Molecular Mechanisms of Chronic Kidney Transplant Rejection via Large-Scale Proteogenomic Analysis of Tissue Biopsies

Aleksey Nakorchevsky,* Johannes A. Hewel,*† Sunil M. Kurian,‡ Tony S. Mondala,§ Daniel Campbell,§ Steve R. Head,§ Christopher L. Marsh,‖ John R. Yates, III,* and Daniel R. Salomon‡

*Department of Chemical Physiology, ‡Department of Molecular and Experimental Medicine, and §DNA Microarray Core, Scripps Research Institute, La Jolla, California; †Center for Cellular and Biomolecular Research, University of Toronto, Toronto, Ontario, Canada; and ‖Scripps Center for Organ and Cell Transplantation, Scripps Health, La Jolla, California

ABSTRACT

The most common cause of kidney transplant failure is the poorly characterized histopathologic entity interstitial fibrosis and tubular atrophy (IFTA). There are no known unifying mechanisms, no effective therapy, and no proven preventive strategies. Possible mechanisms include chronic immune rejection, inflammation, drug toxicity, and chronic kidney injury from secondary factors. To gain further mechanistic insight, we conducted a large-scale proteogenomic study of kidney transplant biopsies with IFTA of varying severity. We acquired proteomic data using tandem mass spectrometry with subsequent quantification, analysis of differential protein expression, validation, and functional annotations to known molecular networks. We performed genome-wide expression profiling in parallel. More than 1400 proteins with unique expression profiles traced the progression from normal transplant biopsies to biopsies with mild to moderate and severe disease. Multiple sets of proteins were mapped to different functional pathways, many increasing with histologic severity, including immune responses, inflammatory cell activation, and apoptosis consistent with the chronic rejection hypothesis. Two examples include the extensive population of the alternative rather than the classical complement pathway, previously not appreciated for IFTA, and a comprehensive control network for the actin cytoskeleton and cell signaling of the acute-phase response. In summary, this proteomic effort using kidney tissue contributes mechanistic insight into several biologic processes associated with IFTA.


Kidney transplantation success has steadily improved since the first efforts in 1954¹ in identical twins. Nonetheless, it is now evident that long-term graft survival has not advanced nearly as dramatically as 1-yr graft survival and the reductions in acute rejection rate.² Chronic allograft nephropathy (CAN), defined as a clinical entity by a progressive deterioration of kidney transplant function, is the most common cause of graft loss in surviving patients. Despite more than a decade of intense effort, there is no single mechanistic explanation for the clinical entity of CAN; rather, it seems to represent a spectrum of interrelated processes between the host and the allograft, resulting in progressive kidney tissue injury including immune- and non-immune-mediated injury mechanisms.

Received June 17, 2009. Accepted October 13, 2009.

Published online ahead of print. Publication date available at www.jasn.org.

A.N. and J.A.H. contributed equally to this work

Correspondence: Dr. Daniel R. Salomon, Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA 92037. Phone: 858-784-9975; Fax: 858-784-2121; E-mail: dsalomon@scripps.edu

Copyright © 2010 by the American Society of Nephrology
More recently, the clinical entity of CAN has been renamed and redefined in histologic terms as interstitial fibrosis and tubular atrophy (IFTA) of unknown etiology. By biopsy histology, the incidence of IFTA is as high as 50% of kidney transplants at 1 yr after transplantation, 70% at 2 yr, and nearly universal after 10 yr, with clear evidence that IFTA progresses and correlates with progressively diminished renal function. Conversely, it is also clear that IFTA does not always progress in a linear, predictable manner, consistent with the hypothesis that elements of IFTA are dynamic. Thus, there is a pressing medical need to develop new strategies to unravel the complex mechanisms of tissue injury producing this clinical/pathologic entity of CAN/IFTA.

In this study, we have done the first investigation of kidney transplant biopsies in patients with documented and histologically graded IFTA using shotgun proteomics. The principal goal of this study was a comprehensive and discovery-driven analysis of protein expression in transplanted kidneys comparing those with normal renal function and biopsy histology with transplants with mild to moderate/severe stages of IFTA. One immediate goal of IFTA proteomics is discovery of specific proteins that can be linked to functional molecular pathways to yield early diagnostic information and serve as metrics for disease progression. Another goal of these studies was to test the hypothesis that mechanistically relevant information can be obtained by shotgun proteomic analysis of a complex clinical/pathologic entity in an equally complex and affected human tissue. We have also correlated our proteomic expression results with parallel genome-wide, gene expression profiling to explore the connections between the transcriptome and the proteome. Our results demonstrate the success of shotgun proteomics in characterizing the histologic stages of IFTA progression and that proteins identified using these technologies can be mapped to molecular networks with functional significance defined by relevant literature as novel insights into the mechanisms driving IFTA in kidney transplantation. Specifically, we demonstrate a strong set of immune rejection-associated proteins and transcripts consistent with the hypothesis that an ongoing chronic allograft rejection is one important mechanistic element of IFTA.

