0041-1337/03/7507-1/0
TRANSPLANTATION
Vol. 75, No. 7, April 15, 2003

INCREASED EXPRESSION OF ENDOARTERIAL VASCULAR CELL ADHESION MOLECULE-1 mRNA IN AN EXPERIMENTAL MODEL OF LUNG TRANSPLANT REJECTION: DIAGNOSIS BY PULMONARY ARTERIAL BIOPSY

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Background. **Early detection of rejection after lung transplantation may prevent allograft failure. This study determines if mRNA from the cell adhesion molecules intercellular adhesion molecule-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin in pulmonary endovascular tissue samples could be markers of early rejection.**

Methods. **Single left lung transplants were performed in five dogs. Each dog was treated for 2 weeks with immunosuppression, after which rejection was allowed to occur. Percutaneous biopsies from 2- to 3-mm distal branch pulmonary arteries were obtained in each dog from the normal and the transplanted lungs at the end of immunosuppression therapy and periodically (2–4 times) for 1 to 3 weeks until euthanasia. Levels of cell adhesion molecule mRNA in the biopsy samples were quantitated by reverse-transcriptase polymerase chain reaction and normalized to β-actin mRNA levels.**

Results. **Between three and five pulmonary endoarterial biopsy samples were obtained from each lung at each catheterization procedure. There was a significant increase in VCAM-1 mRNA levels in the biopsies of the transplanted lungs (which were undergoing rejection) compared with the native right lungs in all dogs. Progressive increases in VCAM-1 mRNA were observed with longer rejection times. VCAM-1 mRNA changes were detected earlier than histologic changes of rejection.**

Conclusions. **In pulmonary endoarterial biopsy samples obtained in a canine lung transplant model, there was a progressive increase in VCAM-1 mRNA levels with increasing rejection. Changes in VCAM-1 mRNA were observed earlier than histologic changes of rejection. VCAM-1 quantitation by endoarterial biopsy may be useful in surveillance and early diagnosis of rejection in patients who undergo lung transplantation.**

In the first 6 months after lung transplantation, most recipients have at least one episode of acute rejection, and approximately 25% of long-term survivors develop chronic rejection (*1*). Rejection needs to be differentiated from infection because the therapy is different and erroneous treatment may result in a fatal outcome. Currently available methods to diagnose acute rejection, including transbronchial biopsy, have been associated with variable institutional results, suboptimal diagnostic yield, low specificity, and important complications (*2–4*). Moreover, the target sample of biopsy is tissue that interacts primarily with antigens in the air rather than with the host's immune system.

Pulmonary vascular changes have long been known to be associated with rejection (*5*). In theory, sampling of the endovascular component of the allograft, the site where foreign tissue is first encountered by the body's circulating immune cells, should demonstrate early changes associated with rejection. In addition, the endovascular layers may be affected more by rejection than by infection. We have previously described a novel endoarterial biopsy catheter that can percutaneously sample pulmonary arteries measuring approximately 2 to 3 mm in diameter (*6*, *7*).

Cell surface adhesion molecules belonging to the selectin, immunoglobulin, and integrin families have been associated with episodes of allograft rejection (*8*). Vascular cell adhesion molecule (VCAM)-1 gene expression has been shown to increase in endomyocardial biopsy samples of patients with cardiac allograft rejection (*9–11*), renal arterial smooth muscle cells of patients with kidney allograft rejection (*12*), hepatic vascular and sinusoid endothelium of patients with liver allograft rejection (*13*), and animal models of cardiac transplant rejection (*14*, *15*).

We tested the feasibility of percutaneously obtaining pulmonary endovascular samples in a canine lung transplant model and assessed whether intercellular adhesion molecule (ICAM)-1, VCAM-1, or E-selectin mRNA levels in these biopsy samples could serve as markers of rejection.

MATERIALS AND METHODS

Dogs

By the use of standard surgical techniques and general anesthesia with isoflurane, we performed left single lung transplants in five mongrel dogs, leaving their native right lungs in situ. Postoperatively, the dogs were treated for 2 weeks with an immunosuppressive regimen of cyclosporine (10 mg/kg twice per day), azathioprine (2 mg/kg/day), and prednisone (1 mg/kg/day). At the end of 2 weeks, the immunosuppressive medications were stopped, and the dogs were allowed to reject the transplanted lungs. All animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health.

