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MECHANISMS OF POST-TRANSPLANT OBLITERATIVE BRONCHIOLITIS

STUDY IN A PORCINE HETEROTOPIC BRONCHIAL TRANSPLANTATION MODEL

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Academic dissertation

To be publicly discussed with the permission of the Medical Faculty of the Helsinki University in Lecture Hall 3, Meilahti Hospital, on May 27th, 2005, at 12 noon.

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals and on unpublished data presented in the results.


## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>Aza</td>
<td>azathioprine</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporine A</td>
</tr>
<tr>
<td>CTGF</td>
<td>connective tissue growth factor</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin-eosin</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MP</td>
<td>methyl prednisolone</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>OAD</td>
<td>obliterative airway disease</td>
</tr>
<tr>
<td>OB</td>
<td>obliterative bronchiolitis</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PDGFR</td>
<td>platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>RAD</td>
<td>rapamycin-derivative (everolimus)</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling</td>
</tr>
</tbody>
</table>
ABSTRACT

Although lung transplantation has become a therapeutic option in treating end-stage lung disease, its long-term results in lung transplantation are still modest. The most threatening complication after lung transplantation is obliterative bronchiolitis (OB). Histologically, OB manifests as epithelial injury, inflammation, fibrosis, and finally obliteration of the small airways. The exact pathogenesis of OB is still in part unknown, but it is primarily considered a manifestation of chronic allograft rejection. The purpose of this thesis was to further elucidate the mechanisms of OB in a heterotopic porcine bronchial transplantation model. The focus was on the occurrence of apoptotic cell death in respiratory epithelial injury (I), on the role of tumor necrosis factor (TNF-α) (II), of platelet-derived growth factor (PDGF), transforming growth factor (TGF-β), and connective tissue growth factor (CTGF) (III) in graft inflammation and fibrosis, and of the expression of collagen types I and III (IV).

In a heterotopic porcine bronchial transplantation model, small peripheral bronchi were implanted subcutaneously. Bronchial autografts showed preservation of normal histological structures, whereas in nonimmunosuppressed allografts, respiratory epithelial damage and total bronchial obliteration occurred within three weeks (I-IV). These histopathological changes were similar to those observed in human OB.

The apoptotic rate of graft epithelial cells was analyzed by labeling fragmented DNA (TUNEL-staining) (I). In nontreated allografts and in allografts with inadequate immunosuppression, the apoptotic indexes of the epithelium were high (>1% of the cells apoptotic), and increased with progressing epithelial damage preceding bronchial obliteration. In allografts receiving effective immunosuppression to prevent alloimmune activation, the rate of epithelial apoptosis was similar to that observed in control autografts (<1% of the cells apoptotic). These results offer further evidence that the apoptotic death of epithelial cells in OB is an important mechanism in events leading to graft deterioration.

Immunohistochemical stainings revealed TNF-α expression in epithelial and inflammatory cells, in endothelium, and in fibroblasts (II). TNF-α expression was significantly up-regulated in the allografts in comparison to that in autografts. TNF-α inhibition with infliximab, an anti-TNF-α antibody, reduced inflammation, rate of epithelial loss, fibrosis, and obliteration early in OB development. It reduced the numbers of TNF-α-positive epithelial and inflammatory cells.
cells and invasion of CD8+ lymphocytes during the first week. This study further confirms that TNF-α plays a role in the development of OB by promoting inflammation and epithelial injury, and suggests that in lung transplant recipients, inhibition of TNF-α may prove beneficial.

PDGF, TGF-β, and CTGF protein expression was analyzed by immunohistochemistry (III). Up-regulation of PDGF-A, PDGF receptor α and β, and TGF-β expression occurred in bronchial allografts, whereas in autografts and in allografts PDGF-B and CTGF expression was similar. This study confirms PDGF and TGF-β as mediators of OB. The protein tyrosine kinase inhibitor, imatinib, targeting PDGF receptors, reduced recruitment of inflammatory cells and modified expression patterns of PDGF-A and PDGF receptors. This indicates the importance of the pathways signaled through PDGF receptors in OB development.

The temporal and spatial activation of type I and III collagen genes was detected by in situ hybridization. In nontreated allografts obliterating within 3 weeks, a preponderance of fibroblasts showing positivity for collagen III mRNA existed in the obliterative plug and bronchial wall. Predominance of fibroblasts expressing collagen III mRNA increased in parallel with progressive bronchial wall fibrosis and luminal obliteration. In autografts and in allografts with immunosuppression adequate to prevent OB, only a minor degree of collagen gene activation occurred without changes in the genetic activity between type I and III collagen. These findings suggest a positive association between OB development and type III collagen mRNA expression in fibroblasts.

Findings presented in this thesis provide further understanding of the mechanisms of OB and offer insights into future development of treatment options.
INTRODUCTION

Lung transplantation has become a therapeutic option in treating end-stage lung diseases. The first human lung transplantation was reported in 1963 by Hardy et al. Ever since, survival rates after lung transplantation have continually improved due to the development of surgical and organ-preservation techniques, immunosuppressive regimens, and rejection diagnosis. However, the long-term results are still relatively modest; the survival rate 5 years after transplantation is 50% (Trulock et al. 2004).

In comparison to other organ transplants, lung transplants are especially susceptible to acute and chronic rejection and infection. This feature is a result of the immunologic activity of the lung tissue and of its constant exposure to inhaled pathogens. The most life-threatening complication after lung transplantation is chronic rejection manifesting as obliterative bronchiolitis (OB) (Milne et al. 1992). Clinically, this is called bronchiolitis obliterans syndrome (BOS), presenting as airflow obstruction and decrease in lung function capacity (Boehler et al. 2003). Its diagnosis is based mainly on exclusion of other possible causes for allograft deterioration. Histologically, in OB, inflammation of the small airways occurs, leading to epithelial damage and occlusion of the airway lumen (Tazelaar et al. 1994). To date, no effective treatment option is available.

The exact pathogenesis of OB is still in part unknown, but the respiratory epithelium is considered to be a potential target for immunologic effector mechanisms (Mauck et al. 1996). Progressive damage and loss of airway epithelium is accompanied by increased production of cytokines and growth factors that augment the inflammatory and fibroproliferative events (Hertz et al. 1992, Boehler et al. 1999, El-Gamel et al. 1999, Kallio et al. 1999, Neuringer et al. 2000).

The aim of this study was to further explore the mechanisms leading to post-transplant obliterative bronchiolitis in a porcine heterotopic bronchial transplantation model.
1. Clinical lung transplantation

After the first human lung transplantation by Hardy’s team in 1963, lung transplantation has become a viable treatment option for patients with advanced and end-stage lung disease. Single-lung, double-lung, and heart-lung transplantations are performed on both adult and pediatric patients. Mainly for children, an option is living-donor lobar lung transplantation. According to the registry of the International Society for Heart and Lung Transplantation (ISHLT), approximately 1600 adult and 60 pediatric lung transplantations are performed each year worldwide (Boucek et al. 2004, Trulock et al. 2004). In Finland, 10 to 15 adult lung transplantations are performed annually. Advances in surgical techniques, organ preservation, and in rejection diagnosis and treatment, together with improved pre- and postoperative care, have contributed considerably to better survival and quality of life (DeMeo et al. 2001a). However, that lung allografts are particularly susceptible to infection and acute and chronic rejection continues to limit the long-term success of lung transplantation.

1.1 Indications

The conditions for which lung transplantation is considered a treatment option fall mainly into the following categories: septic lung disease (cystic fibrosis, bronchiectasis), restrictive lung disease (idiopathic pulmonary fibrosis, other interstitial lung diseases), obstructive lung disease (chronic obstructive lung disease, alpha-1-antitrypsin deficiency emphysema), and pulmonary vascular disease (primary or secondary pulmonary hypertension) (DeMeo et al. 2001a). Globally, the four main indications are for chronic obstructive lung disease, 39.0%, idiopathic pulmonary fibrosis, 17.0%, cystic fibrosis, 16.0%, and alpha-1-antitrypsin deficiency emphysema 9.0% (Trulock et al. 2004). In Finland, the indications are mainly the same, with the exception of cystic fibrosis, which in the Finnish population is rare (Salmela et al 2004). For obstructive and restrictive lung diseases, single-lung transplantation is the commonest treatment option; for septic diseases and pulmonary vascular diseases, double-lung transplantation is almost exclusively performed. If cardiac failure co-exists, the treatment of choice is heart-lung transplantation (DeMeo et al. 2001b). For transplantation, the disease process should be symptomatic and progressive despite optimal medical or surgical treatment. In patients with end-stage lung disease, the relative contraindications for lung transplantation have changed along with improvement in management. The absolute contraindications are
concurrent smoking, alcohol or drug abuse, bone-marrow failure, hepatic cirrhosis, active malignancy, and HIV infection (DeMeo et al. 2001b).

1.2 Outcome

Survival rate after lung transplantation according to international registers at the 1-year, 3-year, and 5-year points are 74%, 58%, and 47% (Trulock et al. 2004). In Finland, this rate is 69% at 1 year and 46% at 5 years (Salmela et al. 2004). Survival is threatened by complications of the lung transplantation procedure itself, infection, and acute, and chronic rejection, as well as complications from the immunosuppressive agents. Early complications occurring within hours to a few weeks after lung transplantation are: direct complications of surgery, airway anastomosis dehiscence and stenosis, and ischemia-reperfusion injury followed by acute graft dysfunction. Airway stenosis may also occur as a late complication. The rate of bacterial, viral, and fungal infections in lung transplant patients in the first two months is high and continues to be high compared to rates for other solid-organ recipients. Immunosuppressive treatments also leave patients prone to infection and malignancy.

1.3 Immunosuppression

As in other solid organ transplantation, lung transplant patients need life-long immunosuppressive therapy. Therapy should be individualized to effectively prevent allograft rejection, but not to subject the patient to a high risk for opportunistic infection and malignancy. The most common immunosuppressive drugs and their actions are presented in Table 1.
Table 1. Common immunosuppressive agents in lung transplantation

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Induction</strong></td>
<td></td>
</tr>
<tr>
<td>Anti-thymocyte globulins</td>
<td>Reduces number of effector T cells, and blocks their function.</td>
</tr>
<tr>
<td>IL-2 receptor antagonists (daclizumab, basiliximab)</td>
<td>Target IL-2 receptors on T cells and block their IL-2-dependent activation.</td>
</tr>
<tr>
<td><strong>Maintenance</strong></td>
<td></td>
</tr>
<tr>
<td><em>Calcineurin inhibitors</em></td>
<td></td>
</tr>
<tr>
<td>Cyclosporine A</td>
<td>Specific inhibitor of T cell activation: inhibits IL-2 synthesis via calcineurin inhibition.</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>Specific inhibitor of T cell activation: inhibits IL-2 synthesis via calcineurin inhibition.</td>
</tr>
<tr>
<td><em>Purine synthesis inhibitors</em></td>
<td></td>
</tr>
<tr>
<td>Azathioprine</td>
<td>Non-specific inhibitor of proliferating cells, acts by alkylating DNA-precursors.</td>
</tr>
<tr>
<td>Mycophenolate mofetil</td>
<td>Inhibits “de novo” purine synthesis; specifically inhibits proliferation and responses of T and B cells.</td>
</tr>
<tr>
<td><em>Corticosteroids</em></td>
<td></td>
</tr>
<tr>
<td>Prednisone, prednisolone, methyl</td>
<td>Multiple anti-inflammatory and immunomodulatory effects, inhibiting arachidonic acid cascade, interfering with macrophage function, and inhibiting cytokine transcription and adhesion molecule expression.</td>
</tr>
<tr>
<td>prednisolone</td>
<td></td>
</tr>
<tr>
<td><em>Proliferation signal inhibitors</em></td>
<td></td>
</tr>
<tr>
<td>Sirolimus (rapamycin)</td>
<td>Inhibits proliferative response of T cells to IL-2 and other cytokines and smooth muscle cell (SMC) proliferation.</td>
</tr>
<tr>
<td>Everolimus (rapamycin-derivative)</td>
<td>Inhibits growth factor-stimulated cell-cycle progression of hematopoietic and vascular smooth muscle cells via mammalian target of rapamycin (mTOR) inhibition.</td>
</tr>
</tbody>
</table>

Preference for induction therapy varies by lung transplant center, and currently is shifting from use of anti-thymocyte/lymphocyte globulins to interleukin-2 (IL-2)-receptor antagonists (Brock et al. 2001, Trulock et al. 2003). General use of induction therapy is diminishing (Trulock et al. 2003). Maintenance immunosuppressive therapy, started intraoperatively, includes at the majority of centers a calcineurin inhibitor, purine synthesis antagonist, and corticosteroid. The classical triple-therapy combination is cyclosporine (CsA), azathioprine (Aza), and methyl prednisolone (MP). Acute rejection usually responds to high-dose steroid
treatment or administration of poly- or monoclonal antibody preparations, or to alteration in maintenance therapy.

Switching from CsA to tacrolimus and especially from Aza to mycophenolate mofetil (MMF) diminishes acute rejection episodes and reverses refractory rejections, and currently tacrolimus and MMF are recommended for primary maintenance treatment (Treede et al. 2001a-b, Izbicki et al. 2002, Vitulo et al. 2002). A common side-effect of calcineurin inhibitors is renal impairment, and for these patients a potential alternative drug is sirolimus (Snell et al. 2002, Ussetti et al. 2003, Venuta et al. 2004); an increasing number of centers include sirolimus in their maintenance regimens (Trulock et al. 2004). However, combined with calcineurin inhibitors, sirolimus may promote their nephrotoxic effects, a fact which has to be taken into account in using these agents simultaneously.

2. Lung allograft rejection

Rejection is defined as an immune response that mediates injury and destruction of transplanted tissue (Colvin 1990). In clinical transplantation, rejection remains the most common cause of graft dysfunction and loss (De Vito Dabbs et al. 2000). Lung allograft rejection is divided into three categories: hyperacute, acute, and chronic.

