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APPLICATIONS OF MICROARRAYS TO RENAL TRANSPLANTATION: PROGRESS AND POSSIBILITIES

Mei-Sze Chua, Elaine Mansfield and Minnie Sarwal

Department of Pediatrics, Stanford University School of Medicine, G320, 300 Pasteur Drive, Stanford, CA 94305

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1. ABSTRACT

By rapidly generating global views of gene expression profiles, microarray technology offers a great advantage over traditional methods of studying gene expression. This technology is gaining rapid and widespread use in many areas of science and medicine because it can be easily adapted to study many experimental questions. This article will review the current applications of microarray technology in the field of renal transplantation, and discuss the potential impact of this technology on transplantation medicine.

2. INTRODUCTION

Since its introduction in the mid 1990s, microarray technology has gained widespread acceptance and use in many areas of biomedical research (1), with the ultimate goal of finding candidate genes for diagnostic, prognostic and therapeutic purposes (2). By providing a global view of the gene expression signatures underlying disease states, microarrays are a valuable tool in mechanistic studies, especially when used in conjunction with other molecular and biochemical techniques. In oncology, for example, microarray technology has generated insightful data regarding the molecular mechanisms of cancer development, the classification of molecular sub-classes of malignancies (3), the identification of prognostic markers (4,5) and the identification candidate gene targets for the design of novel therapeutic agents (6,7). Other disciplines such as immunology (8), nephrology (9) and cardiovascular disease (10) research are beginning to exploit this powerful and flexible technology. This article will provide an overview of the basic principles of DNA microarrays, their limitations and their clinical applications with particular focus on the field of renal transplantation.

3. MICROARRAY TECHNOLOGY

In general, the term microarray refers to a high-density array of complementary DNA (cDNA) or oligonucleotide immobilized onto a structural support; it is based on the principle that complementary sequences of DNA can be used to probe and hybridize to the immobilized DNA molecules. Thus, unlike traditional methods of quantifying mRNAs by northern blotting or quantitative PCR which can only measure a few genes at a time, DNA microarrays allow the rapid and accurate analysis of global gene expression in an overnight hybridization. Because of the extreme versatility of this technology, it can be readily adapted for use in many investigations, ranging from the basic (11) to clinical sciences (12-14).

DNA microarrays are generally made by spotting solutions of individual PCR-amplified double-stranded cDNA fragments (about 500 base pairs) as small spots on microscopic glass slides; this allows approximately 50,000 genes to be printed on a single glass slide. In contrast, oligonucleotide arrays consist of short fragments (20-25 nucleotides) of single-stranded DNA that are either directly synthesized on a solid surface or pre-synthesized and then printed onto glass slides. Very high density arrays of greater than 250,000 oligonucleotide spots/cm^2 can be made repetitively, but this approach incurs a high cost and does not readily allow flexibility in design (15). The more popular technology in academic institutions is the use of

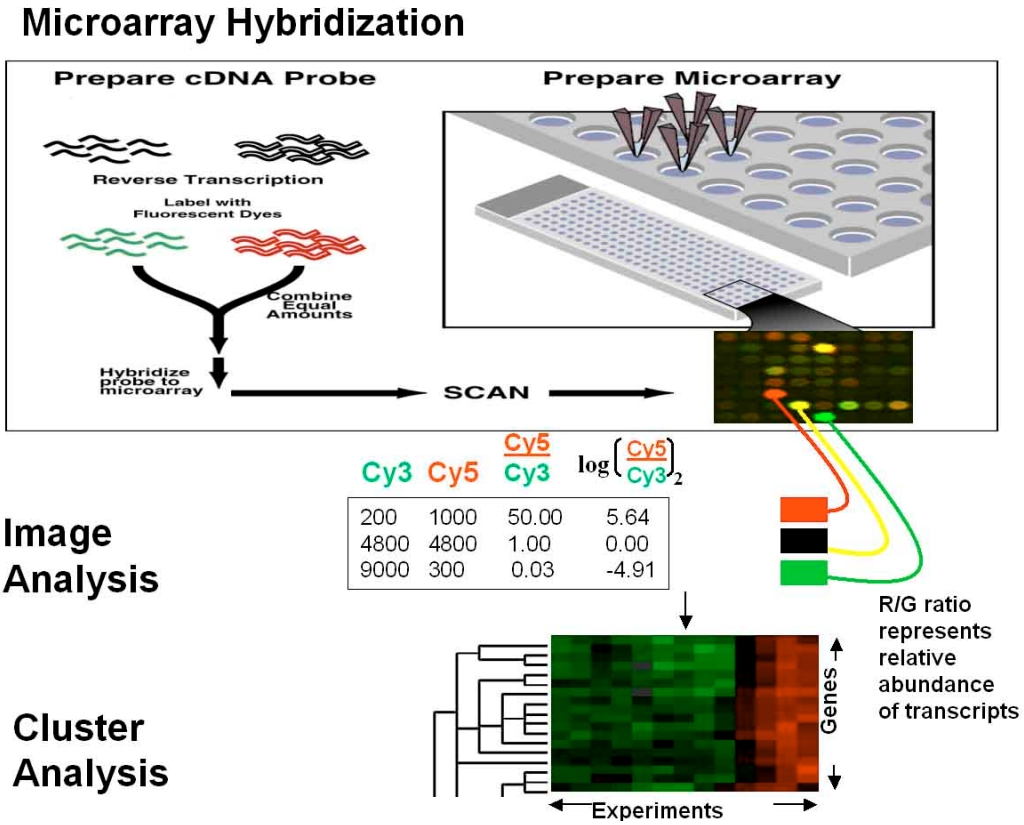

Figure 1. Schematic representation of DNA microarray technology. Typically, total RNA is first isolated from the samples of interest; this test RNA and a reference RNA are then differentially labeled with fluorescent dyes and then competitively hybridized onto a printed DNA microarray. Images that are generated are then scanned and the resulting fluorescent intensities used to generate a hierarchical cluster and for further data analysis.

DNA microarrays, since each slide may be custom designed for specific investigations, and the manufacture is less expensive.

