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The Role of Heat Shock Protein 27 in Bronchiolitis Obliterans Syndrome after Lung Transplantation

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Abstract

Background—Heat shock proteins (Hsps) are a family of evolutionary conserved proteins classified according to their size as small and large Hsps. They have a cytoprotective role and have been shown recently to be immunogenic molecules. In addition, self-reactivity to Hsps has been implicated in various autoimmune diseases and in the development of alloimmunity. The purpose of this study was to examine the relationship of large and small Hsps and anti-Hsp antibodies in recipients of lung transplantation (LTX) who have bronchiolitis obliterans syndrome (BOS).

Methods—Anti-Hsp27 and Hsp70 antibodies and Hsp27, Hsp60 and Hsp70 protein expression levels were evaluated in serum and bronchoalveolar lavage samples collected from 8 LTX recipients with established BOS and 8 recipients without BOS (controls). Serum from 8 normal subjects was examined for Hsp levels as a comparison.

Results—Elevated serum Hsp27 levels were observed in recipients with BOS compared to controls or normal subjects, whereas Hsp70 and Hsp60 expression showed no difference. Anti-Hsp27 antibody levels were significantly higher in BAL of recipients with BOS as compared to those without. In contrast, anti-Hsp70 antibodies levels in serum or BAL showed no difference between groups.

Conclusions—These results support the novel concept that Hsp27 but not the classical Hsp60 and Hsp70 may be associated with the development BOS. The expression of anti-Hsp27 antibodies found only in the BAL fluid suggests a local response occurring at the level of the alveoli and terminal airways.

Chronic allograft rejection following lung transplantation (LTX) is characterized pathologically by the development of obliterative bronchiolitis (OB). Bronchiolitis obliterans

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syndrome (BOS) refers to the clinical findings of progressive airflow obstruction after transplantation, with a decrease in FEV1 by 20% of post transplant baseline being required for stage 1 disease. The development of BOS contributes substantially to the mortality rate after LTX affecting up to 50% of all transplanted recipients by 5 years post-transplant. (1) BOS, thought to be a form of chronic rejection, is treated with both an increase and change in the immunosuppressive regimen. Additionally, non-specific agents are used including HMGCofA inhibitors, dietary supplements and azithromycin. Unfortunately these changes can often slow the process but put the patient at increased risk of additional complication and do not reverse the allograft dysfunction. To date the cause of BOS is unknown, but multiple causes have been hypothesized to play a role including multiple episodes of acute rejection, and therefore a result of alloimmunity as well as cytomegalovirus infection, ischemia reperfusion injury, gastroesophageal reflux and diabetes, . (2,3)

One class of molecules that have been implicated in both autoimmunity and alloimmunity are the heat shock proteins (Hsps). Hsps are classified according to molecular weight as large (Hsp 60, Hsp70, Hsp90) or small such as Hsp27 and α B-crystallin. There has been much interest in defining the role of larger Hsps in immunity; however, their specific role remains unclear. Even less research has been done examining the smaller Hsps and immunity. Hsps prevent cell injury and death after various injuries including oxidative stress and ischemia reperfusion injury. (4) Lower pre-transplant levels of Hsps were linked to early graft loss after liver transplant, supporting a protective role of Hsps in response to the cellular stress that develops at the time of transplantation (5) Others have suggested the larger Hsps may be correlated with allograft rejection after solid organ or bone marrow transplantation (BMT). (6,7) Elevated levels of anti-Hsp70 antibodies have been correlated with graft versus host disease after BMT (8,9) Hsp40 and Hsp70 have been shown to be in biopsy specimens after LTX and have correlated with rejection. (10) The smaller heat shock proteins have been less investigated as source of alloimmunity, but Hsp27 expression has been correlated with allograft rejection after cardiac and renal transplantation. (11–13) However, the research as of now does not clearly indicate whether the increased expression of Hsps is a cytoprotective result of the cellular stress induced by transplantation or whether the increased expression is an antigenic stimulus eliciting allograft rejection.

Using a data registry and sample repository, we examined blood and bronchoalveolar lavage (BAL) from lung transplant recipients. We hypothesized that the smaller Hsps may play a role in the development of allograft rejection after lung transplantation. Therefore, we compared level of heat shock proteins and antibodies to Hsps in recipients with and without evidence of BOS.