Hyperacute lung allograft rejection develops rapidly, within minutes to hours of engraftment, and is caused by pre-formed antibodies against donor major histocompatibility complex (MHC) or ABO blood groups (Frost et al. 1996). This complement-mediated injury to graft endothelium activates inflammatory and coagulative cascades, thus causing extensive thrombosis of graft vessels (Baldwin et al. 1995). Hyperacute rejection is fatal, but fortunately very rare. Acute rejection usually occurs within the first months after transplantation, although late episodes are frequent (Chakinala et al. 2003). The majority of lung transplant patients have at least one acute rejection episode. Chronic rejection occurs in half of lung transplant patients within 5 years (Estenne et al. 2002, Trulock et al. 2004), being the most common cause of late graft failure. Both acute and chronic rejection of the lung are classified and graded according to the ISHLT working formulation (Yousem et al. 1996). Table 2 summarizes the grading of rejection and the main histopathological features. Signs of acute and chronic rejection may exist concomitantly. Chronic vascular rejection probably contributes less to chronic allograft dysfunction in the lung than in other solid organ transplantation (Radio et al. 1996). The gold standard in rejection diagnosis is biopsy.
(Higenbottam et al. 1988, Yousem et al. 1996), and specimens may be obtained either trans- or endobronchially, or rarely, in open lung biopsy. Target areas for biopsies may be identified by computed tomography (CT) (Ikonen et al. 1996, 1997). Other diagnostic tools such as bronchoalveolar lavage (BAL) samples and lung function tests may be additionally employed.

Table 2. Grading and histological features of lung allograft rejection

<table>
<thead>
<tr>
<th>Grade</th>
<th>Histological features</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - none</td>
<td>Normal pulmonary parenchyma without evidence of mononuclear infiltration or of alveolar hemorrhage.</td>
</tr>
<tr>
<td>1 - minimal</td>
<td>Infrequent (A1) or frequent (A2) perivascular cuffing of venules and arterioles by predominately small mature lymphocytes, occasional plasma cells, macrophages, and neutrophils.</td>
</tr>
<tr>
<td>2 - mild</td>
<td>Inflammatory cell infiltrate extending out to alveolar septae and airspaces, expansion of alveolar septae. Lymphocytes, neutrophils, and eosinophils common.</td>
</tr>
<tr>
<td>3 - moderate</td>
<td>Inflammatory process becoming more diffuse with parenchymal necrosis, hemorrhage, and hyaline-membrane formation.</td>
</tr>
<tr>
<td>4 - severe</td>
<td>Inflammatory cell infiltrates cuffing small vessels in the submucosa and adventitia of airways. May occur in the presence or absence of perivascular infiltrates.</td>
</tr>
</tbody>
</table>

A  
Acute rejection (all with or without Grades B, C, D)

Grade B  
Lymphocytic bronchitis/bronchiolitis

<table>
<thead>
<tr>
<th>Grade</th>
<th>Histological features</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - absent</td>
<td>Submucosal and intraluminal scarring of membranous and terminal bronchioles. Initially, perhaps loose myxoid granulation tissue with variable numbers of inflammatory cells. At later phase, eccentric, confluent dense hyalinized collagen plaques.</td>
</tr>
<tr>
<td>1 – present</td>
<td>Fibrosis present with intra- and/or peribronchial mononuclear cell infiltrate.</td>
</tr>
<tr>
<td>a - active</td>
<td>Bronchial scarring without inflammatory component</td>
</tr>
<tr>
<td>b - inactive</td>
<td>Patchy atherosclerosis involving arteries and veins. Circumferential intimal proliferation of myofibroblasts and smooth muscle cells. With extensive disease, internal elastica may become fragmented with thinning of underlying muscular wall.</td>
</tr>
</tbody>
</table>

2.1 Obliterative bronchiolitis (OB)

The main limitation on long-term survival after lung transplantation is OB, a condition first described in 1984 by Burke et al. The pathogenesis of OB is still uncertain, although it is considered a manifestation of chronic rejection (Yousem et al. 1990, Milne et al. 1992, Sakiyama et al. 1994). Many factors may predispose to its development (Table 3).

### Table 3. Risk factors for OB

<table>
<thead>
<tr>
<th>Probable</th>
<th>Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute rejection</td>
<td>CMV infection (without pneumonitis)</td>
</tr>
<tr>
<td>Lymphocytic bronchiolitis</td>
<td>Long graft-ischemic time</td>
</tr>
<tr>
<td>Cytomegalovirus (CMV) pneumonitis</td>
<td>HLA-mismatching</td>
</tr>
<tr>
<td></td>
<td>Bacterial/fungal/non-CMV viral infection</td>
</tr>
</tbody>
</table>

Data modified from Estenne et al. 2002 and Sharples et al. 2002.

The prevailing hypothesis is that an alloreactive immune response is central in OB in initiating the irreversible changes (Milne et al. 1992, Duncan et al. 1996, Heng et al. 1998, Maasilta et al. 2001, Sharples et al. 2002). However, allograft-independent factors such as ischemia-reperfusion injury, infections, and gastroesophageal reflux with aspiration may also contribute to progressive injury of the graft (Keenan et al. 1991, Tullius et al. 1995, Fiser et al. 2002, Vilchez et al. 2003, Cantu et al. 2004).

Histologically, OB manifests as epithelial injury, inflammation, fibrosis, and finally, as obliteration of the small airways (Tazelaar et al. 1994). The disease process begins with a peribronchiolar leukocyte infiltration that invades the basement membrane, submucosa, and luminal epithelium. Subsequently, mesenchymal cell proliferation, extracellular matrix deposition, and granulation tissue formation occur around the lumen of the bronchioli. Ultimately, fibroblasts and mature collagen obliterate the airway (Paradis et al. 1993, Kelly et al. 1997).

### 2.2 Clinical manifestation and diagnosis of OB

Clinically, OB manifests as decline in respiratory function. The term bronchiolitis obliterans syndrome (BOS) connotes graft deterioration secondary to progressive airway disease with no
other cause (Cooper et al. 1993). Infections, acute rejection, bronchial hyperresponsiveness, and anastomotic complications can confound the diagnosis and should be excluded. BOS stage 1 is diagnosed by a 66 to 80% decline in forced expiratory volume in one second (FEV1), but an earlier, potential-BOS stage is considered as a 10% to 19% decrease in FEV1 (Estenne et al. 2002). BAL fluid may show neutrophilia and elevated levels of several inflammatory mediators (Tiroke et al. 1999, Slebos et al. 2002). Transbronchial biopsy sampling is insensitive and difficult because of the patchy distribution of OB lesions (Estenne et al. 2002) and, actually, the designation of BOS does require no histological confirmation. The term OB means a histologically proven diagnosis. The histologic activity of OB does not always reflect the clinical activity monitored by pulmonary function tests, however. In long-term follow-up of lung transplant patients, repeated CT scanning is a sensitive and specific method of diagnosing BOS (Ikonen et al. 1996). CT images of BOS patients reveal air trapping, peripheral bronchial narrowing, central bronchial dilatation, and changes in peripheral vasculature, hyperlucency, and mosaic phenomena (Ikonen et al. 1996, Lee et al. 2000).

3. Immunological basis of OB

Alloreactive T cells are requisite mediators of the alloimmune response (Bolton et al. 1987, Bolton et al. 1989). Antigens provided by the allograft trigger the activation and proliferation of these cells. As a consequence, effector elements are generated that mediate graft injury and are responsible for the clinical manifestations of allograft rejection.

3.1 Alloantigens

Organ donors and recipients usually differ at several genetic loci, some of which the recipient’s immune system may recognize as foreign. The most important determinant in transplantation immunology is the major histocompatibility complex (MHC) (Bach et al. 1976, Gaston et al. 1995). The MCH consists of class I and II proteins, which present antigen to T cells in the form of peptides. The principal products of human MCH, the human lymphocyte antigen (HLA) complex, are the class I molecules HLA-A, -B, and –C, and the class II molecules DR, DQ, and DP. Some studies have proposed an association between OB and an HLA mismatch (Harjula et al. 1987, Hosenpud et al. 1996, Chalermskulrat et al. 2003b). This risk may be mediated through an increased rate of acute rejection (Heng et al. 1998).
MHC class I proteins are expressed on most cell types, and abundantly in endothelial cells and leucocytes. Class II protein expression is restricted to B cells, macrophages, dendritic cells, and endothelial cells (Daar et al. 1984, Page et al. 1992). Interferon-γ (IFN-γ) and other cytokines can also induce other cell types to express class II molecules (Pober et al. 1986, Auchincloss Jr. 1995, Mauck et al. 1996). Patients with OB have shown enhanced expression of class II molecules in bronchial epithelial cells (Taylor et al. 1989). It is also suggested that the transplantation procedure may break the tolerance to autoantigens (Fedoseyeva et al. 1999), such as the collagen V in lung tissue (Mares et al. 2000, Haque et al. 2002) that may subsequently generate an immune response.

3.2 Allorecognition

Recipient T cells are capable of recognizing donor antigens on donor cells (direct pathway) or donor antigens processed and presented by the recipient’s own MHC molecules (indirect pathway) (Shoskes et al. 1994, Auchincloss et al. 1996, Rogers et al. 2001). Both CD4+ (helper) and CD8+ (cytotoxic) T cells are primed to allorecognize by direct and indirect pathways (Heeger 2003). Cells presenting peptides in MHC molecules are called antigen-presenting cells (APC). These cells consist mainly of dendritic cells and monocyte-macrophages, but other cells may also act as APC. Classically, CD8+ T cells act with class I molecules presenting intracellular foreign antigens, while CD4+ T cells act with extracellular antigens in class II molecules. Cross-reactivity to class I and II MHC molecules may, however, occur (Auchincloss et al. 1996), and MHC class I may also present extracellular antigens to indirectly primed recipient CD8+ T cells (Yewdell et al. 1999). CD4+ alloreactivity to both donor HLA class I and class II molecules in OB is believed to be important (Lu et al. 2003).

T cells that are directly primed may mediate rejection early post-transplantation, and this pathway would thus be involved in acute rejection (Baker et al. 2001, Saiki et al. 2001). Over time, donor antigen-presenting cells are destroyed, and in chronic rejection a shift occurs towards the indirect recognition (Vella et al. 1997, Baker et al. 2001). A correlation exists between OB in patients and indirect recognition of donor mismatched class I and II molecules (Sivasai et al. 1999, Reznik et al. 2001). In experimental OB, however, both indirect and direct allore cognition patterns can cause obliterate changes (Chalermskulrat et al. 2003a, Richards et al. 2003).
3.3 T cell activation

T cells react to donor antigens through T cell receptors (TCR). To fully activate alloreactive T cells, a second or co-stimulatory signal between the APC and T cell is required after recognition of an antigen. The lack of these signals renders T cells unresponsive to further antigenic stimulation (Schwartz 1990). The co-stimulatory signal may be additional cell-cell interaction through cell surface molecules or be a stimulus by various cytokines, like TNF-α (Joseph et al. 1998). Important co-stimulatory pathways are CD28-B7 (Linsley et al. 1993, Sayegh et al. 1998) and CD40-CD145 (CD40 ligand) (Sho et al. 2002, Yamada et al. 2002) interactions. After ligation of the TCR with antigens presented by APC, CD28 on T cells binds the B7 molecules on APC. Inhibition of the CD28/B7-2 co-stimulatory pathway delays development of experimental OB and reduces proinflammatory cytokine responses (Tikkanen et al. 2002).

3.4 T cell responses

Following activation of an immune response, T cells produce cytokines and growth factors and stimulate other mediator cells. To maintain the rejection response, communication is required between infiltrating leucocytes, endothelium, parenchymal cells, and extracellular matrix. These events are mediated through generation of adhesion molecules, chemokines, and cytokines (Rocha et al. 2003).

T helper cells are considered crucial in initiating the organ rejection process. These cells are mainly CD4+ cells, but CD8+ T cells may also act as T helper cells. T helper cells produce cytokines that drive the immune response towards a cell-mediated response (Th1 pattern), or towards an antibody-dominated response (Th2 pattern). Polarization of T helper cells into either Th1 or Th2 types is influenced by various factors, including the nature and strength of antigenic stimuli, the type of antigen-presenting cells, the site of the response, and the cytokine milieu (Shirwan 1999, Le Moine et al. 2002). In the direct presentation, T cells encounter APCs that present high levels of allopeptides, resulting in differentiation into Th1 cells (Illigens et al. 2002). In the indirect presentation, only limited amounts of allopeptides are presented to T cells, resulting in Th 2 differentiation (Constant et al. 1995).

Multiple studies have suggested that the Th1 type alloimmune response mediates the OB pathogenesis (Sundaresan et al. 1995, Koskinen et al. 1997, Boehler et al. 1999, Gu et al.
2000, Tikkanen, et al. 2002), although chronic rejection in general is more closely associated with Th2 responses (Shirwan 1999). Cytokines mediating the Th2 responses are IL-4, IL-5, and IL-10 (Strom et al. 1996, Joosten et al. 2003). Overexpression of Th1 cytokines such as IL-1, IL-2, IL-8, IFN-γ, and TNF-α have all been implicated in OB development (Sundaresan et al. 1995, Koskinen et al. 1997, Gu et al. 2000, Neuringer et al. 2000, Scholma et al. 2000, Smith et al. 2001, Tikkanen et al. 2002). The Th2 type cytokines IL-4, IL-6, and IL-10 (Neuringer et al. 2000, Scholma et al. 2000) are also involved, suggesting that multiple T cell pathways contribute to OB development.