Typically, a two-color hybridization scheme is used to visualize and measure the gene expression levels reproducibly when comparing the gene expression profiles of multiple samples using spotted DNA microarrays (1,2) (Figure 1). The first step involves RNA extraction from the test and reference samples, followed by fluorescent dye labeling of the RNA during a reverse transcription step. Common dyes used are Cy3 (for labeling the reference RNA green) and Cy5 (for labeling the test RNA red); labeled RNAs are then competitively hybridized to each DNA microarray and the ratio of red to green fluorescence (R/G ratio) measured by scanning the microarray slide using two different wavelengths specific to each dye (532 nm for Cy3 and 635 nm for Cy5). The color of each spot reflects the relative abundance of test versus. reference RNA: green if reference RNA is more abundant than test RNA, red if the gene is more abundant in the test RNA than reference RNA, and yellow if they are equally abundant (Figure 1). In order to allow comparisons across a large number of samples, a common reference sample of constant composition is usually used in all experiments to provide a consistent comparison standard (16-18).

4. DATA ANALYSIS

Since an overnight hybridization of a single microarray generates thousands of data points, the meaningful analysis of the large data sets involves the use of sophisticated software tools, which are currently available from either public sources (e. g. http://genome-www4.stanford.edu/MicroArray/SMD/restech.html) or from commercial suppliers (such as GeneSpring from SiliconGenetics). Typically, data is first normalized to allow expression levels across samples to be effectively compared; it is then filtered to remove genes that are expressed below a defined threshold value. Finally, clustering and visualization programs such as hierarchical clustering and K-means clustering are used to generate fundamental gene expression patterns inherent in the massive data sets, thereby allowing possible biological or clinical relevance to be inferred.

Among the microarray data analysis tools used in microarray analysis at Stanford University and supported through the Stanford Microarray Database (19) are ones for performing Hierarchical Clustering, Disease or Class Prediction using Prediction Analysis of Microarrays (PAM), Significance Analysis of Microarrays (SAM), and

Singular Value Decomposition (SVD). The use of each of these tools will now be described in further detail.

Class Discovery using Hierarchical Clustering: A reliable unsupervised method (no prior knowledge of the true functional classes) for studying gene expression patterns is hierarchical clustering (20). This method uses similarity or distance measures to distinguish between samples. Genes with similar expression profiles across a set of experimental samples are clustered together on the vertical axis, whereas experimental samples are clustered together on the horizontal axis based on the overall similarity in expression behavior across a filtered list of genes. The data is displayed in a tabular form with each row representing the data for a single gene and each column representing the data for a single experimental sample (See Figure 1 for example display).

A hierarchical tree or dendrogram is displayed next to the clustered genes and above the clustered experimental samples to graphically denote the degrees of relatedness between adjacent samples and genes. The closer two samples are together, the greater the similarity between them (Figure 1). In this colored tabular scheme, the fold-deviation from average expression of each gene across the set of samples studied is represented by an intensity scale of colors from red (above average level of mRNA present for that gene) through black (average expression of that gene) to green (below average level of mRNA present for that gene). The biological or clinical significance of differential gene expression across experimental samples may then be inferred and further tested. Although very useful and popular, hierarchical clustering has a number of shortcomings which can be overcome by the using Self-Organizing Maps (SOMs) (21), which allows the imposition of partial structure on the clusters and facilitate easy visualization and interpretation. SOMs have features which are suited for the clustering and analysis of gene expression patterns; have good computational properties; are easy to implement; and are reasonably fast and scalable to large data sets.

Disease or Class Prediction using Prediction Analysis of Microarrays (PAM): One exciting application of microarray technology is the ability to classify and predict the diagnostic category of a sample based on its gene expression profile. This problem of classification is particularly challenging because of the large number of genes from which to predict classes and the relatively small number of samples. Additionally, it is important to identify the genes that are most characteristic of, and therefore contribute most to, the classification. To this end, a class prediction algorithm, PAM, was developed based on an enhancement of the simple nearest prototype (centroid) classifier (22). PAM is useful in identifying minimal subsets of genes that characterize each cluster, and has proven to be effective and accurate in classifying different subsets of small round blue cell tumors. These small round blue cell tumors of childhood (comprising neuroblastoma, rhabdomyosarcoma, non-Hodgkin lymphoma and Ewing family of tumors) are conventionally difficult to distinguish by light microscopy due to their similar histology. Accurate diagnosis is critical because the choice and outcome of therapy vary widely depending on the diagnosis; however, current clinical diagnostic techniques are limited (23). The ability to distinguish these tumors into their subgroups based on their gene expression signatures therefore represents an important step towards improved diagnosis and treatment. This analytical tool can be further applied to similar problems which are otherwise difficult by conventional parameters of clinical pathology.

Significance Analysis of Microarrays (SAM): Hierarchical clustering of microarray data produces coherent patterns of gene expression but provides little information about statistical significance. Conventional statistical T-tests are limited in that they provide the probability that a difference in gene expression occurred by chance in only a small numbers of genes. SAM (http://www-stat-class.stanford.edu/SAM/SAMServlet) was therefore specifically developed for analyzing microarray data (24); it allows identification of genes with statistically significant changes in expression by assimilating a set of gene-specific T-tests. Each gene is assigned a score on the basis of its change in gene expression relative to the standard deviation of repeated measurements for that gene and genes with scores greater than a user-defined threshold are considered potentially significant. SAM uses random permutations of the sample labels to estimate the false discovery rate (FDR) of the significant gene list. The FDR is the expected proportion of false positive calls among the genes called significant. With large numbers of tests inherent in microarray experiments (genes), this approach is more powerful than the usual Bonferroni method for p-value adjustment (Robert Tibshirani, personal communication). This analytical method is robust, straightforward and can be adapted to a broad range of experimental situations and has been applied with good success to identification of markers for specific classifications of cancer (25, 26).

