Activation of Eosinophil CCR3 Signaling and Eotaxin Using a Bioinformatics Analysis of a Mouse Model of Obliterative Airway Disease

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The role of eosinophils in the development and progression of chronic allograft rejection is recognized in multiple organ transplantation settings. The CCR3 signaling pathway is one of the key regulatory pathways in eosinophil migration to the engrafted tissue. Eotaxin is a ligand for CCR3 and reflects eosinophilic inflammation, which can lead to fibrosis. We hypothesized that the CCR3 pathway would be upregulated in obliterative airway disease (OAD) in an established model of chronic airway allograft rejection. The mouse gene microarray data from a heterotopic mouse model of OAD in the NIH Gene Expression Omnibus (GEO) repository were analyzed for differentially expressed eosinophil pathways, using the Partek Suite and Ingenuity Pathway Analysis. A P value of <0.005 was defined as significant for differential expression, and P value of <0.05 for pathways. Day 25 allografts were defined as chronic allograft rejection and day 4 as acute allograft rejection. The isografts and allografts at day 25 showed significant upregulation of the eosinophil CCR3 pathway ($P=0.04$), based on the analysis of 1,299 uniquely expressed genes. The isografts at day 4 were compared with those at day 25 based on the identification of 1,859 unique genes, and there was a trend toward the CCR3 pathway upregulation over time ($P=0.06$). CCR3 pathways were not upregulated during the progression of alloimmune rejection in the allografts at day 4 versus day 25 in comparison, based on the analysis of 1,603 genes. Eotaxin was upregulated in chronic allograft rejection by 2.5-fold. The eosinophil signaling pathway CCR3 and eotaxin were significantly expressed in chronic allograft rejection and our results imply a role in controlling early alloimmune damage in controls.

Introduction

THE SUCCESS OF heart–lung and lung transplantation continues to be limited despite advances in immunosuppressive regimens and advances in the molecular understanding of allograft rejection. Obliterative bronchiolitis (OB) is a result of an injury response to a multitude of factors ranging from the development of lymphocytic bronchiolitis, viral infection, vascular rejection, and recurrent acute rejection episodes.

Microarray analysis of an established mouse model of OB is termed obliterative airway disease (OAD), and has identified key cytokines such as interleukin (IL)-2, -4, -10, and interferon gamma (IFN- γ) involved in the development of OAD. Analysis has previously identified upregulation of chemokine receptor activity and chemokine expression ($P<0.08$); (Lande and others 2005). CXC ligand 9 expression was noted to increase 64-fold in this model, but the analysis did not include pathway analysis of the entire CCR3 pathway.

Prior human studies have identified eosinophil activation in lung lavage fluid, using the eosinophil activation product,

eosinophilic cationic protein (ECP), as a surrogate marker for eosinophil activation. In 50 bronchoalveolar (BAL) samples obtained from 38 lung transplantation recipients, BAL ECP was found to be higher in concentrations among patients with acute allograft rejection (Dosanjh and others 1997).

T helper cells type 2 (Th2) release IL-5 and IL-9, which in turn, activate eosinophil pathways. Eosinophils constitutively express CCR3 and its receptor. CCR3 signaling is responsible for migration of eosinophils to the graft. It is the primary regulatory molecule in eosinophil chemotaxis and found in a variety of inflammatory cells, including Th2 cells. Its expression in the setting of allograft rejection may indicate that the level of immunosuppression is not sufficient to suppress expression of inflammatory pathways. Eotaxin (CCL11) is a specific ligand for CCR3 and serves as a potent chemoattractant for eosinophils (DeLucca 2006). The CCL11/CCR3 interaction potentially therefore results in eosinophil migration and sequestration into an injured lung tissue, which can lead to fibrosis. This study was undertaken to provide a focused mechanism-driven approach in the

TABLE 1. CHRONIC INJURY AND ACTIVATION OF THE CCR3 PATHWAY

	<i>Isograft</i>	<i>Allograft</i>	<i>P value</i>
Day 4	Early	Early	ns
Day 25	Chronic	Chronic	0.04
<i>P value</i>	0.06	ns	

A comparison of early (day 4) versus late (day 25) allograft rejection and CCR3 gene expression is shown. A time-related trend of differential upregulation of CCR3 gene expression is demonstrated ($P=0.06$).

ns, non significant.

bioinformatics analysis of microarray data in GEO, obtained from the murine model of OAD. We hypothesized that based on the literature demonstrating a role for eosinophil activation in immune-regulated fibrotic responses, the CCR3 pathway may be upregulated in an established murine model of chronic allograft rejection (OAD).

Methods

For this study, the mouse gene microarray data from a heterotopic mouse model of OAD in the NIH Gene Expression Omnibus (GEO) repository were analyzed for differentially expressed eosinophil pathways (Khatn and others 2011), using the Partek Suite and Ingenuity Pathway Analysis. A P value of <0.005 was defined as significant for differential expression, and P value of <0.05 for pathways. Day 25 allografts were defined as chronic allograft rejection and day 4 as acute allograft rejection.

