Review article: uptake of pepsin at pH 7 – in non-acid reflux – causes inflamatory, and perhaps even neoplastic, changes in the laryngopharynx

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SUMMARY

We describe herein how pepsin causes laryngeal epithelial cell damage at pH 7, and thus in non-acidic refluxate. Our data may help explain why some patients have refractory symptoms on maximal proton pump inhibitor therapy, and help explain the reported symptom association with non-acidic reflux events. We report mitochondrial and Golgi damage in laryngeal epithelial cells exposed to pepsin at pH 7. Cell toxicity was also demonstrated using the MTT cytoxicity assay. Pepsin at pH 7 significantly alters the expression levels of multiple genes implicated in stress and toxicity. We also report that pepsin (0.1 mg/mL, pH 7) induces a pro-inflammatory cytokine gene expression profile in hypopharyngeal FaDu epithelial cells *in vitro* similar to that which contributes to disease in gastro-oesophageal reflux patients. Moreover, using a Human Cancer PathwayFinder SuperArray, we have shown that pepsin (0.1 mg/mL, pH 7) significantly alters the expression of 27 genes implicated in carcinogenesis. Collectively, these data suggest a mechanistic link between exposure to pepsin, even in non-acidic refluxate, and cellular changes that lead to laryngopharyngeal disease including cancer. In this context, our unexpected finding that pepsin is taken up by human laryngeal epithelial cells by receptor-mediated endocytosis is highly relevant. Pepsin has been previously assumed to cause damage by its proteolytic activity alone, but our discovery that pepsin is taken up by laryngeal epithelial cells by receptor-mediated endocytosis opens the door to a new mechanism for cell damage, and downstream, the development of new therapies for reflux disease – receptor antagonists and/or pepsin inhibitors.

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INTRODUCTION

Laryngopharyngeal reflux (LPR) contributes to voice disorders, otolaryngological inflammatory disorders, and is associated with upper airway neoplasia. Treatment is currently focussed on increasing the pH of the refluxate as it was thought that the refluxate would not cause injury at higher pH. However, many patients with reflux-attributed laryngeal injury/disease have persistent symptoms despite maximal acid suppression therapy. Recent studies using combined multichannel intraluminal impedance (MII) with pH monitoring showed a positive symptom association with non- and weakly acidic reflux and an association between non-/weakly acidic reflux and refractory symptoms on proton pump inhibitor (PPI) therapy. Thus, the role of acid alone in the development of reflux related laryngeal pathology is being questioned and studies examining the effects of the other components of the refluxate are needed. Crucially, our data supports a role for pepsin in reflux-attributed laryngeal injury/disease, independent of the pH of the refluxate.

There is substantial evidence in the literature demonstrating a significant association between reflux of gastric

contents into the laryngopharynx (LPR) and laryngeal inflammatory diseases, voice disorders and even neoplastic diseases of the laryngopharynx.[1–9] It has been estimated that up to 50% of patients with laryngeal and voice disorders have significant symptoms of LPR.[5] However, the exact role of LPR in injury and disease remains controversial. Several factors complicate this area of research.

First, while there is general agreement that PPIs are effective in treating gastro-oesophageal reflux disease (GERD), their efficacy for the treatment of LPR remains in doubt. Because many patients with reflux-attributed laryngeal symptoms and endoscopic signs do not respond to acid suppression therapy as well, or at all, compared with patients with GERD, some believe that LPR can not be the cause of their symptoms and injury. It has been suggested that the laryngeal mucosa is more sensitive to the damaging effects of gastric refluxate than the oesophagus and thus these patients require higher doses and a longer trial of PPIs.[4, 6, 10–12] In 2006, Vaezi *et al.*[13] reported a prospective multicentre, randomised study which evaluated the efficacy of PPI's in treating LPR. They found no difference in LPR response to PPI or placebo. However, it has been suggested that these data may be inconclusive because the inclusion criteria could have produced a dilution effect. Of the 145 subjects included, most were marginal cases with minimally troubling symptoms based on their LPR–health-related quality of life assessment and absence of pharyngeal acid reflux on pH monitoring. More recently, Reichel *et al.*[14] reported that patients with symptoms and endoscopic signs of LPR showed a statistically significant improvement in both symptoms and physical findings on esomeprazole vs. placebo for 12 weeks. A substantial placebo effect was noted at 6 weeks; however, this was no longer evident at 12 weeks.

