Deficiency of 5-Lipoxygenase Accelerates Renal Allograft Rejection in Mice

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Acute renal allograft rejection is characterized by infiltration of the kidney parenchyma by a mixed population of inflammatory cells. Intense renal vasoconstriction also occurs during rejection, causing marked reductions in glomerular filtration rate and renal blood flow and eventually producing ischemic damage (1–3). Furthermore, the degree of functional impairment is often greater than expected from the morphologic appearance of the allograft (1–3). As a result, it has been suggested that infiltrating inflammatory cells produce locally acting compounds that impair renal hemodynamic function (2, 4). Previous studies have implicated products of the cyclooxygenase (COX) and 5-lipoxygenase (5-LO) pathways of arachidonic acid (AA) metabolism, released by these infiltrating cells, as important mediators of renal dysfunction and injury in acute rejection (4–10). Tissue ischemia is an unavoidable component of organ harvest and transplantation. In addition to potential immunomodulatory actions, LTs and lipoxins, which also depend on 5-LO for their synthesis, play key roles in the pathogenesis of ischemia-reperfusion injury (11).

Metabolism of AA by the 5-LO pathway produces two major biologically active species: LTB₄ and the cysteinyl-LTs, LTC₄, LTD₄, and LTE₄ (12, 13). LTB₄ is synthesized predominantly by neutrophils and interacts with high-affinity G protein-coupled receptors that mediate a number of leukocyte functions. In particular, LTB₄ is the most potent chemotactic and chemokinetic factor for neutrophils and stimulates adhesion of leukocytes to vascular endothelia for extravasation into adjacent tissue (14–16). LTB₄ also initiates neutrophil aggregation and degranulation (16), and has been shown to enhance production of cytokines by T cells and monocytes, including IL-1, IL-2, and IFN-γ (16–20). LTC₄, and its metabolites, LTD₄ and LTE₄, are produced primarily by eosinophils, mast cells, and macrophages. They are potent vasoconstrictors (21–24) and can cause contraction of endothelial cells, resulting in increased permeability of postcapillary venules (13, 14, 16). Cysteinyl-LTs also stimulate contraction of glomerular mesangial cells (25–27) and may alter production of other inflammatory mediators (28, 29). Based on their known biologic properties, enhanced synthesis of LTs during transplant rejection could therefore promote renal inflammation and injury through a variety of direct and indirect mechanisms.

Several studies by our group and others have suggested that LTs may be important in the process of transplant rejection (6, 7, 9, 10). Enhanced synthesis of LTB₄ and cysteinyl-LTs has been observed in rat (9) and dog (6) models of acute renal allograft rejection. Data also suggest that LTs produced within grafts during rejection affect the function and survival of the transplants. Previously, we determined that administration of a specific LT inhibitor or cysteinyl-LT receptor antagonist significantly improved renal hemodynamic function in rat allograft recipients (9). Mangino et al. (7) found that treatment with a combined COX and LO inhibitor, but not a COX inhibitor alone, maintained glomerular filtration rate and renal blood flow at levels similar to isograft controls, and reduced both the severity of inflammatory cell infiltrates and the degree of tissue damage in a dog model of renal allograft rejection.

Until recently, defining the contribution of LTs to transplant rejection has depended upon the use of pharmacological agents that selectively block LT activity, either by inhibiting LT biosynthesis...
or by antagonizing receptor binding. The development of mouse lines deficient in the ability to synthesize LTs, by introducing mutations into the 5lo gene (29, 30), has provided another means by which the role of these inflammatory mediators can be examined in vivo. The objective of the present study was to use 5LO-deficient mice in a model of renal allograft rejection to further investigate the importance of LTs in transplantation. Based on previous data, we hypothesized that the loss of 5-LO would have beneficial effects on the survival of renal allograft recipients, suggesting that 5-LO metabolites play a key role in maintaining kidney allograft function.