Singular Value Decomposition (SVD): The use of SVD additionally provides a useful mathematical framework for processing and modeling genome-wide expression data (27). SVD linearly transforms the expression data in the form of genes x arrays space to 'eigengenes' x 'eigenarrays' space, where the eigengenes or eigenarrays are unique orthonormal superpositions of the genes or arrays. Normalizing the data using identified eigengene vectors enables meaningful comparison of the expression of different genes across different arrays in different experiments. The interpreted sets of eigengenes (and the eigenarrays) help with the discovery of groups of genes that appear to be classified into groups of similar regulation and function (27). Further, the method identifies systematic biases in the data that are inferred to represent noise or experimental artifacts when apparent measurements form replicate samples differ from one another. These differences are minimized after the data is normalized by the SVD algorithm.

5. LIMITATIONS

Despite the advantages associated with the use of microarrays, many limitations currently exist, most of which relate to the technology itself. Some of the common

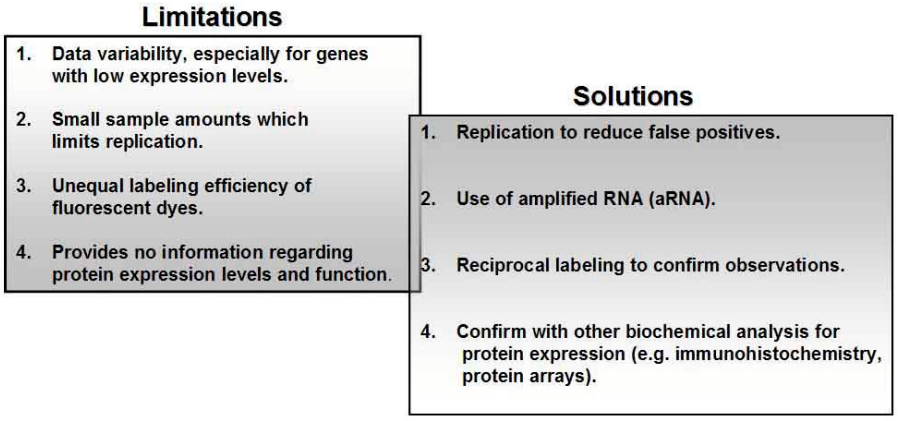

Figure 2. Common limitations and solutions to DNA microarray methodology.

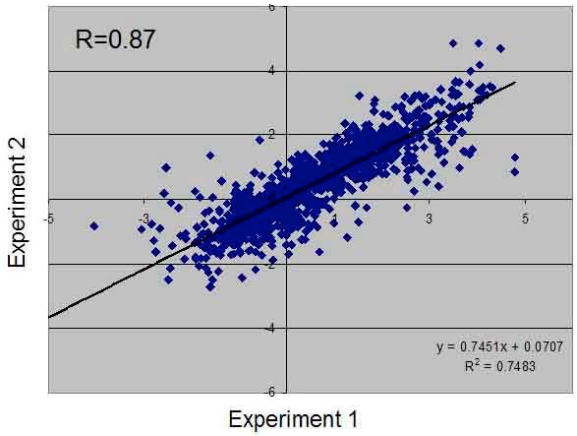

Figure 3. Correlation of gene expression patterns between replicate microarray experiments of amplified RNA prepared from the same sample. Replicate microarray experiments of two samples processed from the same patient yield a high correlation in measurements (R^2=0.7483), suggesting good reproducibility of the microarray hybridization protocol and little biological variability.

limitations and their solutions are listed in Figure 2. Here we will discuss in greater depth some of the more predominant problems.

Controlling variability: The variability of microarray results can be significant, especially for genes with low expression levels. Replication is recommended to establish a high degree of confidence, and to reduce the number of potential false positive results. However, this may be difficult due to high cost or insufficient sample amount. Factors specific to microarray experiments that add to data variability include: 1. insufficient total RNA from samples therefore requiring amplification steps that may introduce bias (see further discussion later in this review); 2. unequal efficiency of fluorescent dye labeling during reverse transcription; 3. reduced ability or failure of certain DNA elements on the array to detect the right transcripts as a result of cross-hybridization or adverse secondary structure. Alternative and more conventional techniques such as northern blotting, RNase protection or real-time PCR, may be used to verify a subset of results thereby helping to establish an estimate of the variability of a given experimental system.

As with other types of scientific experiments, microarray experiments are subject to random fluctuations resulting from either experimental procedures or inherent biological variations (28-30). Fluctuations due to variations in microarray production and their hybridization can be mitigated by re-sampling a single cell type or tissue; whereas fluctuations due to variability across different biological samples can be mitigated by sampling from similar cell types or tissues. In our hands, repeated samples from the same patient gave highly reproducible gene expression patterns (Figure 3), as shown by these two replicate samples being clustered next to each other in our hierarchical cluster (31). Occasionally, the use of different batch prints of microarrays can give rise to "false clustering" of samples; such batch effect can be filtered out using SVD (27), which normalizes the data by filtering out experimental artifacts to allow more meaningful comparison of the expression data.

Sample Amplification for Array Analysis: As the amount of total RNA extracted from either blood or biopsy samples is generally insufficient for DNA microarray hybridization, the extracted RNA is amplified to produce

