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0041-1337/03/7603-539/0
TRANSPLANTATION
Vol. 76, 539–547, No. 3, August 15, 2003
Printed in U.S.A.

EXPRESSION PROFILING ON CHRONICALLY REJECTED TRANSPLANT KIDNEYS[1]

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Background. **Chronic transplant nephropathy remains a poorly defined inflammatory process that limits the survival rate of most renal transplants. We analyzed the gene profile of chronically rejected kidney transplants to identify candidate genes that characterize chronic transplant nephropathy.**

Methods. **To distinguish genes present in normal renal tissue or specific for end-stage renal failure, we compared the gene profiles of 13 chronically rejected kidney transplants with 16 normal kidneys and 12 end-stage polycystic kidneys using a 7K human cDNA microarray. After elimination of genes with signals close to background, 2,190 genes were available for statistical analysis.**

Results. **More than 20% of the examined genes were significantly regulated when compared with the expression level of normal renal tissue ($P<0.0003$). Hierarchic clustering based on 571 genes differentiated normal and transplant tissue, and transplant and polycystic kidney tissue. Most of these genes encoded proteins involved in cellular metabolism, transport, signaling, transcriptional activation, adhesion, and the immune response. Notably, comprehensive gene profiling of chronically rejected kidneys revealed two distinct subsets of chronically rejected transplants. Neither clinical data nor histology could explain this genetic heterogeneity.**

Conclusions. **Microarray analysis of rejected kidneys may help to define different entities of transplant nephropathy, reflecting the multifactorial cause of chronic rejection.**

Chronic transplant nephropathy remains an unresolved problem in allograft transplantation. Recent studies demon-

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Received 27 February 2003. Accepted 2 April 2003.

DOI: 10.1097/01.TP.0000079459.89608.B7

strated that the advances in immunosuppression have reduced early graft rejection (*1*) and improved organ survival within the first year after transplantation (*2*). However, the median survival of cadaveric renal allograft has basically remained unchanged, and the two main causes of graft loss remain chronic transplant nephropathy and death of recipient with functioning graft. The current strategy to combat chronic transplant nephropathy is a combinatorial use of calcineurin inhibitors (i.e., cyclosporine and FK506), newer immunosuppressives such as rapamycin (*3*), and glucocorticoids or antimetabolites (i.e., azathioprine and derivatives of mycophenolic acid) (*4*). Although these strategies are targeted at patients with early signs of chronic graft loss, the diagnosis of chronic transplant nephropathy is currently limited to fairly advanced stages and relies on the detection of interstitial fibrosis, tubular atrophy, and transplant arteriopathy (*5*). In addition, it seems difficult to monitor the progression of chronic rejection (*6*).

The pathogenesis of chronic transplant nephropathy remains elusive, and various theories have been entertained to explain the progressive loss of renal function after organ transplantation (*7–9*). Several immunologic and nonimmunologic factors have been identified that clearly contribute to chronic graft dysfunction, including human leukocyte antigen (HLA) mismatches (*10*, *11*); acute cellular and humoral rejections (*12*); tissue damage during organ retrieval, storage, and surgery; insufficient renal mass and glomerular hyperperfusion (*13*); toxicity of calcineurin-inhibitors (*14*); arterial hypertension (*15*); and dyslipoproteinemia (*16*). These concepts are not mutually exclusive, and chronic transplant nephropathy likely results from a combination of several mechanisms. Each of these factors has distinct targets and may require individual interventions that have to be implemented early after transplantation to prevent the irreversible damage associated with chronic transplant nephropathy.

Detection and classification of chronic transplant nephropathy, particularly at the early stages, remains cumbersome and relies mainly on histologic changes that are often irreversible and signal progressive deterioration of renal function (*17*). Because the complications of long-term immunosuppression are well known, only those patients who experience chronic transplant dysfunction as the result of immunologic mechanisms should be exposed to the risks of increased immunosuppression. To help define chronic transplant nephropathy with more accuracy and potentially identify early causes of this complication, we performed genetic profiling of renal transplants that were removed at the time of chronic renal allograft failure.

MATERIALS AND METHODS

Microarrays

cDNA microarrays were produced and processed essentially according to the Stanford protocol described by Eisen and Brown (*18*). Approximately 7,000 annotated genes from the RZPD (Resource Center and Primary Database, Berlin, Germany) were obtained as bacterial stocks. Plasmids were purified using the Qiagen 96-well Turbo Kit (Qiagen, Hilden, Germany), and inserts were purified by polymerase chain reaction (PCR) using vector primers flanking the individual inserts (5'-CTG CAA GGC GAT TAA GTT GGG TAA C -3' and 5'-GTG AGC GGA TAA CAA TTT CAC ACA GGA AAC AGC-3'). PCR products were purified by ethanol precipitation and resuspended in H_2O. Aliquots were transferred into 384-well plates, dried, and resuspended in 3× standard saline citrate or dimethyl sulfoxide 10% to a final concentration of approximately 40 ng/μL. Printing was performed on aminosilane-coated slides (CMT-GAP II Slides, Corning, NY), using an arrayer that was assembled according to specifications by the Stanford group with software provided by J. de Risi (http://cmgm.stanford.edu/pbrown).

