Transfer of lymphocytes from mice with renal ischemia can induce albuminuria in naive mice: a possible mechanism linking early injury and progressive renal disease?

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Burne-Taney, Melissa J., Manchang Liu, Dolores Ascon, Roshni R. Molls, Lorraine Racusen, and Hamid Rabb. Transfer of lymphocytes from mice with renal ischemia can induce albuminuria in naive mice: a possible mechanism linking early injury and progressive renal disease? Am J Physiol Renal Physiol 291: F981–F986, 2006. First published June 6, 2006; doi:10.1152/ajprenal.00229.2005.—Severe ischemia–reperfusion injury (IRI) predisposes to long-term impairment in kidney function both in patients and experimentally through unknown mechanisms. Given emerging evidence implicating lymphocytes in the pathogenesis of early injury to kidney, liver, and lung after IRI, we hypothesized that kidney IRI would potentially release or expose normally sequestered antigens that would lead to proliferation of antigen-recognizing lymphocytes. This, in turn, would directly participate in progressive kidney injury. To test this hypothesis, we purified splenic lymphocytes from C57BL/6 mice with severe renal IRI or sham operation 6 wk postischemia and transferred these cells to normal mice. Donor mice with IRI had significant fibrosis and cellular inflammation. The recipient mice were followed for 6 or 12 wk. Donor lymphocytes were found to traffic into recipient kidney. Twelve weeks after transfer, kidneys from mice which received IRI-primed lymphocytes exhibited significantly increased urinary albumin excretion compared with lymphocytes from sham mice. Splenic CD3+, CD4+, CD3+CD4+, and CD4+CD44+ counts were significantly increased in mice after lymphocyte transfer from IRI mice vs. mice with lymphocytes from sham mice. These data demonstrate that lymphocytes from IRI mice can traffic to recipient kidney and directly mediate albuminuria. These data identify a novel mechanism by which initial kidney injury predisposes to long-term dysfunction and identify lymphocytes as potential therapeutic targets for progressive renal diseases.

Acute kidney injury has been associated with progressive renal disease following an ischemic insult (2, 3, 8). However, it is unclear exactly how acute injury leads to long-term tissue dysfunction. One possibility is that renal injury could potentially release or expose normally sequestered antigens. This, in turn, may cause proliferation of T cells that recognize these antigens and, if given the opportunity, might target the kidney in an autoimmune response, participating in the development of progressive renal disease. T cells play a role in ischemia–reperfusion injury (IRI) in both kidney and other organs (7, 11, 16), and a change in T cell IFN-γ production and trafficking occurs long term after severe renal IRI (8). Based on these data, plus data from a myocardial ischemia model where splenic lymphocytes were able to cause myocardial changes in recipient rodents (17), we hypothesize that lymphocytes from mice that have undergone a severe ischemic insult could cause changes associated with progressive renal disease after the initial ischemic injury.

To test this hypothesis, mice underwent 60 min of unilateral renal IRI and were maintained for 6 wk. At 6 wk postischemia, lymphocytes from these mice were transferred to normal C57BL/6 mice which were monitored for 6 or 12 wk. Transferred lymphocytes trafficked to recipient mouse kidneys, measured using a Thy1.1 congenic mouse approach to tracking cells. Mice that received lymphocytes from IRI mice, but not those receiving lymphocytes from nonischemic control mice, showed a significant long-term increase in albumin excretion in urine. Mice receiving ischemia-primed lymphocytes also demonstrated a systemic increase in activated and memory T cells. However, minimal changes were seen in kidney fibrosis.

METHODS

Mice. C57BL/6 male mice and B6 Thy1.1 congenic male mice (B6.PL-Thy1+/CyJ; both 5–7 wk old) were purchased from the Jackson Laboratories (Bar Harbor, ME). All animal procedures were approved by the institutional IACUC.

Renal ischemia model. A model of severe renal IRI in mice was used (10). Briefly, 25- to 35-g mice were anesthetized with inotrperitoneal pentobarbital sodium (75 mg/kg), had abdominal incisions, and then the right renal pedicle was bluntly dissected. A microvascular clamp was placed on the right renal pedicle for 60 min while the animal was kept at a constant temperature (≈37°C) and well hydrated. After 60 min, the clamp was removed, wounds were sutured, and the animal was allowed to recover. Animals were maintained for 6 wk before death. Sham animals underwent the same surgical procedures.

