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Human REV1 Modulates the Cytotoxicity and Mutagenicity of Cisplatin in Human Ovarian Carcinoma Cells

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ABSTRACT

REV1 interacts with Y-type DNA polymerases (Pol) and Pol ζ to bypass many types of adducts that block the replicative DNA polymerases. This pathway accounts for many of the mutations induced by cisplatin (*cis*-diamminedichloroplatinium II, DDP). This study sought to determine how increasing human REV1 (hREV1) affects the cytotoxicity and mutagenicity of DDP. Human ovarian carcinoma 2008 cells were transfected with an hREV1 expression vector and 4 sublines developed in which the hREV1 mRNA level was increased by 6.3- to 23.4-fold and hREV1 protein by 2.7- to 6.2-fold. The sublines were 1.3- to 1.7-fold resistant to the cytotoxic effect of DDP and 2.3- to 5.1-fold hypersensitive to the mutagenic effect of DDP. The hREV1-transfected sublines were 1.5- to 1.8-fold better than the parental 2008 cells at managing DDP adducts as assessed by their ability to express *Renilla reniformis* luciferase from a vector that had been extensively loaded with DDP adducts before transfection. Increased hREV1 expression was associated with a 1.5-fold increase in the rate at which the whole population acquired resistance to DDP during sequential cycles of drug exposure. Increasing the abundance of hREV1 thus resulted in both resistance to DDP and a significant elevation in DDP-induced mutagenicity. This was accompanied by an enhanced capacity to synthesize a functional protein from a DDP-damaged gene and, most importantly, by more rapid development of resistance during sequential cycles of DDP exposure that mimic clinical schedules of DDP administration. We conclude that hREV1-dependent processes are important determinants of DDP-induced genomic instability and the development of resistance.

The damage-induced mutagenesis pathway is an important mechanism which generates mutations when a genome that is burdened with lesions is replicated. Most mutations induced by UV irradiation are generated when DNA containing residual unrepaired damage is replicated during S phase of the cell cycle. Such lesions perturb the structure of DNA (Rice et al., 1988; Park et al., 2002) and are likely to block replicative DNA polymerases that have stringent base-pairing requirements. In *Saccharomyces cerevisiae,* mutagenic bypass of DNA damage is equivalent to error-prone translesion replication (Broomfield et al., 2001). Virtually all mutations induced by UV irradiation are dependent on the activity of DNA polymerase ζ (Pol ζ), acting in concert with a protein encoded by the *Rev1* gene (Lawrence and Maher, 2001; Lawrence, 2002). REV1 is required for UV mutagenesis in vivo and interacts with Pol ζ in vitro to stimulate translesion replication activity (Lawrence, 2002). It has been proposed that in human cells, hREV1 protein functions in this pathway through its interaction with Pol ζ and possibly with other members of the Y family of DNA polymerases (Murakumo et al., 2001; Gibbs et al., 2005; Ross et al., 2005).

The damage-induced mutagenesis pathway is conserved evolutionarily. The genes of this pathway identified in yeast have mammalian homologs that include HHR6A, HHR6B, hRAD18, hREV1, hREV3, and hREV7. hREV1 is highly distributive and catalyzes the insertion of a deoxycytidine when it encounters a guanine, a guanine bearing a large chemical adduct, or an abasic site, but it is unable to bypass UV photoproducts (Zhang et al., 2002). In addition to hREV1, higher eukaryotic cells have at least three other DNA polymerases in the Y family, including Pol η, Pol ι, and Pol κ. These enzymes are characterized by their low fidelity when copying undamaged templates and their ability to bypass lesions that block DNA polymerases belonging to other families (Friedberg et al., 2002). Mouse REV1 has been shown to bind to Pol κ, Pol η, and Pol ι, and all the interactions occur at the same site on REV1 protein (Guo et al., 2003). This suggests that in mammalian cells, hREV1 may have a role in supporting translesional synthesis carried out by multiple

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ABBREVIATIONS: Pol, polymerase; DDP, cisplatin (*cis*-diamminedichloroplatinium II); PCR, polymerase chain reaction.

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DNA polymerases. However, the relative contribution of most of these enzymes to DNA damage-induced mutagenesis in genomically unstable tumor cells in vivo, if any, is unknown.