Tissue Samples

Informed consent was obtained from all patients according to the study protocol approved by the ethics committee of Freiburg University. Elective transplant removal was performed on all renal graft recipients at the Freiburg transplant center after transplant failure and reinitiation of renal replacement therapy. Normal renal tissue was obtained from tumor nephrectomies; tumor infiltration was excluded by histology. Tissue samples (approximately 1×1×2 cm) were removed from the cortex and medulla of the kidneys and snap-frozen in liquid nitrogen immediately after organ removal. Samples from kidneys with end-stage polycystic kidney disease (PKD) were obtained from organs removed during transplant surgery. After exclusion of insufficient or tumor-contaminated samples, 12 kidneys with end-stage PKD, 13 chronically rejected renal allografts, and 16 normal renal tissues were analyzed by microarrays.

RNA Preparation

Frozen tissue was homogenized in 4 M guanidinium isothiocyanate with 0.72% β-mercaptoethanol using a Polytron (PT-MR2100, Kinematica AG, Lucerne, Switzerland). Total RNA was subsequently purified on a cesium chloride gradient. After ethanol precipitation, RNA was resuspended at a concentration of 0.4 to 4 μg/μL and stored at −80°C.

Hybridization

All hybridizations were performed in the presence of an equal amount of reference RNA (Stratagene, La Jolla, CA) as recently described by Boldrick et al. (*19*). Twenty-four micrograms of tissue or reference RNA were transcribed into cDNA in the presence of Cy3- and Cy5-labeled dUTP, using Superscript II reverse transcriptase (RT) (Invitrogen, Carlsbad, CA). All other steps, including hybridization, were performed following the protocol published by P. Brown et al. (http://cmgm.stanford.edu/pbrown for details); a PCR-purification kit (Qiagen, Hilden, Germany) was used for cDNA purification after dye labeling.

Data Analysis

Signal intensities were measured by an Axon 4000A scanner using GenePix 3.0 software (Axon Instruments Inc., Union City, CA). The experimental design included a color-reversal experiment for every tissue sample to correct for dye-specific effects. Initially, the log ratio of measured Cy3 and Cy5 values obtained from the image analysis software was computed. Global normalization of expression values was performed by adjusting the data to zero median and unit variance to obtain an identical distribution of overall gene expression. Taking the mean of the expression values of the dye-swap experiments allows correction for dye-specific effects. Following an approach proposed by Dudoit et al. (*20*), the computed expression ratios should not depend on the intensity of the spots. Thus, a smooth nonlinear least squares fit was computed to correct for an intensity-dependent bias (Fig. 1). To exclude artifacts near background range, all genes were eliminated with a signal less than threefold over background in at least 80% of specimens within a group. Applying these criteria, 2,190 genes were subjected to statistical analysis. A two-sample t test was used for a statistical analysis of differentially expressed genes. To adjust the obtained P values, the method by Benjamini and Hochberg (*21*) was applied to control for multiple testing. Hierarchical clustering was performed using the R statistical software package (www.r-project.org).

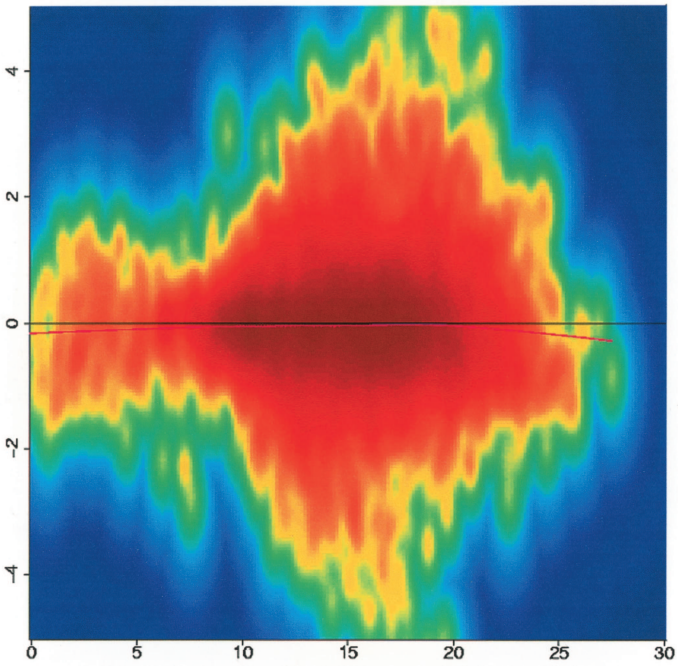

FIGURE 1. Correction of intensity-dependent bias. Data of one representative array after linear global normalization. The measured log ratios of each spot on the y-axis versus signal intensities on the x-axis are shown. A smooth, nonlinear least-squares fit was computed and used to correct for intensity-dependent bias.

Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction

Total RNA (1 μg) from all 13 transplant and 12 normal kidneys was purified with the RNase free DNase set (Qiagen, Hilden, Germany) and reverse transcribed into cDNAs using oligo (dT) 12 to 18 primer and Superscript II RT (Invitrogen, Carlsbad, CA). Each PCR was performed with GAPDH as an internal control in each reaction vial. The following primers were used: (5'-3'): GAPDH: tggaaatcccatcaccatct, gtcttctgggtggcagtgat; Z34893: gcctcagtgaaggtctcctg, ctccatgtaggctgtgctga; M15881: ccaatgacatgaaggtgtcg, tgtaagtggcatgggttcca; L13275: cgacctgaggaaaaagatgc, cttcagcagagggaagttgg; X57809: accacaccctccaaacaaag, tgggatcctgcagctctagt; M31523: tcttctcatgctccgtgatg, gcagtggtgggtgctttatt; X07743: gaaggggagcgtgttcaata, agtggtcctgctgtttggtc; J03037: caatggtcatgctttcaacg, ccccatatttggtgttccag; M14564: ccgcacaccaactatcagtg, tgccactccttctcattgtg; M59305: gattgccatgactgatgtgg, cactgccgattcttctaggc; and AF038451: cagcattcttgctccttgtg, gggtttgttgcttgtcttgg. The PCR reaction was initiated by a denaturation for 5 min at 94°C, followed by 28 cycles of denaturation for 30 sec, annealing for 1 min at 55°C, and elongation for 2 min at 72°C. The PCR products were run on a 3% agarose gel and evaluated in relation to the corresponding GAPDH band using the Scion Image software (Scion, Frederick, MD).