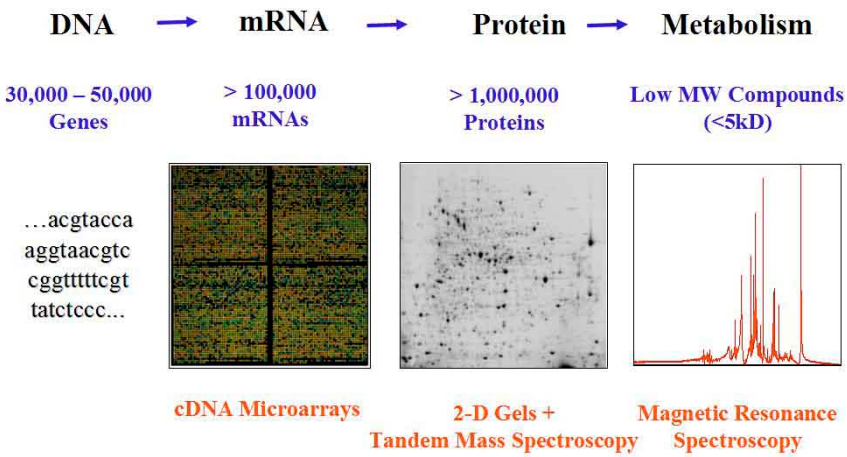

Figure 4. Structural and functional analysis of the human genome. High-density cDNA microarrays have revolutionized the understanding of biology and opened the field of genomics, a study of the transcriptosome at the genome-scale. The 30-50,000 genes stored in genomic DNA are selectively transcribed to over 100,000 different mRNA molecules which result from alternatively spliced variants in different tissues. The relative abundance of all mRNA species can be measured simultaneously using microarrays. Similarly, post-translational modification of translated proteins results in an even higher abundance of different proteins in the living organism. High-resolution two-dimensional gels (2D-gel) and tandem mass-spectroscopy can be used to structurally identify gene products at the protein level. Functional analysis of cellular metabolism requires the analysis of low-molecular weight metabolites and recent advances in magnetic resonance spectroscopy have been applied to these analyses on a high-throughput, automated basis.

enhanced quantities of antisense RNA (aRNA) for subsequent hybridization. Amplification may be done in one or two successive rounds depending on the amount of starting material available. Biopsy samples often need two rounds of amplification in order to produce enough aRNA for labeling. Typically, the amplification protocol produces sufficient aRNA for up to 3 hybridizations, each using 5 microgram of RNA (32). The systematic bias that may be introduced by RNA amplification has been assessed by comparing the expression profiles generated by aRNA vs. that generated by total RNA, and very strong correlation between the two were obtained (31, 33). We have also proven the robustness of this amplification strategy (R^2 = 0.87 between first versus second round amplification aRNA, Sarwal *et al.*, unpublished data) and have been routinely using double amplified aRNA, for both biopsy and blood samples, due to the paucity of starting material (1/2 -1 core of an 18 gauge biopsy needle from allograft biopsy specimens and 2.5 ml of peripheral blood). When samples have been double amplified, the common reference used as a denominator in these experiments is also double amplified (31).

Sampling Source: Renal biopsy samples used for microarray analysis contain a mixture of different cell types. Thus, with the exception of cell type-specific genes (e. g. E-selectin), the source of mRNA is unknown and limits our ability to interpret the cellular signatures relating to the gene expression patterns of our data. To address this concern, laser-capture microdissection of cellular subtypes of interest and microarray analysis after RNA amplification has been attempted with success (34, 35). Alternatively, the gene expression profiles from specific cell types can be compared with that of the whole tissue; data has been generated from resting and activated T and B cells (data extracted from ref. 26), the major group of cells infiltrating the graft during the alloimmune response. An additional method is to study the gene at the protein levels by immunohistochemistry for genes of interest in specific samples of interest (36) or by the use of tissue microarrays (37). The latter allows for the simultaneous examination of hundreds of tissues of interest with numerous different antibodies per sample. Comprehensive systems for high-throughput analysis and storage of tissue microarray data are available at http://genome-www.stanford.edu/TMA/index.shtml.

Further Functional Analyses: DNA microarrays provide results on mRNA expression levels which do not necessarily correlate with protein expression levels or function (38). Thus, these results provide only an incomplete view of the functional significance of differentially expressed genes in the experiments. Techniques for protein analysis such as western blotting, two-dimensional polyacrylamide gel electrophoresis, radioligand receptor binding, chromatographic separation and detection, as well as mass spectrometry (Figure 4) remain indispensable for elucidating protein levels or function (39). With the rapid advance of enabling technologies (i.e. consistent antibody library production and cost-effective slide production methods), the development and use of protein microarrays to address these questions may soon be possible.

6. THE IMMUNE SYSTEM IN TRANSPLANTATION

Renal transplantation is the standard procedure, which also provides the optimum therapy, for patients with

end-stage kidney disease. Several risk periods follow a typical organ transplant procedure. The early period is associated with non-specific injury, inflammation, host immune recognition, and frequent rejection. In recent clinical trials, acute rejection has been almost exclusively confined to the first 6 months, provided patients are compliant with their maintenance immunosuppression therapy. A poorly understood adaptation period then occurs, which renders acute rejection episodes infrequent in compliant patients, although inflammation may persist. The significance of inflammation observed on protocol biopsies remains controversial.

Late rejection episodes are sometimes associated with a worse prognosis, perhaps because they represent a failure of adaptation or compliance (40). Overall outcomes have improved significantly for recipients of renal allografts with better immunotyping and histocompatibility matches; shorter ischemia times; increasing use of living-related donors; and improvements in recent immunosuppressive drug profiles. Nevertheless, certain questions remain elusive and pose a challenge to the physician with regards to diagnosis, treatment and outcome prediction. Single molecule analyses have defined crucial pathways controlling the alloimmune response, and the advent of microarray technology now challenges us to piece together the entire puzzle, urging us to further understand the mysteries shrouding the immunobiology of differential responses in acute rejection, the pathogenesis of chronic rejection, and the development of sustained tolerance. The current tools available to us now allow for structural and functional analysis of the human genome, relating to specific diseases of interest. These range from genomic and complementary DNA sequencing for structural analysis; DNA microarrays for expression analysis; tissue, antibody and protein arrays; and mass spectrometry for more detailed functional analysis (Figure 4).

7. APPLICATION OF MICROARRAY TECHNOLOGY TO RENAL TRANSPLANTATION

7.1. Acute renal allograft rejection

Acute renal allograft rejection is clinically heterogeneous yet histopathologically indistinct. Despite efforts at systemization using the Banff method, it is still difficult to individualize therapy and predict graft outcome based on current criteria. Presumably, the pathophysiological diversity of acute rejection stems from heterogeneity at the molecular level. Recently, Akalin *et al.* have tested this hypothesis by using oligonucleotide arrays to screen for candidate genes that may be involved in the underlying mechanisms of acute rejection and which may be useful for the diagnosis of these episodes (41). We further strengthened this hypothesis by using DNA microarrays to generate gene expression profiles of 21 allograft biopsies from pediatric patients experiencing acute rejection post-transplantation. Indeed, 3 sub-groups of acute rejection were identified: AR-I, AR-II and AR-III (31). Whereas AR-I consists only of acute rejection samples, AR-II and AR-III co-clustered with samples from patients with features of drug toxicity and chronic allograft nephropathy respectively. This molecular sub-classification of acute rejection has potential clinical significance: survival analysis indicates that patients belonging to AR-I had significantly poorer graft function recovery than patients in other sub-groups of acute rejection. This suggests that patients in AR-I require more aggressive therapy and surveillance than patients in other sub-groups. When analyzing a larger data set of post-transplant renal allograft biopsy samples from patients with chronic allograft nephropathy, normal function or acute rejection, acute rejection samples show a cluster of genes that are significantly up-regulated compared to chronic allograft nephropathy and normal samples (Figure 5); the significance of these differences will be further analyzed, and expected to shed light on the molecular mechanisms leading to acute rejection.

