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Airway epithelium in obliterative airway disease

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Chapter 1

General Introduction

1. Obliterative bronchiolitis and obliterative airway disease

1.1. *Obliterative Bronchiolitis*

Lung transplantation is currently the only available treatment for end-stage lung disease patients. Despite the success of improved modern lung transplantation with the introduction of new surgical techniques, improved immunosuppressive agents and innovations in managing of acute rejection and infection, the survival rate of recipients is 75% at 1 year and less than 50% at 5 years. (1) One of the most severe complications after lung transplantation is obliterative bronchiolitis (OB) which affect over 40% of the recipients within 5 years during the post-transplantation period.(1).

OB is a chronic disease that develops from months and mostly years after lung transplantation (2-4). It is characterized by progressive bronchial inflammation, epithelial injury and luminal fibrosis.(5-7). There is no treatment for human OB and insight into the understandings of its mechanism is still lacking. Presently, all clinical efforts are directed at slowing down the process. These efforts include pre-transplant treatment to the donor lung to reduce inflammatory inducing factors, employment of aggressive peri-transplant administration with antibiotics (3;8-11) and improved immunosuppressive regimens. Only re-transplantation appears to be a curable solution, but is mostly not possible due to the limited availability of donor organs. Clinical investigation of OB in humans is restricted by the limited amount of patient material available for research. This makes the development of new treatments a difficult and time consuming task (12). Thus, a simple animal model that resembles the development of OB in human is a desirable goal.

1.2. *Animal transplant models of obliterative airway disease (OAD) for human OB*

As pointed out above an animal model is needed for studying the pathophysiology and possible treatment of OB. A rat lung transplantation model was developed in Groningen and described by Jochum Prop and colleagues in 1984 (13). Using this technique they

were the first to show that OB was secondary to acute pulmonary rejection (14). Also, OB-like airway damage was seen during chronic rejection and viral infections in this rat lung transplantation model. (15) Hertz and his colleagues published a much simpler model in 1993, where they transplanted murine heterotopic tracheas subcutaneously (16), and found that allogeneic tracheas were rejected with massive cellular infiltration and epithelium loss as opposed to isografts which did not show these changes. This led by day 21 to obliteration of the transplanted trachea lumen by the fibroproliferation. This pathological process is called the obliterative airway disease (OAD) and is supposed to reflect the changes seen in OB. Large animal OAD models have also been studied in the past decades. Porcine, canine or even primate tracheas (17) and lungs (18-21) were transplanted and shown to mimic the human OB, since similar histological findings such as graft infiltration, epithelium loss and fibrotic luminal occlusion were observed. Although large animals may be close to humans in size and body structure, the limitations of these large animal models are also obvious: the lack of inbred strains, the cost of purchasing large animals and the peri-transplantation management. The biggest obstacle, however, is the unstable results (22). In summary, OAD in small animal models share histopathological similarities to human OB and is presently the most useful tool for human OB study.

1.3. *The histopathology and immunology of OB/OAD*

Clinical OB is a chronic inflammatory disease of the airways, involving the bronchial epithelium and leading to the gradual obliteration of small and large airways by inflammatory infiltrates, proliferating fibroblasts, mature collagen and extracellular matrix (3). Although the mechanisms underlying the development of OB are not clear yet, the syndrome may be divided into two distinct phases; an acute alloimmune phase with lymphocytic infiltration of the bronchiolar structures followed by a chronic fibroproliferative phase leading to partial or total occlusion of the airway lumen (3;4;10). These pathogenic phases appear to mimic a tissue 'injury-repair' type of pattern in which episodes of potentially reversible acute rejection (injury) lead to the irreversible chronic state of rejection (insufficient repair leading to scar tissue formation).

Transplant injury is caused by the repeated immune-mediated damage inflicted to the airways through allorecognition during acute rejection involving activation of inflammatory mediators (23). Both the humoral and cellular arm of the immune system may be involved. Donor-HLA-specific alloantibodies can be present in the recipient serum as evidence of a humoral response (24;25). More importantly, allo-specific T cells and a plethora of other immune cells observed in the donor lung indicating a cellular response (26-29). These immune processes may lead to the injury of airways. If the injury is not properly balanced by repair processes, excessive migration and proliferation of pulmonary mesenchymal cells, smooth muscle cells and fibroblasts may occur. These processes are driven, at least in part, by the growth factors platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF). PDGF and bFGF have been shown to be upregulated in bronchoalveolar lavage fluid from OB patients in the clinic (30). Fibroproliferation indicates a repair phase of the injured grafts, though it may eventually lead to an overgrowth of fibrotic tissue.

