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Microarray technology in the investigation of diseases of myocardium with special reference to infection

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1. ABSTRACT

Microarrays are now routinely employed to characterize gene expression of thousands of genes from a single hybridization. The genome wide gene expression profile aids in the understanding of genes that may be regulated in a particular pathological condition. This paper provides an overview of microarray technology and its recent developments followed by its usage in studies of cardiovascular disease and how it pertains to viral and parasitic infections of the heart.

2. INTRODUCTION

In the era of "whole genome studies", DNA microarrays are increasingly gaining importance, as a high throughput technology to integrate sequencing information with biological function. DNA microarrays form a generic platform for characterizing gene expression. The gene expression profile determined from such array studies is an efficient approach to assess normal cell functioning because a diseased or pathological condition is often associated with an altered gene expression pattern. Of the thousands of genes in the mammalian genome, only a specific set may be regulated in a particular pathologic condition. Often these genes are associated in a cascade, and are interconnected in a complex network. To understand a disease condition at the molecular level, one has to look at the total gene expression profile or "transcriptome". Even a decade ago research on a particular disease/phenotype was limited to suspected candidate genes, undermining the importance of unidentified novel genes and the interplay between various gene families. The only methodologies that were available by which one could identify differentially expressed genes were subtractive hybridization, differential display and serial analysis of gene expression (SAGE) (1). Although these traditional techniques were able to identify changes at the global level, none of them were as robust, comprehensive and high throughput as DNA microarrays. Thus, this technology

promises to monitor the whole genome on a single chip so that researchers can have a global picture of the interactions among thousands of genes simultaneously, thus providing an insight into the underlying mechanisms of disease pathogenesis.

The two major applications of microarray technology are identification of gene mutation/ change in sequence and whole transcriptome analysis. Microarrays are now regularly used to "fill in the blanks" left by traditional hypothesis driven research. Importantly, microarray technology can identify overlaps in molecular pathways that lead to a common phenotype. Identification of these overlapping genetic modifiers is essential for interdicting various pathological conditions, thus providing a novel tool for diagnosis, prognosis and clinical management of disease. Perhaps the most important use of microarray technology is in the identification of differentially regulated genes in complex pathophysiological syndromes such as cancer, cardiovascular diseases and host parasite interactions. Etiologies of complex disorders are due to multiple genetic factors and their interplay with the environment. The characterization of host cell defense strategies involved in the complex interaction between microbial pathogen and the host reveals the mechanism by which they are regulated. This information is necessary in the designing of advanced medical tools to combat the pathogens.

3. OVERVIEW OF THE MICROARRAY TECHNOLOGY

Microarrays are variously termed DNA chips, DNA microarrays, Biochips, Gene arrays or Genechips (a registered trademark of Affymetrix Inc, Santa Clara, CA). In recent years there has been an explosive increase in the availability of genomic sequence information. In 1995, *Hemophilus influenzae* was the first bacteria to be completely sequenced, today more then 70 prokaryotic genomes have been completely sequenced and another hundred are in various stages of completion (http://www.tigr.org/tdb/). Recent international efforts in genomic projects have produced a burst in knowledge of the gene sequences of various higher organisms, which has given a huge boost to the development of microarrays. So far, complete genomic sequences of 17 organisms have been produced including human, mouse and yeast. Recent findings show that the human genome has 25,000-30,000 genes, out of which 19,599 are confirmed protein-coding genes with another 2,188 suspected protein coding DNA segments (http://www.ornl.gov/sci/techresources/Human_Genome). The next step in this sequencing project is to identify the function, expression and regulation of these genes (more than 80% of which are not known). Microarrays are therefore becoming the choice of technology as they can handle thousands of genes simultaneously. Each technology benefited from the other, as without the sequence knowledge, it would have been impossible to fabricate a chip and without microarrays the goal of sequencing projects would be incomplete.

3.1. The Principle of Microarray Technology

The basic principle of all microarray experiments is that the labeled nucleic acid molecules in solution hybridize to their complementary sequences immobilized on a solid substrate (matrix) with high sensitivity and specificity. Thousands of probe DNAs are immobilized on a matrix as spots. Each spotted nucleic acid sequence is free to hybridize with labeled complementary sequence in the sample, thus enabling profiling expression of tens of thousands of genes or ESTs simultaneously (2, 3). Based on the type of probe deposited on the matrix, microarrays are of basically two types, cDNA and oligonucleotide chips. Microarrays are now commercially available as are smaller customized arrays designed for specific investigation. Both oligonucleotide and cDNA arrays are also produced at most major universities.

3.2. Preparation of the microarray slide

In DNA microarray technology, a grid of micron-sized spots of nucleic acid is printed at a high density on a solid base, which could be glass, plastic, silicon, gel, nitrocellulose or gold. Glass based microarrays are most common and are typically made using microscope slides coated with polylysines or aminosilanes (4), which increase the adherence of the deposited DNA. Generally the spot size ranges between 80 and 150 micron and spots are placed at a regular interval of 100 micron on the slide. The deposition of DNA on the slide is called 'printing' and is performed with special pins attached to a robotic head with x, y, z motion controls. Various technologies are now available for the deposition of the nucleic acids. The original method of contact printing was developed by Patrick Brown and collaborators at Stanford University (5, 6). In contact printing, a robotic head is equipped with capillary tubes (printing tips) so that each takes a small volume of sample (at least 0.5 µl) and deposits 1-5 nl to the matrix with a low but constant pressure. Generally one sample uptake is sufficient to print at least 100 slides. The tips are briefly sonicated and washed several times before the next round of use. In the non-contact method, either piezoelectric or ink jet devices are used for printing. Ink-jet technology was developed by Rosetta Inpharmatics and licensed to Agilent Technologies (http://www.rii.com). Small oligonucleotides could be either synthesized *in situ* (on chip) by photolithography (light directed chemistry) (7, 8), by alternate *in situ* synthesis technologies, or directly deposited over the matrix by ink jet printing (Blanchard et al., 1996). Affymetrix uses the photolithographic technique (http://www.affymetrix.com) in their chip fabrication, employing a large number of masks per nucleotide synthesis. Singh-Gasson *et al* (10) described a maskless array synthesizer (MAS) in which a digital micromirror array creates a virtual mask on the active glass surface followed by programmed chemical coupling and light exposure to synthesize desired oligonucleotides. By this method about 480,000 oligonucleotides could be spotted on a 10x14 mm surface area.