RESULTS

Kidney biopsies were classified as one of four stages of IFTA severity, Banff 0 through Banff 3 (designated b0, b1, b2, and b3 in text). The b0 samples show no histologic evidence of IFTA, in other words, an essentially normal kidney transplant biopsy. The b1 samples represent mild IFTA and reflects the early stages of a progressive tissue injury and fibrosis. The b2 and b3 samples represent moderate to severe IFTA, respectively, and identify a patient population with progressive injury and a high risk for graft loss. We pooled the results for b2 and b3 IFTA in the interest of sample size and compared protein expression changes across the three histologic stages: b0, b1, and b23.

Table 1 lists the clinical and demographic data of the patients in this study. In data set 1, there is no significant difference in serum creatinine in any group, and all patients underwent biopsy by protocol at 2 yr. The only significant difference was that the b0 (no IFTA) group was not treated with calcineurin inhibitor (CNI) immunsuppression but rather had been randomly assigned to rapamycin. One interpretation is that CNI-mediated nephrotoxicity is the predominant cause of IFTA. Alternatively, de novo sirolimus-based immunosuppression is more effective in preventing IFTA. It is important to note that in data set 2, all patients studied were on CNIs. Thus, to avoid any possible drug therapy–related bias, we focused on proteins common to both data sets, which also represent the majority of proteins identified. In data set 2, the percentage of deceased donors was significantly less in the b23 group, surprising in that long-term outcomes are typically better for living-donor recipients. As expected, time to biopsy was significantly different between groups in data set 2, and creatinine was significantly higher for b3. The last difference was a higher steroid use in b1 and b23 as compared with b0. Some believe that steroids are antifibrotic in kidney transplants, but this correlation is not evident here.

The two independent proteomic data sets of kidney transplant biopsies were collected during a period of 2 yr, data 1 (green) and data 2 (blue; Figure 1). Figure 1A illustrates the significant overlap between the data sets for all three categories of IFTA despite the difference in immunosuppression. As noted, our analysis focuses only on the overlaps where protein identifications were confirmed in several technical replicates in both data sets. These identifications consist of two classes of proteins: Those that were differentially expressed and met our significance criteria and proteins that were uniquely identified in one or more IFTA categories. Figure 1B represents differentially expressed and unique proteins via a Venn diagram. Overlaps between categories represent significant differentially expressed proteins, and nonoverlapping portions of the diagram represent unique protein identifications. There are 86 unique proteins in b0, 73 in b1, and 333 in b23. There are 184 significant differentially expressed proteins among all three categories of IFTA, 182 between b0 and b1, 281 between b0 and b23, and 257 between b1 and b23 categories. The relative abundance levels of the differentially expressed population span three orders of magnitude, whereas the majority (97%) of the differentially expressed proteins vary within two orders of magnitude (Figure 1C). In summary, we have identified a total of 904 significantly differentially expressed and 492 unique proteins across three histologic categories of IFTA.

Differentially Expressed Proteins

We divided all differentially expressed proteins into groups (expression profiles) that reflect the expression level and/or presence in b0, b1, and b23. For a complete list of the differentially expressed categories, see the data set key in Figure 2. First, we clustered proteins identified at all IFTA stages but differentially expressed (b0-b1-b23; Figure 1). Cluster_b0, cluster_b1,
Table 1. Clinical and demographic data of the kidney biopsy donors

