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Genomic profiling of kidney ischemia reperfusion reveals expression of specific alloimmunity-associated genes: linking "immune" and "non-immune" injury events

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Abstract

Increased organ ischemia time leads to delayed graft function (DGF), increased acute rejection (AR), enhanced chronic allograft nephropathy (CAN) and reduced long term allograft survival. The mechanisms by which IRI predisposes to AR and CAN are unknown. We hypothesized that gene expression profiling of IRI-affected kidney would identify how IRI predisposes to AR and CAN. Furthermore, we examined how current immunosuppressive drug molecular targets are altered by IRI.

C57BL/6J mice were exposed to 30 (n=3) or 60 (n=3) min of bilateral kidney ischemia or sham surgery (n=5). At 36 hr kidney tissue was collected and analyzed using Affymetrix 430MOEA (22626 genes) array and GC-RMA-SAM pipeline. Genes with the false discovery rate (q<1%) and ±50% fold change (FC) were considered affected by IRI. Genes coding for histocompatibility and antigen presenting factors, calcineurin and mTOR pathway-associated proteins were selected using Gene Ontology (GO) analysis. GO analysis identified 10 and 17 alloimmunity-related genes affected by IRI induced by 30 and 60 min of ischemia, respectively including Traf6 (FC=2.99) and H2-D1 (FC=2.58). We also detected significant IRI genomic responses in calcineurin and mTOR pathways represented by Fkbp5 (FC=4.18) and Fkbp1a (FC=2.0); Eif4ebp1 (FC=16.8) and Akt1 (FC=3.64), respectively.

These data demonstrated that IRI upregulates expression of several alloimmunity-associated genes, which can in turn enhance alloimune responses. Our discovery of IRI-induced upregulation of genes associated with calcineurin and mTOR pathways are consistent with clinical observations that FK506 and Rapamycin can alter course of DGF. Further validation and dissection of these pathways can lead to novel approaches by which improved management of early "non-immune" transplant events can decrease susceptibility to more classic "immune" changes and CAN.

INTRODUCTION

There is accumulating evidence for direct correlation between duration of donor organ ischemia and rejection rates (1, 2). Increased organ ischemia time leads to delayed graft function (DGF), increased acute rejection (AR), enhanced chronic allograft nephropathy (CAN) and reduced long term allograft survival (3). This is of increased importance with the discrepancy between available organs and the growing transplant waiting list, leading to use of expanded criteria

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donor organs. Although, involvement of multiple inflammatory pathways in ischemia reperfusion injury (IRI) has been demonstrated, the mechanisms by which IRI predisposes to AR and CAN are poorly understood. Allograft injury pathways are represented by two major types of responses, antigen-dependent and antigen-independent. The antigen-dependent IRI-response is characterized by upregulation of major histocompatibility complex (MHC) antigen expression (4,5) which is interconnected with antigen-independent pathways (6-8) represented by innate-immunity (9,10), complement (11,12) and pro-inflammatory cytokine/chemokine (13-15) driven responses. We therefore hypothesized that global gene expression profiling of immune-related genes during development of IRI could reveal key mechanisms how alloantigen-independent injury enhances alloreactivity. We further postulated that analysis of IRI-implicated genes that are targeted by commonly used immunosuppressive drugs (16) will both identify possible mechanisms of IRI-induced alloimmunity as well as provide clues how these agents alter recovery from IRI. Previous reports on prolonged DGF observed in rapamycin-treated patients (17) and the reduction of IRI by experimental pharmacological preconditioning with FK506 (18) provided focus towards genes associated with calcineurin and mTOR signaling. To test theses hypotheses we utilized a well established murine model of kidney IRI and used current genomic techniques and in-depth computational analysis of molecular responses of kidney tissues to both moderate and severe IRI.