The deposited DNA probes are in most cases cross–linked to the matrix by ultraviolet irradiation and the residual amines bound to the matrix are reacted with succinic anhydride to reduce the positive charge at the surface. Finally the slide is treated with heat or alkali to melt some of the DNA double strands. After printing, the slides are allowed to dry for 24 hrs and are ready for use.

The slides are usually stored at room temperature and remain stable for at least one year.

3.3. Synthesis of cDNA probes

Prior to microarray printing, the cDNA probes are generated by PCR amplification from purified plasmids containing the clones or directly from clones in bacterial culture using either oligo dT or random hexamer primers or by individual gene specific primers. Chip designed for higher eukaryotes are typically based on expressed sequence tags (ESTs), whereas for yeast and prokaryotes, probes are usually generated by amplifying genomic DNA with gene–specific primers. Chosen 3′ cDNAs (500-3000 bases long) are deposited by either contact or non-contact printing. The main advantage of cDNA microarrays is that the whole genome is represented with all the ESTs and prior knowledge of the gene sequence is not required in its construction. Thousands of genes are analyzed per experiment with a low cost per chip and the spotted cDNA clones are readily available for signal validation. However cDNA microarrays have disadvantages as the cDNA chip manufacture is labor intensive and an expensive laboratory set up is required. Relatively large amounts of RNA are required for hybridization and cross hybridization between family members of a gene is always a possibility. Also, large cDNA sequences may have intra stand cross-link or multiple contacts with the matrix, therefore making it far less accessible for hybridization to labeled cDNAs.

3.4. Synthesis of oligonucleotide probes

In oligonucleotide arrays, 20-80-mer oligonucleotides or peptide nucleic acids (PNA) are immobilized on a solid surface. A 25-mer oligonucleotide is sufficient to distinguish closely related genes. Moreover, oligonucleotide arrays can be designed with 11-20 different oligonucleotides to represent each gene on the array, enabling precise identification of each gene. In this technology, cloning and PCR amplification are not required. Sequences used in the fabrication of the array are selected from GenBank (11), dbEST (12), UniGene (13) or RefSeq. The short oligonucleotide chains are much more accessible for hybridization than lengthy cDNA chains and their uniform length provides similar hybridization (or capture) efficiency. Moreover, during chip fabrication, there is no need to melt the DNA. However, short chain lengths are not without problems; the variations in melting temperature due to AT-GC composition are more acute here. Also reduction in size may compromise the specificity of hybridization. Moreover, if the length of the probe oligonucleotide is less than 30 nucleotides (as in Affymetrix chips), only a single sample can be measured because of which it is often referred to as ‘single channel hybridization’. One actually needs two separate chips and two hybridizations to get an estimate of differential gene expression between the two samples. However, if the length of the deposited oligonucleotide is above 60 nucleotides, ‘two channel hybridization’ like the cDNA chips is possible. Oligonucleotide based chips for more than twenty genomes are now commercially available from Affymetrix Inc. In Affymetrix arrays, each oligonucleotide has a partner with a single base mismatch to control nonspecific binding. The degree of hybridization to the probe compared to the mismatched probe allows Affymetrix software to determine hybridization errors, which in turn improves the quality of the data.

3.5. Sample preparation and hybridization

RNA from either the test tissue (e.g., diseased tissue) and from the reference tissue (e.g., normal, control) is isolated most commonly using the TriZol (Invitrogen) method (14) and cleaned with resins (15). As with any other RNA based assay, the purity and quality of the starting RNA has a significant effect on the results. The purified RNA is used as a template to synthesize single-stranded cDNA in the presence of labeled nucleotides. If the test RNA is labeled with a fluorescent dye such as cyanine 5 (Cy-5), the reference RNA is labeled with a different fluorescent dye such as cyanine 3 (Cy-3). The fluorescent dyes that are chosen should have widely separated excitation and emission spectra, good photo stability and be incorporated at high efficiencies by reverse transcriptase. Alternatively, radioactively labeled cDNAs with higher intrinsic detection limit could be used.

For cDNA microarrays relatively large amounts of RNA are required; typically about 60-100 μg of total RNA or 3-5 μg of poly (A) RNA. If starting sample RNA is limited in amount or the abundance of specific transcript is too low (about 1 in 100,000 transcripts), the RNA is first amplified by *in vitro* transcription and then used for hybridization. For cDNA or long length oligo microarrays, both labeled cDNAs are mixed in equimolar amounts and hybridized to a single slide, allowing simultaneous two-color hybridization with a large linear range of 4-5 orders (Figure 1). However, in Affymetrix oligonucleotide microarrays, different labeled cDNAs are hybridized to different slides. Hybridization results in competitive binding of differentially labeled cDNAs to the corresponding printed probe DNAs. An overnight incubation at 40-50°C in the dark is generally sufficient for hybridization. The hybridized slide is then washed several times to remove unhybridized labeled cDNAs, labeled nucleotides and other debris and dried. Alternatively, the double stranded cDNA generated could carry a T7 promoter attached to it. During *in vitro* transcription and synthesis of cRNA, biotinylated nucleotides are incorporated and each target sample is hybridized to a separate probe. The hybridization signal is detected with a streptavidin-phycoerythrin conjugate that binds to biotin, which when excited with a specific laser, emits fluorescent light. This fluorescence is measured by automatic image analysis software to calculate the relative mRNA abundance for the corresponding gene.

