Advances in Brief

Pleiotropic Resistance to DNA-interactive Drugs Is Associated with Increased Expression of Genes Involved in DNA Replication, Repair, and Stress Response

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Abstract

A combination of four genetic suppressor elements (GSEs), two of which are derived from putative transcriptional regulators, was previously found to increase resistance to drugs inhibiting DNA replication in HT1080 fibrosarcoma cells. In the present study, two GSE-transduced cell lines, isolated with and without cytotoxic selection, were found to be resistant to a diverse group of DNA-interactive agents, including aphidicolin, hydroxyurea, cytarabine, etoposide, doxorubicin, and mafosfamide. Changes in gene expression associated with GSE-induced drug resistance were analyzed by cDNA array hybridization and reverse transcription-PCR. Twenty genes were found to be up-regulated in both of the resistant cell lines. These include genes involved in DNA replication and repair (e.g., PCNA, XRCC1, B-MYB, and GADD45), transcriptional regulators associated with stress response, and cell cycle checkpoint control (e.g., YB-1, DBPA, and ATF4), and genes for signal transduction proteins (e.g., protein tyrosine phosphatase 1B and regulatory subunits α and β of cAMP-dependent protein kinase). The observed changes in gene expression may play a role in pleiotropic resistance to different classes of DNA-targeting drugs.

Introduction

Many anticancer drugs target DNA, by inducing DNA damage through chemical interactions, by promoting the formation of cleavable topoisomerase complexes, or by interfering with DNA replication. To investigate the molecular determinants of tumor cell death induced by inhibition of DNA replication, we have previously (1) isolated GSEs^3, short biologically active cDNA fragments, which conferred resistance to aphidicolin, an inhibitor of DNA replication with a precisely defined mechanism of action (2). Starting from a normalized human cDNA fragment library in a retroviral expression vector, we have selected four GSEs that, when used as a combination rendered human HT1080 fibrosarcoma cells resistant not only to aphidicolin, but also to doxorubicin and hydroxyurea. These GSEs were derived, respectively, from an Expressed Sequence Tag without a known function, from the mitochondrial subunit 3 of cytochrome c oxidase, and from two genes encoding potential transcriptional regulators. The latter includes a Ring3-related gene (ORFX) that encodes a mitogen-activated nuclear kinase and a WIZ gene that encodes a widely interspersed zinc finger protein (1). In the present study, we have found that cells transduced with this combination of four GSEs and isolated with or without cytotoxic selection are resistant to different classes of DNA-interactive drugs. This form of pleiotropic drug resistance is associated with the induction of a specific set of genes involved in DNA replication, repair, cell cycle control, stress response, and signal transduction.

Materials and Methods

Derivation of Cell Lines and Drug-resistance Assays. Aphidicolin selection of GSEs A1, A8, B5, and C5 and of GSE-transduced cell line APH5, isolated by aphidicolin selection from HT1080 clone E14 carrying ecotropic retroviral receptor (3), has been described previously (1). All four GSEs were recloned into retroviral vector LnECX, carrying GFP as a selectable marker (3), and the resulting retroviruses were used for coinfection of HT1080 E14 cells, as described (1). Cells with the brightest GFP fluorescence were isolated by FACS and subcloned. One of the resulting clones, M125, was found by PCR to contain all four GSEs. For control studies, the parental HT1080 E14 cells were transduced with an insert-free retroviral vector, LNCX, as described (1).

Mafosfamide was provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment (National Cancer Institute, NIH, Bethesda, MD); all of the other drugs were from Sigma Chemical Co. Colony assays for drug resistance were carried out in duplicates, by plating 500 cells per 10-cm plate. Twenty-four h after cells were treated with different doses of aphidicolin or hydroxyurea for 48 h and with doxorubicin, cytarabine, etoposide, or mafosfamide for 24 h. Cells were then washed and allowed to form colonies in drug-free media.

cDNA Array Hybridization and RT-PCR. Nylon membranes containing Atlas Human Broad-Coverage cDNA Array 1.2 were obtained from Clontech. Total cellular RNA extraction, ^32P-labeled cDNA probe preparation, and filter hybridization were carried out as recommended by Clontech. At least three cDNA probes from two independent RNA preparations were prepared and used for each of the three cell lines. The results of array hybridization were evaluated using phosphorimager (Molecular Dynamics) and AtlasImage 1.0 software package (Clontech). Signal normalization was carried out relative to the cDNA for L13A (23k) ribosomal protein.

