Altered Gene Expression by Cisplatin in a Human Squamous Cell Lung Carcinoma Cell Line

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Abstract. *Background: Substantial evidence has disclosed that some cytotoxic agents have complex activities in influencing signal transduction pathways in cells. Materials and Methods: cDNA microarray analysis was performed after exposing a human squamous cell carcinoma cell line, RERF-LC-AI, to low-dose cisplatin for 5 days. Up-regulated gene expressions were suppressed by small interfering RNA to investigate phenotypic alteration of the cells. Results: Among 30,000 genes screened, 42 genes showed increases or decreases in expression of more than 2-fold with cisplatin treatment. They included genes with functions involved in apoptosis, cell cycle regulation and DNA metabolism/repair. Suppression of the 5 most significantly altered genes by small interfering RNA resulted in partly reduced apoptosis without altering cytotoxicity of cisplatin. Conclusion: Besides direct cytotoxic effects on cells, cisplatin may have indirect effects involving drug resistance, and synergistic effects with other agents.*

Cytotoxic agents kill cancer cells *via* various mechanisms, including direct effects on nucleic acids, metabolizing enzymes and microtubules. In the majority of current clinical applications, cytotoxic agents are combined with other cytotoxic agents, molecular-targeted agents or radiation. The rationale for combination chemotherapy with multiple drugs, and combination modality therapy with chemotherapeutic agents plus radiation, includes therapeutic advantages by virtue of the combination of these direct effects with different agents or modalities. They are supra-additive, additive or sometimes sub-additive, probably depending on mechanisms of drug–drug or drug–radiation interaction, although the precise mechanisms are not always known (1-7). In such cases, some effects by cytotoxic agents that are not directly related to cytotoxicity may have significant influence on the effects by other agents or radiation. For example, some cytotoxic agents cause cell cycle shift, resulting in modified sensitivity of the cells to other agents or radiation. In other cases, prolonged or repeated exposure to cytotoxic agents can induce multiple drug-resistant mechanisms, making target cells resistant to other agents. As examples for the former, 5-fluorouracil radiosensitizes by acting on cells at the S-phase, resulting in a cell cycle shift that is more sensitive to radiation (8); fludarabine also has a radiosensitizing effect by accumulating cells in the radiosensitive G_2/M-phase (9). As examples of the latter, induction of p-glycoprotein or non-p-glycoprotein-mediated multiple drug resistance (10, 11) and modulation of apoptosis (12-14) by cytotoxic agents have been already reported. In addition, altered gene expression of DNA repair enzymes by some cytotoxic agents, including platinum compounds, may influence the effects of other drugs or radiation (15).

Although such alteration of chemo- and radiosensitivity of cells may require relatively long periods, *in vitro* experiments elucidating drug–drug or drug–radiation interaction, in many cases, have been performed by exposing cells to a cytotoxic agent for a short period. In the clinical setting, however, cytotoxic agents are usually administered repeatedly and even continuously. Gene expression induction to cause an alteration in drug sensitivity would be possible during the clinical use of cytotoxic agents, because even a single administration of an agent, in most cases, contributes to a sustained serum concentration for several hours or more, depending on its pharmacokinetics characteristics.

Cisplatin is one of the most active and frequently used agents for many solid tumors, either as a single agent, in combination with other cytotoxic agents, radiation, or both.

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In addition to its direct effect on DNA that causes potentially lethal damage, it also triggers a variety of signal transduction pathways including those of cell cycle control, DNA repair processes, apoptosis, and the production of some growth factors, with *p53* as a central player (16, 17). This alteration of signal transduction pathways might influence cytotoxicity when cisplatin is administered as a single agent, or as a part of combination therapy with other agents or radiation. Therefore, exploring altered gene expressions in the process of cisplatin exposure would provide us with important information regarding genes involved in its cytotoxicity or its interaction with other agents.

The recently developed cDNA microarray methodology enables us to screen expressions of approximately thirty thousand genes at a time. In cancer research, this will provide valuable insights into carcinogenesis, individual tumor characteristics and resistance mechanisms to therapy. It will also serve as a powerful tool in finding prognostic biomarkers, developing therapeutically relevant genomic classifiers, and identifying novel therapeutic targets (18). Therefore, genes with altered expressions due to relatively extended exposure to cisplatin were exhaustively screened by cDNA microarray, and some, appearing promising, were investigated in terms of their function in the cytotoxicity of the agent.

