Review Article

Apoptosis and Vocal Fold Disease: Clinically Relevant Implications of Epithelial Cell Death

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Purpose: Vocal fold diseases affecting the epithelium have a detrimental impact on vocal function. This review article provides an overview of apoptosis, the most commonly studied type of programmed cell death. Because apoptosis can damage epithelial cells, this article examines the implications of apoptosis on diseases affecting the vocal fold cover.

Method: A review of the extant literature was performed. We summarized the topics of epithelial tissue properties and apoptotic cell death, described what is currently understood about apoptosis in the vocal fold, and proposed

several possible explanations for how the role of abnormal apoptosis during wound healing may be involved in vocal pathology.

Results and Conclusions: Apoptosis plays an important role in maintaining normal epithelial tissue function. The biological mechanisms responsible for vocal fold diseases of epithelial origin are only beginning to emerge. This article discusses speculations to explain the potential role of deficient versus excessive rates of apoptosis and how disorganized apoptosis may contribute to the development of common diseases of the vocal folds.

The true vocal folds are involved in voice production by modulating airflow from the lungs to generate sound. Listeners then internally synthesize the physical production of the voice signal, which is perceived as normal or dysphonic voice quality (Kreiman & Sidtis, 2011). Investigators have implemented a variety of approaches to study the mechanics of normal and abnormal vocal fold vibration. A traditional method relies on theoretical models to explain the physiology of phonation.

In an effort to build upon theory, research has progressed to use of computational and physical models to depict mechanical stress distributions during simulated vocal fold vibration (Cveticanin, 2012; Miri, 2014). To bridge vocal fold tissue mechanics with clinically relevant metrics, scientists have focused on vibration dosimetry techniques using accelerometers, instruments that attach to the neck to detect and estimate vocal fold vibration during continuous speech (Titze & Hunter, 2015; Titze, Svec, & Popolo, 2003). Despite substantial scientific advancements that have been made with these approaches, these methods cannot be used to explain the underlying tissue changes that lead to dysphonia. To fill this need, basic science approaches have gained popularity for investigation of the influence of vibration on direct structural damage to the vocal folds.

The vocal folds are composed of epithelium, lamina propria, and muscle. Stratified squamous epithelium constitutes the superficial cellular surface of the vocal folds (Gray, 2000). Underneath the basal cells, or innermost epithelial cells, is the basement membrane zone, a type of specialized lamina propria (Titze, 1994). There are three layers of lamina propria that include a superficial, intermediate, and deep layer. The bulk of the vocal fold is made up of the thyroarytenoid muscle. The structure of the vocal fold lamina propria is important for determining tissue viscoelasticity and vibratory output (Long, 2010). Because normal voice

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quality is characterized by periodic or regular vocal fold vibration that depends on the integrity of the tissue, disruption to the layered structure results in irregular vibration and often dysphonia. Cells located in the lamina propria have received the greatest attention because of their importance in maintaining tissue homeostasis. However, the outermost layer of the vocal fold is particularly interesting because of its important role in protecting the underlying lamina propria. Thus, investigation of the epithelium may be useful for better understanding the biological mechanisms underlying vocal fold diseases of epithelial origin. The following section describes the major biological properties of epithelial tissue and the relevant literature regarding the vocal fold epithelium.

Properties of Epithelial Tissue

Epithelium is tissue that covers external body surfaces, primarily the epidermis (i.e., skin). Epithelium also lines glands and the internal cavities of the respiratory, digestive, reproductive, and urinary tracts (Ganz, 2002). Epithelium is composed of sheets of closely adjoined epithelial cells. Special properties distinguish epithelial cells from other cells in the body (Martini, 1998; Ross, Kaye, & Pawlina, 2003). Cell polarity directs cell movement and permits specific functions to correspond to three cell membrane domains or directions (Dowdall et al., 2015; Rodriguez-Boulan & Macara, 2014; Ross et al., 2003). The apical or free domain orients to the lumen of internal cavities or external body surfaces, the lateral domain is located inferior to the apical surface, and the innermost basal domain faces the basement membrane (Ross et al., 2003). Another property of epithelial tissue is cell-to-cell adhesion via the assembly of cell junctions. The apical junctional complex seals the paracellular pathways (pathways between epithelial cells) via tight junctions (Marchiando, Graham, & Turner, 2010). At the lateral domain, the adherens junction assists with assembling the tight junctions. The entire junctional complex requires a carefully regulated degree of penetration that depends on the function of the tissue. A highly permeable apical junctional complex may threaten the underlying tissue by allowing unwanted toxins to pass through neighboring structures. However, an impermeable apical junctional complex can lead to impaired nutrient absorption in organs such as the small intestine (Marchiando et al., 2010).