<table>
<thead>
<tr>
<th></th>
<th>Data Set 1</th>
<th></th>
<th></th>
<th></th>
<th>Data Set 2</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Banff 0</td>
<td>Banff 1</td>
<td>Banff 2,3</td>
<td>Significance</td>
<td>Banff 0</td>
<td>Banff 1</td>
<td>Banff 2,3</td>
<td>Significance</td>
</tr>
<tr>
<td>Number</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>NS</td>
<td>4</td>
<td>5</td>
<td>10</td>
<td>NS</td>
</tr>
<tr>
<td>Recipient age (years)</td>
<td>38.7 ± 15.9</td>
<td>42.7 ± 11.2</td>
<td>41.2 ± 16.3</td>
<td>NS</td>
<td>55.5 ± 10.2</td>
<td>51.4 ± 11</td>
<td>45.1 ± 12.1</td>
<td>NS</td>
</tr>
<tr>
<td>Recipient gender (% female)</td>
<td>0</td>
<td>25</td>
<td>20</td>
<td>NS</td>
<td>75</td>
<td>40</td>
<td>60</td>
<td>NS</td>
</tr>
<tr>
<td>Recipient race (% AA)</td>
<td>0</td>
<td>25</td>
<td>20</td>
<td>NS</td>
<td>0</td>
<td>20</td>
<td>10</td>
<td>NS</td>
</tr>
<tr>
<td>PRA &gt;20 (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>NS</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>NS</td>
</tr>
<tr>
<td>HLA mismatch</td>
<td>2.8 ± 2.2</td>
<td>1.5 ± 0.6</td>
<td>2.2 ± 2.3</td>
<td>NS</td>
<td>3.7 ± 3.2</td>
<td>4.4 ± 1.5</td>
<td>3.4 ± 1.3</td>
<td>NS</td>
</tr>
<tr>
<td>Deceased donor (%)</td>
<td>75</td>
<td>50</td>
<td>80</td>
<td>NS</td>
<td>75</td>
<td>100</td>
<td>30</td>
<td>NS</td>
</tr>
<tr>
<td>Donor age (years)</td>
<td>42 ± 20.2</td>
<td>44.8 ± 10.3</td>
<td>40.2 ± 11.4</td>
<td>NS</td>
<td>33.7 ± 22.9</td>
<td>35.8 ± 20</td>
<td>41.4 ± 12.9</td>
<td>NS</td>
</tr>
<tr>
<td>Donor gender (% female)</td>
<td>25</td>
<td>0</td>
<td>60</td>
<td>NS</td>
<td>25</td>
<td>60</td>
<td>60</td>
<td>NS</td>
</tr>
<tr>
<td>Donor race (% AA)</td>
<td>0</td>
<td>25</td>
<td>20</td>
<td>NS</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>NS</td>
</tr>
<tr>
<td>Induction (%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>NS</td>
<td>75</td>
<td>100</td>
<td>100</td>
<td>NS</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td>1.45 ± 0.44</td>
<td>1.33 ± 0.45</td>
<td>2.14 ± 0.88</td>
<td>NS</td>
<td>2.28 ± 1.69</td>
<td>2.02 ± 0.24</td>
<td>3.14 ± 1.33</td>
<td>NS</td>
</tr>
<tr>
<td>Time to biopsy (days)</td>
<td>726 ± 125</td>
<td>704 ± 46</td>
<td>719 ± 94</td>
<td>NS</td>
<td>550 ± 514</td>
<td>1571 ± 1256</td>
<td>2767 ± 992</td>
<td>NS</td>
</tr>
<tr>
<td>CNI (%)</td>
<td>0</td>
<td>25</td>
<td>80</td>
<td>Banff 0 versus 2,3 P &lt; 0.05</td>
<td>100</td>
<td>100</td>
<td>90</td>
<td>NS</td>
</tr>
<tr>
<td>MMF (%)</td>
<td>100</td>
<td>75</td>
<td>100</td>
<td>NS</td>
<td>75</td>
<td>100</td>
<td>80</td>
<td>NS</td>
</tr>
<tr>
<td>Steroids (%)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>NS</td>
<td>25</td>
<td>100</td>
<td>90</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard deviation unless otherwise indicated. NS, not significant; CNI, calcineurin inhibitor treatment; MMF, mycophenolate mofetil treatment; AA, African Americans; HLA, Human Leukocyte Antigen; PRA, Panel Reactive Antibody.

and cluster_b23 represent groups of upregulated proteins. The b0_b1_b0up group is composed of proteins that are differentially expressed between b0 and b1 sets (182 proteins; Figure 1) and upregulated in b0 compared with b1 (39 of 182 proteins). Unique protein groups are designated uniqu_b0, uniqu_b1, and uniqu_b23. Finally, we combined all of the differentially expressed and unique proteins into all_b0, all_b1, and all_b23. We used Ingenuity Pathway Analysis for functional annotations where the proteins were assigned to either biologic function (Figure 2A) or canonical pathways (Figure 2B) and hierarchically clustered. The same holds true for canonical pathways where b0 expression profiles represent a gamut of metabolic pathways whereas the b1 and b23 expression profiles are considerably enriched for proteins that belong to phosphatidylinositol-3 kinase (PI3K)/AKT signaling, acute-phase response signaling, complement system, actin cytoskeleton signaling, fibrosis, and integrin signaling (Figure 2B).

b0-b1-b23 Comparison

We examined the differentially expressed proteins for presence of known renal disease markers as a function of the continuum b0 to b23. The 184-protein set (b0-b1-b23 overlap; Figure 1B) consists of three clusters: Cluster_b0, cluster_b1, and cluster_b23 (Figure 2). It is evident that whereas proteins with the strongest expression in cluster_b0 display biologic functions such as lipid metabolism and small-molecule biochemistry associated with normal kidney function, the balance shifts toward “cellular distress” functions such as cancer, gastrointestinal disease, endocrine systems disease, and metabolic disease from cluster_b1 to cluster_b23. Moreover, cluster_b1 and cluster_b23 are enriched for proteins known to be involved in renal apoptosis, necrosis, fibrosis, and cell death: angiotensinogen (AGT), ATPase β subunit, β1 integrin (ITGB1), and plasminogen (PLG). Clusters representing later stages of IFTA are also enriched for cathepsin D associated...
with apoptosis and keratin 8, known to provide resistance to Fas-mediated apoptosis. The downregulation of cathepsin B in b1 and b23 is also linked in the literature with decreased apoptosis. Annexin A1, reported to play a role in decreasing ischemic injury, is upregulated in b1 and b23. Fibrinogen β chain is upregulated in b1 and b23, supporting the notion that one of the distinguishing features of late-stage IFTA is fibrosis and tissue remodeling. In sum, these protein changes suggest that progressive IFTA represents a losing balance between destructive and reparative mechanisms, a conclusion that is consistent with current views of tissue injury and remodeling in other clinical entities such as congestive heart failure. The 184 proteins and their clusters are listed in Supplemental Table 1.