MATERIALS AND METHODS

All animal protocols have been approved by the Johns Hopkins Animal Care and Use Committee. Six to eight week-old mice (C57BL6/J), weighing approximately 25-30 grams were anesthetized with a 0.03 ml intraperitoneal injection of 10:1 ketamine (100 mg/ml or 135 mg/kg) and acepromozazine (10 mg/ml or 1.5 mg/kg). The flank regions were shaved, and the animals placed on a heating pad to keep a constant temperature that was monitored with a rectal thermometer. Bilateral flank incisions were made, and the kidneys exposed. The renal pedicles were bluntly dissected, and a nontraumatic vascular clamp (Roboz microaneurysm clamp, Roboz Surgical Instrument, Washington, DC) was applied across the pedicles for 30 min or 60 min. The animals received 100 ml/kg of warm saline instilled into the peritoneal cavity during the procedure. After the clamps are released, the flanks were closed and animals allowed recover with free access to food and water. At 36h after ischemia, the animals were reanesthetized blood collected by cardiac puncture, kidneys harvested and snap-frozen. 36 hrs was chosen because pilot studies revealed that many of the 60 min ischemia animals died at 48 hrs.

Blood samples were tested for serum Creatinine (SCr) levels (mg/dl) using Roche Cobas Fara automated system (Roche, Nutley, NJ, USA) and Creatinine 557 kit (Sigma Diagnostics, St Louis, MO, USA).

Total RNA (7-10 μg) from kidney tissues was isolated and reverse transcribed (primers oligo-dT coupled to a T7 RNA polymerase binding site). Double-stranded and biotinylated cRNA was synthesized using T7 polymerase. Unincorporated nucleotides were removed and the cRNA quantitated. This cRNA was randomly sheared to approximately 50 nucleotides and hybridized (16 hours) to the Affymetrix MOE430A (22,626 transcripts) GeneChip.. The quality of each step was monitored on an Agilent 2100 Bioanalyzer. The hybridized biotinylated cRNA was detected with phycoerythrin-streptavidin and quantitated by scanning (Agilent GeneArray Scanner). IRI affected genes were identified using GC-Robust Multichip Average (GC-RMA) and Significance Analysis of Microarrays (SAM) software. Genes with the false discovery rate ($q<1\%$) and ±50% fold change (FC) were considered affected by IRI. Genes coding for *histocompatibility* and *antigen presenting* factors were identified by Gene Ontology (GO) analysis using GenMAPP (19) and MAPPFinder (20) tools. Relation of IRI-implicated genes to FK-506 and Rapamycin was established using PubMatrix automated

biomedical literature search engine (21). Clustering analysis of candidate genes identified by all three approaches was conducted using the MultiExperiment Viewer (MeV) of the microarray software suite TM-4 (22).

RESULTS

Either 30 min or 60 min ischemia followed by reperfusion was used for identification of effects of ischemia and duration-related genomic changes in kidney tissues. Significant ($p<0.01$) increase in serum creatinine (2.31±0.7 mg/dl and 2.84±0.1 mg/dl in 30 and 60 min clamped mice, respectively) versus sham operated control (0.83±0.1 mg/dl) confirmed kidney injury and dysfunction. Gene expression analysis of IRI-affected kidney tissues demonstrated that extended exposure to ischemia affected a larger pool of genes with higher mean of gene expression fold changes (mFC) after 60 min ischemia (3615 genes, mFC=3.47) compared to 30 min ischemia (2150 genes, mFC=2.89). Gene ontology analysis identified 10 and 17 histocompatibility and antigen presenting genes affected by IRI after 30 and 60 min ischemia, respectively. The pathway analysis detected significant transcriptional changes of 24 genes involved in the calcineurin and 11 genes related to mammalian target of rapamycin (mTOR) signaling. The immunosupressant-association analysis of IRI-affected genes identified 97 FK-506/Tacrolimus-associated and 159 Rapamycin/Sirolimus-associated genes where 52 genes were linked to both pharmacological agents. The combined hierarchical clustering of candidate genes (identified by all three approaches) revealed several clusters of genes that were upregulated by IRI induced by 30min and/or 60 min ischemia (Figure 1).