3.5 Adhesion molecules and chemokines

The process of leucocyte extravasation involves sequential adhesive interactions between the leucocyte and endothelium. The complex process of recruitment, activation, adhesion, and migration of leucocytes is coordinated by adhesion molecules and chemokines (Butcher et al. 1999). These molecules also play a role in antigen presentation. Their expression is upregulated by proinflammatory cytokines, with different effects from distinct cytokine patterns (Collins et al. 1995).

The adhesion molecules are traditionally divided into three categories: selectins, integrins, and the immunoglobulin superfamily. In terms of chronic lung rejection, upregulation of multiple adhesion molecules precedes OB in biopsy specimens (Devouassoux et al. 2001), and overexpression of E-selectin (endothelial-leucocyte adhesion molecule, ELAM-1) correlates with OB (Shreeniwas et al. 1996). In addition, the intercellular adhesion molecule-1 (ICAM-1) is present in biopsies and in BAL fluid, but with no direct correlation with OB (Milne et al. 1994, Shreeniwas et al. 1996, Reynaud-Gaubert et al. 2002). In murine tracheal allografts, however, brief treatment with an antibody against leukocyte function-associated antigen-1 (LFA-1), a ligand of ICAM, leads to prolonged OB inhibition (Morikawa et al. 2001). In experimental OB, at the time of maximal cellular rejection, ELAM-1 expression is upregulated (Maasilta et al. 2001). Blocking selectin-based cell adhesion significantly reduces allograft rejection in rat lung transplantation (Brandt et al. 1997), supporting the association between OB and increased expression of ELAM-1. Recently, detection of increased expression of integrin αVβ6 in healing rat tracheal isografts (Dosanjh et al. 2004), has suggested a role for this molecule in cell-cell interactions in graft re-epithelialization.
The chemokine superfamily is divided into four subfamilies (C, CC, CXC, and CX3C). C and CXC chemokines attract lymphocytes, while CC chemokines predominantly recruit mononuclear cells (Baggiolini et al. 1997). In OB, the role of several chemokines has been characterized. BAL fluid of OB patients shows increased levels of monocyte chemoattractant protein-1 (MCP-1) (Reynaud-Gaubert et al. 2002), CXCL8 (Riise et al. 1999, Elssner et al. 2000), CCR2 (Belperio et al. 2001), CXCL9, CXCL10, CXCL11, and CXCR3 (Agostini et al. 2001, Belperio et al. 2002). Broad-spectrum chemokine inhibition reduces inflammation and attenuates epithelial injury and luminal obliteration (Naidu et al. 2003). A similar effect is evident with inhibition of CCL5 chemokine (Farivar et al. 2003).

3.6 Macrophages

In chronic allograft rejection, tissue macrophages are important effector cells. They phagocytose foreign antigens and present them to T cells (Shirwan 1999). Macrophages interact with T cells in generating an alloimmune response, and Th1 type cytokines are strong stimulators of macrophages (Romagnani 1999). In transplanted human and animal lungs, alveolar macrophages secrete inflammatory mediators, chemoattractants, and growth factors (Hertz et al. 1992, Fattal-German et al. 1996, Magnan et al. 1996, Sekine et al. 1997, Fattal-German et al. 1998, Rizzo et al. 2000). These mediators stimulate endothelial and epithelial cells, leucocytes, and fibroblasts to express adhesion molecules and release cytokines and growth factors to facilitate cell recruitment and proliferation. In experimental OB, macrophages infiltrate into tissue (Neuringer et al. 1998, Maasilta et al. 2001). In BOS patients’ biopsy samples, higher amounts of tissue macrophages are evident than in stable recipients (Leonard et al. 2000).

3.7 B cells and humoral immunity

In chronic allograft rejection, B cells produce antibodies directed against MCH class I and II antigens, and non-MHC antigens. B cells recognize antigen through their cell-surface antigen receptors. Activation of antigen receptor with co-stimulatory signals from T cells or cytokines results into B cells’ differentiation into plasma cells that secrete large amounts of antibody. In lung transplant recipients, the presence of antibodies against HLA indicates higher risk for OB (Jaramillo et al. 1999, McKenna et al. 2000, Reznik et al. 2000, Palmer et al. 2002). Anti-HLA antibodies may be the cause of epithelial cell damage and thus contribute to development of OB (Reznik et al. 2000, Jaramillo et al. 2003). Moreover, non-HLA
antibodies against airway epithelial cells have also been detected in OB patients (Jaramillo et al. 2001).

Activation of the complement cascade results in recruitment and activation of neutrophils and macrophages as well as amplification of the immune response. Deposition of complement into alveolar tissue and in capillaries may occur (Magro et al. 2002). In experimental OB, inhibition of complement leads to reduced graft inflammation and fibrosis (Kallio et al. 2000).

### 3.8 Other immune cells in OB

Because neutrophils clear invading micro-organisms, they are important for the innate immune system. They are recruited and activated by macrophage- and lymphocyte-derived proinflammatory mediators. Neutrophils generate and release reactive oxygen species and several proteases (Sibille et al. 1990, Chabot et al. 1998). When activated in the transplanted lungs, neutrophils can cause significant lung damage (Elssner et al. 2001). Elevated numbers of neutrophils within the airways of OB patients is a general feature, and in OB is considered to be one diagnostic hallmark (DiGiovine et al. 1996, Elssner et al. 2000, Reynaud-Gaubert et al. 2000, Zheng et al. 2000). Lungs of BOS patients show impaired antiprotease defense mechanisms and increased oxidant activity (Riise et al. 1998, Hirsch et al. 1999, Behr et al. 2000).

Cytotoxic natural killer (NK) cells are also part of the innate immunity, and in lung transplant recipients, increased levels of NK cells can appear in BAL fluid (Crim et al. 1996, Ward et al. 2001). In canine lung transplantation, these cells were found to be elevated in peripheral blood (Nguyen et al. 1993). Eosinophils are associated with allergies, hypersensitivity reactions, and asthma (Allen et al. 1994), and are capable of producing cytotoxic proteins. Tissue and BAL fluid eosinophils are associated with aggressive acute rejection of the lung, a relationship, however, not clearly defined (Riise et al. 1996, Dosanjh et al. 1997, Dosanjh et al. 1998, Bewig et al. 1999, Mogayzel Jr. et al. 2001). Mast cells produce and store cytokines, vasoactive agents, growth factors, and enzymes (Wershil et al. 1988, Roberts et al. 2000) and degranulate upon stimulation. In postmortem samples of patients with OB, numbers of mast cells are significantly increased (Yousem 1997).
4. Mechanisms in OB

In pathogenesis of OB, alloimmune-induced inflammatory cell invasion, epithelial injury, fibroblast proliferation, extracellular matrix deposition, and finally obliteration of the bronchiolar lumen are the key events. These events are suggested to occur or to be mediated by various mechanisms.

4.1 Apoptosis

One of the mechanisms of cellular death is apoptosis, an energy-dependent active process that is genetically regulated (Steller 1995). It is a physiological phenomenon necessary to tissue homeostasis, but defects in its regulation may cause numerous diseases. In apoptotic cell death, the cell shrinks, plasma membrane undergo blebbing, mitochondria release cytochrome c, and the DNA fragments. Ultimately, the cell breaks into small apoptotic bodies which are cleared through phagocytosis by neighboring cells (Delhalle et al. 2003). In cells undergoing apoptosis, the plasma membrane structure is conserved throughout the process, minimizing the damage to neighboring cells. This is in contrast to necrotic cell death, characterized by cellular swelling, disruption of the plasma membrane, and leakage of intracellular contents (Assuncao Guimaraes et al. 2004).

Apoptosis is a strictly regulated process in which several steps are controlled by various families of proteins. The morphologic changes are initiated by the activation of proteases called caspases. These exist in cells as inactive zymogens, and require proteolytic cleavage for their activation (Salvesen et al. 1997). Many pathways for activating caspases exist, but two of the most investigated are the extrinsic (death receptor) and the intrinsic (mitochondrial) pathway (Reed 2000). The extrinsic pathway is triggered by activation of TNF receptor superfamily members (Wang et al. 2003). These include the TNF-α/TNF receptor and Fas/Fas ligand (FasL) interactions (Locksley et al. 2001). The intrinsic pathway is triggered by mitochondrial release of various molecules (e.g., cytochrome c) in response to intracellular injuries like DNA damage. The caspase activity is modulated by several pro- and antiapoptotic proteins (e.g., the Bcl-2 family of proteins, inhibitors of apoptosis proteins) (Delhalle et al. 2003).

In transplant rejection, apoptosis is suggested to be one of the mechanisms of cellular loss (Kabelitz et al. 1998). Alloreactive T cells may induce apoptosis of the target cells by
releasing perforin and granzyme B, and cytotoxic cytokines like TNF-α and INF-γ. Cytotoxic T cells may also bear the FasL, a transmembrane protein which induces apoptosis after binding to Fas protein on target cells (Kroemer et al. 1995, Nagata 1999, Ross et al. 1999, Zavazava et al. 2000).

In acute and chronic liver, kidney, and heart allograft rejection, apoptotic epithelial and parenchymal cell death may contribute to graft deterioration (Krams et al. 1995, Ito et al. 1995, Laguens et al. 1996, Szabolcs et al. 1996, Laine et al. 1997). In the context of lung transplantation, apoptotic cell death has been investigated in ischemia-reperfusion injury, and in acute and in chronic lung allograft rejection, both in human samples and in rodent models (Hansen et al. 1999, Hansen et al. 2000, Fischer et al. 2000, Keshavjee et al. 2000, Schmid et al. 2000, Stammberger et al. 2000, Neuringer et al. 2002). Ischemia-reperfusion injury enhances apoptotic activity (Fischer et al. 2000a-b, Keshavjee et al. 2000, Stammberger et al. 2000), and in acute lung rejection the number of apoptotic cells correlates with rejection grade (Hansen et al. 1999). In the lungs of OB patients, increased apoptotic activity occurs (Hansen et al. 2000), meaning that apoptotic cell death may contribute to the disease process.

4.2 TNF-α

Currently, the TNF superfamily comprises at least 18 distinct members with numerous effects on cell proliferation, survival, and apoptosis (Gaur et al. 2003). TNF-α is a pleiotropic cytokine with strong proinflammatory and immunomodulatory properties as well as fibrogenic effects (Beutler et al. 1988, Strieter et al. 1996). It is involved in the development of cytotoxic lymphocytes, in enhancing T-cell responses, and in regulating leukocyte movement. It also shows direct cytotoxic activity (Scheurich et al. 1987, Sedgwick et al. 2000, Aspalter et al. 2003). TNF-α induces the release of other inflammatory mediators and of angiogenic factors and chemokines. Adhesion molecule expression in endothelial cells is upregulated by TNF-α, enabling leukocyte extravasation. TNF-α also promotes fibroblast proliferation and chemotaxis (Miyazaki 1995, Strieter et al. 1996, Chou et al. 1996, Zhang et al. 1997, Eidelman et al. 2001). TNF-α exists in tissues either as a soluble or as a membrane-bound form and exerts its effects through two specific cell-surface receptors, TNF-R1 and TNF-R2 (Bazzoni et al. 1996, MacEwan 2002). In the lung, epithelial and endothelial cells, alveolar macrophages, and mesenchymal cells are capable of producing TNF-α (Fattal-German et al. 1996, Ermert et al. 2003, Shinbori et al. 2004).

Studies in rodent models of OB reveal that TNF-α inhibition attenuates acute (Saito et al. 1993, DeMeester et al. 1993) and chronic rejection (Smith et al. 2001, Aris et al. 2002). Clinically, blockade of the proinflammatory effects of TNF-α by either anti-TNF-α antibodies or soluble TNF-α receptor fusion protein has significantly changed the therapy for diseases with chronic inflammation as a key feature in their pathophysiology (Feldman et al. 2001, Suryaprasad et al. 2003, Rutgeerts et al. 2004).

4.2.1 Infliximab

Infliximab is a chimeric human mouse monoclonal antibody targeting both soluble and membrane-bound forms of TNF-α (Scallon et al. 1995, Valle et al. 2001). Currently, it is approved as a therapy for refractory cases of rheumatoid arthritis and inflammatory bowel diseases (Lipsky et al. 2000, Rutgeerts et al. 2004). In lung diseases, infliximab therapy has been successful in individual cases of rheumatoid arthritis-associated pulmonary fibrosis (Vassallo et al. 2002) and in sarcoidosis (Baughmann et al. 2003). Infliximab is suggested to have potential in the treatment of organ allograft rejection (Feldman et al. 1998). In two intestinal transplant patients, reversal of refractory acute cellular rejection occurred after infliximab treatment (Pascher et al. 2003), and in renal transplantation, single-center trials are ongoing (Vincenti 2003).

4.3 PDGF, TGF-β, and CTGF

PDGF, TGF-β, and CTGF are peptide growth factors that regulate and participate in multiple molecular and cellular events, including inflammatory and fibroproliferative processes (Heldin et al. 1999, Ihn 2002, Brigstock 2003). The PDGF family consists of five different dimers built up of four different polypeptide chains. These chains can form disulfide-bonded
hetero- or homodimers consisting of PDGF-AA, PDGF-BB, PDGF-AB (Heldin et al. 1999), and the novel members PDGF-CC and PDGF-DD (Li et al. 2000, Bergsten et al. 2001, Heldin et al. 2002). PDGF ligands act via two receptor tyrosine kinases: PDGF receptors (PDGFR) \( \alpha \) and \( \beta \) (Heldin et al. 1999). PDGF-AA ligand binds only to an \( \alpha-\alpha \) receptor dimer, PDGF-AB binds to \( \alpha-\alpha \) and \( \alpha-\beta \) receptors, and PDGF-BB can bind to \( \alpha-\alpha \), \( \alpha-\beta \), and \( \beta-\beta \) receptors. PDGF-CC binds to \( \alpha-\alpha \) receptors, and PDGF-DD to \( \beta-\beta \) receptors (Bergsten et al. 2001, Heldin et al. 2002).

