ABSTRACT

Drug resistance in cancer is a major obstacle to successful chemotherapy. Cancer cells exposed to antitumor drugs may be directly induced to express a subset of genes that could confer resistance, thus allowing some cells to escape killing and form the relapsed resistant tumor. Alternatively, some cancer cells may be expressing an array of genes that could confer intrinsic resistance, and exposure to cytotoxic drugs select for the survival of these cells that form the relapsed tumor. We have used cDNA microarray to monitor the expression profiles of MCF-7 cells that are either transiently treated with doxorubicin or selected for resistance to doxorubicin. Our results showed that transient treatment with doxorubicin altered the expression of a diverse group of genes in a time-dependent manner. A subset of the induced genes was also found to be constitutively overexpressed in cells selected for resistance to doxorubicin. This distinct set of overlapping genes may represent the signature profile of doxorubicin-induced gene expression and resistance in cancer cells. Our studies demonstrate the feasibility of obtaining potential molecular profile or fingerprint of anticancer drugs in cancer cells by cDNA microarray, which might yield further insights into the mechanisms of drug resistance and suggest alternative methods of treatment.

INTRODUCTION

The development of cancer is accompanied by genetic alterations of multiple oncogenes and tumor suppressor genes. Understanding the complexity of the array of genetic, hormonal, and dietary factors that contribute to the etiology of cancer is confounded by the lack of information on the expression of specific genes associated with the initiation and progression of the disease. In addition, treatment of cancer with chemotherapy is often empirical based more on the histological appearance of the tumor than on an understanding of drug-resistant phenotypes in relapsed and metastatic cancers. Cancers are either primarily resistant to chemotherapy (intrinsic resistance), or respond to chemotherapy but later recur to form a multidrug-resistant tumor (acquired resistance; Ref. 1). Several mechanisms of drug resistance in tumors are understood and include overexpression of the multidrug resistance gene (MDR1; Ref. 2), the multidrug resistance-associated protein (3), and increased DNA repair (4). Various regulatory genes in the cell targeted for genetic alterations during tumorigenesis may also influence cellular sensitivity to chemotherapeutic drug (5). These genetic alterations involve a diverse group of gene products that include tumor suppressor genes, oncogenes, cell cycle regulators, transcription factors, growth factor receptors, DNA repair factors, and cell death regulators. Mechanisms of development of intrinsic drug resistance are not thoroughly understood and may involve the expression of multiple genes during tumor progression. Therefore, a single mechanistic pathway cannot explain the genesis of resistance in cancer. Rather, drug resistance likely involves the altered expression of a diverse group of genetic factors influencing various biochemical pathways. The emergence of acquired resistance, on the other hand, may be associated with either drug induction or drug selection of tumor cells during chemotherapy, resulting in relapses that are refractory to treatment.

The advent of DNA microarray technology and its capacity for simultaneous probing of the genome on high-density microarrays in yeast and man has enabled the analysis of the expression profiles of thousands of genes (6–17). In view of the complex array of genetic factors contributing to drug resistance, DNA microarray should be useful for examining the development of drug resistance in cancer. These analyses ultimately may enable us to use the signature expression profiles of drug-resistant tumors to predict response to drugs and to design therapeutic regimens to circumvent drug resistance.

In this study, we used cDNA microarray to monitor mRNA expression in breast cancer cells that were either transiently treated with or selected for resistance to doxorubicin. We found altered expression of a large number of genes in response to doxorubicin exposure and also in the doxorubicin-resistant cells. A signature profile of doxorubicin induction and resistance is suggested.

MATERIALS AND METHODS

Fabrication of Microarrays. The cDNA microarray filter, obtained from Research Genetics, Inc. (Huntsville, AL), is a 5 × 7-cm Nylon membrane containing 5760 spots corresponding to 3801 functionally known genes and 1379 ESTs with high and moderate similarity with known genes in human or other species. The array also contains 576 spots of total genomic DNA, which serve as reference points for the image analysis software Pathway (Research Genetics Inc.), for normalization purposes and for verifying the homogeneity of the hybridization. The clones selected contain the 5′ untranslated region, with an average size of 1 kb, and have been sequence verified. After PCR amplification, 10 ng of insert DNA are printed on a charged Nylon membrane by a custom made robot.

