Biomarkers, August 2008; 13(5): 486–495

informa
healthcare

Thioredoxin as a biomarker for graft rejection in lung transplant recipients

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Abstract

Primary graft dysfunction and rejection are common complications in lung transplant recipients. Increased expression of thioredoxin-1 (Trx), a 12-kDa redox-regulatory protein, has been reported in multiple lung pathophysiological conditions involving oxidative and inflammatory mediated injury including graft rejection in canine and rat models of lung transplantation. Our objective was to determine whether increased Trx expression is associated with progression of rejection pathophysiology in human lung transplant recipients. Bronchoalveolar lavage (BAL) fluid and transbronchial biopsy samples were collected as a routine part of post-transplant clinical care from 18 lung transplant patients from our adult lung transplant programme. Lung transplant recipient profile included age/sex, ethnic background, days on ventilator, total ischaemic time, and cytomegalovirus (CMV) status. Based on histopathological grading criteria, patients were divided into two groups, rejecting (A1/A2 or B1) and non-rejecting (A0/B0). Rejecting and non-rejecting group total BAL cell counts and differential cell counts for neutrophils, macrophages, lymphocytes and eosinophils as well as total BAL cell Trx levels were analysed. Total BAL cell counts were significantly ($p < 0.05$) elevated in graft rejecting versus non-rejecting patients. Differential BAL macrophage counts were comparable in rejection and non-rejection groups, whereas there were significant increases in neutrophils and lymphocytes but not eosinophils in patients with rejection versus non-rejection pathology ($p < 0.05$). Total ischaemic time and days on ventilator in rejection and non-rejection groups were comparable. However, Trx levels were significantly elevated in BAL cells from graft-rejecting patients compared with non-rejecting patients ($p < 0.05$). These data suggest that surveillance monitoring of BAL Trx levels after lung transplantation can serve as a biomarker to assess severity of graft rejection.

Keywords: *Lung transplant, BAL cell differential, thioredoxin, graft rejection*

(Received 9 November 2007; accepted 17 March 2008)

Introduction

Lung transplantation has emerged as a valid therapeutic approach for a variety of end-stage pulmonary diseases (Trulock 1997, DeMeo & Ginns 2001, Meddeloff et al.

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ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500802061822

2002). Despite continued improvement in surgical procedures and therapeutic approaches, early graft dysfunction and acute cellular rejection remain important causes of morbidity and mortality after lung transplantation (Bando et al. 1995, Christie et al. 1998, De Hoyos et al. 1992). A number of human and animal studies have described multiple factors including early ischaemia–reperfusion (I/R) injury, generation of inflammatory mediators and progressive infiltration of inflammatory and immune cells at the site of inflammation which lead to acute and chronic rejection in lung transplant recipients (Chatila et al. 2003, Pierre et al. 1998, Hirt et al. 1999, King et al. 2000, de Perrot et al. 2003). In general, I/R responses are represented as early collective events arising from cold-ischaemic storage and warm reperfusion. These events incite generation of reactive oxygen species (ROS), which can inflict vascular endothelial cell injury directly or indirectly via increasing sequestration of circulating leukocytes and neutrophils, leading to enhanced generation of mediators such as cytokines and increased expression of the redox-regulatory cytoplasmic protein thioredoxin-1 (Trx) (Pober & Cotran 1990, Briscoe et al. 1998, de Perrot et al. 2003, Calvin et al. 2006, Cottini et al. 2006).

Trx is a potent antioxidant, cell growth promoter, and unique chemoattractant to neutrophils, monocytes/macrophages, and T cells (Schenk et al. 1996, Bertini et al. 1999, Nordberg & Arner 2001, Rancourt et al. 2007). In fact, causal association between increased Trx expression and graft rejection has been shown in canine and rat models of lung transplantation (Wada et al. 1995, Patel et al. 2001). However, the link between graft rejection and increased expression of Trx in human lung transplant recipients remains to be established. This study was designed to examine whether post-transplant increases in bronchoalveolar lavage (BAL) cell Trx levels can serve as a surveillance biomarker to assess graft rejection pathophysiology in lung transplant recipients.