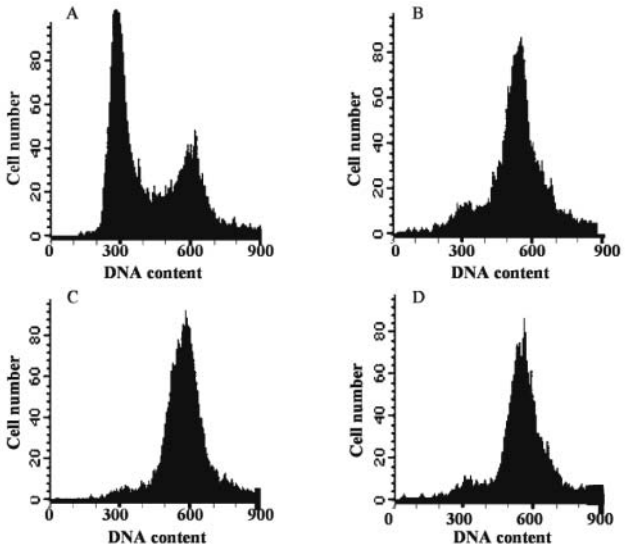

Figure 1. *Effect of cisplatin treatment on the growth of a human squamous cell lung carcinoma line RERF-LC-AI. Compared to untreated cells (A), cisplatin treatment of cells at a dose of 0.6 μg/ml for 5 days caused a significant cell cycle shift predominantly at the G_2/M phase (B). To adjust cell cycle distributions, untreated cells with cisplatin were treated with colcemid and cultured further. The cell cycle distribution after 16 hours from the completion of colcemid treatment (C) most resembled that of cisplatin-treated cells (B) among these after 12, 14, 16, 18, 20 and 22 hours of culture. For cDNA microarray analysis, RNA extracted from cisplatin-treated cells (D) and -untreated cells (C), both treated with colcemid before RNA extraction was used.*

Materials and Methods

Cells, cell culture, drug exposure and colonogenic assay. A human squamous cell lung carcinoma cell line, RERF-LC-AI (LCA) was purchased from Riken Cell Bank (Tsukuba, Japan). These cells were cultured as a monolayer in a humidified atmosphere including 5% CO_2 at 37˚C with Eagle's minimum essential medium (Invitrogen Co., Carlsbad, CA, USA) supplemented with 292 mg/l L-glutamine, 0.225% sodium bicarbonate, 100 units/ml penicillin, 100 mg/ml streptomycin and 10% heat inactivated fetal bovine serum. A preliminary experiment with polymerase chain reaction-single strand conformation polymorphysm covering exons 5, 6, 7 and 8 of *p53* disclosed that the cell line had wild-type *p53* (data not shown).

For colonogenic assay, the cells were treated with different concentrations, ranging from 0.001 to 1.0 μg/ml of cisplatin (a kind gift from Bristol-Myers Squibb Japan, Tokyo, Japan) for 4 days without medium exchange, then washed with complete medium twice, and finally trypsinized to make cell suspensions, resulting in 4-fold cell washes to completely remove cisplatin. Defined numbers of suspended cells, that is, 100 cells per plate for cisplatin at a concentration of 0, 0.01 and 0.1 μg/ml, and 300, 1,000 and 10,000 cells per plate for cisplatin concentrations of 0.2, 0.6 and 1.0 μg/ml, respectively, were immediately re-plated onto culture dishes in triplicate and cultured for 10 days with the above mentioned complete medium. Colonies were counted with the aid of 1% crystal violet staining and a dissecting microscope.

Extraction of mRNA after cell cycle adjustment. As a previous experiment demonstrated a significant cell cycle shift predominantly to G_2/M-phase of the cells by cisplatin treatment for 4 or 5 days (7), a simple comparison between cisplatin-treated and -untreated cells seemed difficult. To overcome this problem, cell cycles of the two groups were adjusted by synchronizing them with the use of colcemid (Invtrogen Co.). After culture with or without cisplatin of 0.6 μg/ml for 4 days, the cells were treated with colcemid at a concentration of 0.03 μg/ml for 4 hours, followed by gentle shaking of the culture dishes to obtain detached cells, and then washing twice to remove colcemid before cells were re-plated on new dishes for further culture, again with or without cisplatin of 0.6 μg/ml. The majority of the detached cells were confirmed to be in G_2/M-phase by propidium-iodine single-color method with flow cytometry (FACScan, BD Bioscience, San Jose, CA, USA). Cisplatin-untreated cells treated with colcemid were examined chronologically as to cell cycle, after further culture for 12, 14, 16, 18, 20 and 22 hours. Among them, the cell cycle distribution of the cisplatin-untreated cells cultured for 16 hours after colcemid treatment resembled that of cells with cisplatin treatment for 4 days most closely, and that of cells with cisplatin plus similar colcemid treatment (Figure 1). Based on this observation, cells treated or untreated with cisplatin for 4 days were further treated with colcemid for 4 hours. After the completion of colcemid treatment, the cells were additionally cultured for 16 hours with or without cisplatin, resulting in an approximately 5-day culture in total. Finally, their total RNAs were extracted for cDNA microarray. The concentration of cisplatin was 0.6 μg/ml for 5 days, at which the cell-surviving fraction was approximately 0.1.

cDNA microarray. Total RNA was extracted with Trizol reagent (Invitrogen Co.) according to the manufacturer's instructions. The microarray chips, Human OligoChip30K of AceGene (DNA Chip Research Inc., Yokohama, Japan) were the oligo DNA type for 30,000 genes.