**b0-b1 Comparison**

We analyzed three categories of differentially expressed proteins, b0-b1, b0-b23, and b1-b23. For example, the b0-b1 group contains proteins differentially expressed between b0 and b1. The most significant biologic functions upregulated in b0 (b0b1_b0up; Figure 2B) are healthy biologic functions including amino acid and drug metabolism and molecular transport; however, the proteins upregulated in b1 (b0b1_b1up; Figure 2B) are functionally linked to cancer, gastrointestinal disease, and genetic disorders. These include tubulins, fibrinogens, thrombin, biglycan, ITGB1, clusterin, profilin 1, heparin sulfate proteoglycan 2 (HSPG2), and angiotensin I–converting enzyme (ACE). The ACE enzyme is a key component of the renin-angiotensin system (RAS) and an angiotensin II regulator. RAS activation is one of the contributors to IFTA progression, and ACE inhibitors are commonly used clinically to attenuate decline of renal function and increase graft survival. Upregulated biglycan and ITGB1, a common chain of one class of integrin adhesion molecules, have been reported to decrease apoptosis of kidney cells, mesangial cells, glomerular cells, and proximal tubules. The cell adhesion protein HSPG2 was previously reported to increase glomerular and mesangial kidney proliferation. The 182 differentially expressed proteins between b0 and b1 are listed in Supplemental Table 2.

**b0-b23 Comparison**

There are 281 differentially expressed proteins in b0-b23 (b0-b23 overlap; Figure 1B). Similar to the b1 stage, the proteins upregulated in b23 (b0b23_b23up; Figure 2) represent biologic functions such as cancer, gastrointestinal disease, and cellular movement. Some of the proteins upregulated in b23 are cathepsin D, profilin 1, the tubulins, decorin, ITGB1, AGT, and clusterin. AGT is further overexpressed in b23 samples compared with b1. Decorin, ITGB1, AGT, and clusterin are linked with renal necrosis and apoptosis. We have also identified a large set of b23 upregulated proteins that belong to the complement system: Complement components 3, 5, 6, and 9; complement factors D and H; and serpin peptidase inhibitor. The complement system plays an important role in immunity and inflammation, and, specifically, C3, C5, and complement factor H are linked to renal damage in mouse models. The full list of 281 differentially expressed proteins between b0 and b23 is shown in Supplemental Table 3.

**b1-b23 Comparison**

There are 257 differentially expressed proteins between b1 and b23 stages of IFTA (b1-b23 overlap; Figure 1B). The proteins

---

**Figure 1.** (A) overlap between independently collected data sets, each composed of multiple technical replicates (data 1, green; data 2, blue) for the b0, b1, and b23 classes represents the consensus proteomic data. (B) Venn diagram showing the overlap between consensus b0, b1, and b23 protein identifications. (C) Relative abundance distribution of differentially expressed proteins. The binary overlaps (b0-b1, b0-b23, and b1-b23) contain proteins that are differentially expressed at P = 0.05 level of significance (two-tailed independent t test), whereas the b0-b1-b23 overlap represents differentially expressed proteins at P = 0.05 level of significance as determined with one-way ANOVA. The areas marked with arrows represent total pair-wise differentially expressed proteins between Banff categories.
upregulated in b23 include PLG, AGT, hemopaxin, annexin 1, complement factors 3 and H, β-2 microglobulin, and HSPG2. Thus, although proteins that belong to the acute-phase response signaling and complement system are upregulated in both b1 and b23, most are further upregulated in b23 compared with b1. These results further support their potential mechanistic importance and the ongoing connection between these pathways and progression of IFTA. The full list of 257 differentially expressed proteins is shown in Supplemental Table 4.

Unique Proteins
There are also a significant number of unique proteins in each category of IFTA (Figure 1B, unique proteins). One of the dominant proteomes uniquely expressed in b23 samples was the actin cytoskeleton pathway including multiple acts, fibronectin, mitogen-activated protein kinases (MAPK1 and MAP2K1), Rac2, Ras (RHOA and RHOC), vasodilator-stimulated protein, complements 7 and 8, and complement factors B and I. We also identified tissue PLG exclusively in b23 samples. PLG along with its inhibitor (plasminogen activator inhibitor 1) have been linked to CAN/IFTA, in which plasminogen activator inhibitor 1 upregulation is associated with renal fibrosis and thrombosis. The complete list of unique proteins is shown in Supplemental Table 5.

