Rejection and Expression of Thioredoxin in Transplanted Canine Lung*

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Thioredoxin (TRX) production has been shown to be induced by a variety of stresses. In this study, we examined TRX expression in lung tissues after canine lung transplantation to determine whether it could be induced by allogeneic stimulations and could be used to diagnose early rejection. Thirty-five adult mongrel dogs were used in all. In group A, 24 dogs underwent allotransplantation of the left lung with no immunosuppressant and were sacrificed at various times; 5 were sacrificed on postoperative day (POD) 1; 5 on POD 2; 7 on POD 3; and 7 on POD 5. In group B, 5 donor right lungs were used for negative control. In group C, 3 dogs underwent autotransplantation. In group D, 8 dogs underwent allotransplantation of the left lung with optimal immunosuppression and were sacrificed at POD 28. Lung tissues were stained with anti-TRX antibody, and the TRX high-producer (TRXh) cells in a randomly chosen field were counted as the index of TRX expression. In group A, the number of TRXh cells were as follows: 1.68±1.14 in grade 0; 4.87±1.07 in grade 1; 10.42±4.24 in grade 2; 27.34±17.96 in grade 3; and 50.90±17.36 in grade 4. In group B, the number of TRXh cells was 1.82±1.01. There was a significant difference between each rejection grade in group A and group B (p<0.01), and we could observe TRXh cells in the early stage of rejection. These results suggest that analysis of TRXh cells in lung tissues may be useful in the early diagnosis of rejection. (CHEST 1995; 108:810-14)

AM=alveolar macrophage; ADF=adult T cell leukemia derived factor; CsA=cyclosporine; TRX=thioredoxin; TRXh=TRX high-producer cells; POD=postoperative day

Key words: canine lung transplantation; thioredoxin; adult T cell leukemia derived factor (ADF); rejection

Lung transplantation has become an accepted treatment for patients with end-stage lung disease, and an early diagnosis of rejection is essential in the management of these patients. Thioredoxin (TRX [molecular weight: 11,734]) consists of 104 amino acids with characteristic two -SH radicals, -Cys-Gly-Pro-Cys-. A coenzyme catalyzing the reduction of proteins in prokaryotic systems, TRX has various activities including radical scavenger activity and protein-refolding activity. In the other hand, adult T cell leukemia derived factor (ADF) is a humoral factor produced by T cell leukemia cells infected with the retrovirus. The ADF has interleukin-2 receptor induction ability. The cDNA cloning of ADF has shown a remarkable homology between ADF and Escherichia coli-derived TRX; ADF is now considered to be human TRX.

Thioredoxin expression can also be induced by a variety of stresses, including x-rays, ultraviolet irradiation, and hydrogen peroxide (H2O2). In gel retardation assays, we have shown that recombinant TRX markedly enhances the binding of NF-κB to the target sequence in α chain promoter, suggesting that TRX may be required in the process of activating NF-κB systems. These findings suggest that TRX expression might be induced by allogeneic stimulation. Thus, we hypothesized that cells producing high levels of TRX would increase in number as rejection progressed. To examine this hypothesis, we performed left lung allotransplantation in adult mongrel dogs and observed the expression of TRX in biopsy specimens of the lung.

**MATERIALS AND METHODS**

**Animals and Anesthesia**

Thirty-five mongrel dogs weighing between 6 and 25 kg were used. General anesthesia was performed by the method described previously. The dogs were pretreated with an intramuscular injection of ketamine, 7.5 mg/kg, and anesthetized with an intravenous injection of pentobarbital, 20 mg/kg. Anesthesia was maintained with an inhalation oxygen concentration (FiO2) at 0.5, N2O at 0.5, and halothane at 1% during the operation, and mechanical ventilation was performed with a respirator (Harvard type pump; Shinano, Nagano, Japan) with fraction of inspired oxygen of 0.5; frequency, 20 times per minute; 25 mL/kg of tidal volume; positive end-expiratory pressure, 5 cm H2O.
All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the National Academy of Science published by the National Institutes of Health (NIH publication No. 85-23, revised 1985).