Methods

Definition of patient groups

Using the OSU Lung Transplant Repository, 8 lung transplant patients meeting the criteria of initial onset of BOS, with a reduction in FEV1 of at least 20%, were identified (BOS group). (14,15), A group of 8 lung transplant recipients from the same time period not having BOS were also identified (Controls). All patients were transplanted between 2003 and 2009. All studies were IRB approved. Patients received 1 g of mycophenolate mofetil and Tacrolimus (10 mg/kg) pretransplant and 1g of solumedrol at time of initial implantation. Post operatively all patients were maintained on prednisone, Tacrolimus and Cellcept. All patients were prophylaxed with Bactrim three times weekly, voriconazole and valcyte. Patients followed a very stringent follow up protocol which included bronchoscopy and transbronchial biopsy and pulmonary function testing every 1 month for the first year, every 3 months for the second year, every 6 months for the third year and yearly thereafter unless clinical changes mandated an increased frequency.

The normal volunteers utilized for this study had not undergone transplantation, had no history of lung disease, and were not receiving immunosuppressive medications.

Specimen collection and processing

At the time of bronchoscopy, peripheral blood for all study participants was collected in anticoagulant tubes (EDTA) and fractionated by centrifugation at 3000 rpm for 10 – 15 minutes. The resulting plasma layer was removed and 1 to 2 ml aliquots were stored at - 80°C. Additionally, bronchoalveolar (BAL) fluid was obtained during the bronchoscopy. Without prior suctioning, the tip of the bronchoscope was secured in a subsegment of either the right middle lobe or lingual where 100 cc of sterile saline was then instilled and recovered in 20 cc aliquots. The recovered BAL fluid was divided, with one portion being sent to the clinical lab where it underwent pathologic cellular examination and was cultured and assayed for bacterial and viral pathogens. The other portion was processed for the specimen repository where it was initially gauze filtered to remove mucus and other large debris. The specimen then was centrifuged for 15 minutes at 1500 rpm. The resulting supernatant was stored at - 80°C. None of the samples utilized for this investigation revealed any evidence of microbial infection of the lung allograft.

Laboratory analysis

ELISA (Enzyme-Linked Immunosorbent Assay) was used to measure three heat shock proteins (Hsp27, Hsp60, and Hsp70). In addition we examined the serum concentrations of antibodies to Hsp27, and Hsp70. Commercially available ELISAs (Stressgen, Ann Arbor, MI) were used to measure Hsp 27, 60, 70 and anti-Hsp70 antibodies. No commercially available ELISA kit was available for anti-Hsp27 antibody, so we developed an ELISA. Microtiter plates were coated with rHsp27 (Assay Designs, Ann Arbor, MI) overnight and then blocked and washed. Samples were added and then horseradish peroxidase conjugated anti-human IgGAM (Assay Designs, Ann Arbor, MI) secondary antibody was added, and the assay was developed with tetramethylbenzidine (TMB) substrate (R&D Systems, Minneapolis, MN). Absorbance was read on a spectrophotometer.

Samples were diluted 1:2 – 1:200 in PBS or “sample diluent” from the commercial kits and run in duplicate. Serum concentrations of Hsp27 and Hsp60 are reported in ng/mL. No Hsp27 antibody standard was available, so results were reported as optic density. For ease of comparison, anti-Hsp70 ELISA values were also reported as a measure of OD.

Statistical analysis

ANOVA was used to test for differences in the log transformed heat shock protein levels. Transformation was required to meet the normality and equal variance assumptions necessary for ANOVA. Significance was defined as a $p < 0.05$.

Results

Eight LTX recipients met the criteria for inclusion in the BOS group while 8 other contemporaneous recipients who did not have established BOS were designated as the ‘control’ group. Table 1 lists demographic information for the 8 recipients in each group. The serum sample selected from the repository for analysis was the first available sample obtained following the establishment of the BOS diagnosis. This sample collection date averaged 324 days following the establishment of the BOS diagnosis. The average elapsed time from transplant for this serum collection was 1357 days. Since the control group, by definition, did not exhibit BOS, the available repository sample closest to this post-transplant date (day 1357) was chosen for comparative analysis. Because LTX recipients are more likely to develop BOS as they progress further from their transplant date, with 50% of these patients having BOS by

5 years, those who did not manifest BOS tended to be earlier in their post-transplant course. Thus, the repository sample that could be obtained for analysis from the control group averaged 467 days from the time of transplant. Because there were 8 recipients in each LTX group, 8 normal volunteers were randomly selected and had serum obtained for the purposes of comparison.

We found statistically higher levels of Hsp27 in serum from recipients with BOS as compared to control recipients (124.4 vs. 5.1, p -value = 0.042, Figure 1A). Differences in levels of Hsp27 in BAL fluid from recipients with BOS and control recipients did not reach statistical significance (Figure 1B). In contrast, there were no differences in the levels of Hsp60 or Hsp70 found in serum or BAL fluid of recipients with BOS compared with controls (Figure 1B and 1C respectively). Only serum samples (not BAL) were available from normal subjects and they were measured as a reference. Interestingly, levels of Hsp27 (in ng/ml) were much higher than those seen for the larger Hsp70 in both the serum and BAL fluid in all groups. Levels of Hsp 70 were measured to be less than 1 ng/ml.