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Received 20 September 2002. Revision requested 20 October 2002. Accepted 11 December 2002.
DOI: 10.1097/01.TP.0000057530.32397.6D

Biopsies

Beginning with the last day of immunosuppressive medication (day 0), biopsies were performed every 2 to 3 days through a jugular vein using an endoarterial biopsy catheter. Under general anesthesia with isoflurane, biopsy samples were obtained from 2- to 3-mm distal pulmonary arteries of both lungs of each dog (Figs. 1 and 2). Samples were subsequently either frozen in tissue biopsy freezing solution at −70°C or placed in formaldehyde for histologic examination.

Isolation of RNA and Reverse-Transcriptase Polymerase Chain Reaction

Biopsy tissue samples were thawed from storage and homogenized by a STAT-60 mRNA isolation protocol (Tel-Test "B", Inc., Friendswood, TX). The resultant mRNA pellet was suspended in 12 μL diethylpyrocarbonate-treated water and used in the reverse-transcriptase (RT) procedure. The cDNA sample was split for use as ICAM-1, VCAM-1, or E-selectin and β-actin templates. β-actin is constitutively expressed in smooth muscle cells; therefore, it provided an internal comparison standard for ICAM-1, VCAM-1, and E-selectin.

The following forward and reverse primers, respectively, were used: (1) ICAM-1: 5'-ATCATACTCCGAGGAGGCTCTGTG-3' and 5'-ATTCTCGTCTCCCAGTGTCACCTC-3'. (2) VCAM-1: 5'-CTCTTGGAGAACCCAGATAGACAGTC-3' and 5'-ATGTTCCAGAATCTTCCAGCCTCATAGCAATTA-3'. (3) E-selectin: 5'- CTTCTCCTATTTAAAGAGGGTGGAGC-3' and 5'- CCTTCTTCACACTCAAAGGTACATG-3'. (4) β-actin: 5'-TCATGAAGTGTGACGTTGACATCCGT-3' and 5'-TCTAGAAGCATTTGCGATGGACGATG-3'.

The polymerase chain reactions (PCRs) on the samples were run for 38 cycles for ICAM-1, VCAM-1, and E-selectin and 35 cycles for β-actin, with aliquots removed such that samples were in the exponentially increasing stages of PCR before reaching their respective plateaus. The products of PCR were run on 2% agarose gel electrophoresis with ethidium bromide stain. Bands were visualized under ultraviolet light, photographed with a Kodak digital camera (Kodak, Rochester, New York), imported into Kodak's Digital Science software, and quantitated. A ratio of cell adhesion molecule to β-actin was calculated for each sample and plotted.

Gross Pathology

The dogs were euthanized on the development of significant clinical signs of rejection, including severe listlessness, respiratory distress, cough, fever, and no appetite. At necropsy, the main and branch pulmonary arteries were dissected free to the distal lung, and the opened biopsied and non-biopsied vessels in the normal and transplanted lung arteries were examined. Sections from the arteries and lung parenchyma were prepared for histologic examination or frozen and stored.

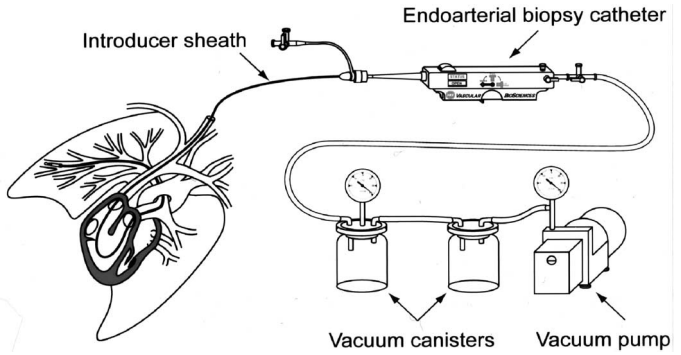

FIGURE 1. Endoarterial biopsy approach. The biopsy catheter is connected externally to a vacuum pump placed distally to two suction canisters in series. The biopsy catheter is advanced through a long 8F introducer sheath, through the right heart, into a distal pulmonary artery branch.