Materials and Methods

Mice

The generation of 5LO-deficient mice has been previously described (30, 32). The 5lo-/ mice and corresponding wild-type animals are on an inbred 129 (H-2b) genetic background. CByD2F1/J (BALB/cByJ X DBA/2JF1) (H-2b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were males at least 8 wk old, and were bred and maintained in specific pathogen-free animal barrier facilities at the University of North Carolina and the Durham Veterans Affairs Medical Center.

Renal transplants

For the allograft groups, kidneys from 5lo+/+ CByD2F1/J (CBy) mice were transplanted into fully allogeneic 129 recipients. As nonrejecting isograft controls, kidneys from CBy donors were transplanted into CBy recipients. Renal transplants were performed as previously described (4, 33). Briefly, the donor kidney, ureter, and bladder were harvested en bloc, including the renal artery with a small aortic cuff and the renal vein with a small caval cuff. Anastomoses were created between the vascular cuffs and the recipient abdominal aorta and vena cava, respectively. Donor and recipient bladders were anastomosed to the ureter. The entire anastomosis was flushed with warm PSS for 30–40 min. The right native kidney was removed at the time of transplantation, and the left native kidney was removed through a flank incision 3–5 days later. Overall surgical mortality was ~25–30% and was not different between the experimental groups.

Renal hemodynamic studies

To precisely assess renal allograft function, clearances ofulin were measured 2 wk after transplantation, as previously described (n = 7 for isografts, n = 8 for 5lo+/+ , and n = 7 for 5lo–/– ) (4, 33). Inulin clearance is a measure of glomerular filtration rate (GFR). Briefly, on the day of study, animals were anesthetized with 0.04 mg/g pentobarbital, and a polyethylene catheter (PE-90) was inserted into the trachea to facilitate spontaneous ventilation. The left carotid artery and left jugular vein were cannulated en bloc, including the renal artery with a small aortic cuff and the renal vein with a small caval cuff. Anastomoses were created between the vascular cuffs and the recipient abdominal aorta and vena cava, respectively. Donor and recipient bladders were anastomosed to the ureter. The entire anastomosis was flushed with warm PSS for 30–40 min. The right native kidney was removed at the time of transplantation, and the left native kidney was removed through a flank incision 3–5 days later. Overall surgical mortality was ~25–30% and was not different between the experimental groups.

Histopathologic studies

Following the renal hemodynamic studies, a portion of the transplanted kidney was removed and placed in 10% buffered Formalin. After Formalin fixation, kidneys were sectioned and stained with H&E. All of the tissues were examined by a pathologist (P.R.) masked to the experimental groups. The overall severity of rejection was determined by examining the pattern of inflammatory cell infiltration in both glomeruli and tubulo-interstitial areas, and glomerular, tubular, and vascular abnormalities. As previously described, grading was performed using a semiquantitative scale, in which 0 represented the absence of histologic abnormalities, and 1, 2, 3, and 4 represented mild, moderate, moderately severe, and severe abnormalities, respectively. An overall histologic score for each kidney was obtained by summing the individual grades for the glomeruli, tubules, interstitium, and vasculature (34).

Immunohistology

Immunohistopathology was performed, as previously described (33). Briefly, separate portions of kidney allograft tissue were embedded in OCT and snap frozen in precooled 2-methylbutane. Tissue sections (4 μm) were cut with a cryostat, air dried, and fixed in acetone. Fixed sections were washed with PBS (pH 7.4), blocked with normal mouse serum, and stained with appropriately diluted primary mAb. Binding of the primary Ab was detected with affinity-purified biotin-goat anti-rat IgG, followed by a secondary layer of affinity-isolated FITC rabbit anti-goat IgG (Cappel, Durham, NC). The primary rat mAbs used included: TIB126 (anti-MHC class I), TIB120 (anti-MHC class II), GK1.5 (anti-CD4), and 3.155 (anti-CD8), prepared as hybridoma supernatants (American Type Culture Collection, Manassas, VA), along with 30-H122 (anti-Thy-1.2; Boehringer Mannheim, Indianapolis, IN), RA36B2 (anti-B220), and M170/HL (anti-CD11b/Mac-1; BD Pharmingen, San Diego, CA). The intensity of immunostaining were evaluated by an individual (J.L.P.) who was masked to the experimental groups.

