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RESEARCH ARTICLE

Human neutrophil peptide in lung chronic allograft dysfunction

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Abstract

Context: Our previous case-control study identified human neutrophil peptide (HNP) as a potential biomarker for bronchiolitis obliterans syndrome (BOS) in lung transplant recipients.

Objective: To prospectively validate HNP as a biomarker for BOS.

Materials and methods: HNP was measured by ELISA in bronchoalveolar lavage (BAL) fluid in lung transplant recipients.

Results: The first HNP measurement after reaching baseline pulmonary function was predictive of developing BOS ≥2 ($p = 0.0419$). HNP remained elevated in those that developed BOS. The effect of potential confounders did not significantly impact BOS-free survival time.

Conclusion: HNP levels are elevated early and persistently in those that develop BOS.

Keywords: Biomarkers, rejection, lung transplant

Introduction

Lung transplantation is an effective treatment for end-stage lung disease, with 2-year survival rates approaching 70% (Anyanwu et al. 1999). However, long-term survival remains low compared to most other solid organ transplants, mainly due to the development of chronic allograft rejection. Chronic rejection is manifested histologically by obliterative bronchiolitis (OB) with bronchiolitis obliterans syndrome (BOS) the clinical surrogate. BOS is diagnosed by a progressive decline in pulmonary function, specifically forced expiratory volume in one second (FEV1) and/or mid-expiratory flow rates (FEF_{25-75}) (Estenne et al. 2002). However, the pathobiology of BOS appears to be heterogeneous, as obliterative bronchiolitis is only one of many different factors contributing to loss of lung function after transplantation. Regardless of the etiology, development of chronic allograft dysfunction confers significant morbidity and subsequent mortality.

Certain markers of inflammation and fibroproliferation have been associated with BOS development; however, none of these putative biomarkers have been validated prospectively (Belperio et al. 2002a, Belperio et al. 2001; Belperio et al. 2002c, Charpin et al. 2000; DiGiovine et al. 1996; Elssner and Vogelmeier, 2001; Kelly et al. 1998; Meyer et al. 2001). We have previously identified the antimicrobial peptide, human neutrophil α-defensin, also called human neutrophil peptide (HNP), as a potential biomarker of BOS in a small case control study using BAL fluid samples collected between 1993–96 (Nelsestuen et al. 2005; Zhang et al. 2005). HNP is an essential member of the innate immune system and belongs to a family of anti-microbial peptides referred to as defensins (Cole and Ganz, 2000; Ganz, 2002b). Four of the six α-defensins are present in neutrophils and some lymphocytes, and can be found in lungs. The release of HNP is stimulated by microbial products and certain cytokines, and they act directly against many bacteria,

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fungi, and certain viruses (Borregaard et al. 2000). In addition to their role in innate immunity, defensins have concentration-dependent roles in both cellular repair and injury, and cell proliferation (Austin et al. 2000; Ganz, 2002a). Both HNP and BAL neutrophilia have been independently reported to be elevated in lung transplant recipients at risk for and those who have already developed BOS (Nelsestuen et al. 2005; Riise et al. 1999; Riise et al. 1998; Elssner and Vogelmeier, 2001; DiGiovine et al. 1996; Zheng et al. 2000; Anderson et al. 2008).This present study was designed to validate BAL fluid HNP as a biomarker of BOS. HNP was measured prospectively in BAL fluid from lung transplant recipients undergoing either surveillance or clinically indicated bronchoscopies over a 6-year period.

Methods

Subjects

Between January 2002 and December 2009, 244 patients underwent lung transplantation at the University of Minnesota. From these patients, 149 individuals had 577 BAL fluid samples collected after reaching baseline post-transplant pulmonary function tests (FEV1 or $FEF_{25/75}$) (Estenne et al. 2002), and these patients and samples are the basis of this study (Table 1). The median age of these subjects was 61, and the most common diseases were COPD and idiopathic pulmonary fibrosis. Our primary outcome was the development of BOS grade 2 or higher (BOS ≥ 2) (Estenne and Hertz, 2002). BOS grades and baseline pulmonary function date were assigned, based on International Society for Heart and Lung Transplantation (ISHLT) guidelines (Estenne et al. 2002). Baseline pulmonary function is defined as the average of the two highest measurements, not necessarily sequential, obtained at least 3 weeks apart(Estenne et al. 2002).