The cDNA microarray analysis was performed as reported elsewhere (19). Briefly, an RNA probe was synthesized from an extracted total RNA of 5 μg using the Message AMP aRNA kit (Ambion Inc., Austin, TX, USA). First, the single-stranded cDNA was synthesized by appling T7 oligo (dt) primer and was converted to double-stranded DNA. Amino allyl modified aRNA from the double-stranded cDNA templates was then generated. This was labeled with either Cy3 or Cy5, and hybridized to the DNA microchip for 16 hours, followed by washing. Images were analyzed with QuantArray (GSI Lumonics, Nepean, Canada) and DNASIS Array (Hitachi Software Engineering, Tokyo, Japan) software. The means and standard deviations (SD) of background levels were calculated and genes with intensities of less than mean ± 2 SD of background were excluded from further analysis. The Cy5/Cy3 ratios of all spots on the microarray were normalized using the global normalization method. An RNA probe from untreated cells with Cy3 label and one from cisplatin-treated cells with Cy5 label were coupled for one experiment, and the reverse pair for another experiment. Signals for any gene from the 2 independent experiments were judged as an informative data set, and the average of the two experiments with more than 2-fold ratio of gene expression with cisplatin treatment, compared to that without cisplatin, was used for further steps.

Annotation of gene name, symbol and function. Selected genes by cDNA microarray were annotated with gene names, symbols, and established or speculated functions by the DNASIS Array software.

Quantitative reverse transcriptase-PCR (RT-PCR). Among genes with altered expression by cisplatin treatment, the five genes with the most altered expression were further investigated in the following experiments; these five genes were serine/cysteine protease inhibitor (serpin B2), connective tissue growth factor (CTGF), lymphocyte antigen 96 (Ly96), spermidine/spermine N_1-acetyltransferase (SAT) and procollagen-lysyl hydroxylase (PLOD 2).

The mRNA levels were measured by TaqMan 5'-nuclease fluorogenic quantitative PCR analysis with normalization to 18S rRNA. Primers and probes for the five genes were designed by software (Primer Express; Applied Bio-systems, Foster City, CA, USA) based on the sequence database of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA). The TaqMan probe and primer sets for the five genes were provided by Applied Bio-systems. Quantitative RT-PCR amplification was performed using the ABI PRISM 7000 Sequence Detection System (Applied Bio-systems) with the amplification conditions of preincubation at 48˚C for 30 min and at 95˚C for 10 min, during which reverse transcription was processed, with 40 cycles consisting of 95˚C for 15 s and 60˚C for 60 s in each cycle.

Suppression of gene expression by small interfering RNA (siRNA). To suppress these five gene expressions, a corresponding double-stranded siRNA for each gene was synthesized by Quiagen (Hilden, Germany) with primers of the following sequences: 5'-GGG UCA AGA CUC AAA CCA A dTdT-3' for serpin B2; 5'-AGG UUA GUA UCA UCA GAU A dTdT-3' for CTGF; 5'-CAA UUU CAA UUA AUG UUA A dTdT-3' for Ly96; 5'-ACG UGG UGU GAU CUU AAU A dTdT-3' for SAT; 5'-CAG AUA AAU UAU UAG UCA U dTdT-3' for PLOD 2; and 5'-UUC UCC GAA CGU GUC ACG U dTdT-3' for the negative control. They were inoculated into the culture medium at a final concentration of 5 nM from the start of culture with cisplatin until cell harvest for each experiment.

Apoptosis assay. Apoptosis was evaluated by caspase 3 activity, detection of annexin V with FACS, and Hoechst staining. For determining caspase 3 activity, a colorimetric assay monitoring the absorbance at 405 nm to measure cleaved *p*-nitroanilide (*p*NA) from synthetic substrates with cell extracts was used. Caspase 3 activities were thus evaluated with synthetic substrates and DEVD-*p*NA with Colorimetric Assay Kit APOPCYTO (Medical & Biological Laboratories Co., Nagoya, Japan).

For detecting annexin V, cells in apoptosis were detected by annexin V-propidium iodide double-color method with FACScan, according to the manufacturer's instructions. Briefly, non-adherent and trypsinized adherent cells were washed twice with ice-cold phophate-buffered saline (PBS) and resuspended in binding buffer (10 mM HEPES at pH 7.4, with 140 mM NaCl and 2.5 mM $CaCl_2$) at a concentration of $1x10^6$ cells/ml. A cell suspension of 100 μl was mixed with 5 μl annexin V-FITC (BD Biosciences) and 0.2 μg/ml propidium iodide, followed by 15-min incubation at room temperature in the dark and admixing with 400 μl of the binding buffer, before analyzing by FACScan within an hour. The data were analyzed by CELL Quest software (BD Biosciences).