In mammals, three isoforms of TGF-\( \beta \) exist; TGF-\( \beta \)1, TGF-\( \beta \)2, and TGF-\( \beta \)3 (Kingsley 1994). In addition, the TGF-\( \beta \) superfamily comprises many other factors, each playing a role in tissue differentiation and morphogenesis. Of the isoforms, TGF-\( \beta \)1 is the most ubiquitous in all tissues. TGF-\( \beta \) signal transduction is initiated by binding of two cell-membrane serine-threonine kinase receptors, termed TGF-\( \beta \) receptor type I and II. In addition, a type III receptor exists that is suggested to function as a reservoir of TGF-\( \beta \) (Derynck et al. 1997).

CTGF belongs to the connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) protein family (Brigstock 2003). CTGF’s mode of action is mediated by integrins, e.g., \( \alpha v \beta 3 \), \( \alpha IIb \beta 3 \), \( \alpha M \beta 2 \), identified as its cellular receptors (Babic et al. 1999, Jedsadayanmata et al. 1999, Schober et al. 2002). The cellular sources PDGF, TGF-\( \beta \), and CTGF, their biological responses, and observations in organ transplants are summarized in Table 4.
Table 4. Cellular sources of PDGF, TGF-β, and CTGF in the lung, their biological response, and appearance in organ transplants

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Cellular sources</th>
<th>Biological response</th>
<th>In organ transplants (clinical and experimental)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β</td>
<td></td>
<td>Acts as chemoattractant and stimulator for connective tissue cells</td>
<td>Overexpression attenuates acute heart and lung rejection (Qin et al. 1994, Mora et al. 2000).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Up-regulates integrins, enhances cell adhesion</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Has stimulatory, but also inhibitory effects on inflammatory cell proliferation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Promotes angiogenesis</td>
<td></td>
</tr>
<tr>
<td>CTGF</td>
<td>Promotes fibroblast proliferation and migration, cellular adhesion, and extracellular matrix formation, and angiogenesis</td>
<td></td>
<td>Expressed in a mouse model of chronic lung rejection (Ramirez et al. 2004).</td>
</tr>
</tbody>
</table>


In lung-transplant patients with OB, levels of PDGF and TGF-β have appeared as elevated in BAL fluid or in transbronchial biopsies (Hertz et al. 1992, El-Gamel et al. 1999). In rodent models of OB, expression of PDGF and TGF-β is also elevated (Kallio et al. 1999, Aris et al. 2002). Daily injections of PDGF induce fibroproliferation (Al-Dossari et al. 1995), whereas
inhibition of PDGF receptor slows development of OB (Kallio et al. 1999, Tikkanen et al. 2003). CTGF contributes to various lung diseases (Allen et al. 1999, Moussad et al. 2000, Atamas et al. 2003, Ning et al. 2004), but in the context of lung transplantation, only one study exists. Recently, CTGF was detected in mouse tracheal allografts (Ramirez et al. 2004).

4.3.1 Imatinib

Of the agents capable of interrupting growth factor signaling, imatinib mesylate (STI571) is a protein tyrosine kinase inhibitor currently used in clinical oncology (George 2003). It acts on signal transduction of the PDGF receptors (Buchdunger et al. 2000, Apperley et al. 2002) and additionally inhibits the binding site of stem cell receptor c-Kit, and tyrosine kinases V-Abl and ARG (Heinrich et al. 2000, Druker et al. 2001, Okuda et al. 2001). In experimental organ transplantation, imatinib has shown promise in preventing chronic rejection in rodent kidney, heart, and aorta transplantation models (Savikko et al. 2003, Sihvola et al. 2003).

4.4 Collagen I and III

Collagens, the major constituents of connective tissue (Byers 2000), play a crucial role in maintaining the structural integrity of many tissues and organs. Additionally, collagens participate in numerous other biological functions, e.g., in cell attachment, chemotaxis, platelet aggregation, and filtration through basement membrane (Midwood et al. 2004). Lung extracellular matrix consists mainly of interstitial collagens type I and III (Kirk et al. 1984). Fibroblasts are the major synthetizers of type I and III collagens in the lung; however, but endothelial, epithelial, alveolar type II, and smooth muscle cells also produce these collagens (McAnulty et al. 1995). Type I collagen exists as a heterotrimer composed of two different \( \alpha \)-chains \([\alpha 1(I)]_2 \alpha 2(I)\), and type III collagen as a homotrimer of three identical \( \alpha \)-chains \([\alpha 1(III)]_3\) (Linsenmayer 1991). Collagen gene regulation is a complex interplay between upregulatory factors and extracellular matrix-degradating proteins and their tissue inhibitors (Ghosh 2002, Pardo et al. 2002). Imbalance between synthesis and degradation of collagen characterizes fibrotic disorders (Myllyharju et al. 2001).

In most tissues, type III collagen is co-expressed with type I collagen (Linsenmayer 1991). Changes in the distribution and increase in the production of collagens I and III occur in various diseases, like pulmonary fibrosis (Shahzeidi et al. 1993, Raghu et al. 1995). In the airways of OB patients, collagens I and III protein exists in subepithelial fibrotic lesions.
(Yousem et al. 1992, Coers et al. 1999). In transbronchial biopsies, increased amounts of type III collagen deposition appear in clinically manifest OB (Zheng et al. 1999).

4.5 Other pathways and molecules contributing to OB

OB pathogenesis has been under active investigation, with several other pathways and molecular mechanisms found or suggested as taking part. These are presented in Table 5.

5. Treatment options for OB

As yet, for most OB patients, no therapeutic option is able to influence the progress of the disease. Although chronic rejection is strongly believed to be the main cause of OB, augmentation of immunosuppressive therapy rarely induces a longstanding positive effect (Boehler et al. 2003). Treatment results for OB in lung-transplant patients are still disappointing, and various strategies such as modification of the maintenance regimen (Knoop et al. 2004), addition of inhaled immunosuppressants (Iacono et al. 1996, De Soyza et al. 2001), total lymphoid irradiation (Diamond et al. 1998), photopheresis (O’Hagan et al. 1999), allopurinol (Scott et al. 1992), methotrexate (Dusmet et al. 1996), and statins (Johnson et al. 2003), have undergone investigation.

Use of newer immunosuppressive agents such as tacrolimus, MMF, sirolimus, and everolimus is increasing (Trulock et al. 2004). Tacrolimus and MMF have been effective in treatment of acute rejection, but their role in preventing OB long-term remains unclear (Treede et al. 2001a-b, Bhorade et al. 2003, Knoop et al. 2004). In addition to their effect on lymphocyte proliferation, sirolimus and everolimus also show an antiproliferative impact on smooth muscle cells and on fibroblasts (Nair et al. 1997, Azzola et al. 2004). Thus far, studies conducted on these agents in OB prevention have included only small numbers of patients, but the results have been promising (Cahill et al. 2003, Ussetti et al. 2003). However, despite the advances in immunosuppressive therapies, BOS/OB has a high incidence among lung transplant recipients, remaining, in the field of lung transplantation, the leading challenge.
<table>
<thead>
<tr>
<th>Molecule or agent</th>
<th>Function</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NO</strong></td>
<td>Mediator of immune defense, leucocyte adhesion, and vasorelaxation</td>
<td>Decreased production in endothelium and in airway epithelium in OB patients (McDermott et al. 1997). Increased exhaled levels in lung transplant recipients with lymphocytic bronchiolitis and early OB (Fisher et al. 1998). Induction inhibits SMC proliferation and modulates immune responses in rat model of OB (Kallio et al. 1997).</td>
</tr>
<tr>
<td>ET-1</td>
<td>Vasoconstrictor, growth factor for mesenchymal cells, inducer of adhesion molecule expression</td>
<td>In human lungs upregulated with OB (Jeppsson et al. 1998), increased in rat model of OB (Aris et al. 2002), and blockade of ET receptors attenuates development of OB (Tikkanen et al. 2004). Upregulated in OB patients (Al-Dossari et al. 1995) and in rat model of OB (Aris et al. 2002).</td>
</tr>
<tr>
<td>FGF</td>
<td>Mitogens and stimulators of fibroblasts</td>
<td>Upregulated in BAL samples of BOS patients, and suggested as an early marker of OB (Charpin et al. 2000).</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Mitogens and stimulators of fibroblasts</td>
<td></td>
</tr>
<tr>
<td>MMP</td>
<td>Key enzymes involved in extracellular matrix degradation and tissue remodeling</td>
<td>Increase in sputum concentrations of MMP-9, and decrease in its tissue inhibitor-1 (TIMP-1) inversely correlates with airflow obstruction in lung transplant patients (Beeh et al. 2001). MMP-2 and its activator membrane type 1 (MT1)-MMP localized in lymphocytes and in fibrotic areas in rodent model of OB (Inaki et al. 2004). MMP-2 and MMP-9 activity increases with concomitant rejection in porcine model of OB (Eerola et al. 2005)</td>
</tr>
<tr>
<td>HO-1</td>
<td>Cytoprotective protein, identified as a graft survival gene</td>
<td>Increased in murine heterotopic airway rejection; deficiency accelerates development of OB lesions (Visner et al. 2003).</td>
</tr>
<tr>
<td>ACE</td>
<td>Increased in fibrotic disorders</td>
<td>In rodent model of OB, presence of ACE in the fibroproliferative lesion demonstrated, and inhibition of ACE by captopril limits development of obliteration (Maclean et al. 2000).</td>
</tr>
<tr>
<td>Phosphodiesterase-4</td>
<td>Mediator of inflammation</td>
<td>Inhibition reduces macrophage infiltration and mesenchymal-cell proliferation in rat tracheal allografts (Roth-Eichhorn et al. 2001).</td>
</tr>
</tbody>
</table>

**NO**=nitric oxide, **iNOS**=inducible nitric oxide synthase, **ET-1**=endothelin-1, **FGF**=fibroblast growth factor, **IGF-1**=insulin-like growth factor, **COX-2**=cyclooxygenase-2, **MMP**=matrix metalloproteinases, **HO-1**=heme oxygenase, **ACE**=angiotensin-converting enzyme
6. Experimental models of OB

Clinical investigation of OB in human beings is restricted by the limited amount of biological material available from patients. To address this problem, several experimental animal models have been developed with either ortho- or heterotopic transplantation of lung, bronchial, or tracheal structures (Hertz et al. 1993, Al-Dossari et al. 1994, Huang et al. 1995, Ikonen et al. 1998, Hausen et al. 1999). Orthotopic rat and porcine lung allografts exhibit changes similar to those in human OB (Al-Dossari et al. 1994, Ikonen et al. 1995, Hirt et al. 1999), but orthotopic transplantation techniques are very demanding and time-consuming, and development of OB lesions is slow and variable (Uyama et al. 1992, Al-Dossari et al. 1994, Ikonen et al. 1995). Heterotopic transplantation models are considered feasible and cost-effective (Hele et al. 2001). In these models, the graft is usually implanted into the omentum or into subcutaneous pouches: one widely employed example is a model of heterotopic tracheal transplantation in rodents.

The actual site of human OB is small bronchi and bronchioles, and in order to study smaller airways, porcine (Ikonen et al. 1998) and primate (Hausen et al. 1999) models have been developed for transplantation of bronchi and bronchioles. The porcine heterotopic bronchial transplantation model developed by our group exhibits histological changes similar to those of human OB following lung transplantation. Total epithelial destruction and permanent luminal obliteration occur rapidly in allografts without immunosuppression (Ikonen et al. 1998).
AIMS OF THE STUDY

The focus of this study was to elucidate mechanisms in the development of post-transplant OB, employing a porcine heterotopic bronchial transplantation model. The specific aims of this study were to investigate in OB:

- epithelial cell apoptosis (I)
- the role of TNF-α (II)
- the roles of PDGF, TGF-β, and CTGF (III)
- the expression and distribution of collagens I and III (IV)
METHODS

1. Porcine heterotopic bronchial transplantation model

The animals were 48 non-related, random-bred domestic pigs weighing initially 20 kg. They 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 Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No.86-23, revised 1985). The study protocol was accepted by the institutional committee for animal research and by the Uusimaa Provincial Administration, Finland. Special attention was given to anesthesia and pain relief during surgical procedures.

1.1 Anesthesia

Anesthesia was induced with intramuscular ketamine sulphate (10-15 mg/kg) (Pfizer, Brooklyn, NY, USA) azaperone (10-15 mg/kg) (Janssen Pharmaceutica, Beerse, Belgium), atropine sulphate (0.05 mg/kg) (Leiras, Turku, Finland), and intravenous sodium pentobarbital (6-12 mg/kg) (Orion, Turku, Finland). Intravenous diazepam (0.25 mg/kg) (Orion Pharma, Espoo, Finland) was given prior to intubation, and later, muscle relaxation was maintained with intravenous pancuronium bromide (2-4 mg) (Organon, Oss, Holland) (I, II, IV). In Study III, intravenous prophophol (1.5-2.5 mg/kg) (Abbott, Espoo, Finland) was used instead of pentobarbital and pancuronium bromide. During the surgical procedure the animals were ventilated with 40% oxygen and enflurane (Abbott). When the bronchial grafts were harvested, the pigs were anesthetized with 6 mg/kg ketamine sulphate and 6 mg/kg azaperone intramuscularly. At the end of follow-up, they were euthanized with a high intravenous dose of sodium pentobarbital.

1.2 Surgery

The surgical procedure consisted of left thoracotomy performed for removal of the caudal lobe (Figure 1A). Peripheral bronchial segments (1-2 cm in length and 1-2 mm in diameter) were dissected free of the surrounding lung parenchyma (Figure 1B). Bronchial implants were transplanted subcutaneously into the ventral side of each recipient (Figure 1C), each serving as both donor and recipient. Both autograft and allograft transplantations were performed.
1.3 Post-operative medication

Postoperative pain was controlled with diclophenic acid 37.5 mg (Novartis, Basel, Switzerland) intramuscularly, and keftriaxone 500 mg (Roche, Basel, Switzerland) was given for 3 days intramuscularly for infection control. For ulcer prophylaxis, perioperatively administered intravenous ranitidine 50 mg (Orion Pharma) was continued at a daily oral dose of 150 mg for 3 weeks. Oral kefalexin 500 mg (Orion) was given for 2 to 3 weeks.