The clinical effectiveness of DDP is limited by the rapid development of resistance. The most abundant lesions that DDP produces are intrastrand cross-links, and these are believed to account for the majority of both the cytotoxic and mutagenic effects of the drug. Its mutagenic potential has been well documented in both bacterial (Yarema et al., 1994, 1995) and mammalian cells (Turnbull et al., 1979; Johnson et al., 1980; Cariello et al., 1992; Lin and Howell, 1999). We have reported that many of the mutations induced by DDP adducts seem to result from error-prone translesional DNA synthesis mediated by DNA polymerase ζ and/or hREV1 (Wu et al., 2004; Okuda et al., 2005). Several investigators have reported previously that a loss of hREV1 function markedly reduces UV-induced hypoxanthine guanine phosphoribosyl transferase mutations in human cells engineered to contain reduced levels of hREV1 mRNA through the expression of an *hREV1* antisense RNA (Gibbs et al., 2000) or a ribozyme that cleaves endogenous hREV1 mRNA (Clark et al., 2003). It has also been demonstrated that inactivation of the *REV1* gene in chicken DT40 cells renders them hypersensitive to a wide variety of mutagens, including DDP (Simpson and Sale, 2003). We demonstrated recently that suppression of *hREV1* expression by a short hairpin RNA targeted to hREV1 mRNA in human ovarian carcinoma cells, whereas it moderately increases DDP sensitivity and markedly reduces its mutagenicity and the rate at which human ovarian carcinoma cells acquire resistance to DDP during repeated cycles of exposure (Okuda et al., 2005). This indicates an important role for hREV1 in DDP-induced mutagenesis and identifies hREV1 as being of particular interest with respect to the mechanism underlying the emergence of the multidrug-resistant phenotype that so frequently accompanies the development of DDP resistance.

To further explore the role of hREV1 in the genesis of resistance to DDP, we have molecularly engineered human ovarian carcinoma 2008 cells to express increased amounts of hREV1. We report here that such forced expression of hREV1 results in increased resistance to DDP and a significant elevation in DDP-induced mutagenicity and that these phenotypes are associated with an enhanced hREV1-mediated capacity to express a gene containing DDP adducts.

Materials and Methods

Drugs. DDP was a gift from Bristol-Myers Squibb (Princeton, NJ). A stock solution of 1 mM cisplatin in 0.9% NaCl was stored in the dark at room temperature. 6TG was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in 0.2 N sodium hydroxide to form a 20 mM stock solution and stored at −20°C.

Vector Construction. The 3.8-kilobase, full-length human REV1 cDNA was removed from the yeast expression vector pEGLh6-hREV1 (Lin et al., 1999) with SalI and was inserted into the SalI site of pBluescript II SK(+) (Fermentas Inc., Hanover, MD). The insert was then removed from pBluescript II SK(+) by digesting with NotI and ApaI and cloned into pcDNA3.1(−) (Invitrogen, Carlsbad, CA) by sticky-end ligation. The resulting vector containing full-length hREV1 cDNA and expressing a geneticin resistance marker was sequence-verified and designated pcDNA3.1-hREV1.

Cell Lines, Transfection, and Selection. The human ovarian carcinoma cell line 2008 was grown in RPMI 1640 supplemented with 5% fetal bovine serum. Cells were transfected with pcDNA3.1-hREV1 using Fugene 6 (Roche, Indianapolis, IN) according to the manufacturer's recommendations. Transfected cells were then selected by continuous exposure to 400 μg/ml geneticin. Geneticin-resistant colonies were isolated, expanded, and screened for hREV1 mRNA expression level by real-time PCR and for hREV1 protein level by Western blotting. Four clones, designated 2008-hREV1-C1, -C7, -C8, and -C9, in which the steady-state hREV1 protein level was at least 2-fold increased, were chosen for all subsequent experiments.

