Review

DNA Array-Based Gene Profiling in Tumor Immunology

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ABSTRACT

Recent advances in tumor immunology have fostered the clinical implementation of different immunotherapy modalities. However, the alternate success of such regimens underscores the fact that the molecular mechanisms underlying tumor immune rejection are still poorly understood. Given the complexity of the immune system network and the multidimensionality of tumor–host interactions, the comprehension of tumor immunology might greatly benefit from high-throughput DNA array analysis, which can portray the molecular kinetics of immune response on a genome-wide scale, thus accelerating the accumulation of knowledge and ultimately catalyzing the development of new hypotheses in cell biology. Although in its infancy, the implementation of DNA array technology in tumor immunology studies has already provided investigators with novel data and intriguing hypotheses on the cascade of molecular events leading to an effective immune response against cancer. Although the principles of DNA array-based gene profiling techniques have become common knowledge, the need for mastering this technique to produce meaningful data and correctly interpret this enormous output of information is critical and represents a tremendous challenge for investigators. In the present work, we summarize the main technical features and critical issues characterizing this powerful laboratory tool and review its applications in the fascinating field of cancer immunogenomics.

TUMOR IMMUNOLOGY IN THE POST-GENOMIC ERA

Recent years have witnessed important breakthroughs in the understanding of tumor immunology (1). In particular, the identification of the genes encoding tumor-associated antigens (TAAs) and the development of therapies for immunizing against these antigens have opened new avenues for the development of an effective anticancer immunotherapy (2). Nevertheless, although the regression of established cancer has been obtained in humans by a variety of immunotherapeutic strategies (3–7), cancer immunotherapy appears to have reached a plateau of results. To further explore the anticancer potential of the immune system, a better understanding of the finely orchestrated molecular mechanisms governing tumor–host interactions is very much needed. Only when the molecular matrix governing immune responsiveness of cancer is deciphered, will new therapeutic strategies be designed to fit biologically defined mechanisms of cancer immune rejection.

Traditional molecular analyses are “reductionist” because they assess the expression of only one or a few genes at a time. Thus, the output of single-gene analysis is hardly applicable to biological models whose outcome is likely to be governed by the combined influence of a global gene network (8). The development of other molecular methods, such as comparative genomic hybridization (CGH; Ref. 9), differential display (10), serial analysis of gene expression (SAGE; Ref. 11), and DNA arrays (12), together with the sequencing of the human genome, has provided an opportunity to monitor and investigate the complex cascade of molecular events that regulate tumor-host interactions. The availability of such large amounts of information has shifted the attention of scientists from a hypothesis-driven approach to biological phenomena (the analysis of one event at a time) to a “non-reductionist” approach, in which thousands of observations are recorded at once (13). In particular, the novelty of functional genomics lies in the double opportunity to give a holistic genetic basis to hypothesis-driven approaches as well as to make unbiased observations first and then generate new, unanticipated hypotheses from those observations. Global gene-expression analysis should be of great use in the field of immunology, because it has been shown clearly that the study of a single immunological parameter at one time is not sufficient to generate a general view of how the immune system fights a given pathogen or tumor, maintains self-tolerance, or “memorizes” past encounters with antigens. High-throughput technologies can be used to follow changing patterns of gene expression over time. Among them, DNA arrays have become prominent because they are easier to use, do not require large-scale DNA sequencing, and allow the parallel quantification of thousands of genes across multiple samples. Although this technology provides no information on the biologically active products of genes (i.e., proteins), functional genomics studies have demonstrated a tight correlation between the function of a protein and the expression patterns of its gene (12), which represents the rational for a gene profile-based formulation of scientific hypotheses. Once a gene or (more frequently) a set of genes have been identified in a DNA array-based experiment, investigators commonly confirmed the results with more accurate low-throughput techniques, such as quantitative real-time PCR (14). To further validate gene profiling data, the expression of proteins coded by the genes of interest is generally assessed by standard immunohistochemistry or Western Blot techniques. Because translational gene expression regulation and posttranslational protein modifications are also of crucial
importance in determining cell functions, DNA array technology should be complemented with other recently developed high-throughput assays, such as tissue microarray (15) and proteomics (16). Hopefully, by integrating these powerful analytic tools, investigators will be able to comprehensively describe the molecular portrait of the biological phenomena underlying tumor development and progression.