In animal OAD models, a number of pathophysiology features are observed that mimic human OB. A growing body of evidence suggests that development of OAD after trachea or lung transplantation is caused by the allogeneic immune response against cell surface antigens of the allospecific major histocompatibility complex (MHC) antigens, expressed on parenchymal cells of the allograft (24;29;31;32). In different animal OAD models, T lymphocytes, macrophages and granulocytes were found in the allografts preceding epithelium loss and complete luminal obliteration at day 21-28 were observed (CD4+, CD8+ T cells) (5;33-35). Several studies in recent years have indicated that indirect allorecognition of donor MHC-derived peptides by CD4 T cells is one of the most important factors leading to the development of chronic allograft rejection. (36-39) As a result of alloantigen recognition by CD4 (T-helper) cells, which recognize class II MHC antigens expressed by the graft's cells, an alloimmune response is started. Cytotoxic T cells (CTLs), mainly CD8+ cells which recognize class I MHC antigens, may directly kill target cells (40). But CD4+ cells may be more harmful by recruiting also the cells belonging to the innate immune system.

The airway epithelium has been mentioned several times already in the pathogenesis of OB/OAD. We think that it may play a

role both in the induction of the injury and in the repair process. In the following part we will focus on this aspect.

2. Airway epithelium in transplantation

2.1. Airway epithelium structure and function

ANATOMY Mammalian airways are lined by a pseudostratified layer of epithelial cells. These include the highly differentiated ciliated cells that cover the airway luminal surface and the less differentiated basal cells bearing a high capacity for proliferation and regeneration (41;42). Other types of cells in the epithelium layer are present in lower number. Some of these are only present at scattered locations, such as goblet cells and Clara cells. These epithelial cells are involved in a number of critical functions related to normal homeostasis.

FUNCTION Classically, epithelium was considered to be a passive barrier between the external environment and the inner tissues of the lung. However, it is now clear that the epithelium plays a pivotal role in controlling many airway functions. Its function as a barrier is essential through tight junctions (zonula occludens) located between the apices of adjacent cells, which restrict paracellular diffusion of electrolytes and other molecules. Desmosomes, intermediate and gap junctions are also involved in maintaining the structural integrity of the epithelium (43-45). The epithelium has also a secretory function and can produce a diverse array of lipid mediators, growth factors, and bronchoconstricting peptides as well as chemokines and cytokines (45). In addition, the epithelium is a major source of arachidonic acid metabolites which help to regulate airway smooth muscle tone, epithelial mucus secretion, neurotransmitter release and, is also involved in inflammation (44-46).

EPITHELIAL RESPONSE TO INJURY Tissue repair is dependent on a structured progression of events that re-establish the integrity of the damaged tissue. The precise mechanisms involved in regeneration of the airway epithelium are still a matter of debate. Much of what is known about epithelium injury and repair (46) either comes from *in vivo* studies in which the epithelium is experimentally injured, such as by chemical (47), drugs (48), physical factor (49) and repair processes are followed histologically over time, or from cell

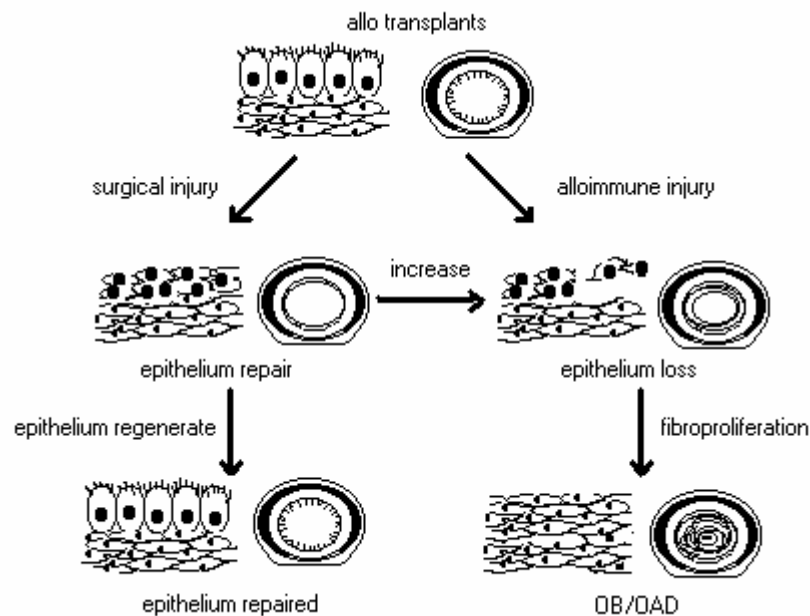
culture models (50). It is clear that the airway epithelium has a tremendous capacity to repair itself after injury. In a recent *in vivo* study, Erjefalt et al. showed that in as little as 15 min after an 800- μ m wide wound was made in the tracheal epithelium of guinea pigs, epithelial cells on the damaged margin (including secretory and ciliated cells) began to dedifferentiate, flatten and migrate over the denuded area (51). In this study, the denuded area was completely covered by a thin layer of flattened undifferentiated epithelium. Proliferation of epithelial cells was not observed until about 30 hours after the initial stimuli. Within 5 days a fully differentiated epithelium was present again. At approximately the same time as cells begin to differentiate, a plasma exudate was observed which might be an indication of functional repair (52-55).