RESULTS

Data Collection and Quality Control

To characterize end-stage chronic nephropathy, we used surgical specimens of chronically rejected transplants (Table 1) that yielded a minimal amount of 50 μg of total RNA. Thirteen transplants fulfilled this criterion and were included in our analysis (Table 1). To determine whether the profile of 7,000 genes differentiates these organs from normal renal tissue and other states of chronic renal failure, we used nonaffected renal tissue removed during 16 tumor nephrectomies and 12 samples from patients with end-stage PKD. To compare individual kidneys, each microarray analysis was performed against a standard reference RNA. In addition, each hybridization was repeated with inversed dyes to correct for the effects of different dye incorporation and to reduce the statistical error.

Hierarchical Clustering Differentiates Chronic Transplant Nephropathy from Normal Renal Tissue and Polycystic Kidney Disease

The microarray analysis of 16 control kidneys, 13 renal transplants, and 12 polycystic kidneys, entailing more than 550,000 single measurements, clearly distinguished the gene expression pattern of chronically rejected kidney transplants from the expression patterns of normal kidneys and polycystic kidneys with end-stage renal disease (ESRD). More than 20% of the examined genes meeting the inclusion criteria were significantly regulated when compared with the expression level of normal renal tissue ($P<0.0003$). We performed two-dimensional hierarchical cluster analysis using genes with significant

TABLE 1. Clinical characteristics of transplant patients

Tx	Sex	Age	Renal disease	Time since transplantation (mo)	Best serum creatinine (mg/dL)	HLA mismatches A-B-DR
1	F	28	Chronic glomorulonephritis	106	1.0	1-1-1
2	F	50	Mesangioproliferative glomerulonephritis	26	1.2	NK
3	F	47	IgA nephropathy	168	1.3	1-1-1
4	F	21	Congenital renal hypoplasia	66	1.3	1-1-0
5	M	47	Chronic pyelonephritis	174	1.0	1-1-0
6	M	44	Membranous glomerulonephritis	107	1.2	0-1-0
7	M	41	Mesangioproliferatative glomerulonephritis	97	1.2	0-0-0
8	F	40	Lupus nephritis type V	132	1.6	2-1-1
9	F	40	Chronic extracapillary glomerulonephritis	102	1.2	2-1-1
10	F	21	Juvenile nephronophitisis	67	0.6	0-1-1
11	F	61	Membranous glomerulonephritis	181	0.9	1-1-1
12	F	38	Diabetic nephropathy	133	1.2	NK
13	M	34	Mesangioproliferatative glomerulonephritis	158	1.3	2-1-1

HLA, human leukocyte antigen; Ig, immunoglobulin; Tx, transplantation; NK, not known.