Epithelial tissue is characterized by rapid cell turnover. Cell turnover refers to continual cell self-renewal and is a life span cycle involving cell death, division, and renewal (Pellettieri & Sanchez Alvarado, 2007). In epithelial tissue, frequent cell turnover promotes a strong barrier against insults from the external environment (Hooper, 1956; Pellettieri & Sanchez Alvarado, 2007). Cell death terminates differentiated epithelial cells from the apical surface. As apical cells detach, basal cells proliferate (reproduce rapidly) in the deeper layers (Hooper, 1956). As a result, rapidly renewing cells assist with maintaining normal epithelial tissue homeostasis. In certain tissues, the rate of cell turnover may serve a specific purpose. For instance, endometrial epithelial cells undergo higher rates of cell turnover during phases of the menstrual cycle

corresponding with shedding of the uterine lining (Ellis, Yuan, & Horvitz, 1991).

Scientists have described major features of the epithelium of the vocal folds. Nonkeratinized, stratified squamous cell epithelium constitutes the surface cell layer of the membranous true vocal folds (Gray, 2000). The epithelium of the vocal folds is considered an active layer. Epithelial cells are capable of transporting ions and water and can readily respond to a variety of environmental challenges. The vocal fold epithelium is the primary recipient of environmental challenges during inhalation (e.g., air pollutants), gastric content from the digestive tract (e.g., acid reflux), and biomechanical stresses during vibration (e.g., phonotrauma; Levendoski, Leydon, & Thibeault, 2014). Stratified squamous epithelium is thick and most flattened at the superficial layer and typically covers structures that undergo more severe forms of biomechanical trauma (Maximow & Bloom, 1952). Gray (2000) speculated that in addition to tolerating trauma the vocal fold epithelium provides shape to the underlying lamina propria.

Because a major role of the vocal fold epithelium is to protect, epithelial cells must form a selective barrier. This epithelial barrier forms the boundary between the external environment and the underlying connective tissue (Ross et al., 2003). The primary function of the epithelial barrier is to prohibit water, ions, and large solutes from freely passing through the barrier (Marchiando et al., 2010). The vocal fold epithelial barrier is maintained by the tight intercellular junctional complex, which includes tight junction proteins located at the apical junctions of the epithelium (Levendoski et al., 2014). Researchers have speculated that the vocal fold epithelium is primarily responsible for protecting the underlying connective tissue. Emerging evidence also suggests that the vocal fold epithelium plays a role in vibratory function (Murray & Thomson, 2012; Tse, Zhang, & Long, 2015; Xuan & Zhang, 2014). Thus, normal epithelium is an important factor in maintaining normal voice quality.

Research on cell turnover in the vocal fold epithelium has received limited attention. Given that the vocal folds undergo significant mechanical trauma during vibration, it is important to understand how cell turnover may assist with maintaining vocal fold tissue homeostasis. A logical mechanism to examine during turnover is cell death, given that the regulation and dysregulation of cell death is indicated in a variety of human diseases. The next section provides a review of the most commonly studied form of cell death, apoptosis, and research on apoptosis in the vocal folds.