DISCUSSION

The current gene expression profiling studies of moderate and severe IRI demonstrate specific gene expression alterations in response to IRI in mouse kidney detectable by microarray techniques. These experiments revealed a complex genomic response during IRI and led to identification of candidate genes associated with an IRI-induced alloimmunity. Our data demonstrated that IRI upregulates several alloimmunity-associated genes including Histocopatability 2 and 13, and Traf6 (23), thus increasing expression of antigen presenting molecules (Figure 1) and enhancing alloimune responses. We also identified significant IRI-induced transcriptional changes of genes involved in calcineurin pathway including Fkbp1a, Fkbp5, and Nfat5; and mTOR pathway including Akt1, Eif4ebp1, and Rps6ka1 (Figure 1). This discovery is consistent with clinical observations that FK506 and Rapamycin can alter course of DGF (24), and can reveal potential underlying mechanisms. Furthermore, association studies of non-specific effects of FK506 and Rapamycin on genes relation of which to calcineurin and mTOR pathways is not yet established identified additional IRI-induced candidate genes that could be involved in worsening allograft survival. The most upregulated representatives of this group (ICAM-1, Sdc1, Ptgs2) are shown in Figure 1. It has been reported that expression of ICAM-1 gene coding for cell surface adhesion molecule was not only affected by FK-506 (25) but also was linked to antigen presenting processes by enhancing presentation of cognate peptide MHC-I complexes to cytotoxic T cells. (26). The responsiveness of another surface molecule (syndican) coding gene Sdc1 to FK-506 treatment was identified in mouse tumor model. It has been demonstrated that Sdc1 expression was directly regulated by Fkbp1a (27). Notable, both these genes were upregulated in our IRI model (Figure 1). We also identified IRI-induced upregulation of Ptgs2 gene which is known to be responsive to calcineurin inhibitor treatment. This gene is coding for inflammatory regulator cyclooxygenase-2 (COX-2) and expressed in a regulated fashion in the kidney. The recent discovery of the binding site for the nuclear factor of activated T cells (NFAT), the member of calcineurin pathway (Figure 1) explains inhibition of COX-2 by Tacrolimus (28). The evaluation of these novel IRI-affected candidate genes and dissection of their corresponding

pathways can lead to new approaches by which improved management of early “non-immune” transplant events can decrease susceptibility to more classic “immune” changes and CAN.

In summary, our results suggests that diverse gene expression analysis is a useful tool in selecting candidate genes involved in IRI-associated pathophysiological processes with clear advantages of this complex approach over the single method analysis. Further investigation of the dysregulated alloresponse – related genes that we have identified in IRI could help develop new therapies for IRI and also modify how immunosuppressive medicines are used during delayed graft function.

REFERENCES

1. Tilney NL, Guttmann RD. Effects of initial ischemia/reperfusion injury on the transplanted kidney. Transplantation 1997;64(7):945. [PubMed: 9381538]
2. Terasaki PI, Cecka JM, Gjertson DW, Takemoto S. High survival rates of kidney transplants from spousal and living unrelated donors. N Engl J Med 1995;333(6):333. [PubMed: 7609748]
3. Boros P, Bromberg JS. New cellular and molecular immune pathways in ischemia/reperfusion injury. Am J Transplant 2006;6(4):652. [PubMed: 16539620]
4. Waddell TK, Gorczynski RM, DeCampos KN, Patterson GA, Slutsky AS. Major histocompatibility complex expression and lung ischemia-reperfusion in rats. Ann Thorac Surg 1996;62(3):866. [PubMed: 8784021]
5. Penfield JG, Wang Y, Li S, et al. Transplant surgery injury recruits recipient MHC class II-positive leukocytes into the kidney. Kidney Int 1999;56(5):1759. [PubMed: 10571784]
6. Savransky V, Molls RR, Burne-Taney M, Chien CC, Racusen L, Rabb H. Role of the T-cell receptor in kidney ischemia-reperfusion injury. Kidney Int 2006;69(2):233. [PubMed: 16408111]
7. Rabb H. The T cell as a bridge between innate and adaptive immune systems: implications for the kidney. Kidney Int 2002;61(6):1935. [PubMed: 12028434]
8. Nikbakht-Sangari M, Qayumi AK, Keown PA. The role of inflammatory mediators in the mechanism of the host immune response induced by ischemia-reperfusion injury. Immunol Invest 2000;29(1):13. [PubMed: 10709843]
9. Wu HS, Zhang JX, Wang L, Tian Y, Wang H, Rotstein O. Toll-like receptor 4 involvement in hepatic ischemia/reperfusion injury in mice. Hepatobiliary Pancreat Dis Int 2004;3(2):250. [PubMed: 15138120]
10. Kim BS, Lim SW, Li C, et al. Ischemia-reperfusion injury activates innate immunity in rat kidneys. Transplantation 2005;79(10):1370. [PubMed: 15912106]
11. de Vries B, Walter SJ, Peutz-Kootstra CJ, Wolfs TG, van Heurn LW, Buurman WA. The mannose-binding lectin-pathway is involved in complement activation in the course of renal ischemia-reperfusion injury. Am J Pathol 2004;165(5):1677. [PubMed: 15509537]
12. Sacks SH, Zhou W. Locally produced complement and its role in renal allograft rejection. Am J Transplant 2003;3(8):927. [PubMed: 12859526]
13. Molls RR, Savransky V, Liu M, et al. Keratinocyte-derived chemokine is an early biomarker of ischemic acute kidney injury. Am J Physiol Renal Physiol 2006;290(5):F1187. [PubMed: 16368740]
14. Kaminska D, Tyran B, Mazanowska O, et al. Cytokine gene expression in kidney allograft donor biopsies after cold ischemia and reperfusion using in situ RT-PCR analysis. Transplant Proc 2003;35 (6):2155. [PubMed: 14529873]
15. Chen D, Ding Y, Schroppel B, et al. Differential chemokine and chemokine receptor gene induction by ischemia, alloantigen, and gene transfer in cardiac grafts. Am J Transplant 2003;3(10):1216. [PubMed: 14510695]
16. Treska V, Molacek J, Kuntscher V, et al. Immunosuppresive agents have an influence on ischemia-reperfusion injury in kidneys procured from a non-heartbeating donor: experimental study. Transplant Proc 2004;36(10):2931. [PubMed: 15686663]
17. Loverre A, Ditonno P, Crovace A, et al. Ischemia-reperfusion induces glomerular and tubular activation of proinflammatory and antiapoptotic pathways: differential modulation by rapamycin. J Am Soc Nephrol 2004;15(10):2675. [PubMed: 15466272]