For Hoechst staining, trypsinized cells, together with floating cells, were harvested, fixed with 1% glutaraldehyde and stained with 1 mM *bis*benzimide H33258 fluorochrome trihydrochloride (Hoechst 33258, Nakalai Tesque Inc., Kyoto, Japan). They were examined under fluorescence microscopy. Aggregating cells and cells with fragmented chromatin were regarded as apoptotic. In each experiment, 500 cells were counted, and the ratio of apoptotic cells to intact cells was calculated.

Statistical analysis. Comparisons of RT-PCR-assessed mRNA levels between cells treated or untreated by cisplatin, caspase 3 activites, annexin V detection and Hoechst staining between the groups were performed by non-parametric Mann-Whitney *U*-test. Differences were judged as statistically significant when *p*-values were less than 0.05 (two-sided test).

Results

Altered gene expression and their characteristics. Among 30,000 genes screened after cell exposure to cisplatin for 5 days, DNA microarray provided informative results for 26,505 genes. Among them, 52 genes showing increases or decreases in expression of more than a two-fold ratio compared to control cells were identified. Genes with names and symbols not assigned by the NCBI database were excluded, resulting in 38 genes with expressions elevated more than two-fold, and 4 genes with expressions declining to less than half of their original value. These genes are listed in Table I together with their known or speculated functions. Among the 42 genes selected, the five genes with the greatest degree of expression alteration, serpin B2, CTGF, Ly 96, SAT and PLOD 2, were further investigated.

Table I. *Genes regulated by cisplatin in RERF-LC-AI cells.*

Gene symbol	Gene name	Biological process	Signal ratio
Up-regulated			
SERPINB2	Serine/cysteine proteinase inhibitor, clade B, member 2	Anti-apoptosis	7.269
CTGF	Connective tissue growth factor	Epidermal differentiation, cell growth/maintenance, response to wounding, cell adhesion, cell motility, DNA metabolism, regulation of cell growth	3.391
LY96	Lymphocyte antigen 96	Cell surface receptor linked signal transduction, cellular defense response, inflammatory response, antibacterial humoral response	3.166
SAT	Spermidine/spermine N1-acetyltransferase		3.133
PLOD2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	Protein modification, protein metabolism	2.939
MMP1	Matrix metalloproteinase 1	Collagen catabolism	2.906
TXNRD1	Thioredoxin reductase 1		2.747
AWP1	Protein associated with PRK1	Biological process unknown	2.653
IFI16	Interferon, gamma-inducible protein 16	Response to virus, regulation of transcription, DNA-dependent, immune response, cell proliferation, monocyte differentiation	2.599
RECQL	RecQ protein-like (DNA helicase Q1-like)		2.572
PSAT1	Phosphoserine aminotransferase 1	L-serine biosynthesis, metabolism, pyridoxine biosynthesis	2.555
GGH	Gamma-glutamyl hydrolase		2.552
RAP1B	RAP1B, member of RAS oncogene family	Small GTPase mediated signal transduction	2.544
MAP1LC3B	Microtubule-associated protein 1 light chain 3 beta		2.516
CSF2RA	Colony stimulating factor 2 receptor, alpha, low-affinity		2.503
GADD45A	Growth arrest and DNA-damage-inducible, alpha	Cell cycle arrest, regulation of cyclin dependent protein kinase activity, protein biosynthesis, DNA repair, apoptosis	2.476
FLJ14681	Hypothetical protein FLJ14681	Lipid catabolism	2.474
CAV1	caveolin 1, caveolae protein, 22 kDa		2.409
DDIT3	DNA-damage-inducible transcript 3	Cell cycle arrest, response to DNA damage stimulus, cell growth and/or maintenance, regulation of transcription, DNA-dependent	2.390
MK-STYX	Map kinase phosphatase-like protein MK-STYX	Intracellular signaling cascade, protein amino acid dephosphorylation	2.309
MGC9913	Hypothetical protein MGC9913		2.308
TNFRSF19L	Tumor necrosis factor receptor superfamily, member 19-like		2.291
FLJ23518	Hypothetical protein FLJ23518		2.231
TFPI2	Tissue factor pathway inhibitor 2	Blood coagulation	2.227
HMMR	Hyaluronan-mediated motility receptor (RHAMM)	Cell motility	2.190
RIG-I	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide		2.188
ATP6AP2	ATPase, H+ transporting, lysosomal accessory protein 2		2.183
PTDSS1	Phosphatidylserine synthase 1	Phospholipid biosynthesis, phosphatidyl-serine biosynthesis	2.170
MN7	D15F37 (pseudogene)		2.155
C6orf55	Chromosome 6 open reading frame 55		2.147
ERCC6	Excision repair cross-complementing group 6	Perception of sound, regulation of transcription, DNA-dependent, DNA repair, transcription from Pol II promoter	2.091
TFPI	Tissue factor pathway inhibitor	Blood coagulation	2.088
JM5	JM5 protein		2.066
PTDSS1	Phosphatidylserine synthase 1	Phospholipid biosynthesis, phosphatidyl-serine biosynthesis	2.053
ALEX3	ALEX3 protein		2.052
SLC38A1	Solute carrier family 38, member 1	Amino acid transport	2.044
LOC51248	Hypothetical protein LOC51248		2.041
EPLIN	Epithelial protein lost in neoplasm beta		2.003
Down-regulated			
ID1	Inhibitor of DNA binding 1	Eevelopment, regulation of transcription from Pol II promoter	2.738
SFRS3	Splicing factor, arginine/serine-rich 3	Nuclear mRNA splicing, via spliceosome (mRNA splicing, pre-mRNA splicing)	2.052
HNRPA1	Heterogeneous nuclear ribonucleoprotein A1		2.042
U2AF1	U2(RNU2) small nuclear RNA auxiliary factor 1	RNA splicing, nuclear mRNA splicing, *via* spliceosome	2.009