Measurement of capacity for renal eicosanoid production

A portion of the transplanted kidney was removed and prepared for the measurement of the capacity for renal eicosanoid production, as previously described (9, 34). Briefly, the transplanted kidney was removed and then bisected, a central slice was obtained, and cortex was separated from medulla by macrodissection (n = 6 for isografts, n = 6 for 5lo+/+ , and n = 7 for 5lo–/– ). Portions of cortex were uniformly homogenized with a Tenbroeck tissue grinder and suspended in 2 ml of Kreb’s buffer containing the Ca2+–ionophore A23187 (20 μg/ml). The suspensions were incubated for 30 min at 37°C in 95% O2/5% CO2. Samples (measured in duplicate) were centrifuged at 3000 rpm for 10 min at 4°C, and supernatants were stored at −70°C until eicosanoids were measured as described below. The tissue pellets were resuspended in 2 ml of Kreb’s buffer and stored at −20°C until protein concentrations were measured using the Coomassie brilliant blue dye-binding assay (35).

Concentrations of thrombomodulin (TX) B2 and LT B4 in unextracted supernatants from incubations of renal cortex were measured by direct RIA, as previously described (4). Antisera and standards for TX B2 were obtained from AMI-Seragen (Boston, MA). Antisera and standards for LT B4 were obtained from Amersham (Piscataway, NJ). Samples (measured in duplicate) and standards were incubated for 20 min at 4°C with a mixture of antisera and known quantities of tritiated standard. After incubation, free, unbound eicosanoids were removed from the mixture with a suspension of dextran-coated activated charcoal, and tritium remaining in the supernatants was measured with a liquid scintillation counter (TM Analytical). Sample concentrations were determined by a standard curve in which the logarithm of the concentration was plotted against the logit of the B/B0 value. The concentration of TX B2 was normalized for protein content of the cortical homogenates and is expressed as picograms per milligram of protein. Lipoxin A4 levels in the renal cortical homogenates were measured by using direct enzyme immunoassay analysis (Oxford Biomed, Oxford, MI).

Cytokine mRNA expression

A portion of the allograft was removed, and total RNA was prepared using TRIzol according to the manufacturer’s directions (Life Technologies, Gaithersburg, MD). mRNA expression for cytokines was quantified by RNase protection assay using two multiprobe template sets (BD Pharmingen), as described previously (36). Riboprobes were prepared from one template set containing the cDNA for murine IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-15, GAPDH, and L32; and a second template set containing IL-12p35, IL-12p40, TNF-α, IL-1α, IL-1β, IL-1RA, migration inhibition factor, IL-6, and IFN-γ. Probes were prepared simultaneously using [γ-32P]ATP and T7 reverse transcriptase according to the manufacturer’s directions (BD Pharmingen). A total of 10 μg total RNA was assayed in each sample using the RiboQuant kit, as outlined by the manufacturer (BD Pharmingen). The protected fragments were size fractionated on an 8% acrylamide/8 M urea sequencing gel. Dried gels were placed on Kodak XAR film. Autoradiograms were scanned using a semiautomated densitometer, and the protected fragments were quantitated using an in vitro transcription kit according to the manufacturer’s instructions (Stratagene). The protected fragments were size fractionated on an 8% acrylamide/8 M urea sequencing gel. Dried gels were placed on Kodak XAR film. Autoradiograms were scanned using a semiautomated densitometer.
Statistical analysis

Data are presented as the mean ± SEM. For the hemodynamic studies, data points for each animal represent the mean of the values measured during at least two clearance periods. For quantitative data, statistical significance for comparisons between groups was assessed using an unpaired two-sample t test. Survival analysis was performed using the SAS software package (SAS Institute, Cary, NC), and statistical significance was determined using the Mann-Whitney U test.

