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REVIEW ARTICLE

Biomarkers and laryngopharyngeal reflux

J M WOOD, D J HUSSEY, C M WOODS, D I WATSON, A S CARNEY

ENT Unit, Department of Surgery, Flinders University and Flinders Medical Centre, Bedford Park, South Australia, Australia

Abstract

Laryngopharyngeal reflux is a controversial but increasingly made diagnosis used in patients with a collection of often non-specific laryngeal symptoms. It is a clinical diagnosis, and its pathophysiology is currently poorly understood.

Previous reflux research has focused on injurious agents, acid, pepsin and biomarker expression. Failure of intrinsic defences in the larynx may cause changes in laryngeal epithelia, particularly alterations in carbonic anhydrases and E-cadherin. Carbonic anhydrase III levels vary in the larynx in response to laryngopharyngeal reflux, depending on location. Expression of E-cadherin, a known tumour suppressor, is reduced in the presence of reflux. Mucin expression also varies according to the severity of reflux.

Further research is required to define the clinical entity of laryngopharyngeal reflux, and to identify a definitive mechanism for mucosal injury. Understanding this mechanism should allow the development of a comprehensive model, which would enable future diagnostic and therapeutic interventions to be developed.

Key words: Larynx; Mucins; Pepsin A; Gastroesophageal Reflux; Carbonic Anhydrases; Cadherins

Introduction

Throat symptoms and gastroesophageal reflux are common clinical complaints which may be linked, with at least a proportion of some throat symptoms occurring secondary to laryngopharyngeal reflux. However, laryngopharyngeal reflux is a highly controversial issue for otolaryngologists, gastroenterologists and upper gastrointestinal surgeons, with widely varying views held about its prevalence, aetiology, diagnosis and treatment. A better understanding of this condition, and of the role of various investigations and treatments, is essential for progress in this area.

In this paper, we review the evidence supporting the diagnosis of laryngopharyngeal reflux, focusing on the role of molecular biology and on the possible ways this may assist the clinician.

Gastroesophageal reflux disease is one of the commonest diseases in the Western world,[1,2] affecting up to 50 per cent of Western adult populations. Extra-oesophageal manifestations of gastroesophageal reflux disease have progressively attracted attention over the last 15 years, and have been linked to asthma, non-cardiac chest pain and chronic cough. Otolaryngological manifestations attributed to laryngopharyngeal reflux may include dysphagia, dysphonia, hoarseness, globus pharyngeus and altered salivation.[3]

Laryngopharyngeal reflux is increasingly 'diagnosed' in ENT practice, and often suspected in patients who present with chronic or intermittent laryngopharyngeal symptoms. These patients are typically identified by their medical history, clinical examination and fibre-optic laryngoscopy results. Changes attributed to laryngopharyngeal reflux include erythema and oedema of the posterior commissure, laryngeal granulomata, subglottic stenosis, vocal fold nodules, and laryngeal pseudosulcus. Both pre-malignant and malignant transformations have also been attributed to laryngopharyngeal reflux.[4] However, a history of non-specific laryngeal symptoms, and examination findings with poor inter-observer reliability,[5] make definitive diagnosis difficult.

From clinical and molecular biology research, it is becoming apparent that the pathophysiology of laryngopharyngeal reflux may actually be different to that of gastroesophageal reflux disease.[4] Patients with laryngopharyngeal reflux are thought to suffer more upright (daytime) reflux, whereas patients with gastroesophageal reflux disease tend to reflux more in the supine (nocturnal) position.[4] Additionally, it is likely that mucosal acid exposure is prolonged in gastroesophageal reflux disease when compared with laryngopharyngeal reflux.[4] This is because distal oesophageal acid exposure is always greater than proximal exposure, and the mechanism underpinning laryngopharyngeal reflux probably depends on shorter periods of exposure to refluxate.

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Despite this, definitive diagnosis has proved elusive, and there is no consistently reliable diagnostic tool currently available.[6] Hypopharyngeal pH monitoring has a reported diagnostic sensitivity of only 40 per cent,[7] and there is no pathognomonic laryngopharyngeal reflux finding on laryngoscopy.