Second, there are many nonspecific symptoms and findings of LPR. This has resulted in an over-diagnosis of LPR, and subsequently an inappropriate use of PPIs in patients exhibiting similar symptoms and findings which are unrelated to LPR. As a result, this has likely increased the number of patients included in studies investigating the efficacy of PPI therapy, who do not actually have LPR.[15, 16]

Third, combined MII with pH monitoring (MII-pH), has been introduced to our field relatively recently as a method of measuring and supporting with the diagnosis of LPR especially in identifying those patients (around 20%) who do have a reflux/symptom relationship. It should be noted that the majority of MII-pH studies (approximately 80%) have a negative symptom association with non-acid or weakly acidic reflux and extra-oesophageal symptoms. However, a significant association between non- and weakly acidic reflux and persistent symptoms on PPI therapy[15–17] has been shown in approximately 20% of patients. Patients with signs and symptoms associated with non-acidic and weakly acidic reflux would likely have a negative pH test and would not benefit from PPI therapy. Diagnosis and treatment have focused on the acidity of the refluxate because it was thought that the other components of the refluxate would not be injurious at higher pH. However, it is now known that certain bile acids are injurious at higher pH,[18, 19] and our data support a role for pepsin in reflux-attributed laryngeal injury and disease, independent of the pH of the refluxate.[20–23] Given: (i) the multiple reports of refractory reflux-attributed laryngeal symptoms and endoscopic findings on maximal PPI therapy; (ii) that studies using MII-pH reveal a positive symptom association with non- and weakly acidic reflux events; and (iii) we now know that pepsin and bile acids are injurious at higher pH, the role of acid alone in reflux-attributed signs and symptoms has to be questioned and subsequently the efficacy of acid suppression therapy for treating such.

The objective of our ongoing studies are to: (i) elucidate pepsin as a causal agent involved in early events in carcinoma of the laryngopharynx; (ii) isolate and identify the receptor with which pepsin interacts on the surface of human laryngeal epithelial cells; and (iii) further delineate the effects of receptor-mediated uptake of pepsin on the biochemistry and biology of laryngeal epithelium. Our long-term goal is to develop more effective, better targeted, therapeutics for patients with reflux disease, specifically for that large population that have persistent symptoms despite maximal acid suppression therapy. The potential protective effect of irreversible inhibitors of peptic activity is currently being investigated. Following identification of the receptor with which pepsin interacts, antagonists will be developed and tested using *in vitro* and *in vivo* models, to determine whether they prevent pepsin uptake and injury.

DIAGNOSIS OF LPR

For the diagnosis of LPR, most physicians rely on a combination of the patients' symptoms,[24, 25] laryngeal findings[26, 27] and reflux testing results.[28–30] Ambulatory 24 h double-probe (simultaneous oesophageal and pharyngeal), pH monitoring and impedance testing are the most widely applied. There are several disadvantages to

using double-probe pH monitoring. This technique cannot detect non-acidic reflux events, which are now known to be associated with laryngeal symptoms and endoscopic findings.[15–17, 31] Furthermore, calculations of the sensitivity of dual-probe pH monitoring for the detection of LPR range from 50% to 80%.[4] MII was introduced to our field more recently as a method of measuring and supporting with the diagnosis of LPR. The MII system measures changes in electrical conductivity of intraluminal content as a bolus more through the oesophagus and into the laryngopharynx. In Alternating Current circuits the resistance to electrical current flow is called impedance. MII permits not only identification of liquid, gaseous, or mixed intra-oesophageal/intra-pharyngeal materials, but also the direction of their travel. Furthermore, MII technology in conjunction with a pH sensor allows discrimination of acid (pH < 4.0) from weakly acidic (pH 4.0–6.5) and non-acidic (pH 7 and above) reflux.