Apoptosis

Cells throughout the body continually self-renew via turnover. To maintain tissue morphology and function, differentiated cells (cells that become specialized to perform predetermined functions) are regularly replaced with proliferating cells (Denecker, Vercammen, Declercq, & Vandenabeele, 2001). Epithelial tissue maintains homeostasis and self-organizes

by cell death, that is, the physiological elimination of cells (Macara, Guyer, Richardson, Huo, & Ahmed, 2014). The most commonly studied type of programmed cell death is apoptosis, which is a genetically regulated mechanism that determines which cells are eliminated (Ashkenazi & Dixit, 1998; Denecker et al., 2001; Elmore, 2007). Apoptosis has been described as intentional cell death or physiological cell suicide. This notion is emphasized by the Greek origin of the word apoptosis (*apo* meaning “from” and *ptosis* meaning “a fall”), which provides imagery of leaves falling naturally from a tree (Majno & Joris, 1995). Macroscopically, cell death is most obvious during desquamation (shedding of the outermost layer of tissue) of the superficial layer of the epidermis (Hooper, 1956). An example of desquamation is skin peeling from a sunburn. During the induction phase, a stimulus triggers apoptosis via transmitting signals to the cell or the cell recognizes an internal defect (e.g., DNA damage) and initiates apoptosis. As the cell identifies the signal to die, it subsequently commits to apoptosis during the detection phase. The effector phase then begins, in which downstream effectors and caspases activate. The final removal phase permanently eliminates the cell (Rai, Tripathi, Sharma, & Shukla, 2005).

Apoptosis is considered an organized method of cellular degradation for several reasons. During apoptosis, the plasma membrane is maintained and cellular constituents of apoptotic cells are not disbursed throughout the surrounding tissue. Thus, apoptosis generally does not initiate an inflammatory response. The process of phagocytosis, in which a cell engulfs a solid particle, occurs relatively quickly, thereby reducing the chances of reactive necrosis. No proinflammatory cytokines are associated with cells during phagocytosis (Elmore, 2007). Although macrophages do not release proinflammatory cytokines during phagocytosis, Gregory and Devitt (2004) reported that macrophages are related to the production of anti-inflammatory cytokines. Apoptosis generally affects only single or small clusters of cells (Elmore, 2007).

Morphological criteria typically identify the type of cell death. Apoptosis begins with cell shrinking and increased cell density (Majno & Joris, 1995). During the early phases of apoptosis, another readily identifiable feature is pyknosis, in which chromatin condenses and becomes more concentrated. Next, a budding phenomenon results in rupturing of the cell nucleus (karyorrhexis). The cell extrudes fragments via plasma membrane blebbing (Majno & Joris, 1995), and these cellular fragments subsequently transform into apoptotic bodies. Apoptotic bodies consist of cytoplasm with tightly organized organelles and an intact plasma membrane that maintains the integrity of the organelles. Phagocytosis removes the apoptotic bodies by surrounding cells such as macrophages, parenchymal cells, or neoplastic cells (Elmore, 2007; Majno & Joris, 1995).

Regulation of Apoptosis

Cysteine-aspartic proteases (caspases) control apoptosis. Caspases are a family of cysteine proteases (enzymes

that degrade proteins) and are cell death proteases. When a single caspase is activated, a chain reaction activates multiple caspases (Cory & Adams, 2002). An initiator caspase is first activated to trigger the apoptotic cascade, which activates downstream effector caspases (i.e., caspase-3 and caspase-7). Effector or executioner caspases cause the morphological signs of apoptosis (Fiers, Beyaert, Declercq, & Vandenabeele, 1999). Apoptosis is activated by intrinsic and extrinsic signaling pathways that both conclude with the execution pathway of caspase-3, -6, or -7 cleavage.