Currently, commencement of empirical antireflux medication (typically proton-pump inhibitors (PPIs)) has been used as an alternative diagnostic modality, with a favourable response taken as 'confirming' the diagnosis. The literature on PPIs and other antireflux medication is variable in quality, and different outcomes have been reported.[8]

Laparoscopic fundoplication is a well established surgical treatment for gastroesophageal reflux disease, with reliable and reproducible results;[9] however, its role in the management of laryngopharyngeal reflux is uncertain. Recent research reviewing a large series of patients following fundoplication found that patients with throat symptoms in addition to typical (gastroesophageal) reflux symptoms had a similar improvement to those with only typical reflux symptoms.[9] In contrast, patients with only throat symptoms, but with objective evidence of reflux on 24-hour pH monitoring, had a much poorer outcome,[9] indicating a possible non-reflux-related cause of symptoms in many of these patients.

However, an incomplete understanding of the pathophysiology and accurate diagnosis of laryngopharyngeal reflux makes high quality evaluation of any medical or surgical management difficult. Consequently, an understanding of the molecular basis of laryngopharyngeal reflux is an important first step.

Damaging events

The luminal environment of the pharynx is pH-neutral, at 7.0,[10] whilst the stomach secretes acid at a pH of 1.5 to 2.0. Consequently, reflux can lead to a significant decrease in laryngeal pH. Damage may occur due to this drop in pH and also due to exposure to noxious elements in the refluxate, including pepsin, bile salts and pancreatic enzymes.[11] In order for refluxate to reach the oesophagus and larynx, there needs to be failure of the anatomical and physiological barriers to reflux. Whilst it is normal for individuals to experience some 'physiological' oesophageal reflux, the amount of laryngopharyngeal reflux required to cause injury is uncertain.

Acid

Whilst up to 50 oesophageal reflux episodes per day can be considered normal,[12] as few as three episodes of laryngeal reflux may cause mucosal injury.[3] Consequently, techniques for diagnosing laryngeal reflux episodes based on oesophageal reflux may lack the sensitivity to identify such infrequent events. Acid reflux is recognised as leading to oesophagitis, which increases in severity with increasing acid exposure. However, the effect of acid on the larynx is uncertain, with some research suggesting that a combination of acid and pepsin is required to cause laryngeal damage.[11]

Pepsin

Non-acidic reflux has increasingly been implicated in leading to inflammation in both laryngopharyngeal reflux and gastroesophageal reflux disease. Multichannel intra-luminal pH monitoring impedance studies have identified episodes of gastric reflux that are either non-acidic or weakly acidic, in symptomatic patients,[13] suggesting that mucosal injury may be caused by non-acid refluxate components such as bile salts and pepsin. The damaging effects of pepsin in an acidic environment have been well described previously,[3] with an optimum activity at a pH of 2.0.[14] Recent research has proposed that pepsin is a causative agent of laryngeal damage in non-acidic reflux.[11–13,15]

Whilst pepsin is inactive at a pH of 6.5,[14] it is irreversibly inactivated at a pH of 8.0.[16] Recently, it has been shown that at 37°C pepsin remains stable at a pH of 7.0 for more than 24 hours, retaining nearly 80 per cent of its original activity on re-acidification. With a mean pH of 6.8,[15] the larynx may contain stable pepsin, which may potentially cause more damage with subsequent reflux episodes. Additionally, there is evidence that such pepsin is actively transported into, and remains within, laryngeal epithelial cells.[16] Intracellular structures such as Golgi bodies and lysosomes have a lower pH (of 5.0 and 4.0, respectively); therefore, pepsin could be acting by causing intracellular damage[16] even if the larynx itself is only exposed to inactive pepsin.

Furthermore, research on patients with reflux-attributed laryngeal disease has found a significant association between the presence of pepsin in laryngeal epithelia and the depletion of two laryngeal protective proteins, carbonic anhydrase isoenzyme III (CA III) and Sep70 (a squamous epithelial stress protein).[16] It is of note that both these proteins are depleted after exposure to pepsin, and not in response to low pH alone, suggesting a specific role for pepsin in laryngeal damage.