TREATMENT OF LPR

Treatment of LPR depends on the type and severity of symptoms and signs and is usually empirical. Patients with LPR are typically prescribed PPIs, such as Nexium, to control the acidity of the refluxate. PPIs inhibit the H^+/K^+ ATPase enzyme that catalyses acid secretion in parietal cells in the stomach and thus are potent gastric acid suppressing agents. However, PPI therapy appears to have limited ability to protect patients from reflux-attributed symptoms and injury. In fact, it has been suggested that 25–50% patients have refractory symptoms on maximal PPI therapy. These patients can be subdivided into three groups: (i) Patients with symptom association with breakthrough acid reflux: This patient population may benefit from an increase in dose of their PPI or an H_2-receptor antagonist at bedtime. (ii) Patients who have symptom association with non-acidic reflux events: These patients would likely have a negative pH test and would not benefit from PPI therapy. Surgery is one of the few options for these patients. Several studies in the literature report resolution of reflux-attributed voice disorders and laryngeal symptoms and endoscopic findings after fundoplication.[32] (iii) Patients who have no symptom association: Reflux is unlikely to be the cause of symptoms and injury in this population and thus other causes should be investigated. Combined MII with pH monitoring (MII-pH) is now being used to correlate symptoms with reflux events to help identify potentially PPI-responsive acid reflux patients, who should be distinguished from both non-acid reflux and nonreflux laryngitis patients unlikely to respond to acid suppression therapy.

Using MII-pH monitoring, Tamhankar *et al.*[17] showed that PPI therapy decreases the H^+ ion concentration in the refluxed fluid, but does not significantly affect the frequency or duration of reflux events. Kawamura *et al.*[31] reported on a comparison of GER patterns in 10 healthy volunteers and 10 patients suspected of having reflux-attributed laryngitis. Using a bifurcated MII-pH reflux catheter, the investigators found that gastric reflux with weak acidity (above pH 4.0), is more common in patients with reflux-attributed laryngitis compared with healthy controls. Oelschlager *et al.*[33] demonstrated that the majority of reflux episodes into the pharynx are in fact non-acidic. More recently, Sharma *et al.*[15] reported that 70/200 (35%) patients on at least twice daily PPI had a positive symptom index for non-acidic reflux. Tutuian *et al.*[16] also recently reported that reflux episodes extending proximally are significantly associated with symptoms irrespective of the pH of the refluxate. Here, we present a hypothetical paradigm to explain these observations.

ROLE OF PEPSIN IN INFLAMMATORY DISEASE OF THE LARYNGOPHARYNX

Pepsin is a proteolytic enzyme produced only in the stomach, initially secreted in zymogen form as pepsinogen by gastric chief cells. Hydrochloric acid in the stomach causes the pepsinogen to unfold and cleave itself in an autocatalytic fashion, generating pepsin – the active form. Pepsin is maximally active at pH 2.0, but can cause tissue damage above this pH, with complete inactivation not occurring until pH 6.5.[11, 34, 35] While pepsin is inactive at pH 6.5, it remains stable until pH 8.0 and thus can be reactivated when the pH is reduced. Pepsin is not irreversibly inactivated until pH 8.0.[34, 35] Thus, even if the pepsin which we have detected in, for example, laryngeal epithelia is inactive[21, 22] (mean pH of the laryngopharynx is 6.8) it would be stable and thus could sit inactive/dormant in the laryngopharynx and have the potential to become reactivated by a decrease in pH. Using a specific and sensitive antibody against human pepsin, we have demonstrated the presence of pepsin in laryngeal epithelial biopsy specimens taken from patients with reflux-attributed laryngeal disease; not detected in normal control subjects.[11, 21, 22] In these studies, we also report a significant association between the presence of pepsin and depletion of laryngeal protective proteins; carbonic anhydrase isoenzyme III (CAIII) and squamous epithelial stress protein Sep70. Using an established

porcine *in vitro* model, we have demonstrated that exposure of laryngeal mucosa to pepsin, though not to low pH alone, causes depletion of CAIII and Sep70 protein levels. These findings suggest that the pepsin present in the laryngeal epithelia of patients with reflux-attributed laryngeal disease is likely to be the causal factor for the observed depletion of CAIII and Sep70 proteins in these same patients.