Table 1. Subject characteristics.

Category	Suspects with HNP levels after baseline pulmonary function (N=149 (%))
Age	27–74 (median 61]
Female	68 (46%)
Underlying disease	
COPD/Emphysema	57 (39%)
Idiopathic pulmonary fibrosis	30 (20%)
α-1 Antitrypsin	
Deficiency	22 (15%)
Cystic fibrosis	15 (10%)
Pulmonary hypertension	7 (5%)
Sarcoidosis	5 (3%)
Bronchiectasis	2 (1%)
Miscellaneous	11 (17%)
BOS grade	
0	73 (49%)
1	33 (22%)
2	25 (17%)
3	18 (12%)

All patients with a BOS diagnosis had the assigned BOS grade for a minimum of 6 months at the time of study closure. Acute rejection was defined using the 2007 consensus criteria (Stewart et al. 2007). The study was approved by the University of Minnesota Institutional Review Board (Protocol 0107M04822).

Bronchoalveolar lavage

An average of 3.87 BAL fluid samples/subjects were collected either as part of standard surveillance for acute rejection or when clinically indicated (Gimino et al. 2003). Standard surveillance bronchoscopies started at 1 month post-operatively and then continued for every 2 months until the recipient completed 1 year without any episodes of acute rejection. BAL fluid was sent for routine clinical evaluations including cytology, viral rapid antigen detection (CMV and seasonal RSV and influenza), culture (bacterial, fungal, viral) and differential cell counts. The remainder of the BAL fluid was placed on ice and transported to the laboratory, where it was centrifuged; supernatant was stored in aliquoted samples at –80˚C until use. HNP ELISA was performed on all samples in batches of 40. Samples obtained after baseline pulmonary function was established, were used for our primary outcomes.

HNP Measurements

A commercial ELISA kit (HyCult Biotechnology, Uden, Netherlands) was used to measure cumulative HNP1-3 in all samples as previously reported (Nelsestuen et al. 2005).

Statistical methods

While this cohort study was powered for detecting differences in BOS-free survival rates, we employed several different statistical approaches to fully understand the nature of the relationship between this potential biomarker and development of BOS. Our primary analysis was based on examining differences in BOS-free survival between those with HNP levels exceeding and those less than the median after reaching baseline pulmonary function. In addition to this primary analysis, we tested for a more immediate prognostic effect of HNP using a time varying Cox proportional hazards model to predict time to BOS ≥ 2 given the most recent HNP level. The value of this approach is that it is similar to how HNP would be used as a clinical tool; however, it entails assumptions such as proportional hazards and the functional form of the relationship between HNP and the hazard for developing BOS ≥ 2. A third *mixed model approach* adds considerable insight into the nature of the relationship between HNP levels and the development of BOS ≥ 2. This approach utilizes a linear model to assess the effect of time since transplantation and if a patient develops BOS 2 on the level of HNP. We constructed a receiver operating characteristics (ROC) curve using hold-one-out cross-validation in the context of our time varying Cox model. Specific statistical methods are available in the online supplemental material.