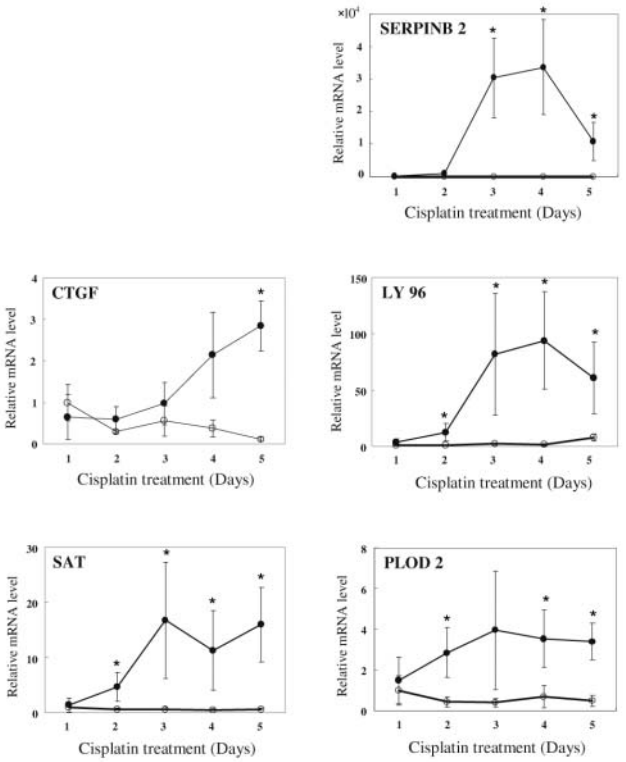

Figure 2. *Serial quantitative RT-PCR, from the start of cisplatin treatment, of the five genes found to be most up-regulated by cDNA microarray confirmed sustained elevation of the expression by cisplatin treatment (closed circles), compared to untreated cells (open circles). Each point and bar represent mean and SD of 5 independent experiments. Asterisks represent data sets with statistically significant differences between treated and untreated cells ($p<0.05$).*

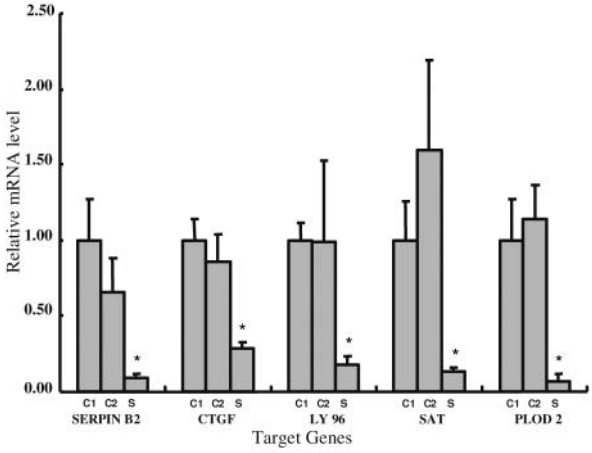

Figure 3. *Corresponding siRNAs for the five genes were successful in repressing the elevation of gene expression by cisplatin. Quantitative RT-PCR clearly showed significantly ($p<0.05$) lower expression of each gene with treatment of the corresponding siRNA (S) compared to cells treated with cisplatin alone (C1) and cisplatin plus negative siRNA (C2). Cells were treated by cisplatin with or without siRNA for 5 days until analysis. Columns and bars represent mean and SD, respectively ($n=3$).*

Another DNA microarray analysis was carried out by exposing cells to cisplatin for 4 days but not adjusting for the cell cycle stage by omitting the colcemid treatment, resulting in 13 genes with expressions elevated to more than double, and 110 genes with expressions which declined to less than half of their original value (data not shown). Again, serpin B2, Ly 96, CTGF and PLOD 2 were identified among the 13 genes, with their elevated signal ratios being 6.9, 4.0, 3.1 and 2.1, respectively. SAT was not included in the 13 genes, its signal ratio being 1.7.