Quantification of hREV1 mRNA by Reverse-Transcriptase PCR. Total RNA was extracted with TRIzol reagent (Invitrogen). First-strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) and random primers. Real-time PCR was performed using the Bio-Rad iCycler iQ detection system in the presence of SYBR-Green I dye (Bio-Rad, Valencia, CA). For the hREV1 gene expression, the forward (5′-AAGGCTGATGCAATCG-3′) and reverse (5′-CCACCTGGACATTGTCAAGAATAA-3′) primers were used for amplification with an iCycler protocol consisting of a denaturation program (95°C for 3 min) and amplification and quantification program repeated 40 times (95°C for 10 s and 55°C for 45 s) and melting curve analysis. A melting-curve analysis immediately followed amplification and was executed using 95°C for 1 min then 55°C for 1 min, followed by 80 repeats of heating for 10 s starting at 55°C with 0.5°C increments. The data were analyzed by using the comparative Ct method, where Ct is the cycle number at which fluorescence first exceeds the threshold. The ΔCt values from each cell line were obtained by subtracting the values for 18S Ct from the sample Ct. A 1-unit difference in Ct value represents a 2-fold difference in the level of mRNA.

Western Blot Analysis. The nuclear proteins were extracted as described previously (Schreiber et al., 1989), heated, and a sample containing 10 μg was then subjected to electrophoresis on a 4 to 15% SDS-polyacrylamide gel electrophoresis gel. The proteins were then transferred to a nitrocellulose membrane that was blocked with 5% skimmed milk in buffer (0.35 M NaCl and 10 mM Tris-HCl, pH 8.0) containing 0.05% Tween 20 for 1 h at room temperature and then incubated overnight at 4°C with a 1:100 dilution of polyclonal antibody against hREV1 and a 1:500 dilution of a goat polyclonal antibody against histone H1 (Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was then washed and exposed for 1 h to a 1:500 dilution of a horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology), and bands were detected using the ECL Western blotting detection system (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and analyzed densitometrically by a ChemiImager (Alpha Innotech Corporation, San Leandro, CA).

Clonogenic Assay. Clonogenic assays were performed by seeding 250 cells into 60-mm plastic dishes in 5 ml of complete media. After 24 h, appropriate amounts of DDP were added to the dishes, and the cells were exposed for 1 h. Thereafter, the cells were washed, and fresh drug-free medium was added. Colonies of at least 50 cells were visually scored after 10 to 14 days. Each experiment was performed a minimum of 3 times using triplicate cultures for the drug concentration. IC_{50} values were determined by log-linear interpolation.

Measurement of DDP Mutagenicity. The 2008 and its hREV1 overexpressing sublines were grown in medium containing 0.4 μM aminopterin, 16 μM thymidine, and 100 μM hypoxanthine for a minimum of 14 days to remove pre-existing hypoxanthine guanine phosphoribosyl transferase mutants and were then exposed for 1 h to 10 μM DDP. Thereafter, the cells were washed twice and recultured in regular medium for 8 days during which the cultures were split 2:1 as needed to keep them from becoming confluent. All of the cells were then trypsinized and seeded into each of 10 100-mm tissue culture dishes at 100,000 cells/dish in the presence of 10 μM 6-thioguanine. At the same time, aliquots of 250 cells were seeded into each of three 60-mm dishes in drug-free medium for the determination of cloning efficiency. After 14 days, colonies were counted after staining with 0.1% crystal violet. The frequency of highly drug-resistant variants

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was calculated as follows: variant frequency = $a/(b \times 10^6)$, where a is the number of colonies present in the 10 drug-treated dishes and b is the cloning efficiency. Each experiment was performed a minimum of three times, and the data are presented as mean ± S.D.

Relative Rate of Resistance Development to DDP. The rate at which a cell population became resistant to DDP during repeated cycles of exposure to DDP was determined by measuring the IC_{50} value for DDP using a clonogenic assay after each round of selection. The DDP concentration used for selection was the IC_{90} value for the population under study. For each round of selection, 10^6 cells were exposed to DDP for 1 h. When the cells had recovered to 90% confluence, an aliquot was used to determine the cell number and the slope of the DDP concentration-survival curve in a clonogenic assay, and another aliquot was again exposed to DDP. Total cell number and plating efficiency were determined at each step; this information, along with the exact number of cells subcultured, was used to calculate population doubling (PD) according to the following equation: PD = (ln[total number of cells] − ln[number of cells plated × plating efficiency])/ln2. The rate of acquisition of resistance to DDP was then calculated by plotting the slope of the DDP concentration-survival curve as a function of population doubling. The slope of the latter plot yields the rate of relative resistance development. This experiment was performed on three independent populations of each cell type.