RT-PCR was carried out using gene-specific primers as recommended by Clontech. The L13A (23k) ribosomal protein was used as a normalization control for different cDNA preparations. For quantitation, ^32P-dCTP was added to each PCR reaction (0.2 μCi per reaction). To assure exponential amplification, four aliquots were removed from each PCR assay at different cycle numbers, starting from 20, 25, 30, or 35 cycles (as determined in preliminary experiments to produce the weakest detectable PCR product for each gene) and increasing by 2, 4, and 6 cycles. PCR products were separated in 2% agarose or 7% polyacrylamide gels, bands were cut out of the gel, and the incorporated radioactivity was determined by scintillation counting. The average yield of RT-PCR products in the reactions falling in the exponential range was calculated for each gene relative to the L13A (23k) control.

Results and Discussion

We have previously described cell line APH5, a derivative of HT1080 E14 cells, which carries the combination of GSEs A1, A8, B5, and C5 and has been isolated after aphidicolin selection (1). To avoid potential artifacts associated with drug selection, we reintroduce...
duced the combination of these four GSEs into E14 cells using a GFP-containing retroviral vector. GFP-expressing transductants were isolated by FACS and subcloned, without cytotoxic selection. One of the transduced cell lines, M125, contained all four GSEs and, like APH5, was found to be resistant to aphidicolin. Clonogenic assays for drug resistance (Fig. 1) indicated that both APH5 and M125 cell lines were resistant not only to aphidicolin, but also to other DNA replication inhibitors (hydroxyurea and cytarabine), to topoisomerase II-poisoning drugs (doxorubicin and etoposide) and to an alkylating agent (mofosfamide, an in vitro active analogue of cyclophosphamide). APH5 cell line showed no difference from the control cells in its resistance to cisplatin or Taxol (data not shown). Transduction of E14 cells with an insert-free retroviral vector, LNCX, had no effect on the resistance to any of these agents (data not shown). It remains to be determined whether all four GSEs are required for resistance to all of the drugs in Fig. 1, as has been previously found for aphidicolin resistance (1). We have shown earlier that the resistant phenotype of APH5 is not associated with a decreased accumulation of a fluorescent drug (doxorubicin; Ref. 1). We have now found by FACS analysis that M125 cells also accumulate the same amount of doxorubicin as the parental E14 cells (data not shown), indicating that the resistance phenotype is not mediated by decreased drug uptake.

Because two of the GSEs used for transduction were derived from putative transcriptional regulators, we hypothesized that GSE-induced drug resistance could be due to alterations in cellular gene expression. We, therefore, investigated changes in gene expression in GSE-transduced cell lines APH5 and M125, relative to the parental E14 cells. This analysis was carried out by hybridizing 32P-labeled total cDNA probes from each of the three cell lines with Atlas Human Broad-Coverage cDNA Array 1.2 (Clontech), containing 1176 genes with different biological functions. All of the genes showing at least a 2-fold difference in signal intensity between the parental and the resistant cell lines were individually analyzed by semiquantitative RT-PCR. Parental E14 cells transduced with LNCX were used as a control. Examples of RT-PCR analysis are shown in Fig. 2.

None of the genes in the cDNA array showed a reproducible over 2-fold decrease in hybridization signal intensity in the resistant cell lines APH5 and M125. In each sample, aliquots were collected at different cycle numbers (see "Materials and Methods"). A L13A (23K) ribosomal protein was used as a normalization control.

Fig. 1. Colony formation assays for resistance to the indicated drugs in cell lines APH5 (□), M125 (□) and E14 (■). Clonogenic survival is plotted as a fraction relative to untreated cells. The drugs and the concentration units are: A, aphidicolin (μg/ml); B, cytarabine (μM); C, hydroxyurea (mM); D, doxorubicin (μM); E, etoposide (μg/ml); F, mofosfamide (μM).