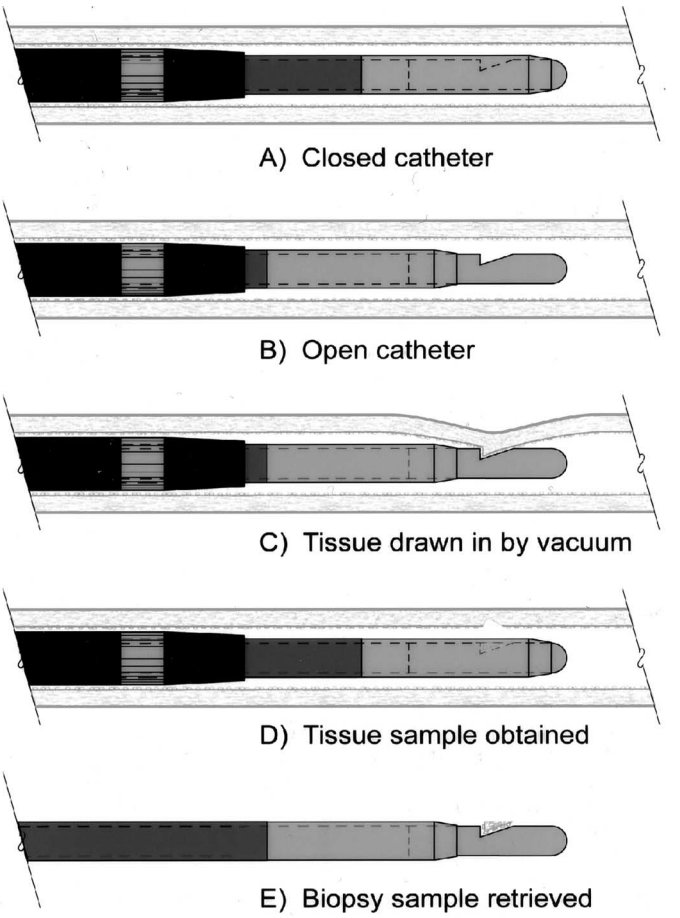

FIGURE 2. Schematic sequence of the biopsy sampling procedure. The biopsy catheter consists of two flexible polymeric tubes that slide with respect to each other. The inner tube has a stainless steel distal end with a beveled opening that accommodates arterial tissue. The outer tube terminates in a stainless steel cutting tube. (A) The catheter is placed in a distal pulmonary artery branch with the outer tube covering the inner tube end. (B) The outer tube is pulled back, exposing the beveled opening on the inner tube. (C) Endoarterial tissue is drawn into the beveled opening by the vacuum. (D) The outer tube is advanced over the inner tube, severing the tissue sample. (E) After the catheter is removed from the body, the outer tube is retracted back, exposing the retrieved tissue sample.

Histology

Endoarterial samples for histology were formalin fixed, routinely processed, and embedded in paraffin, sectioned, stained with hematoxylin-eosin, and mounted on slides. The technicians and pathologist were blinded as to the side from which the samples were taken and the timing of the samples after cessation of immunosuppression.

Statistics

Unpaired *t* tests were used to assess the statistical significance of the cell adhesion molecule to β-actin ratios in both left and right lungs and in the left lung with increasing time of rejection.

RESULTS

Biopsy Procedure

Between three and five pulmonary endoarterial biopsy samples from each lung were obtained at each catheterization procedure. The dogs remained hemodynamically stable during the biopsy procedures and recovered easily from the general anesthesia. There were no significant complications.

Biopsy Samples

The samples were generally adequate in size and quality for both histologic examination and assessment of mRNA levels. In only 2 of 19 biopsy procedures, the biopsy samples did not provide detectable cell adhesion molecule or β-actin mRNA levels. On histology, the biopsy samples from these two procedures were composed largely of thrombus, with variable amounts of endoarterial tissue. These two sets of biopsy samples were obtained late in the rejection period. At necropsy, the pulmonary vascular tree showed variable amounts of intraluminal thrombus, including vessels that had not been subjected to previous biopsy.