Plasmid Reactivation Assay. The pRL-CMV mammalian expression vector (Promega, Madison, WI) containing the 935-base pair *Renilla reniformis* luciferase cDNA produces high-level *R. reniformis* luciferase expression in transfected mammalian cells. Thirty micrograms of plasmid DNA was dissolved in buffer containing 10 mM Tris and 1 mM EDTA, pH 7.4, and incubated with 5 μM DDP at 37°C for 3 h. The platinated DNA was then purified by ethanol precipitation, and unbound free drug was removed. This procedure resulted in plasmid DNA that was >90% supercoiled, as verified by gel electrophoresis. The platination procedure yielded 1.5 ± 1.4 pg/μg DNA (S.D.), which is equivalent to 9.3 adducts per plasmid or 3.2 adducts per Luc coding region and promoter as we reported previously (Cenni et al., 1999). Similar levels of platination have been shown previously not to affect the efficiency of transfection (Eastman et al., 1988). One microgram of randomly platinated or unplatinated pRL-CMV vector was transfected for 6 h into the cells, as described above. Thereafter, DNA was washed off and fresh medium was added. Twenty-four hours after transfection, duplicate samples were washed with ice-cold phosphate-buffered saline and then lysed in a *R. reniformis* luciferase assay lysis buffer (Promega). Cell lysates were stored at −70°C until tested for luciferase activity using Promega's *R. reniformis* luciferase assay system. In brief, using the automatic injector of the Monolight 2010 Luminometer (Analytical Luminescence Laboratory, San Diego, CA), 20 μl of cell extract was mixed sequentially with 100 μl of *R. reniformis* luciferase assay buffer containing luciferase substrate; the light output was measured for 10 s after an initial 2-s preintegration delay.

Statistics. The data were analyzed by the use of a two-sided paired Student's t test with the assumption of unequal variance. Correlation coefficients were calculated using the correlation function of Excel (Microsoft Corp., Redmond, WA).

Results

Characterization of Ovarian Carcinoma Cells Engineered to Overexpress hREV1. To generate a series of clones expressing high levels of hREV1, the ovarian carcinoma 2008 cell line was transfected with the pcDNA3.1-hREV1 vector, which expresses the full-length hREV1 coding sequence and a Geneticin-resistance marker. Forty-eight clones resistant to geneticin were isolated, expanded, and tested for hREV1 mRNA level by reverse transcriptase PCR. As shown in Fig. 1A four clones, 2008-hREV1-C1, -C7, -C8, and -C9, were found to express substantially higher levels of hREV1 mRNA (17.1-, 10.5-, 6.3-, and 23.4-fold, respectively) than the parental untransfected 2008 cells. To determine whether the increase in mRNA was accompanied by an increase in hREV1 protein level, the clones were subjected to Western blot analysis using an antibody that detects hREV1. Figure 1B shows that the level of the 138-kDa form of the REV1 protein was 4.1-, 3.3-, 2.7-, and 6.2-fold higher in the 2008-hREV1-C1, -C7, -C8, and -C9 cells than that in the parental 2008 cells after normalization for the level of expression of histone H1. Thus, the higher level of hREV1 mRNA was proportionally accompanied by an increase in the level of hREV1 protein.

Effect of hREV1 Expression on Sensitivity to the Cytotoxic Effect of DDP. Clonogenic assays were used to determine whether the overexpression of REV1 could modulate cellular sensitivity to DDP. The DDP concentration-survival curves for the parental untransfected 2008 cells and the four clones that overexpressed hREV1 are shown in Fig. 2. The 2008 cells were the most sensitive with an IC_{50} value of 8.5 ± 0.4 μM (S.E.M.). All four REV1 overexpressing sublines were less sensitive to the cytotoxic effect of DDP with the IC_{50} values of 13.2 ± 0.5, 12.2 ± 0.1, 10.8 ± 0.1, and 14.0 ± 0.2 μM (S.E.M.), respectively, for the 2008-hREV1-C1, 2008-hREV1-C7, 2008-hREV1-C8, and 2008-hREV1-C9 cells. Thus, overexpression of hREV1 rendered the cells 1.3- to 1.7-fold resistant to DDP ($p < 0.05$ for each clone compared with the parental 2008 cells). Prior studies have documented no effect of either transfection with the vector containing no insert or geneticin selection on sensitivity to DDP (Holzer et al., 2004). It is noteworthy that there was an excellent correlation between the relative increase in hREV1 protein level and the degree of resistance ($n = 5$, $r = 0.90$, $p < 0.05$). This observation is consistent with the concept that hREV1 is