Correlation to Gene Expression
We have compared gene expression with protein expression across the three stages of CAN for the subpopulation of proteins we found to be differentially expressed across the b0, b1, and b23 stages (184 proteins in Figure 1B, b0-b1-b23 overlap). Using Pearson correlation coefficient, we determined that the

Figure 2. (A and B) Hierarchical clustering outcome for the biologic function (A) and canonical pathway (B) annotations of differentially expressed proteins is shown. Protein identifications were split into 15 groups (data set key) that represent expression programs in different stages of CAN (b0 upregulated, green; b1 upregulated, yellow; b23 upregulated, red). Both functional categories and differential groups of proteins were clustered using Spearman rank average linkage metric. Each data point is colored according to the probability score (expressed as P value) of a functional category in a particular differentially expressed group of proteins.
average correlation between the gene and protein expression is 0.13 on the scale of $-1$ to 1. The low overall correlation between the transcripts and the proteins can be explained by considering the effects of posttranslational regulation and regulatory degradation of gene products during IFTA. We list the individual correlations in Supplemental Table 6; however, in contrast, the transcript-to-protein correlations within the frameworks of specific molecular pathways that we have identified during analysis are high (see the Discussion section). This high correlation at the pathway level emphasizes the value of filtering unbiased genome-wide proteogenomic data sets during analysis by defined molecular networks with functional significance on the basis of existing literature.

### Validation of Identified Proteins

We have carried out a novel validation strategy for the proteins unique to b0 and b23 stages of IFTA. We have selected 23 most abundant unique b23 proteins and 21 most abundant unique b0 proteins for targeted validation using single-reaction ion monitoring (SRM) mass spectrometry (MS; see the Concise Methods section). We were monitoring 96 peptides from b23 samples and 92 peptides from b0 samples. As part of the control data sets, we also monitored b23 targets in b0 samples and vice versa. Finally, we have acquired data sets in which we monitored b0 and b23 targets using a blank sample to establish the intensity baseline threshold. The results are summarized in Table 2.

We have confirmed 100% of the selected b23 protein targets with 75 unique peptides and 19 proteins confirmed by two or more unique peptides (Supplemental Table 7). The average coverage of the peptide fragments was 0.94, and the average intensity of the fragments was 10,478. We have confirmed 81% of the b0 proteins with 39 unique peptides and 12 proteins confirmed by two or more unique peptides (Supplemental Table 7). The average coverage of the peptide fragments was 0.88, and the average intensity of the fragments was 4780. We also attempted to find the b0 targets in b23 samples and vice versa to measure the selectivity of the large-scale analysis. When looking for the b0 targets in b23 samples, we did find 20 peptides and 13 proteins with only six proteins confirmed by multiple peptides (Supplemental Table 9); however, when looking for b23 targets in b0 samples, we could find only one protein confirmed by multiple peptides (Supplemental Table 10). Identification of proteins with bottom-up MS is a concentration-dependent process whereby the most abundant proteins yield the highest concentration of peptides, which are sampled more frequently by the instrument and identified with higher confidence scores. A key point is that the sensitivity of SRM validation is higher by 2 to 3 orders of magnitude than that of the large-scale method. Thus, it is not surprising that we discover some of the “normal” b0 proteins in the b23 sample; however, the near absence of b23 markers in the b0 sample demonstrates that the unique b23 proteins discovered in the large-scale study using MudPIT proteomics are highly correlated with IFTA as we have claimed.

### DISCUSSION

The clinicopathologic entity of CAN/IFTA represents an outcome reflecting a balance of many cellular processes, destructive and reparative, affecting the kidney transplant. It is evident from Figure 2 that overexpressed or unique proteins reveal IFTA as a shifting landscape of biologic functions from housekeeping to increasingly stress-related, immune/inflammatory, and tissue injury functions. Figure 3 highlights these changes at the level of biologic functions (Figure 3A) and canonical pathways (Figure 3B). For example, the percentage of differentially expressed proteins annotated as cancer biology increases dramatically from 17 to 40% as a function of IFTA severity. Another example is the acute response-signaling pathway represented in b1 and b23 by four and 32 identified proteins, respectively, and none in b0. The following pathways become upregulated during later stages of IFTA: Acute-phase response signaling, actin cytoskeleton and chemokine signaling, coagulation and complement systems, regulation of actin-based motility by Rho, integrin signaling, hepatic fibrosis, and PI3K/AKT signaling.