Cell Culture and RNA Preparation. MCF-7 and the doxorubicin-resistant MCF-7/D40 cells were cultured in RPMI media supplemented with 10% fetal bovine serum and containing glucose and the antibiotics penicillin and streptomycin (Life Technologies, Inc., Gaithersburg, MD). Cells were cultured in 15-cm Petri dishes and then treated with 1 μg/ml doxorubicin for the various times indicated. RNAs were then harvested from the cells using Trizol reagent (Life Technologies, Inc.), as specified by the manufacturer.

Labeling, Hybridization, and Scanning of Microarray. The labeling and hybridization procedures were conducted as specified by the manufacturer, and details of the protocols are downloadable from the Research Genetics, Inc. web site. cDNA probes were synthesized from total RNA with 32P-dCTP by oligo dT-primed polymerization using Superscript II reverse transcriptase (Life Technologies, Inc.). The pool of nucleotides in the labeling reaction was 0.5 mM dGTP, dATP, and dTTP, and 0.2 mM dCTP. Probes were purified by gel chromatography (BioSpin 6; Bio-Rad) and ethanol precipitated, and then resuspended in 100 μl of 10 mM Tris (pH 8)-1 mM EDTA buffer. Prior to hybridization, the solution was boiled for 2 min, then allowed to cool to room temperature. Hybridization was
conducted overnight at 42°C with the microarrays, and then the filters were washed for 20 min in 2× SSC, 0.2% SDS at room temperature, and then for 30 min in 0.1× SSC, 0.2% SDS, at 60°C. Washed filters were then exposed on phosphorimager screens. The phosphorimager screens were scanned on the Molecular Dynamics Storm Imager at 100-µm resolution, and the scanned files containing the microarray were analyzed with the Pathways software (Research Genetics, Inc.). Verification of cDNA expression was performed with the QuantumRNA RT-PCR kit (Ambion Inc., Austin, TX), using the 18 S rRNA as internal standard according to the manufacturer’s specifications.

RESULTS

Drug resistance occurs in cancer by various mechanisms. In acquired drug resistance, the emergence of a relapse drug-resistant tumor following chemotherapy suggests that the resistance phenotype may be either induced or selected in a population of tumor cells exposed to chemotherapy. In this study, global expression profiles of the human breast carcinoma MCF-7 cells treated with doxorubicin and also MCF-7 cells selected for resistance to doxorubicin were examined by microarray. The MCF-7 cells were transiently treated with doxorubicin (1 µg/ml) for various times, ranging from 1–15 h. Total RNA prepared from these cells was used to synthesize 33P-labeled cDNAs by reverse transcription, followed by hybridization to the human cDNA microarray from Research Genetics, Inc., as described in “Materials and Methods.” The experiments were conducted independently for three times on different filters.

Typical scanned phosphorimages of one of the experiments with MCF-7 cells treated with doxorubicin for 15 h in comparison to the vehicle-treated control are shown in Fig. 1. The scanned image demonstrated alterations in expression of cDNAs after 15 h of induction (indicated in matching colored circles for the corresponding genes). The measured intensities of the array elements are represented in Fig. 2 as simple bivariate scatterplots, comparing the profiles of the doxorubicin-treated samples at various times (Y axis) to the vehicle-treated control MCF-7 cells (X axis). Approximately 1 h after treatment with doxorubicin, there were few changes in gene expression (Fig. 2A). As time progresses following exposure to doxorubicin, an increasing number of changes in gene expression were observed, as indicated by the shifts of the data points toward (indicating decreased expression) or away from the X axis (indicating increased expression; Fig. 2, B–E). Simultaneous alterations in the expression of a large number of genes following induction were observed. The patterns of expression of some of these genes, as shown in Fig. 3, exhibited an orderly time-dependent alteration after doxorubicin treatment, as indicated by the gradual changes in expression. Approximately 500 ESTs, representing about 10% of the total DNA elements on the array, had substantially altered levels of expression after induction with doxorubicin for 15 h. The changes ranged from ~2–30-fold. The entire data set of genes analyzed in this experiment can be found at a web site maintained by our laboratory.5