Methods

Study patients

This study was conducted as a routine part of the post-transplant clinical care of lung transplant patients representing both genders and minority subjects from our adult lung transplant programme between October 2004 and November 2005. A total of 18 patients who signed an informed written consent and were <90 days post-transplant were included in this study. All patients who had undergone a single or bilateral lung transplant during the study period were included. The study protocol (no. 460–03) was approved by the Institutional Review Board (IRB).

Collection of BAL from lung transplant patients

Bronchoscopy for BAL was performed as previously described. (Baz et al. 1996). In brief, after local anaesthesia with lidocaine, a fibreoptic bronchoscope was wedged into the right middle lobe or lingula and sterile saline (5×20-ml aliquots) was instilled and immediately recovered by gentle suction. The average return BAL fluid was approximately 50% of the instilled volume. The BAL fluid was processed for differential cell counts including macrophages, neutrophils, eosinophils and T lymphocytes.

BAL cell differentiation

Immediately after collection, BAL fluids were centrifuged (1300*g*, 5 min, 4°C) to remove membranous debris. Lavage cell suspensions (100 μl) were used to prepare slides for differential counting of nucleated cells. Cytospin slides were stained with Leukostat (Fisher Diagnostics, Orlando, FL, USA) and differentiated by standard haematological procedures.

Transbronchial biopsy samples from lung transplant patients

Transbronchial biopsy was performed by wedging the bronchoscope in the middle lobe segment, passing the biopsy forceps through the side channel of the bronchoscope, and obtaining 6–10 small pieces (1–2 mm each) of lung tissue as previously described (Baz et al. 1996). The complication rate for this procedure is less than 5% (Baz et al. 1996). The slides prepared from the fixed tissue blocks were used to evaluate morphological evidence of rejection. The standard post-transplant surveillance bronchoscopy/BAL procedures scheduled at our institution include 1–7 days to 36 months (±30 days) and were performed postoperatively. For this study, up to 90 day postoperative samples were used.

Histopathological grading for rejection of grafts

The biopsy specimens were formalin-fixed, routinely processed, and paraffin-embedded. Slides 5 μm thick were obtained from the paraffin blocks and stained with haematoxylin-eosin for microscopic examination of morphological evidence of rejection. The degree of rejection was evaluated using the following classification system described in the 1996 lung allograft rejection working formulation (Yousem et al. 1996). Briefly, acute cellular rejection was determined by the intensity of perivascular lymphocytic infiltration and classified and graded as A0 to A4: A0, none; A1, minimal; A2, mild; A3, moderate; and A4, severe. Airway rejection was determined by the degree of lymphocytic bronchiolitis and marked as B0 to B4: B0, none; B1, minimal; B2, mild; B3, moderate; and B4, severe. Based on histopathological grading, patients were divided into two groups, rejecting (A1/2 or B1) and those without rejecting (A0/B0).

BAL Trx levels

Total BAL cell Trx content was determined by Western blot analysis as previously described Zhang et al. (1998, 1999). In brief, cell lysate proteins (30 μg) and human recombinant purified Trx (50 ng; USB Corporation, Cleveland, OH, USA) as a standard were fractionated by 7.5% SDS-PAGE and electroblotted onto nitrocellulose membranes. The blots were incubated in blocking solution (0.2% non-fat milk in TBS (20 mM Tris–HCl, pH 7.5, 5 mM NaCl)) and then hybridized with human anti-Trx monoclonal antibody (1:1000 dilution) in antibody buffer (0.2% non-fat milk and 0.1% Tween-20 in TBS) at room temperature for 1 h. After washing with buffer (0.1% Tween-20 in TBS), the membranes were incubated in 1:3000-diluted antirabbit IgG, alkaline phosphatase-linked whole antibody for 1 h. The immunoreactive bands were visualized by enhanced chemiluminescence as described previously (Zhang et al. 1998, 1999). The blots were scanned to determine the optical density of

sample and standard Trx contents using a Fluor-S MultImager system (Bio-Rad, Richmond, CA, USA). An optical density arbitrary unit of 1 was assigned to the standard Trx blot.