Serum and BAL fluid concentrations of antibodies to Hsp27 and Hsp70 are demonstrated in Figure 2. Anti-Hsp27 antibody levels were significantly higher in BAL in patients with BOS as compared to control recipients (25.9 vs. 43.7, p -value = 0.034, Figure 2). There were no differences in Hsp70, Hsp60, or anti-Hsp70 levels in serum or BAL between groups.

Conclusions

Heat shock proteins constitute a family of conserved proteins well recognized for their role in protein folding. They also are induced in response to stressful stimuli such as heat, and are cytoprotective. They also play a role in apoptosis. Hsp27 has recently been described as anti-apoptotic and able to regulate cellular apoptosis through several pathways. Hsp27 inhibits caspase 3 activation by interacting with pro-caspase 3, and is also able to inhibit the Fas apoptotic pathway by blocking interaction with DAXX. (14–16) We have described Hsp27 as an anti-apoptotic regulator in monocytes and CD8+ lymphocytes. (17,18) In addition to their roles in protein folding and apoptosis, Hsps have been reported to help with antigen presentation as well as cross-presentation. Intracellular peptides are transported to class I molecules by Hsps. (19)

While Hsps can be thought of as cytoprotective, they also have been shown to be immunogenic molecules and self-reactivity to Hsps have been implicated in playing a role in various autoimmune diseases, including autoimmune diabetes, autoimmune arthritis, atherosclerosis and many others. (20–22) One mechanism that may be responsible for this is molecular mimicry, where T cells induced by microbial Hsps may cross react with self Hsps. Microbial Hsp65s possess epitopes that are cross-reactive with self Hsp65. (19,23) However, the role of Hsps remains unclear. Other studies, in the same diseases such as autoimmune arthritis and autoimmune diabetes, suggest that the Hsps may play a protective role in the immune response. (24,25) One study suggests that part of the antigen presentation properties of Hsps are due to their binding of antigenic molecules and this may account for Hsp induced cytokine production by immune cells. (19)

Studies examining the role of larger heat shock proteins in alloimmunity have also had conflicting results, suggesting possibly a dual role for Hsps. Several groups have shown relations between the larger Hsps and graft versus host disease (GVHD) after peripheral blood stem cell transplantation. (6,8,9,26) An in-vitro model demonstrated levels of Hsp70 correlated with the GVHD response. (6) In patients with acute GVHD there were higher levels of antibody to Hsp70 and/or Hsp90, but not Hsp60, and preceded the development of chronic GVHD. Other studies have failed to show a relationship between alloimmunity and Hsps. One study found

detectable antibodies to Hsps after BMT but with no correlation to GVHD. (27) In an acute skin allograft rejection model no role of Hsp70 was detected in the immune response. (28) In cardiac transplantation, there were no differences between pre- or post-cardiac transplant anti-Hsp65 antibodies and outcomes. (29) Another study looked at human kidney allograft recipients and found no differences in anti-Hsp60, and anti-Hsp70 antibodies before or after transplant. (30)

While most studies looking at Hsps and immune response have dealt with the larger Hsps, it is even less clear if smaller Hsps are capable of eliciting an immune response. Some studies have suggested an anti-inflammatory role for Hsp27. Recently Hsp27 has been shown to inhibit the differentiation from monocytes to dendritic cells, and monocytes to macrophages, consistent with anti-inflammatory properties. (31) Serum levels of Hsp27 have been found to be elevated in other chronic inflammatory states such as pancreatitis and pancreatic carcinoma. (32) Hsp27 expression confers resistance of breast cancer cells to doxorubicin by inhibiting apoptosis. (33,34) Also, serum antibodies to Hsp27 have been shown to be elevated and correlate with improved survival in breast cancer. (35) Levels of Hsp27 in lymphocytes and monocytes decrease with age. (36)

In studies of transplantation most studies also support an anti-inflammatory role of Hsp27. Hsp27 overexpressing mice are protected against liver ischemia reperfusion injury. (37) It is thought the cellular protective effects of Hsp27 may contribute to its protective effect around the time of transplantation. Increased levels of Hsp27 were found in cardiac biopsy specimens associated with acute rejection after transplantation, but its cellular effects are uncertain. (13) Late after transplantation also, Hsp27 expression in vessels was found to correlate with freedom from cardiac allograft vasculopathy after cardiac transplantation. (11)