### Complement Pathways

Figure 4 shows the components of the complement pathway and highlights proteins either overexpressed or unique to b23. Proteins unique to b23 IFTA are highlighted in gray, whereas the differentially expressed proteins are colored in shades of red and labeled with their relative abundance (fold change relative to b0 and b1). Here we also integrated mRNA expression levels from these same samples obtained by gene expression profiling. Thus, identified proteins are also labeled with colored rectangular boxes that show correlations between protein abundance and gene expression across b0, b1, and b23. In these comparisons of complement pathway proteins, our results are consistently in agreement with the gene expression results from parallel microarray analysis, and with the exception of C7 and C9, all have a strong positive correlation with their mRNA transcript levels.

Some complement pathway proteins in kidney transplants are expected. For example, C4d staining is one parameter in the histologic Banff classification; however, the number of...
upregulated complement system proteins in b23 stage biopsies emphasizes the importance of this pathway during IFTA. Although it has been shown that the process of doing cadaveric, brain-dead donor transplants is a source of activated complement components such as C3, these events are confined to the early days of the posttransplantation period. In contrast, the ongoing upregulation of complement proteins documented in our studies from b0 to b23 identifies donor-independent upregulation of complement as a chronic progressive injury process happening over years after the transplantation surgery. Thus, we were able to identify and map a significant number of the complement system members (12 total) and all of the known members of the membrane attack complex. In addition, proteins unique to b23 are complement factors B and I, C4BP, C7, C8, and C9. Our evidence suggests activation of complement via both the classical (serpin peptidase inhibitor and C4BP) and the alternative pathways (complement factors B, D, H, I, and C3) but not the lectin pathway. Furthermore, we observed overexpression of CD59 at b0 and b1 stages (Supplemental Data) that is a known inhibitor of the complement membrane attack complex. The activation of the classical complement system in late IFTA suggests ongoing antibody-mediated chronic rejection, despite the evidence that <10% of these patients were C4d positive by histology (data not shown). The activation of the alternative pathway could serve as an amplifier circuit for low levels of donor- or tissue-specific antibodies or could represent a presently unknown, antibody-independent mechanism of complement activation linked to tissue injury and/or endothelial activation. Thus, we present direct proteomic evidence and describe correlative gene expression evidence that complement activation is one of the key processes in graft injury and IFTA progression.

Actin Cytoskeleton and Lymphocyte/Macrophage Cell Signaling
Two overlapping processes, —actin cytoskeleton signaling and regulation of actin-based motility by Rho—are also upregulated in late IFTA compared with b0 and b1 (Figure 5). Among the proteins exclusively expressed in b23 are RhoA (including ROCK-Rho–associated kinase), Rac, mitogen-activated kinases MEK and ERK, several isoforms of actinin, CD14 antigen, myosin and myosin kinases, and several subunits of the actin-related protein 2/3 complex, all of which are linked to macrophage and lymphocyte activation in immunity. Several of the identified differentially expressed proteins have also been linked to disruption of podocyte structure in the glomerulus (α-actinin-4, synaptopodin, CD2AP, CapZ, and RhoA), consistent with the development of transplant glomerulopathy as one element of IFTA. Also, in the glomerulus, endothelial and mesangial cell proliferation via a Rho-depen-
dent actin mechanism\textsuperscript{52} alters glomerular architecture and filtration function. Indeed, upregulation of actin cytoskeleton signaling as a function of progressively severe IFTA stages is also consistent with a progressive tissue remodeling and fibrosis, in effect suggesting one unifying mechanistic network.

We have conducted the first large-scale, unbiased proteomic profiling of kidney transplant biopsies from patients whose CAN/IFTA was diagnosed at various stages of increasing severity. Our results support the notion that IFTA represents a progression of multiple ongoing biologic processes characterizing each stage of severity, some expected by the current knowledge in the literature and many novel and worthy of further mechanistic investigations. The proteins identified and/or upregulated at b0 represent mainly metabolic and normal regulatory pathways consistent with and defining the proteome of healthy, functioning kidney transplants. In contrast, the proteins upregulated in b1 and b23 biopsies belong to multiple processes involved in cell stress, tissue injury, immune responses, inflammation, signal transduction, and tissue remodeling. The b1 stage is revealed here as a transition period in the progression of IFTA when upregulation of several immune rejection-associated processes such as PI3K/AKT signaling, actin cytoskeleton/Rho kinase signaling, RAS, and the coagulation system begins. The b23 late stage of IFTA is highlighted by dramatic upregulation of proteins involved in distress pathways such as the alternative complement pathway, actin cytoskeleton signaling, Rho kinase signaling, and acute-phase responses. A clinical challenge raised by our results is whether the many immune and inflammatory gene networks identified at the mRNA expression and protein levels here are under the control of the immunosuppressive drugs taken by all transplant patients. One possibility is that progressive tissue injury from other mechanisms results in a downstream common pathway of reactive and nonspecific immunity and inflammation that is a secondary phenomenon. Alternatively, progression of IFTA represents an ongoing donor-specific and/or tissue-specific immune response and represents a failure to maintain adequate levels of immunosuppression long term. If the latter is true, then a primary driver of IFTA is chronic rejection, a conclusion with significant clinical implications for therapy. These results demonstrate the success of shotgun proteomics to identify proteins that can be mapped to functionally significant molecular networks creating novel, testable, and clinically relevant insights into the mechanisms producing IFTA in kidney transplantation.

