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CISPLATIN-INDUCED IMBALANCES IN THE PATTERN OF CHIMERIC MARKER GENE EXPRESSION IN HELA CELLS

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The effects of the antitumor drug cisplatin on gene expression have been measured during transient transfections in HeLa cells. The results indicate a surprising diversity of response. Expression from two promoters is strongly induced, both in non-replicating plasmids and cellular integrants, whereas expression from two other promoters is strongly inhibited. The results suggest that a drug-induced imbalance in gene expression may contribute to the antitumor properties of cisplatin.

Cis-diamminedichloroplatinum(II) (cisplatin) is a chemotherapeutic agent used commonly in the clinical treatment of a variety of tumor types (1,2). This drug has been proposed to work by attacking chromosomal DNA and causing arrest of replication probably by introducing intrastrand crosslinks involving adjacent guanosines (G strings) on the same DNA strand (3,4). That such lesions can arrest replication, at least temporarily, is not in doubt. However, a variety of recent studies have suggested that alterations in the balance of gene expression may contribute importantly to the cytotoxicity of the drug. These include targeting of the drug to GC-box-type sequences that directs strong expression from some promoters (5,6) and correlations of cytotoxicity with events in the cell cycle (7) and with the activation of programmed cell death or apoptosis (8).

Recently, we have investigated the ability of cisplatin to alter gene expression in cultured, non-transformed mouse and monkey cells (Evans and Gralla, manuscript submitted), and now extend this work to transformed human cells. Human HeLa cervical carcinoma cells have been shown to incur a typical pattern of lesions upon treatment with cisplatin (3,9). The current experiments use transient and stable transfection assays where non-replicating plasmids carry genes into HeLa cells. The cells are then challenged with cisplatin to measure the effects on marker gene expression. Transient expression assays have previously been used to expose altered capacities for plasmid DNA repair in *cis*-DDP-sensitive Walker rat tumor cells (10), and in *cis*-DDP-resistant murine L1210 leukemia cells (11), and in cells

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derived from xeroderma pigmentosum patients (12), and in our previous studies of gene expression in non-transformed mouse 3T3 and monkey CV-1 cells.

Previous studies of the effect of cisplatin on gene expression have indicated that bulk RNA and protein synthesis is relatively insensitive to cisplatin inhibition. Preexisting proteins are also poor targets for cisplatin modification (3). When individual enzymes are studied, most are relatively insensitive to cisplatin in vivo (13-15). Very recent studies have shown that the production of at least one growth control enzyme and several marker test genes driven by oncogene promoters can be inhibited by very low, pharmacologically relevant doses of cisplatin in mouse and monkey cells (Evans and Gralla, manuscript submitted). Thus it appears that the production of many proteins is not affected by low doses of cisplatin, but that there are exceptions that could be of pharmacological relevance. In this report we show that in HeLa cells there is considerable diversity of response to cisplatin challenge, including a strong and unexpected induction of gene expression in certain cases.

MATERIALS AND METHODS

Human HeLa cervical carcinoma cells were obtained from the American Type Culture Collection (ATCC). Cells were from (16) and E3-CAT cells from (17). For transient and stable expression experiments, cells were seeded at $7x10^5$ per 10 cm dish and incubated for 24 h before drug addition. Cisplatin was added to duplicate dishes, incubated for 24 h, and then for 24 h in the absence of drug.

Plasmid expression used pHIVLTRcat (18), SVER-CAT with pSV2cat (19), MLP-CAT with pSVOcatMLP (20), E3-CAT with pE3cat(-236) (21), and background used pSVOcat (19).

Transient transfections were as in Shen et al. (22), except that the plasmid DNA concentration was 5-10 μg/ml. Immediately after the addition of transfection cocktail, cisplatin was added to duplicate dishes. After 4 h at 37°C, the medium was aspirated and the cells were (PEG)-sucrose shocked at room temperature for 2 min (22) and processed as described.

CAT assay was as described (19). Incubations were at 37°C for 0-120 min such that no more than 50% of the [^{14}C]CAM was converted to its acetylated derivatives. Background was determined by assay of CAT activity in extracts of cells either mock-transfected without plasmid DNA or transfected with promoterless pSVOcat plasmid DNA.