In this study, we examined levels of Hsps and antibodies to Hsps in patients after lung transplantation with and without obliterative bronchiolitis. We are unaware of any other studies that have examined the role of antibodies to Hsps in the pathogenesis of allograft rejection after lung transplantation. One study showed exposing donor animals to heat pretreatment before harvest results in increased synthesis of Hsp70, which protected against subsequent ischemia-reperfusion injury in the lung isograft. (38) The same authors found donor adenovirus-mediated gene transfer of Hsp70 decreased ischemia-reperfusion injury in rat lung isografts. (39) These studies support a protective effect of Hsps in lung injury at the time of transplantation. Another study looked at Hsp40, Hsp60, and Hsp70 in biopsy specimens from human lung transplant recipients and found increased expression of Hsp40 and Hsp70 in subjects undergoing rejection, however, Hsp70 could not differentiate between rejection and infection. (10)

The etiology of BOS remains unknown and remains a clinical dilemma in lung transplantation. It is thought that acute lung rejection (which can be characterized by lymphocytic bronchiolitis) and resultant lung injury leads to chronic rejection which is manifested as BOS and therefore BOS is an end stage of alloimmunity. This is supported by the fact that recurrent episodes of acute rejection are a risk for BOS, and also BOS is much more common after lung transplantation than in other patients. However many more recent theories suggest that multiple injuries promote continued collagen deposition resulting in terminal airway obliteration. (40) CMV infection, other respiratory viral infections, gastroesophageal reflux disease have all been suggested to upregulate the alloimmune response after lung transplantation. (40) In the present study we found elevated levels of Hsp27 in the serum of patients with BOS and elevated levels of antibody to Hsp27 only in the BAL fluid suggesting a localized immune response occurring at the level of alveoli and terminal airways. An intriguing hypothesis to explain our findings is that an alloimmune generated necrosis of cells (possibly initiated by one of the previously mentioned potential insults) leads to exposure of normally intracellular Hsp27 resulting in a stimulus that further participates in the development of BOS.

In summary, this pilot study demonstrates elevated levels of Hsp27 protein in the serum in patients that have developed BOS after lung transplantation, as well as elevated levels of antibody to Hsp27 in their BAL fluid. While not significant, higher levels of HSP 27 were also found in the BALF. Considering our low number of samples, the failure to show a statistical difference could have been the result of a Type II error. These results need to be expanded with a larger population and with serial studies due to the small sample size in the current population. However, it is intriguing to speculate that Hsp27 may play a role as a marker of alloimmunity after transplantation or even in the pathogenesis of bronchiolitis obliterans syndrome.

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Figure 1. Hsp expression in lung transplantation recipients with BOS, without BOS (Controls), and normal volunteers. A. Serum Hsp27 (ANOVA p -value = 0.042) B. BAL Hsp27 C. Serum Hsp60 D. BAL Hsp60 E. Serum Hsp70 F. BAL Hsp70

Figure 2. Hsp27 and Hsp70 antibodies of lung transplantation recipients with BOS, without BOS (Controls), and normal volunteers. A. Serum Anti-Hsp27 (ANOVA p -value = 0.034) B. BAL Anti-Hsp27 C. Serum Anti-Hsp70 D. BAL Anti-Hsp70.

Table 1

Demographics of Lung Transplant Recipients

OB Group: N=8			
Gender	Pre-LTX Diagnosis	Days since LTX	Days following OB Diagnosis
M	COPD/EMPHYSEMA	2219	1282
M	IDIOPATHIC PULMONARY FIBROSIS	2160	64
M	COPD/EMPHYSEMA	718	60
M	COPD/EMPHYSEMA	1852	754
F	IDIOPATHIC PULMONARY FIBROSIS	1372	0
F	COPD/EMPHYSEMA	1000	26
M	ALPHA - 1 - ANTITRYPSIN DEFICIENCY	950	199
M	IDIOPATHIC PULMONARY FIBROSIS	586	210
Control Group (non-OB): N=8			
Gender	Pre-LTX Diagnosis	Days since LTX	
M	ALPHA - 1 - ANTITRYPSIN DEFICIENCY	586	
F	SARCOIDOSIS	566	
F	COPD/EMPHYSEMA	284	
M	COPD/EMPHYSEMA	1054	
F	IDIOPATHIC PULMONARY FIBROSIS	473	
F	IDIOPATHIC PULMONARY FIBROSIS	520	
M	IDIOPATHIC PULMONARY FIBROSIS	139	
M	IDIOPATHIC PULMONARY FIBROSIS	120	