**Allotransplantation and Autotransplantation of the Lung**

Transplantation of the left lung was performed by a modification of the method of Veith and Richards.\(^\text{15}\) The average warm ischemic time was 78.6±18.0 min. Antibiotics were administered to prevent infection for 7 days.

**Experimental Groups**

Group A (allotransplantation group) was comprised of the left allotransplanted lungs from 24 dogs. No immunosuppressant was administered. To observe TRX expression in the various grades of rejection, the dogs were sacrificed and biopsies were performed at various times: on postoperative day (POD) 1, n=5; POD 2, n=5; POD 3, n=7; and POD 5, n=7.

Group B (control group) was comprised of the donor right lungs from five of the allotransplanted dogs in group A.

Group C (autotransplantation group) was comprised of the autotransplanted left lung from 3 dogs, which did not receive any immunosuppressant therapy and were sacrificed on POD 5.

Group D (immunosuppressed group) was comprised of eight mongrel dogs which were allotransplanted. Immunosuppressants were administered every day according to our previous report.\(^\text{11,12}\) FK-506 (tacrolimus), 0.1 mg/kg/d, was administered intramuscularly in 4 dogs; and cyclosporine (CsA), 20 mg/kg/d, was given orally in 2 dogs; and 1/2 FK-506, 0.05 mg/kg/d, was given intramuscularly plus 1/2 CsA, 10 mg/kg/d, orally in 2 dogs. The dogs were killed on POD 28, and biopsies were performed.

**Thioredoxin Antibody**

C-terminal synthetic oligopeptides of recombinant TRX protein (28-mer) conjugated with bovine serum albumin, along with Freund’s adjuvant, were injected subcutaneously into rabbits. After three immunizations, serum from the rabbits was purified by saturated ammonium sulfate precipitation, followed by application to a bovine serum albumin-sepharose column to remove antitoxin serum albumin components. Finally, the serum was purified with the use of an immobilized TRX column. The specific antibody was dialyzed against phosphate-buffered saline solution and stored frozen at -20°C. By immunohistochemical analysis with the use of this anti-TRX antibody, adult T cell leukemia-2 cells that were determined originally to produce TRX were stained red, although they were not stained with normal rabbit immunoglobulin. The specificity of this antibody was also confirmed by Western blotting with the use of recombinant TRX and purified TRX obtained from condition medium of adult T cell leukemia-2.\(^\text{16,17}\) Western blot analysis of recombinant TRX and dog’s serum showed the same 13KD band (data are not shown).

**Thioredoxin Expression**

Lung tissues were fixed in Bouin’s solution for 4 h and embedded in paraffin. Sections were cut 5 μm thick and set on slides. The slides were dewaxed in toluene and hydrated in graded ethanol solution. Slides of cells and tissues were stained as follows: endogenous peroxidase activity was blocked with H₂O₂ (0.3% in methanol for 6 min); nonspecific reactions were blocked with normal goat serum; the staining procedure was performed with a DAKO LSAB Kit (DAKO; Carpinteria, Cal). The slides were incubated with 0.4 μg/mL anti-TRX antibody for 90 mins at 37°C, or normal rabbit immunoglobulin as a negative control, then incubated with a mixture of avidin–biotin horseradish peroxidase complex, and developed with 3-amino-9-ethylcarbazol. Counterstaining was performed with hematoxylin. Ten colored microphotographs (magnification, x100) were randomly taken of each anti-TRX-stained sample. The TRX high-producer (TRXh) cells in each microphotograph were counted, and the average number of TRXh cells was used as the index of TRX production.

**Histologic Examination**

Serial sections were stained with hematoxylin and eosin, and TRXh cells were observed morphologically. The degree of rejection was classified according to “the grading of acute rejection by International Society for Heart Transplantation.”\(^\text{18}\) The histologic examination and the count of the number of TRXh cells in this study were performed by two pathologists blindly and independently.

**Anti-Human-Lysozyme Antibody Stain**

Based on the morphologic analysis, it appeared that TRXh cells were mainly alveolar macrophages (AMs). Examining the cell type, three sections from three dogs in group A were stained with anti-human-lysozyme antibody (A099; DAKO; Copenhagen, Denmark). Then these sections were cut off the antibody with trypsin buffer and stained again with anti-TRX antibody.

