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Pepsin like activity in Bronchoalveolar lavage is suggestive of gastric aspiration in lung allografts.

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Running head: Gastro Oesophageal Reflux in lung allografts

Key words

Lung allograft, Gastro Oesophageal Reflux (GOR), Pepsin.

Abstract

Introduction: A biologically plausible link between gastro-oesophageal reflux (GOR), aspiration and lung allograft dysfunction has been suggested, but there is no systematic evidence indicating the presence of gastric contents in the lung. We have tested the hypothesis that pepsin, as a marker of aspiration, is detectable in bronchoalveolar lavage (BAL) of allograft recipients who had not reported symptoms of GOR.

Methods: Standardised 3x60mL surveillance BAL samples from thirteen chronologically sequential, stable lung allograft recipients without chronic rejection (ten patients treated with a prophylactic proton pump inhibitor) were studied. Lavage supernatants were assayed by an ELISA, based on a monospecific goat antibody for pepsin/pepsinogen. Pepsin levels were compared with a group of four normal volunteer controls.

Results: Pepsin levels were measurable in all allograft recipients, in keeping with gastric aspiration. Median 109ng/mL (range 35-1375). Pepsin levels were below the limit of detection in the control group. Treatment with proton pump inhibitor was not correlated with pepsin levels. There was no correlation between BAL neutrophils and pepsin level.

Conclusion: Our data demonstrate lung epithelial lining fluid concentrations of pepsin in lung allograft recipients which are much higher than blood reference levels, with no detectable pepsin in controls. This provides direct evidence of gastric aspiration, which is potentially injurious to the allograft.

Introduction

Human lung transplantation is a well accepted therapeutic option for selected patients with advanced cardio-pulmonary disease but long term survival is limited by the development of obliterative bronchiolitis (OB), the physiological hallmark of which is the bronchiolitis obliterans syndrome (BOS) (1). The pathophysiology of OB is poorly understood, but it is increasingly recognised to represent immunological and non-immunological mechanisms, and an aberrant response to injury (1, 2).

Gastro-oesophageal reflux (GOR) has been implicated as a possible cause of non-immunological allograft injury (3). Allograft recipients have a number of risk factors for GOR. Lung allograft surgery causes significant damage to vagal innervation of the gastro-intestinal tract and the immunosuppressant drugs cyclosporine and tacrolimus reduce gastric motility(4). In addition, cough reflexes and muco-cilliary clearance, the normal defence mechanisms against aspiration, are attenuated (5). All the above make reflux more likely and this is why many allograft recipients are put on prophylactic acid suppression therapy.

Despite high clinical suspicion regarding GOR in lung allografts, the literature is small and largely retrospective. However, formal oesophageal pH studies, when carried out, do indicate that GOR may be a significant problem in lung allografts and fundoplication surgery is associated with improved allograft function (6).

We are unaware of any literature to date indicating gastric aspiration into the lung and we have therefore tested the hypothesis that pepsin, as a marker of aspiration, is detectable in the lungs of allograft recipients, who did not present with obvious symptoms of GOR.

Methods

The study was approved by the Local Research Ethics Committees for Newcastle and North Tyneside, with separate applications for prospective studies in lung allografts and normal volunteer controls.

Following informed consent, thirteen chronologically sequential, unselected subjects undergoing either routine or symptom driven transbronchial biopsy (TBB) and bronchoalveolar lavage (BAL) were recruited (table 1), with the research sample intercalated within this procedure. Research samples were taken at least one month after any preceding bronchoscopic procedure and in the absence of any clinical evidence of prior microaspiration. All patients were receiving a standard long term maintenance regimen of immunosuppressive therapy comprising cyclosporin, azathioprine and prednisolone. The patients were not formally investigated for GOR, but did not report symptoms suggestive of GOR. In particular, there were no reports of heartburn, stomach ache, sour taste in the mouth or pain on swallowing. Ten patients were treated with a prophylactic proton pump inhibitor, which is a common empirical therapy in this patient population.

Four normal, non smoking controls (one female, median age 39, range 32-46) were recruited from volunteer hospital staff for a research BAL. These subjects were recruited nine months following the transplant patients, as soon as full ethical approval had been gained for bronchoscopic investigations in normal volunteers.