7.2. Chronic allograft nephropathy

Apart from acute rejection, another poorly understand and equally important process is chronic allograft nephropathy (CAN), which is a complex process resulting from as yet undefined etiology with both an immune and non-immune components. Being able to define the cause of a CAN episode can be greatly beneficial. To this end, we and others have generated animal models of vascular injury, and have been studying gene expression patterns of a non-immune, primate (baboon) vascular model of chronic injury (42,43). The baboon was chosen as the model system in our studies for several reasons: 1. human samples are difficult to obtain and sample volume is often inadequate for study; 2. the baboon carotid (unlike the rodent vessel) has a defined intima area similar to that in humans; 3. the baboon is genetically homologous to humans and thus cross hybridization of baboon samples to human arrays would be likely.

To date, we have identified multiple factors involved in the early, intermediate and late phases of chronic vascular injury and eventually hope to correlate these results to human chronic vascular injury in CAN biopsy samples. Interestingly, many of the pathways involved in chronic injury and fibrosis are regulated very early in the course of the injury (manuscript in preparation), when the downstream effects of these alterations are still not evident by pathology. Results from these studies could help to suggest molecular targets for intervention to abrogate vessel injury as well as the appropriate timing for these interventions.

7.3. Peripheral blood markers for rejection

Traditionally, graft outcome has been monitored by follow-up biopsies. To obviate the need for this invasive monitoring technique, research efforts have been aimed at identifying peripheral blood markers of graft dysfunctions such as acute rejection. Considerable progress has been made in this aspect, using the technique of RT-PCR to identify non-invasive markers of acute rejection in the peripheral blood and urine (44-46). Using DNA microarrays, we have validated the expression of some of these marker genes, such as granulysin, RANTES and perforin, to be preferentially expressed in biopsy

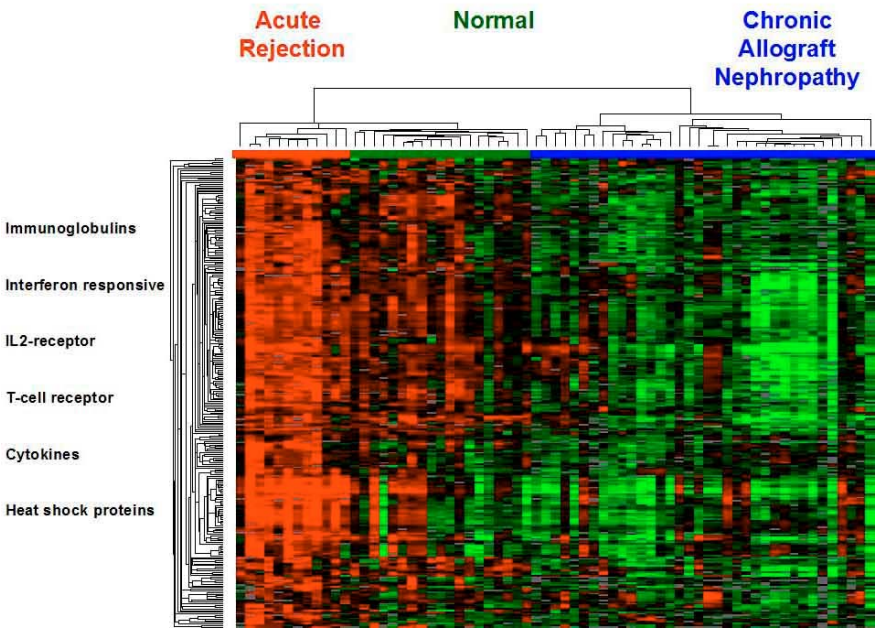

Figure 5. A hierarchical cluster of gene expression patterns across a set of biopsy samples obtained from patients with acute rejection, stable graft function, or chronic allograft nephropathy. Genes (in rows) and patients (in columns) are grouped together based on the similarities identified in their gene expression patterns. In this color scheme, genes that are up-regulated or down-regulated relative to the average expression of that gene across all the samples are represented in red or green respectively. Black represents expression level equal to the average expression of that gene, whereas grey represents missing data. Among the genes most highly-differentially expressed in this study are clustered with immunoglobulins, interferon responsive genes, the IL2-receptor or T-cell receptor, cytokines, and heat shock proteins.

samples from patients with acute rejection. Additionally, gene expression profiles of peripheral blood lymphocytes isolated from patients with acute rejection have been generated; correlation between these two data sets is currently underway and will be valuable in highlighting genes that may serve as markers of acute rejection from peripheral blood samples.

7.4 Analysis of ancillary pathways affected during acute rejection

Compromised renal function following renal allograft transplantation often results in anemia in the recipient. Although inadequate erythropoietin production and iron deficiency have been reported to be the main underlying causes of anemia, the complete picture has yet to be fully understood. By studying the gene expression level at a genome-wide scale using DNA microarrays, insightful and valuable information was obtained to supplement our understanding of the molecular events underlying the etiology of anemia in acute renal allograft rejection (47). Specifically, a cluster of genes was identified to be related to hemoglobin synthesis and/or erythropoiesis that was altered in kidneys with renal allograft rejection compared to normal kidneys. The possible relationship between alterations in the expression of this cluster, reduced renal function, the alloimmune process itself, and other influences on the renal transplant awaits further analysis. These analyses can help to suggest alternative therapeutic and potentially cost-saving approaches (such as aggressive iron and folate replacement and reduced exposure to calcineurin inhibitor drugs) for the correction of anemia in acute rejection, apart from the traditional use of erythropoietin.