1.4 Graft harvesting

Depending on the study protocol, the follow-up time ranged from 21 days to 3 months. On the pre-determined postoperative days, 1-3 bronchial segments were removed serially from each animal. Each harvested bronchial segment was cut into three portions. Portions for histological staining, immunohistochemistry of paraffin-sections, and for in situ hybridization were fixed in 10% normal buffered formalin solution. Portions for immunohistochemistry of frozen sections were embedded in Tissue-Tek™ and snap frozen in liquid nitrogen. Sample portions for determination of total collagen and for RNA isolation were also snap frozen. All frozen specimens were stored at -70°C.

2. Study groups

Study groups comprised control autografts and allografts, and allografts treated with various drug regimens. Each group comprised four animals. The following drugs were investigated either as a single treatment or in combinations to prevent OB (Table 6):
Cyclosporine A (CsA) (Novartis) was used in combination with Aza and MP or with everolimus and MP. The dose was 10 mg/kg/day orally and CsA administration began 3 days prior to the transplantation procedure.

Everolimus (RAD, 40-O-(2-hydroxyethyl)-rapamycin) (Novartis) was used either with CsA and MP or as a single treatment at an oral dose of 1.5 mg/kg/day, started 3 days prior to transplantation. Dosage was based on a pharmacokinetic study (Maasilta et al. submitted).

Methyl prednisolone (MP) (Pharmacia, New York, NY, USA) was given in combination with CsA and Aza or with CsA and everolimus at an oral dose of 20 mg daily.

Azathioprine (Aza) (GlaxoSmithKline, Philadelphia, PA, USA) was given with CsA and MP at an oral dose of 2 mg/kg/day.

Infliximab (Schering-Plough, Kenilworth, NJ, USA), a chimeric human/mouse anti-TNF-α monoclonal antibody was administered intravenously at a dose of 5 mg/kg. A single dose infusion was given preoperatively; 5 mg/kg was based on previous experiments in rats and pigs (Olmarker et al. 2001, Kulmatycki et al. 2001) and in human pharmacokinetics (Kavanaugh et al. 2000, Cornillie et al. 2001).

Imatinib (Novartis), a tyrosine kinase inhibitor that targets PDGF receptors, c-kit, and V-abl was given as a daily oral dose of 10 mg/kg. The treatment was started 3 days prior to transplantation. The dose was based on clinical use (Druker et al. 2001) and on studies in rodent models (Savikko et al. 2003, Sihvola et al. 2003).
Table 6. Study groups

<table>
<thead>
<tr>
<th>Study</th>
<th>Groups</th>
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<tbody>
<tr>
<td>I</td>
<td>Autografts</td>
</tr>
<tr>
<td></td>
<td>Nontreated allografts</td>
</tr>
<tr>
<td></td>
<td>CsA, Aza, MP - allografts</td>
</tr>
<tr>
<td></td>
<td>CsA, everolimus, MP - allografts (I, IV)</td>
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<tr>
<td></td>
<td>Everolimus - allografts</td>
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<td>II</td>
<td>Autografts</td>
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<td>Nontreated allografts</td>
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<tr>
<td>IV</td>
<td>Autografts</td>
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<tr>
<td></td>
<td>Nontreated allografts</td>
</tr>
<tr>
<td></td>
<td>CsA, everolimus, MP - allografts (I, IV)</td>
</tr>
</tbody>
</table>

3. Histologic analysis

Formalin-fixed, paraffin-embedded samples were cut into 4-µm sections and hematoxylin-eosin (H&E) stained. From each sample, 1 to 3 bronchi were analyzed. Histologic alterations (Table 7) were semiquantitatively graded on a scale from 0 to 3. Furthermore, in fibrotic areas, the relation between cellular and extracellular matrix components was scored as equal, <1, or >1. Percentages of normal epithelial cells, epithelial cells with metaplastic atypia (ciliated respiratory cells changing into squamous cells), and the pure basal cell layer of the remaining epithelium were also evaluated. In Study IV, luminal obliteration was evaluated by measurement of the area of the luminal plug and total luminal area with Olympus DP-Soft Version 3.1 software, and by calculating the relation between these areas.
Table 7. Grading of histologic alterations

<table>
<thead>
<tr>
<th>Alterations</th>
<th>Scale used</th>
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<tbody>
<tr>
<td>Epithelial destruction</td>
<td></td>
</tr>
<tr>
<td>Bronchial wall (area from beneath the epithelium down to the cartilage)</td>
<td>0 = no alteration</td>
</tr>
<tr>
<td>• inflammation, necrosis, fibrosis</td>
<td>1 = mild alteration including a minor portion of the observed area</td>
</tr>
<tr>
<td>Cartilaginous</td>
<td>2 = moderate, in which areas of pathologic alterations are equal to areas of normal tissue</td>
</tr>
<tr>
<td>• necrosis, proliferation</td>
<td>3 = severe alterations, in which pathologic changes are the predisposing component</td>
</tr>
<tr>
<td>Pericartilaginous (area surrounding the cartilaginous structures)</td>
<td></td>
</tr>
<tr>
<td>• fibrosis, inflammation</td>
<td></td>
</tr>
<tr>
<td>Total fibrosis (bronchial wall fibrosis and pericartilaginous fibrosis)</td>
<td>0 = no obliteration, (0-1 = incipient obliteration)</td>
</tr>
<tr>
<td></td>
<td>1 = approximately one-third of the lumen occluded</td>
</tr>
<tr>
<td></td>
<td>2 = two-thirds occluded</td>
</tr>
<tr>
<td></td>
<td>3 = total occlusion</td>
</tr>
</tbody>
</table>

4. Analysis of apoptotic cells

The apoptotic index % (apoptotic cells/total number of cells) of the graft bronchial epithelium was evaluated preliminarily in H&E-stained sections based on apoptotic morphology. To further confirm the appearance of apoptotic cells in the epithelium of bronchial airways, we used in situ 3'-end labeling of apoptotic DNA (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, TUNEL) with the ApopTag Peroxidase Kit (Oncor, Gaithersburg, MD, USA). Sections 4 µm thick were cut from paraffin–embedded bronchial implants and placed on glass slides (SuperFrost® Plus, Menzel-gläser, Germany). These sections were dewaxed, rehydrated, and pretreated in 20 µg/ml proteinase K at room temperature (RT) for 15 min. Endogenous peroxidase activity was consumed by incubation in 2% hydrogen peroxide in phosphate-buffered saline (PBS), pH 7.2, for 20 min. Terminal deoxynucleotidyl transferase (TdT) enzyme was used to catalyze the addition of digoxigenin-labeled nucleotides to the 3'-OH ends of the fragmented DNA. After this, antidigoxigenin-peroxidase solution was applied to the sections. Sections for which the TdT-enzyme was
omitted served as negative controls. A color reaction was developed with diaminobenzidine (DAB) and hydrogen peroxide. The sections were counterstained with methyl green.

The counting of apoptotic cells in the epithelium was performed in 10 microscopic visual fields at x40 objective magnification. Only cells or cellular fragments with intense brown staining were considered positive. If several apoptotic bodies were visible in the same cluster, they were counted as one cell. Over 1% of apoptotic cells of the epithelium was considered a high apoptotic index.

5. Immunohistochemical analysis

5.1 Frozen sections

Sections 4 µm thick were cut and placed on glass slides, air-dried, fixed in acetone at -20°C for 20 min, and stored at -20°C until used. A three-layer indirect immunoperoxidase method was used with the following antibodies: mouse monoclonal antibodies against pig CD4+ and CD8+ cells (clones 74-12-4 and PT81B) (diluted at 5µg/ml and 1µg/ml) (VMRD Inc., Pullman, WA, USA), against human monocytes/macrophages, also reactive with porcine macrophages (clone MAC387) (5µg/ml) (Serotec, Oxford, UK), and against pig TNF-α (clone 9B4) (10µg/ml) (Endogen, Woburn, MA, USA). Before staining, the slides were re-fixed in chloroform for 30 min at room temperature and then air-dried. The primary antibodies were diluted in 0.1% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) in TRIS-NaCl buffer (TBS), pH 7.4. The sections were first incubated with the primary antibody, (against CD4, CD8, and macrophages for 30 min, at RT, and against TNF-α for 18 hours at +4°C), then with peroxidase-conjugated, rabbit anti-mouse Ig antibody (Dako A/S, Denmark) for 30 min at RT, and thereafter with a peroxidase-conjugated, goat anti-rabbit Ig antibody (Zymed, San Fransisco, CA, USA) for 30 min at RT. The secondary and tertiary antibodies were diluted in 50% normal pig serum in TBS. Between incubations, the slides were washed with TBS. The reaction was revealed by 3-amino-9-ethyl carbazole (AEC; Sigma) solution containing hydrogen peroxidase. Sections were counterstained with Mayer’s hemalun, and coverslips were aquamounted (Aquamount; Gurr, Poole, UK). Controls for TNF-α immunostaining were performed with mouse monoclonal IgG1 (X931, Dako) as a primary antibody. None of the controls showed positivity.
5.2 Paraffin-embedded samples

Rabbit polyclonal antibodies against PDGF-A (sc-7958) (diluted at 4 µg/ml), PDGF-B (sc-7878) (4 µg/ml), PDGFR-α (sc-338) (0.5 µg/ml), and PDGFR-β (sc-339) (0.67 µg/ml) (Santa Cruz Biotech. Inc, Santa Cruz, CA, USA), TGF-β1 (CPT001) (3.3 µg/ml) (Cell Sciences, Inc. Norwood, MA, USA), and CTGF (ab6992) (10 µg/ml) (Abcam, Cambridge, UK) served as primary antibodies in immunostaining with Vectastain Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA). In the protocol, the samples were cut into 5-µm sections, and placed on glass slides. They were deparaffinized and hydrated through descending ethanol concentrations, and then pretreated in a microwave oven and incubated in 1.6% H2O2 in methanol to block endogenous peroxidase. After this, the sections were incubated in blocking (goat) serum for 30 min, followed by overnight incubation at +4ºC with the primary antibody. Subsequently, they were incubated with biotinylated goat anti-rabbit antibody at RT for 30 min and finally with avidin/biotinylated enzyme complex for 30 min. All dilutions were made in Tris-buffered saline (TBS), pH 7.4, and between each step, the slides were rinsed in TBS. The reaction was revealed with AEC solution containing hydrogen peroxidase. The sections were counterstained with Mayer’s hemalun. For rabbit polyclonal antibodies, controls for immunostaining were performed with rabbit immunoglobulin fraction (X 0936, Dako) as a primary antibody. None of the controls showed positivity.

5.3 Quantitation of immunohistochemistry

The number of macrophages and cells positive for CD4, CD8, and TNF-α were counted separately in the epithelium, in the bronchial wall, and in the obliterative plug. Number of positive cells was counted in 10 randomly chosen microscopic fields in the epithelium and in five fields in the bronchial wall and obliterative plug. Number of TNF-α-positive epithelial cells and inflammatory cells among the epithelium were calculated separately. In addition, TNF-α-positive epithelial cells were divided according to weak or strong staining. The intensity of PDGF-A, PDGF-B, PDGFR-α, PDGFR-β, TGF-β1, and CTGF staining was scored from 0 to 3 as follows: 0=no visible staining, 1=cells with mild staining, 2=moderate intensity with multifocal staining, 3=intense diffuse staining. Staining intensity was scored separately for the epithelium and for fibroblasts, inflammatory and endothelial cells, and for the smooth muscle cell ring surrounding the bronchi. In each sample, 3 separate bronchi (if present) underwent analysis, the same bronchi being evaluated in histologic and immunohistochemical analyses.
6. Determination of total tissue collagen

Total tissue samples were homogenized in distilled water and the hydroxyproline content determined from the homogenate. These homogenates were hydrolyzed in 6 N HCl for 3 hours at 130°C, the HCl was evaporated, and the residue was dissolved in 20 mM HCl. The hydroxyproline was analyzed with high-pressure liquid chromatography (HPLC, Alliance System, Waters, MA, USA) and pre-column derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. The value obtained for hydroxyproline was then used to estimate the total collagen per mg of wet tissue, assuming that hydroxyproline comprises 13.7% collagen by weight (Hamlin et al. 1971).