Bronchoscopy, BAL and TBB

Bronchoscopy was carried out in accordance with published guidelines (7). Subjects were pre-medicated with intravenous midazolam. 4% lignocaine was applied topically to the nose, pharynx and larynx and below the cords in 1ml aliquots, as required, up to a maximum dose of 8mg/kg body weight. Bronchoscopy was carried out with patients in a semi-reclined position.

Bronchoalveolar lavage was standardized to a 3x60mL procedure with oxygen saturation routinely measured during the procedure. The BAL sample was split, and assessed for clinical microbiology and differential cell counts on Giemsa stained cyto-centrifuge preparations. Cell free BAL supernatants were prepared by centrifugation (10 mins, 1,500rpm, 10 minutes), aliquots snap frozen by immersion in liquid N_2, and stored at -80°C prior to ELISA. Transbronchial biopsies were obtained from the allograft patients only.

Pepsin/pepsinogen ELISA

A locally developed ELISA was performed using 100µl of un-concentrated BAL supernatants. The assay, based on a monospecific antibody to porcine pepsin, measured both pepsin and total pepsinogens, referred to henceforth as "pepsin", with a lower limit of detection of <100pg/mL (8, 9). All assays were performed by one individual and the coefficient of variation for the assay was 13%. Serum reference levels for pepsin are (49·8–86·6 µg/L),(8). The normal subjects were recruited after the transplant patients, when full ethical approval had been gained and the ELISA on these samples were performed nine months after the transplant patients.

Transbronchial biopsy processing.

Five to seven TBB were taken at each allograft bronchoscopy, fixed in 10% formalin, embedded in paraffin and then stained with haematoxylin and eosin to assess acute or chronic rejection according to standard criteria (10).

Statistical analysis

Non parametric methods were used throughout using Minitab statistical software. The median allograft pepsin levels were compared to median control levels by the Mann Whitney U test (two tailed).

Results

Patient demographics, BAL and pathological rejection assessments are summarised in table 1.

5 of the 13 subjects had clinically significant, mild to moderate (A2) acute rejection but all were free from long term, irreversible loss of lung function.

BAL data.

Median BAL return was 90mL (range 55-100) in the allografts and 80 mL (range 55-90) in the controls, indicating technically satisfactory procedures.

As in our previously published allograft data, median BAL PMN % were variable and elevated compared to our normal range. Median 2.0% (range 0.2-35.6) versus 1.6% (range 0-2), p=0.03 (11).

BAL Pepsin

BAL Pepsin levels were measurable in all allograft BAL samples (Fig 1), in keeping with gastric aspiration. Median 109ng/mL (range 35-1375). Pepsin levels were below the limit of detection (<1ng/mL) in the control group. Treatment with a maintenance dose of proton pump inhibitor did not correlate with pepsin levels. There were no correlations between BAL neutrophils, acute rejection, and pepsin level.

Discussion

Limited previous reports, largely retrospective but some with formal objective oesophageal pH monitoring, have suggested GOR is a significant problem in lung allografts (6) and treatment of gastro-oesophageal reflux has been cited as a new therapeutic option to treat patients with BOS (12).

In this study we have shown that high and variable levels of pepsin are detectable in allograft recipients, with no pepsin detected in normal control BAL samples. To our knowledge this is the first systematic, direct evidence of gastric aspiration into lung allografts. This may be a continuing and cumulative potential injury to allografts and we provide mechanistic support for this contention.

Absolute determination of the dilution of the peri-cellular epithelial lining fluid (ELF) sampled by BAL is not possible (13) but estimations are practicable, based on the morphometric data of Weibel (14). These considerations suggest our BAL procedure represents approximately a 1 in 200 dilution of the ELF sampled, with our present data consistent with epithelial lining fluid concentrations of pepsin 10-10^3 times higher than serum reference levels (8). In contrast, our published data on BAL albumin in allografts are consistent with ELF levels substantially *lower* than those found in serum (15). Overall our data indicate a gastric source of the pepsin detected.