A similar genome-wide approach can be adopted to study other processes post-transplantation, such as hyperlipidemia, hypertension and immunosuppression usage, and thus help to provide a complete understanding of the systemic effects of renal transplantation in concert with perturbations in the innate and alloimmune response.

7.5. Profiling immunosuppressive therapies

Corticosteroids have been extensively used as immunosuppressive agents in transplantation, despite their multi-systemic side effects. Their use is particularly limited in pediatric patients who have to take these medications long-term. In an effort to improve patient compliance and possibly post-transplant morbidity, an alternative, steroid-free immunosuppressive regime was recently implemented using extended dosing of daclizumab (a humanized anti-IL-2 receptor antibody), mycophenolate mofetil (an inhibitor of T- and B-lymphocyte proliferation in response to allospecific stimulation) and tacrolimus (an inhibitor of calcineurin and therefore cytokine production) (48). Patients on these alternative immunosuppressive drugs have improved renal function and decreased incidence of clinical and sub-clinical rejection. These differences in graft outcome between a steroid-based and steroid-free patient cohort could possibly be related to differences at the gene expression level, as understandably, different immunosuppressive agents will induce different gene expression profiles. By using DNA microarrays,

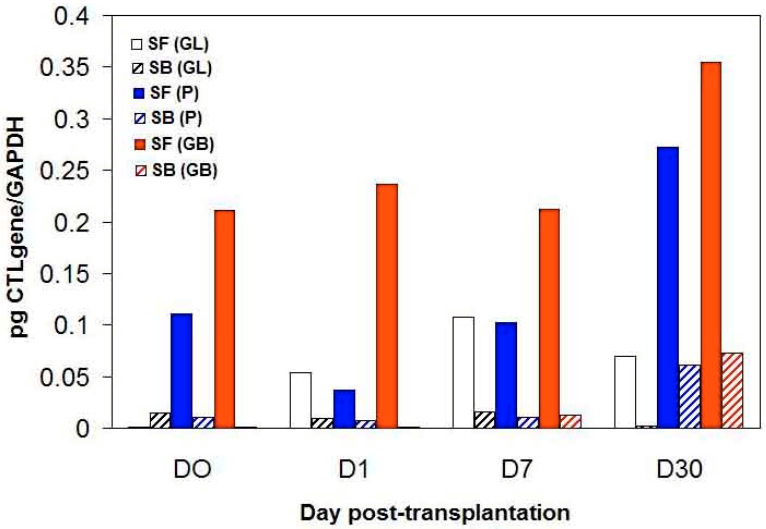

Figure 6. Quantitative RT-PCR verifies enhanced expression of cytotoxic T-lymphocyte (CTL) effector molecules. Peripheral blood samples were obtained from renal allograft recipients on either steroid-free (n=16) or steroid-based (n=24) immunosuppression protocols in the early post-transplantation period. Total RNA was extracted from the blood samples and the levels of gene expression measured using gene-specific primers. Among the differentially expressed genes identified by microarray screening and verified with quantitative reverse-transcriptase PCR are granulysin (GL), perforin (P), and granzyme B (GB).

these differences are being dissected to gain further insight into aspects of transplant immunopharmacology that will help us to provide better post-transplantation care to the patients. A preliminary analysis has shown some differences in the expression of cytotoxic T lymphocyte (CTL) effector molecules between steroid-free and steroid-based patients post-transplantation (49) (Figure 6); in our laboratory, we are currently using microarray analysis to investigate other pathways, separate from the CTL response, that also show differential regulation with steroid usage.

7.6. Graft tolerance

The ultimate goal of organ transplantation is allograft acceptance to the point that minimum or no immunosuppressive therapy is required. As yet, the mechanisms leading to graft tolerance is only vaguely defined and understanding the underlying molecular processes of tolerance will have tremendous impact on transplant medicine. We have approached this through microarray technology, since this can readily provide a global gene expression snap shot of tolerant patients, and thus aid in the identification of markers of tolerance which can be further investigated. Additionally, candidate gene markers of tolerance, once identified, could be used to custom design a 'tolerance chip', which could be used to specifically profile patients in tolerogenic regimes prior to and after complete immunosuppression withdrawal. These efforts are also currently being supported by the Immune Tolerance Network (http://www.immunetolerance.org/).

8. PERSPECTIVE

The use of DNA microarrays is undoubtedly beneficial in providing rapid and global views of the gene expression profiles of different disease states, thereby allowing improved understanding of the molecular mechanisms of the diseases. Further, this technology has been used to help to identify attractive and potentially important diagnostic, prognostic and therapeutic markers. Although the cost of microarray technology and the complexity of the data set may preclude it from being used as a general monitoring tool in the clinic, it is nevertheless a very useful screening tool to help highlight genes that may be further studied as surrogate markers of disease states and progression. A combination of genomics and the emerging proteomics technologies will prove to be invaluable in helping to address many unanswered questions relating to transplantation medicine.

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10. REFERENCES

1. Schena M, D Shalon, RW Davis, and PO Brown: Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270, 467-470 (1995)

2. Brown PO, D Botstein: Exploring the new world of the genome with DNA microarrays. *Nature Genet (Suppl)* 21, 33-37 (1999)

3. Alizadeh AA, DT Ross, CM Perou, and M Van de Rijn: Towards a novel classification of human malignancies based on gene expression patterns. *J Pathol* 195, 41-52 (2001)

4. Ginestier C, E Charafe-Jauffret, F Bertucci, F Eisinger, J Geneix, D Bechlian, N Conte, J Adelaide, Y Toiron, C Nguyen, P Viens, MJ Mozziconacci, R Houlgatte, D Birnbaum, J Jacquemier: Distinct and complementary information provided by use of tissue and DNA microarrays in the study of breast tumor markers. *Am J Pathol* 161, 1223-33 (2002)