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without clamping of the renal artery. Sixty minutes of ischemia was chosen because it produces severe damage to the kidney. The unilateral model was chosen to prevent mortality of mice, since they would not survive 60 min of bilateral renal ischemia for 6 wk.

Isolation of splenic lymphocytes. Spleens were removed intact and teased apart in HBSS using a sterile technique. Spleens were then homogenized and washed in 2 ml HBSS and spun at 1,000 rpm for 10 min at 4°C. Spleen cells were then passed through nylon filters and washed. Cells were then placed over 5 ml of lympholyte M (Cedarlane Laboratories, Ontario, Canada) to purify the lymphocyte population and spun for 20 min at 1,000 g at room temperature. The central dense layer was collected, washed twice with 10 ml HBSS, and spun at 1,000 rpm for 10 min at 4°C.

Concanavalin A activation. Isolated lymphocytes at a concentration of 3.5 × 10^6 cells/ml were cultured in T-25 flasks containing 1 μg/ml of the T cell mitogen concanavalin A. The flasks were placed in a 37°C incubator at 5% CO2 for 3 days, after which they were transferred to a 50-ml tube and spun down at 1,400 rpm for 15 min. The cell pellets were finally resuspended into PBS and used for adoptive transfer experiments. The compositions of lymphocytes in these transferred cell preparations were analyzed by flow cytometry.

Adoptive transfer of activated T cells. Activated T cells from mice with ischemic injury and sham-operated control mice were injected via the tail vein. Cells were injected at doses 8.0–8.5 × 10^6 cells, and recipient mice were monitored for up to 6 or 12 wk.

Postischemic histological injury quantification. Kidneys were harvested at 6 or 12 wk posttransfer and analyzed for interstitial fibrosis by Masson’s Trichrome staining (6). Peritubular interstitial fibrosis was scored by a renal pathologist (LR) in a blinded fashion based on average percentages of high power fields.

Mouse urine albumin and creatinine. Mice were placed in specially designed metabolic cages overnight at pre (before transfer), and 1, 3, 6, and 12 wk posttransfer. Urine collected during this period was analyzed for mouse urine albumin excretion using an ELISA kit (Bethyl Laboratories, Montgomery, TX) according to the instructions. To correct for effects of urine volume on the individual albumin concentration, mouse urine creatinine concentrations were measured by an automated chemistry analyzer (Cobras Mira plus, Roche Diagnostics, Indianapolis, IN) with a 557A creatinine kit (Sigma Diagnostics, St. Louis, MO), and the mouse urine albumin excretion was presented as a ratio of urine albumin concentration to urine creatinine concentration.

Renal mononuclear cell isolation. Kidney tissue was disrupted mechanically in 10 ml of RPMI 1640 medium supplemented with 5% of newborn calf serum using a Stomacher 80 Biomaster (Seward). To remove debris, samples were passed through a glass wool column, and the resulting cell suspension was centrifuged at 300 g for 10 min to pellet the cells. The pellet was suspended in 36% Percoll (Amersham Pharmacia Biotech, Piscataway, NJ), gently overlaid onto 72% Percoll, and centrifuged at 1,000 g for 30 min. Cells were washed twice, then resuspended for staining.

Analysis of activated T lymphocytes by flow cytometry. Spleens were collected from euthanized mice, homogenized, and treated with ammonium chloride to remove red blood cells. Washed cells were then filtered through a 70-μm nylon cell strainer and counted in a hemocytometer. One million cells were treated with Fc Block for 30 min and then stained with appropriate FITC- or PE-labeled antibodies CD3 (clone 17A2), CD4 (clone L3T4, GK1.5), CD19, CD25, Thy1.1, Thy1.2, and CD44 (clone IM7; Pharmingen, San Diego, CA) for 1 h on ice. Cells were then washed twice and resuspended in 1% formaldehyde. Flow cytometry was performed using Epics II (Coulter, Hialeah, FL) gating on appropriate unstained and isotype controls.