Fig. 2. Semiquantitative RT-PCR analysis of gene expression in HT1080, APH5, and M125 cells. In each sample, aliquots were collected at different cycle numbers (see "Materials and Methods"). A L13A (23K) ribosomal protein was used as a normalization control.
CHANGES IN GENE EXPRESSION IN DRUG-RESISTANT CELLS

In contrast, 20 genes showed increased expression in both APH5 and M125 relative to the parental line. Two other genes showed increased expression in APH5 but not in M125 cells. These genes, their biological functions, and their fold up-regulation in the resistant lines (as determined by RT-PCR relative to LNCX-transduced E14 cells) are listed in Table 1.

Because APH5 and M125 cells are resistant to several chemotherapeutic drugs targeting DNA, it is particularly interesting that the products of several genes, overexpressed in these cell lines, are involved in DNA replication or repair. These include: PCNA, which functions in DNA replication, repair, and cell cycle control (4); DNA repair protein XRCC1, associated with resistance to ionizing radiation (5); damage-inducible protein Gadd45, which plays a role in DNA repair and G2 checkpoint arrest (6); and DNA mismatch repair protein MLH1 (overexpressed only in APH5 cells), which also plays a role in G2-M checkpoint arrest (7) and has been associated with resistance to cisplatin (8).

A large group of proteins in Table 1 acts as transcription factors or cofactors. Interestingly, several of these proteins regulate the onset of DNA replication, including B-Myb (9), PC4 (10), ATF4 (11), and C-1 (12). Several other proteins are involved in cell cycle checkpoint controls, including c-Myc, which promotes cell entry into mitosis (13); RanGAP1, which regulates mitotic spindle assembly (14); and heat shock protein HSPA8, which may affect G1-S transition through binding a cyclin-dependent kinase inhibitor p27kip1 (15).

It is especially interesting that one of the strongest up-regulated genes in both of the resistant cell lines is the YB-1 transcription factor, which was shown to play a role in cellular resistance to several DNA-damaging agents (16) and to act as a positive regulator of MDR1 (multidrug resistance) gene expression (17). Another up-regulated transcription factor, dbpA, is also involved in stress response and drug resistance and binds to the same Y-box element as YB-1 (18). Cytokine-inducing transcription factor DB1 is another stress-related gene, which is regulated by RhoB GTPase (19). The transcriptional regulator ATF4 is also involved in stress response and has been shown to activate the transcription of the damage-inducible protein Gadd153, implicated in cell growth arrest and apoptosis (20).

The products of two other genes overexpressed in the resistant cell lines are known as positive regulators of apoptosis, including c-Myc (21) and apoptosis adaptor protein Cradd/Raidd (22). Drug-treated HT1080 cells are less prone than most other cell lines to undergo apoptosis (23). In the present study, however, we have observed that HT1080 E14 cells, detached from solid support after treatment with all of the tested drugs, showed features of apoptosis: nuclear condensation, DNA ladder formation, and, in mafosfamide-treated cells, apoptosis-specific cleavage of poly (ADP-ribose) polymerase (data not shown). In light of these observations, the finding that HT1080 derivatives resistant to these drugs overexpress positive regulators of apoptosis is rather surprising and warrants additional investigation.

The final clearly identifiable group of genes that are up-regulated in the resistant cell lines is involved in signal transduction. This group includes both regulatory subunits (PRKAR1α and PRKAR1β) of the cAMP-dependent protein kinase; PRKAR1α has been shown to play a role in cisplatin resistance (24). Interestingly, some transcriptional regulators that are overexpressed in the resistant cell lines have also been associated with the cAMP response. These include ATF4, which interacts with multiple domains of cAMP-responsive element-binding protein (25) and is transiently down-regulated (at the RNA level) by