**Dogs With Pneumonia**

Accidentally, we found two dogs with pneumonia which were resistant to chemotheraphy; one of the dogs had never had an operation and the other had left lung transplantation which was well immunosuppressed.

**Statistical Analysis**

All the numeric values are expressed as the mean ± SD. Data were analyzed by Student’s t test with analysis of variance, and a probability value of less than 0.05 was considered to show a statistically significant difference.

**Results**

**Group B (Control Group)**

The number of TRXh cells per field was 1.82±1.01.

**Group A (Allotransplantation)**

The grades of rejection were as follows: grade 0 in 5 dogs; grade 1 in 4 dogs; grade 2 in 5 dogs; grade 3 in 5 dogs; and grade 4 in 5 dogs. The numbers of TRXh cells in allotransplanted lungs in grades 0 through 4 were 1.68±1.14, 4.87±1.07, 10.42±4.24, 27.34±17.96, and 50.90±17.36, respectively (Fig 1). The number of TRXh cells in group A increased as rejection progressed. There was a significant difference between the control group (group B) and each rejection grade (p<0.05). Some phagocytizing cells were stained red in one of the grade 4 lungs from group A (Fig 2).

**Group C (Autotransplantation)**

The number of TRXh cells was 1.66±0.28 on POD 5. There was no significant difference between groups B and C.

**Group D (Immunosuppressed)**

Two of the 8 lungs showed grade 0 rejection, 3 showed grade 1 rejection, and 3 showed grade 4
These findings suggest that normal healthy dogs express no TRXh cells, that operative stress does not affect evaluation of the expression of TRX, and that allogenic stimulation increases the number of TRXh cells in lung tissues. The TRXh cells increased significantly (p<0.01) even in grade 1. This is a critical finding because it is necessary to start treatment in the early stages of rejection (grade 1 or 2) to prevent serious consequences of rejection. Thus, we believe that the evaluation of TRXh cells in transplanted lung tissue obtained during thoracotomy or bronchoscopic lung biopsy may supply useful diagnostic information. In group D, there was no significant difference between group B (control group) and grade 1 rejection. We assume that in group D there was relatively mild grade 1 rejection because the animals were immunosuppressed. So there was a significant difference between group B (control group) and grade 1 rejection in group A; however, there was no significant difference between group B (control group) and grade 1 rejection in group D.

We thought that morphologically some of the TRXh cells appeared to be AMs and thus we used immuno-
histrochemical staining to confirm this suspicion. Some phagocytizing AMs were stained red with anti-TRX antibody and anti-lysozyme antibody. These results suggest that some of the TRXh cells may be AMs. We tried another staining technique to confirm the cell type, using anti-human macrophage antibody (KP-1; DAKO; Tokyo, Japan). However none of the cells were stained with this antibody. This may be because canine macrophage has no cross activity with this antibody.

The TRX expression can be induced by a variety of stresses. The mechanism by which activated AMs express TRX is not obvious, but there is much evidence to suggest a requirement for diithiol-related reducing conditions for the in vitro proliferation of lymphoid cells. We have shown in gel retardation assays that recombiant TRX markedly enhances the binding of NF-κB to the target sequence in a chain promoter. So TRX may play some role in the process of activating NF-κB. We hypothesize that a similar phenomenon occurs in the process of antigen presentation in AMs under the allogenic stimulation.

Of particular importance are studies to determine whether measurement of TRXh cells can distinguish between rejection and infection, the usual problem facing clinicians. By chance, we had two dogs with pneumonia which were resistant to chemotherapy. Although the number of animals was small, we observed very few TRXh cells. Whether measurement of TRXh cells can distinguish between rejection and infection is unclear, and thus further clinical studies are required.

ACKNOWLEDGMENTS: The authors thank Drs. Yasuhiro Yokomise and Kenji Imai for assistance and advice with this study. They are also grateful to Naomi Teramoto for technical expertise.

REFERENCES