Results

During the early phase of graft injury, isografts and allografts did not demonstrate significant differential expres-

sion of the CCR3 pathway as shown in Table 1. Isografts were compared with allografts at day 4 (early injury) and showed only minimal changes in eosinophil-related pathways in this model, as shown in Table 1.

The isografts and allografts during the late fibrotic phase though showed significant upregulation of the eosinophil CCR3 pathway ($P=0.04$), based on analysis of 1,299 uniquely expressed genes. When the isografts at day 4 were compared with those at day 25, based on the identification of 1,859 unique genes (Fig. 1), there was a trend toward the CCR3 pathway upregulation over time ($P=0.06$). In contrast, this was not the case when allografts at day 4 were compared with those at day 25, based on the analysis of 1,603 genes.

The isografts at day 4 were compared with those at day 25 based on the identification of 1,859 unique genes (Fig. 1), and there was a trend toward the CCR3 pathway upregulation over time ($P=0.06$). This time-related regulation was not demonstrated in the allografts at day 4 versus those at day 25 in comparison, based on the analysis of 1,603 genes.

Eotaxin, a key chemokine ligand for CCR3 was 2.5-fold increased in expression for day 25 allografts compared with isografts. Further analysis of genes related to allergic inflammation, in particular, the IL-17 family, IL-4, and IL-5 did not show significant changes utilizing our bioinformatics approach.

Discussion

Our bioinformatics study indicates that in an established animal model of OAD, the eosinophil-related CCR3 pathway and eotaxin gene expression were significantly differentially expressed. This is one of the first descriptions of the expression of this pathway in OAD, and the first to utilize a

FIG. 1. A Venn diagram showing the number of genes uniquely expressed. Based on the analysis of 1,299 uniquely expressed genes, there was significant upregulation of the eosinophil CCR3 pathway ($P=0.04$).

bioinformatics approach in elucidating the eosinophil signaling pathways involved in the progression of allograft rejection. Our study supports the differential activation of the CCR3 signaling pathway once the lesion has been established in allografts, and perhaps in maintaining the isograft airway lumens. This study suggests that CCR3 signaling may be controlling or responding to fibroproliferative changes in the graft.

As the graft injury progresses from the early inflammatory stage, the differential upregulation of the CCR3 pathway was demonstrated only among isografts. Whereas upregulation occurred over time in both isografts and allografts, only the isografts demonstrated early to late injury differential expression. Interestingly, the CCL11 (Eotaxin) ligand for CCR3 increased 2.5-fold at day 25.

These results imply that the CCR3 signaling regulation is important in the control of airway fibrotic responses in an established murine model of OAD. Further study is needed to better understand the pathogenesis of OAD in relation to the production of CCR3 ligand CCL11 (eotaxin). The results suggest that the ligand is produced, but did not result in significant upregulation early in allograft injury.

The study of eosinophil signaling pathways in the development of early and late allograft dysfunction has yielded variable results. Most studies, depending on the organ allograft, support the role of eosinophils in the regulation of fibroproliferation and collagen deposition in the graft. CCR3 is a receptor for eotaxin, and our study provides evidence that eotaxin and CCR3 signaling pathways are differentially expressed in chronic allograft rejection (OAD).

In one of the first studies to describe eosinophil infiltration and activation in cardiopulmonary transplantation, 886 cases of moderate to severe cardiac or pulmonary acute allograft rejection were reviewed. Among the biopsies analyzed, 12% demonstrated tissue eosinophilia not associated with peripheral eosinophilia, atopy, or infection. The patients with tissue allograft eosinophilia also demonstrated higher tissue ECP concentrations and lower fractional shortening ($P < 0.01$). The eosinophil scores did not correlate significantly with forced expiratory volume or room air partial pressure of oxygen (mm) (Dosanjh and others 1998).

In a study of human cardiac allograft rejection, 169 endomyocardial biopsies were studied. The authors reported that there was no significant alteration in eotaxin, the ligand for CCR3, but that the CCR3 pathway correlated with T-cell CD3-positive infiltrates (OR 2.98, $P < 0.001$) and acute rejection (OR 1.88, $P < 0.06$). The results indicated that CXCR3-positive cells were significantly associated with acute allograft cardiac rejection ($P = 0.01$, OR 19) (Melter and others 2001).

In another study utilizing a murine model of vascularized heterotopic cardiac transplantation, cluster algorithms and self-organizing mapping identified 9 differential genes. Among these, CCR3 was found to have a unique profile of transcriptional expression in the process of heterotopic cardiac allograft rejection in day 1 mice compared with day 7 (Damrauer and others 2002).