Suppression of gene expression by siRNA. Serial RT-PCR during the exposure to cisplatin disclosed remarkably elevated expressions of the five genes selected above, whereas their expressions in the untreated cells were significantly lower (Figure 2). Figure 3 shows that each siRNA strongly suppressed its corresponding gene expression after cisplatin exposure for 5 days, whereas the negative siRNA had no effect.

Effect of siRNA on cytotoxicity and apoptosis induced by cisplatin. None of the siRNAs for the selected five genes significantly modified sensitivity of the cells to cisplatin as measured by colonogenic assay after exposure to cisplatin for 5 days, when compared to the control experiments without siRNA or with negative siRNA (Figure 4). Effects of the siRNAs on apoptotic cell induction by cisplatin were rather complex. That is to say, inhibition of serpin B2 and Ly 96 enhanced apoptosis by cisplatin when assessed by caspase 3 activity; in addition to these two genes, SAT and PLOD 2 were also suggested to be related to apoptosis induction by cisplatin when assessed by annexin V, in contrast, assessment with Hoechst staining showed no significant alteration of apoptosis by cisplatin *via* suppression of any of the five genes (Figure 5). Suppression of serpin B2 resulted in enhanced apoptosis even without cisplatin treatment when measured by annexin V, although no similar change was observed when assessed by caspase 3 and Hoechst staining.

Discussion

The cDNA microarray chips covering 30,000 genes highlighted 52 genes whose expressions were elevated or had declined by more than a 2-fold ratio to the original expression after 5-day exposure of a human squamous cell carcinoma cell line to cisplatin at a dose causing a cell survival fraction of 0.1. Moreover, 42 genes with NCBI-assigned gene names and symbols, including 20 genes having

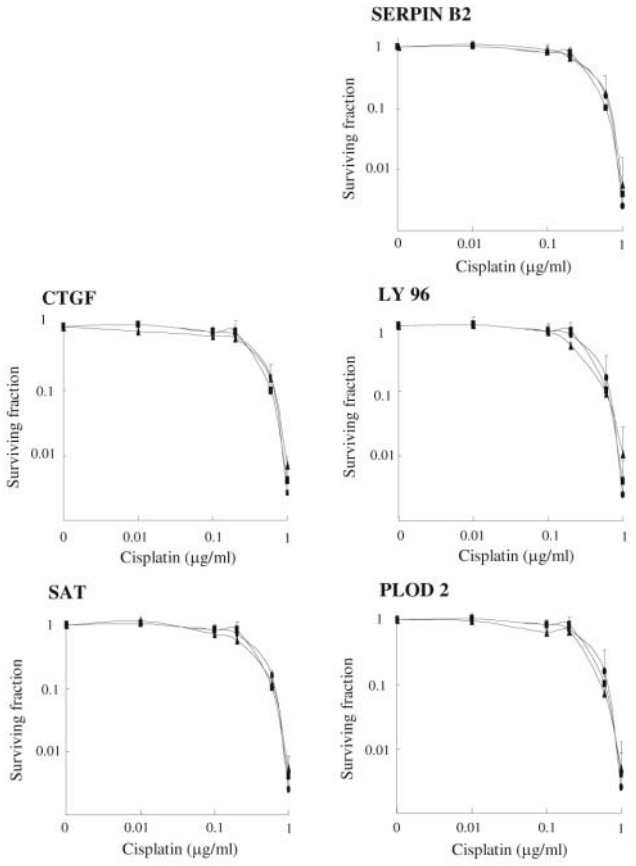

Figure 4. *Survival curves of cells with cisplatin treatment. Cells were treated with various doses of cisplatin for 5 days, with or without siRNA, and then exposed to a colonogenic assay. Suppression of each gene with the corresponding siRNA (triangles) did not modify the survival curves of cells with cisplatin alone (circles) or with cisplatin plus negative siRNA (squares). The dots and bars represent mean and SD, respectively. Independent experiments were repeated 5 times with triplicates in each.*

