of serous to mucous acini did not change as a function of age, atrophy was most frequently observed in the mucous acini.

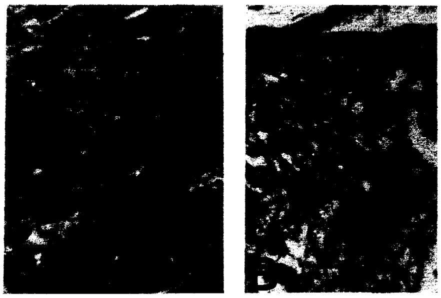

FIG. 7. A: Dense connective tissue in the parenchyma of the vestibular fold surrounding seromucous acini in a 16-year-old boy. Compare this with the disorganization and fragmentation of connective tissue in a 78-year-old man (B). Safron-phloxine-hematoxylin; ×60 original magnification.

The submucousal glands have been implicated as major contributors to respiratory secretions (6,20). Data from the present study suggest that loss of mucous acini in the vestibular folds is a principal involutional change. This change would be expected to make laryngeal secretions less viscous and thus likely to compromise protection and lubrication of the vocal folds. Rheostatic properties are also likely to be altered. This may be particularly important in light of recently reported data from Fukuda et al. (23) who suggested the importance of complex movement and coating action by laryngeal glandular secretions of the vocal folds during sound production. The works of Bless and Shaikh (8) and Finkelhor et al. (9) support this assumption. They found that vocal fold vibration, voice quality, and vocal effort can be adversely affected when hydration and lubrication of the vocal folds are significantly diminished.

Age-related decreases in vocal fold hydration may also contribute to the reported increases in fundamental frequency observed in older male voices (24) that are thought to occur after age 50. Dryness of the vocal fold epithelium, resulting in increased stiffness of the cover, may contribute to this change in fundamental frequency as well as increased perturbation (jitter), as reported by Honjo and Isshiki (25).

Connective tissue changes

In association with changes in the secretory components of the vestibular glands, results from the present study have shown that there are accompanying changes in the connective tissue stroma. It is not possible, however, to say whether they develop concurrently. In fact, this may not be the case as connective tissue changes were found in a 28-

year-old specimen that exhibited intact glandular structure. It is readily acknowledged that the small size of the present sample does not permit this observation to be generalized into a trend. However, it appears important to note that connective tissue changes may occur earlier than previously thought and may precede glandular changes. It is logical to assume that compromised nutritive function provided by the connective tissues via exchange of nutrients and waste products may ultimately compromise function of the glands as well.

Age-related changes in the elastic and collagenous fibers of the vocal folds have been demonstrated by several investigators (2,12,14,15,26–28). Findings in the present study reveal similar patterns of change in the connective tissues of the vestibular folds surrounding the submucosal glands as reflected in decreases in density and disorganization and separation of fibers.

The functional significance of these connective tissue changes appears to relate to support and trophic functions of the glands and the periglandular area. The fibers serve as a mechanical support for the lobules of the gland and ducts, while the aqueous portion of the matrix is a medium through which nutrients and metabolic wastes are diffused and exchanged. The consistency, composition, and state of hydration of the matrix "exert an important influence upon this vital exchange" (21). Thus, important trophic activities of the gland acini and their immediate environments may be affected by changes in contiguous connective tissues.

Future research

This report suggests a trend for the decrease in functional glandular components in the vestibular fold, which is basic to the pattern of intrinsic change and problems of the aging larynx. The size of the sample used in this study has made it possible to suggest but not confirm the association between connective tissue changes and the decline of glandular function. There reportedly exists a disassociation between connective tissue fibers and muscle fibers in the true vocal fold in aged specimens (15) that is similar to the separation of connective tissue fibers from glandular acini observed in the study. Do these observations suggest a similar response to biological aging? Do they exist as part of a larger pattern of connective tissue change? Are connective tissue changes in the true vocal fold always associated with the same changes in the false fold? Do changes in the false fold precede changes in the true fold? These questions must be answered using larger groups of subjects within each decade of life. Further research should include female subjects, since females reportedly experience a different rate of advancement of laryngeal aging in general (26).

Indeed, another interesting question regards the constituent components of the secretions found in the larynx. How is the delicate balance maintained? It is known that exogenous influences may trigger the goblet and intraepithelial cells to produce their product. Submucosal glands are stimulated via sympathetic and parasympathetic nervous stimuli. If the submucosal glands are suppressed or function at a suboptimum level, do the goblet cells at the surface of the epithelium, secondary to drying, eventually begin to produce more product in compensation? Careful chemical analysis of each product, its stimulus, and source may ultimately explain the dilemma of the elderly patient who presents with secretory disturbance secondary to change in quality or quantity of secretions.

The interaction of secretory components at the level of the larynx is a final and crucial question. It is apparent that laryngeal hydration is important for phonation. The less obvious question regards the interaction of fluids within the vocal fold as well as external to the epithelium. The questions raised by Finkelhor et al. (9) regard the internal as well as the external tissue viscosity. How patent is the epithelium as a barrier to fluid transport? What are the compensatory strategies of the mucosa in the presence of dehydration? Once the chemical and transport factors are known, the optimum tissue viscosity at the level of the vocal fold may be discovered. Ultimately, these known values may help in the development of chemical agents to restore or enhance vibratory characteristics in patients who suffer from phonatory disorders secondary to alterations in the status of hydration of the laryngeal tissues.

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