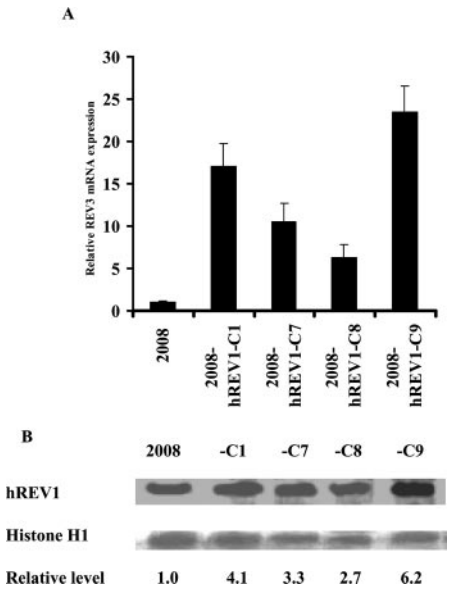

Fig. 1. Generation of cell clones expressing different levels of REV1. A, relative hREV1 mRNA levels in the parental 2008 cells and the 2008-hREV1-C1, -C7, -C8, and -C9 sublines. The expression of hREV1 mRNA was assessed by real-time PCR. The relative expression level of *hREV1* was determined by normalizing the ΔCt value to the 2008 cell value. The level of hREV1 mRNA in the 2008 cells was arbitrarily set to 1. Vertical bars, S.D. B, Western blot analysis of hREV1 expression. The level in the 2008-hREV1-C1, -C7, -C8, and -C9 cells is expressed relative to that in the 2008 cells after normalization to the level of histone H1.

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required for DNA damage tolerance probably through its ability to enhance hREV1-mediated bypass replication.

Effect of hREV1 Overexpression on the Ability of DDP to Generate Resistant Variants. DDP is a mutagen in human cells; it generates mutations that result in high-level resistance to both DDP itself and many other classes of drugs (Lin and Howell, 1999; Lin et al., 1999, 2001). To determine the effect of increasing hREV1 expression on sensitivity to DDP-induced mutagenicity, cells were exposed to 10 μM DDP for 1 h, and then 21 days later, the fraction of clonogenic cells in the surviving population that demonstrated high-level resistance to 6TG was determined. As shown in Fig. 3, overexpression of hREV1 increased the frequency of 6TG-resistant variants in all four independent sublines by a factor of 2.3- to 5.1-fold compared with control parental 2008 cells. There was a good correlation between the frequency of 6TG-resistant variants and the magnitude of the increase in hREV1 protein level ($n = 5$, $r = 0.94$, $p < 0.05$). Thus, DDP was able to generate variants in the surviving population that were highly resistant to 6TG, and increasing hREV1 levels augmented this mutagenic effect. This indicates that hREV1 plays a role in generating mutations that lead to drug resistance when DDP adducts are present in DNA.

Effect of hREV1 Overexpression on Plasmid Reactivation. The relative activity of hREV1 cannot readily be measured directly in intact cells; however, the net effect of REV1 on the capacity of the cell to successfully use a gene in which a DDP adduct has formed can be quantified indirectly by determining the ability of the cell to successfully express the *R. reniformis* luciferase from a vector that has been extensively loaded with DDP adducts by treatment with DDP before transfection. Such activity reflects the overall ability of the cell to deal with DDP adducts through translesion synthesis, transcription-coupled repair, or other repair mechanisms. Figure 4 shows that the parental 2008 cells and all four hREV1 overexpressing sublines exhibited similar transfection efficiencies and levels of luciferase activity after transfection with the nonplatinated vector. When the platinated vector was transfected into the parental 2008 cells, the luciferase activity was significantly reduced by a factor of 2.9 ± 0.2-fold (S.E.M.). In contrast, when the same platinated vector was transfected into the hREV1 overexpressing sublines, there was much less impairment (1.5- to 1.8-fold) of the generation of luciferase activity, indicating the augmented capacity of the cell to manage the impediments introduced by the presence of DDP adducts. A weak correlation between the relative increase in reactivation ability and -fold-increase of hREV1 protein levels was identified ($n = 5$, $r = 0.81$, $p = 0.09$).