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Results

Initial HNP level after reaching baseline pulmonary function

In our previous study we observed transiently high HNP levels within the first several months after transplantation, possibly due to post-operative changes and/or infection (Nelsestuen et al. 2005; Zhang et al. 2005). Therefore, to determine if HNP is predictive of developing BOS we chose the first HNP level after the baseline pulmonary function date was established in order to avoid early post-operative changes. In this study we had 149 patients with a BAL sample obtained after reaching baseline pulmonary function. The time to reach baseline pulmonary function varied from 0.16 to 0.65 years (Table 2), and all of the subjects had reached their baseline pulmonary function by 1 year after transplant. Using a log rank test to compare those subjects with an HNP level below the median to those above the median we found that the initial HNP value after reaching baseline pulmonary function is significantly predictive of developing BOS ≥ 2 ($p = 0.0419$), whereas the first HNP level after transplantation is not statistically significant ($p = 0.086$). In the subjects that developed BOS ≥ 2, the time from their first HNP measurement after reaching baseline pulmonary function until developing BOS ranged from 0.07 to 4.32 years (median 0.88 with quartiles 0.61 and 2.12). Figure 1 displays the associated Kaplan-Meier curves of individuals that remain free from BOS ≥ 2 using values above the median (bottom line) versus those below the median (top line).

Table 2. Time from lung transplantation to baseline pulmonary function tests.

BOS grade	Time to baseline PFT (Years)
0	0.65 (0.28, 1.6)
I	0 36 (0.12, 0.83)
2	0.16 (0.12, 0.41)
3	0.65 (0.28, 1.4)

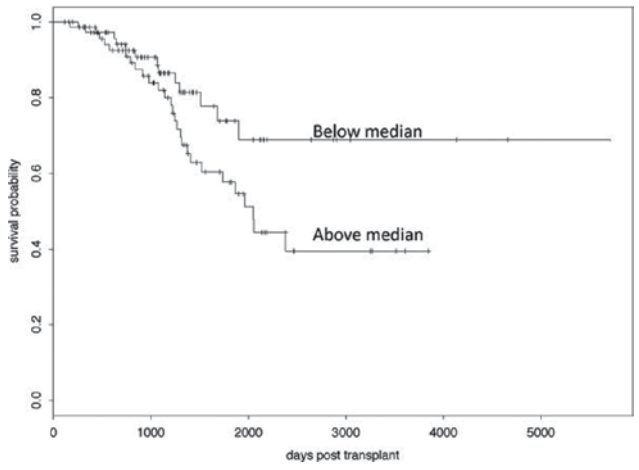

Figure 1. Kaplan Meier curve for HNP value after baseline pulmonary function date comparing BOS ≥2 free-survival for those with values below versus above the median HNP value.

A time varying Cox proportional hazards model also confirmed a statistically significant impact of the most recent HNP measurement after baseline pulmonary function on the time to development of BOS ≥ 2 ($p = 0.0490$). A 95% confidence interval for the risk associated with each unit increase in the logarithm of the HNP level is (1.0, 1.2) and indicates that higher levels of HNP are associated with an increased risk of developing BOS ≥ 2. The Grambsch-Therneau test for proportional hazards gave a p value of 0.663 indicating that the data are not at odds with the proportional hazards assumption. Although HNP was predictive of developing BOS ≥ 2, we did not find HNP to be predictive of developing BOS grade 1. BOS grade 1 was documented in 33/43 individuals (77%) that developed BOS ≥ 2. Therefore, the smaller sample size was likely underpowered to detect a correlation.

HNP trends

Using a mixed-model approach we found the log HNP values to be significantly higher in patients that developed BOS ≥ 2 compared to those that did not ($p = 0.0013$ 95% CI: 0.563, 2.274) throughout all time points; and the effect of days since transplantation not significant ($p = 0.113$). The p-value for the interaction between time since transplantation and BOS ≥ 2 status was 0.899, indicating that there is no significant difference in the HNP trajectories over time among patients who developed BOS ≥ 2 versus those who did not. This model found that the log HNP values were elevated in the first bronchoscopy after baseline pulmonary function was reached and remained persistently (17%) higher in those that developed BOS ≥ 2 compared to those that did not develop BOS ≥ 2 (C.I. of 6–26%).