The remainder of the biopsy samples were composed predominantly of smooth muscle cells, with a thin layer of endothelial cells, consistent with intima and a portion of arterial media (Fig. 3). Early rejection samples demonstrated normal medial architecture and mild endothelial cell swelling (Fig. 4). Late rejection samples showed transmedial infiltration of lymphocytes and more significant endothelial cell damage (Fig. 5). Histologic rejection of the transplanted lung, including vasculitis in arteries accessible to biopsy, was confirmed at necropsy (Fig. 6). There was no evidence of infection (inclusion bodies, collections of neutrophils, or consolidation) on examination of biopsy samples or at necropsy.

Intercellular Adhesion Molecule-1 mRNA

There was no significant change in the level of ICAM-1 mRNA between the left and right lung biopsy samples. In addition, there was no increase in ICAM-1 mRNA levels with longer rejection times.

E-selectin mRNA

No significant E-selectin mRNA signal was detected by RT-PCR in any of the biopsy samples tested.

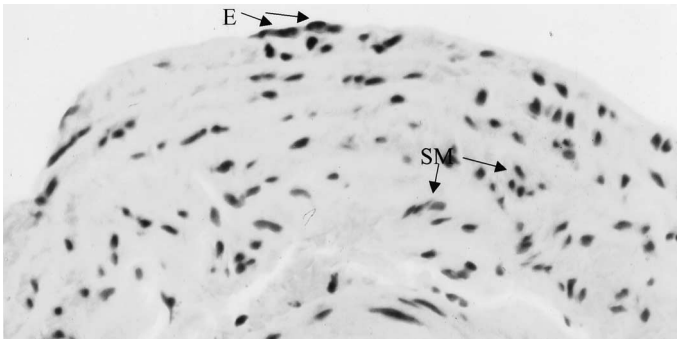

FIGURE 3. Typical normal endoarterial specimen including a flat endothelial cell layer (E) and underlying media (smooth muscle, SM). Smooth muscle nuclei appear spindle shaped in longitudinal section or round on cross section.

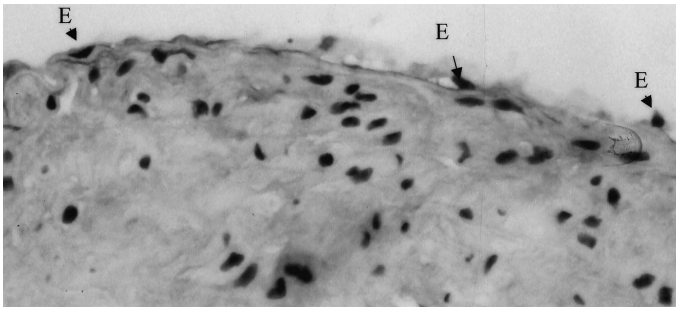

FIGURE 4. Endoarterial biopsy during "early rejection" showing minimal reactive endothelial cell (E) changes, with rounded, plump nuclei.

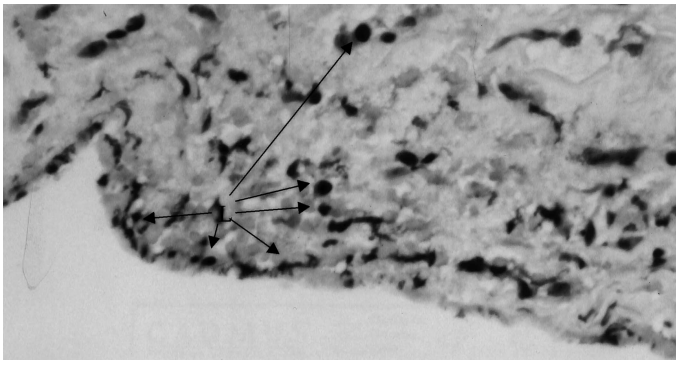

FIGURE 5. Subendothelial and medial inflammation consistent with "late rejection." Numerous lymphocytes (L) are present immediately under the endothelium and within the smooth muscle layer.