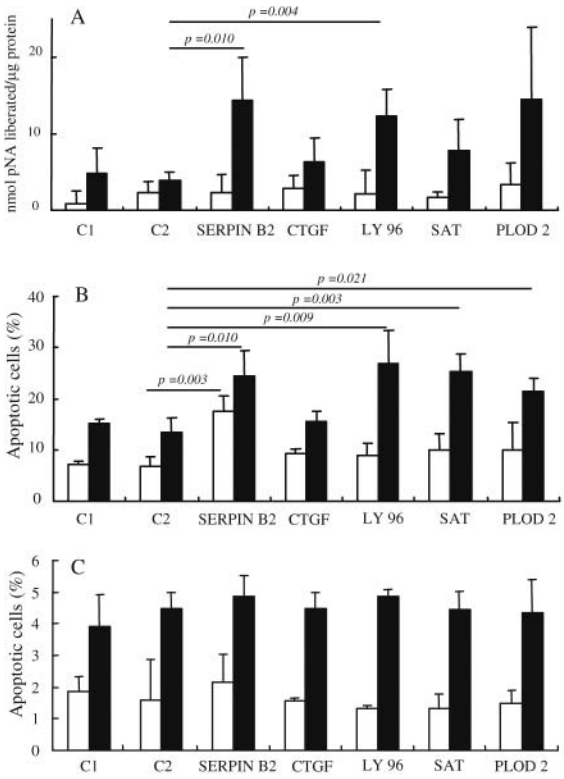

Figure 5. *Suppression of serpin B2 and Ly 96 by the corresponding siRNAs significantly enhanced cisplatin-induced apoptosis (closed columns) in comparison with control cells treated with cisplatin plus negative siRNA (C2), when assessed by caspase 3 activity. They had no effect, however, on baseline apoptosis without cisplatin (open columns), with the same assay (A). FACS analysis with annexin V staining (B) disclosed enhanced cisplatin-induced apoptosis by suppression of serpin B2, Ly 96, SAT and PLOD 2. Suppression of serpin B2 also resulted in enhanced apoptosis without cisplatin in this assay (B). In contrast, assay with Hoechst staining showed no alteration of apoptosis induction by cisplatin and baseline apoptosis by suppressing these genes (C). Cells were treated with or without 0.6 μg/ml cisplatin for 5 days. Duration of the treatment with siRNAs was also 5 days. C1 represents another control cell not treated by either cisplatin or negative siRNA. Columns and bars represent mean and SD of the percentages of apoptotic cells, respectively (n=4).*

known functions, were identified as varying their expressions with cisplatin exposure for 5 days. Many of them appeared to have functions involved in apoptosis, cell cycle regulation, nucleic acid metabolism and DNA repair. Needless to say, these genes with altered expressions by cisplatin are not necessarily related to the sensitivity or resistance of cells to cisplatin. Selected gene expression alterations by cDNA microarray inevitably include incidental alteration (20).

Several studies on cDNA microarray analysis by cisplatin have been already reported. For example, Qin *et al.*, examining gene expression profiles related to cell cycle regulation and apoptosis, reported up-regulation of cell cycle regulators, pro-apoptotic genes, growth receptors and signal transduction players in a human hepatocellular carcinoma cell line after treatment with cisplatin for 12 hours (21). Huerta *et al.* identified 15 genes and 9 expressed sequence tags significantly altered in a metastatic colon cancer cell line by cisplatin exposure for 4 hours, including some known genes related to cell cycle regulation and DNA repair (22). Schweyer *et al.*, screening 205 apoptosis-related genes in a human germ cell tumor cell line after treatment with cisplatin for 6 hours, found significantly elevated expression of 12 genes, including those involved in cell cycle regulation, apoptosis and signal transduction (23). Kerley-Hamilton *et al.* also treated a human germ cell tumor cell line with cisplatin for 6 hours and examined gene expression in harvested cells at 24 hours after completion of the treatment. The resulting 46 up-regulated and 5 down-

regulated genes included those implicated in the apoptotic cell death receptor pathway and mediators of reactive oxygen species generation, and 54% of the up-regulated genes were established or suspected downstream targets of *p53* (24). In contrast to these studies, we treated cells with cisplatin for a much longer period, namely for 5 days. In addition, we adjusted the cell cycle distribution of the untreated cells so that it was comparable to that of the treated cells, because a previous experiment demonstrated a significant cell cycle shift to the G_2/M-phase by the treatment of cisplatin with this protocol (7). Without adjusting the cell cycle shift, many gene expressions would very likely be altered by cell cycle-specific gene expression. Moreover, the present study employed cDNA microarray chips covering 30,000 genes representing almost the entire human genome, instead of chips designed for specific gene groups. This kind of exhausting screening, however, might have the disadvantage of underevaluating some important genes, in spite of the potential promise of finding some unexpected genes. Consequently, the present study again emphasized the up-regulation of genes involved in apoptosis, cell cycle regulation and DNA repair.