5. Ross DT, U Scherf, MB Eisen, CM Perou, C Rees, P Spellman, V Iyer, SS Jeffrey, M Van de Rijn, M Waltham, A Pergamenschikov, JC Lee, D Lashkari, D Shalon, TG Myers, JN Weinstein, D Botstein, and PO Brown: Systematic variation in gene expression patterns in human cancer cell lines. *Nature Genet* 24, 227-235 (2000)

6. Li Y and F Sarkar: Down-regulation of invasion and angiogenesis-related genes identified by cDNA microarray analysis of PC3 prostate cancer cells treated with genistein. *Cancer Lett* 186, 157 (2002)

7. Tracey L, R Villuendas, P Ortiz, A Dopazo, I Spiteri, L Lombardia, JL Rodriguez-Peralto, J Fernandez-Herrera, A Hernandez, J Fraga, O Dominguez, J Herrero, MA Alonso, J Dopazo, and MA Piris : Identification of genes involved in resistance to interferon-alpha in cutaneous T-cell lymphoma. *Am J Pathol* 161, 1825-37 (2002)

8. Staudt LM, and PO Brown: Genomic views of the immune system. *Annu Rev Immunol* 18, 829-859 (2000)

9. Hsiao LL, RL Stears, RL Hong, and SR Gullans: Prospective use of DNA microarrays for evaluating renal function and disease. *Curr Opin Nephrol Hypertens* 9, 253-258 (2000)

10. Witowski NE, C Leiendecker-Foster, NP Gerry, RC MacGlenney, and G Barany: Microarray-based detection of select cardiovascular disease markers. *BioTechniques* 29, 936-938 (2000)

11. Duggan DJ, M Bittner, Y Chen, P Meltzer, and JM Trent. Expression profiling using cDNA microarrays. *Nature Genet (Suppl)* 21, 10-14 (1999)

12. Scherf U, DT Ross, M Waltham, LJ Smith, JK Lee, L Tanabe, KW Kohn, WC Reinhold, TG Myers, DT Andrews, DA Scudiero, MB Eisen, EA Sausville, Y Pommier, D Botstein, PO Brown, and JN Weinstein: A gene expression database for the molecular pharmacology of cancer. *Nature Genet* 24, 236-244 (2000)

13. Sorlie T, CM Perou, R Tibshirani, T Aas, S Geisler, H Johnson, T Hastie, MB Eisen, M van de Rijn, SS Jeffrey, T Thorsen, H Quist, JC Matese, PO Brown, D Botstein, P Eystein Lonning, and AL Borresen-Dale : Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* 2001; 98:10869-10874.

14. Heller RA, M Schena, A Chai, D Shalon, T Bedilion, J Gilmore, DE Woolley, and RW Davis. Discovery and analysis of inflammatory disease-related genes using cDNA microarrays. *Proc Natl Acad Sci USA* 94, 2150-2155 (1997)

15. Chee M, R Yang, E Hubbell, A Berno, XC Huang, D Stern, J Winkler, DJ Lockhart, MS Morris, and SP Fodor. Accessing genetic information with high-density DNA arrays. *Science* 274, 610-614(1996)

16. Bard JB. A bioinformatics approach to investigating developmental pathways in the kidney and other tissues. *Int J Dev Biol* 43, 397-403 (1999)

17. Stanton LW, LJ Garrad, D Damm, BL Garrick, A Lam, AM Kapoun, Q Zheng, AA Protter, GF Schreiner, and RT White. Altered patterns of gene expression in response to myocardial infarction. *Circ Res* 86, 939-945 (2000)

18. Chan V, N Hontzeas, and V Park. Gene expression. Preprint article available at http://www.cbi.pku.edu.cn/mirror/wli/microarray.

19. Brown MP, WN Grundy, D Lin, N Cristianini, CW Sugnet, TS Furey, M Ares Jr, and D Haussler. Knowledge-based analysis of microarray gene expression data by using support vector machines. *Proc Natl Acad Sci USA* 97, 262-267 (2000)

20. Eisen MB, PT Spellman, PO Brown, and D Botstein. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* 95, 14863-14868 (1998)

21. Tamayo P, D Slonim, J Mesirov, Q Zhu, S Kitareewan, E Dmitrovsky, ES Lander, and TR Golub. Interpreting patterns of gene expression with self-organizing maps: Methods and application to hematopoietic differentiation. *Proc Natl Acad Sci USA* 96, 2907-2912 (1999)

22. Tibshirani R, T Hastie, B Narasimhan, G Chu. Diagnosis of multiple cancer types by shrunken centroids of gene expression. *Proc Natl Acad Sci USA* 99, 6567-6572 (2002)

23. Khan J, Wei JS, Ringner M, Saal LH, Ladanyi M, Westermann F, Berthold F, Schwab M, Antonescu CR, Peterson C, Meltzer PS. Classification and diagnostic prediction of cancers using gene expression profiling and artificial neural networks. *Nature Medicine* 7, 673-679 (2001)

24. Tusher VG, R Tibshirani, G Chu. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* 98, 5116-5121 (2001)

25. Golub TR, TK Slonim, P Tamayo, C Huard, M Gaasenbeek, JP Mesirov, H Coller, ML Loh, JR Downing, MA Caligiuri, CD Bloomfield, and ES Lander. Molecular classification of cancer: Class discovery and class prediction by gene expression monitoring. *Science* 286, 531-537 (1999)

26. Alizadeh AA, MB Eisen, ER Davis, C Ma, IS Lossos, A Rosenwald, JC Boldrick, H Sabet, T Tran, X Yu, JI

Powell, L Yang, GE Marti, T Moore, J Hudson Jr, L Lu, DB Lewis, R Tibshirani, G Sherlock, WC Chan, TC Greiner, DD Weisenburger, JO Armitage, R Warnke, R Levy, W Wilson, MR Grever, JC Byrd, D Botstein, PO Brown, and LM Staudt. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 403, 503-511 (2000)

27. Alter O, PO Brown, and D Botstein. Singular value decomposition for genome-wide expression data processing and modeling. *Proc Natl Acad Sci USA* 97, 10101-10106 (2000)