DNA ARRAY TECHNOLOGY

High-throughput DNA array technology allows for the simultaneous measurement of the expression level of thousands of genes in a single experiment. Each array consists of a solid support (usually nylon or glass) on which cDNA or oligonucleotides (i.e., target) are arrayed in an addressable and miniaturized configuration, which minimizes the requirement of source material. Fluorescent, chemiluminescent, or radioactive labeled genetic material (i.e., probe) derived from cell lysate mRNA is hybridized to the target on the array. The fluorescent, chemiluminescent, or radioactive emissions of specifically bound probes are detected using an appropriate scanner that provides a quantitative estimate of each gene expression.

Two main implementations of DNA arrays have been applied with success. The first uses arrays of cDNA clones robotically spotted on a solid surface in the form of PCR products. Several versions exist, depending on the type of support (nylon, glass) and the type of target labeling (radioactivity, chemiluminescence, fluorescence; Ref. 17). This approach is flexible, allowing researchers to make arrays with their own gene sets, but it requires accurate annotation, collection, and storage of cDNA clones and PCR products, as well as avoidance of cross-contamination.

The second technological platform (Fig. 1) uses arrays of oligonucleotides either directly synthesized in situ on a support (18, 19) or robotically spotted (20). In this case, targets design requires knowledge of gene sequences. Oligonucleotide length, which varies from 20 to 80 bp, allows for alternative transcripts not distinguishable with full-length cDNA arrays. The main drawback remains the elevated cost.

The final step of a DNA array-based assay is the conversion of the image acquired with the scanner into a numeric table that associates multiple values to every gene (or oligonucleotide set) in the array. This is achieved with analysis packages that automatically recognize the position of each spot in the image and convert the distribution of pixel intensities into mean/median signal intensity.
DATA ANALYSIS

The analysis, interpretation, and meaningful display and storage of the large volume of data generated by DNA array experiments are particularly challenging. When looking at gene expression changes between samples, no consensus exists as to the best approach to testing statistically significant difference. The Student t test with the Bonferroni correction is generally perceived as too stringent given the low number of replicates in most microarray experiments. Alternative techniques may be more appropriate, including parametric and nonparametric ANOVA and permutation-based significance analysis of microarrays (SAM). If the experiment is aimed at describing a molecular phenotype, the more conservative SAM may reduce the chance of type I error. For hypothesis-generating experiments, parametric ANOVA will most likely generate a larger, less stringent data set that can be subjected to independent experimental validation.

The true strength of high-throughput experiments in revealing the complexity of tumor–host relation derives from the mathematical identification of similar expression patterns (called “signatures”) within profiling data. Dedicated software developed for this task includes the “unsupervised” and “supervised” varieties (21, 22). Unsupervised methods [e.g., cluster analysis (23), self-organizing map (SOM; Ref. 24), and principal component analysis (PCA; Ref. 25)] define classes without any a priori intervention on data, which are organized by clustering genes and/or samples simply according to similarities in their expression profiles. Among investigators, cluster analysis is probably the most popular method of DNA array data analysis (23, 26–30). Depending on the way in which the data are clustered, one can distinguish between hierarchical and nonhierarchical clustering (23, 31, 32). Hierarchical clustering allows for the detection of higher-order relationships between clusters of profiles (Fig. 2), whereas the majority of nonhierarchical classification techniques work by allocating gene expression profiles to a predefined number of clusters. The possibility of exploring different levels of the hierarchy has led many authors to prefer hierarchical clustering to the nonhierarchical alternatives.

The resulting sample classification provided by unsupervised methods often correlates with a general characteristic of the sample, as defined by large sets of genes, but not necessarily with the particular feature of interest, generally identified by a smaller set of genes. By defining relevant classes before analysis, supervised techniques [e.g., support vector machines (33), weighted votes (34), and supervised neural networks (35)] bypass this issue. These algorithms incorporate external information related to samples studied to identify the optimal set of genes that best discriminate between experimental samples.