We have recently documented co-localisation of pepsin with clathrin in laryngeal epithelial cells[36], a widely accepted marker of the receptor-mediated pathway.[37] This supports our previous findings of co-localisation of pepsin with transferrin, another marker of the receptor-mediated pathway.[23] Together, these immuno-electron microscopy data strongly suggest that pepsin is taken up by laryngeal epithelial cells by receptor-mediated endocytosis. However, molecules taken up by fluid phase endocytosis can also rarely be detected in clathrin coated pits. Thus, it was necessary to confirm real receptor-type behaviour. We performed competitive binding experiments with unlabelled ligand (pepsin) in the cold to ascertain whether binding is saturable and can be competed for, characteristics of receptor-mediated uptake. Using pepsin labelled with tetramethyl rhodamine isothiocyanate (TRITC) we documented uptake of pepsin by laryngeal epithelial cells and its presence inside the cell after incubation at 37 °C for 5–10 min. In competitive binding experiments, where cells were exposed to an excess of free/unlabelled pepsin at 4 °C prior to incubation with pepsin-TRITC, pepsin-TRITC was not detected inside the cells even after 30 min at 37 °C. If pepsin-TRITC was being taken up by general fluid-phase endocytosis, prior incubation with an excess of unlabelled pepsin at 4 °C would not have significantly affected uptake. One would have expected to see uptake of pepsin-TRITC at the same rate as before – in intracellular vesicles after 5–10 min at 37 °C. However, in the case of specific receptor-mediated uptake, the high concentration of unlabelled ligand (pepsin) saturated the receptors at 4 °C and was taken up when warmed to 37 °C. Only once receptors recycle to the cell surface, will you see labelled pepsin (pepsin-TRITC) inside the cells. These competitive binding experiments confirm that uptake of pepsin is saturable and thus unequivocally receptor mediated. This is further supported by our finding that pepsin remains on the cell surface in the presence of wortmannin, an inhibitor of receptor-mediated endocytosis, but is detected inside intracellular vesicles in the presence of DMA, an inhibitor of fluid phase but not receptor-mediated endocytosis.

Pepsin is thought to cause damage by its proteolytic activity alone, digesting the structures that maintain cohesion between cells. Our discovery that pepsin is taken up by laryngeal epithelial cells by receptor-mediated endocytosis is a novel scientific finding which could also have important clinical implications. If pepsin taken up by the cell was merely targeted to lysosomes for degradation, a role for pepsin in reflux-attributed injury would seem unlikely. However, we have shown that pepsin can be detected in late endosomes 6 h following a 20 min exposure, revealing that it is not merely targeted to lysosomes for degradation. Our preliminary investigations also suggest that intracellular pepsin is intact. When cultured FaDu cells are incubated with pepsin-TRITC (10 ng/mL) at 4 °C for 1 h and then warmed to 37 °C, a single band is detected at 35 kDa (corresponding to the correct molecular weight of pepsin) by sodium dodecyl sulphate – polymerase gel electrophoresis (SDS–PAGE). A polypeptide band was not detected when cells were incubated at 4 °C. At 4 °C, endocytosis is stopped and thus any pepsin present remains on the cell surface. When the cells are warmed to 37 °C, pepsin is taken up by the cell by receptor-mediated endocytosis and can be detected in intracellular vesicles. Detection of a single band at 35 kDa by SDS–PAGE when pepsin is inside the cell, suggests that intracellular pepsin is intact. We intend to isolate intracellular organelles via differential centrifugation and analyse the intracellular pepsin by SDS–PAGE to confirm that it is intact.