HNP sensitivity and specificity

From these data we generated a ROC curve using the time varying Cox model approach. The resulting ROC curve demonstrates an area under the curve (AUC) of 0.73, which is in close agreement with our previously reported value of 0.79 (Figure 2) (Nelsestuen et al. 2005). Although a higher AUC is desirable, this lower AUC likely reflects the heterogeneous etiology of BOS, and that no single biomarker will reflect the diversity of causes for organ dysfunction following lung transplantation. Using this information, we can use the most recent HNP level to estimate sensitivities and specificities (Table 3).

HNP and neutrophil counts

To test for an association between HNP levels and neutrophil counts we used a mixed effect model using the log of HNP and neutrophil counts. From this model we found that HNP levels were associated with elevated neutrophil counts ($p < 0.0001$) (Figure 3). This suggests that elevated HNP levels in BOS are secondary to an elevation in neutrophils along with neutrophil activation; however we did not find neutrophil counts to be predictive of time to BOS ≥ 2 in a statistically significant

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fashion ($p = 0.513$). This discrepancy may reflect that this study was not powered to look at effects of neutrophilia and BOS, and thus, the power for detecting a difference is lacking.

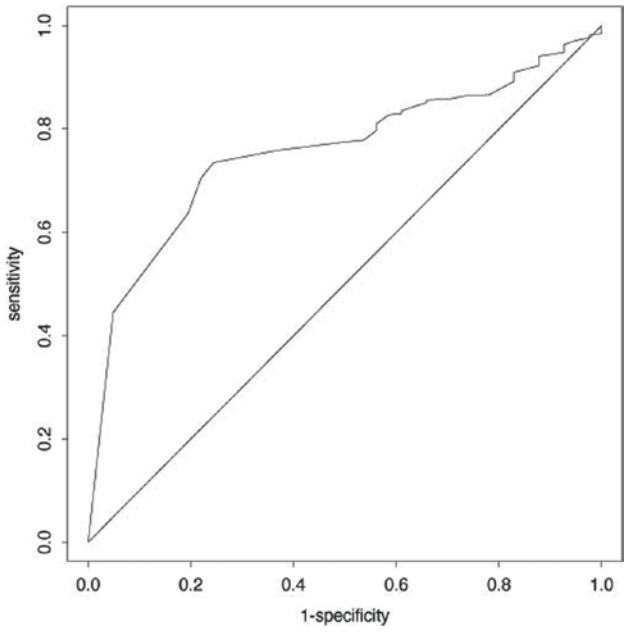

Figure 2. Receiver operator curve for HNP levels and BOS ≥2 based on a time varying Cox proportional hazards model. The ROC area under the curve is 0.73.

Table 3. The sensitivity and specificity with specific HNP cut-off levels on the first bronchoscopy after the baseline pulmonary function date to predict development of BOS ≥2.

Raw 1 INT level	Logarithmic transformed HNP level	Sensitivity	Specificity
4881.6	8.49	0.71	0.44
12215.9	9.41	0.66	0.56
27824.0	10.2	0.54	0.65

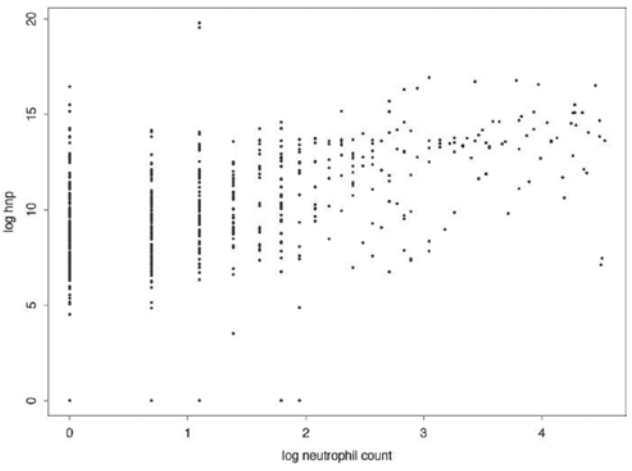

Figure 3. Scatterplot of HNP versus neutrophils. The estimated variance of the random effects was near 0 and not statistically different from 0, hence we summarized the association with Pearson's correlation coefficient. The value of the correlation coefficient was 0.52 and the usual p-value for testing if the correlation is 0 is less than 0.0001.