Fig. 2  Hierarchical aggregative clustering. The color codes, the measured fluorescence ratios: black, genes with unchanged expression levels; increasingly intense red, genes with increasingly positive expression; increasingly intense green, genes with increasingly negative expression. Accordingly, the darker the color, the closer to unchanged expression. The figure shows an example with the color-coded expression values of five genes (gene 1–5) in five different experimental conditions (C1, C2, C3, C4, C5). In the aggregative method, the closest pair of profiles is chosen based on a given metric. Then, an average of both profiles is constructed. This defines a relationship of closeness between both profiles that remain tied by the corresponding branch of the tree. Thus, the linked profiles are substituted by the average profile, and the process continues until all of the profiles are linked. The linkage relationship defines the hierarchy of the tree. Asterisks link corresponding rows of genes during the clustering process.
source of genes over/underexpressed after cell lysis (for RNA extraction) of heterogeneous samples (Fig. 3). In fact, PBMC and solid tumor specimens contain several cell types (leukocyte subpopulations, normal/malignant cells) in different proportions and functional status. An expression profile from such samples represents a snapshot of the genes expressed by all cell types present in the specimen at that moment. A solution may come from confronting, with clustering techniques, expression profiles of heterogeneous specimens with those of cell lines that represent the cell types present in the sample (virtual microdissection; Refs. 27 and 29). A more accurate but also more difficult and labor-intensive strategy lies in the use of tissue microdissection, which allows the procurement of pure/near-pure cell subpopulations from frozen- or fixed-tissue specimens (36, 37).

**RNA Abundance.** DNA array experiments require large amounts of high-quality RNA. Fine-needle aspirate material (38) and many clinical specimens from early diagnoses and new minimally invasive diagnostic procedures (e.g., sentinel node biopsy for melanoma or breast cancer) can provide critical ex vivo biological information using high-content screen technology but are limited by the amount of material obtained (Fig. 3). Most DNA array platforms work with a few micrograms (3–5 μg) of mRNA except for nylon membranes with radioactive...
for the former, it is generally appropriate to take steps to vary sources of variability are biological and technical variation. As contributions of multiple sources of variation (43). The main issues that should be kept in mind while dealing with technical variability should also be addressed while designing DNA array-based experiments. Although this can be achieved by repeating the experiment, high-throughput DNA array experts suggest that the use of spot replicates within the same array is the best way to deal with this issue (44, 45). In particular, biostatistical analysis has shown that a minimum of three replicates should be used to reduce the number of false-positive and false-negative results generated by studies performed without replication (46).

Finally, a highly challenging issue common to all high-throughput technologies is the biological interpretation of the results, which is limited by our lack of knowledge on the relationships among signaling pathways, transcriptional regulation, and metabolic stability of cells. To address this issue, various software programs have been developed to connect experimental results with available data bases or literature-based information (47). These data bases can directly link individual genes to other genes with known relationship and can help construct biological hypotheses.

**CANCER IMMUNOGENOMICS**

**Tumor Escape from Immune Surveillance.** Despite the evidence that immune effectors can play a significant role in controlling tumor growth in natural conditions or in response to therapeutic manipulation, it is evident that cancer cells can survive their attack as the disease progresses. Several mechanisms underlying immune escape have been proposed (48), such as down-regulation of HLA molecules/TAA on tumor cell surface, the production of immunosuppressive cytokines, and the expression of lymphotoxic molecules (i.e., FAS ligand) by malignant cells (49). However, these mechanisms cannot be advocated in many cases of immunotherapy failure (48) and some of the existing hypotheses have been questioned (50).

Gene expression profiling led Toulouse et al. (51) to hypothesize that a tumor suppressor gene [i.e., retinoic acid receptor β2 (RARβ2)] exerts an anticancer activity through the stimulation of the immune system. RARβ2, which is inactivated in many epithelial tumors and their derived cell lines, has frequently been shown to be the principal mediator of the tumor suppressive effects of retinoic acid. Searching for genes regulated by this receptor, the authors found that several of them code for proteins favoring an effective antitumor immune response, suggesting that down-regulation of these genes in RARβ2-deficient tumor cells may contribute to immune system evasion. In this paradigmatic experience, DNA array technology allowed investigators to formulate and corroborate their hypotheses by simultaneously screening several gene pathways potentially influenced by a given gene.