<table>
<thead>
<tr>
<th>Gene product</th>
<th>GenBank no.</th>
<th>Fold increase</th>
<th>Functions</th>
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<tr>
<td>PCNA</td>
<td>M15796</td>
<td>7.5</td>
<td>Replication, repair, cell cycle control</td>
</tr>
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<td>DNA repair protein XRCC1</td>
<td>J04718</td>
<td>6</td>
<td>DNA repair, resistance to ionizing radiation, regulation of replication</td>
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<td>Y-box binding protein 1 (YB-1/NSEP)</td>
<td>M36089</td>
<td>6</td>
<td>Cold-shock domain transcription factor associated with drug resistance and stress response</td>
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<td>C-1 helix-loop-helix protein</td>
<td>M83234</td>
<td>6</td>
<td>Putative transcription factor associated with G1-S transition</td>
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<td>Activating transcription factor 4 (ATF4/TAXREB67/CREB2)</td>
<td>U41816 (D90209)</td>
<td>6</td>
<td>Transcription activating factor involved in stress response and G1-S transition</td>
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<td>Matrix metalloproteinase 16 (MMP16/MT3-MMP)</td>
<td>D50477 (3.5)</td>
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<td>Extracellular matrix degradation</td>
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<td>DB1 (zinc finger protein 161)</td>
<td>D28118</td>
<td>3</td>
<td>RhoB-regulated transcription factor, cytokine expression</td>
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<tr>
<td>Major prion protein precursor (PrP)</td>
<td>M13667</td>
<td>3</td>
<td>Cell membrane glycoprotein related to signal peptidases</td>
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<td>Death domain containing protein Cradd/Raidd</td>
<td>U84388</td>
<td>3</td>
<td>Positive regulator of apoptosis</td>
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<td>DNA-binding protein A (DbpA/NF-GMB)</td>
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<td>Cold-shock domain transcription factor associated with stress response</td>
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<td>Myb-related protein B (B-MYB)</td>
<td>X13293</td>
<td>2</td>
<td>Regulation of signal transduction by cytoskeletal and extracellular factors</td>
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<tr>
<td>Protein-tyrosine phosphatase 1B (PTP1B)</td>
<td>M31724 (2.0)</td>
<td>6.2</td>
<td>Regulation of cell differentiation</td>
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<tr>
<td>c-Myc</td>
<td>V00568</td>
<td>1.9</td>
<td>Transcription factor, positive regulator of mitosis and apoptosis</td>
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<td>Positive cofactor 4 (PC4/PC4CTD/p15)</td>
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<td>1.6</td>
<td>Transcriptional coactivator, regulates DNA replication, growth-suppressing</td>
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<td>Ran GTPase activating protein 1 (RanGAP1)</td>
<td>X82260</td>
<td>1.6</td>
<td>Regulation of nucleocytoplasmic transport and mitotic spindle assembly</td>
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<td>cAMP-dependent protein kinase type 1-α regulatory chain (PRKAR1α)</td>
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<td>1.5</td>
<td>Regulatory subunit of protein kinase A</td>
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<td>Heat shock cognate M, 71,000 protein (HSPA8/HSP73/HSC70)</td>
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<td>Heat shock protein, binds to p27kip1 during G1-S transition</td>
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<td>cAMP-dependent protein kinase type 1-β regulatory chain (PRKAR1β)</td>
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<td>DNA mismatch repair protein MLH1</td>
<td>U07418</td>
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<td>DNA repair; G1-M checkpoint arrest, apoptosis</td>
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<td>Leukemia inhibitory factor (LIF; cholinergic differentiation factor)</td>
<td>X13967</td>
<td>3.8</td>
<td>Regulation of cell differentiation</td>
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* Fold-increase in RNA levels relative to LNCX-transduced HT1080 E14 cells was determined by RT-PCR, as described in “Materials and Methods.”

MLH1 expression was not detected in HT1080 cells.

ND, no detectable expression in M125.
cAMP (26), and PCNA, transcription of which is activated by cAMP-responsive element-binding protein (27). Another up-regulated gene involved in signal transduction encodes protein-tyrosine phosphatase 1B, which was reported to be up-regulated in doxorubicin-resistant breast carcinoma cells (28). The up-regulated major prion protein precursor, PrP, shares homology with signal peptidases (29) and, therefore, may also be involved in signal transduction.

It seems reasonable to hypothesize that changes in gene expression observed in the resistant cells may represent direct or indirect effects of the GSEs derived from putative transcriptional regulators ORFX and WIZ (1). ORFX, in particular, encodes a mitogen-activated nuclear kinase that shares substrate specificity with myosin light-chain kinase and cAMP-dependent protein kinase (30), both of which are up-regulated in GSE-transduced cells (Table 1). It is conceivable that overexpression of these kinases might represent a feedback-regulatory response to the inhibition of ORFX by the antisense-oriented GSE C5. Although the relationship between these kinases and the drug-resistant phenotype is not readily apparent, genes that are activated in GSE-transduced cell lines include those that are directly involved in DNA replication, repair, and stress response. These functions suggest that up-regulation of such genes may be causally involved in the observed pleiotropic resistance to different classes of DNA-targeting drugs.

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References

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