Etoposide (VP-16-213)-induced gene alterations: Potential contribution to cell death

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ABSTRACT We have shown previously a good correlation between etoposide-induced sister chromatid exchanges (SCE) and cytotoxicity. A semisynthetic derivative of podophyllotoxin, etoposide is also called Vepesid (Bristol; code designation VP-16-213, abbreviated VP-16). Since SCE represent DNA recombinational events, we hypothesized that VP-16-induced SCE might result in nonhomologous recombination in which segments of DNA were either deleted or added, leading to an alteration of gene sequences responsible for essential cell proteins. Alterations of such essential genes and consequent interference with formation of their products could consequently lead to cell death. To evaluate whether VP-16 treatment caused sufficient levels of DNA sequence alterations to interfere with gene product formation, we isolated hypoxanthine (guanine) phosphoribosyltransferase (HPRT)-deficient mutants from Chinese hamster V79 cells grown in the presence or absence of VP-16. DNA from 3 spontaneous mutants and 10 VP-16-induced mutants was analyzed by Southern blot hybridization to a full-length Hamster HPRT cDNA probe. Most of the VP-16-induced mutants showed partial deletions and/or rearrangements of the HPRT gene. In contrast, spontaneous mutants showed negligible deletions or rearrangements. These results provide strong support for our hypothesis that deletion of genetic sequences may constitute an important component of the mechanism of VP-16-induced cell death.

Cytotoxicity of etoposide, a semisynthetic derivative of podophyllotoxin that also is called Vepesid (Bristol; code designation VP-16-213, abbreviated VP-16), is mediated by its interaction with DNA topoisomerase II (Topo II), an ATP-dependent nuclear enzyme that regulates DNA topology by transiently breaking and rejoining double-stranded DNA to allow DNA strand passage (1-3). Under normal conditions, the formation of a DNA strand break by Topo II is followed by precise realignment and resealing of the broken ends. A large body of evidence suggests that VP-16 toxicity is related to its ability to cause protein-crosslinked DNA double-strand breaks by inhibiting the resealing activity of Topo II, thereby trapping the enzyme in a transient state referred to as a "cleavable complex" in which the enzyme is bound through a phosphodiester linkage at the 5' termini on opposing sides of the DNA double-strand break (4-6). VP-16 action does not result from blocking Topo II activity but rather by subverting the enzyme in such a way as to utilize it to initiate a lethal process (6, 7).

While there is strong circumstantial evidence that the "cleavable complex" triggers VP-16-induced cytotoxicity, there is little information in mammalian cells regarding the subsequent steps by which the cleavable complex results in cell death. After VP-16 removal, the DNA breaks rapidly reverse; however, the cells continue to become arrested in G2 phase of the cell cycle, and many of them subsequently die (4, 8, 9). These observations raise several important ques-

TIONS. First, what are the biochemical consequences of cleavable complex formation and second, how do they result in cell death?

In our studies aimed at obtaining a better understanding of VP-16-induced cell death, we have previously shown that there is a clear dissociation between VP-16-induced DNA strand breaks and cytotoxicity (10). In contrast, we found a good correlation between drug-induced sister chromatid exchanges (SCE) and cytotoxicity (10). SCE usually represent transfer of genetic material between homologous segments of sister chromatids. SCE also represent opportunities for nonhomologous recombination in which unequal exchange of genetic material may occur. In the latter situation, genetic sequences may be gained or lost from progeny cells, potentially leading to elimination of genetic sequences from some daughter cells or inactivation of genes by insertion of pieces of DNA with the potential for interruption of normal sequences. In either case, nonhomologous recombination could result in interruption of the normal sequence of any particular gene and the subsequent loss of the genes' protein from daughter cells. Thus, if the eliminated gene coded for an essential protein, then subsequent daughter cells would eventually run out of that protein, as it was eliminated by its own spontaneous decay process. If the protein were not replaced because the gene was interrupted by nonhomologous recombination, the cells eventually would lose function of that particular enzyme or protein, and elimination of that vital function ultimately would lead to cell death. Thus, induction of SCE with nonhomologous recombination affecting an essential enzyme function could account for cell death at some delayed time after initial exposure to an agent such as VP-16.

To determine whether this process could occur with VP-16 exposure, we examined cells treated with VP-16 for loss of a particular enzyme function. We selected hypoxanthine (guanine) phosphoribosyltransferase (HPRT) as an indicator enzyme for this process because of the ease of selecting for development of resistance to 6-thioguanine as a positive selection technique for loss of enzyme function. In these experiments we exposed cells to VP-16 and then selected for 6-thioguanine resistance. Resistant colonies derived from VP-16 treatment were then compared to spontaneously resistant mutant cell lines for alterations in the HPRT gene. We clearly demonstrate that VP-16-treated cells show a loss of enzyme function in association with deletions of large segments of genetic material, whereas spontaneous mutations in HPRT were not associated with significant alterations in the HPRT gene.

MATERIALS AND METHODS

Chemicals, Cell Culture, and Mutant Isolation. VP-16 was a gift from Bristol Laboratories. Preparation of VP-16 solutions was as described (10).

Abbreviations: VP-16, VP-16-213 code designation for etoposide (Vepesid); Topo II, topoisomerase II; SCE, sister chromatid exchanges; HPRT, hypoxanthine (guanine) phosphoribosyltransferase; APRT, adenine phosphoribosyltransferase.