Statistical analysis

A multivariate regression analysis was applied to determine the significance of the effects of variables including ischaemic time, differences in BAL cell differentiation, and Trx levels in rejection and non-rejection groups. Significant effects were further analysed by post-hoc Student's *t*-test; $p < 0.05$ was considered significant.

Results

Profile of rejecting and non-rejecting lung transplant recipients

As shown in Table I, 18 adult lung transplant recipients enrolled in this study. There were 60% male and 40% female subjects of which 78% were Caucasian and 11% each were Hispanic and African-American. The mean ages of six non-rejecting (17% female) and 12 rejecting (50% female) patients were 51.3 ± 16 and 51.2 ± 19 years, respectively. Due to the small group sizes, differences in ethnic background between the two groups could not be analysed. None of the samples in this study showed histopathological changes consistent with C or D grading. All patients received triple immunosuppressive regimen consisting of azathioprine/serolimus, tacrolimus and prednisone. Eighty per cent of the patients in both groups received a single lung transplant due to interstitial pulmonary fibrosis. There was no evidence of infection in any of the study patients. Donor and recipient lung CMV status is provided in Table I. There was no clear difference in cytomegalovirus (CMV) status between graft rejecting and non-rejecting pathology. Similarly, declines in FEV_1 values were $<10\%$ and not different in the lungs of rejecting and non-rejecting patients. There was also no association between ischaemic time and post-transplant days on a ventilator, and rejection. Figure 1 show that total ischaemic time (cold + warm) (panel A) as well as post-transplant number of days on a ventilator (panel B) were comparable in non-rejecting and rejecting patients.

BAL cell differentiation in allograft rejecting versus non-rejecting patients

Figure 2 shows representative light micrographs ($400\times$) of BAL cell morphology of non-rejecting and rejecting patients (panel A). Total BAL cell contents in the rejecting group were significantly ($p < 0.05$) greater than in the non-rejecting group (panel B). BAL cell differential analysis indicated that macrophage counts were comparable in patients in the rejecting and non-rejecting groups. However, here were significant increases in neutrophils and lymphocytes but not eosinophils in patients with acute rejection pathology (panel C) which primarily reflects increased inflammatory cellular infiltration in the allografts.

Trx levels in allograft rejecting versus non-rejecting patients

A representative Western blot of BAL total cell lysate Trx contents of two separate rejecting and non-rejecting patients shows a single band at 12 kDa which is identical

Table I. Profile of lung transplant recipients.

Age (years)/ sex	Transplant type	Disease	Ethnic background	Rejection: A/B	Ischaemic time (min)	CMV (don/rec)	Post-transplant (weeks)
Rejection (+)							
47/M	Single	IPF	Caucasian	A2/B1	173	−/+	8
56/M	Single	IPF	Caucasian	A2/B1	271	+/+	12
59/M	Single	IPF	Caucasian	A1/B1	176	−/+	3
63/M	Single	IPF	Caucasian	A2/B1	180	+/−	10
66/M	Single	IPF	Caucasian	A2/B1	233	−/−	4
64/M	Single	IPF	Caucasian	A1/B1	247	−/+	6
52/F	Bilateral	Bronchiectasis	Hispanic	A1/B1	350	+/+	4
23/F	Single	CF	Caucasian	A1/B1	219	−/−	2
32/F	Single	IPF	Caucasian	A2/B1	217	−/+	12
32/F	Bilateral	IPF	Caucasian	A2/B1	440	+/+	6
59/F	Single	IPF	Caucasian	A2/B1	278	+/+	12
62/F	Single	COPD	Caucasian	A2/B1	153	−/−	8
Rejection (−)							
44/M	Single	IPF	African-American	A0/B0	223	+/+	6
47/M	Single	IPF	Caucasian	A0/B0	240	+/+	8
58/M	Single	IPF	Hispanic	A0/B0	219	−/+	12
59/M	Single	IPF	Caucasian	A0/B0	153	−/−	4
65/M	Bilateral	IPF	Caucasian	A0/B0	257	−/+	10
35/F	Single	Sarcoidosis	African-American	A0/B0	297	−/+	12