Effect of Increased hREV1 Expression on the Rate of Development of DDP Resistance. Sequential cycles of

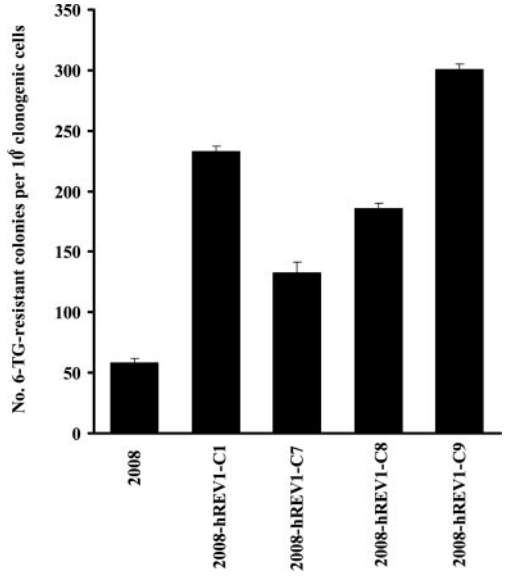

Fig. 3. Effect of REV1 expression on the ability of DDP to generate drug-resistant variants in the surviving population. The number of 6TG-resistant colonies per 10^6 clonogenic cells was scored on day 21 after a 1-h exposure to 10 μM DDP. Each data point represents the mean of three experiments. Vertical bars, S.E.M.

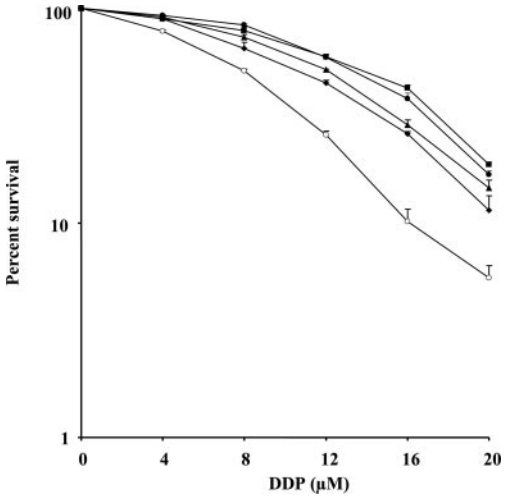

Fig. 2. Comparison of cellular sensitivity to the cytotoxic effect of DDP by clonogenic assay (○, 2008 cells; ●, 2008-hREV1-C1 cells; ▲, 2008-hREV1-C7 cells; ♦, 2008-hREV1-C8 cells; ■, 2008-hREV1-C9 cells). Results are the mean ± S.E.M. of three experiments, each performed with triplicate cultures. Cloning efficiency (mean ± S.E.M.) was 44 ± 1.4, 52 ± 1.4, 51 ± 1.5, 49 ± 2.0, and 46 ± 2.0%, respectively, for the 2008, 2008-hREV1-C1, 2008-hREV1-C7, 2008-hREV1-C8, and 2008-hREV1-C9 cells.

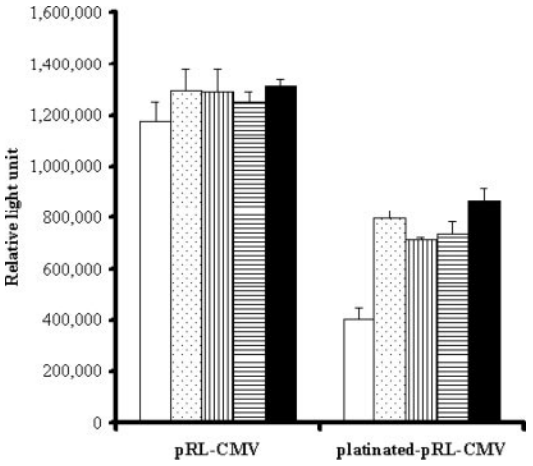

Fig. 4. Efficiency of the generation of luciferase activity from an unplatinated or platinated plasmid. □, 2008 cells; ▨, 2008-hREV1-C1 cells; ▥, 2008-hREV1-C7 cells; ▤, 2008-hREV1-C8 cells; ■, 2008-hREV1-C9 cells. Cells were transfected with nonplatinated or platinated pRL-CMV vector. Luciferase activity is expressed as relative light units. Data points represent the mean ± S.E.M. of three independent experiments, each performed with duplicate transfections.