3.6. Confocal scanning

Following hybridization, the slide is usually scanned with a high-resolution confocal fluorescence scanner using two different wavelengths corresponding to the dyes used. For scanning of Cy-3 and Cy-5 labeled samples, a Helium Neon laser operating at 633 nm (Cy-5) and 534 nm (Cy-3) is used. The slides are first scanned for Cy-5 and then for Cy-3 since Cy-5 is more susceptible to photo degradation than Cy-3. The relative signal intensities given by both the dyes for each individual spots are

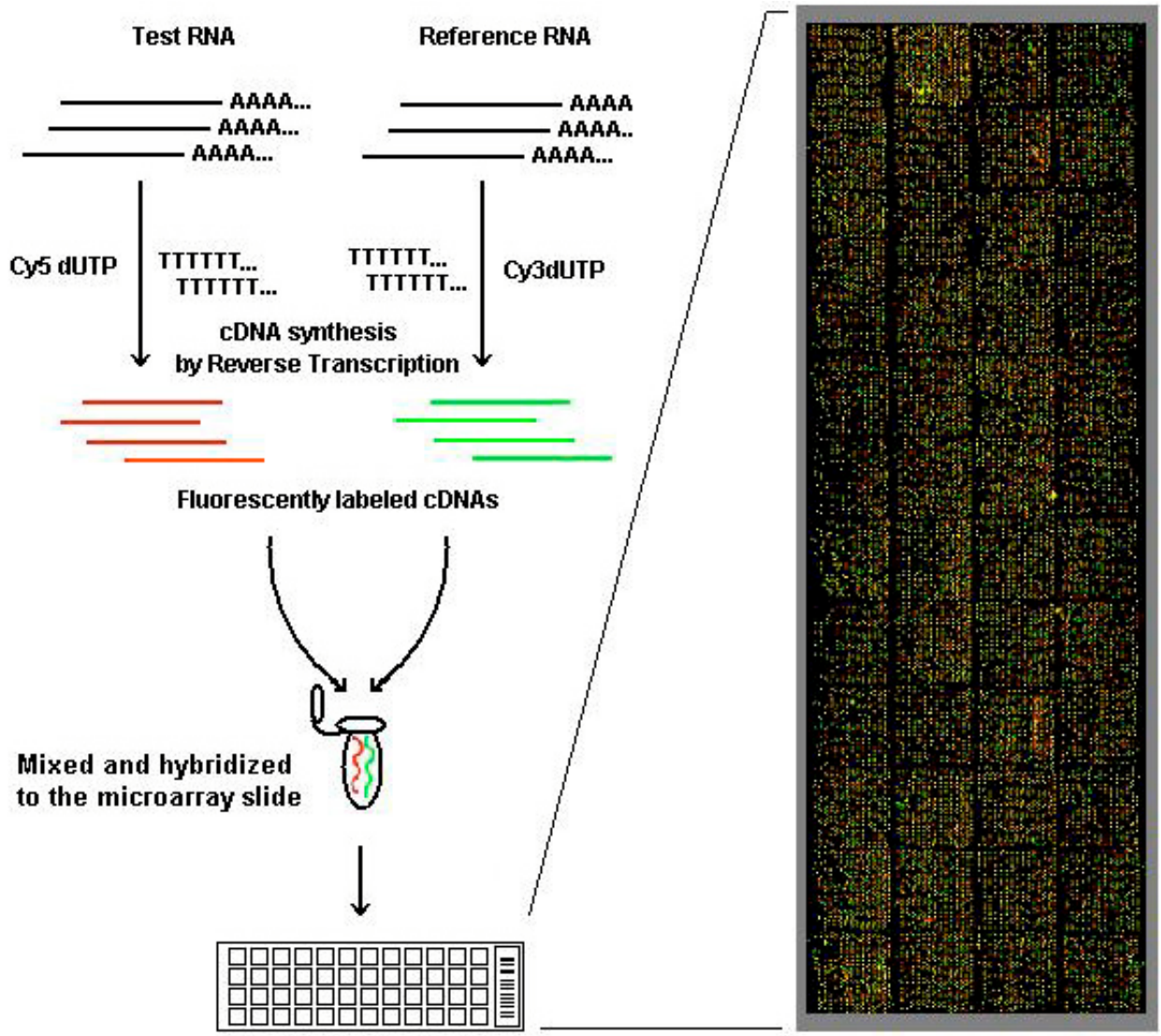

Figure 1. Microarray Preparation. Schematic diagram illustrating the procedure for generating fluorescently-labeled cDNA and hybridization to a microarray slide.

calculated, which gives an estimate of the ratios of mRNA abundance for each printed gene. A yellow spot indicates a Cy5/Cy3 ratio close to 1, a result of equal expression of that gene in both the test and the control RNA (Figure 1).

3.7. Image analysis and normalization

The image obtained from confocal scanning is aligned to a specific grid corresponding to the specific microarray platform used. This is an essential step as only a proper aligning ensures correct position of the spots to the corresponding genes. Spurious signals and other hybridization artifacts are manually rejected and the local background noise for each spot is estimated. The background subtracted intensities for each spot are calculated using either the median or the mean intensity of the spot. While Cy-3 and Cy-5 dyes are mostly used for labeling cDNAs, this approach suffers from differential incorporation of Cy-3 and Cy-5 labeled nucleotides; therefore, the data generated from microarray is subjected to normalization to reduce any discrepancy in incorporation efficiency and dye specific hybridization artifacts (Figure 2). Normalization also helps to control the differences in the quantity of the starting RNA from the two samples. An alternative approach to overcome this problem is to label the cDNA with amino allyl nucleotides for both the samples followed by covalent coupling to NHS-ester of the appropriate Cyanine dye (16). Following normalization the genes that are minimum 1.5 fold up or down regulated is selected. The cut off value depends on the experiment and the quality of the hybridization.