7. Construction of cDNA clones for porcine proα1(I) collagen and proα1(III) collagen mRNAs

Total RNA was extracted by an established method (Chomczynski et al. 1987) from experimental granulation tissue induced for 15 days by implantation of sterilized viscose cellulose sponges (5 x 5 x 10 mm, dry weight ~10mg, Cellomed Oy, Turku, Finland) into subcutaneous pockets of recipient pigs. Random hexamers and oligo(dT) were used to prime reverse transcription of 1 µg of total RNA by Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase under conditions suggested by the supplier (GIBCO BRL, Gaithersburg, MD, USA). Aliquots of cDNA were used for amplification by polymerase chain reaction (PCR) (AmpliTaq™, Perkin Elmer, Branchburg, NJ, USA) with oligonucleotide primers based on existing human, mouse, and rat sequences (Bernard et al. 1983, Genovese et al. 1984, Ala-Kokko et al. 1989, Metsäranta et al. 1991, Glumoff et al. 1994) covering the 3’-untranslated sequence of the mRNA. The reactions were cycled through denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 2 min. After 30 amplification cycles, aliquots of the reactions were fractionated on 1.0 % agarose gels, and the specific fragments purified and cloned into the pGEM™-T Easy vector (Promega, Madison, WI, USA). Identification of the clones was performed by sequencing with the ABI Prism 377 and dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA, USA). The sequences were deposited in the EMBL/GenBank database under accession numbers AJ289757 (pPCol1a1-1 for pig type I collagen mRNA) and AJ289758 (pPCol3a1-1 for pig type III collagen mRNA).
8. Northern blotting

Total RNA was extracted from the bronchial samples by the method of Chomczynski et al. (1987). Aliquots of total RNA (10 µg) were fractionated by electrophoresis on agarose gels and then transferred by blotting onto a nylon transfer membrane (MagnaGraph Nylon Transfer membrane; Micron Separations Inc., Westborough, MA, USA). cDNA clones specific for pig procollagen α1(I)- and procollagen α1(III) mRNA served as hybridization probes, with clone pRGAPDH for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference probe. The clones were amplified by PCR and labeled with $^{32}$P-dCTP by random priming (High Prime DNA Labeling Kit, Boehringer Mannheim, Mannheim, Germany). After blotting, the membranes were UV-crosslinked and prehybridized. The hybridizations were performed at 42°C for 16 to 18 hours in hybridization solution (50% formamide, 1 M NaCl, 1% sodium dodecyl sulfate (SDS), 5x Denhardt's solution, 10% dextran sulfate, and 100 µg/ml herring sperm DNA). After hybridization, the filters were washed three times in 2x standard saline-citrate (SSC), (1x SSC is 0.15M NaCl and 0.015M sodium citrate), once in 0.1% SDS at room temperature, and twice in 0.1x SSC and 0.1% SDS at 55°C. The bound probe was detected by autoradiography at -70°C with Kodak X-omat films. The relative intensity of the bands of pig procollagen α1(I)- and procollagen α1(III) mRNA were analyzed by scanning of exposed films by a densitometer (HP Scanjet IIc Scanner, Hewlett Packard Co., Edina, MN, USA), and the bands were quantified (Software, Bio Image, Ann Arbor, MI, USA). Results were corrected for minor variations in the amount of GAPDH mRNA in respective samples.

9. In situ hybridization

Vectors containing the cDNA inserts for pig procollagen α1(I)- and procollagen α1(III) mRNA were linearized by appropriate restriction enzymes to synthesize antisense and sense probes with Sp6 and T7 RNA polymerases. Probes were labeled with digoxigenin-11-UTP with the DIG RNA labeling kit (Boehringer Mannheim). Concentrations of the transcripts were determined by serial dilution of the color reaction against known concentrations of labeled control RNA. Paraffin sections 4 µm thick were deparaffinized and hydrated through descending ethanol concentrations, and then pretreated in buffered proteinase K solution (10 µg/ml) at 37°C for 30 min and fixed with 4% paraformaldehyde. For hybridization, the labeled complementary RNA (cRNA) probes were diluted in hybridization solution (20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.3 M NaCl, 50% formamide, 10% dextran sulphate, 1x Denhardt's solution, 200
µg/ml sheared herring sperm DNA, yeast tRNA 200 µg/ml). Hybridization was performed in a humidified chamber at 50°C for 18 hours. After hybridization, the sections were sequentially washed in 5x SSC at 50°C for an hour, in 2x SSC/50% formamide at 65°C for 30 min, and three times in NTE buffer (0.5 M NaCl, 10 mM Tris-HCl, 5 mM EDTA) at 37°C for 10 min to reduce non-specific binding by the probe. For digestion of the unbound probe, the sections were treated with RNAase A (20 µg/ml in NTE buffer) at 37°C for 30 min, washed in NTE buffer at 37°C for 15 min, in 2x SSC/ 50% formamide at 65°C for 30 min, in 2x SSC at RT for 15 min, and finally in 0.1x SSC at RT for 15 min. Digoxigenin-labeled probes were detected following the methods of the DIG-detection Kit (Boehringer Mannheim) by use of antidigoxigenin antibody, and incubation at RT for 30 min. After digoxigenin detection, the sections were incubated for 16 hours in color substrate. The sections were then counterstained with Mayer's hemalum and cover-slipped with aqueous-based mounting medium.

9.1 Quantitation of in situ hybridization

Of each tissue section hybridized with the antisense probe, a control was hybridized with the sense probe. A cell was defined as positive when cytoplasmic red-brown staining against the pale staining background was visible with the antisense but not with the sense probe. Distribution and number of cells positive for proα1(I) and proα1(III)collagen mRNA were analyzed separately in the obliterative plug, in the bronchial wall, and in the pericartilaginous area. Positive cells were counted in five randomly chosen microscopic fields from each area.

10. Statistical analysis

All data are expressed as mean ± SEM. Variations between any two groups were analyzed with the Mann-Whitney U-test, and variations between multiple groups were calculated by the nonparametric Kruskal-Wallis one-way analysis by ranks. The rank sums were then used for Dunn's test at a significance level of 5% (Medstat, Astra Group A/S, Copenhagen, Denmark). Values of p<0.05 were considered significant. For correlation analysis, Spearman’s rank correlation (Statistica v. 5, StatSoft Inc., Tulsa, OK, USA) was used.
RESULTS

1. Porcine heterotopic bronchial transplantation model (I-IV)

Subcutaneously transplanted bronchial segments, 1 to 2 cm in length and 1 to 2 mm in diameter were serially removed in the porcine heterotopic bronchial transplantation model, for study of autografts and allografts either nontreated or treated with various drug regimens. No serious adverse effects occurred during the drug treatments.

1.1 Autografts

Normal ciliated bronchial epithelium was maintained through the study periods with no luminal obliteration in autograft bronchial transplants after post-ischemic injury on day 3 (Figure 2). Bronchial wall inflammation and fibrosis as well as pericartilaginous inflammation were mild. Bronchial cartilage remained viable, and formation of new cartilage was apparent. In the bronchial wall, CD4+ and CD8+ lymphocytes as well as macrophages appeared in only small amounts.

1.2 Nontreated allografts

Nontreated allografts lost their epithelial covering almost completely within one week. Concomitantly, inflammatory cell infiltrates consisting mainly of lymphocytes and macrophages in the bronchial wall increased. Fibroproliferative tissue protruding into the bronchial lumen was first seen on day 7, and luminal obliteration was complete within 3 weeks (Figure 2). In nontreated allografts, bronchial wall fibrosis consisted of fibroblasts and extracellular matrix components in equal proportions. It increased from mild to moderate by day 10, persisting thereafter. Pericartilaginous inflammation and cartilaginous necrosis proceeded gradually to severe within one month. These histological changes differed significantly (p<0.05) from those of autografts. In correlation analyses, degree of epithelial destruction from day 4 onwards correlated with that same day’s obliteration (r=0.42-0.99, p<0.001) and future obliteration (r=0.67-0.96, p<0.001). Numbers of CD4+, CD8+ lymphocytes, and macrophages were elevated in comparison to the numbers of these cells in autografts (p<0.05). CD8+ lymphocytes were predominant throughout follow-up.
Figure 2. Autografts, nontreated allografts, and allografts immunosuppressed with CsA, everolimus, and MP. On day 3, all grafts showed epithelial injury due to initial ischemia. Thereafter, epithelium recovered and remained intact in autografts, and in immunosuppressed allografts (days 7 and 90). In nontreated allografts, the epithelium was denuded, and luminal obliteration gradually developed (days 7 and 21).

1.3 Effect of immunosuppression

The effect of different immunosuppressive agents on epithelial preservation, obliterative changes, and degree of bronchial wall inflammation are compared in Figure 3. In allografts treated with the triple therapy of CsA, Aza, and MP, development of pathological changes was otherwise similar to that of nontreated allografts but somewhat slower. In most allografts treated solely with everolimus, total epithelial destruction and luminal obliteration occurred by day 28, but in sporadic samples these processes were prevented up to days 75 and 90, respectively. Triple therapy of CsA, everolimus, and MP totally prevented development of OB over an up to 3-month study period. The bronchial lumen stayed patent, with intact epithelial coverage consisting mainly of normal epithelium (p<0.05 in comparison to
nontreated allografts). In these grafts, mild to moderate bronchial wall inflammation was encountered, but mural fibrosis remained mild, consisting mainly of cellular components. Similarly to autografts, bronchial cartilage remained viable, and cartilaginous proliferation occurred.

2. Epithelial apoptosis in a porcine bronchial model of OB (I)

Only on day 3, in autografts and in the allografts on triple therapy of CsA, everolimus, and MP, were metaplastic epithelial atypia and pure basal cell layers, and moderate epithelial destruction observable, and the apoptotic index was at its highest (1.0+0.5). From day 7 onwards, normal ciliated epithelial coverage prevailed through the 3-month follow-up, and the apoptotic indexes remained low (<1%).

In nontreated allografts, the epithelium underwent total destruction within 7 days, and the apoptotic index analyzed only on day 3 was slightly higher than in autografts. In the group receiving the triple therapy of CsA, Aza, and MP, mainly atypic epithelial cells or pure basal cell layers were present on day 3, after which the remaining epithelium recovered slightly. In some grafts even normal epithelial cells were detected up to day 14. Apoptotic indexes were elevated at all observation points, being highest on days 7 (2.8+0.9, p<0.05 in comparison to autografts) and 14 (3.4+2.6). In most allografts treated solely with everolimus, normal epithelial coverage prevailed at most observation points, but on day 3 and at late time-points, a more atypical type epithelium or the pure basal cell layer was encountered. The proportion of apoptotic cells in the epithelium on day 3 (1.4+0.6) was similar to that of other groups. Apoptosis of epithelial cells began to increase significantly on day 21 (2.7+1.1, p<0.05 compared with autografts) together with progressive epithelial destruction. The apoptotic index remained high in the samples with assessable epithelium on days 28 (1.9+.4) and 45 (2.2+0.1, p<0.05 compared with autografts).
Figure 3. Effect of immunosuppression in bronchial allografts. Histological changes are graded on a semiquantitative scale of 0 to 3. Data shown until total obliteration or until 3 months. All data expressed as mean±SEM.

3. TNF-α in a porcine bronchial model of OB (II)

Epithelial cells and inflammatory cells among the epithelium showed positivity for TNF-α. In the bronchial wall, macrophages, lymphocytes, occasional endothelial cells, smooth muscle cells, and fibroblasts expressed TNF-α. TNF-α expression was up-regulated in the allografts in comparison to autografts (p<0.05 from day 7 onwards). Infliximab treatment led to slowed epithelial loss and luminal obliteration early in the disease process (p<0.05), and the normal epithelium prevailed longer in the treated allografts. Treated allografts showed milder bronchial-wall (p<0.01 on days 4, 7, and 21) and pericartilaginous inflammation (p<0.05 on days 4, 7, and 11) and milder fibrosis (p<0.05 on days 11 and 14) than in nontreated allografts.

The number of TNF-α-positive epithelial cells and inflammatory cells among the epithelial cells was lower in treated than in nontreated allografts (p<0.05 on day 4). Recruitment of CD8+ lymphocytes in the epithelium was diminished during the first week (p<0.05), and
numbers of macrophages were lower on day 4 (p<0.05). In the bronchial wall, the number of CD8+ lymphocytes was similarly reduced on days 2, 4, and 7 (p<0.05), and numbers of macrophages were significantly lower on day 7.

The number of TNF-α-positive epithelial cells on days 4, 7, and 11 correlated with the epithelial damage (r=0.62, p<0.001) and obliteration (r=0.67, p<0.001) on the same day or at future time points (r=0.39-0.74, p<0.05) (r=0.33-0.86, p<0.001). In the bronchial wall, the number of TNF-α-positive cells on days 7, 11, 14, and 21 correlated with obliteration on the same day or at future time points (r=0.43-0.62, p<0.05) (r=0.49-0.82, p<0.01).

4. PDGF, TGF-β, and CTGF in a porcine bronchial model of OB (III)

In autografts retaining normal bronchial histology, epithelial expression of PDGF-A and of PDGF receptors was mainly mild, whereas PDGF-B and TGF-β expression was moderate. In the bronchial wall, the expression of growth factors was generally mild, except that of CTGF, which ranged from moderate to intense.

In nontreated allografts, epithelial destruction was almost complete by day 7, being slightly less extensive in the treated allografts. Obliteration of the bronchial lumens was slower in the treated group than in the nontreated one (p<0.05 on day 11). Bronchial wall fibrosis increased gradually in both allograft groups from mild to moderate. Imatinib treatment reduced inflammatory cell infiltrates in the bronchial wall and in the pericartilaginous area (p<0.01 on day 11 in comparison to nontreated allografts). Furthermore, it reduced recruitment of CD4+ lymphocytes (p<0.001 on days 11 and 14), of CD8+ lymphocytes (p<0.001 on days 7 and 14), and of macrophages (p<0.05 on days 4, 7 and 14).

In allograft epithelium on day 4, expression of growth factors was moderate, except that treated grafts showed mild expression of PDGF-A (p<0.01 in comparison to nontreated allografts), and of TGF-β. Expression of PDGF-A, PDGF receptors, and of TGF-β was elevated (p<0.05) in the nontreated allografts when compared to autografts in most cell types at all time points. In the bronchial wall of all allografts, fibroblasts and smooth muscle cell rings showed moderate positivity for PDGF-A. In endothelial cells it was mild. In inflammatory cells of nontreated allografts, moderate positivity for PDGF-A appeared, whereas in treated allografts it was mild (p<0.05 on days 4, 7, 11). PDGF-B expression was
similar in all groups, being mainly mild. Fibroblasts and inflammatory cells showed milder positivity for the PDGF receptors in the treated than in the nontreated allografts (for fibroblast p<0.05 through the follow-up for PDGFR-α, and for PDGFR-β on days 7 and 11, and for inflammatory cells p<0.05 on days 4 and 14 for PDGFR-α, and on day 7 for PDGFR-β).