The biochemical functions of the genes in this expression profile...
are diverse and include transcription factors, protein kinases and phosphatases, cell cycle regulators, proteases, apoptotic and antiapoptotic factors, as well as a large number of metabolic genes (Fig. 3; see “Discussion”). Altered expression of the transcription factors includes down-regulation of the general transcription factor RNA polymerase II, the transcription corepressor Dr1-associated protein, and the enhancer binding proteins AP-3 and AP-4 (Fig. 3A). These changes seemed to be consistent with a general shut down of transcription in response to doxorubicin treatment. Changes in the expression of a group of zinc finger transcription factors were also noted. Increased expression of cytochrome c, which triggers apoptosis by activating the caspases and down-regulation of Bcl-2, an antiapoptotic factor, were consistent with the cytotoxic effects of doxorubicin (Fig. 3B). In addition, a cluster of genes involved in the ubiquitin-proteasome pathway was also prominently targeted for up-regulation (Fig. 3F) by doxorubicin treatment. To confirm the changes observed by microarray, we measured the expression of several representative genes by quantitative RT-PCR. The RNA from the array experiments was used in the RT-PCR assay. We found the MDA-7 gene, which is involved in melanoma cell growth and differentiation and progression, to be down-regulated. In addition, CDC28 protein kinase 1 and the heat shock chaperone DNAJ were also down-regulated. The 26S proteasome regulatory subunit 4 and the epoxide hydrolase genes were induced. The expressions of these genes were altered in a time-dependent manner similar to that observed in the microarray (Fig. 4). Overall, the success rate of validation by RT-PCR is ~58%. The other primer pairs yielded products of unanticipated sizes or no products at all. These failures in validation could be attributed to various experimental factors including the sequence context and optimal PCR conditions for these gene specific primers, physical parameters of the PCR reactions, and other factors that we do not understand at present.

We next examined the expression profile of a human doxorubicin-resistant breast carcinoma cell line, MCF-7/D40 (18). The MCF-7/D40 cells exhibit cross-resistance to a number of drugs, including the Vinca alkaloids and some topoisomerase II inhibitors. They have an increased expression of P-glycoprotein, decreased drug accumulation relative to the parental cells, and show reversal of drug accumulation and drug resistance by verapamil (18). Results in Fig. 5A show that ~300 genes exhibited altered levels of gene expression in MCF-7/D40 cells compared with the MCF-7 parental cells. P-glycoprotein, which is overexpressed in these cells, was also found by microarray to be overexpressed. When MCF-7/D40 cells were treated with doxorubicin, the changes in gene expression were far less than observed after exposure of MCF-7 parental cells (Fig. 5B). This is not surprising because these cells are resistant to doxorubicin and the genes that confer drug resistance are already altered in their expression, therefore, transient treatment of the cells with doxorubicin did not induce cell death nor further altered the expression profile of a large number of genes. Further analysis revealed that some of the genes that are overexpressed in the MCF-7/D40 cells were also induced in MCF-7 cells after treatment with doxorubicin (Table 1). These results suggest

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K. Kudoh and K-V. Chin, unpublished data.
that cancer cells may recruit and activate the expression of a distinct set of genes in transient induction or when selected for resistance to doxorubicin. The expression of this subset of genes may be the molecular signature of doxorubicin resistance.

To further evaluate the specificity of the signature profile of the doxorubicin-resistant MCF-7/D40 cells, we asked whether the expression pattern of MCF-7 cells resistant to other chemotherapeutic drugs have distinct signature profiles from the MCF-7/D40 cells by studying the expression profile of cisplatin-resistant MCF-7/CP cells. Cisplatin, an anticancer agent that reacts with the N7 position of purines to form DNA adducts, and doxorubicin, a topoisomerase II inhibitor, have different mechanisms of action. Microarray analysis revealed the altered expression of about 400 genes in the cisplatin-resistant MCF-7/CP cells, with expression ratios greater than 2.6 Comparison of the expression profiles between MCF-7/CP and MCF-7/D40 cells showed a subset of about 40 genes that are overlapping (Table 2). These genes are distinct from the changes observed in the doxorubicin-resistant and the doxorubicin-induced cells (Table 1). These results demonstrated that the signature profiles of the doxorubicin- and the cisplatin-resistant cells are different.