Interestingly, the proteolytic activity of pepsin is not essential for receptor-mediated uptake, as inactive pepsin is taken up by receptor-mediated endocytosis.[23] Receptors and their ligands are typically sorted in late endosomes or the TRG. Using antibodies against Rab-9 (a marker of late endosomes) and TRG-46 (a marker of the TRG) we have confirmed the presence of pepsin in these intracellular compartments (Johnston *et al.*, in press). As our SDS–PAGE data suggest that intracellular pepsin is intact, it is possible that it could become reactivated in either of these intracellular compartments, which are approximately pH 5. Pepsin, even when inactive, remains stable below pH 8.0.[34, 35] Thus, pepsin below pH 8 taken up by the cell is stable and thus has the potential to become reactivated by a subsequent decrease in pH as in late endosomes or the TRG. It should be noted, while pepsin is maximally active at pH 2.0, it has 40% of its maximum activity at pH 5.0.[34, 35] We intend to both reversibly and irreversibly inhibited pepsin in an indirect approach to investigate whether inactive pepsin (pepsin at pH 7) taken up by receptor-mediated endocytosis

causes damage by becoming reactivated inside the cell. If pepsin does becomes reactivated intracellularly *in vivo*, a reversible, but not an irreversible, inhibitor of peptic activity would be expected to prevent pepsin from becoming reactivated inside the cell and subsequently causing damage. Alternatively, it may be that activation of the cell surface receptor by pepsin results in a cell signalling cascade ultimately having a negative effect on normal cell function. The process of signal transduction, whereby binding of a ligand to its receptor initiates a signalling cascade, is often dysregulated in disease. It is unlikely that there is a specific cell surface receptor for pepsin, but perhaps it is more plausible that pepsin somehow exploits another receptor on laryngeal epithelial cells. One would presume that a receptor antagonist would be required to prevent peptic injury by this mechanism. Our long-term goal is to elucidate this novel mechanism for peptic injury and to test pepsin inhibitors and receptor antagonists using *in vitro* and *in vivo* models.

To test our hypothesis that inactive pepsin can be taken up by laryngeal epithelial cells and cause intracellular damage, perhaps by becoming reactivated inside the cell in late endosomes or the TRG (compartments of lower pH) or by initiating a cell signalling event following interaction with a cell surface receptor, we exposed cultured epithelial cells to pepsin (0.1 mg/mL human pepsin 3b) at pH 7, for either 1 or 12 h at 37 °C, washed three times briefly and examined by transmission electron microscopy.[20] The cells remained viable following a 1- and 12-h incubation with pepsin at neutral pH. Cell and nuclear membranes were intact. However, both mitochondria and Golgi were clearly damaged. Mitochondria were swollen and the cristae degraded in cells exposed to pepsin (0.1 mg/mL) at pH 7 for 1 h at 37 °C. Further mitochondrial damage was evident in cells exposed to pepsin for 12 h. Golgi were also swollen in cells exposed to pepsin for 12 h. Control cells, which were incubated for the same time period, in the absence of pepsin, showed no signs of mitochondrial or Golgi damage. The mitochondrial damage we observed in human FaDu epithelial cells exposed to pepsin (0.1 mg/mL) at pH 7 is probably an early indicator of necrosis and supports our hypothesis that pepsin can cause injury to laryngeal epithelial cells in non- and weakly acidic refluxate. There is no doubt that pepsin will be more injurious to the laryngeal epithelium in acidic refluxate. However, our data reveals that it could also cause damage in non-acidic refluxate. In support of a pepsin effect on mitochondria, initially observed by transmission electron microscopy, we also report cell toxicity measured by a MTT cell toxicity colorimetric assay kit (Sigma-Aldrich Corp., St Louis, MO, USA). The key component of this kit is 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide or MTT, which can be used to measure mitochondrial activity in living cells. A decrease in absorbance, compared with control, is indicative of damage. We exposed cultured FaDu cells to pH 7 or 5.5 ± pepsin (0.1 mg/mL) for 1 h at 37 °C. Data from three biological replicates, read in triplicate, were analysed using one-way analysis of variance. Importantly, a significant increase in toxicity was detected following exposure to pepsin at pH 7 compared with pH 7 control ($P < 0.01$). This finding supports our electron microscopy data showing mitochondrial damage by pepsin at neutral pH.