18. Yang CW, Ahn HJ, Han HJ, et al. Pharmacological preconditioning with low-dose cyclosporine or FK506 reduces subsequent ischemia/reperfusion injury in rat kidney. Transplantation 2001;72(11): 1753. [PubMed: 11740384]

19. Dahlquist KD, Salomonis N, Vranizan K, Lawlor SC, Conklin BR. GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. Nat Genet 2002;31(1):19. [PubMed: 11984561]

20. Doniger SW, Salomonis N, Dahlquist KD, Vranizan K, Lawlor SC, Conklin BR. MAPPFinder: using Gene Ontology and GenMAPP to create a global gene-expression profile from microarray data. Genome Biol 2003;4(1):R7. [PubMed: 12540299]

21. Becker KG, Hosack DA, Dennis G Jr. et al. PubMatrix: a tool for multiplex literature mining. BMC Bioinformatics 2003;4:61. [PubMed: 14667255]

22. Saeed AI, Sharov V, White J, et al. TM4: a free, open-source system for microarray data management and analysis. Biotechniques 2003;34(2):374. [PubMed: 12613259]

23. Kobayashi T, Walsh MC, Choi Y. The role of TRAF6 in signal transduction and the immune response. Microbes Infect 2004;6(14):1333. [PubMed: 15555541]

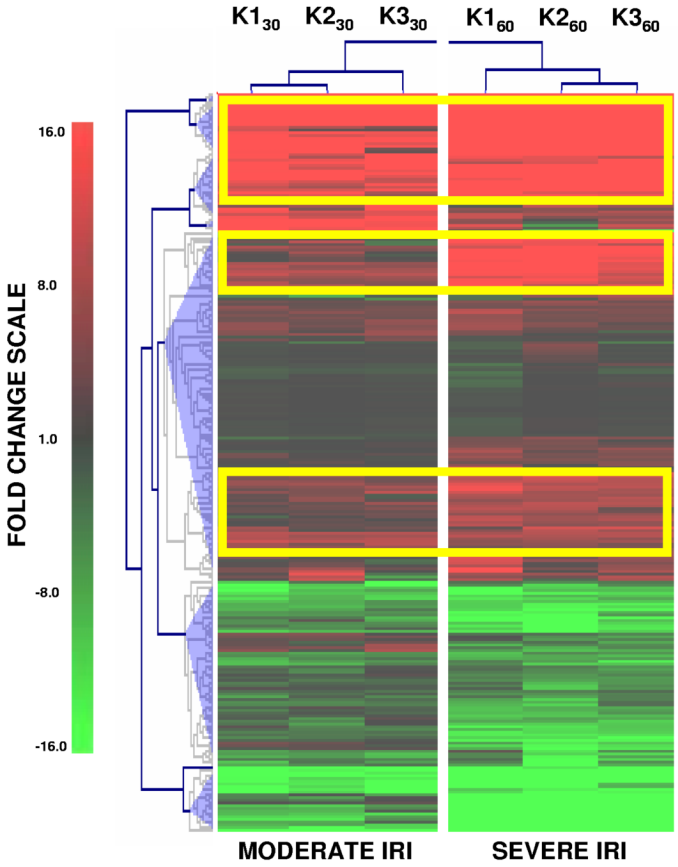
24. Mendez R, Gonwa T, Yang HC, Weinstein S, Jensik S, Steinberg S. A prospective, randomized trial of tacrolimus in combination with sirolimus or mycophenolate mofetil in kidney transplantation: results at 1 year. Transplantation 2005;80(3):303. [PubMed: 16082323]