2.2. Airway epithelium injury during transplantation

Airway epithelium injury is frequently observed upon transplantation. Airway epithelium damage may be already induced before transplantation by the donor's brain death (56), and by ischemia-reperfusion (57-59) during the transplantation procedures. Evidence of inflammatory cytokines up-regulation such as interleukin (IL)-1, IL-2, IL-6, tumor necrotic factor alpha (TNF- α) and interferon gamma (IFN- γ) is shown in peripheral organs including lung tissue from brain-dead donors shortly after harvesting (60). It is known that TNF- α and IL-1 are the "early response cytokines" produced by alveolar macrophages in acute lung injury through activation of the transcription factor nuclear factor-kappa B (NF- κ B) (61). Upon transplantation, these cytokines induce inflammatory responses towards donor epithelial cells by attracting recipient macrophages and other inflammatory cells (62). This may contribute to the acute injury seen in donor tissue after transplantation. Furthermore, donor tissue that expresses allo MHC antigens induces cellular and humoral alloimmunity in the recipient. Human epithelial cells are known to continuously express MHC antigens in high density (63,64). A recent study shows that the binding of MHC alloantibodies to human epithelial cells induces apoptosis of these cells (65). Epithelial cells apoptosis was also observed in animal airway transplant OAD (66).

Clinical pathologic features of OB suggest that in particular, the injury of epithelial cells by persistent inflammation in small airways hampers epithelium regeneration and stimulates fibroproliferation due to aberrant tissue repair. In animal tracheal transplants, airway epithelium is involved in the rejection process as one of the targeted tissue leading to OAD (67). The loss of airway epithelium by transplant rejection in allografts but also by enzymatic removal in isografts resulted in OAD (68;69). This stresses the importance of epithelium for the development of OB/OAD.

Figure 1. Hypothesis of airway epithelium in OAD/OB occurrence



In the illustration, airway allograft is transplanted and rejection occurs. There are two type of rejecting process: 1. Graft-independent process (left down arrows). It is caused by normal grafting surgical procedures and foreign body responses. It occurs to every type of grafting and is not dependdnt on the type of grafts. Epithelium mostly experiences ischemia-reperfusion damage and could quickly recover. 2. Graft-dependent process (right down arrows). It is caused by alloimmune responses that recognize allo-MHC antigens expressed in allografts. It occurs only in the allgraft transplantation and is suggested to be the main cause of graft injury and dysfunction.

3. Hypothesis and experimental models

3.1. *Hypothesis of OB/OAD*

Studies in patients and animal models indicate that donor airway epithelium injury is playing a role in the process of OB (6;70) and OAD (71). We hypothesize that transplant injury causes airway epithelium damage and that excessive and persistent loss of epithelium results in fibrosis that eventually leads to the occurrence of OB/OAD.

Firstly, airway epithelium is one of the primary target tissues during alloimmune responses that are graft-dependent. It is also the target of graft-independent factors, such as general inflammatory response and transplant surgical injury. This type of injury is presented among all types of airway tissue (67) and is largely dominated by alloimmunity. Loss of epithelium during injury 'denudes' the transplanted airway, resulting in loss of the defense barrier and, generally, epithelium dysfunction. As a result, the submucosa tissue may directly encounter foreign pathogens that increase the risk of infection. Secondly, damaged airway epithelium may lose its own functional role in regulating the airway repair process during injury (72). In response to injury, airway epithelial cells are capable to dedifferentiate and to regenerate to replace the injured cells, as indicated above. In vitro (73;74) and vivo (75) studies have shown that airway epithelial cells also regulate fibroblast proliferation. Normal epithelial cells inhibit fibroblast proliferation in vitro probably by excretion of inhibitory cytokines (72;76). More specific investigation of the response of epithelium to transplant injury seems justified. At the moment, however, we have no good model directly focusing on role of airway epithelial cells in the development of OB/OAD.

3.2. *Transgenic animal model for epithelium specific immune response in OAD study*

Trachea transplantation is a convenient technique for investigation of epithelial responses. A drawback of studying OAD in an allogeneic tracheal transplant is that immune responses are directed against all cell types of the graft tissue. This is so, because the recipient's immune system recognizes alloantigens on all cells expressing these

antigens. So all cells in the trachea grafts are attacked and it is impossible to investigate specifically the airway epithelium injury and its effects in OAD development after transplantation.