Gene profiling of melanoma biopsies allowed us to observe that the tumor microenvironment is naturally rich in expression of immunomodulating molecules (e.g., cytokines, growth factors; Ref. 52). Because of their concomitant pro-inflammatory properties, many of these factors might trigger a dormant host immune system otherwise tolerant toward the poorly immunogenic malignant cells by enhancing TAA immunogenicity in vivo (53). This interpretation fits well the “danger” model postulated by Matzinger (54), according to which, TAA recognition by CTLs must be preceded by a nonspecific immunological alarm (i.e., danger signal) for an effective immune response to take place. When the level of immunostimulatory molecules within the tumor microenvironment reaches the threshold required to induce an immune response, tumors spontaneously regress, as observed with relatively high frequency in melanoma and renal cancer patients. If, however, the level of immune or inflammatory stimulation is below the threshold required for immune rejection, the balance struck between the host immune system and cancer enables their coexistence. In this case, sys-
temic cytokine [(e.g., interleukin (IL)-2) administration and/or TAA-specific immunization might shift the balance in favor of the host by enhancing the ongoing immune/inflammatory response, the result being tumor rejection (3).

**New Targets for Anticancer Vaccines.** DNA array technology has been extensively used to identify gene patterns specific for normal cells (e.g., lymphocyte subsets), as well as pathological tissues (e.g., cancer; Refs. 55, 56). In particular, investigators are using the gene fingerprint of cells not only to differentiate between normal and pathological samples (diagnosis) but also to better define the phenotype of neoplasms (onco-cotranscriptome), which in turn might be particularly useful in subclassifying tumor types according to their different clinical outcomes (prognosis). A corollary of such research is the identification of novel TAAs suitable for cancer immunotherapy. Classically, the identification of TAA-derived T-cell epitopes requires patient-derived T cells and either a gene expression approach (57) or a mass spectrometry-based sequencing of the recognized peptides (58). More recently, “reverse immunology” has been proposed as a novel approach to select HLA class I-restricted epitopes from a given TAA (59). Main hurdles of this strategy are the time-consuming culture techniques and, more importantly, the low frequency of preexisting epitope-specific T cells. Comparative expression profiling of a tumor and the corresponding autologous normal tissue enabled by DNA array technology (19, 60) is an excellent method for identifying large numbers of candidate TAAs from individual tumor samples (61–63). Using this strategy, Mathiasson et al. (64) have found that several genes were overexpressed by transplantable thymomas established from an inbred p53−/−mouse strain. Mice were then immunized with mixtures of peptides representing putative cytotoxic T-cell epitopes derived from one of the gene products identified by DNA array analysis. Interestingly, such immunized mice were protected against subsequent tumor challenges, showing that this gene profile-based strategy is suitable for the screening of new TAA-derived immunogenic peptides. Similar findings have been already reported in humans (65). Therefore, it appears appealing to screen the entire transcriptome of any given tumor to identify genes encoding potential tumor specific antigens suitable for peptide-based cancer vaccines. A potential development could be the utilization of DNA array technology for designing patient-tailored TAA-based vaccination. To this aim, Weinschenk et al. (66) have recently proposed the integration of high-density oligonucleotide array with mass spectrometry, quantitative real-time PCR, and HLA-tetramer technology to identify patient-specific candidate peptides suitable for anticancer vaccination. After sorting out genes selectively expressed or overexpressed in malignant tissues (e.g., renal cell carcinomas), these investigators identified HLA class I-restricted peptides from tumor specimens by mass spectrometry. Then, peripheral CD8+ T cells from tumor patients and healthy individuals were tested for reactivity toward the candidate peptides using quantitative real-time PCR (14) and HLA-tetramer-based flow cytometry (67), thus allowing the investigators to identify TAA epitopes potentially suitable for clinical implementation.