25. Squadrito F, Altavilla D, Squadrito G, et al. Tacrolimus limits polymorphonuclear leucocyte accumulation and protects against myocardial ischaemia- reperfusion injury. J Mol Cell Cardiol 2000;32(3):429. [PubMed: 10731442]

26. Lebedeva T, Dustin ML, Sykulev Y. ICAM-1 co-stimulates target cells to facilitate antigen presentation. Curr Opin Immunol 2005;17(3):251. [PubMed: 15886114]

27. Fong S, Mounkes L, Liu Y, et al. Functional identification of distinct sets of antitumor activities mediated by the FKBP gene family. Proc Natl Acad Sci U S A 2003;100(24):14253. [PubMed: 14612567]

28. Hocherl K, Dreher F, Vitzthum H, Kohler J, Kurtz A. Cyclosporine A suppresses cyclooxygenase-2 expression in the rat kidney. J Am Soc Nephrol 2002;13(10):2427. [PubMed: 12239231]



Group	Gene Symbol	Fold Change	
		IRI_{30}	IRI_{60}
Calcineurin and mTOR pathway associated	Eif4ebp1	10.98	16.80
	Fkbp5	3.63	4.18
	Akt1	1.98	3.64
	Rps6ka1	1.49	2.81
	Nfat5	1.43	2.60
	Fkbp1a	2.16	2.00
Histocompatibility and Antigen presenting	Traf6	1.26	2.99
	H2-D1	1.17	2.58
	H13	1.53	1.74
Tacrolimus and Sirolimus responsive	Sdc1	8.37	16.40
	Icam1	8.94	10.65
	Ptgs2	1.74	4.22

Akt1-thymoma viral proto-oncogene 1, **Eif4ebp**-eukaryotic translation initiation factor 4E binding protein 1, **Fkbp 5** and **1a** - FK506 binding protein 5 and 1a, **H2-D1**-Histocompatibility 2, D region locus 1, **H13**-Histocompatibility 13, **Icam1**-intercellular adhesion molecule 1, **Nfat5**-nuclear factor of activated T-cells 5, **Ptgs2**-prostaglandin-endoperoxide synthase 2, **Rps6k a1**-ribosomal protein S6 kinase polypeptide a1, **Sdc1**-syndecan1, **Traf6**-Tnf receptor-associated factor 6.

Figure 1.

Heatmap of hierarchical clustering of candidate genes expression of which was significantly affected by IRI. Genes, expression of which was significantly (q<1%) affected by IRI in moderately (30 min of ischemia) or severely (60 min of ischemia) injured kidney tissues were combined and clustered using MeV software. Each column represents an experimental condition of corresponding tissue sample. Severity hierarchical clustering (top dendrogram) correctly grouped experimental conditions into two large clusters of moderately and severely injured kidney tissues. Hierarchical clustering of genes identified 5 major clusters (blue triangles) and three regions (encompassed with yellow rectangles) which demonstrated clear induction of gene expression by moderate and/or severe IRI. The most representative genes and their corresponding pathways are listed on the right where deep red highlights genes from the most upregulated (top) cluster, the pink marks genes from the middle cluster, and the lightest red represents genes from the bottom cluster. Red color on the heatmap indicates up-regulation and green color indicates down-regulation of gene expression relative to corresponding sham operated controls. The color intensity depicts the fold-change amplitude (fold-change scale shown on the left). Sample coding: IRI-ischemia reperfusion injury, K-kidney, $_{30, 60}$ – duration of ischemia, 1,2,3- number of the animal.