In a study of a rat model of acute cardiac rejection, eotaxin protein concentrations were elevated at day 28 in donor organs compared with control recipient explanted organs. The authors reported that quantitative real-time polymerase chain reaction (RT-PCR) showed an increase in mRNA expression of CCR3, the receptor for eotaxin during

the acute and early allograft rejection. The primary cells identified with increased eotaxin mRNA expression were within infiltrating macrophages, but not in mast cells (Zweifel and others 2009).

In a study of 47 consecutive heart transplant recipients, with a median follow-up period of 90 days, the role of CCR3 chemokines, including eotaxin, in predicting acute allograft rejection was studied (Trull and others 2004). The authors reported that changes in peripheral eosinophil concentrations in the early postoperative period are directly related to plasma eotaxin levels. The levels and pharmacokinetics of immunosuppressive medications such as cyclosporine and prednisolone have inhibitory effects on eotaxin mRNA expression and protein production. The study showed that eosinophil concentrations were inversely related to prednisolone dosage (Trull and others 2004). The authors suggest that eosinophils may be a surrogate marker of the state of immunosuppression and useful in predicting acute allograft rejection (Trull and others 2004). Other studies have suggested that CCR3 is important in regulating eosinophil migration.

In a study of human skin xenografts, from atopic individuals, engrafted in a humanized mouse model of allergic cutaneous reactions, the authors used a blocking antibody to CCR3, which resulted in a reduced number of eosinophils in the graft. These findings suggest that one potential role of CCR3 is to regulate eosinophil migration to the graft (Senechal and others 2002).

In a murine model of acute graft versus host disease, the expression of 30 chemokines or chemokine receptors were studied in the lung using RT-PCR at 1, 2, 3, and 6 weeks after allogeneic bone marrow transplantation. Based on this study, CCR3 was not identified as associated with acute injury (Bouazzaoui and others 2009).

The CCR3 pathway is associated with T helper subtype 2 responses in the setting of renal allograft rejection. In a study of 31 human renal biopsy specimens, CCR3 was absent in the biopsies of acute cellular rejection. In contrast, CXCR3 ligand IP-10, CCR5 and its ligands were upregulated. The authors concluded that in the process of renal allograft rejection, Th1 responses predominated (Seeger and others 2001). In a review of 29 consecutive patients with renal allograft dysfunction and allograft nephrectomies, significant interstitial graft eosinophilic infiltrates (SIGEI) were diagnosed when eosinophils represented 10% or more of interstitial inflammatory infiltrates in the graft. SIGEI was noted in 13 of 29 patients, but was absent in 16. Banff II vascular rejection was present in 11 of the 13 patients with SIGEI, and a statistically significant association was present ($P = 0.04$), compared with only 3 or 9 grafts with both SIGEI and vascular rejection (Meleg-Smith and Gauthier 2005). The authors concluded that the presence of high numbers of eosinophils may be a helpful criterion in the pathologic diagnosis of renal allografts. This study lends support to our findings that the CCR3 signaling pathway associated with Th2 inflammatory mediators and eotaxin gene upregulation is involved in the development of OAD.

Infections may predispose the graft to the development of allograft dysfunction. The role of another chemokine family, CXCR3, was studied during community-acquired lung infection among 51 lung transplant recipients. There were 34 patients without community-acquired respiratory viral infection as controls. Among the 17 patients with a

community-acquired viral infection, 47% patients had early grade 3 graft dysfunction compared with only 29% in the control group. The differences in graft dysfunction were not studied, however, in relation to absolute CXCR3 levels.

The lower airway lavage sample analysis of IFN- γ inducible CXC chemokine ligands revealed that CXCR3 chemokine ligands though increased at the time of infection diagnosis, the authors suggested that this may be related to chronic allograft dysfunction (Weight and others 2012). Further study to evaluate the diagnostic and predictive value of chemokine ligands in establishing the diagnosis of chronic lung allograft dysfunction is needed.

Our study utilized a bioinformatics approach to study the eosinophil-related signaling pathways and ligand of CCR3, eotaxin, in an established murine model of OAD. The acute phase in this model is characterized by epithelial loss and regeneration (day 4) and the late phase at day 25 is associated with obliteration of the airway and fibroproliferation (Lande and others 2005). Our analysis identified additional differentially expressed eosinophil regulatory signaling in the development of OAD.

One of the drawbacks of this approach is that there are limitations in studying human bronchiolitis obliterans in a murine model. This particular model utilizes the trachea rather than whole or small airway grafts. Other models of airway injury in OAD have included human–mouse chimeric l grafts. These models may offer more similarity to the human process of OB when studying immune effectors and pathway regulation (Xue and others 2011).

Conclusions

Using a bioinformatics approach, the eosinophil signaling pathway CCR3 and eotaxin were significantly expressed in chronic allograft rejection animal models. Our study demonstrates the use of bioinformatic mining of animal model data related to OAD. This approach may be very useful in other transplantation animal models. Future studies in the area of bioinformatics and lung transplantation are needed to better understand the pathogenesis of OB.

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