Results

To evaluate the role of 5-LO in the recipient immune system during the course of renal allograft rejection, kidneys from CBByD2F1/J (CBy) mice were transplanted into fully mismatched 5-LO-deficient and wild-type 129 mice. As shown in Fig. 1, the 5lo mutation had a significant detrimental effect on survival of renal allografts. ~60% (11 of 18) of the 5-LO-deficient recipients died before week 3. In contrast, only 15% (3 of 19) of the wild-type allograft recipients died by the third week, and many of these animals survived for 5–6 wk after transplantation (Fig. 1, p = 0.0047). Moreover, mean survival time of 5lo–/– recipients was 27 ± 3.9 days, which was significantly less than that of wild-type recipients (39 ± 3.6 days; p = 0.015). All isograft controls survived 8 wk or more following transplantation.

To determine whether differences in histopathology or graft function could be detected at a time point before graft failure occurs, we assessed renal function and morphology 2 wk after transplantation. Allograft function was determined by directly measuring GFR in transplanted animals. As shown in Fig. 2, GFR was substantially reduced in wild-type (2.9 ± 0.5 ml/min per kg) and 5-LO-deficient (2.4 ± 0.4 ml/min per kg) allografts compared with nonrejecting isografts (6.2 ± 0.2 ml/min per kg; p < 0.005). However, at 2 wk posttransplantation, the 5lo mutation had no significant effect on renal allograft function. The levels of GFR in the 5-LO-deficient allografts were similar to those in the wild-type group (Fig. 2, p = 0.220) and, in both groups, these levels are sufficient to support normal health and survival.

To investigate whether the increased mortality of 5-LO-deficient allograft recipients was associated with discernible changes in graft histomorphology at an early time point, we compared the severity of renal morphologic abnormalities between the experimental groups at 2 wk after transplantation (Table I). In the isograft control group, renal histomorphology was normal and there was no evidence of infiltrating inflammatory cells, although mild to moderate levels of hypercellularity were detected in the glomeruli of some of the isografts. In contrast, wild-type and 5-LO-deficient allografts were markedly abnormal with histologic evidence of acute cellular rejection. In both allograft groups, the predominant finding was intense infiltration of inflammatory cells in interstitial and perivascular regions. Both focal and diffuse patterns of infiltration were seen. Glomerular and tubular pathologic changes were variable and less pronounced. As shown in Table I, there were no obvious differences in the pattern and/or severity of histomorphologic abnormalities between 5-LO-deficient and wild-type allograft groups when glomerular, tubular, interstitial, and vascular abnormalities were graded separately using a semiquantitative scale. To explore this issue further, we also performed immunostaining of allograft sections with Abs specific for T cells, CD4+ and CD8+ cells, macrophages, and B cells. There were no differences in the intensity and pattern of immune cell infiltration between allografts in wild-type and 5-LO–/– recipients at both 1 and 2 wk.

To determine the effects of the 5lo mutation on the capacity for renal LT production, we measured levels of LTB4 in A23187-stimulated preparations of cortex from the transplanted kidneys. These data are illustrated in Fig. 3. We find that LTB4 production by the allografts in 5lo+/+ recipients (12.3 ± 1.7 pg/min per mg protein) is significantly higher than that of isografts (6.1 ± 0.5 pg/min per mg protein; p = 0.011 vs 5lo+/+) or allografts in 5lo–/– recipients (7.4 ± 0.8 pg/min per mg protein; p = 0.02 vs 5lo+/+). There was no significant difference in the capacity for LTB4 generation between the isografts and allografts in 5lo–/– recipients (p = 0.26).

We and others have previously shown that the eicosanoid TXA2 is generated during rejection and mediates a substantial portion of the renal dysfunction observed in this model (4, 5, 8, 10). Additional studies have demonstrated that the loss of 5-LO may result in enhanced synthesis and activity of other eicosanoids, particularly COX metabolites (30, 32). Therefore, we explored the possibility that eicosanoid production may be altered in 5-LO-deficient allografts by measuring levels of TXB2 (the stable metabolite of TXA2) in cortical homogenates prepared from the transplanted kidneys and stimulated with the calcium ionophore A23187. As depicted in Fig. 3, the capacity for renal production of TXB2 was
increased in both wild-type (14.6 ± 4.7 pg/min per mg protein) and 5lo−/− (15.1 ± 2 pg/min per mg protein) allografts compared with isografts (6.4 ± 1.2 pg/min per mg protein; p = 0.06 and 0.002, respectively). However, we found no significant differences in TXB2 levels in renal cortical homogenates from 5-LO-deficient allografts compared with the wild-type group (Fig. 3, p = 0.461).