**CONCISE METHODS**

**Clinical Samples**

Biologic samples consisted of kidney transplant biopsies obtained by protocol or "for cause" in transplant recipients treated with either CNI-based immunosuppression (e.g., FK506, cyclosporine) or a de novo rapamycin therapy.\textsuperscript{53–55} Data set 1 represents 16 individual renal biopsies pooled according to histologic IFTA severity, whereas data set 2 represents 17 individual biopsies, each with biopsy-documented IFTA scores (Table 1). Severity was determined for both sets using the Banff '97 classification schema\textsuperscript{23} used originally for data set 1 so that there was diagnostic uniformity. b0 represents no histologic evidence of IFTA, b1 represents mild IFTA, and b2 and b3 grades are assigned to grafts with advanced IFTA. Diagnosis was done first by local pathologists and reviewed in a blinded manner by Drs. Kim Solez (data set 1) and Lillian Gaber (data set 2). All of the research done at the Scripps Research Institute and the participating clinical centers were under institutional review board–approved human subjects protocols.

**Sample Preparation and Data Acquisition**

Total protein (Trizol; Invitrogen) was denatured, alkylated, and trypsin-digested similar to as described previously.\textsuperscript{56} A total of 50 µg of digested protein sample (BCA; Pierce) was used for each experiment. Each clinical sample was analyzed in technical triplicates. Mass spectrometry data were acquired using an LTQ LX linear ion trap mass...
spectrometer (ThermoFisher Scientific) interfaced in-line with two-dimensional HPLC. The chromatography was set up as MudPIT, whereby discrete fractions from a front-end strong cation exchange column were loaded onto and eluted from a reverse-phase (RP) analytical column. MudPIT is an implementation of orthogonal chromatographic separation whereby columns with different properties (affinities toward the analyte molecules) are used in succession, thereby improving resolving power of the separation. MudPIT was implemented using strong cation exchange material as the first dimension followed by RP column. Each experiment consisted of 14 discrete strong cation exchange fractions followed by a 130-min linear RP gradient from 0 to 50% acetonitrile. Sample was introduced using nanospray ESI at the flow rate of approximately 250 nl/min. Data sets were acquired in a data-dependant tandem manner whereby the top three peptide ion peaks from each full scan were fragmented via collision-induced dissociation. The full-scan data provide the mass-to-charge ratio of peptide ions, whereas the fragmentation pattern from subsequent MS/MS scans provides the sequence information of that peptide ion. Only approximately 15% of the acquired MS/MS scans are identified as peptides as a result of the fast scanning rate of the instrument, concentration-dependent nature of peptide fragmentation, and presence of chemical noise.

Gene Expression Profiling

Total RNA was extracted from graft biopsies, and biotinylated cRNA was prepared using Ambion MessageAmp Biotin II and hybridized to Affymetrix Human Genome U133 Plus 2.0 GeneChips according to standard protocols (http://affymetrix.com/index.affx). Intensities of individual probe sets were quantile-normalized and log base 2-transformed. Genes represented by more than one probe set were limited to the probe set with the highest coefficient of variance across all categories of IFTA. Pair-wise comparison between differentially expressed proteomic and genomic data were done using Pearson correlation whereby the normalized protein expression across the b0, b1, and b23 stages was correlated to its gene expression across the same stages of IFTA.

Proteomic Data Analysis

Raw data were searched against the human EBI database (December 1, 2006, release) supplemented with a decoy database in which each entry of the original protein contains its reversed sequence. The data-
base search was carried out using a PBS parallelized version of SE-QUEST (v27). MS/MS search involves comparison of the observed fragmentation patterns of unknown peptides from MS/MS scans to the predicted patterns given the peptide sequences in annotated FASTA protein databases. The highest scoring known peptide sequence from the database gets assigned to the MS/MS scan. Search outcomes were postprocessed and filtered using DTASelect 2.0 (in preparation). DTASelect 2.0 uses a quadratic discriminant analysis to adjust dynamically the XCorr and DeltaCN parameters to meet a required false-positive rate (chance probability of 0.05). Postprocessing is required to establish statistical confidence in primary identifications and is usually done using a reversed or shuffled protein database that will yield false-positive identifications when applied to the experimental data. Protein identifications were extracted, and a measure of normalized amino acid coverage was used as label-free quantification. The exact formula used to calculate relative protein abundance is as follows:

\[ A_{\text{protein}} = \frac{\sum_{i=1}^{n} \text{peptide}_i}{\sum_{j=1}^{k} \text{scan}_j} \]