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DDP exposure may result in the development of resistance because of either enrichment for resistant clones already present in the population or DDP-induced generation of new resistant clones, or both effects. As shown above, increasing the expression of hREV1 increased the ability of DDP to generate drug-resistant variants in the surviving population. If this effect of DDP drives the development of acquired DDP resistance in the whole population, then elevated hREV1 expression would be expected to increase the rate at which resistance emerges. The rate of development of resistance was measured in the whole population of the parental 2008 cells and the sublines that expressed the highest level of hREV1 protein among the four hREV1 overexpressing clones, 2008-hREV1-C9. Starting with a total 500,000 cells, the population was exposed to an IC_{90} concentration of DDP for 1 h, and the exposure was repeated again as soon as log-phase growth resumed at between 7 and 10 days. After each round of drug treatment, the sensitivity of the whole population to DDP was measured by determining survival over 2 logs of cell kill as a function of DDP concentration in a clonogenic assay. The degree of resistance after repeated cycles of DDP exposure was expressed as the ratio of the IC_{50} values for the treated cells relative to the untreated cells for each line separately. Figure 5 shows that the 2008-hREV1-C9 cells acquired resistance to DDP at a faster rate than the 2008 cells. On the basis of the ratio of the slopes of the plot of resistance as a function of population doubling, increased expression of hREV1 increased the rate of development of resistance to DDP by 1.5 ± 0.1-fold (S.E.M.) ($p < 0.05$) relative to that observed in the 2008 cells. This result demonstrates that hREV1 plays a role in controlling the rate of development of DDP resistance at the population level. This result is consistent with the concept that mutagenic translesional synthesis across DDP adducts is responsible for generating drug-resistant variants that become enriched in the population by subsequent rounds of DDP exposure.

Discussion

Translesional synthesis is fundamentally important to the survival of a cell whose genome is burdened with various types of adducts. Recruitment of specialized DNA polymerases capable of translesional synthesis to stalled replication forks allows the completion of genome replication. The result is enhanced cell survival but, in the case of DDP adducts, this occurs at the cost of an increased number of mutations as a result of the error-prone nature of the bypass mechanism. The results of the studies reported here indicate that hREV1 plays a role in producing these genetic changes and that increasing its abundance reduces cellular sensitivity to the cytotoxic effect of DDP, increases the mutagenicity of DDP, enhances the ability of the cell to synthesize proteins from a reporter gene burdened with DDP adducts, and increases the rate at which the population acquires resistance during repeated cycles of drug exposure.

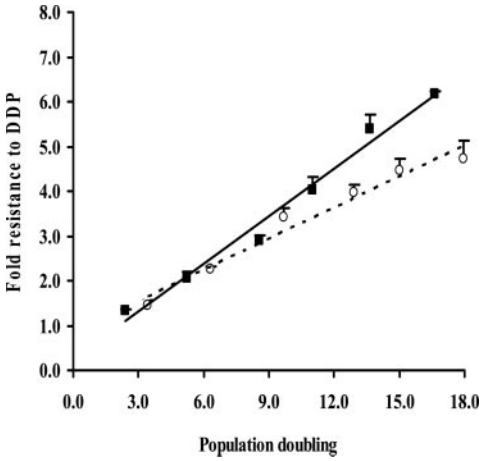

Fig. 5. Effect of hREV1 expression on the rate of development of DDP resistance. Y, 2008 cells; ■, 2008-hREV1-C9 cells. Each data point represents the mean of three independent experiments. Vertical bars, S.E.M.