A1 or A2, perivascular lymphocytic infiltration; B1, lymphocytic bronchiolitis; A0/B0, no rejection; CMV, cytomegalovirus; IPF, interstitial pulmonary fibrosis; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; ischaemic time, total (cold+warm); don/rec, donor/recipient. All patients were on a triple immunosuppressive regimen (azathioprine/siroliumus, tacrolimus and prednisone). Days on ventilator: rejection group 1–5 days; non-rejection group 1–6 days.

to the human purified standard Trx (Figure 3A). The optical densities of Trx Western blots of total BAL cells from rejecting and non-rejecting patients as well as standard Trx blots were determined. An optical density arbitrary unit of 1 was assigned to the human purified standard Trx blot. Figure 3B shows significant elevation of Trx levels in BAL cells from graft-rejecting patients compared with the Trx content of BAL cells from non-rejecting lung transplant patients ($p < 0.05$ vs non-rejection). The increased BAL cell Trx levels in rejecting grafts appear to be associated with increased BAL neutrophils and lymphocytes (Figure 2C).

Discussion

Experimental evidence suggests that increased lung Trx levels in post-transplant lungs are associated with allograft rejection in canine and rat models of lung transplantation (Wada et al. 1995, Patel et al. 2003). The results of the present study using morphological grading criteria for degree of graft rejection demonstrate for the first time a post-transplant elevation of Trx levels in BAL cells in human allografts and lymphocyte infiltration. Although the molecular mechanisms of increased expression of Trx in animal and human lung transplant models remain to be determined, generation of multiple mediators from donor organ preservation to transplant events occurring within 24 h following transplant may play a critical role. For example, excessive oxidative stress from unavoidable I/R is known to increase activation and infiltration of inflammatory cells in allografts (Pierre et al. 1998, Hirt et al. 1999, King et al. 2000, Chatila et al. 2003, de Perrot et al. 2003). Activation of multiple signalling

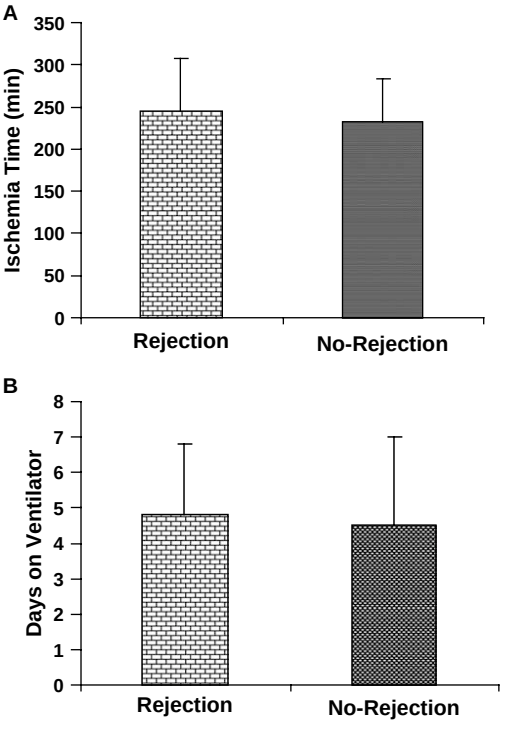

Figure 1. Effects of total ischaemic time and post-transplant days on a ventilator in allograft rejecting ($n = 12$) and non-rejecting patients ($n = 6$). Data represent mean $\pm$ SD.

events by oxidative stress and inflammatory mediators is known to enhance transcription factor nuclear factor (NF) κB/DNA-binding activity and expression of an exceptionally large number of genes including genes encoding Trx and inflammatory cytokines (Siebenlist et al. 1994, Ghosh et al. 1998). Increased levels of multiple cytokines have been reported in patients with post-transplant reperfusion oedema from our lung transplant programme (Mathur et al. 2006).