Intrinsic Apoptosis Signaling Pathway

In the intrinsic apoptosis signaling pathway (e.g., mitochondrial pathway), the B cell lymphoma 2 (Bcl-2) protein family is the primary regulator (Lalaoui, Lindqvist, Sandow, & Ekert, 2015). Several other family members also share regions of homology, referred to as the Bcl-2 homology domains, and promote survival (e.g., Bcl-XL, Bcl-w, and Mcl-2). Bcl-2 proteins that promote apoptosis include a group that shares all four Bcl-2 homology domains (i.e., BAX, BAK, and BOK) and a group that shares only the Bcl-2 homology-3 domain (i.e., BIM, PUMA, BID, BAD, NOXA, BIK, HRK, and BMF; Lalaoui et al., 2015). Bcl-2 family proteins balance whether a cell's fate is to survive or die. When a cell is programmed to survive, anti-apoptotic (i.e., prosurvival) Bcl-2 family members bind to the prodeath family members to neutralize and ultimately prevent their action (Walensky, 2006). However, a cell is committed to undergo apoptosis when BAX and BAK are activated. BAX and BAK oligomerize and create pores in the mitochondrial membrane to allow the release of proteins such as cytochrome *c*. Cytochrome *c* combines with apoptosis protease activating factor (i.e., Apaf1), which recruits caspase-9 to form a protein complex termed the apoptosome. Once the complex is formed, caspase-9 becomes active, subsequently activating caspase-3 and caspase-7. These caspases cleave their substrates (i.e., target of an enzyme), one of which is the inhibitor of caspase-activated deoxyribonuclease (DNase). Cleaving the inhibitor of caspase-activated DNase activates caspase-activated DNase, and this step causes the hallmark characteristic of DNA fragmentation in cells undergoing apoptosis (Lalaoui et al., 2015). DNA fragmentation is the foundation for many experimental approaches to detect apoptosis via the intrinsic apoptosis signaling pathway. Figure 1 depicts the overall schematic of the intrinsic apoptosis signaling pathway.

Extrinsic Apoptosis Signaling Pathway

In contrast, the extrinsic apoptosis signaling pathway, or death receptor pathway, involves a subcategory of death receptors. Death receptors are cell surface receptors that belong to the tumor necrosis factor receptor gene superfamily (Fiers et al., 1999). For instance, triggering the death receptor CD95 (i.e., Fas and Apo1) ultimately leads to the generation of the death-inducing signaling complex (Lalaoui et al., 2015). Following CD95 ligation, death domain

Figure 1. Schematics of the B cell lymphoma 2 (Bcl-2) family of proteins (Panel A) and the intrinsic apoptosis signaling pathway (Panel B). A cell is committed to undergo apoptosis when BAX and BAK are activated (circled in Panel A).

receptors cluster and the adaptor protein Fas-associated death domain binds to the clustered death receptor domains. Fas-associated death domain contains a death effector domain that recruits caspase-8 to activate by self-cleavage, triggering downstream effector caspases. Activation of these caspases also results in formation of mitochondrial membrane pores by BAX oligomerization, releasing cytochrome *c* and further activating effector caspases. This is the point at which the cell is committed to undergo apoptosis (Ashkenazi & Dixit, 1998). Figure 2 depicts the overall schematic of the extrinsic apoptosis signaling pathway.

Figure 2. Schematic of the extrinsic apoptosis signaling pathway.

Extrinsic Apoptosis Signaling Pathway

Other Forms of Cell Death

Additional forms of cell death include necrosis, programmed necrosis, secondary necrosis, autophagy, and aponecrosis. Necrosis (i.e., oncosis), or accidental cell death, is viewed as a disordered manner in which cells die and is associated with an inflammatory reaction (Denecker et al., 2001; Elmore, 2007). Necrosis is characterized by cell swelling, karyolysis or complete disbanding of chromatin by the enzymatic action of endonucleases, plasma membrane rupture, and eventual dispersal of cytoplasmic contents into the surrounding tissue (Denecker et al., 2001; Elmore, 2007). In contrast, signaling pathways intentionally regulate programmed necrosis (i.e., necroptosis and necrapoptosis; Edinger & Thompson, 2004). Secondary necrosis can occur after apoptosis is initiated when apoptotic bodies are unable to be cleared by neighboring cells. When surrounding cells are unavailable or defective, secondary necrosis is activated via autolysis. Autolytic degradation, or self-digestion, of the dying cell then completes the process of cell death (Silva, 2010). Autophagy or Type II cell death is nonapoptotic and refers to cellular “self-eating.” Aponecrosis has morphological characteristics of both apoptosis and necrosis and is identified by decreased adenosine triphosphate (Formigli et al., 2000; Papucci et al., 2004). Although researchers continue to expand the study of different types of cell death, apoptosis remains the most commonly studied form.