DISCUSSION

The emergence of drug resistance poses a significant obstacle to the success of cancer chemotherapy. Mechanisms of development of resistance in cancer are not clearly understood and may arise intrinsically from the plethora of genetic alterations during tumor progression. Alternatively, drug resistance may also be acquired through either induction or selection in cancer during chemotherapy (1). We used DNA microarray to evaluate these complex changes associated with drug resistance. Our results show that transient exposure to doxorubicin elicits a gradual change in the expression profile of a large number of genes (Figs. 2 and 3). A subset of these transiently induced genes intersected with a distinct set of genes that are constitutively overexpressed in a cell line stably selected for resistance to doxorubicin (Table 1). This set of genes may represent the signature profile of doxorubicin-resistant cells. Furthermore, the signature profile of doxorubicin resistance differed from the signature profile of cisplatin-resistant cells (Table 2).

Our results provide a genome-wide analysis of the cellular response to doxorubicin as well as the mechanisms of resistance to doxorubicin. The cytotoxic response to doxorubicin in MCF-7 cells involves the induction, as well as the repression, of genes of various biochemical and regulatory pathways (Fig. 3). These include genes that have specific functions in transcription, cell cycle regulation, various protein kinases and phosphatases, and other factors involved in signal transduction, apoptosis, protein degradation, and a large number of metabolic regulators. Alterations in the expression of these genes occurred in a time-dependent manner in the MCF-7 cells upon treatment with doxorubicin.
The transcriptional response involves both transcription activators as well as repressors (Fig. 3A). Doxorubicin exposure leads to the down-regulation of the general transcription factor RNA polymerase II, the global transcription corepressor Dr1-associated protein (19), and enhancer binding proteins (AP-3 and AP-4). Decreased expression of these genes may trigger a general repression of transcription in the cells in response to the deadly insult of a cellular poison. In contrast, the activation of serum response factor may represent a stress response of the cells to the cytotoxic effects of doxorubicin. Moreover, it is also intriguing that the expression of a previously unknown putative zinc finger transcription factor, was prominently targeted for alterations in response to doxorubicin treatment (Fig. 3A). Although the functional consequences of these alterations are unclear, we speculate that the downstream target genes of these zinc finger transcription factors may either be a response to the cytotoxic insult or have impact on the emergence of drug resistance.

Another cluster of genes that showed striking changes after doxorubicin treatment were the genes involved in proteolysis. We found that some ubiquitin-associated factors and subunits of the proteasome including Pohl1, were up-regulated after doxorubicin treatment (Fig. 3F). Furthermore, the regulatory subunit 4 of the 26 S proteasome, which is induced after doxorubicin treatment, is also constitutively overexpressed in the doxorubicin-resistant cells (Fig. 3F and Table 1). Protein degradation is recognized to be critical in the regulation of cell cycle, transcription, and signal transduction (20). It has also been shown that overexpression of a 26 S proteasome subunit, Pohl1, confers a multidrug resistance phenotype (21, 22). The 26 S proteasome, a multiprotein complex, mediates protein degradation through the ubiquitin pathway. These results reveal that expression of the ubiquitin-dependent proteolysis pathway is activated in response to doxorubicin and suggest the involvement of the ubiquitin-proteasome system in drug resistance. In this case, DNA microarray confirmed and extended previous observation on the ubiquitin pathway in drug resistance. The precise mechanisms by which protein degradation may affect drug resistance remain to be determined.