**Dendritic Cell Biology and Cancer.** Despite the strong preclinical evidence supporting the use of dendritic cells (DCs) for anticancer vaccination in humans, the results of clinical trials thus far carried out do not appear to meet expectations (4, 68–72), probably because the physiology of these cells is only partially understood. Immature DCs capture TAAs in the peripheral tissues, process them into peptides bound to HLA molecules, and then migrate to lymphoid organs in which they present HLA-peptide complexes to T lymphocytes. After the interaction with TAA-specific T-helper lymphocytes, DCs become activated through the CD40 signaling pathway, up-regulate HLA and costimulatory molecules expression on their surface, and acquire a mature phenotype characterized by the expression of new markers such as CD83 and by the secretion of pro-inflammatory and chemotactic cytokines (73). Gene profiling studies have recently broadened the spectrum of genes that distinguish immature versus mature DCs (74). Mature DCs prime CTLs, thus polarizing the effector arm of cell-mediated immunity against the noxious agent (75, 76). By contrast, DCs conditioned by regulatory T-suppressor cells are “licensed” to inhibit the initiation of the immune response by inducing T-helper lymphocyte anergy (77–80). To characterize the molecular changes occurring in tolerogenic DC, Socia-Foca Cortesini et al. (81) investigated the mRNA profile of DCs exposed to allospecific T-helper and T-suppressor cells, showing that immature DCs conditioned by T-suppressor cells differentiate into tolerogenic DCs with a distinct phenotype as compared with mature nontolerogenic DCs. The identification of DC gene pathways induced by suppressor lymphocytes could be of paramount importance to dissect the molecular mechanisms underlying immune tolerance toward malignant cells and, consequently, to identify new strategies to tackle this problem.

Nevertheless, using DNA array technology, Chen et al. (82) described the molecular portrait characterizing DC at different stages of maturation. In an animal model, these authors could link two different DC gene patterns with two levels of effectiveness in inducing tumor regression mediated by DC-based vaccine. If confirmed in a human model, these results might explain some vaccination failures observed in the clinical setting and might indicate new avenues of research in the design of more effective DC preparation protocols for antitumor vaccines.

**T-Cell Biology and Cancer.** In animal models, it has been demonstrated that the activated tumor-specific effector T cells mainly comprise type 1 CD4+ and CD8+ lymphocytes, both of which are important for an effective antitumor immune response (83). Thus, the cellular and molecular biology of these T-cell subsets is of substantial interest in the context of both basic and clinical tumor immunology. Using DNA array technology, Zhang et al. have started exploring the mRNA steady state of such tumor-specific T-cells as compared with naïve T-cells in mice (84). Gene expression profiling has been also applied to the study of the mechanisms of partial T-cell activation, which accounts for different cytotoxic capabilities and might determine the clinical outcome of vaccinated cancer-bearing patients (85). To mimic a suboptimal CTL activation, Verdeil et al. (86) developed a model of naïve CD8+ T-cells from transgenic mice expressing an alloreactive T-cell receptor for which a mutant alloantigen behaved as a partial agonist, inducing only some of the effector functions induced by the native alloantigen. To ascertain the molecular bases for the establishment of divergent fates within the same naïve CD8+ T-cells, they used cDNA microarrays to monitor sequential gene
expression patterns in conditions of full or partial response of these naive CD8⁺ T cells. Clusters of genes encoding costimulatory molecules and genes controlling cytolytic function, cytokine production, and chemokines were found to discriminate between partially and fully activated lymphocytes, providing new insights on the gene pathway potentially leading to an effective immune reaction against cancer.