Bile acids

While acid and pepsin are important in the development of oesophageal mucosal injury, there is evidence to suggest that duodeno-gastro-oesophageal reflux contributes bile acids and pancreatic secretions to the refluxate. Duodenal secretions have been shown in clinical studies to be capable of refluxing into the stomach and oesophagus,[17,18] and of causing damage to the larynx.[19] Conjugated bile causes mucosal injury at a low pH (1.2–1.5).[20]

Interestingly, the unconjugated component of bile, chenodeoxycholic acid, is activated at pH 7.0 but not at pH 2.0. Consequently, in the experimental setting, conjugated bile acids are more injurious to mucosa at an acidic pH, whereas chenodeoxycholic acid is more active at pH 5.0–8.0.[19] Recent research exposed rat

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laryngeal mucosa to taurocholic acid and chenodeoxycholic acid at a pH range of 1.5–7.4, with normal saline as a negative control, and found that taurocholic acid is injurious to laryngeal mucosa at a pH of 1.5, whereas chenodeoxycholic acid causes maximum inflammation at a pH of 7.4.[19] This suggests that bile has a mechanism for generating laryngeal injury in both acid and non-acid environments, although it remains to be determined whether the same mechanism occurs in the human larynx.

Reflux biomarkers

Inflammatory cytokines

Multiple inflammatory cytokines have been implicated in oesophageal mucosal inflammation caused by reflux. It has been well documented that gastroesophageal reflux disease leads to changes in interleukin-6 (IL-6) messenger RNA expression, and this correlates with reflux-induced mucosal inflammation.[21] Interleukin-6 is a cytokine with roles in multiple processes, including acute-phase responses and inflammation and immune responses.[22] It is recognised that oesophageal levels of IL-6 increase as the grade of reflux pathology increases, and decrease following treatment of gastroesophageal reflux disease. Consequently, it would be reasonable to consider IL-6 to be an indicator of reflux-related inflammation in both the oesophageal and laryngeal mucosa. Despite this, few studies of laryngopharyngeal reflux have directly included IL-6 as a marker of inflammation.[23]

Interleukin-8 has also been implicated in the inflammatory process associated with gastroesophageal reflux disease, and its expression has been found to increase with such reflux. The greatest expression levels have been found in the oesophageal mucosa of patients with reflux-related complications, including Barrett's dysplasia and adenocarcinoma. Following anti-reflux surgery, IL-8 levels decrease significantly.[24] Activation of this cytokine is of importance, given its role in tumour progression. Tumour-derived IL-8 is recognised as having an autocrine mechanism, and can activate endothelial cells in tumour vasculature to promote angiogenesis; it can also enhance the proliferation and survival of cancer cells. Furthermore, it can induce tumour-associated macrophages to secrete additional growth factors that can increase the rate of cell proliferation.[25] The involvement of IL-8 in laryngopharyngeal reflux is still uncertain. However, *in vitro* experiments have demonstrated increased expression of this and other inflammatory markers, when exposed to pepsin.[13]

Carbonic anhydrase

The actual mechanism of damage caused by reflux is elusive. However, recent research has focused on the failure of anti-reflux barriers. Such failure could allow increased exposure of epithelia to refluxate, and in laryngopharyngeal reflux this occurs in an area considered to be more sensitive than the oesophagus to such injury.[12] Such exposure may have greater effects because the larynx lacks some of the extrinsic defences which are normally present in the oesophagus. Carbonic anhydrase (CA) is an integral component of this defence, and acts by catalysing the reversible hydration of carbon dioxide. This produces bicarbonate ions which are then actively pumped into the extracellular space, enabling neutralisation of acidic refluxate. In the oesophagus, this process plays a significant role, with CA capable of increasing the pH of gastroesophageal refluxed residual acid from 2.5 to close to neutral.[26]

There are 11 identified CA isoenzymes,[27] with demonstrated differences in activity, inhibitor susceptibility and tissue distribution. Carbonic anhydrase isoenzymes I to IV have been demonstrated to be expressed by oesophageal epithelium, and changes in distribution have been found in inflamed oesophageal biopsy specimens.[27]