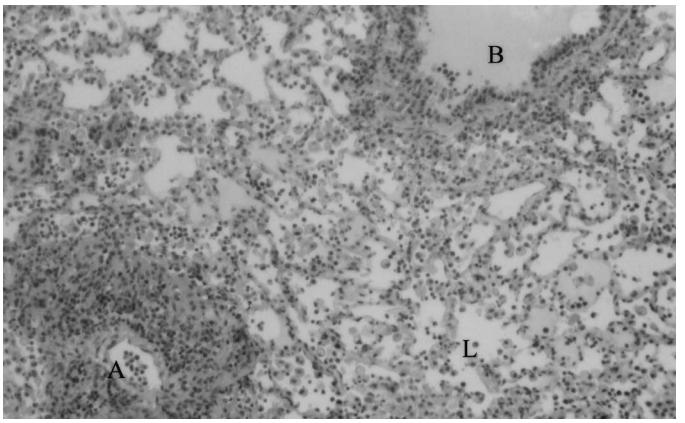

FIGURE 6. Section of transplanted lung showing typical changes of acute rejection including perivascular and subendothelial mononuclear infiltrates and peribronchial lymphocytic inflammation and bronchitis. Small artery (A), bronchus (B), and lung parenchyma (L).

Vascular Cell Adhesion Molecule-1 mRNA

On the last day of immunosuppression therapy, the biopsy samples from both the transplanted and normal lung in each dog contained no detectable levels or low levels of VCAM-1 mRNA. The ratio of VCAM-1 to β-actin mRNA in the transplanted lung ranged from 0 to 0.11 with a mean of 0.05 ± 0.04.

On the last biopsy day, which was the same day the dogs were euthanized, the ratio of VCAM-1 to β actin mRNA in the transplanted lung ranged from 0.36 to 1.89 with a mean of 0.94 ± 0.60 ($P < 0.05$ compared with the last day of immunosuppression).

In each dog, there was usually an increase in VCAM-1 mRNA levels in the biopsies of the transplanted lung with increasing days off immunosuppressive therapy (Figs. 7 and 8). However, the rate of increase and the final VCAM-1 levels were variable from animal to animal.

On the last day of immunosuppression therapy, the native lung showed no detectable or trace levels of VCAM-1 mRNA. On the last biopsy day, which was the time of most rejection, low levels of VCAM-1 mRNA were present in all native lung biopsies. The ratio of VCAM-1 to β-actin mRNA was always higher in the transplanted (0.40 ± 0.58) compared with the native (0.07 ± 0.13) lung biopsies of each dog.

DISCUSSION

Rejection, infection, and bronchiolitis obliterans continue to limit the survival of patients who undergo pulmonary transplantation (*16*). The methods for surveillance and diagnosis of these lung transplant complications vary among different transplant institutions. Although some centers perform surveillance transbronchial pulmonary biopsies with the aid of bronchoalveolar lavage, others perform only diagnostic pulmonary biopsies, and still others perform no invasive diagnostic procedures but treat on the basis of clinical, radiographic, and hematologic information. Among the biopsy routes, the most commonly used is transbronchial, with endobronchial and open lung procedures also described (*17*, *18*). Although transbronchial biopsy has been considered the gold standard, the technique has been limited by variable diagnostic yields and complications.

We have previously reported the results of the use of a novel endoarterial biopsy catheter, which is capable of obtaining pulmonary endoarterial samples from 2- to 3-mm vessels. In a canine model of normal and elevated pulmonary arterial pressures, the biopsy procurement success rate was 83% to 93%, with a low complication rate (*6*, *7*).

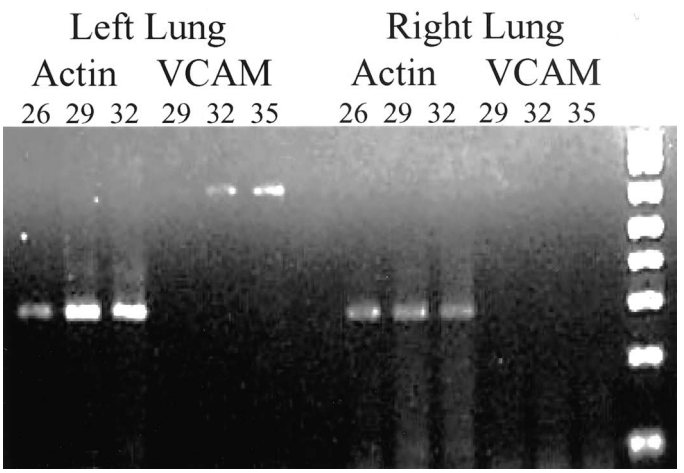

FIGURE 7. Representative quantification of vascular cell adhesion molecule (VCAM)-1 and β-actin mRNA levels by reverse-transcriptase polymerase chain reaction (RT-PCR) in endoarterial biopsy samples from an early rejection time point. Aliquots were sequentially removed at 26, 29, and 32 PCR cycles for β-actin and 29, 32, and 35 PCR cycles for VCAM-1. Left lung=transplanted, right lung=native, Actin=β-actin, VCAM=VCAM-1.