3.8. Verification of gene expression alterations

The data generated by the microarray are confirmed by at least three replicate experiments on independent samples. The individual gene of interest is further verified by conventional techniques like RT-PCR or

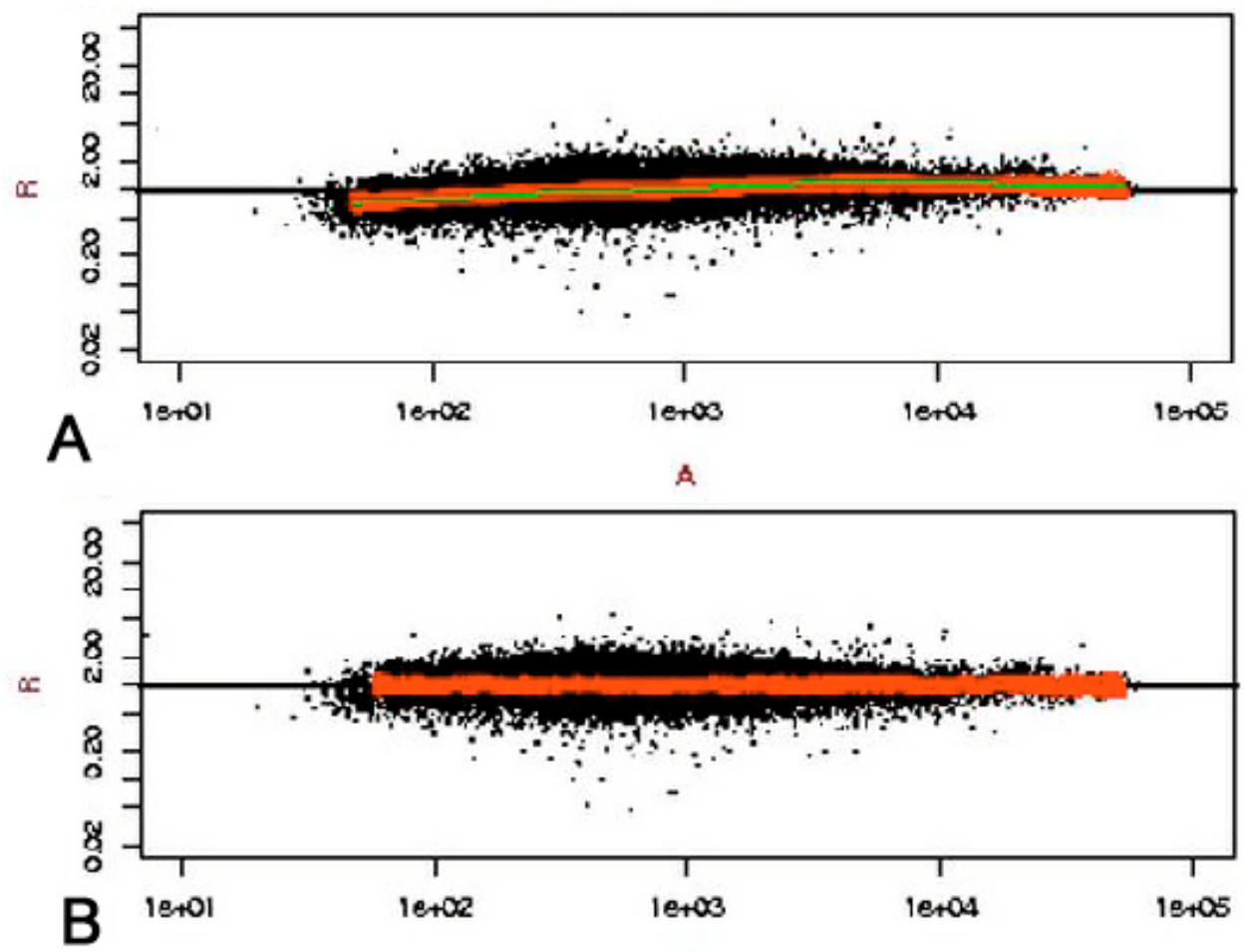

Figure 2. Intensity dependent normalization of microarray data. This eliminates any possible bias effects in ratio measurements due to intensity of the signal being measured. The dots represent gene expression values for all of the genes in the microarray. Panel A represents data before normalization whereas Panel B represents data after normalization.

real time PCR, both of which gives a fair estimate of the abundance of mRNA and the change in protein synthesis is estimated by immunoblotting. Microarrays could be by itself used to verify the hybridization pattern by 'dye flipping' or reverse labeling. To perform dye flipping, oppositely labeled cDNAs from the reference RNA and test RNA are mixed and hybridized to a separate microarray slide and both the slides are compared for the labeling pattern. A red spot in one slide gives a green spot in the other slide at the corresponding position (Figure 3). Although this gives a strong and clear verification of the hybridization pattern, often the intensities of the fluorescence between the dyes are not the same. This is mainly because of nonstoichiometric incorporation of the two dyes during cDNA synthesis and also due to the differential fluorescence decay.

4. USE OF MICROARRAY IN CARDIOVASCULAR DISEASE

Microarray and serial expression in gene analysis (SAGE) have been utilized to study the pathogenesis of human cardiovascular diseases. In this section we will discuss the use of microarray technology in the investigations of common myocardial and vascular diseases.