We have shown recently that suppression of hREV1 expression results in enhanced DDP cytotoxicity and reduced mutagenicity in ovarian carcinoma 2008 cells (Okuda et al., 2005). The reduction in DDP-induced mutagenicity was accompanied by a 2.8-fold reduction in the rate at which the entire cell population acquired DDP resistance during repeated cycles of drug exposure. If one reduces the level of a critical member of a protein complex, then the activity of the whole complex is likely to be impaired. However, if one increases the level of such a protein, it is not apparent that the activity of whole complex will be enhanced. In the current study, the availability of a set of cell clones that differed in the extent to which the REV1 level was increased after stable transfection of an hREV1 expression vector permitted a quantitative analysis of the relationship between hREV1 protein level and sensitivity to the cytotoxic effect of DDP. There was a remarkably good correlation between these two parameters, which, in combination with our earlier study (Okuda et al., 2005), indicates that either suppression or enhancement of the level of hREV1 modulates DDP sensitivity and that the abundance of REV1 is rate-limiting in some manner. It is of interest that even quite small increases in the level of hREV1 were accompanied by reduced killing of 2008 cells by DDP. Although modest in degree, such low-level resistance is nevertheless sufficient to result in clinical failure of treatment of ovarian cancer (Andrews et al., 1990). This raises the possibility that tumor-to-tumor variance in REV1 levels, or alteration in the activity of REV1 caused by the single nucleotide polymorphisms that have been identified in the *REV1* coding sequence, may account for some fraction of the variance in DDP sensitivity observed between tumors of the same or differing histological type. REV1 levels have not been quantified by Western blot in very many types of malignant human cells, so it is uncertain whether the basal level in 2008 cells is representative of that found in other tumors. No substantial differences were found between 2008 cells and HCT116 colon carcinoma cells (data not shown), but additional studies are required to address this point.

Whereas hREV1 helps preserve viability by allowing the completion of DNA synthesis, the consequence is that additional mutations are introduced into the genome, some of which result in drug resistance. The observation that increasing the level of hREV1 enhanced the ability of DDP to generate highly 6TG-resistant clones in the surviving population provides strong evidence that the translesional synthesis pathway in which hREV1 functions is error-prone when it bypasses DDP adducts in mammalian cells. It seems that this pathway normally facilitates the development of resistance to DDP both by permitting the survival of cells

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that contain mutagenic adducts in their DNA and by generating new mutations in genes that mediate the resistant phenotype. There is substantial heterogeneity in the rate at which patients' tumors become resistant to DDP during treatment. The good correlation between change in REV1 level and change in DDP mutagenicity suggests that this may be due in part to differences in REV1 expression.

Our working hypothesis is that the reduced sensitivity and enhanced mutagenicity of DDP in hREV1-overexpressing cells is attributable to increased mutagenic translesion synthesis. Translesion synthesis activity cannot be quantified directly in whole cells, but the effect of REV1 on the overall ability to express a protein from a gene containing DDP adducts can be quantified using the *R. reniformis* luciferase assay. Whereas platination of the plasmid substantially reduced the expression of luciferase in the parental cells, the effect of platination was significantly smaller in the cells which expressed increased levels of REV1. Increased luciferase expression may reflect enhanced translesion synthesis, transcription-coupled repair, transcriptional bypass, or the contribution of several other types of repair. In fact, one of its major advantages is that the assay measures the ability of the cell to complete all steps in the process and actually generate a functional protein, and this has been well-validated for DDP adducts (Sheibani et al., 1989; Jennerwein et al., 1991; Parker et al., 1991; Ali-Osman et al., 1994; Cenni et al., 1999; Chang et al., 2005). Two pieces of evidence suggest that the effect of REV1 on this assay in fact reflects a mutagenic process, presumably translesion synthesis. First, transcription-coupled nucleotide excision repair is largely error-free, yet increased expression of REV1 was clearly associated with an increase in mutagenesis, as measured by the generation of 6TG-resistant variants. Second, we have shown previously that a reduction in REV1 expression did not impair the early phase of removal of DDP adducts from genomic DNA, a process mediated largely by nucleotide excision repair. There is currently no other evidence that REV1 modulates the function of the nucleotide excision repair system or components of the apoptotic pathway, although this remains a possibility that needs to be addressed in future studies.

The results of the current study provide further evidence that hREV1 modulates DDP-induced mutagenicity, a role that has substantial clinical importance because DDP mutagenicity is directly linked to the emergence of DDP resistance (Wu et al., 2004; Okuda et al., 2005). It is now of substantial interest to determine the extent to which REV1 expression differs in DDP-sensitive and -resistant cell lines and between tumors of the same histological type that rapidly develop resistance and those that do not. This study highlights the potential importance of single-nucleotide polymorphisms that might affect REV1 activity. Finally, the current results further validate hREV1 as an attractive pharmaceutical target whose inhibition would be expected to simultaneously render tumors more sensitive to DDP and reduce the risk of generating resistance.

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