In addition, we measured the capacity for lipoxin A4 production by allografts from the experimental groups. Significantly higher amounts of lipoxin A4 were detected in both wild-type (22.2 ± 3.9 pg/min per mg) and 5-LO-deficient (24 ± 4 pg/min per mg) allografts compared with nonrejecting CBy isografts (12.9 ± 1.9 pg/min per mg; p < 0.05). However, lipoxin A4 levels in renal cortical homogenates from 5lo−/− recipients were similar to those seen in the wild-type allograft group (p = 0.374).

To further examine the potential mechanism by which the 5lo mutation accelerates renal allograft rejection, we analyzed cytokine mRNA expression within the allografts at 1 wk following transplantation by RNase protection assay. Using a multiprobe template set, we evaluated a panel of cytokines that have been suggested to play a role in allograft rejection, including the cytokines IL-1α, IL-1β, IL-2, IL-10, IL-15, IFN-γ, TNF-α, TGF-β1-3, and migration inhibition factor, along with the chemokines RANTES, macrophage-inflammatory protein (MIP)-1α, MIP-1β, MIP-2, and monocyte chemoattractant protein-1 (37, 38). Expression of mRNAs for these inflammatory mediators was increased in allografts compared with isografts, but the levels of expression were similar in the 5lo+/+ and 5lo−/− groups (data not shown).

Thus, accelerated rejection by 5-LO-deficient transplant recipients cannot be attributed to an altered profile of cytokine or chemokine expression.

Discussion
In the present study, we examined the role of LTs in kidney transplantation using mice that are genetically deficient in the 5-LO enzyme. Previous reports have suggested that LTs are involved in allograft rejection (6, 7, 9, 10, 34). Based on these findings and on the well-characterized proinflammatory activities of LTs, we expected that the 5lo mutation might have beneficial effects on the course of rejection, perhaps by improving renal function and histopathology and/or prolonging the survival of grafts. However, we have shown in this study that the absence of a functional gene for 5-LO results in a marked acceleration of the allograft rejection process. These findings are similar to those from a previous study in which we showed that the 5lo mutation decreased the survival of male MRL/MpJ-Fas−/− mice, a model of autoimmune disease (39). In those experiments, we also demonstrated that, despite the increased mortality, glomerular filtration rate and renal histopathology measured at an early time point in the disease were similar between 5-LO-deficient and wild-type MRL/MpJ-Fas−/− mice. In those studies, the major impact of 5-LO deficiency was seen in male mice. Sex differences in physiological responses have also been observed in LTB4 receptor-deficient mice (39). Because of technical constraints related to the transplant surgery procedure, we have used only male mice in the studies presented in this work, and potential sex differences in the contribution of LTs in this model of allograft rejection could not be assessed.

As we have described previously (9), the capacity for LTB4 production by kidney allografts in 5lo+/+ recipients is significantly higher than that of isografts. Levels of LTB4 were also higher in the allografts in 5lo+/+ recipients than 5lo−/− recipients consistent with the absence of functional 5-LO in the recipient mice. However, detectable LTB4 production was present in the allografts in 5lo−/− recipients. Since the kidney grafts came from 5lo+/+ donors, we assume that 5-LO associated with the donor tissue mediates LT production in this setting. As the levels of LTB4 in these allografts were virtually identical to those of isografts, we assume that these levels reflect the capacity of the kidney, along with its passenger leukocytes, dendritic cells, and macrophages, to generate LTs. Furthermore, we infer that the enhanced capacity for LTB4 generation by the 5lo−/− allografts is due to the influx of inflammatory cells that are associated with rejection. The generation of 5-LO products by these cells may contribute to the differences in transplant outcomes between the two allograft groups.