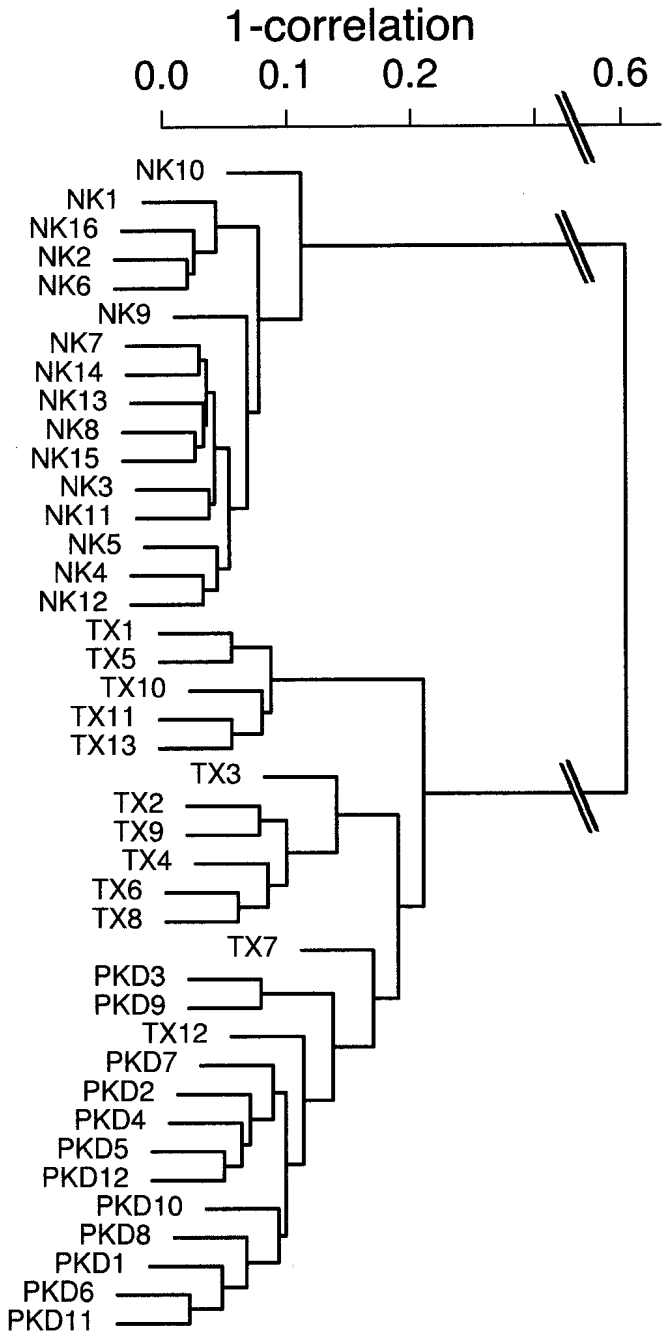

FIGURE 2. Hierarchical cluster analysis of 16 normal kidneys, 13 transplant kidneys, and 12 kidneys with polycystic kidney disease (PKD). Hierarchical clustering was performed on the basis of 571 genes differentiating the three tissues at a significance level of *P* value less than 0.0003 (normal vs. transplant kidney tissue) and *P* value less than 0.05 (transplant vs. PKD tissue). The dendrogram has two major branches. One branch contains all 16 normal samples, whereas kidneys with end-stage renal disease (ESRD) are sorted into the other branch. The cluster analysis revealed that the gene expression profiles of normal, cystic, and rejected kidneys are clearly distinct.

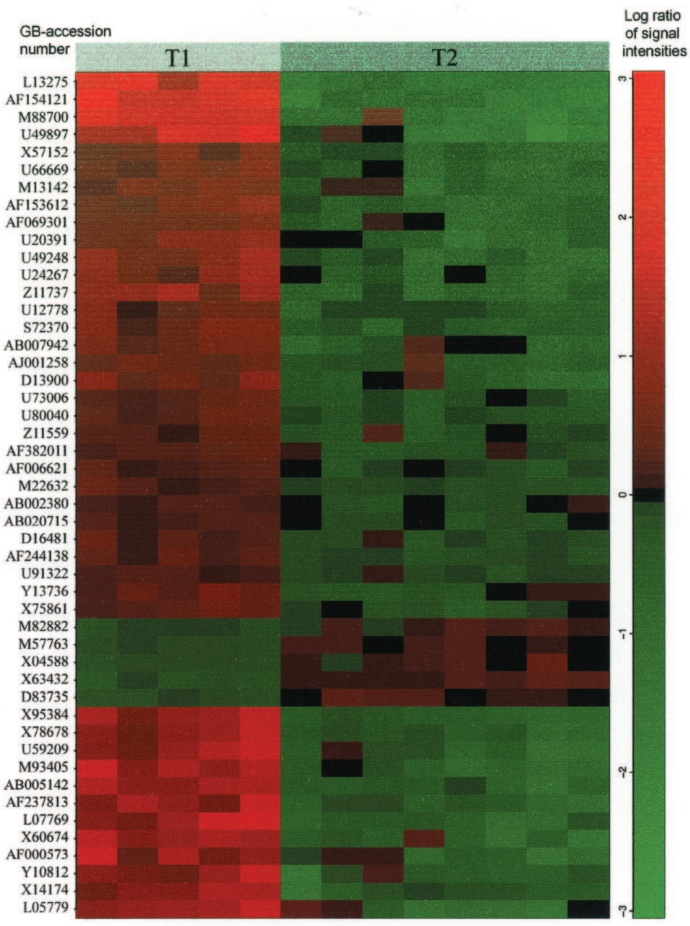

FIGURE 3. Genetic heterogeneity of chronic transplant nephropathy. Comprehensive gene profiling of chronically rejected kidneys revealed two distinct subsets of chronic transplant nephropathy that were not apparent by clinical or histologic data. These two subgroups were designated T1 (Tx1, Tx5, Tx10, Tx11, and Tx13) and T2 (Tx2, Tx3, Tx4, Tx6, Tx7, Tx8, Tx9, and Tx12). There were 48 genes separating these two groups with a significance level of 1% for the false discovery error rate. These genes encode for a variety of biologic functions, including cellular metabolism, signaling, and the immune system.

differences between transplants and normal kidney tissue ($P<0.0003$) or transplants and PKD tissue ($P<0.05$). A total of 571 genes fulfilled these criteria and were included in the cluster analysis. On the basis of these 571 genes, the hierarchical clustering allocated transplants with chronic rejection, normal renal tissue, and polycystic kidneys with ESRD in distinct groups. In two patients, a bilateral PKD nephrectomy was performed with a delay of several months. The corresponding kidneys (PKD 9 and 3, and PKD 12 and 4) were identified as closely related tissues by the hierarchical cluster algorithm. As shown in Figure 2, the dendrogram has two major branches. One branch contains all 16 normal samples, whereas kidneys with ESRD constitute the other branch. The ESRD branch is further divided into two major branches, one containing a group of five transplants (Tx1, Tx5, Tx10, Tx11, and Tx13). The second branch divides the remaining kidneys into two subgroups. The first subgroup consisted of the transplants Tx2, Tx3, Tx4, Tx6, Tx8, and Tx9, revealing a similar expression profile. The transplants Tx7 and Tx12 are found in a second subgroup together with all polycystic kidneys (Fig. 2). Thus, the dendrogram revealed that chronically failing transplants form a het-

TABLE 2. Regulated genes that differentiate chronic transplant failure from renal failure of polycystic kidneys