In the endothelium, receptor expression was mild in both groups, except on day 7, when it was moderate in nontreated allografts (p<0.05 for PDGFR-α, p<0.001 for PDGFR-β). In smooth muscle cell rings, mild to moderate PDGF receptor expression existed in both groups. On day 7, PDGFR-β expression was more intense in nontreated allografts (p<0.05). In both groups, TGF-β positivity in fibroblasts was moderate and in inflammatory cells moderate to intense. In smooth muscle cells it peaked by day 11, decreasing thereafter. In the endothelium, TGF-β expression was mild, except on day 7, when it peaked at moderate in nontreated allografts. CTGF expression in both groups ranged from moderate to intense.

5. Collagens I and III in a porcine bronchial model of OB (IV)

In situ hybridization revealed type I and III collagen mRNA nearly exclusively in fibroblasts and in occasional smooth muscle cells. In autografts, the number of cells expressing type I and III collagen transcripts decreased by day 14 and remained low thereafter. In early obliterative lesions of nontreated allografts, cells positive for collagen I and III mRNA existed in equal numbers. From day 14 onwards, the majority of the positive cells were expressing type III collagen mRNA. In the bronchial wall, fibroblasts expressing collagen III mRNA started to increase on day 14 and were significantly (p<0.05) augmented on days 21 and 30 compared to numbers of autografts. The dominance of fibroblasts showing a positive signal for type III collagen mRNA was evident at all assessment points. In immunosuppressed allografts, the pattern of fibroblast numbers expressing type I and III collagen mRNAs resembled that of autografts. No similar increase appeared in number of collagen III mRNA-positive cells compared to collagen I mRNA-positive cells as it did in nontreated allografts. In immunosuppressed allografts, at some assessment points cells positive for collagen I and III mRNA were abundant in the pericartilaginous area, even in the total absence of positivity in the bronchial wall.

Quantification of collagen I and III mRNA of the grafts showed no significant differences between the collagen types between groups. In all groups, however, in situ hybridization
detected the majority of positive cells in the pericartilaginous area. Comparison of absolute cell numbers in this area in autografts and in immunosuppressed allografts revealed a preponderance of collagen I mRNA to type III mRNA expression in fibroblast-like cells, whereas nontreated allografts showed the opposite. Total collagen in nontreated allografts decreased during follow-up to below the level of total collagen in native bronchial tissue. An increased total collagen content was evident in autografts and in immunosuppressed allografts at all assessment points, with $p<0.05$ on days 7, 21, and 60 between nontreated allografts and autografts.
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<td>In nontreated and in inadequately immunosuppressed allografts, apoptotic indexes of the epithelium increased with concomitant epithelial destruction. In allografts with immunosuppression adequate to prevent epithelial destruction, and in autografts, well-preserved epithelium was maintained with low apoptotic indexes.</td>
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<td><strong>TNF-α (II)</strong></td>
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<td><strong>PDGF, TGF-β, CTGF (III)</strong></td>
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<td>Few fibroblasts expressed type I and III collagen mRNA in autografts and in allografts with CsA, everolimus, and MP therapy. In nontreated allografts, number of cells expressing collagen III mRNA increased parallel to the developing obliteration and fibrosis.</td>
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DISCUSSION

Obliterative bronchiolitis in lung transplants is considered primarily to be the manifestation of chronic rejection. It remains the major barrier to long-term success after lung transplantation. Although clinical and experimental studies have provided substantial knowledge of the pathophysiology of OB, options for its prevention are still limited. Better understanding of the development of OB will lead to insights for future therapeutic interventions and preventive measures. To address this, the study focused on the mechanisms of OB in a porcine heterotopic bronchial transplantation model.

1. Porcine heterotopic bronchial transplantation as a model of OB

Because OB lesions in man take months to years to develop (Boehler et al. 2003), detailed studies of the pathophysiological mechanisms of OB are difficult to carry out. Our group has developed a heterotopic porcine bronchial transplantation model in which changes similar to human OB occur (Ikonen et al. 1998, Uusitalo et al. 1999). In subcutaneously implanted bronchial allografts, total obliteration develops within 3 weeks, but with immunosuppressive regimens this process is delayed or prevented. Neovascularization of the transplanted bronchi is observable within a few days (Salminen et al. 2000). Despite an initial anoxic period, autografts show normal bronchial structures, indicating that the obliterative changes in allografts are not due to ischemic injury. In nontreated allografts, the epithelium sloughs off within a week with a simultaneous influx of inflammatory cells. Before total epithelial loss, the epithelial cell type changes from cuboidal to squamous. This is followed by development of human OB-like fibrotic lesions (Tazelaar et al. 1994, Wallace et al. 2003); fibrous tissue plugs protrude into the bronchial lumen and gradually fill it, forming a dense scar. Similar to this, airway inflammation and epithelial injury are suggested to initiate the development of OB in human lung allografts (Hruban et al. 1988, Paradis et al. 1993, Reichenspurner et al. 1995). In bronchial grafts, the inflammatory cell infiltrates consisted of CD4+, CD8+ lymphocytes and macrophages. CD8+ cells predominated, a feature characterized also in porcine orthotopic lung transplantation models (Al-Dossari et al. 1994, Allan et al. 2002) and in OB patients (Holland et al. 1990, Milne et al. 1994, Whitehead et al. 1995).

In bronchial allografts, prevention of the obliterative process was achieved with the combination therapy of CsA, MP, and everolimus. Conventional triple therapy of CsA, Aza, and MP failed, however, to prevent OB in our model, despite the relatively high whole-blood
levels of CsA (583±225 ng/ml) (Salminen et al. 2000). The everolimus dose of 1.5 mg/kg was high in comparison to dosage in clinical transplantation, but this dose or even higher has been used in several experimental lung transplantation models (Serkova et al. 2000, Hausen et al. 2000a-b), and we chose it based on our pharmacokinetic and safety study in pigs (Maasilta et al. submitted).

Models of heterotopic lung transplantation are technically less demanding and less time-consuming than are models of orthotopic whole lung transplantation. In contrast to the widely used rat or mouse tracheal heterotopic transplantation models (Hertz et al. 1993, Huang et al. 1995, Koskinen et al. 1997), we transplanted small bronchi close to the bronchioles, the actual site of OB. The main difference between bronchi and bronchioles is the presence of cartilaginous structures and submucosal glands in bronchi. As an advantage over rodent models, this experimental set-up provided the opportunity to investigate the development of OB in serial samples from the same animal. In the porcine model, we have also explored bronchi of larger diameter (Maasilta et al. 2000), terminal bronchioles without cartilage (Salminen et al. 2002), and lung tissue implants (Ikonen et al. 1998, Salminen et al. 2000). However, evaluation of small bronchi has proven the simplest and most reliable method to assess the developing histopathologic changes in our model.

All experimental models have limitations when compared to clinical lung transplantation. In our model, disruption of bronchial vasculature occurs until neovascularization takes place, and this contributes to the early changes observed in all grafts. Due to this initial graft avascularity, the administration of immunosuppressive drugs should begin a few days prior to transplantation. Furthermore, bronchial grafts lack an air-epithelial surface, constant airflow, and clearance of secreted mucus. The pace of OB is accelerated in comparison to the slow disease progression in actual lung transplant patients. On the other hand, this feature is beneficial to orthotopic lung transplantation models, in which OB changes take months to develop (Al-Dossari et al. 1994, Ikonen et al 1995). Thus, with its characteristics of invariable and rapid airway obliteration and histopathologic changes similar to those in human OB, our large-animal model provides a useful tool for exploration of the pathogenesis of OB.
2. Apoptotic death contributes to epithelial cell loss in OB

In OB pathogenesis, epithelial loss is considered a prerequisite for luminal obliteration (Mauck et al. 1996, Ikonen et al. 2000, Adams et al. 2000). We found a high proportion of apoptotic epithelial cells prior to total epithelial destruction in bronchial allografts with inadequate immunosuppression. In contrast, similar to control autografts, allografts with adequate immunosuppression showed only a few apoptotic epithelial cells.

Activated recipient T cells infiltrating the graft are the primary initiators of the apoptotic cascade in donor endothelial, epithelial, and parenchymal cells (Zavazava et al. 2000). In our study, nontreated allografts exhibited an influx of CD4+ and CD8+ lymphocytes, and of macrophages, with a predominance of CD8+ cells (II, III). In allografts developing bronchial obliteration, inflammatory cell infiltrates were present in abundance in the bronchial wall and also to some extent among the epithelial cells.

One possible mechanism of T cell-induced apoptosis in OB might be a Fas/FasL interaction and the perforin/granzyme-mediated pathway. Increased expression of Fas on bronchial epithelial cells, together with up-regulated FasL on infiltrating mononuclear cells, appears in a rat model of OB (Yagyu et al. 1997). These findings are supported by biopsy specimens of acute lung rejection that show a similar increase in Fas/FasL expression (Bittmann et al. 2004). Granzyme B and perforin expression is associated with acute lung rejection (Clement et al. 1994, Bittmann et al. 2004), and in experimental OB, allografts produce more granzyme B than do isografts (Neuringer et al. 2000). Furthermore, T cells produce IL-2, which in turn induces the release of proapoptotic cytokines like TNF-α (Squier et al. 1995, Ross et al. 1999). We found that the inflammatory cells in bronchial allografts undergoing rejection showed high levels of TNF-α expression (II), suggesting that epithelial cell apoptosis in these grafts were at least partly mediated via TNF-α signaling.

In adequately immunosuppressed allografts, the bronchial epithelium remained intact, and apoptotic indexes resembled those of autografts, although degree of inflammation was histologically similar to that of other allograft groups. CsA and everolimus both inhibit IL-2-driven T cell stimulation and have a synergic effect (Schuler et al. 1997, Schuurman et al. 1997). Even though T cells were present in CsA- and everolimus-treated grafts, it is likely that their activity was sufficiently reduced to prevent initiation of epithelial apoptosis and
destruction. This observation would point to the central role of epithelium-preserving therapies in prevention of OB.

Graft reperfusion after lung transplantation is considered a strong inducer of epithelial and pneumocytic apoptosis (Fischer et al. 2000a-b, Stammberger et al. 2000, Shaw et al. 2001, Rivo et al. 2004, Yamashita 2004). On day 3, degrees of epithelial destruction and extent of epithelial cell apoptosis in allografts and in autografts were relatively similar. Because neovascularization also occurs by day 3 (Salminen et al. 2000), we speculate that graft reperfusion may have an impact on the high initial apoptotic indexes. High early apoptotic rate may also reflect initial injury caused by the transplantation procedure. In allografts regaining epithelial integrity, the apoptotic activity was low thereafter, whereas in nontreated allografts epithelial loss proceeded rapidly after initial injury. Inadequately immunosuppressed allografts underwent more slowly progressing epithelial destruction, and in these allografts, apoptotic activity of the epithelium remained elevated, implying epithelial damage and development of OB.

Lung transplant patients with OB have been reported to have more apoptotic epithelial cells in transbronchial biopsy specimens than do those without OB (Hansen et al. 2000). In a rodent tracheal model, the number of apoptotic epithelial cells in rejected allografts was elevated. This portion was under 5% of the total epithelial cell number (Neuringer et al. 2002). We observed similar phenomena in our bronchial transplants. Because phagocytes rapidly clear the apoptotic bodies, the number of apoptotic cells at a certain observation point may lead to underestimation of the actual apoptotic rate. It is thus likely that even minor increases in the apoptotic rate contribute significantly to graft loss.

In experimental models, epithelial cell necrosis occurs after lung transplantation (Fischer et al. 2000b, Neuringer et al. 2002). It is inevitable that necrotic cell death also contributed to loss of epithelium in the bronchial grafts. However, we demonstrated that high apoptotic activity in conjunction with the changes in epithelial morphology and destruction signaled OB development. Immunosuppression that was effective in preventing alloimmune activation also led to a reduced rate of epithelial apoptosis, indicating that in our model increased apoptotic activity is primarily a result of alloimmune injury. This is further evidence that in OB, apoptotic death of epithelial cells is an important mechanism in the events leading to graft deterioration.
3. **TNF-α mediates epithelial injury and inflammatory and fibroproliferative changes in OB**

A correlation appeared between expression of TNF-α and epithelial injury and obliteration. Inhibition of TNF-α delayed onset of OB by reducing inflammation, especially the CD8+ lymphocytes, and delayed the process of epithelial destruction and luminal occlusion.

The multiple functions of TNF-α are considered crucial in the initiation and maintenance of the inflammatory cascades (Kollias et al. 1999). By binding TNF-α, infliximab reduces leukocyte trafficking and diminishes expression of adhesion molecules and chemokines (Taylor et al. 2000, Smeets et al. 2003), and also inhibits angiogenesis (Maini et al. 1999). Furthermore, by binding membrane-bound TNF-α, infliximab is capable of inducing apoptosis in T lymphocytes (Van den Brande et al. 2003, Di Sabatino et al. 2004). On the other hand, in patients with Crohn’s disease, infliximab reduces intestinal epithelial cell apoptosis (Zeissig et al. 2004). In OB, inflammatory cell invasion is one of the key events (Reinsmoen et al. 1993, Neuringer et al. 1998, Boehler et al. 1999, Neuringer et al. 2000, Maasilta et al. 2001); in our model, this was attenuated in infliximab-treated bronchial allografts, and better graft preservation was evident. Likely explanations for the lower grade of rejection were the ability of infliximab to down-regulate chemokines and adhesion molecules as well as directly to inhibit the lymphoproliferative and cytotoxic actions of TNF-α. Induction of T cells and inhibition of epithelial cell apoptosis may also have occurred.