**Immune Response within the Tumor Microenvironment.** Until recently, most studies addressing the immunological effects of vaccination in cancer patients have looked at variations in the level of TAA-specific reactivity in circulating lymphocytes (87). Results from clinical trials have shown that vaccination can be quite effective in inducing tumor-specific T-cell responses that can be easily observed among circulating lymphocytes. However, the identification of such immune responses could not be consistently correlated with tumor regression (88). Thus, it is questionable whether the immunogenic wave, induced systemically by the vaccine, reaches the tumor microenvironment. Complementing the analysis of immune responses in circulating lymphocytes with the study of the tumor microenvironment may yield information about the quality and intensity of the elicited immune response within the relevant arena (88). Using fine-needle aspiration material from melanoma metastases (38), we found that tumor nodules undergoing complete regression in response to peptide-IL-2-based vaccination were characterized by a different transcript signature as compared with those progressing (89, 90). Interestingly, many genes overexpressed in responding melanoma metastases were immune-related. Among them, we focused on TIA-1 and IL-10. TIA-1 codes for a Mr 15,000 cytotoxicity-related protein expressed by CTLs and natural killer (NK) cells and is characterized by proapoptotic properties (91). IL-10 is generally considered an immunosuppressive molecule that can anergize CTLs, acting both directly (92) and through its inhibitory effects on DCs (93). However, several preclinical models have shown that IL-10 can mediate tumor regression, also by stimulating NK...
cells activity (94, 95). Furthermore, using cDNA microarray, we observed that, in vitro, IL-10 induced NK cell (but not CTL) expression of cytotoxicity-related genes, including TIA-1 (95). These observations led us to hypothesize that, in the presence of high levels of IL-10 in the tumor microenvironment, NK cells might be stimulated to lyse cancer cells, thus increasing TAA availability and “danger signal” delivery (54) required by DCs to be activated, ultimately favoring CTLs priming against TAs (Ref. 96; Fig. 4). If this theory were proved to be correct, future antitumor immunotherapy strategies should address the challenging task of stimulating both innate and adaptive immunity in a timely fashion.

Because systemic IL-2 administration significantly increases the frequency of tumor regression induced by peptide-based vaccination of melanoma patients (3), we also investigated the role of this cytokine in facilitating an effective immune response. It has been postulated that the antitumor effects of IL-2 are mediated through in vivo activation and expansion of cytotoxic lymphocytes (97) and/or promotion of their migration within target tissues (3), but it has become apparent that IL-2 at the doses used therapeutically has broader immune/pro-inflammatory effects (98, 99). Which of these effects has a critical role in mediating tumor regression remains enigmatic. In our study, we compared early changes in transcriptional profiles of PBMCs with those occurring within the microenvironment of melanoma metastases after systemic IL-2 administration (100). The results of this work suggested that IL-2 administration induces three predominant effects: (a) activation of antigen-presenting monocytes; (b) a massive production of chemoattractants that may recruit other immune cells, among which are the chemokines MIG and PARC, specific for T cells, to the tumor site; and (c) the activation of lytic mechanisms ascribable to monocytes (calgranulin, grancalcin) and NK cells (e.g., NKG5, NK4). These findings suggest that systemic IL-2 administration may facilitate T-cell effector function in the target organ not by sustaining their proliferation, as generally believed, but rather by promoting their migration and by providing a milieu conducive to their activation in situ through the activation of antigen-presenting cells. If this hypothesis were correct, then adoptive transfer of effector T cells should follow, rather than precede, administration of systemic IL-2.

CONCLUSIONS

In the near future, the DNA-array approach will be extremely prolific in identifying and characterizing biological phenomena and will provide, as a consequence, biologically targeted therapies. This will be particularly the case for cancer immunotherapy. Obviously, attention should be devoted to the profiling of tumor and not only of the host. Molecular profiling has been successfully used to identify specific phenotypes that may help in the subpathological diagnosis of diseases and allow forecasting of their clinical response and outcome. However, this strategy has been, thus far, applied only in very limited circumstances for the understanding of immune-mediated cancer rejection in humans. Because a detailed mechanism of how immunotherapy actually works is still not known, the interactions of various cell types should be taken into account; these cell types include not only T cells but also other components of the immune system (e.g., innate immunity mediators), as well as tumor cells. In fact, the failure of current immunotherapeutic strategies might depend on the unresponsiveness of immune sentinels to the therapeutic manipulation and/or to the resistance of malignant cells to the immune response evoked by the treatment. Tools are now available to study, in real-time, tumor-host interactions before, during, and after immunotherapy in humans (while leaving tumor lesions undisturbed) and, consequently, to identify gene patterns underlying therapeutic mechanisms or to explain the phenomenon of tumor immune escape (38).

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