Fold-change	Gene name	ID	Category
−2.36	Aquaporine 2 water channel	D31846	Homeostasis
−2.21	Lipoprotein lipase	M15856	Lipid metabolism
−1.88	PML-2	M79463	Unknown
−1.78	Aquaporine 3 water channel	AB001325	Homeostasis
−1.67	Napsin 1 precursor	AF098484	Protein metabolism
−1.54	Flotillin-1	AF089750	Signaling
1.54	Aortic-type smooth muscle alpha-actin	M33216	Cytoskeleton
1.58	Sarcospan-2 (SPN2)	AF016028	Cytoskeleton
1.60	Proteasome activator hPA2	D45248	Protein metabolism
1.63	Ribosomal protein L39	D79205	Protein metabolism
1.66	Ceramide glucosyltransferase	D50840	Signaling
1.68	Aortic carboxypeptidase-like protein	AF053944	Cytoskeleton
1.71	Ribosomal protein S15	X84407	Protein metabolism
1.74	DKFZp586J021	AL110197	Unknown
1.85	Type IV collagenase	M55593	Adhesion/ECM
1.87	Hepatocyte growth factor activator inhibitor	AB000095	Signaling
1.87	RIG-like 7-1	AF034208	Unknown
1.89	Proteasome subunit MECI-1	Y13640	Protein metabolism
1.94	Prostaglandin E receptor EP3 subtype	D86096	Immune response
1.97	TEM8 (tumor endothelial marker 8 precursor)	AF279149	Unknown
2.49	MHC class 1	AI565209	Immune response
2.61	KIAA0201	D86956	Inflammation
2.77	Diubiquitin	Y12653	Protein metabolism
2.88	DNA-binding protein	M91196	Transcription
3.01	FYN binding protein	AF001862	Signaling
3.08	Complement subcomponent C1s	M18767	Inflammation
4.93	Immunoglobulin heavy chain	AJ239383	Immune response

Genes are listed that were significantly different from normal and polycystic kidneys ($p < 0.05$) and at least 1.5-fold up- or down-regulated. The largest subgroup were nine genes involved in metabolism; the other genes belonged to the categories immune response, inflammation, transcription, signaling, cytoskeleton, adhesion, and homeostasis. All genes were sequence verified, and genes that could not be verified were omitted. Some genes were spotted in duplicate and showed nearly identical regulation, of which only one is shown the result tables 2 or 3.

MHC, major histocompatibility complex.

erogeneous group with some transplants more closely related to other kidneys with ESRD.

Hierarchical Clustering Reveals Two Subgroups Among Chronically Rejected Transplant Kidneys

The dendrogram defined two distinct groups of chronically rejected transplants that were different from normal or polycystic kidneys, designated T1 (Tx1, Tx5, Tx10, Tx11, and Tx13) and T2 (Tx2, Tx3, Tx4, Tx 6, Tx 8, and Tx 9), and were subjected to further analysis. There were 48 genes separating these two groups with a significance level of 1% for the false discovery error rate. Thus, the expected number of falsely selected genes, 0.48, is much smaller than the actual number of genes found. This indicates the statistical significance of the two subgroups. Figure 3 demonstrates the expression pattern of the 48 discriminating genes that encode for a variety of biologic functions, including cellular metabolism, signaling, and the immune system. Several clinical criteria, including donor characteristics, HLA mismatches, immunosuppression, reason for explantation, and length of time after initiation of hemodialysis, failed to distinguish the two subgroups, and no histologic differences between the two subgroups could be found according to the Banff classification (Table 4).

Specifically Regulated Genes that DifferentiateChronic Transplant Failure from Renal Failure of Polycystic Kidneys

To identify genes specific for chronic transplant nephropathy, we evaluated all genes that were significantly ($P<0.05$) different from normal and polycystic kidneys. We further limited our analysis to genes that were at least 1.5-fold up-regulated or down-regulated and eliminated genes generally altered in ESRD by including only genes significantly ($P<0.05$) different between rejected transplants and polycystic kidneys. As shown in Table 2, 27 genes met these criteria, 22 of which were up-regulated and six of which were down-regulated in the transplant group. Genes of interest were subsequently annotated according to their biologic function. The largest subgroup was composed of nine genes involved in metabolism. The other genes belonged to the following categories: immune response, inflammation, transcription, signaling, cytoskeleton, adhesion, and homeostasis.

Genes Characteristic for ESRD

We chose a similar strategy to identify genes that were differentially regulated in both transplant and polycystic kidneys in comparison with normal renal tissue. To identify genes characteristic for ESRD, we selected genes that were significantly ($P<0.05$) different from normal tissue and at least 1.5-fold up-regulated or down-regulated; 429 genes met these criteria. In Table 3, 49 potential ESRD "marker genes" are depicted that were more than fourfold regulated in the ESRD group, 11 of which were up-regulated and 37 of which down-regulated. Notably, 8 of the 11 up-regulated genes belong to the immune response group, whereas the down-regulated genes mainly encode for various metabolic functions. It is noteworthy that immune response genes are up-regulated in the PKD and transplant groups, indicating that

TABLE 3. Genes differentially regulated in ESRD.