Increased Trx expression has been shown to control a wide spectrum of biological activities ranging from cell growth to death (Holmgren 1985, Powis et al. 1995). Clinically, increased Trx levels have been reported in multiple inflammatory diseases including patients with acute lung injury, acute respiratory distress syndrome, rheumatoid arthritis, and HIV disease (Nakamura et al. 1996, Gromer et al. 2004, Burke-Gaffney et al. 2005, Callister et al. 2006, Maurice et al. 1997). Trx also functions as a unique chemoattractant to inflammatory and immune cells (Schenk et al. 1996, Bertini et al. 1999). In contrast, several *in vitro* and *in vivo* studies have shown that exogenous supplementation of purified Trx or overexpression of human Trx attenuate oxidative injury and inflammatory responses (Okubo et al. 1997, Takagi et al. 1999, Nakamura et al. 2001, Billiet et al. 2005). We recently reported that priming of donor lungs with recombinant purified human Trx prior to transplantation attenuates post-transplant biochemical and morphological changes in allografts using a rat model of lung transplantation (Hu et al. 2007). Despite multiple known biological functions of Trx, very little is known about its roles as a potent antioxidant, a cell growth promoter and a unique chemoattractant to inflammatory and immune

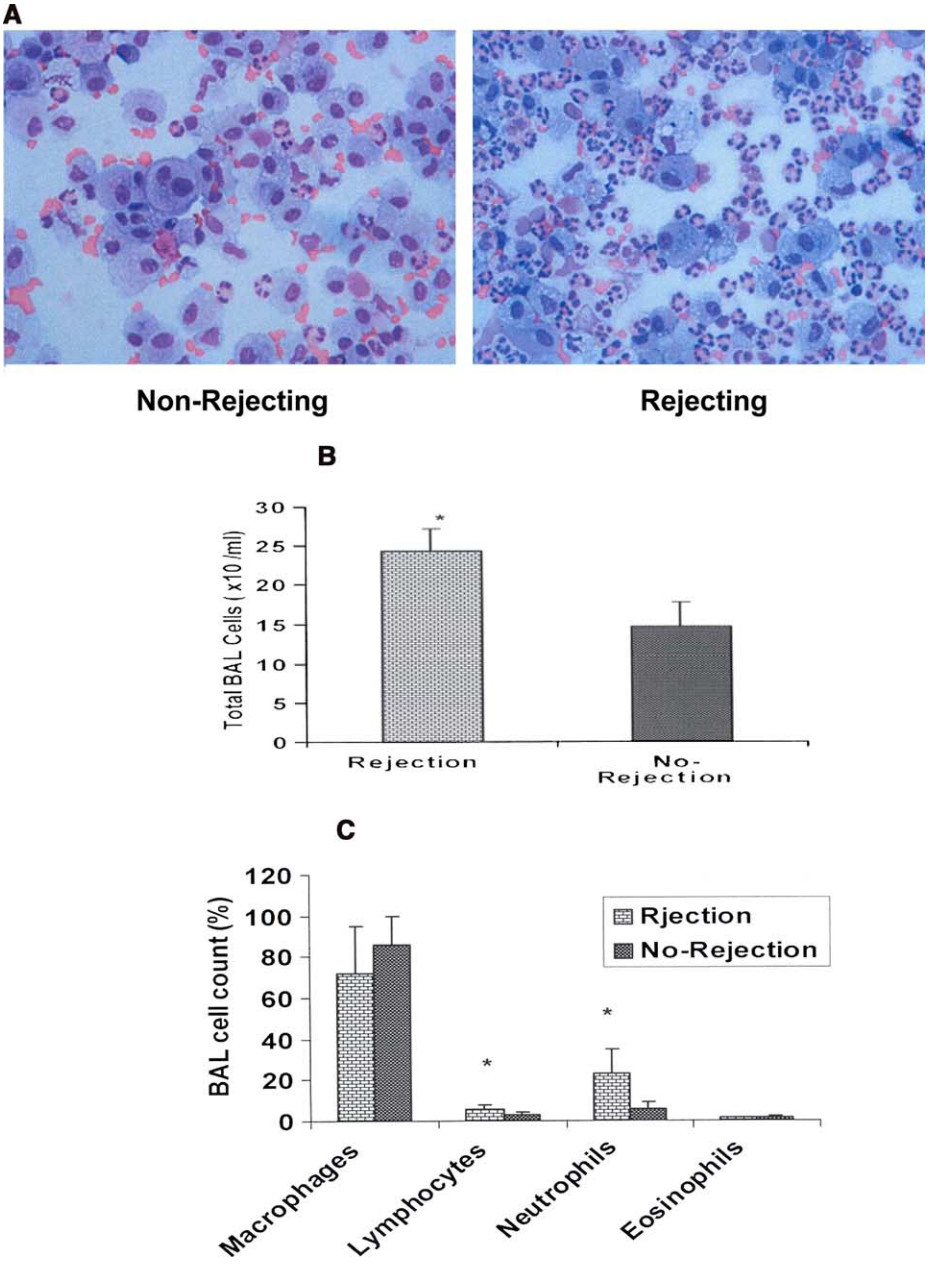

Figure 2. BAL cell differentiation in allograft rejecting ($n=12$) and non-rejecting patients ($n=6$). Panel A shows representative light micrographs (400×) of BAL cell morphology of rejecting and non-rejecting patients. Total BAL cells (panel B) and differential cell counts for macrophages, neutrophils, lymphocytes and eosinophils (panel C) were determined as described in Methods. Differential cell counts are expressed as percentage of total cell population. Data represent mean ± SD. $^{*}p<0.05$ vs no-rejection.