Impact of potential confounders

We examined the role of infection and other potential confounders on the association between HNP levels and time to the development of BOS ≥ 2 by including these variables as covariates in our time varying covariate Cox proportional hazards model in conjunction with HNP levels. In addition to infection we also included acute rejection and cystic fibrosis as an underlying disease as variables, since elevated HNP levels have been reported in these populations. CMV was defined as a positive rapid antigen test or culture. A positive bacterial culture was defined as a positive culture other than normal oral flora. In models that included each of these variables and HNP no statistically significant effect was detected for any of these variables (Table 4). We did not investigate if these variables by themselves were predictive of time to BOS ≥ 2, since the goal was to determine HNP role and this study was not powered to investigate these factors.

Discussion

BOS is a leading cause of morbidity and mortality in lung transplant recipients and occurs in 60–70% of long-term survivors. We previously identified HNP in BAL fluid as a potential biomarker of BOS using mass spectrometry. In this current study, we sought to prospectively validate these findings in patients undergoing surveillance bronchoscopy using a standard ELISA. In our previous study we observed that many individuals had transiently elevated HNP levels within the first year following lung transplantation. We felt this was likely due to early post-operative changes influencing HNP levels such as wound healing and infection. To avoid this confounder we used the first HNP level obtained after reaching baseline pulmonary function in this study and all samples were obtained within the first post-transplant year. We found that HNP levels were elevated in the first BAL fluid obtained after reaching baseline spirometry and remained consistently higher throughout the entire observation period in those patients that eventually developed BOS 2 or higher. These elevated levels were evident up to 4 years prior to the clinical diagnosis of BOS. This suggests that events early after transplantation are setting the stage for the subsequent development of BOS, and these conditions persist throughout the post-transplantation period until BOS manifests itself clinically.

HNP is a small neutrophil peptide that is key in the host innate immune response that exhibits antimicrobial activity and is seen in lung diseases associated with infection (Aarbiou et al. 2002b, Ashitani et al. 2002; Bals and Hiemstra, 2004; Boyton and Openshaw, 2002; Ganz, 2002a, Hiemstra et al. 1998; Hiratsuka et al. 2003; Ihi et al. 1997; Mukae et al. 2002; Borregaard et al. 2000). Elevated HNP levels have also been associated with non-infectious lung diseases including Idiopathic Pulmonary Fibrosis, sarcoid, and Chronic Obstructive Pulmonary Disease (Mukae et al. 2002; Hiemstra et al. 1998). Although infections, particularly CMV infection,

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Table 4. Summary of time varying covariate models that include HNP and potential confounding variables. All models included just one of these covariates in addition to HNP levels.

	CMV	Bacterial infection	Fungal infection	Acute rejection	CF
p-value	0.45	0.84	0.58	0.58	0.18
HR (95% CI)	0.71 (0.30, 1.71)	0.92 (0.40, 2.09)	1.24 (0.63, 2.44)	1.51 (035,650)	2.06 (0.72.5.86)

have been identified as risk factors for developing BOS, we did not find that viral, fungal and bacterial infection were predictive of the time to the development of BOS ≥ 2 given the effect of HNP. Elevated HNP levels have also been measured in individuals with cystic fibrosis (Aarbiou et al. 2002b), presumably secondary to chronic inflammation and infection. In this study, we did not find that cystic fibrosis as an underlying disease was predictive of time to BOS ≥ 2 given the effect of HNP. Previous studies have identified BAL neutrophilia as a risk factor for BOS. Although we found an association between HNP levels and neutrophilia, neutrophilia itself did not reach statistical significance in this study to predict BOS. Elevated HNP levels may reflect neutrophil activation and therefore, be a more sensitive biomarker.