Functions of Apoptosis

In addition to maintaining tissue morphology and function, cell death serves as a defense mechanism when cells are damaged or injured (Elmore, 2007). Such mechanisms serve both a physiologic role in normal states and a

pathologic role in disease. A number of conditions can trigger cell death, such as when a cell loses contact with its surroundings or when there are too many conflicting signals at one time (Ashkenazi & Dixit, 1998). Many environmental insults cause apoptosis. Biomechanical trauma is a common injury that induces apoptosis to maintain homeostasis in a number of biological systems (Que & Gores, 1996; Ren & Wilson, 1997; Serbest, Horwitz, Jost, & Barbee, 2006; Watson, Duckworth, Guan, & Montrose, 2009; Wernig & Xu, 2002; Wilson & Kim, 1998). External biomechanical stresses present a significant risk to a variety of cell types throughout the body. Thus, to maintain homeostasis cells must rapidly adapt to the constant changes in mechanical forces. Apoptosis is one specific cellular mechanism for regulating cells and tissues. For instance, corneal epithelium is subjected to mechanical injury such as eye blinking, eye rubbing, and poorly fitted contact lenses (Wilson & Kim, 1998). Using an in vitro rabbit model to simulate eyelid blinking, Ren and Wilson (1997) induced shear stresses to corneal epithelia via magnetic stirring in tear solution with stirring in static tear solution as the control. Results revealed that the mean rate of apoptosis in corneas exposed to shear stresses was significantly higher than that in the control. The rate of apoptosis significantly increased with increasing stress time, suggesting that biomechanical stresses caused the epithelial surface to exfoliate and remove a greater number of cells (Ren & Wilson, 1997).

The cardiovascular system is also vulnerable to mechanical forces such as stretch and shear stresses. In animal models of hypertension, increased rates of apoptosis were present in the heart and brain (Wernig & Xu, 2002). Apoptosis and necrosis also were observed in neuronal cells after mechanical forces simulated damage from traumatic brain injury (Serbest et al., 2006). In the gastrointestinal tract, cell turnover is frequent. During turnover, apoptosis is a key process as the lining of the intestinal wall sheds (Que & Gores, 1996; Watson et al., 2009). Although researchers disagree regarding the precise functional role that apoptotic cell death plays during intestinal cell shedding, associations between apoptosis and epithelial barrier function have been found in intestinal disease (Watson et al., 2009). Overall, regular damage from biomechanical trauma seems to result in apoptosis. In contrast, severe mechanisms of injury such as infections and toxins are more often associated with necrosis. Tissues may signal cells to initiate apoptosis after sustaining mild damage in the form of biomechanical stress, which appears to be a critical component in maintaining normal tissue regulation. Other common stimuli to activate cell death are toxic agents, heat, radiation, hypoxia, and ischemia (Denecker et al., 2001; Elmore, 2007).

Irregularities and disruptions in the process of cell death can lead to adverse consequences in such disorders as cancer, autoimmune disease, and neurological disease (Ashkenazi & Dixit, 1998; Cory & Adams, 2002). In the intestine and cornea, an emerging threat to the epithelial barrier is apoptotic cell shedding (Marchiando et al., 2011; Ren & Wilson, 1997). Irregular rates of apoptosis, either excessive or deficient, occur under conditions of disease or

trauma (Elmore, 2007; Meresman et al., 2000). Increased rates of apoptotic cell shedding can lead to gaps in the epithelial surface layer. Such gaps may significantly increase the permeability of the epithelial barrier (Gitter, Bendfeldt, Schulzke, & Fromm, 2000) and increase its susceptibility to invasion by pathogens, thereby compromising the barrier function, that is, protection from the external environment (Watson et al., 2009).

Given that epithelial tissue is found in many different types of surfaces and internal cavities, the primary function of the epithelium must be considered when evaluating normal versus abnormal tissue homeostasis. Although a large body of literature about apoptosis exists, different cellular properties guide different cell types (Majno & Joris, 1995). Because the vocal folds are susceptible to several types of injury (e.g., biomechanical and iatrogenic), the role of apoptosis in the vocal fold tissue should be evaluated. The following section describes what is currently known about apoptosis in the vocal folds.