As the next step, the five genes with the highest ratios of increased expression were further investigated. Their gene expressions were serially quantified by RT-PCR during a 5-day exposure to cisplatin. The results disclosed sustained increases in gene expressions of all five genes, a fact that suggested their close associations with the pharmacological action of cisplatin. In addition, another DNA microarray analysis of the cells treated by cisplatin, but without adjustment of the cell cycle with colcemid, again found four genes out of the five genes, with the remaining one still elevated to a 1.7 ratio, assuring reproducibility of the results. Among the five genes, *serpin B2*, also known as plasminogen activator inhibitor-2 or PAI-2, is expressed in many normal and transformed cell types, particularly by stimulation with proinflammatory cytokines, and has a role in protecting cells from apoptosis in TNF-α-mediated inflammatory processes (25, 26). It was also shown to interact with the transcriptional regulator protein, Rb1, and to stabilize it (27). The second gene, *CTGF* or *CCN2*, belongs to the *CCN* family and codes for a secreted growth factor that can bind to integrins on the cell surface. The molecule is involved in growth, proliferation and differentiation of chondrocytes, proliferation and migration of endothelial cells, and regulation of apoptosis in breast cancer cells and aortic smooth muscle cells (28). Its role in cancer tissue seems controversial, as its overexpression was reported to correlate with some malignant propensity in some types of cancer (29), although other studies reported its involvement in metastasis inhibition and its clinical usefulness as a good prognostic factor in colorectal cancer (29) and lung adenocarcinoma (28). The third gene, *SAT*, coding for a polyamine catabolic enzyme, has been connected to anti-proliferation activity and apoptosis, and was also identified by other investigators with cDNA microarray analysis as a top 10 gene up-regulated by oxaliplatin, one of the other platinum compounds, and as a top 20 gene up-regulated by cisplatin (30). The fourth gene, *Ly 96*, a synonym of MD-2, acts as a co-receptor in toll-like receptor 4 (TLR4) signaling. It is physically associated with TLR4 on the cell surface, and the TLR4/MD-2 complex is responsive to bacterial components. Recently, this mechanism was shown to be involved in the anticancer effect of dendritic cell-mediated tumor immunity (31). Finally, an isoform of procollagen lysyl-hydroxylase, *PLOD 2*, is crucially involved in collagen fiber formation and is significantly induced by hypoxia (32). Although its direct involvement in cancer stromal formation has not been established, it seems likely that the gene is induced and contributes to the tumor-related stroma in cancer tissues that are generally hypoxic. In any case, these five genes very possibly have strong connections to tumor growth, cell death and anti-tumor immunity.

To investigate the functional roles of the five genes in the process of cisplatin cytotoxicity, corresponding siRNAs for suppressing each were synthesized. Although each siRNA seemed effective in suppressing its corresponding gene expression on cisplatin exposure, none of them modified the sensitivity of the cells to cisplatin, when assessed by colonogenic assay. To confirm this phenomenon, apoptotic induction was evaluated by the measurement of caspase 3 activity, annexin V and Hoechst staining. The results differed according to the evaluation methods. These discrepancies are to be expected, however, as these different assay systems represent different aspects of the apoptotic process (33-35). Suppression of *serpin B2* and *Ly 96*, together with *SAT* and *PLOD 2*, is likely to cause enhanced apoptosis by cisplatin. In general, the main mechanisms of cell death by genotoxic agents, including most cytotoxic agents and radiation, are apoptosis and mitotic cell death, of which the latter is the most common form (36). This fact may explain the discrepancy between the colonogenic assay and apoptosis assay in the present study.

It might be important that suppression of *serpin B2* caused enhanced apoptosis even without cisplatin treatment, when assessed by annexin V. This seems natural because *serpin B2* reportedly protects cells from apoptosis. The absence of the same effect as measured by caspase 3 and Hoechst staining may suggest that its anti-apoptosis activity might be *via* a pathway that is not related to caspase 3-involved pathways. Extensive elucidation of the very complex pathways relating to apoptosis would be needed to confirm this hypothesis. Although the present study failed to demonstrate a direct relationship of the five selected genes to cytotoxicity mechanisms by cisplatin as far as they

were evaluated by cell survival with the colonogenic assay, the possibilities of other functional deviations, including apoptosis induction, cannot be excluded. In addition, it is entirely likely that other genes listed in the present study in Table I may very possibly have important roles in the cytotoxicity caused by cisplatin.

Conclusion

cDNA microarray analysis of cells with cisplatin exposure for a relatively long duration, 5 days, yielded 38 up-regulated and 4 down-regulated gene expressions of more than 2-fold ratio alteration, after adjusting for the cell cycle distributions of the cisplatin-treated and untreated cells. These genes included those involved in apoptosis, cell cycle regulation and DNA metabolism/repair, suggesting their possible importance in the process of cytotoxicity of cisplatin. We envision that the present data will be of some use for future disclosures of molecular mechanisms of cisplatin cytotoxicity and resistance mechanism in a single-agent administration, or in combination with other drugs or radiation.

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