Fold-change Tx	fold-change PKD	Gene name	Gene ID	Category
−21.73	−25.88	Glutathione S-transferase A3	L13275	Oxygen and radical metabolism
−15.75	−20.94	Phenylalanine hydroxylase	U49897	Protein metabolism
−12.23	−19.62	Serum albumin gene	M12523	Homeostasis
−11.32	−13.52	Glucose-6-phosphatase	U01120	Carbohydrate metabolism
−9.59	−11.69	Glutathione S-transferase subunit 1	M21758	Oxygen and radical metabolism
−9.54	−12.21	C19-steroid specific UDP-gluconuryl-transferase	U59209	Steroid metabolism
−9.03	−12.82	Na-dependant high affinity dicarboxalate transporter	AF154121	Homeostasis
−8.95	−14.20	Dopa decarboxylase (DDC)	M88700	Protein metabolism
−8.32	−11.73	D-amino acid oxidase	X13227	Protein metabolism
−7.89	−7.60	Uromodulin (Tamm-Horsfall glycoprotein)	M15881	Unknown
−6.35	−6.95	4-hydroxyphenylpyruvic acid dioxygenase	D31628	Protein metabolism
−5.89	−8.40	Galectin 7	L07769	Cell cycle
−5.86	−9.67	AK3 pseudogene	X60674	DNA metabolism
−5.52	−7.98	Translational inhibitor protein p14.4	X95384	Protein metabolism
−5.52	−7.88	Maltase-glucoamylase	AF016833	Carbohydrate metabolism
−5.50	−7.13	Klotho	AB005142	Signaling
−5.41	−8.47	Metallothionein	X64177	Homeostasis
−5.34	−9.69	Common acute lymphoblastic leukemia antigen	J03779	Immune response
−5.32	−7.42	Aldolase C	X05196	Carbohydrate metabolism
−5.29	−6.51	Methylmalonate semialdehyde dehydrogenase	M93405	Carbohydrate metabolism
−5.14	−5.53	Putative glycine-N-acyltransferase	AF023466	Protein metabolism
−5.08	−6.95	Ketohexokinase	X78678	Carbohydrate metabolism
−5.01	−7.90	Alpha-methylacyl-CoA recemase	AF047020	Lipid metabolism
−4.91	−6.28	Zn-alpha2-glycoprotein	X59766	Adhesion/ECM
−4.82	−5.85	Argininosuccinate synthetase	X01630	Protein metabolism
−4.81	−4.03	Atrial natriuretic peptide clearance receptor	M59305	Homeostasis
−4.73	−4.87	Fructose-1,6-biphosphatase (FBP1)	U21931	Carbohydrate metabolism
−4.73	−5.13	Apolipoprotein H	M62839	Lipid metabolism
−4.50	−4.41	Cytosolic epoxide hydrolase	L05779	Oxygen and radical metabolism
−4.40	−4.43	F-actin capping protein beta subunit	U03271	Cytoskeleton
−4.25	−5.49	GIF-growth inhibitory factor	S72043	Homeostasis
−4.18	−7.06	Apolipoprotein E	M12529	Lipid metabolism
−4.16	−6.24	NPD009	AF237813	Lipid metabolism
−4.11	−7.73	Phosphenolpyruvate carboxykinase	L05144	Carbohydrate metabolism
−4.10	−5.69	MTIL	AJ011772	Homeostasis
−4.05	−5.33	Liver-type alkaline phosphatase	X14174	Differentiation
−4.01	−5.38	Enyol-CoA	L07077	Lipid metabolism
4.47	6.17	HE4 extracellular proteinase inhibitor homologue	X63187	Protein metabolism
5.09	5.96	Nicotinamide N-methyltransferase	U08021	DNA metabolism
5.26	4.54	Immunoglobulin heavy chain	AF013620	Immune response
6.69	6.25	Ig rearranged gamma chain	M63438	Immune response
6.78	4.25	CD53 glycoprotein	M37033	Immune response
7.06	4.71	Secreted cement gland protein	AF038451	Steroid metabolism
7.35	5.40	Ig lambda heavy chain	Y14737	Immune response
8.48	7.18	SNC73 protein	AF067420	Immune response
9.11	9.98	Ig kappa chain	M11937	Immune response
9.20	6.66	Rearranged immunoglobulin heavy chain	U19885	Immune response
9.81	9.44	Ig germline kappa-chain C18	X72815	Immune response

To identify significantly regulated genes in ESRD, we selected genes that were significantly different from normal tissue ($P < 0.05$) and at least 1.5-fold up- or down-regulated. A group of 430 genes met these criteria. This table lists all genes that were more than 4-fold regulated in the ESRD group.

PKD, polycystic kidney disease; ESRD, end-stage renal disease; Ig, immunoglobulin.

hematopoietic cells infiltrating the failing kidneys probably cause this up-regulation.

Verification of Differentially Expressed Candidate Genes by Reverse Transcriptase-Polymerase Chain Reaction

To demonstrate that differentially regulated genes were correctly identified by microarray analysis, we performed semiquantitative RT-PCR of several candidate genes. Although this method may underestimate differences in gene expression, the results obtained by RT-PCR closely mirror the microarray data (Fig. 4) except for one gene, M27749, which is an immunoglobulin light chain carrying the MER 22 repetitive element. The up-regulation of M27749 seen in several array experiments could not be verified with RT-PCR specific for the M27749 sequence and is most likely the result of the cross-reactivity with several different light chains that are not amplified using specific primer sets for M27749.