4.1. Myocardial ischemia and infarction

Myocardial ischemia is the consequence of a reduction in blood flow and the availability of oxygen to the myocardium. Ischemia may be associated with chest pain although many individuals have silent ischemic episodes and subsequent infarction. Importantly, it has been observed that brief exposure of the myocardium to ischemia initiates several adaptive responses in the heart, which greatly enhance tolerance to a subsequent ischemic injury (17, 18). The molecular mechanism by which such adaptive response is attained is largely unknown. Onody *et al* (19) used cDNA microarrays (3200 genes) to investigate changes in genes during coronary ischemia/reperfusion and preconditioning in rat heart. They found that 28 genes were significantly up-regulated and 21 genes were down-regulated due to ischemia/reperfusion. Genes with altered expression included those encoding for tubulin,

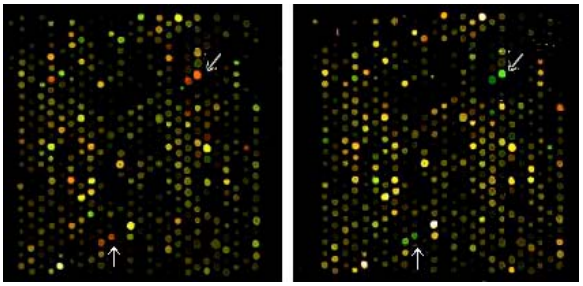

Figure 3. Validation of microarray results by flip array as described in the text and shown in this figure. The genes that are upregulated in one array (e.g., red spot) appeared green in the flipped array. The genes that remained unchanged appeared orange in both the slides (note arrows).

procollagen, several metabolic enzymes, apoptosis, heat shock proteins and chaperonin subunit ε. The repressed genes included several mitochondrial genes and the enzyme aconitase, necessary for the citrate cycle. When a comparison was made between ischemic rat hearts with and without preconditioning, genes encoding for chaperonin subunit ε, anion exchange protein 2 and metallothionein II were highly induced; natriuretic peptide precursor type B was moderately induced; peroxisome proliferator activator receptor γ (PPARγ) and betaine-homocysteine S-methyltransferase showed a repressed transcription level. This is interesting since PPARγ is known to be involved in atherosclerosis and ischemic heart disease, and pharmacological activation of PPARγ even protects the ischemic heart (20).

Stanton *et al* (21) prepared ~7000 cDNAs from surgically induced infarcted rat heart and printed these on microarrays for expression analysis in the left ventricle (LV), free wall and the interventricular septum (IVS). Myocardial infarction was induced by ligating the coronary artery and mRNA was collected from samples 2-16 weeks after infraction. They identified a set of 731 differentially expressed cDNAs in the left ventricle or interventricular septum at least in one time point. There was increased expression of a large number of extracellular matrix proteins (ECM) such as collagen, fibronectin, laminin and fibulin in the infracted heart as compared with controls. Moreover, the expression in LV was greater than that observed in the IVS. They also observed elevated expression of TIMP-3, a metalloproteinase inhibitor that favors deposition of structural components in extracellular matrix. SPARC (secreted protein acidic and rich in cysteine), a protein that controls the interaction of endothelial cells to ECM is uniquely altered in the left ventricle and not in the interventricular septum. The pattern of gene expression in left ventricle and the interventricular septum indicates that although there are uniquely up regulated genes in left ventricle, none exist for interventricular septum, indicating the degree of cardiac remodeling varies between these two regions of the heart.

4.2. Cardiac hypertrophy

Myocardial hypertrophy is an adaptive response due to a variety of factors including reduced cardiac function, stress and injury. Myocardial hypertrophy invariably increases the cardiac mass and cardiac myocyte volume. This structural change in cardiac myocyte is compensated by increased myocardial force-generating capacity and favorable geometry to permit increased cardiac work (22). Since stress in a broad sense can include a myriad of pathological stimuli including mechanical/volume load, hypertension, myocardial infarction, cardiac arrhythmias, endocrine disorders, and genetic mutations in cardiac contractile protein genes, transcriptome analysis of each case should be different from the other, ultimately leading to the same pathophysiological phenotype. It has been observed that naturally occurring cardiac hypertrophy induced by pressure/volume overload and ischemic damage are often associated with activation of fetal cardiac genes, induction of immediate-early genes, such as *c-fos*, *c-myc, c-jun and Erg1* (23-26) and increased expression of atrial natriuretic peptide, β -myosin heavy chain and α-skeletal actin (27, 28).

Friddle *et al* (29) compared the expression changes during induction and regression of hypertrophy in a murine hypertrophic model. They administered angiotensin II and isoproterenol by osmotic mini-pump to induce hypertrophy, which was reversed after the drug was withdrawn. The RNA obtained from the left ventricle from the induced and regressed mice were then compared using DNA microarray technology. They found alteration of 32 genes during induction and 8 genes during regression including those for atrial natriuretic factor, *BNP*, *ECE-2*, *COMT*; all of which are known to participate in hypertrophic pathways. This study also identified 30 novel genes not previously associated with hypertrophy, even though they used a microarray representing only 3% of the mouse genome. This indicates that only a fraction of the candidate genes were identified and suggests the possibility of finding several hundred such genes when employing a genome wide chip. This is interesting, as it indicates that distinct genes that are differentially regulated in hypertrophy and during regression. These studies underscore the notion that this technology can not only confirm preexisting concepts but also provide the opportunity to create new hypotheses.

The molecular mechanisms of cardiac hypertrophy were studied by microarray in four different genetically induced mouse models (30). In these mice cardiac hypertrophy was transduced through over expression of specific cardiac genes, known to induce hypertrophy. Their hypertrophied mouse models include (a) mice over-expressing the protein kinase C-ε activation peptide (ψε RCK) that leads to hypertrophic cardiac remodeling while retaining normal contractile function. (31); (b) mice over-expressing the calcium-dependent phosphatase calcineurin (CN), which activates NF-AT3 to translocate into the nucleus and in turn induces cardiac zinc finger transcription factor GATA4 to synergistically induce cardiac transcription. This leads to cardiac hypertrophy and heart failure (32); (c) mice over-expressing calsequestrin (CSQ), a high capacity Ca^{2+}-binding protein in the sarcoplasmic reticulum (SR) lumen that causes cardiac