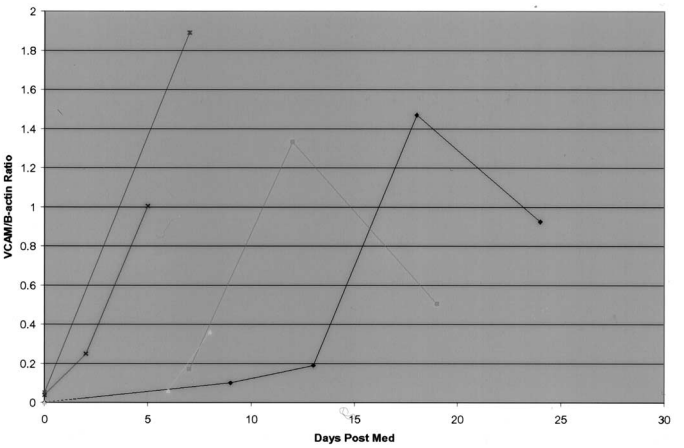

FIGURE 8. Ratio of VCAM-1 to β-actin mRNA in endoarterial biopsy samples from the left lung. Days postmed=days after the immunosuppressive medications were discontinued. Each line represents a separate dog.

This study determined whether pulmonary endoarterial samples could be obtained effectively and safely in a canine pulmonary transplant model and tested whether ICAM, VCAM-1, or E-selectin mRNA levels in pulmonary endoarterial biopsies could serve as markers of pulmonary rejection.

We showed that endoarterial biopsies could be procured effectively and safely in this experimental model. Biopsy samples of adequate size and number were generally obtained from the normal and transplanted lung in each dog. Samples from early rejection revealed either normal histology or minimal endothelial reactive changes. In contrast, biopsy samples obtained in the last or next to last biopsy day showed transmural migration of white cells and more destructive cellular and architectural changes.

Allograft rejection was confirmed by postmortem histologic examination. There was extensive lymphocytic infiltration, including vasculitis (in arteries of a size amenable to endoarterial biopsy) and destruction of pulmonary parenchymal architecture. However, because the early rejection biopsy samples did not show significant histologic changes, we examined whether the levels of ICAM, VCAM-1, or E-selectin mRNAs could serve as markers of rejection. In this study, there was no change in ICAM-1 or E-selectin mRNA levels with rejection. In contrast, VCAM-1 mRNA levels did increase early with rejection.

Several reports have shown an association between VCAM-1 mRNA levels and cardiac, renal, and hepatic rejection (*19–23*). In endomyocardial biopsy samples from cardiac transplant patients, VCAM-1 expression decreased in postrejection biopsies from patients who had been adequately treated (*21*). After cardiac and renal transplantation, persistent elevation of VCAM-1 levels after treatment of a rejection episode was predictive of recurrent rejection and poor clinical outcome (*22*, *24*). Furthermore, antibodies directed against VCAM-1 have been demonstrated to prolong graft survival (*25*).

ICAM-1 and VCAM-1 levels have been measured in circulating blood and tissues of animals and humans after organ transplantation. In most instances, tissue levels showed better correlation with rejection than blood levels of these cell adhesion molecules (*26*, *27*). These data lend support to the method of obtaining pulmonary endoarterial samples, as described in this study, rather than measuring circulating levels of adhesion molecules for the diagnosis of lung transplant rejection.

Although the studies mentioned previously show a positive relationship between VCAM-1 levels and rejection after cardiac, renal, and hepatic transplantation, other studies have shown that VCAM-1 mRNA may also increase with ischemia-reperfusion, nonspecific inflammation, and allograft infection (*26*, *28*). In addition, in an ovine renal transplantation model, anti-VCAM-1 antibodies did not prolong graft survival (*29*). Therefore, the value of VCAM-1 mRNA levels in differentiating rejection from infection and in predicting graft survival remains to be explored.