Apoptosis in Vocal Folds

Research is emerging regarding apoptotic cell death in the vocal folds. Apoptosis occurs during the embryologic development of murine vocal fold epithelium. Lungova, Verheyden, Herriges, Sun, and Thibeault (2015) investigated the mechanisms by which the vocal fold epithelium is formed during major developmental events. Their work revealed that during the fusion of the lateral epithelial walls and separation of the epithelial lamina, epithelial cells of prospective vocal folds die by apoptosis. Lungova et al. (2015) found that although apoptotic cell death was observed during embryogenesis, rates of apoptosis did not seem to be associated with any of the investigated developmental events. Thus, apoptosis may play a primary functional role in eliminating vocal fold epithelial cells to assist with tissue remodeling and sculpting.

In addition to normal development, biomechanical forces applied to the vocal folds can cause apoptosis. For instance, Bartlett et al. (2015) used a bioreactor to study the effect of vibration on the rate of cell death in cultured human vocal fold fibroblasts. Following vibration, 2%–7% of fibroblasts underwent apoptotic cell death. Gaston, Quinchia Rios, Bartlett, Berchtold, and Thibeault (2012) also confirmed that cultured human vocal fold fibroblasts undergo apoptosis. Their work revealed that the rate of apoptosis in vocal fold fibroblasts that underwent vibration exposure in a synthetic extracellular matrix for 8 hr was not different from that in nonvibrated controls. Although fibroblasts underwent apoptotic cell death in response to biomechanical stimulation, apoptosis appeared to occur at the same rate as in fibroblasts that were not exposed to biomechanical stimulation.

Some evidence of apoptosis in the vocal fold epithelium has been found in response to biomechanical trauma. After in vivo rabbit vocal fold vibration and approximation, ultrastructural characteristics of apoptotic cell death were observed in vocal fold epithelial cells (Novaleski, Mizuta, &

Rousseau, 2016). Specifically, biomechanical vibration and vocal fold adduction and abduction without vibration resulted in condensed chromatin and apoptotic bodies. The vocal fold epithelium also expressed an immunohistochemical marker for apoptosis that labeled fragmented DNA. Further work using transmission electron microscopy quantified the immunohistochemical marker and morphological sign of cellular shrinkage (Novaleski, Kimball, Mizuta, & Rousseau, 2016). Results revealed that apoptosis occurred in the vocal fold epithelium after 120 min of in vivo vibration exposure compared with the same duration of vocal fold approximation only. The results of these two studies suggest that the vocal folds respond to mechanical forces by undergoing apoptosis. In contrast to standardized measurements of apoptosis, the literature reveals common descriptions that seem to allude to apoptosis in the vocal folds. Several researchers have characterized the microscopic morphology of benign vocal fold lesions as having shrinking nuclei and altered chromatin (Dikkers, Hulstaert, Oosterbaan, & Cervera-Paz, 1993; Kotby, Nassar, Seif, Helal, & Saleh, 1988). Although these descriptions allude to cellular features consistent with apoptosis, a degree of uncertainty remains because of a lack of standard apoptosis measures.

Apoptosis is involved in several voice disorders. Common voice disorders related to the epithelium include laryngeal squamous cell carcinoma, leukoplakia, keratosis, epithelial hyperplasia or thickening, human papilloma virus (HPV), and benign lesions. Surgical and medical interventions for voice disorders also can adversely affect the epithelial layer of the vocal folds. The most notable interventions impacting the epithelium are phonosurgery to remove lesions and radiation therapy to treat head and neck cancers. Thus, cell death by apoptosis may be involved, at least in part, in the pathogenesis of diseases affecting the vocal fold epithelium.