hypertrophy and induces fetal gene expression (33) and d4) mice over-expressing the wild-type Gαq in the heart using the α-myosin heavy chain promoter, leading to increase in heart weight, atrial naturietic factor, β-myosin heavy chain and α-skeletal actin expression leading to cardiac hypertrophy (34). These phenotypically similar, but pathophysiologically dissimilar genetic hypertrophic models were tested for differentially expressed genes. Aronow et al. (30), found that the total number of regulated genes was proportional to the severity of the model tested. For example, mice over expressing Gαq had the highest hypertrophy as compared to over-expressing CN, which was followed by over expressing CSQ and ψεRACK models. With the exception of atrial natriuretic peptide, no common hypertrophic gene expression profile was observed in all four models, suggesting that transcriptional alterations are highly specific to individual genetic causes of hypertrophy. This is interesting since many studies pointed to single determinants like Gαq over-expressors but this result shows that each hypertrophic model has its own molecular signature, specific to the hypertrophy-inducing process and should be dealt with using individual therapeutic strategies.

4.3. Dilated cardiomyopathy and congestive heart failure

Dilated cardiomyopathy is a major cause of morbidity, caused when compensatory mechanisms fail to maintain sustained hemodynamic overload. Dilated cardiomyopathy is associated with left ventricular dilation and wall thinning leading to congestive heart failure. How the decomposition process leads to dilated cardiomyopathy is not known (35), but loss of cardiac myocytes due to apoptosis is believed to play a role in decompensation leading to heart failure (36-39). Apoptosis in mature cardiac tissues is regulated by a fine balance between pro-apoptotic pathways and cell survival pathways. To interdict the decompensating mechanisms, the molecular mechanism of myocardial apoptosis must be known. Thus, Yussman *et al* (40) used microarray analysis to identify myocardial apoptosis in Gq-mediated (using cardiac specific Gαq transgenic mouse model) and pressure-overload induced cardiac hypertrophy. Although it is known that cardiac hypertrophy is associated with the induction of embryonic genes, evidence for induction of pro-apoptotic genes is less clear. Pro-apoptotic genes play a vital role in the normal embryonic development of the heart but are barely detectable in terminally differentiated hearts (41). Of the 88-apoptotic genes assayed, only 4 were found to be regulated, including the Nix/Bnip3L gene. Since a specific type of apoptotic gene was observed to be induced, a generalized anti-apoptotic approach to control hypertrophy was judged not to be therapeutically useful at least in this case. They also demonstrated that the expression of Nix dominant inhibitor, sNix (both *in vivo* and *in vitro*) could protect against hypertrophic decompensation in Gαq-over-expressors. There are reports that cardiac specific over-expression of TNF-α leads to dilated cardiomyopathy (42) and that this condition can be reversed by treatment with soluble TNF receptors with improved left ventricular function in patients (43). Recently, Steenbergen *et al* (44) used microarrays to evaluate whether there is a pro-apoptotic shift in apoptotic genes in failing hearts. They selected ~75 apoptosis-related genes for evaluation. Interestingly, they observed a decreased expression of TNF-α and NF-κB induced anti-apoptotic genes and an increased expression of TNF receptor super family genes. Their data strongly suggest a pro-apoptotic shift in the TNF-α pathway during the transition to end stage heart failure. Barrans *et al* (45) used a custom printed CardioChip containing 10,848 human cardiovascular-based EST cDNAs to evaluate the genomic portrait of the failing heart as compared to the normal state. They identified more than 100 transcripts that are differentially regulated >1.5-fold (P<0.05). Notable among them was 19-fold up regulation of atrial natriuretic peptide (ANP), cardiac troponin, tropomyosin, HSP 40, HSP 70 and several transcriptional/translational regulators. Among the down-regulated genes are Ca^{2+}/calmodulin-dependent kinase and inositol 1,4,5-trisphosphate receptor. Their findings suggest that down regulation of calcium signaling may be crucial for the evolution of the heart failure. In another study, Hwang *et al* (46) compared the differentially expressed genes in human dilated and hypertrophic cardiomyopathy. In both the conditions, 192 genes were up regulated and 51 genes were down regulated. Important up regulated genes in both the cases include ANP, CD59, decorin etc while the down-regulated genes include elastin, sarcoplasmic reticulum Ca^{2+}-ATPase etc. Several genes such as those encoding αβ-crystalline, calsequestrin, lipocortin were differentially expressed between dilated and hypertrophic cardiomyopathy.

End stage heart failure is due to severe loss of cardiac myocytes, either by ischemic or non-ischemic origins. It is characterized by an increase in myocyte apoptosis, impaired signal transduction and altered cytoskeleton and myofibrillar organization leading to an impairment of the contractile function. Yang *et al* (47) compared the expression profiles between two non-failing (end stage ischemic) and failing human hearts (dilated cardiomyopathy). Despite the differences between the two diseased conditions, they were able to identify 12 genes that showed similar expression patterns, indicating that these genes are fundamental to the development of heart failure. This includes Gelsolin, Myomesin, EF-2, ubiquitin. The down-regulated genes include, striated muscle LIM protein-1, α1–anti-chymotrypsin, αβ-crystalline and β-actin. They also identified 5 genes (eukaryotic initiation factor 4AII, μ-Crystallin, Pre-B cell stimulating factor homologue, Transcription factor homologue HBZ 17 and ANF) that were only expressed in failing hearts and 2 genes (Plasminogen activator inhibitor and TIM17 preprotein translocase) present only in the non-failing hearts.