Inhibition of TNF-α had the greatest influence on the number of CD8+ lymphocytes and to some extent on macrophages. This may have occurred because the rapid influx of inflammatory cells was largely due to CD8+ lymphocytes. In addition to the decreased inflammation, reduced bronchial wall fibrosis was evident in anti-TNF-α antibody-treated allografts. TNF-α is involved in the development of lung fibrosis (Zhang et al. 1997, Brass et al. 1999), and in OB, TNF-α has been suggested to up-regulate the expression of profibrotic growth factors (Aris et al. 2002). Our findings support the profibrotic role of TNF-α in OB development.
The potential of TNF-α inhibition in acute and chronic lung rejection has previously been explored in rodent models. In studies of acute rejection, either polyclonal anti-TNF-α antibodies (Saito et al. 1993) or neutralizing TNF-α antisera (DeMeester et al. 1993) have been administered. The results were contradictory, with the former study showing no benefit from TNF-α inhibition, while the latter showed significant attenuation of acute rejection in the treatment group. In chronic rejection studies, treatment with serial doses of antibodies against TNF-α (Smith et al. 2001) or with TNF-α-soluble receptor (Aris et al. 2002) prevents or delays OB development. Recently, reduction in OB was linked to diminished intragraft TNF-α mRNA and protein expression after treatment with inhibitors of p38 kinase or poly (ADP)-ribose synthetase (Farivar et al. 2004a-b).

Considering the initial avascularity of our bronchial grafts, we administered the drug infusion prior to the removal of the left lung lobe. Thus, infliximab was already present in the tissue at the time of subcutaneous implantation. After infliximab treatment, we observed a significant delay, but as expected, no prevention of OB. Repeated drug administration may have resulted in extended survival, although in humans, at a 5-mg/kg dose, infliximab is detected in the serum up to 8 to 12 weeks (Kavanaugh et al. 2000, Cornillie et al. 2001). The dosing interval is between 6 and 12 weeks for disease-suppressing activity in chronic inflammatory diseases (Braun et al. 2003).

Because various cytokines participate in the rejection process (Boehler et al. 1999, Neuringer et al. 2000), inhibition of a single cytokine is ineffective in preventing the progression of the obliterative process. Our results demonstrated, however, that inhibition of TNF-α with infliximab, an anti-TNF-α antibody widely used in clinical practice, ameliorated the development of OB in transplanted small airways. This study further confirms that TNF-α may play a role in OB development by promoting inflammation and epithelial injury, and suggests that in lung transplant recipients, inhibition of TNF-α may prove beneficial.

### 4. PDGF and TGF-β play a role in OB pathogenesis

Our study demonstrated up-regulated expression of PDGF-A, of PDGF receptors, and of TGF-β in rejection of bronchial allografts. Imatinib treatment reduced the extent of
inflammatory changes and the rate of the obliterative process, and it modified the expression patterns of PDGF-A and PDGF receptors.

In rodent tracheal allografts, the expression of PDGF-A, of PDGFR-α, and of PDGFR-β is to some extent upregulated, whereas PDGF-B positivity is more prominent in syngeneic grafts (Kallio et al. 1999). In some experimental and clinical studies, however, PDGF-B expression in OB increases (Hertz et al. 1992, Bergmann et al. 1998, Aris et al. 2002). Induction of PDGFR-β synthesis is considered crucial to chronic vascular rejection (Rubin et al. 1988, Savikko et al. 2003). Our finding that, despite the mild expression of PDGF-B, PDGFR-β was markedly upregulated in allografts supports previous observations (Savikko et al. 2001). An explanation for this phenomenon may be the low levels of PDGF-B, which in turn activate PDGFR-β synthesis.

TGF-β has been detected in OB at sites of inflammation and fibrosis (El-Gamel et al. 1999) and is suggested to serve as an OB marker (Charpin et al. 1998, El-Gamel et al. 1999). In rodent tracheas, blocking of TGF-β binding to its receptor type III or blocking TGF-β downstream signaling through Smad3 reduces intraluminal fibrosis (Liu et al. 2002, Ramirez et al. 2004). In our porcine bronchial allografts, that high levels of TGF-β expression were encountered supports the role of TGF-β as an important mediator in OB. TGF-β is thought to function as the main up-regulator of CTGF and can induce a prolonged CTGF response (Xie et al. 2005). Despite the mild staining of TGF-β in autografts, CTGF expression between autografts and allografts was uniform. High CTGF expression associates with various fibrotic disorders, but in contrast, normal tissue expression of CTGF can also be very prominent (Moussad et al. 2000). In our model, the short avascular period may have up-regulated many mediators. The expression of CTGF may be up-regulated also by other growth factors, autoinduction, and physiological factors (Igarashi et al. 1993, Oemar et al. 1997, Riser et al. 2000). It is probable that TGF-β-independent regulation of CTGF occurred in bronchial grafts.

Imatinib had a strong influence on the number of CD4+ and CD8+ lymphocytes and on macrophages, suggesting its having immunosuppressive properties. In vitro studies have also shown that imatinib inhibits activation and proliferation of CD4+ and CD8+ T cells in addition to dendritic cells (Appel et al. 2004, Cwynarski et al. 2004, Dietz et al. 2004,
Seggewiss et al. 2005). In mice, it inhibits the delayed-type hypersensitivity reaction (Dietz et al. 2004). The effect of imatinib on T-cell function is independent of v-Abl, c-Kit, and PDGF receptors (Dietz et al. 2004), and recently it was suggested to occur via inhibition of tyrosine kinase LCK in the TCR signaling cascade (Seggewiss et al. 2005). In addition, imatinib reduced IL-2 production (Seggewiss et al. 2005).

Those findings on imatinib’s effect on T cell signaling in vitro may apply to our work as well. However, PDGF plays a crucial role in inflammatory responses by acting as a significant mediator between macrophages and T cells, and by up-regulating and amplifying inflammatory reactions (Daynes et al. 1991, Brody et al. 1992, Morisaki et al. 1994, Krettek et al. 2001). Furthermore, in experimental OB, depletion of recipient macrophages leads to a significant decrease in graft PDGF mRNA expression and in rate of obliteration (Oyaizu et al. 2003). In our model, in rejected bronchial allografts, inflammatory cells strongly expressed PDGF-A and PDGF receptors, and imatinib significantly reduced this. These findings suggest that attenuation of T cell responses may have occurred through interruption of PDGF signaling between macrophages and T cells and inhibition of PDGF receptor function on T cells. In our imatinib-treated grafts, decreased endothelial expression of PDGF receptors probably diminished the extent of cellular infiltration into the grafts, although inhibition of other T cell signaling mechanisms may also have been involved.

In rat studies, intraperitoneally administered CGP 53716, a precursor molecule of imatinib, with or without CsA, prevents or delays development of OB (Kallio et al. 1999, Tikkanen et al. 2003). CGP 53716 affects solely fibroblasts and smooth muscle cells, and thus the authors suggest that its effect is mediated via PDGF receptor inhibition. In experimental aorta and heart transplantation, imatinib in combination with CsA does not affect the staining intensity of PDGF-A, nor the numbers of macrophages (Sihvola et al. 2003). However, in rodent kidney transplantation, this combination reduces the number of graft-infiltrating CD4+ cells and macrophages and reduces PDGF ligand and receptor expression and chronic rejection (Savikko et al. 2003); the authors detected no staining of c-kit or v-Abl, suggesting that imatinib functions via PDGF receptor signaling. Intraperitoneally injected imatinib reduces pulmonary fibrosis in mice (Daniels et al. 2004). These authors suggest that imatinib also inhibits TGF-β signaling in fibroblast cultures through c-abl, a member of the Abl family of kinases. In our study, the effect of imatinib on fibroblasts was evident as reduced expression
of PDGF receptors and delayed obliteration. Because imatinib did not affect TGF-β levels, we speculate that in bronchial allografts imatinib interferes with PDGF signaling pathways.

This study confirms that in OB, PDGF and TGF-β function as mediators. An agent inhibiting PDGF receptors, imatinib, modified the inflammatory responses and the expression patterns of PDGF-A and PDGF receptors. This supports the concept that in OB, pathways signaled through PDGF receptors are involved.

5. Up-regulation of collagen III mRNA associates with development of OB

We assessed the cells expressing collagen type I and III mRNA during development of OB in bronchial allografts. Development of OB was associated with an increase in fibroblasts expressing collagen III mRNA. This expression increased parallel with luminal obliteration and number of fibroblasts and amount of extracellular matrix in the bronchial wall.

Increase in number of cells expressing collagen I and III genes and enhanced mRNA expression by individual cells are both suggested to contribute to pulmonary fibrosis (Shahzeidi et al. 1993, Shahzeidi et al. 1994, Zhang et al. 1994). In addition, an increase in type III collagen occurs (Vuorio et al. 1989). Elevated levels of the aminoterminal propeptide of type III collagen have been detected in BAL fluid of patients with fibrosing alveolitis (Lammi et al. 1999), in adult respiratory distress syndrome (Marshall et al. 2000), and in pulmonary edema (Chesnutt 1997), predicting poor prognosis. In post-transplant OB, excessive deposition of type III collagen in the bronchial submucosa correlates with poor lung function (Zheng et al. 1999). We compared the expression of collagen I and III genes and found a dominance of type III collagen expression in fibroblasts in the obliterate plug and in the bronchial wall of rapidly obliterating allografts. These findings suggest an association between increased synthesis and deposition of collagen III and the severity of a disorder.

Collagen biosynthesis can be regulated at transcriptional, post-transcriptional, and translational levels (Raghow et al. 1989). Modulation of mRNA stability and translation into protein are important mechanisms in determining net collagen production (Adams 1989). Various exo- and endogenous factors affect this process (Bateman et al. 1981, McAnulty et al. 1995). Influx of inflammatory cells releasing cytokines and growth factors, together with the action of immunosuppressive agents, has diverse effects on fibroblasts. In bronchial allografts, we found upregulation of PDGF-A, TGF-β, and TNF-α (II, III). TGF-β promotes
the transcription and stability of procollagen mRNA (Penttinen et al. 1988, Kenyon et al. 2003), and together with PDGF–A and TNF-α promotes fibroblast replication and chemotaxis (Kovacs et al. 1994, Geremias et al. 2004). Glucocorticoids reduce collagen synthesis by reducing levels of procollagen mRNA (Oikarinen et al. 1986). In addition, inhibition of the mTOR pathway is demonstrated to inhibit collagen mRNA transcription and stability (Shegogue et al. 2004). On the other hand, CsA has been shown to promote type III collagen transcription (Oelligiani et al. 2000). In our immunosuppressed allografts, histological findings and the number of cells producing type I and III collagen mRNA resembled those of autografts. Immunosuppression with CsA, everolimus, and MP not only inhibited immune activation and fibrogenesis, but probably also affects the transcriptional and translational rate of collagen mRNAs in individual cells.

In bronchial autografts, collagen I and III gene expression observed at early time points led to minor fibrotic changes. Nonimmunologic injury caused by the heterotopic method may have resulted in this. CTGF expression observed in autografts (III) may provide a link between this injury and early collagen gene activation, as CTGF can be induced by mechanical stress (Schild et al. 2002). Although expression of type I and III collagen mRNA was detectable both in autografts and in immunosuppressed allografts, only insignificant pathological alterations were evident. An important finding was the unaltered number of cells expressing collagen III mRNA.

Quantitation of total collagen and collagen I and III mRNA did not directly correlate with the increase in positive cells detected by in situ hybridization. In autografts and immunosuppressed allografts, cartilage remained viable and proliferating. Total collagen content probably reflected more the changes in the collagen type II derived from cartilage than the changes in relatively small fibrotic areas of the bronchial wall or lumina in nontreated allografts. Quantification of mRNAs by Northern hybridization does not identify the tissue origin of mRNA, and the strong expression of collagen genes in immunosuppressed allografts at some assessment points may have been detected in the pericartilaginous area.

In conclusion, this study showed that collagen I and III mRNA were expressed in fibroblasts in OB lesions, with a preponderance of and increase in cells expressing type III mRNA. In autografts and in allografts with immunosuppression adequate to prevent OB, only a minor degree of collagen gene activation occurred. In these groups, no changes in genetic activity
between type I and III collagen were observable, in contrast to the situation in obliterateing allografts.

6. Conclusions

Transplanted lung may develop OB as a response to alloimmune injury. In this pathogenic process, epithelial cell injury, inflammatory cell invasion, and fibroblast proliferation and extracellular matrix accumulation take place. The data in this thesis suggest that these pathological changes are associated with increased epithelial cell apoptosis and increased production of TNF-α, PDGF, and TGF-β, as well as synthesis of collagen III mRNA.

In bronchial allografts, high apoptotic activity together with changes in epithelial morphology and destruction signaled OB development. When immunosuppressive therapy was sufficiently effective to prevent epithelial injury, few epithelial cells appeared apoptotic. This indicates that increased apoptotic activity in bronchial grafts is caused primarily by alloimmune injury. These results support the idea that epithelial cell apoptosis is an important mechanism in the events resulting in OB.

This study showed a correlation between increased expression of TNF-α and airway epithelial injury and obliteration. Inhibition of TNF-α by infliximab delayed the process of epithelial destruction and luminal occlusion, suggesting that TNF-α is a crucial mediator of epithelial injury, as well as of inflammatory and fibroproliferative changes in OB.

Up-regulation of PDGF-A, of PDGF receptors, and of TGF-β occurred in rejection of bronchial allografts. These findings confirm the contribution of PDGF and TGF-β to OB. An agent targeting PDGF receptors, imatinib, modified the expression patterns of PDGF-A and PDGF receptors, and had a prominent influence on the number of CD4+ and CD8+ lymphocytes, and of macrophages. This indicates the importance for OB development of the pathways signaled through PDGF receptors.

Predominance of fibroblasts expressing collagen III mRNA increased parallel to progression of bronchial wall fibrosis and luminal obliteration. In autografts and in allografts with immunosuppression adequate to prevent OB, only a minor degree of collagen gene activation occurred without changes in genetic activity between types I and III collagen. This
observation indicates that one of the mechanisms in the fibrotic process of OB is an increase in fibroblasts expressing collagen III mRNA.

The studies presented in this thesis provide further understanding of the mechanisms of OB and offer insights into future development of treatment options.
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