where \( A_{\text{protein}} \) (protein abundance) is expressed as a ratio of the total redundant peptide identifications per protein to the total number of scans identified in the experiment. Each protein identification was annotated by GO association (Revision 1.59; http://www.geneontology.org). Protein identifications across replicate experiments were pooled to represent a union for each category of IFTA (b0, b1, and b23). Relative protein abundance was compared across histologic categories of IFTA for proteins present in more than one category. One-way ANOVA (b0 versus b1 versus b23 comparison) and a two-tailed, independent t-test (pair-wise b0 versus b1 and b1 versus b23 comparisons) were used for hypothesis testing (P value cutoff of 0.05). Only the significant differentially expressed proteins were considered for functional analysis. Proteins identified in more than one technical replicate in a single category and not in any other stage of IFTA were also considered for functional analysis as unique identifications.

Clustering and Functional Annotations

Clustering of the relative expression profiles from the ANOVA set was implemented with k-means algorithm, where \( n \) protein expression profiles were partitioned into \( m \) categories (clusters) using Euclidian distance as a metric. A protein’s expression profile represents its relative abundances across the three categories of IFTA. Hierarchical clustering of the functional annotations (biologic function, canonical pathway) was carried out using Spearman rank average linkage metric. A P value cutoff of 1.3 (chance probability of 0.05) was applied to the functional profiles. Ingenuity Pathway Analysis tool (Ingenuity Systems; http://www.ingenuity.com) was used to generate functional annotations of identified proteins in known molecular pathways. Fisher exact test was used to calculate a p value determining the probability that each biologic function and/or disease process correlation assigned to that data set is due to chance alone. The significance of the canonical pathways defined by identified proteins was measured in two ways: (1) A ratio of the number of proteins from the data set that map to the pathway divided by the total number of molecules that exist in the canonical pathway and (2) a P value (Fisher exact test) determining the probability that the association between the proteins in the data set and the canonical pathway is explained by chance alone.

Validation of Identified Proteins

SRM approach was carried out for validation using a triple-quadrupole mass spectrometer (TSQ Ultra; ThermoScientific). Peptides previously observed in large-scale experiments were selected as validation targets given the following conditions: A peptide was tryptic, a peptide was observed with a charge state of higher than +1, a peptide did not contain either methionine or cysteine residue, and the maximum m/z of a peptide was <1500. A series of unique fragmentation targets (three transitions per peptide) were generated for each peptide, and the collision-induced dissociation energy was m/z dependent similar to that reported by Kuzycz et al. The first quadrupole (Q1) served as a lens programmed to acquire 3 amu wide, 0.075-s scans at the resolution of 0.7 around the m/z of each target precursor ion. The second quadrupole (Q2) served as a collision cell, and the third quadrupole (Q3) was programmed to monitor the three fragments of the precursor ion with the settings similar to that of Q1 except the resolution was set to 0.2. The instrument was operated in-line with a single-dimension RP column containing 100 μg of pooled b0 or pooled b23 sample. The target generation and data analysis were carried out with in-house software for SRM validation. The experiments were run in technical duplicates and included b0 validation; b23 validation; b0 validation with b23 sample (specificity measure); b23 validation with b0 sample (specificity measure); and b0, b23 validation with no sample (blank). The experimentally determined threshold parameters were as follows: Minimum signal intensity, 100; mass accuracy of the fragment ion, unity; minimum number of unique targets observed per peptide, 2; minimum number of peptides per protein, 2.

ACKNOWLEDGMENTS

Funding was provided by National Institutes of Health grants U19 AI52349 and P41 RR011823, the Molly Barber Research Fund, and the Verna Harrah Research Fund. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

A.N. and J.A.H. contributed equally to biopsy sample processing and collection of the proteomic data sets; A.N. took primary responsibility for the writing of the manuscript with D.R.S.; D.R.S. and J.R.Y. designed the original experiments and supervised the data acquisition, analysis, and writing; S.M.K., T.S.M., and S.R.H. supervised sample collections, pathology reviews, and gene expression data acquisition; D.C. performed all gene expression profiling; and C.L.M. participated in sample collection, clinical data analysis, and manuscript writing.

We thank Conrad H. Lu, Tina Tan, and Donald L. Tschirhart (St. Vincent Medical Center); Frank Wesley Hall, David Bylund, and Ed-
ward Kane, Jr. (Scripps Clinic); and Jonathan L. Myles (Cleveland Clinic Foundation) for local pathology reviews from the participating clinical centers and Dr. Lillian Gaber (Texas Medical Center, Houston) for the centralized reviews. We also acknowledge Joanna Sung (Scripps Clinic), Barbara Mastroianni and Kathy Savas (Cleveland Clinic), and Anjela Tsirunyan (St. Vincent Medical Center) for the clinical coordination of this research study.

DISCLOSURES
None.

REFERENCES


Supplemental information for this article is available online at http://www.jasn.org/