Mitochondria are known to play a central role in cell metabolism, and damage – and subsequent dysfunction – in mitochondria is an important factor in a wide range of human diseases.[38] While seemingly unrelated, there is a common thread between the different diseases associated with mitochondrial damage: cellular damage causing oxidative stress and the accumulation of reactive oxygen species. These oxidants then damage mitochondrial DNA, resulting in mitochondrial dysfunction and death.[39] There is evidence that CAIII protects against oxidative damage[40, 41] that has been shown to occur experimentally from reflux.[42, 43] We have shown that CAIII expression levels are depleted in patients with LPR and that laryngeal CAIII levels are depleted following exposure to pepsin *in vitro*.[11, 22] The possible link between reflux-attributed laryngeal injury/disease, depleted levels of protective CAIII by pepsin, and the mitochondrial damage observed following exposure to pepsin, warrants further investigation. Perhaps depletion of laryngeal CAIII by LPR of pepsin results in the accumulation of reactive oxygen species and subsequent mitochondrial damage.

In addition to depletion of CAIII, we have also reported that patients with LPR have depleted levels of laryngeal Sep70, compared with normal control subjects. Furthermore, both CAIII and Sep70 proteins are depleted following exposure to pepsin, but not low pH alone, *in vitro*.[11, 21, 22] More recently, we found that patients with LPR have depleted levels of MUC 2, 3 and 5ac mRNA, and that pepsin prevents production of these mucins *in vitro*.[44] However, given that pepsin is a proteolytic enzyme, it is likely that pepsin would have a more global effect, rather than cause damage by depleting the expression of a select few genes/proteins. To this end, a

Human Stress and Toxicity PathwayFinder PCR Array (SABiosciences, Frederick, Maryland, USA) was used to examine the effect of pepsin, at neutral pH, on the expression of 84 genes whose expression levels is indicative of stress and toxicity. Cultured FaDu cells were incubated with complete growth media ± pepsin (0.1 mg/mL) for either 1 or 12 h at 37 °C, washed and processed for real-time RT–PCR. Data from three biological replicates were analysed using the RT^2 Profiler PCR Array Data Analysis software – student's *t*-test. Our data indicates that pepsin significantly alters the expression levels of multiple genes implicated in stress and toxicity.[20] The expression levels of seven genes, implicated in stress and toxicity, were significantly upregulated following 1 h incubation with pepsin (0.1 mg/mL, pH 7). A time response was observed: the expression levels of 25/84 of these genes were significantly altered following a 12 h incubation with pepsin. A long exposure time was used in these initial experiments to see if an effect could be observed. We anticipated that we would only see an effect by exposure to pepsin at neutral pH after a long time period, compared with pepsin at acidic pH where one would expect to see an effect relatively quickly. The morphological changes we observed would have been missed by simply examining gross morphology (for example, H&E stained sections examined by light microscopy) and require detailed examination of the intracellular structures (using transmission electron microscopy). While damage clearly occurs, the cells do remain viable and thus potentially able to recover from a single insult. It is likely that permanent injury and symptoms would result from multiple uncontrolled reflux events, as is thought to occur in LPR patients. Time course, repeated exposure and pulse chase experiments will now be performed. However, compared with controls, pepsin is clearly injurious to laryngeal epithelial cells at neutral pH. A SuperArray for inflammatory cytokines and receptors was also used to investigate whether pepsin, at pH 7, elicits an inflammatory response.[45] This is important as the consequence of reflux damage and the cause of symptoms is de facto chronic inflammation. The expression of a number of inflammatory cytokines and receptors was altered in human hypopharyngeal epithelial cells following overnight treatment with pepsin at neutral pH >1.5-fold change in gene expression was detected for CCL20, CCL26, IL8, IL1F10, IL1A, IL5, BCL6, CCR6 and CXCL14 ($P < 0.05$). These pro-inflammatory cytokines and receptors are known to be involved in inflammation of the oesophageal epithelium in response to reflux and contribute to the pathophysiology of reflux oesophagitis.[45] These data indicate that refluxed pepsin may contribute to laryngeal inflammation associated with non-acidic gastric reflux including that experienced by patients despite maximal acid suppression therapy.