The most notable vocal fold disease involving apoptosis is laryngeal squamous cell carcinoma. Cancer involves simultaneous interactions of uncontrolled cell proliferation and reduced apoptosis (Evan & Vousden, 2001); incessantly proliferating cells invade and destroy tissue. Much interest has been expressed for exploring the prognostic and diagnostic significance of cell death in laryngeal squamous cell carcinoma to develop assessment tools to more accurately predict clinical outcomes for cancer patients. For instance, primary tumor and resection biopsies from patients with laryngeal squamous cell carcinoma and epithelial hyperplasia revealed evidence of apoptosis (Hellquist, 1997; Hirvikoski et al., 1999). Patients with increased apoptotic events had a significantly poorer prognosis for survival than did patients with fewer apoptotic events (Hirvikoski et al., 1999), which suggests that apoptosis may be a useful clinical biomarker for predicting the survival rate for laryngeal cancer.

Other researchers have argued that several simultaneous molecular markers are necessary to identify patients who would most benefit from aggressive treatment approaches for laryngeal squamous cell carcinoma. Georgiou, Gomatos, Pararas, Giotakis, and Ferekidis (2003) reported that among patients with no expression of the BAX protein, an association

was found between a lower rate of survival and protein expression of proliferating cell nuclear antigen. These results indicate that although the expression of a single protein did not predict the survival rate among patients, the combination of molecular markers improved the ability to find associations. A similar argument was echoed by Hellquist (1997), who observed that the rate of apoptosis did not differ between epithelial hyperplastic laryngeal lesion types that included laryngeal squamous cell carcinoma and lesions from simple and abnormal hyperplasia. This finding led to the hypothesis that a single apoptosis measure alone is insufficient to distinguish cancer from other laryngeal lesion types (Hellquist, 1997).

Strong evidence exists of an association between laryngeal squamous cell carcinoma and HPV. HPV16 is a common high-risk virus and is present in approximately 17% of laryngeal squamous cell carcinomas (Kreimer, Clifford, Boyle, & Franceschi, 2005); therefore, researchers have studied the effect of apoptosis on HPV cells. Du et al. (2004) focused on established laryngeal cell cancer lines that expressed HPV16 E6 and E7. These cells were less sensitive (i.e., cells were less likely to die) to apoptosis-inducing stimuli, suggesting that HPV may be particularly resistant to cell death and therefore more likely to incessantly proliferate (Du et al., 2004). It remains unknown whether HPV vaccinations could be clinically useful in the prevention of laryngeal squamous cell carcinomas (Kreimer et al., 2005). However, studying cellular mechanisms such as apoptosis may provide a better understanding of how HPV is involved in the development of laryngeal cancer and how these mechanisms could ultimately be manipulated to prevent and treat laryngeal cancer.

Tissue-level changes to the vocal folds are indicative of abnormalities at the cellular level. Macroscopic tissue changes often present as clinical signs of vocal fold pathology (Gray, 2000) and the auditory perceptual judgment of dysphonia. In an attempt to speculate about the importance of apoptosis in clinical vocal fold diseases, we propose several possible explanations for how abnormal apoptosis during wound healing may be involved in vocal pathology.

Speculations About Abnormal Apoptosis in Vocal Folds

An increasing amount of evidence indicates that apoptosis plays a critical role during wound healing. When a tissue is subjected to injury, the body responds by initiating a wound healing cascade. Wound healing is a complex process that requires a series of overlapping events that include an inflammatory, proliferative, and remodeling phase (see reviews by Branski, Verdolini, Sandulache, Rosen, & Hebda, 2006; Greenhalgh, 1998). Each phase of healing is contingent on the efficiency of the preceding phase because promptness determines the overall success of a healed wound. If open for a long time, a wound remains persistently inflamed and poor clinical outcomes result (Greenhalgh, 1998). It is believed that apoptosis is primarily responsible for removing

inflammatory cells that are no longer necessary during wound healing. Removal of these inflammatory cells reduces the time that the tissue remains in the inflammatory phase, thereby accelerating the wound healing response and signaling the next stage of cell proliferation (Ellis et al., 1991; Rai et al., 2005). Thus, apoptosis appears to promote optimal healing outcomes by reducing excessive scarring and fibrosis (Ellis et al., 1991; Elmore, 2007; Greenhalgh, 1998).