Recent research has demonstrated that CA isoenzymes I, II and III are present in normal laryngeal epithelial cells to a variable extent.[27,28] Carbonic anhydrase isoenzyme III has been demonstrated in the squamous epithelial cells of the oesophagus and in the posterior commissure area of the larynx.[29] In inflamed oesophageal mucosa from patients with gastroesophageal reflux disease, increased levels of CA III expression have been noted both in the oesophageal squamous epithelia and in the laryngeal commissure, with a redistribution of expression from the basal to the suprabasal cell layers.[27,30] It is thought that these changes are due to refluxate, and represent attempts to counteract damage.[29] It has been proposed that an increase in CA III expression may be due to basal cell hyperplasia, a histopathological sign of oesophagitis.[27]

Carbonic anhydrase isoenzymes I and II have been demonstrated in both the vocal fold and inter-arytenoid areas, while CA III has been found throughout the laryngeal epithelium. In patients with laryngopharyngeal reflux, the expression of CA III has been found to differ between laryngeal biopsy locations.[27] In the presence of laryngopharyngeal reflux, and pepsin in particular, CA III expression in the vocal fold is potentially decreased, allowing further damage to occur from acidic refluxate. Conversely, CA III expression may increase in the posterior commissure in response to laryngopharyngeal reflux,[12] with a correlation between symptom severity and CA III levels.[28] Given that the larynx possesses areas of respiratory-type epithelium in addition to squamous epithelium, there remains the possibility that certain laryngeal areas may vary in response to laryngopharyngeal reflux; however, there is currently no epidemiological research assessing the epithelial type of various laryngeal areas in patients with laryngopharyngeal reflux.

E-Cadherin

The cadherin family of molecules are calcium-dependent, cell–cell adhesion molecules which mediate

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homophilic adhesion. E-cadherin is recognised as having a crucial role in the maintenance of epithelial integrity and barrier functions.[29] Damage to epithelial cell–cell adhesion from refluxate may lead to a breach of the mucosal barrier. Pepsin has been proposed to damage structures by digesting intracellular structures that maintain cohesion between cells.[29] Levels of E-cadherin have been found to decrease in response to laryngopharyngeal reflux,[31] but it is not clear whether this down-regulation is due to the refluxate components (e.g. acid and pepsin) or to an inflammatory response associated with the reflux.

Decreased expression of E-cadherin has been associated with poor prognostic factors in head and neck squamous cell carcinoma patients, including vascular invasion, and with decreased patient survival.[32] There is strong evidence that E-cadherin is a tumour suppressor, and that the loss of E-cadherin expression is a key initial step in tumour invasion.[32] Consequently, decreased E-cadherin expression in the presence of laryngopharyngeal reflux may play a role in the development of laryngeal symptoms, and may contribute to the development of laryngeal carcinoma in the setting of reflux.

TABLE I
MUCIN GENES IN THE AERODIGESTIVE TRACT

Gene	Localisation	Primary tissue expression
MUC1	Transmembrane	Breast, pancreas
MUC2	Secreted	Jejunum, ileum, colon
MUC3	Transmembrane	Colon, small intestine, gallbladder
MUC4	Transmembrane	Airways, colon
MUC5AC	Secreted	Airways, stomach
MUC5B	Secreted	Airways, submandibular glands
MUC6	Secreted	Stomach, ileum, gallbladder
MUC7	Secreted	Sublingual & submandibular glands
MUC8	Secreted	Airways
MUC12	Transmembrane	Colon, airways, reproductive tract
MUC13	Transmembrane	Colon, trachea, kidney, small intestine
MUC15	Transmembrane	Colon, airways, small intestine, prostate
MUC17	Transmembrane	Duodenum, colon, stomach
MUC18	Transmembrane	Airways, lung, breast
MUC19	Secreted	Salivary glands, trachea
MUC20	Transmembrane	Placenta, colon, lung, prostate, liver

Adapted with permission.[33]

Mucins

Mucins are high molecular weight glycoproteins which traditionally have been considered to be gel-forming components of viscoelastic mucus gels. They are expressed by various epithelial cell types that exist in relatively harsh environments exposed to fluctuations in pH, ionic concentration, hydration and oxygenation. Accordingly, their primary functions are protection, lubrication and transport. Mucins have also been implicated in renewal and differentiation of epithelium, modulation of cell cycle progression, adhesion, and signal cell transduction.[33] They have a role in maintaining homeostasis, and consequently promote cell survival. They are classified into two primary classes: secreted gel-forming mucins and transmembrane mucins. Sixteen mucins (see Table I adjustment) have been identified in the aerodigestive tract (Table I). Altered expression of mucins has been reported in a number of inflammatory and neoplastic diseases.