TABLE 4. Clinical characteristics of transplant patients belonging to the transplant subgroups T1 and T2 and the Banff classification of the transplant subgroups T1 and T2

Tx-cluster	Tx	Sex	Time since restart of hemodialysis	Immunosuppression	Donor age	Donor sex	Donor serum creatinine	Cold ischemia time	Warm ischemia time
1	1	F	1 mo	FK506, Steroids	46	F	0.7	37 h 45 min	35 min
1	5	M	8 mo	Steroids, MMF	30	F	1	15 h 57 min	21 min
1	10	F	1 mo	Cyclosporine, Steroids, MMF	57	M	1.3	25 h 24 min	25 min
1	11	F	3 mo	Cyclosporine, Steroids	16	M	0.9	18 h 28 min	26 min
1	13	M	6 mo	Cyclosporine, Steroids	19	M	0.9	14 h 1 min	23 min
		Mean	3.8±3.1		33.6±17.6		1.0±0.2	22 h 19 min±9 h 39 min	26±5 min
2	2	F	4 mo	Steroids	10	F	0.7	16 h 28 min	27 min
2	3	F	19 mo	FK506, Steroids	47	M	1.4	14 h 39 min	33 min
2	4	F	3 mo	FK506, Steroids	14	M	1	15 h 57 min	30 min
2	6	M	10 mo	FK506, Steroids	30	F	0.6	21 h 21 min	25 min
2	7	M	25 mo	Steroids	43	M	1	19 h 22 min	27 min
2	8	F	39 mo	None	NK	NK	NK	35 h 49 min	26 min
2	9	F	2 mo	Cyclosporine, Steroids	57	M	0.6	30 h 7 min	28 min
2	12	F	30 mo	FK506, MMF	NK	NK	NK	NK	NK
		Mean	16.5±13.9		33.5±18.8		0.9±0.3	21 h 58 min±8 h 01 min	28±3 min
		P value	NS		NS		NS	NS	NS

Tx-cluster	Tx	cg	ci	ct	cv
1	1	3	3	3	2
1	5	3	3	3	3
1	10	1	2	2	2
1	11	3	2	2	2
1	13	3	1	2	2
	Mean	2.6	1.8	2.4	2.2
2	2	1	3	2	1
2	3	3	3	3	3
2	4	1	2	2	1
2	6	3	3	3	3
2	7	3	3	3	3
2	8	1	1	1	2
2	9	3	3	2	2
2	12	2	3	3	2
	Mean	2.1	2.6	2.4	2.1
	P	NS	NS	NS	NS

Histologic findings of transplant kidneys belonging to the transplant subgroups T1 and T2. For classification, the revised Banff '97 criteria were used: eg., allograft glomerulopathy; ci, interstitial fibrosis; ct, tubular atrophy; cv, vascular fibrous intimal thickening. No significant differences were found between the two groups. NS, not significant; MMF, mycophenolate mofetil.

DISCUSSION

Chronic transplant nephropathy remains a poorly defined disease entity that accounts for a 3% to 4% annual graft loss after the first year of transplantation and that limits the current half-life of renal allografts (*7–9*, *22*, *23*). Calcineurin inhibitors have significantly improved the short-term survival, but they had little impact on the long-term outcome of renal transplants. During the past years, it has become increasingly apparent that chronic rejection has multiple causes, generally divided into immunologic and nonimmunologic factors. HLA mismatches and preformed donor-specific antibodies, often responsible for acute vascular rejections (*24*), are predictive of subsequent graft dysfunction. At later stages, glomerular hyperfiltration as the result of progressive nephron loss may contribute to progressive renal dysfunction that is no longer amenable to immunosuppressive therapy (*13*, *25*). Optimizing donor conditioning, reducing ischemia times, and treating hypertension and hyperlipidemia are believed to reduce the risk of chronic rejection. However, these factors probably account for less than 40% of the graft dysfunction observed in experimental models of chronic rejection (*26*). Therefore, improving immunosuppressive therapy that prevents acute rejection and subsequent chronic dysfunction without interfering with the development of immune tolerance early after transplantation remains a major task. To optimize current immunosuppressive protocols, clearly defined sensitive and specific markers are needed that reliably predict chronic rejection at an early stage. Currently, the diagnosis of chronic rejection relies mainly on nonspecific histologic findings (*23*), and more sensitive tools are needed to accurately diagnose chronic transplant nephropathy.

We therefore analyzed the pattern of gene expression in renal transplants. Chronically rejected transplants were chosen for two reasons: First, these transplants provide sufficient material for microarray analysis avoiding the bias associated with nonlinear amplification methods. Second, these transplants may more uniformly reveal the typical gene expression pattern of chronic rejection. Microarray analysis from transplant biopsies at earlier stages are confounded by

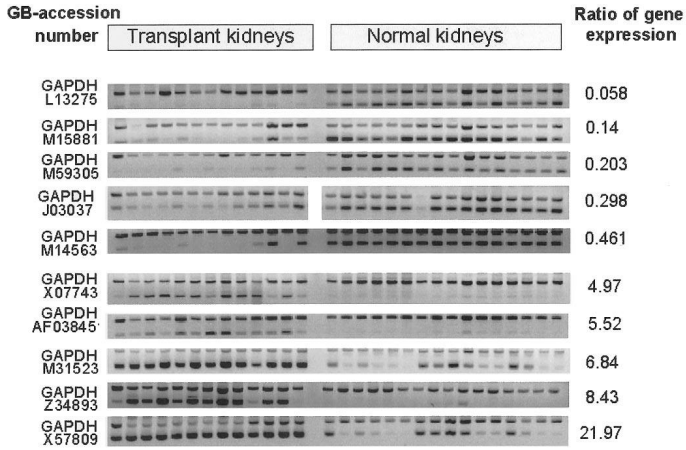

FIGURE 4. Verification of the differential expression of candidate genes by reverse transcriptase-polymerase chain reaction (RT-PCR). Differential expression of candidate genes was verified by RT-PCR. Semiquantitative RT-PCR of several candidate genes was performed for 10 regulated genes. The results obtained by RT-PCR showed comparable results to the microarray data except for one gene, M27749. The mean of the ratios of gene expression was calculated with the Scion Image software (Scion, Frederick, MD).