RESULTS

The response to drug challenge in transient expression assays

Chimeric fusion genes were used to test the effects of cisplatin on gene expression. In each gene chimera, a mammalian virus promoter DNA was hooked directly to the chloramphenicol acetyltransferase (CAT) marker gene. The promoters were varied since they affected sensitivity in CV-1 cells (Evans

and Gralla, unpublished). Included were the human immunodeficiency virus (HIV) LTR promoter-enhancer combination, the Simian virus 40 (SV40) early region (SVER) promoter-enhancer combination, the adenovirus 2 major late promoter (MLP), and the adenovirus 5 E3 promoter. They are all known to be active in HeLa cells (18,20,21). Table 1 shows the relative strength of the four promoters as measured using the same protocol that will be used for assaying response to cisplatin challenge.

Non-replicating plasmids containing these genes were introduced into HeLa cells by transient transfection. Varying amounts of cisplatin were then added to the media for 24 h. The media was then replaced with drug- free media and the cells were incubated for a further 24-h. The cells were then disrupted and CAT enzyme activity was quantitatively determined.

Representative CAT assay results for the HIV and SVER plasmids are shown in Figure 1. SVER-directed CAT activity (Fig. 1B) was steadily inhibited with increasing drug concentration over the range of 0-20 μM, much as we have also seen in similar experiments carried out in CV-1 monkey cells (Evans and Gralla, manuscript submitted). In strong contrast, HIV-directed CAT activity (Fig. 1A) was induced by relatively low doses of cisplatin. This induction of HIV-CAT by cisplatin was not seen in the CV-1 cell experiments. We conclude that the promoter used can determine whether a gene is inhibited or induced by cisplatin challenge. The response depends critically on the type of cells.

The experiments were repeated using either the MLP or E3 promoters. The data for the set of four promoters were analyzed quantitatively and are displayed as dose-response curves in Figure 2. These are expressed as relative CAT enzyme activity from equal plate fractions as a function of the concentration of cisplatin added to the media. The analysis shows that the HIV- and E3-constructs are similar in that CAT activity (Fig. 2A) was moderately induced by low doses of cisplatin. HIV was induced 3-fold relative to control at about 3 μM added drug, and E3 was induced nearly 2-fold at the same drug concentration. In contrast, the stronger SVER- and MLP-directed CAT activity (Fig. 2B) was strongly inhibited by low doses of cisplatin. Under these conditions in HeLa cells, at 3 μM added cisplatin, the effect of the drug was to cause up to a 6-fold relative difference in the transient expression of the different gene constructs studied here.

TABLE 1. PROMOTER ACTIVITY

Promoter	Relative Strength
SV40 Early	20
Adeno MLP	5
HIV LTR	2
Adeno E3	1

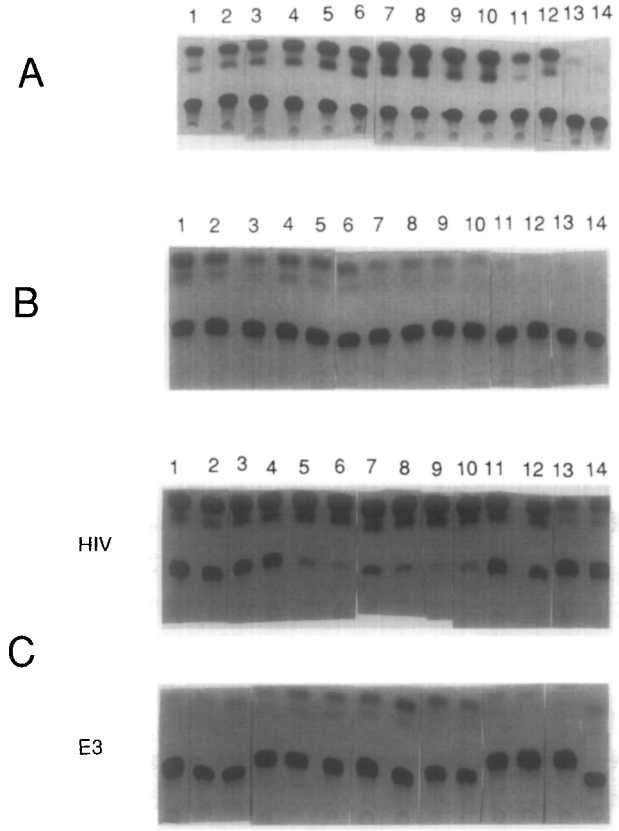

Figure 1. Effects of cisplatin on the expression of chimeric CAT genes in HeLa cells. (A and B) Cells were transiently transfected with HIV-CAT (A) and SVER-CAT (B) plasmid DNAs and were treated with 0 (lanes 1-2), 0.7 (lanes 3-4), 1.0 (lanes 5-6), 2.0 (lanes 7-8), 3.0 (lanes 9-10), 5.0 (lanes 11-12), and 10.0 μM (lanes 13-14) cisplatin. (C) Exponentially growing HIV-CAT HeLa and E3-CAT HeLa cells treated with 0 (lanes 1-2), 1.0 (lanes 3-4), 5.0 (lanes 5-6), 7.5 (lanes 7-8), 10 (lanes 9-10), 15 (lanes 11-12), and 20 μM (lanes 13-14) cisplatin.