Samuels *et al.*[33] studied laryngeal biopsies from three patients with laryngopharyngeal reflux and two controls. MUC1–5, 7, 9, 13, 15, 16 and 18–20 were detected in normal laryngeal epithelium, while mucins 6, 8 and 17 were absent. Of these, MUC1 and 4 were the predominant transmembrane mucins, and MUC2, 5AC and 5B the major constituents of airway mucus in the laryngeal epithelium. In the patients with laryngopharyngeal reflux, there was decreased expression of MUC2, 5AC and 5B. This would lead to an overall decrease in mucin secretion from the laryngeal epithelium, resulting in decreased protection against further reflux episodes. This is consistent with a gastroesophageal reflux disease model in which oesophageal mucin secretion was also reduced in patients with reflux oesophagitis.[34]

Alterations in mucin expression have been identified in a variety of inflammatory conditions, including gastritis, peptic ulcer disease, intestinal neoplasia and inflammatory bowel diseases.

A reduction in MUC3 expression has also been noted in samples from laryngopharyngeal reflux patients. MUC3 has been noted to play an active role in epithelial cell restitution.[35] Specifically MUC3A mucins are thought to play a role in the maintenance of intestinal epithelium during hypoxic conditions and in the modulation of cell migration and apoptosis to promote wound healing.[35]

Recently, it has been suggested that mucins are involved in the pathogenesis of cancer. Recent studies have indicated that MUC1 and 4 may modulate various pathways affecting cell growth.[37] MUC1 is known to be over-expressed in pancreatic and colon cancers, and in over 90 per cent of breast cancers.[38] MUC1 is recognised to have multiple effects on tumourigenesis. Firstly, it is known to act as a natural ligand of galectin-3 in human cancer cells, and the interaction between galectin-3 and cancer-associated mucin 1 enhances adhesion between cancer cells and endothelial cells, which may promote metastasis.[39] Secondly, as a transmembrane protein, its cytoplasmic tail binds with the ErbB family of growth factor receptor tyrosine kinases and potentiates ErbB-dependent signal transduction in the MUC1 transgenic breast cancer mouse model. MUC1 activation is thought to increase cell proliferation by activating extracellular signal-regulated kinases,[37] and it also plays a role in protection against oxidative stress induced cell death.[37]

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One study found high levels of MUC1 expression in patients with laryngeal dysplasia and cancer.[40] High expression levels were also reported in 'control' patients' larynges; however, these were not healthy controls. The role of MUC1 in the context of laryngeal pathology remains unclear, and further research is required to characterise MUC1 expression in patients with pathology ranging from laryngopharyngeal reflux through to laryngeal cancer.

MUC4 is expressed on the epithelial surfaces of the oral cavity, eye, salivary glands and many other epithelial surfaces, where it acts to protect and lubricate. In a retrospective analysis of laryngeal cancer specimens, MUC4 was identified in nearly half the specimens.[41] In this study, the presence of MUC4 was associated with a trend towards better survival in patients with advanced stage, non-metastatic laryngeal cancer. In contrast, other research has shown that pancreatic, bile duct and lung cancers over-express MUC4, and that it is associated with a poorer prognosis in patients with these tumours.[37] Consequently, whilst there are proposed mechanisms for tumour progression in other cancers, the role of MUC4 in laryngeal cancer is still unclear.