the multiplicity of events, including the recovery from ischemia-reperfusion injury, acute rejection, drug toxicity, and infection. We analyzed the differentially regulated genes of chronic rejection in comparison with two different controls: First, we subtracted all housekeeping genes, typically expressed in normal renal tissue. Second, we eliminated genes differentially regulated in ESRD not related to chronic rejection. The second control is problematic because it can eliminate potential genes of interest from further analysis, for example, genes that are nonspecific for transplant nephropathy but predictive of chronic rejection early after transplantation. Therefore, we selected polycystic kidneys with ESRD, because glomerular hyperperfusion and secondary focal sclerosis does not seem to significantly contribute to the pathogenesis of renal failure in this hereditary disease. Gene profiling of each sample was performed compared with a well-defined reference RNA to allow the comparison between the three sets of tissues. To distinguish gene expression patterns of the three sets of samples, we selected 571 genes that were differentially regulated, using the paired t test to calculate statistical significance. These genes were subsequently used for hierarchical clustering, an algorithm that determines how closely related samples are, on the basis of the expression profile of each sample. The results are typically depicted as two-dimensional dendrograms (Fig. 2) in which short vertical lines to the next branch indicate a high level of similarity. Not surprisingly, this analysis revealed that chronically rejected transplants are more closely related to polycystic kidneys than they are to normal tissue. However, it is quite remarkable that the two polycystic kidneys removed from the same two patients, respectively, within several months, were correctly identified as closely related tissues by the hierarchical cluster algorithm (PKD 9 and 3, and PKD 12 and 4). The 13 transplants were identified as distinct entities, different from normal tissue and more closely related to the gene expression pattern in polycystic kidneys with renal failure. Surprisingly, the differential expression of 48 genes further subdivided the transplants into two distinct groups. Correlation with clinical and histologic data did not reveal apparent differences between these two groups, thus indicating that microarray analysis can identify distinct subsets of chronic rejection that are not apparent by clinical or histologic criteria.

A variety of cytokines, profibrotic growth factors, and matrix proteins have been implicated in the pathogenesis of chronic rejection (*7*, *23*, *25*, *27*) and correlate with the development of structural injury (*28–30*). Whether microarray analysis of samples taken as protocol biopsies may reveal new pathogenic or therapeutic targets remains to be shown. However, our pilot study of 13 transplants clearly shows that this approach can define different subsets of chronic transplant dysfunction that may indeed represent different disease entities requiring different therapeutic approaches to prolong graft survival.

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0041-1337/03/7603-547/0
TRANSPLANTATION
Vol. 76, 547–552, No. 3, August 15, 2003
Printed in U.S.A.

SIMULTANEOUS PANCREAS-KIDNEY TRANSPLANT FROM LIVING RELATED DONOR: A SINGLE-CENTER EXPERIENCE

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Background. **Simultaneous pancreas and kidney transplantation (SPK) from cadaveric donors has become a widely accepted therapeutic option for insulin-dependent uremic patients. In 1996 the first SPK from a live donor was performed. This procedure offers the advantage of a better immunologic match, reduced cold ischemia injury, and decreased waiting time. As such, it is an attractive alternative treatment for diabetic patients with end-stage nephropathy with an available living donor.**

Methods. **We performed six SPKs from living-related donors. There were four men and two women among the recipients; median age was 34 (range, 29–39) years. All donors were recipients' siblings with excellent HLA matching. Donors underwent standardized metabolic workup, anti-insulin and anti-islet antibody assays, and computed tomography of the abdomen. Both donors and recipients were treated with octreotide for 5 days perioperatively. After transplantation, the patients were maintained on tacrolimus-based immunosuppression, with the exception of one recipient of SPK from an identical twin, who received cyclosporine monotherapy.**

Results. **All the donors are doing well and have normal renal function and blood glucose levels. One-year patient, renal, and pancreatic graft survival rates were 100%, 100%, and 83%, respectively. Acute kidney rejection was documented in two patients, and both recovered completely after OKT3 therapy. No rejection of pancreatic graft has been documented. Except for one patient who lost the graft because of hemorrhagic pancreatitis, all recipients maintained serum glucose levels at less than 130 mg/dL without insulin therapy. No major surgical complications such as graft thrombosis, intra-abdominal infection, or abscess were reported.**

Conclusions. **Living donor SPK can represent a successful alternative to cadaveric donor SPK. The procedure can be performed safely in the donor and with low morbidity in the recipient.**

The annual incidence of type 1 diabetes has been rising steadily worldwide during the past 70 years. It is now estimated that this disease affects 500,000 to 800,000 people in the United States and a comparable number in Europe (*1*).

To reestablish the euglycemic state, the treatment options for diabetic patients include either multiple daily insulin injections or continuous subcutaneous insulin infusion by external or internal pump. Despite the aggressive exogenous insulin therapy, life-threatening long-term complications, including nephropathy, develop in diabetic patients (*2*). Chronic renal insufficiency leads the patient to dialysis and significantly diminishes day-to-day quality of life. Moreover, diabetic patients with end-stage renal disease (ESRD) manifest the highest mortality of any group of patients with ESRD and the highest rate of severe coronary atherosclerotic events (*3*,*4*). Insulin independence for these patients can only be achieved by transplantation of pancreatic islet beta cells. The resumption of normal glucose homeostasis can be achieved after free islet cell transplantation, which has been introduced to the treatment in recent years (*5*). It is conceptually the most efficient modality, but considering its many

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Received 3 January 2003.
Revision Requested 12 February 2003. Accepted 8 April 2003.
DOI: 10.1097/01.TP.0000076624.79720.14