Transgenic animals expressing a neoantigen on a specific type of tissue may provide a new option to investigate the role of epithelium specific response in OAD. In a recent study, trachea transplants from HLA-class I transgenic mice were shown to induce alloreactive CD4 T cells and alloantibodies against the HLA-class I neoantigens (77). Although this is clearly an improved model to investigate the role of a single alloantigen (HLA-class I) in OAD, it still has a major drawback, since all types of tissue cells may express the neoantigen, making it not suitable for the study of single type of tissue, such as epithelium. To allow investigation of immune injury to be directed against an epithelium specific neoantigen in mouse tracheas, we decided to use the human epithelial glycoprotein-2 (hEGP-2) transgenic mice as donors. In these transgenic mice, hEGP-2 antigens are expressed exclusively on epithelial cells. Using hEGP-2 transgenic mice as donors, we were able to investigate whether the hEGP-2 antigen induces epithelium specific immune response after trachea transplantation and whether the response causes epithelial injury in the transgenic trachea transplants. This model also gave the opportunity to immunize recipients prospectively with the hEGP-2 antigen before transplantation. In this way, a pre-existing immunity directed exclusively to transplanted epithelium was induced.

3.3. *Blockade of immune responses and airway epithelium protection*

In clinical transplantation, immunosuppressive agents such as cyclosporine A (CsA), FK506 and rapamycin have been shown to effectively prolong graft survival (78). Using these drugs, alloreactive T cell responses are either reduced or blocked. Possible side effects, such as the CsA involvement in pro-fibroproliferation (79) or the pro-inflammatory effect of CsA and FK506 (11), are hampering the full exploration of these therapies. Another approach is the treatment by (monoclonal) antibodies against T cells, such as Anti-thymocyte Globulin (80-82), Anti-Cytotoxic T Lymphocyte Antigen-4 (83) or antibodies against CD2 and CD3 T cell common antigens (84). These antibodies have been used for depletion of T cells for prolong graft

survival. However, all these drugs are not specific for alloreactive T cells but modulate other normal T cells as well. This increases the recipient sensitivity to infection as the body defense system is suppressed.

Recently, a novel monotherapy using anti-CD45RB monoclonal antibodies (mAb) to deplete recipient T cells was investigated to block T cell responses and to prolong allograft survival (85). The CD45RB molecule belongs to a family of transmembrane protein tyrosine phosphatases that is expressed by leukocytes and play a critical role in regulating T-cell activation through modulating the activation status of the T cell receptor. In animal studies, mAb MB23G2 against CD45RB prolonged allograft survival through T cell depletion. These antibodies are T cell specific, easy in management and cause no severe side effect in animal models. Prolonged survival has been demonstrated after engraftment of islets (86), hearts and kidneys in MHC disparate mice after the treatment of recipients with anti-CD45RB mAb (87;88). Therefore, it seems worthwhile to investigate if a protective effect on transplant airway epithelium preventing OAD can be found in our mouse trachea transplant model.

4. Main issues addressed and the scope of this thesis

4.1. *Epithelium dynamics during injury and its role in OAD*

It is essential to know the behavior of airway epithelium upon transplantation injury and to establish its role in airway obliteration. We transplanted rat tracheas to observe airway changes post transplantation in a rat model (**chapter 2**). Injury was caused by rejection of the MHC fully mismatched tracheas and by enzymatic denudation of syngeneic transplants. Analyses were focused on epithelium integrity, the time point at which fibroproliferation and obliteration occurred. A series of experiments was carried out to stimulate repair of airway epithelium by isolation and reseeded of epithelial cells in trachea transplants.

4.2. *Epithelium specific immune responses*

In chapter 3, we introduced the hEGP-2 transgenic mouse OAD model for the study of epithelium specific immune responses. The

recipient immune responses against the transgenic grafts were studied in regards of both humoral and cellular reactivity. The effect of the immune responses on epithelium and its subsequent effect on OAD were analyzed. This is for the first time that the effect of a single type of tissue specific immune response is studied in a transplant setting.

4.3. ***OAD development under pre-existing immunity***

In **chapter 4** the OAD development under pre-existing epithelial cell specific immunity was studied further. Recipient mice were immunized before transplantation to induce pre-existing immunity for hEGP-2 grafts. The reasoning is to mimic the immunity in allotransplantation where alloantibodies and T cells may directly recognize alloantigens. In our model, however, the pre-existing immunity was directed exclusively towards epithelium. This allowed us to analyse epithelium behavior and its effect on OAD development.

4.4. ***Anti-CD45RB antibody monotherapy to prevent OAD***

The prevention of OAD by blocking allreactive T cells in an allogeneic trachea transplantation model is evaluated in **chapter 5**. To reduce the recipient T cells, a leukocytes antibody anti-CD45RB was administered in this model and the effect of this antibody on epithelium protection and OAD development was evaluated.

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