Pepsin is a proteolytic enzyme, active at acidic pH. There are no data that we are aware of regarding the pH of allograft ELF, but acidic breath condensate is increasingly reported as a marker of inflammation in asthma, COPD, bronchiectasis, cystic fibrosis and following cardiothoracic surgery (16). These pH levels are consistent with potential proteolytic activity for pepsin. Aspiration of gastric contents into the lung would be anticipated to cause epithelial damage in allografts, stimulation of cytokine production and an airway inflammatory/remodelling response, potentially contributing to irreversible loss of allograft function and eventual failure (1, 2).

It was noteworthy that the majority of the patients we studied were being treated with a prophylactic proton pump inhibitor at a low maintenance dose. This reflects widespread empirical use in allograft recipients in view of concurrent oral corticosteroid use and their role in patients with cystic fibrosis to prevent pancreatic enzyme supplement degradation. Such medication would be expected to suppress symptoms associated with GOR caused by acid, but a potential concern highlighted by our study is that “clinically occult” aspiration of other gastric contents would still be possible.

Our study though novel is preliminary and our control information is limited. However, we specifically adopted a rigorous approach to this by recruiting normal volunteers, and no pepsin was detected in these controls. Our results therefore indicate the presence of unexpectedly high, potentially deleterious levels of pepsin in lung allografts. This may be significant irrespective of aetiology, with lung allografts singularly vulnerable to injury (1, 2). Longitudinal studies are now required to assess whether the presence of BAL pepsin and other markers of GOR are related to long term allograft failure and chronic rejection and such techniques may be broadly useful in studying other patients with GOR who develop lung disease.

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Table 1. Summary of Patient demographics, BAL and pathological rejection assessments

Subject	Age	Diagnosis	Months Post Transplant	BAL Return (mL)	Cell count ($x10^{4/mL}$)	PMN (%)	AM (%)	Lymph (%)	Micro	Biopsy	PPI/H2	[Pepsin] ng/mL
1	19	CF	3	70	36	0.2	99.6	0.2	negative	a2/b1	yes	81
2	45	Bronchiectasis	6	95	67.2	0.2	96.4	3.4	negative	a0/b1	yes	60
3	25	PPH	2.5	100	24.3	1.0	98.8	0.2	negative	a2/3b1	yes	172
4	25	OB	3	100	34.3	3.4	96.2	0.2	negative	a1/bx	yes	68
5	20	CF	0.25	55	56.5	35.6	61.0	3.0	negative	a2/bx	no	129
6	41	A1AT	2.5	85	22.8	7.4	86.4	5.0	negative	a2/b0	no	50
7	44	LAM	6	100	11	1.0	94.6	4.4	negative	a1/b1	yes	34
8	44	VSD-EISEN	87	95	2.6	1.4	93.0	5.6	aspergillus	a0/b0	yes	67
9	38	CF	6	80	4.7	4.6	75.8	18.4	klebsiella	a1/b1	yes	107
10	62	LAM	12	90	39.9	2.0	94.4	3.6	negative	a0/bx	no	1375
11	39	Bronchiectasis	3	75	9.6	8.0	76.0	15.8	negative	a0/b0	yes	225
12	47	A1AT	2	75	22.6	5.6	94.2	0.2	negative	a2/b0	yes	1200
13	21	CF	12	95	8.5	1.0	98.2	0.6	penicillium	a0/b0	yes	237

Key:

DIAGNOSIS: CF=Cystic Fibrosis, PPH=Primary pulmonary hypertension, OB=Obliterative bronchiolitis, i.e patient had a second lung transplant due to failure of the first. A1AT=Alpha 1 anti-trypsin deficiency, LAM= lymphangiomyomatosis, VSD-EISEN=Eisenmengers syndrome.
PMN=Neutrophils, AM=Alveolar Macrophages, Lymph=Lymphocytes.
Micro=Microbiology.
Biopsy=Pathological assessment for rejection according to International Society for Heart and Lung Transplantation criteria. a=acute rejection ≤ a1 non significant, b=airway assessment, axbx=no material for assessment.

PPI/h2= Prophylactic treatment with proton pump inhibitor or H2 receptor antagonist.

Fig 1: BAL Pepsin levels