The response to drug challenge in stable transfectants

Two of these promoter-CAT fusion constructs had previously been used to obtain stably-transfected HeLa cell lines where the genes were permanently integrated into the chromosomal DNA (16,17). To compare with transient HIV-CAT and E3-CAT expression in HeLa cells, the response to cisplatin was measured in the context of stable expression. In these cases the protocol was the same as for the above transient studies. One difference is that the stable transfectants have a pre-existing pool of CAT enzyme and the changes caused by cisplatin must be measured against this background of endogenous enzyme activity. In the transient experiments, all of the CAT activity is made after the cells are exposed to drug.

The results (Fig. 3) show that expression of HIV-CAT, and to a lesser extent E3-CAT, is induced by cisplatin. The HIV-CAT activity levels are

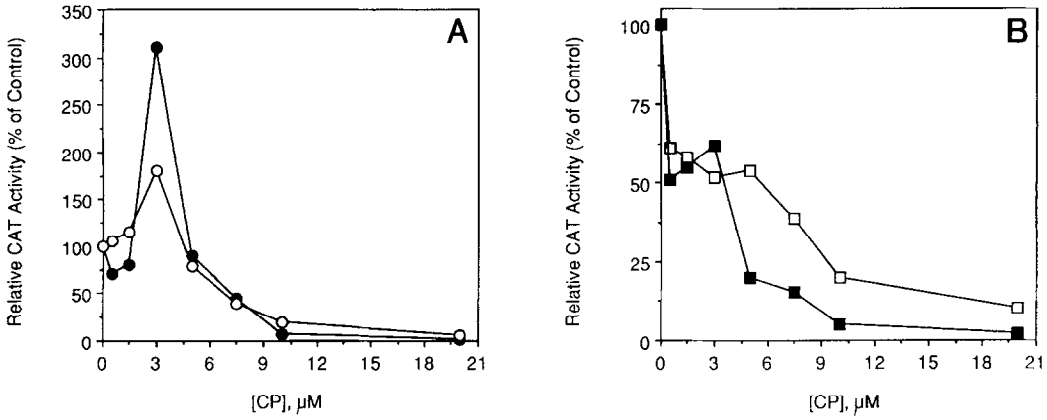

Figure 2. Quantitation of the effects of cisplatin on transient expression of chimeric CAT genes. Quantitative analysis of the CAT activity data represented in Figure 1A-B. (A) HeLa cells transfected with HIV-CAT (●) and E3-CAT (○). (B) Cells transfected with SVER-CAT (■) and MLP-CAT (□). The data shown are averages for two to four independent experiments for each construct.

maximally induced by 7-fold with 10 μM added drug, while E3-CAT levels are maximally induced by about 4-fold at 5 μM added drug. Thus the induction observed in transient assays is also seen in stable assays. The level of induction is somewhat higher in the situation where the constructs are stably integrated into the chromosome. The induction phase from integrated constructs also continues at higher doses where transient expression is inhibited (compare Figs. 2A and 3).

In contrast to the strong induction of HIV- and E3-CAT activity, which likely reflects the induction of the corresponding proteins, the bulk protein

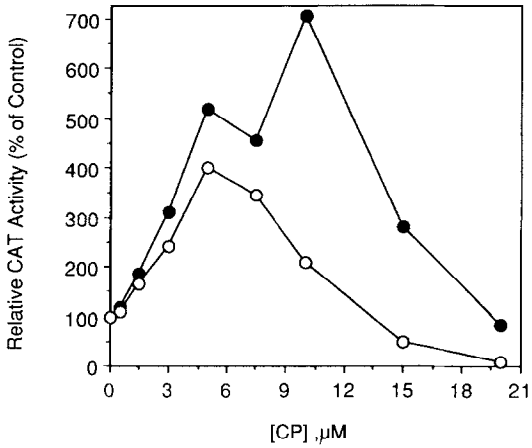

Figure 3. Quantitation of the effects of cisplatin on stable expression of chimeric CAT genes in HeLa cells. Quantitative analysis of the CAT activity data for cisplatin-treated HIV-CAT HeLa (●) and E3-CAT HeLa (○) cells represented in Figure 1C. The CAT data shown are averages for two independent experiments for each cell line.

level in cell extracts is monotonically inhibited in these cells, with an ID_{50} dose of approximately 5 μM added drug (data not shown). Thus in the context of stable cellular gene expression, the effects of cisplatin on the expression of single genes can be quite different from its effect on the average cellular protein as measured by bulk methods.