Table 2: Specificity of microarray. Gene expression profiles in doxorubicin- or cisplatin-resistant MCF-7 cells

<table>
<thead>
<tr>
<th>Up-regulated genes</th>
<th>Down-regulated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBP-associated factor (hTAFII30)</td>
<td>PL-3-kinase associated p85</td>
</tr>
<tr>
<td>G-rich RNA-associated sequence binding factor 1</td>
<td>Arp2/3 protein complex subunit p20-Arc</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>V-crk</td>
</tr>
<tr>
<td>NOTCH4</td>
<td>Peptidylprolyl cis-trans isomerase</td>
</tr>
<tr>
<td>TRAF-interacting protein I</td>
<td>Metallothionein-1le gene (hMT-le)</td>
</tr>
<tr>
<td>Integrin αE</td>
<td>Insulinoma rig-analog</td>
</tr>
<tr>
<td>High mobility group protein isoform I</td>
<td>Estrogen sulfotransferase</td>
</tr>
<tr>
<td>Transcription factor AREB6</td>
<td>β-2-microglobulin precursor</td>
</tr>
<tr>
<td>Clathrin light chain A</td>
<td>Spermidine synthase</td>
</tr>
</tbody>
</table>

*Profiles of the intersecting genes between doxorubicin- and cisplatin-resistant cells are different from those of the doxorubicin-induced transcription program (see Table 1).
ble 1). These genes have diverse functions, and it remains to be determined whether they form a functional relationship. In addition, additional studies will be required to determine whether overexpression of these genes may confer drug resistance. We have also identified from our experiments individual genes in the induced and selected cells that are known to play a role in drug resistance. For example, it has been shown that epoxide hydrolase, a drug metabolizing enzyme, is found to be highly expressed in breast cancer and hepatocellular carcinoma and, therefore, may confer intrinsic resistance in these cancers (27, 28). Our results showed that epoxide hydrolase was induced by doxorubicin and was also overexpressed in doxorubicin-resistant cells. These observations suggest that drug may induce the expression of epoxide hydrolase in cancer cells, thus enabling them to increase the metabolism of doxorubicin and evade killing by chemotherapy. The surviving cells may subsequently undergo clonal expansion to form the relapse tumor, which overexpresses epoxide hydrolase, that no longer response to treatment.

It has already been shown that overexpression of the Pohl1 gene in the ubiquitin-proteasome pathway may confer a multidrug resistance phenotype in cells (21, 22). Increased expression of the 26 S proteasome regulatory subunit 4 gene in MCF-7/D40 cells may contribute to an increase in resistance to doxorubicin. Because the ubiquitin-proteasome pathway affects the functions of a large number of cellular processes, its influence on drug resistance may be an indirect one, most likely resulting from its effects on a downstream target(s) controlled by either transcription, cell cycle, stress response, or metabolic adaptations (29). It is also quite intriguing that the single-stranded DNA repair protein Xrccl1 is also constitutively overexpressed in the drug-resistant cells. The Xrccl1 gene functions in the repair of single-strand DNA breaks in mammalian cells and forms a repair complex with β-polymerase, ligase III, and poly (ADP-ribose) polymerase (30). In addition, Xrccl also specifically binds single-strand break DNA (gap and nicked) and also in a gap DNA-β-polymerase complex. Because topoisomerase II generates a double-stranded DNA break, it is unclear how XRCCL1 protein might participate in the repair of such lesions unless XRCCL1 could also bind to and repair double-stranded DNA gap lesions produced by topoisomerase II. Thus, elevated expression of XRCCL1 may increase the efficiency of the repair of topoisomerase II-generated DNA lesion, which may contribute to an increased resistance. The overexpression of the other members of this subset of overlapping genes and their functional significance in drug resistance are not immediately apparent and need to be further investigated. Nevertheless, their expression may potentially represent a distinct signature profile for doxorubicin resistance. Clearly, further comparisons of expression profiles are necessary to determine the prevalence of the expression of this subset of genes in various other doxorubicin-resistant cells.

The use of DNA microarrays containing ~2500 EST elements in our analysis has provided a global view of the response of breast cancer cells to doxorubicin at the genomic levels. Undoubtedly, when the human genome sequence is completely deciphered, DNA microarrays containing EST elements that encode for all of the genes of the human genome sequence is completely deciphered, DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. Genome Res., 6: 639–645, 1996.


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Monitoring the Expression Profiles of Doxorubicin-induced and Doxorubicin-resistant Cancer Cells by cDNA Microarray

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