cells in allografts. Post-transplant activation of NFκB and associated increased expression of Trx can be a potent combination for enhanced production of biological risk factors associated with acute cellular injury and progression of graft rejection. As

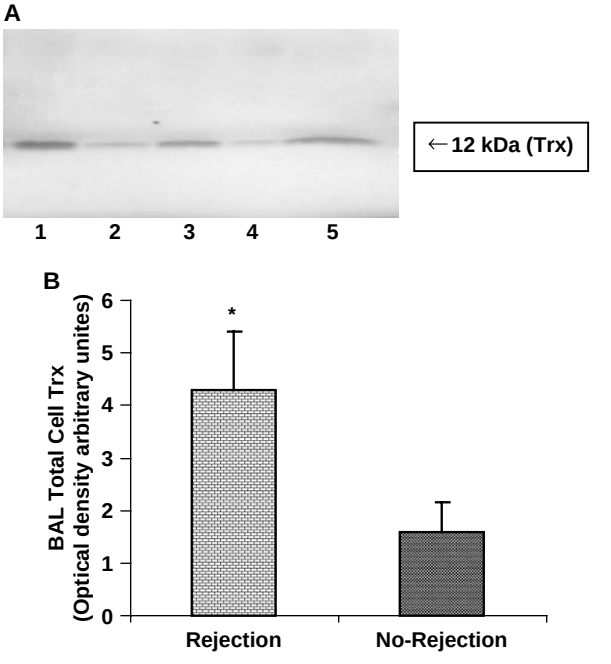

Figure 3. BAL cell Trx levels are increased in allograft rejecting patients ($n=12$). Western blot analyses of total BAL cell lysates were performed using human purified Trx as a standard as described in Methods. Panel A shows a representative Western blot of human purified Trx (lane 1) and two separate non-rejecting (lanes 2 and 4) and rejecting (lanes 3 and 5) patients. Total BAL cell Trx levels (Panel B) were determined by densitometric analysis of Western blots. The optical density arbitrary units are relative to 1 arbitrary unit set for the Western blot representing 50 ng of human recombinant purified Trx as described in Methods. Data represent mean $\pm$SD, non-rejection patients $n=6$. $^{*}p<0.05$ vs no-rejection.

such, our results provide preliminary indication that Trx may be a biomarker with which to identify graft rejection in lung transplant recipients. We are planning a larger study consisting of longitudinal bronchoscopic specimens to measure Trx over a longer period of time and to identify expression levels of Trx in target inflammatory and/or immune cells to confirm our findings and to determine whether Trx can be used as a biomarker for rejection in human lung allografts.

Acknowledgements

We thank Mrs Dihua He for processing BAL samples and Trx analysis. This work was supported in part by NIH grant HL67886 and by the Department of Veterans Affairs (J.M.P.).

Declaration of interest: The authors have no conflict of interest to disclose.

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