Comparison and quantification of data. Data were expressed as means ± SE. ANOVA was used for statistical comparison of means of multiple groups. For paired data, Student’s t-tests were used. Statistical significance was set at P < 0.05.

RESULTS

Severe kidney ischemic injury leads to long-term development of interstitial fibrosis and cellular inflammation in donor mice. Donor mice underwent 60-min unilateral renal warm ischemia followed by reperfusion and were killed 6 wk after ischemia. Both ischemic and contralateral kidneys were sectioned and stained with Masson’s trichrome. Compared with the contralateral kidneys, which appeared enlarged but essentially normal, the ischemic kidneys had extensive tubular atrophy, interstitial inflammation, and fibrosis (Fig. 1).

Composition of adoptively transferred lymphocytes. After 6 wk of renal ischemia or sham operation, both wild-type and Thy1.1 donor mice were killed, and their splenocytes were isolated and activated in vitro. A total of 8–8.5 × 10^6 pooled cells were transferred into each normal recipient mouse with the following compositions: Thy1.1 donors (IRI vs. Sham): CD4, 16 vs. 19%; CD8, 4 vs. 6%; CD19, 72 vs. 66%. Wild-type donors (IRI vs. Sham): CD4, 19 vs. 30%; CD8, 5 vs. 4%; CD19, 72 vs. 58% (Fig. 2, n = 4). There was no significant difference in cell transfer between IRI and sham animals, wild-type, or Thy1.1 strains.

Renal Thy1.1 and Thy1.2 expression in recipient mice. At 6 wk after adoptive transfer, the wild-type recipient mice receiving splenocytes from B6 Thy1.1 mice (congensics) were killed and their renal mononuclear cells were isolated, stained with Thy1.1 or Thy1.2 antibody, and analyzed by flow cytometry to track donor lymphocytes infiltrating into a recipient mouse kidney. At 6 wk of adoptive transfer, the Thy1.1 CD4 and CD8 T cells were (sham vs. IRI, expressed as % of total lymphocytes) 2.74 ± 0.93 vs. 1.94 ± 0.28 and 1.43 ± 0.40 vs. 0.87 ± 0.23% (Fig. 3). There was no difference in adoptively transferred lymphocyte infiltration from IRI or sham donor mice. The bulk of the cells and remainder of the lymphocytes found in the kidneys were wild-type Thy1.2 expressing, and thus from the recipient (data not shown).

Urinary albumin excretion in recipient mice. To assess renal functional changes in the recipient mice, urine albumin was measured from urine collected pre, 1, 3, 6, and 12 wk after adoptive transfer of donor lymphocytes. Urinary albumin excretion, corrected for urine creatinine excretion, was increased in both IRI and sham groups at 1 wk and declined to baseline levels at 3 and 6 wk. However, urine albumin excretion had a trend to be increased 6 wk in mice receiving lymphocytes from IRI mice, which then became significantly increased compared with the sham-transferred lymphocyte mice 12 wk after transfer (Fig. 4; *P < 0.02).

Renal function in recipients long after adoptive transfer. To assess renal functional changes in the recipient mice long after receiving ischemic or sham operation-primed lymphocytes, we measured recipient mice serum creatinine before and at 6 or 12 wk after transfer and found that there were no significant increases in serum creatinine at 6 or 12 wk in either group (serum creatinine in mg/dl: sham vs. IRI, baseline, 0.18 ± 0.03 vs. 0.23 ± 0.06, P = 0.21; at 6 wk, 0.23 ± 0.05 vs. 0.30 ± 0.06, P = 0.36; 12 wk, 0.18 ± 0.03 vs. 0.23 ± 0.06, P = 0.40).

Histology of recipient mouse kidney after adoptive transfer. To assess for evidence of interstitial fibrosis in recipient mice after adoptive transfer of ischemic or sham-primed lymphocytes, kidneys were examined at 6 or 12 wk after transfer. Limited patchy peritubular fibrosis was seen in both IRI and...
sham-operated recipient mice. With the use of semiquantitative evaluation, the scoring of interstitial fibrosis per high-power field was comparable in IRI and sham lymphocyte recipient kidney (Fig. 5).