ROLE OF PEPSIN IN CANCER OF THE LARYNGOPHARYNX

Laryngeal carcinoma accounts for about 1% of all newly diagnosed cancers in the US. Approximately, 11 000 new cases are diagnosed every year and about 4300 deaths per year are attributed to laryngeal carcinoma. Despite a decrease in the number of people who smoke in the US, the incidence of laryngeal cancer actually appears to be rising. Unfortunately, the prognosis remains poor and the mortality rate high, with a 5-year survival rate of 40%.[46–50] Tobacco and alcohol are well-known established risk factors. Other risk factors include human papilloma virus, radiation exposure, occupational exposure and LPR.[4] The latter remains controversial and requires further investigation, especially as it has become one of the most common chronic diseases of adults in the US. For many reasons, it is very difficult to prove that reflux is a causal agent in the development of laryngeal cancer. Many clinical studies have shown a high prevalence of LPR in patients with laryngeal cancer[4, 50]; however, these studies are confounded by the fact that the majority of patients with laryngeal cancer have a significant smoking and alcohol history, and many lack appropriate controls. Another difficulty is the lack of uniformity in establishing the diagnosis of GERD and LPR in the literature.

While it seems logical that chronic laryngeal inflammation could lead to a neoplastic lesion, it remains unclear whether reflux laryngitis is a precursor to laryngeal cancer. It is hoped that research in cell biology of reflux may eventually lead to an answer to this age-old question, since population and other clinical studies have too many confounding variables. Gabriel and Jones[51] were among the first to present evidence suggesting this possibility. Many others have also suggested an association.[4, 7, 52–55] To further explore the association between LPR and laryngeal cancer, several investigators have examined the direct effect of the individual components of gastric refluxate – mainly acid, pepsin and bile acids – on laryngeal cell and molecular biology and pathology.[4, 56, 57] These studies demonstrated a significant role for pepsin and bile acids in carcinogenesis, in a dose-dependent manner with greater toxicity at lower pH. Interestingly, several clinical studies evaluating patients with prior gastrectomy suggest that the components of non-acidic reflux promote the development of laryngeal

cancer.[58–60] We report that exposure of hypopharyngeal epithelial cells to pepsin (0.1 mg/mL, pH 7) causes a significant change in the expression of 27 genes implicated in carcinogenesis (Johnston N, unpublished data). Analysis of these genes strongly suggests that pepsin exposure causes an increase in cell proliferation and thus may contribute to oncogenic transformation by aberrant cell growth. This was investigated further using propidium iodide staining and flow cytometry. Pepsin was indeed found to significantly increase the percentage of cells in S phase in a dose-dependent manner. Growth curve data are consistent with pepsin causing an increase in cell proliferation and thus support our flow cytometry data.

CONCLUSION AND FUTURE DIRECTIONS

Our data strongly suggest that pepsin may be responsible for laryngeal symptoms and injury associated with non- and weakly acidic reflux and help explain why many patients have refractory symptoms on maximal acid suppression therapy. Moreover, our preliminary data demonstrate that pepsin may even initiate neoplastic changes which could result in the development of laryngopharyngeal cancer. We are currently exposing human hypopharyngeal and laryngeal epithelial cells to human pepsin at pH 7 in time-course and dose–response experiments. The effect of pepsin on cell viability and cytotoxicity will be measured using the Vybrant Cell Metabolic Assay. An accurate measurement of cell proliferation will be obtained using the more superior Click-iT Edu Proliferation Assay. The Cell Clonogenic Survival Assay will be used to test the capability of adherent cells to survive and replicate following exposure to pepsin and an Anoikis Assay will be used to measure anchorage-independent growth and monitor anoikis propelled cell death. Finally, using microarray technology, the expression of 113 gene indicators of the 15 different signal transduction pathways involved in oncogenesis will be examined to explore the possible molecular mechanisms by which pepsin dysregulates hypopharyngeal and laryngeal epithelial cells. We are also testing pepsin inhibitors in our *in vitro* models to see if they prevent peptic injury. Once we have identified the receptor with which pepsin interacts, we will also design and synthesise receptor antagonists to test in our *in vitro* models. If our *in vitro* studies demonstrate that pepsin inhibitors and/or receptor antagonists prevent pepsin uptake/injury, an *in vivo* model will be used to investigate the clinical usefulness of such pharmacological agents.

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