There are several potential explanations for the detrimental effects of the loss of a functional 5lo gene on graft rejection in addition to the absence of LTs. For example, the loss of 5-LO might enhance the synthesis and activities of other eicosanoids,
particularly the COX products of AA metabolism, which can adversely contribute to transplant rejection. We have previously reported that macrophages from 5-LO-deficient animals released increased amounts of PGE₂ and TXB₂ compared with wild-type controls (30), and we found an enhanced role for prostanooids in several acute inflammatory processes in 5-LO-deficient mice (30, 32). Furthermore, several studies have demonstrated enhanced production of COX metabolites, specifically TXB₂, in rat (5, 10, 41) and dog (10, 42) models of allograft rejection, and that TXB₂ contributes to transplant rejection (4, 5, 8, 10). However, we found no differences in the levels of TXB₂ measured in renal cortical homogenates from 5-LO-deficient allograft recipients compared with wild-type controls. In addition, expression of other inflammatory mediators, including cytokines such as IL-1, IL-2, and IFN-γ, was similar between both experimental groups.

We next considered the possibility that functional 5-LO might be required for the production of other lipid mediators, such as lipoxins, that may exert antiinflammatory or cytoprotective actions in graft rejection. The major pathways of lipoxin production require 5-LO enzyme activity in combination with one of the other lipoygenases, either 15-LO or 12-LO (43). Recent studies have shown that lipoxins play important immunoregulatory roles in leukocyte trafficking and inflammation, although a role for lipoxins in allograft rejection has not been identified (43). As the biochemical actions of 5-LO are a presumed requirement for lipoxin synthesis, we posited that lipoxin A₄ production should be dramatically reduced in 5-LO-deficient mice. However, we detected similar levels of lipoxin A₄ in allografts from both 5-LO-deficient and wild-type recipients. This suggests that 5-LO expressed in cells provided with the donor kidney is sufficient for lipoxin production. Alternatively, another pathway not requiring 5-LO may be responsible for lipoxin synthesis in this setting. Regardless of the exact source of lipoxin production, our findings do not support a model in which the detrimental affects of 5-LO deficiency on graft survival can be explained by reduced lipoxin production. However, because the renal cortical preparation used in these experiments may not directly reflect lipoxin A₄ synthesis in vivo over the entire posttransplant period, further investigation of this pathway in transplant rejection may be warranted.

The adverse effects of the 5LO mutation in renal allograft rejection suggest an unexpected protective effect of LT in this model. Although the bulk of current evidence indicates that LTs act primarily as proinflammatory lipid mediators, this study along with our previous experiments in the MRL/MpJ-Fas⁻/⁻ model (39) indicates the potential beneficial action of LTs in renal inflammation. Although the mechanism of this effect is not clear, it is possible that these actions may involve interactions with the family of peroxisome proliferator-activated receptors (PPARs) (44). Emerging evidence suggests that various lipid mediators are ligands for PPARs, a family of nuclear receptors that can act as transcription factors. In particular, studies by Devchand et al. (45, 46) have suggested that LTB₄ is a ligand for PPARα and that activation of PPARα by LTB₄ may function to limit the inflammatory response. Since LTB₄ levels were reduced in the allografts in 5LO⁻/⁻ recipients, our findings raise the possibility that interruption of LTB₄ activation of PPARα in this setting might contribute to accelerated graft rejection. With the recent cloning of the receptors for LTB₄ (47–49) and the peptidoleukotrienes (50), precise molecular dissection of the contributions of these different pathways to inflammatory responses such as allograft rejection should be possible. In this way, novel antiinflammatory pathways with strong influences on graft survival may be identified.

In summary, this study has shown that the absence of 5-LO accelerates renal allograft rejection in a mouse model of acute rejection. However, reduced survival was not associated with exaggerated impairment of renal hemodynamic function or enhanced pathological abnormalities at early time points. Similarly, the absence of 5-LO did not alter production of other inflammatory mediators by the allograft. These data demonstrate an unexpected protective role for 5-LO metabolites in allograft rejection.

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References