28. Wu TD. Analyzing gene expression data from DNA microarrays to identify candidate genes. *J Pathol* 195, 53-65 (2001)

29. Lee MLT, FC Kuo, GA Whitmore, J Sklar. Importance of replication in microarray gene expression studies: statistical methods and evidence from repetitive cDNA hybridizations. *Proc Natl Acad Sci USA* 97, 9834-9839 (2000)

30. Bartosiewicz M, M Trounstine, D Barker, R Johnston, A Buckpitt. Development of a toxicolofical gene array and quantitative assessment of this technology. *Arch Biochem Biophys* 376, 66-73 (2000)

31. Sarwal M, X Chen, M-S Chua, N Khambam, S-C Hsieh, T Satterwhite, A Alizadeh, M Masek M, M Diehn, O Salvatierra Jr, and PO Brown. DNA microarray profiling identifies molecular heterogeneity and suggests a role of B-cells in acute renal allograft rejection. In press.

32. Van Gelder RN, ME Von Zastrow, A Yool, WC Dement, JD Barchas, and JH Eberwine. Amplified RNA synthesized from limited quantities of heterogenous cDNA. *Proc Natl Acad Sci USA* 87, 1663-1667 (1990)

33. Wang E, LD Miller, GA Ohnmacht, ET Liu, and FM Marincola. High-fidelity mRNA amplification for gene profiling. *Nature Biotechnol* 18, 457-459 (2000)

34. Alevizos I, M Mahadevappa, X Zhang, H Ohyama, Y Kohno, M Posner, GT Gallagher, M Varvares, D Cohen, D Kim, R Kent, RB Donoff, R Todd, CM Yung, JA Warrington, and DT Wong. Oral cancer *in vivo* gene expression profiling assisted by laser capture microdissection and microarray analysis. *Oncogene* 20, 6196-6204 (2001)

35. Kitahara O, Y Furukawa, T Tanaka, C Kihara, K Ono, R Yanagawa, ME Nita, T Takagi, Y Nakamura, and T Tsunoda. Alterations of gene expression during colorectal carcinogenesis revealed by cDNA microarrays after laser-capture microdissection of tumor tissues and normal epithelia. *Cancer Res* 61, 3544-3549 (2001)

36. Sarwal MM, A Jani, S Chang, P Huie, Z Wang, O Salvatierra Jr, C Clayberger, R Sibley, AM Krensky, and M Pavlakis. Granulysin expression is a marker for acute rejection and steroid resistance in human renal transplantation. *Human Immunology* 62, 21-31 (2001)

37. Liu CL, W Prapong, Y Natkunam, A Alizadeh, K Montgomery, CB Gilks, and M van de Rijn. Software tools for high-throughout analysis and archiving of immunohistochemistry staining data obtained with tissue microarrays. *Am J Pathol* 161, 1557-1565 (2002)

38. Ewton DZ, K Lee, X Deng, S Lim, E Friedman. Rapid turnover of cell-cycle regulators found in Mirk/dyrk1B transfectants. *Int J Cancer* 103, 21-8 (2003)

39. Goodstadt L, CP Ponting. Sequence variation and disease in the wake of the draft human genome. *Human Molecular Genetics* 10, 2209-2214 (2001)

40. Halloran PF. Immunosuppresion in the Post-Adaptation Period. *Transplantation* 70, 3-5 (2000)

41. Akalin E, RC Hendrix, RG Polavarapu, TC Pearson, JF Neylan, CP Larsen, and FG Lakkis. Gene expression analysis in human renal allograft biopsy samples using high-density oligoarray technology. *Transplantation* 72, 948-953 (2001)

42. Aavik E, A Mahapatra, S Chang, D DuToit, P Hayry, and M Sarwal. Application of gene expression patterns using cDNA microarrays in a baboon chronic vasculopathy model. American Society of Transplantation Annual Meeting Abstract 440 (2001)

43. Du Toit D, E Aavik, E Taskinen, E Myburgh, E Aaltola, M Aimonen, S Aavik, J van Wyk, and P Hayry. Structure of carotid artery in baboon and rat and differences in their response to endothelial denudation angioplasty. *Ann Med* 33, 63-78 (2001)

44. Li B, C Hartono, R Ding, VK Sharma, D Kracker, D Dadhania, D Serur, J Mouradian, JE Schwartz, and M Suthanthiran. Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. *New Eng J Med* 344, 947-954 (2001)

45. Vasconcellos LM, AD Schachter, XX Zheng, LH Vasconcellos, M Shapiro, WE Harmon, TB Strom, and D Schachter. Cytotoxic lymphocyte gene expression in peripheral blood leukocytes correlates with rejecting renal allografts. *Transplantation* 66, 562-566 (1998)

46. Strehlau J, M Pavlakis, M Lipman, M Shapiro, L Vasconcellos, W Harmon, and TB Strom. Quantitative detection of immune activation transcripts as a diagnostic tool in kidney transplantation. *Proc Natl Acad Sci USA* 94, 695-700 (1997)

47. Chua M-S, C Barry, X Chen, O Salvatierra, and MM Sarwal. Molecular profiling of anemia in acute renal allograft rejection using DNA microarrays. *Am J Transplantation* 3, 17-22 (2003)

48. Sarwal MM, PD Yorgin, S Alexander, MT Millan, A Belson, N Belanger, L Granucci, C Major, C Costaglio, J Snchez, P Orlandi, and O Salvatierra Jr. Promising early outcomes with a novel, complete steroid avoidance immunosuppression protocol in pediatric renal transplantation. *Transplantation* 72, 13-21 (2001)

49. Satterwhite T, M-S Chua, S-C Hsieh, J Scandling, O Salvatierra, and MM Sarwal. Increased expression of cytotoxic effector molecules: Different interpretations for steroid-based and steroid-free immunosuppression. *Pediatric Transplantation* 7, 53-58 (2003)

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Send correspondence to: Dr Minnie Sarwal, Department of Pediatrics, Stanford University School of Medicine, G320, 300 Pasteur Drive, Stanford, CA 94305, Tel: 650-498-5483, 650-498-5557, E-mail: msarwal@stanford.edu