Splenic activated and memory T lymphocytes in recipient mice. Given that exposure to kidney ischemia could lead to activation of lymphocytes and expansion of a memory population, which in turn could participate in the development of the albuminuria, recipient splenocytes were stained with anti-mouse CD3, CD4, CD25, and CD44 antibodies. CD3+ and CD4+ lymphocytes were increased in mice receiving lymphocytes from IRI donors. In addition, splenic CD3+/CD25+ (activated T cells) and CD4+/CD44+ (memory T cells) were increased in mice receiving lymphocytes from IRI donors (Fig. 6: *P < 0.05, **P < 0.001 vs. sham; Fig. 7: *P < 0.05, **P < 0.001 vs. sham).

DISCUSSION

Many forms of disease that progress toward end-stage organ failure have some degree of mononuclear leukocyte infiltration. It has been shown that the cells recruited into an inflamed interstitium in the early phase of fibrosis consist mainly of CD4+ lymphocytes and macrophages (13). Based on studies in experimental myocardial ischemia where lymphocytes in the spleen could induce cardiac changes in recipient animals (17), we hypothesized that lymphocytes from mice long term after kidney ischemia could induce changes in the kidney when transferred to normal mice. There was trafficking of transferred lymphocytes to recipient mouse kidneys. We found that albuminuria was induced in mice that received lymphocytes from animals with kidney ischemia compared with control animals.
with sham operation, despite equal numbers and populations of cells transferred. In addition, an upregulation of activated T cells and memory CD4 T cells was observed in spleens of mice receiving lymphocytes from IRI donors compared with the mice receiving lymphocytes from sham-operated donors (n = 4, *P < 0.02 vs. sham group).

Even though T cells are found in increased numbers in tissues of many chronic and progressive diseases, their direct role in the pathogenesis of long-term tissue dysfunction and injury is less well understood. Targeting of T lymphocytes has recently been demonstrated to slow the progression of various progressive/chronic diseases such as anti-thy 1 glomerulosclerosis and chronic pancreatitis (19, 20). Both of these studies, which used FTY720, an immunosuppressant that decreases blood lymphocyte counts, demonstrated that decreasing the amount of lymphocyte trafficking to an injured organ can slow the progression of inflammation and fibrosis to the tissue. An episode of IRI can lead to long-term organ dysfunction. This is observed in humans with severe ischemic injury during kidney transplantation, with living unrelated kidneys having a longer half-life than better HLA-matched deceased donor kidneys (22). Experimental kidney IRI leads to long-term development of albuminuria, interstitial fibrosis, and renal lymphocyte infiltration (8). It is unknown whether the lymphocytes directly participate in the development of progressive tissue injury. An elegant study adoptively transferring splenocytes 6 wk after myocardial infarction in a rat model found that recipient rats developed some areas of myocardial inflammation, patchy fibrosis, but no functional changes as measured by echocardiography (17). It was concluded that posts ischemic injury led to unveiling of neoantigens, and a state of autoimmunity developed whereby immune cells then proceeded to progressively damage tissue. Based on the above observations, we closely followed the experimental approach followed in the cardiac ischemia model in rats, but applied this to kidney ischemia in a mouse model. Consistent with the cardiac ischemia study, we found changes in the target organ after transferring lymphocytes that were in a postischemic tissue milieu. However, unlike the cardiac study, we found a significant functional impact on the immune cell transfer, notably an increase in albumin excretion in the urine. Given that increased albumin excretion is an early finding in many progressive diseases, we conclude that similar to after cardiac ischemia, kidney ischemia can incite an immune response, which can in turn lead to progressive kidney dysfunction. However, unlike studies in heart, we did not find major histological changes, despite using
both Masson’s trichrome staining as well as conventional H & E staining. Reasons for this difference between the previous heart study and the current kidney one could be due to differences between heart and kidney injury. Differences could also be because the cardiac study was conducted in rats, whereas the current study was in mice. Furthermore, the cardiac study lasted up to 6 wk in the recipients, while we extended our studies to 12 wk, which was the point when the differences in albuminuria were most pronounced between groups. Experiments using larger numbers of animals will be needed to confirm our current findings. Furthermore, we acknowledge that albuminuria may not necessarily lead to later fibrosis.