4.4. Proinflammatory molecules and reactive oxygen species in cardiac hypertrophy

Recent evidence strongly suggests that inflammation plays a major role in the pathogenesis of ischemic heart disease ultimately leading to cardiac remodeling (48). This is in part due to the activation of various immune cells such as macrophages, lymphocytes, endothelial cells, cardiac myocytes and smooth muscle

cells. These cell types express pro-inflammatory cytokines such as IL-1β, TNF-α, endothelin-1. Takahashi *et al* (49) demonstrated that macrophage migration inhibitory factor (MIF), an important regulator of inflammation (50) is also secreted by cultured cardiac myocytes. They studied the involvement of MIF-responsive genes in cardiac myocytes by microarray analysis. Of 1152 genes screened they showed 52 genes were differentially expressed in response to MIF. The important upregulated genes include cytochrome P-450 isozyme 5 (15.3 fold), insulin like growth factor binding protein (8.4) and CXC chemokine LIX (6.4). This finding is interesting since recently Blocki *et al* (51) reported that MIF is similar to GST and plays an important role in the redox stress system. Therefore this finding indicates that MIF may act as a redox sensitive cytokine in heart.

4.5. Hypertension and mechanical injury

Hypertension is a risk factor for coronary artery disease, stroke and peripheral artery disease (52). In atherosclerosis, the arteries accumulate lipids and extracellular matrix in the vessel wall, leading to inflammation. Macrophages are often associated with human coronary atherosclerotic plaques and are known to secrete proteolytic factors (53, 54) that dissolve the plaques leading to thrombosis. Although it is not known whether macrophages are subjected to mechanical stress in these atherosclerotic lesions, there is no proper way to know how macrophage stress affects macrophage function.

DNA microarrays were employed by Ohki *et al* (55) to identify differentially regulated genes in a human monocyte/macrophage cell line (THP-1). They used a mechanical device (56) that applies a uniform biaxial strain field to cultured cells to induce the cells. Transcription profiling showed that the cells responded by inducing immediate early response gene, *IEX-1*; inflammatory genes like interleukin –8 and a pro-apoptotic gene PAR-1. IL-8 is implicated in variety of diseases (57) and might be responsible for inflammation associated with atherosclerosis. *IEX-1* is an inducible immediate-early gene activated by NFκB, TNFα, Fas and 12-*O*-tetradecanoylphorbol-13-acetate. A splice variant of *IEX-1* gene is anti-apoptotic and induction of this gene in macrophages might promote differentiation and promotion of atherogenesis. Induction of a pro-apoptotic gene like PAR-4 poses a dilemma; it is not known whether pro-apoptosis or anti-apoptosis factor plays a dominant role in stress-induced macrophages. This *in vitro* model might mimic the *in vivo* stress situation and identifies important differential gene expression associated with atherogenesis and instability of coronary-artery plaques.

Geraci *et al* (58) reported that transgenic mice over expressing prostacyclin synthase were protected from the development of pulmonary hypertension after exposure to chronic hypobaric hypoxia. To investigate the mechanism underlying prostacyclin synthase's protective effect, they examined the gene expression pattern of lung tissue from the transgenic mouse in comparison to that in negative control. The mRNA from five transgenic mouse lungs was pooled and compared with five nontransgenic, sex-matched littermates. Using strict criteria (a twofold change in expression), they found that of the 6500 genes surveyed, 32 genes showed an increase in expression and 26 showed a decrease in expression. Among them, peroxisome proliferator-activated receptor (PPAR) γ, RAS GTPase, focal adhesion kinase, keratinocyte growth factor, IL-7, IL-17 receptor, cathepsins C, D, and E are significantly increased while PPAR δ, cyclooxygenase-2, α-catinin, TGF-β and its receptor are significantly decreased. This differentially expressed gene pattern has important implications for the pathogenesis and treatment of severe pulmonary hypertension.

4.6. Aortic aneurysm

Tung *et al* (59) used an Atlas array Human 1.2I (clonetech) to compare the altered transcriptome between abdominal aortic aneurism and the normal aorta. They identified several key genes that are significantly upregulated in aneurysms of the aorta, which includes myeloid cell nuclear differentiation antigen (31-fold), cathepsin H (30-fold), platelet-derived growth factor-A (23-fold), apolipoprotein E (13-fold), gelatinase B/matrix metalloproteinase-9 (12-fold) and interleukin-8 (11-fold). Interestingly, the only gene that was substantially down regulated was that for myosin light chain kinase (39-fold)

5. USE OF MICROARRAY TECHNOLOGY IN INFECTIOUS HEART DISEASES

Microarrays are now increasingly being used to elucidate roles for genes in the pathogenesis of infectious diseases. It is beyond the scope of this review to discuss all such research conducted to date utilizing the microarray technology in all infectious diseases. In this section, we will focus discussion on the use of microarrays in heart diseases associated with infection.

5.1. Viral and bacterial infections

Viral-induced myocarditis is common in North American with nearly 50% of the cases attributable to Picornaviradae infections, such as coxsackievirus B (CVB). Taylor *et al* (60) used cDNA microarray to assess coxsackievirus B3 Infection in a murine model (male adolescent A/J mice), that mimics the human disease process. They harvested RNA from 3, 9, and 30 day infected mice heart and compared them with respective controls. They identified about 169 known genes as having either a significant (1.8-fold) decrease or increase in expression at one or more time points and classified them according to their functional groups such as cell division, structure/motility, signaling, host defense, gene/protein expression, metabolism, mitochondrial sequences, and presently unknown function. The altered genes were then interpreted in terms of heart dysfunction and myocarditis.

Virus mediated gene alteration included increased synthesis of poly (A) binding protein, ubiquitin-specific protease, and inorganic pyrophosphatase. There was also up regulation of the peripheral-type benzodiazepine receptor (PBR) gene on days 3 and 9 after infection, which helps in cell survival by inducing an anti-apoptotic process. On the host defense side, there was

increased expression of glutathione peroxidase, metallothionein, and thioredoxin, all of which help in scavenging reactive oxygen species and in the protection of cardiac myocytes. They also observed a down regulation of genes involved in the β-oxidation of saturated fatty acids, which is well documented in other models of cardiac dysfunction. They also found profound tissue injury and death of cardiac myocytes 2-4 days post infection. After day 5 and up to day 14, the injury was complicated by an inflammatory infiltrate, which helps in clearing of both virus and dying cells. After 14 days post infection, there was a healing phase characterized by ventricular remodeling.