There are limited data in the literature specifically addressing the utility of VCAM-1 mRNA levels and pulmonary rejection. In one study, circulating ICAM-1 and VCAM-1 levels were not helpful in differentiating infection from rejection in human lung transplant recipients (*30*). However, there are no published studies addressing the relationship of pulmonary vascular tissue VCAM-1 levels and rejection after lung transplantation.

In this canine study of transplant rejection, we showed that VCAM-1 mRNA levels obtained from pulmonary endoarterial biopsy samples were elevated early in acute rejection. Moreover, there was a progressive increase in VCAM-1 mRNA levels with longer rejection times. In endoarterial biopsies obtained on the last day of immunosuppressive therapy, there was minimal or no VCAM-1 mRNA detected. This indicates that the surgical procedure itself, lung preservation, and the immunosuppressive drugs themselves did not cause persistent elevation of VCAM-1 mRNA levels. In general, each dog showed an increase in VCAM-1 mRNA levels with longer rejection times. However, the rate of increase was variable among the dogs. This may be the result of variable degrees of antigenic incompatibility between the different pairs of dogs that underwent transplantation, variable rates of rejection in each individual dog, and varying time periods of the biopsy procedures. In this study, three of four biopsy samples from the last day of immunosuppressive therapy showed a VCAM-1 to β-actin mRNA ratio of less than 0.05. In contrast, 20 of 21 biopsy samples obtained during rejection (both "early" and "later") showed a VCAM-1 to β-actin mRNA ratio of greater than 0.05 ($P<0.05$).

In right lung biopsy samples, VCAM-1 mRNA was absent or detectable in only trace amounts on the last day of immunosuppressive medications and on early rejection days. However, in right lung biopsy samples obtained during "later rejection," low levels of VCAM mRNA were present. This finding is likely the result of circulating cytokines produced by the severely rejecting allograft, which can then affect other organs (including the native right lung). Nonetheless, at all stages of rejection, VCAM/β-actin mRNA ratios were higher in the left compared with the right lung biopsy samples.

There are several limitations to this study. Although we used a typical immunosuppressive regimen, discontinuing all the drugs simultaneously does not occur clinically. However, a much slower wean of immunosuppressive drugs would be tedious, expensive, and still not necessarily comparable to the experience in patients. Another limitation of this study was the small number of dogs studied (i.e., 5). The degree of immunologic compatibility may not have been representative of a larger group of dogs or humans. Also, although VCAM-1 seems to be a marker of rejection in dogs, it is not known if similar changes occur in humans or at what stages of rejection. In addition, some studies have shown increases in VCAM-1 levels with infection after transplantation, and the current study did not systematically rule out fungal, bacterial, or viral infections or directly compare rejection with infection effects on VCAM-1 mRNA levels. However, there was no histologic or radiographic evidence of infection (consolidation or infiltrates on chest roentgenograms obtained at the beginning of each biopsy procedure). Finally, although the endoarterial biopsy procedure was safe and effective in dogs, the procedure has not yet been evaluated in humans. However, extensive experimental experience suggests that the procedure should have similar results in human patients. Even if VCAM-1 is not a marker of rejection in patients, the biopsy samples may provide tissue to study other potential markers of rejection, infection, the development of bronchiolitis obliterans, and graft failure.

CONCLUSION

Endoarterial biopsy samples can be safely and effectively obtained from branch pulmonary arteries of dogs that have undergone lung transplantation. VCAM-1 mRNA seems to be a marker of rejection and is detectable earlier than histologic changes associated with rejection. In patients who undergo lung transplantation, the endoarterial biopsy method, perhaps in combination with transbronchial biopsy or bronchoalveolar lavage, may be useful in the surveillance and diagnosis of rejection and infection. Moreover, endoarterial biopsy may aid in detecting early vascular changes associated with bronchiolitis obliterans. The ultimate aim is to improve the survival and quality of life in patients who have undergone lung transplantation.

Acknowledgments. The authors thank Jifeng Zhang, Ron G. Konopka, Peter G Chiles, and Craig A. Pedersen for their technical assistance.

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