An important question is whether the adoptively transferred cells trafficked to the recipient kidney. This question was not addressed in the cardiac ischemia study where transfer of lymphocytes led to changes in recipient hearts (17). We used the Thy1.1 mouse as a donor in one set of studies. This mouse strain has lymphocytes that express a distinct epitope on their T cells compared with wild-type mice and thus can be tracked with a specific antibody. By combining use of this congenic mouse with a kidney lymphocyte isolation and flow cytometric analysis technique, we demonstrated that adoptively transferred lymphocytes homed in on the recipient kidney. However, these were in small quantities compared with normal populations of wild-type cells, and similar numbers were seen in both lymphocytes transferred from IRI and sham mice. Thus it was likely that qualitative rather than quantitative changes occurred in the donor-transferred lymphocytes. In a previous study, splenic lymphocytes from mice with IRI were found to have a higher interferon-γ production than lymphocytes from sham-operated mice (8).

We examined the composition of splenic T cells in recipient mice with albuminuria vs. those without and found higher percentages of CD3+ and CD4+. More important was the finding that spleens of recipient mice that had received lymphocytes from IRI mice had an increase in their activated T cell population. This activated population could be producing proinflammatory and profibrotic factors responsible for the albuminuria.

Immunological memory ensures rapid and strong responses to immune antigens. T cell memory is due to both an increase in frequency of and qualitative changes in antigen-specific T cells (9, 12, 24). In mice, memory T cells express high levels of CD44 (5). We found higher levels of CD44 expression in spleens from mice which received lymphocytes from ischemic mice compared with mice that received lymphocytes from sham-operated mice. A variety of cytokines and cells are responsible for upregulation of CD44 including IL-15, IFN-γ, macrophages, and neutrophils. A recent study also found that CD44 can promote the recruitment of inflammatory cells to sites of chronic inflammation (10), of potential relevance to the current study, as there was increased neutrophil and macrophage infiltration into the kidney.

In a study examining alloantigen responses, as opposed to IRI, primed CD4+ T lymphocytes can induce chronic allograft nephropathy in an athymic rat transplant model (15). This study, however, was found to be dependent on the amount of CD4+ cells that were transferred. The primed CD4+ T cells were also able to induce antibodies against MHC Class I and GBM which eventually contributed to the development of chronic allograft nephropathy. It is possible that if we transferred even higher numbers of lymphocytes from mice with kidney ischemia, we may have found an earlier increase in albuminuria, or development of fibrosis in the recipient mice.

Although we focused on lymphocytes, other mononuclear cells may well play a role in fibrosis after injury. The infiltration of macrophages can be detected in early phases of progressive renal disease. In rats subjected to 5/6 renal ablation, macrophage infiltration started at 1 wk after ablation and increased progressively with time (23). Macrophages can mediate tissue injury through different pathways. Besides the ability to produce proteolytic enzymes, reactive oxygen species, and vasoactive substances, macrophages can synthesize cytokines and fibrogenic growth factors responsible for a sustained inflammatory milieu. The maintenance of these cells in later phases of renal disease suggests that macrophages are also involved in the chronic inflammatory process leading to the development of fibrotic changes. Therefore, in our model, it is possible that neutrophils and macrophages are also potential effectors of the transferred injury.

In summary, we demonstrate induction of albuminuria in naive mice injected with lymphocytes from mice with severe unilateral ischemic renal injury, as well as presence of an activated and expanded memory T cell population in these mice. An understanding of this observation, which suggests a pathogenic mechanism linking acute ischemic injury in the kidney to later albuminuria, could lead to therapies to abrogate progressive renal injury. This supports a model of allo-antigen-independent injury to the kidney leading to long-term dysfunction, with the lymphocyte being an important pathogenic modulator. Future steps include the identification of the exact lymphocyte population that is involved in the transfer of progressive injury along with the analysis of other organs in the recipient to determine whether this response is directly linked to the kidney or is a generalized response throughout the mouse.

GRANTS

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