Reoviruses cause injury to heart and brain *in vivo* by inducing apoptosis. The induction of apoptosis is primarily because of the activation of transcription factors NF-B and c-Jun. De Biasi *et al* (61), used high-density oligonucleotide microarrays to analyze alteration in gene expression in HEK293 cells after infection with apoptosis inducing and myocarditis inducing reovirus strain T3A, with that of minimally apoptosis inducing and non myocarditic strain T1L and uninfected cells. They found that a significant proportion of altered genes were those involved in apoptotic signaling (including death receptor, mitochondrial and ER mediated pathways) and DNA repair.

Acute rheumatic fever (ARF), caused by *Streptococcus* infection, is a common cause of childhood heart disease worldwide. Smoot *et al* (62), sequenced the genome of strain MGAS8232, a serotype M18 organism and causative agent of ARF in US. They compared 36 serotype M18 strains from diverse localities by microarray analysis and found that there was little or no variation in the overall gene content which is in contrast to the data reported in *S. aureus* (63) and *Helicobacter pylori* (64). The only regions of variation lie in the phages or phage-like elements. Their data indicated that M18 strains recovered during two ARF outbreaks in Salt Lake City, occurring 12 years apart, were nearly genetically identical.

5.2. Parasitic heart disease-Chagas' disease

Chagas' disease, caused by infection with *Trypanosoma cruzi*, is a major cause of acute myocarditis and chronic cardiomyopathy in endemic region of Latin America. Ten to 30% of those infected may ultimately lead to cardiac remodeling associated with congestive heart failure and dilated cardiomyopathy. Mukherjee *et al* (65) from our laboratory profiled the global alterations in host gene expression to gain insight into the molecular mechanisms underlying the development of chagasic cardiomyopathy in a murine model (C57BL/6 x 129sv male mice). Cardiac gene expression was examined at the chronic stage (100 days post infection) in mouse heart infected with Brazil strain of *T. cruzi* using murine cDNA microarray chip.

Differentially expressed genes were sorted according to their normalized expression patterns and functional groups including those involved in transcription, intracellular transport, structure/junction/adhesion or extracellular matrix, signaling, host defense, energetics, metabolism, cell shape and death. The regulated genes were interpreted in view of the pathogenesis of chagasic heart disease. Interestingly, the gene for secreted leukocyte protease inhibitor (SLPI) was found to be up regulated in this model. This protein inhibits neutrophil function, cathepsin G and other proteases thereby protecting tissue from self-degradation by these enzymes, thus limiting neutrophil mediated tissue injury. We also observed that activation of important cell adhesion molecules such as procollagen, CD44, ICAM2 and laminins. Up-regulation of transcription factor, GTFIIH and septins underscored the proliferative nature of the cardiac tissues.

Recently, Garg *et al* (66) also examined changes in gene expression in a murine model infected with *T. cruzi*. They compared the infected and control heart at immediate early, acute, and chronic stages of infection. Immediately after infection, there was an increased expression of pro-inflammatory and interferon induced immune genes. In the acute state, the genes encoding the extracellular matrix proteins were over expressed, indicating an active remodeling process. They found that there were 114 genes that were differentially expressed in the myocardium of infected mice over the course of disease development. However, in the progression from acute infection to cardiac disease there was down-regulation of a majority (>70%) of the differentially expressed genes including that for troponins, and the genes encoding components of oxidative phosphorylation pathways. Importantly, they found a reduction in the expression of the complex IV polypeptides and a decline in the cytochrome c oxidase activity with disease development in the myocardium of infected mice and concluded that an overall loss of mitochondrial oxidative phosphorylation capacity in infected mice have an adverse effect on global energy production and subsequent cardiac performance, a similar situation is observed in other experimental models of heart failure. Their findings are supported by the observation of Mukherjee *et al* (67), that *T. cruzi* infection in cultured smooth muscle cells and endothelial cells leads to an increased activation of ERK MAPK, all of which can be induced by oxidative stress. More recently, microarray analysis was performed on the hearts of humans with chagasic cardiomyopathy (69). Their data demonstrates the importance of cytokines in the pathogenesis of this disease.

6. CONCLUSIONS

Both microarrays and SAGE allow researchers to determine the expression pattern of thousands of genes simultaneously. The main advantage of SAGE is the portability of its data, which can be compared directly and does not depend on chip formats. Also, it is not amenable to normalization and filtration. However, in differential display, RNA sequences are amplified by random priming followed by their electrophoretic separation. This technique does not guarantee the representation of all the differentially expressed genes and may miss rare transcripts. Parallel evaluation of genomic scale gene expression provides both static (singular) as well as dynamic information (how the related genes are affected).

Microarrays have been used to analyze the genetic polymorphisms in microbes, in the study of host–pathogen interactions, by pathogens that may be involved in pathogenicity and by identifying genes that are differentially regulated in the host due to infection. Microarrays have also been used to investigate the mechanism of drug action.

There are several limitations in microarray analysis. In this technique, the gene expression is measured indirectly on the basis of fluorescence intensity as measured by the scanner. Also the dye labeling efficiency is not same for all dyes used; this necessitates the normalization of the fluorescence intensities across the array. Since microarray analysis generates a lengthy data set, most of the researchers are content in taking 1.5-2 fold changes as the cut off limit, which seriously undermines genes that are differentially expressed to a lesser extent, but are statistically significant (68). The data obtained by expression profiling are only the blueprint of further research, each corner of which must be resolved by specialized individual experiments. Also it is important to consider that DNA microarrays can detect only the changes at the transcription level and many genes are known that are regulated at the post-translational level as well. Another important aspect is that microarrays in current widespread usage represent only a fraction of the entire genome, so it is likely that a considerable number of important transcripts may be missed in the analysis.

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