Acute rejection is also a known risk factor for developing BOS and is characterized histologically by perivascular and peribronchial lymphocytic inflammation. These lymphocytes are primarily CD8 T-cell lymphocytes that are cytotoxic (Riise et al. 1997). Although localized neutrophilia has been identified in BOS, it has not been associated with acute rejection. In this study we did not find a statistically significant association between HNP levels and incidence of acute rejection, likely a reflection of absence of neutrophilia and different pathological processes.

The role HNP plays in the pathogenesis of BOS is unknown. HNP has also been implicated in adaptive immunity (Biragyn et al. 2002; Chaly et al. 2000; van Wetering et al. 2002; van Wetering et al. 2000). In lung epithelial cells HNP induces expression of co-stimulatory ligands and enhances interaction with CD4+ lymphocytes (van Wetering et al. 2000). HNP can also induce cellular proliferation of lung epithelial cells and fibroblasts, along with increased collagen production (Aarbiou et al. 2002a, Aarbiou et al. 2002b, Aarbiou et al. 2004; Chaly et al. 2000). However, at very high concentrations HNP has been shown to be cytotoxic *in vitro* (Aarbiou et al. 2002b). These high HNP levels were similar to the elevated concentrations measured in this study suggesting that HNP could be cytotoxic in the setting of developing BOS. Interestingly, we found HNP levels to be elevated in the first BAL fluid obtained after reaching baseline lung function. In addition, HNP remained elevated in those who subsequently developed BOS, suggesting early pathological changes occur in those who develop BOS, and that HNP plays a role in the pathogenesis and/or acceleration of airway injury and fibrosis that is seen in chronic lung allograft dysfunction.

The pathogenesis of BOS remains poorly understood and is likely multifactorial. Therefore, it is not a surprise that no individual or set of biomarkers adequately predicts those at risk for developing BOS, and likely explains the relatively modest sensitivity and specificity of HNP as a biomarker. It would be advantageous to identify individuals at risk of developing BOS prior to irreversible loss of lung function to allow for interventions in immuno- or non-immunotherapy to prevent the final common pathway of airway dysfunction. A number of biomarkers in BALF have been described for BOS including neutrophilia (Riise et al. 1999; Riise et al. 1998; Whitford et al. 2001; Zheng et al. 2000; DiGiovine et al. 1996; Elssner and Vogelmeier, 2001; Neurohr et al. 2009) and certain molecular markers (Belperio et al. 2002a, Belperio et al. 2002b, Belperio et al. 2001; Belperio et al. 2002c, Charpin et al. 2000; Meyer et al. 2001). However, to date, none of these biomarkers have been introduced into standard medical practice. Therefore, until precise phenotypes are defined it is likely that multiple biomarkers may be necessary to determine the risk of developing BOS. HNP may be one such biomarker that, when persistently elevated after the recipient has reached baseline pulmonary function, portends an increased risk for developing BOS.

Conclusions

In those that develop BOS, HNP levels are elevated as early as the first bronchoscopy after reaching stable pulmonary functions and are predictive of those that will subsequently develop BOS. In addition, these levels remain elevated until the development of BOS, even up to 4 years. This suggests pathological changes occur early after lung transplantation and that HNP may be a key player in the development of BOS. The sensitivity and specificity of HNP as a biomarker for BOS is relatively low and this may reflect the heterogeneity of BOS as a clinical surrogate for chronic rejection.

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Dr. C. Reilly conducted all the statistical analyses and wrote the manuscript with Dr. C. Wendt. Dr. Cervenka edited the manuscript, was involved in the initial recruitment of subjects, sample collection, establishing the database and measurements. Dr. Hertz edited the manuscript and provided expertise in clinical assessments. Ms. Becker assisted with sample and data collection, including quality assurance of the samples. Dr. Wendt supervised the sample and data collection, wrote the manuscript with Dr. Reilly and assisted with the interpretation of results. The authors would like to thank Dr. Gary Nelsestuen for technical advice and the use of laboratory equipment.

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Declaration of interest

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