DISCUSSION

These results have shown that in HeLa cells the transient expression of chimeric marker genes is differentially altered by the anticancer drug cisplatin. Transient expression driven by the HIV or the human adenovirus E3 regulatory elements was induced by low doses of cisplatin. In contrast, expression driven by the monkey virus SVER or adenovirus MLP elements was inhibited in the same drug concentration range. Relatively strong induction of HIV- and E3-directed CAT expression was also seen when the test genes were stably integrated into HeLa cell chromosomes. If these results are representative of at least a sub-class of cellular genes, then the consequences of cisplatin treatment will be to create an imbalance in the expression of such genes. Despite this potential imbalance, there would be little effect on the level of total proteins made, as has been observed previously in many cell lines.

These results differ somewhat from our previous studies in CV-1 monkey cells, where all genes studied were inhibited, although to quite differing extents. Using these same plasmids and protocols in HeLa cells, HIV-CAT expression is induced 3- to 7-fold at approximately 5 μM drug, which compares to a similar extent of inhibition in CV-1 cells. Induction of HIV expression has previously been reported in these same HIV-CAT HeLa cells using DNA damaging agents such as mitomycin C and UV light (16,23). Viewed in this context, the induction by cisplatin is less surprising since it too is a DNA-damaging agent. Thus the induction may be a component of a more general induction of various genes upon treatment of mammalian cells with cytostatic agents (24-26), perhaps analogous to SOS stress induction in microorganisms. Thus the extent to which cisplatin causes an imbalance in gene expression will likely depend on the types of regulatory pathways operative in the particular cell type. It is interesting that this imbalance is greater in a human tumour cell line than in a monkey kidney cell line.

An imbalance in gene expression caused by cisplatin could have profound consequences for the maintenance of the tumorigenic state. If this balance favors the induction of tumor supressors (27) over tumor promoting genes (28), then cisplatin might interfere with the rapid growth of the tumor cell. Recent results have led to the suggestion that cisplatin acts via induction

of a program of active cell death or apoptosis (8). Independent studies have shown that induction of gene expression can be required for developmentally programmed cell death (29). Thus the imbalance caused by cisplatin could induce such genes (as it does HIV, above) as well as repress genes (as it does adeno MLP, above) that are suppressors of apoptosis (see 27). Recent progress has been made in the identification of genes which induce, or are associated with, active cell death (29-31). It will be interesting to see if the expression of these genes or other genes involved in tumorigenesis is altered by cisplatin.

The molecular basis for the selective alteration of gene expression in HeLa cells by cisplatin has not been identified here. It is interesting that the weaker promoters are stimulated whereas the stronger promoters are inhibited. That is, the MLP- and SVER-CAT promoters were strongly inhibited by cisplatin and are 3- to 10-fold stonger than the weaker HIV and E3 promoters. We have recently found that in viable CV-1 cells treated with cisplatin there is excellent quantitative correlation between inhibition by cisplatin and promoter strength. This supports the suggestion that strong promoters are associated with accessible chromatin, which in turn allows greater attack by cisplatin on the chromosomal DNA. Other characteristics that might alter the sensitivity to cisplatin are the gene length (by changing the target size) and the half-life of the RNA and the protein (Evans and Gralla, manuscript submitted). The potential effects of GC-box-type regulatory DNA (5,6) are not realized in these experiments due to the dominance of the apparent effect of promoter strength dictating chromatin accessibility.

Overall, these considerations support the recent proposals that alterations in gene expression may contribute importantly to the pharmacology of cisplatin (5-8). Perhaps the most important point is that both inductions and inhibitions may be seen in one type of cell (a human tumor cell) whereas a different pattern of differential inhibition occurs in another cell (monkey kidney cell). It may be critical to learn whether distinct alterations in gene expression occur in cisplatin-sensitive tumors.

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