A steady balance between cell death and cell proliferation is critical to epithelial homeostasis and for understanding health and disease (Boccafroschi, Sabbatini, Bosetti, & Cannas, 2010; Hooper, 1956; Wernig & Xu, 2002). For example, autoimmune diseases often cause excessive production of apoptotic immune cells, leading to the inability to effectively protect against infections (Greenhalgh, 1998). Thus, an ideal homeostatic state occurs when cells proliferate at a rate reasonably comparable to that of cells that are dying (Hooper, 1956). However, frequent apoptosis may not always result in disease. In the small intestinal epithelium, the epithelial barrier is maintained during apoptosis because tight junction proteins subsequently rearrange to expand the length of the barrier (Madara, 1990; Marchiando et al., 2011). Such protective mechanisms may be in place to allow for brief disruptions in the rate of apoptosis without resulting in the development of a disease process.

Vocal pathology may be associated with abnormal rates of apoptosis. We speculate that the pathogenesis of vocal pathology may be associated with cell signaling miscommunication that involves apoptosis. Signal disruptions may lead to ineffective communication that inflammatory cells have increased, resulting in the inability to properly initiate apoptosis. For instance, a signal for apoptosis may be accidentally turned off or unable to be detected. A significant decrease in the rate of apoptotic cell death might ultimately lead to an imbalanced cell number. Because epithelial cells cannot be eliminated quickly enough to reach the next stage of wound healing, chronic or poor wound healing may result from this cell signaling problem. Such a chronic nonhealing wound may be vulnerable to further injury during continued vibration exposure. Rather than progressing from granulation to scar, irregularities in apoptosis may eventually evolve into epithelial hyperplasia, benign nodular formations, or subsequent formation of excessive vocal fold scarring (Greenhalgh, 1998; Rai et al., 2005).

Another direction in the rate of abnormal apoptosis may provide an alternative explanation regarding the mechanisms of vocal pathology. If significantly increased rates of apoptosis occur, the tight junction proteins could gradually lose the ability to remodel at a sufficient rate. The change in phenotype of the apical junctional complex could result in enlarged epithelial surface gaps. Failure of epithelial gaps caused by apoptosis to sufficiently reseal presents a substantial threat to the integrity of the epithelial barrier. Greater gaps might increase the epithelium's permeability to pathogen invasion into the vocal fold lamina propria. Sites of pathogen attack and infections may lead to greater vulnerability to further epithelial injury. Therefore,

excessive rates of apoptosis may indicate that that vocal fold tissue has an underlying susceptibility to disease. Thus, dysregulated apoptosis (e.g., deficient or excessive) may be implicated in the pathogenesis of vocal fold disease. Abnormalities in apoptotic cell death, similar to those observed in nonmalignant cases such as autoimmune disease, inflammatory bowel disease, and neurological disease (Ashkenazi & Dixit, 1998; Cory & Adams, 2002; Neurath et al., 2001), may be a predictor of individuals who are more susceptible to developing voice disorders. If so, biomarkers for apoptosis could provide a useful screening tool for identifying individuals at risk for voice disorders (e.g., teachers and singers). Thus, preventive strategies and treatments could be more effectively implemented among individuals who would benefit from early intervention.

Conclusion

In the voice science literature, basic science approaches have gained increasing popularity with the goal of investigating the mechanisms underlying vocal fold repair. Vocal fold diseases affecting the epithelium have a detrimental impact on vocal function. Healthy vocal fold epithelium is necessary to maintain an optimal environment for healthy vocal folds (Murray & Thomson, 2012; Tse et al., 2015; Xuan & Zhang, 2014). In contrast, disorganized vocal fold epithelium presents clinically in many voice disorders. The vocal fold epithelium serves several important biological functions that contribute to maintaining a healthy environment for normal tissue function. Among these functions, apoptosis is responsible for removing faulty cells when something goes wrong during the cell turnover process. Apoptosis is the most commonly studied type of programmed cell death that occurs through complex signaling pathways that are responsible for allowing cells to either die or survive. In the vocal folds, apoptosis occurs during development and after biomechanical trauma and is involved in several diseases of the vocal folds. An increasing amount of evidence indicates that apoptosis plays a critical role during wound healing. Thus, the study of apoptosis in the vocal folds may provide a better understanding of normal vocal fold function and the development of voice disorders.

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