Discussion

Laryngopharyngeal reflux has made a significant impact on the otolaryngological literature over the last 20 years, although scepticism exists about the methods used for its diagnosis, and even whether the condition actually exists. The latter view is widely held by upper gastrointestinal surgeons, who have in general been disappointed with the clinical outcomes of antireflux surgery for patients with a diagnosis of laryngopharyngeal reflux.[9]

Undoubtedly, there exists a wide cluster of symptoms which are attributed to laryngopharyngeal reflux. However, many of these are not restricted to laryngopharyngeal reflux alone. There are a number of laryngological signs which are suggestive of rather than definitive for laryngopharyngeal reflux, and their detection is recognised as having high intra- and inter-observer variability.[42]

Furthermore, the reliability of double-probe 24-hour pH monitoring has also been questioned, and there is no consensus regarding probe placement or interpretation of results.[43]

Currently, a combination of history, examination and, typically, a successful trial of PPI treatment is considered proof of laryngopharyngeal reflux. However, failure of such a trial may suggest an inability to treat the noxious non-acid components of refluxate rather than the acid alone.[42]

When all of this uncertainty is combined with the lack of a definitive diagnostic test, clinicians are left with a conundrum about how best to manage these patients.

What is well accepted is that there is a link between gastroesophageal reflux disease and symptoms suggestive of irritation and inflammation of the structures of the upper respiratory tract in some patients, and that some are 'cured' by antireflux surgery. The most logical explanation for these cases is that of damage caused by refluxate. Research has demonstrated that acid alone is not the only causative agent, with pepsin and bile acids also being contributors.[12,16,44] The actual mechanism for damage to the mucosal surface is not yet apparent. However, pepsin is being increasingly implicated, with research demonstrating its intracellular presence, and its ability to remain stable and to be reactivated at a pH which is not uncommon in the larynx.[13,45]

There have been many studies investigating the presence of, and resultant damage from, potential biomarkers of laryngopharyngeal reflux. The most notable of these affected by laryngopharyngeal reflux is CA III, a component of intrinsic mucosal protection in the larynx.[12] Mucins, which also provide mucosal protection, have altered expression in the larynx in the presence of laryngopharyngeal reflux. Such changes are significant, given the increasing amount of information emerging regarding these biomarkers' roles, not only in mucosal defence but also in tumour progression.

A definitive diagnostic technique for laryngopharyngeal reflux remains elusive. However, research implies that the occurrence of such reflux is indicated by biomarker changes and the presence of pepsin. Therefore, further research is required, in order to better define the clinical entity and spectrum of laryngopharyngeal reflux, and to identify specific patient subgroups (e.g. non-acid refluxers). In addition, as a definitive mechanism for mucosal damage is lacking (although probable causative agents have been identified), deeper understanding of this mechanism may enable the identification of better diagnostic biomarkers, as well facilitating therapeutic developments, particularly for patients unresponsive to PPIs.

Conclusion

Laryngopharyngeal reflux disease is commonly diagnosed in ENT practice, in the presence of a cluster of non-specific laryngeal symptoms and signs.

The mechanism of laryngeal injury is uncertain, but is considered to be caused by a combination of acid and refluxate components, particularly pepsin. The latter is implicated particularly in 'non-acid' or 'weak-acid' reflux, and may remain in a stable, inactive form in the larynx, to be reactivated by further reflux episodes. Receptor-mediated uptake of pepsin may cause damage at an intracellular level.

Carbonic anhydrase isoenzyme III has also been implicated. Expression of this enzyme varies throughout the larynx in response to laryngopharyngeal reflux, with decreased expression in vocal fold epithelium but increased expression in the posterior commissure.

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Laryngopharyngeal reflux is also associated with reduced expression of E-cadherin and mucin, either in response to reflux components (e.g. pepsin) or as a result of the inflammatory response to reflux.

Further studies are required to identify a definitive diagnostic tool for laryngopharyngeal reflux, and to determine the mechanism of mucosal injury, in order to enable therapeutic developments to help manage the full spectrum of laryngopharyngeal reflux pathology.

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Address for correspondence:
Prof A.S. Carney,
Flinders ENT, Department of Surgery,
Room 3D204, Flinders Medical Centre,
Bedford Park,
South Australia 5042, Australia

Fax: +61 8 8204 3987
E-mail: scarney@ent-surgery.com

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