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Culture conditions, maintenance of Chinese hamster V79 cells, and protocols for mutant isolation have been described (11, 12). Briefly, logarithmic-phase V79 cells that had been grown in HAT medium (α modified Eagle’s medium containing 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine) for approximately 1 month to eliminate spontaneous HPRT− cells were treated with 0, 5, or 10 μM VP-16 for 1 hr. These VP-16-treated and -untreated cells were allowed an expression time of 7–8 days during which they were subcultured twice. Finally, cell populations were exposed to 2 μg of 6-thioguanine per ml for 14–18 days to select mutant cell lines deficient in HPRT. Only one colony from each plate was isolated and expanded to avoid any duplication. Five colonies obtained from 5 μM VP-16 treatment were designated SM(1), SM(2), SM(3), SM(4), and SM(5). Similarly, colonies obtained from 10 μM VP-16 treatment were designated 10(1), 10(2), 10(3), 10(4), and 10(5). No HPRT− mutants were found in the HAT-grown cells that were not treated with VP-16, thus indicating that HPRT− mutants obtained from HAT-grown, VP-16-treated populations were truly VP-16-induced and not the result of spontaneous mutations. These results agree with the previous demonstration that VP-16 treatment markedly increases the mutation rate at a number of loci, including HPRT (13). Spontaneous HPRT− mutants were obtained by exposing normal V79 cells to 2 μg of 6-thioguanine per ml for 10–14 days. Three spontaneous HPRT− mutants, each of which was isolated and expanded from independent plates, were designated SM(1), SM(2), and SM(3).

**DNA Isolation and Restriction Enzyme Analysis.** The procedure for DNA isolation has been described (14). Briefly, cells from three confluent 175-cm² flasks were detached and pelleted. The cells were rinsed in phosphate-buffered saline and then resuspended in 12 ml of 100 mM NaCl/10 mM Tris-HCl, pH 8.5/5 mM EDTA. (Unless otherwise specified, all chemicals were from United States Biochemical.) Ten percent SDS in water was added dropwise to a final concentration of 0.5%, and the solution was mixed gently. Proteinase K (Jersey Lab Supply, Livingston, NJ) (25 mg/ml suspension) was added to a final concentration of 0.2 mg/ml. The cell lysate was placed on a rotator at 37°C for 14 hr. The lysate was extracted three times with phenol/chloroform, 1:1 (vol/vol). RNase A (Sigma; previously heated to 100°C for 10 min) was added to a final concentration of 10 μg/ml, and the lysate was incubated at 37°C for 1 hr. The mixture was extracted twice with 1:1 phenol/chloroform and once with 100% chloroform. The DNA was precipitated by adding 0.5 vol of 7.5 M ammonium acetate and 3 vol of ethanol and subsequently was "spooled out" by using a glass pipette. The DNA was rinsed with 70% ethanol and 100% ethanol then dissolved in 1 ml of 10 mM Tris-HCl, pH 8/1 mM EDTA.

The DNA was quantitated by absorption spectroscopy and 20 μg of DNA was treated with the appropriate restriction endonuclease. The relative size of the DNA and the completion of restriction enzyme digestions were confirmed by agarose gel analysis of undigested and digested samples. Restriction enzymes HindIII, Pst I, and EcoRI were purchased from Boehringer Mannheim. Restriction enzyme digests were done according to the manufacturer’s directions. The plasmid pHPT20 was obtained from the American Type Culture Collection (15, 16). The HPRT cDNA was isolated from the plasmid after Pst I restriction endonuclease digestion and agarose gel electrophoresis. The 750-base-pair (bp) insert was purified and labeled by using the random primer DNA labeling kit to a specific activity of 1 × 10⁶ dpm/μg (Boehringer Mannheim).

**Southern Blotting.** The procedure for Southern blotting has been described (17). Briefly, after restriction endonuclease digestion, DNA was analyzed by electrophoresis in a 0.75% agarose gel exposed to 50 V for 16 hr with bromophenol blue dye migration to 20 cm. The 1-kilobase (kb) ladder (Bethesda Research Laboratories) was included on the gel to allow determination of migration distance for specific-size fragments. The gel was stained with ethidium bromide solution (0.002 mg/ml in water), and the migration distance was measured relative to DNA fragments in the 1-kb ladder. The gel was exposed to UV light for 8 min for DNA < 1 kb and for 16 min for DNA ≥ 1 kb. The DNA was denatured by three cycles of 20-min incubation at room temperature in 0.5 M NaOH/1.5 M NaCl. The gel was rinsed with water and neutralized by three cycles of 20-min incubation at room temperature in 1 M Tris-HCl, pH 8/1.5 M NaCl. The DNA was transferred by capillary action to GeneScreen (DuPont/NEN) in double-strength saline sodium citrate (2× SSC = 0.3 M NaCl/0.03 M sodium citrate). The membrane was baked under reduced pressure at 80°C for 2 hr. The membrane was prehybridized for 4 hr at 55°C in 1% bovine serum albumin/0.5 M sodium phosphate buffer, pH 7.2/1 mM EDTA/4% SDS. Hybridization to the 750-bp HPRT cDNA was done at 55°C for 20–40 hr in the same solution containing 0.5 × 10⁶ dpm of labeled DNA per ml. The membrane was washed twice in 0.1% SDS/2× SSC at room temperature for 10 min and twice for 30 min each in 0.1% SDS/0.2× SSC at 55°C and subsequently was exposed to XAR diagnostic film (Eastman Kodak).

**RESULTS**

Fig. 1 shows a Southern blot analysis of parental V79 cells and spontaneous and VP-16-induced mutants deficient in HPRT. When cut with HindIII and hybridized to the hamster HPRT cDNA probe, the parental cells exhibited characteristic DNA fragments at approximately 16,500 bp and 14,500 bp. The small fragment at 6600 bp is consistent with a previously identified pseudogene (18). This "normal" pattern was seen in all three spontaneous mutants (designated SM) but in only 1 of the 10 (5 or 10 μM) VP-16-induced mutants (designated 5 and 10). The largest band in the HindIII digest of SM(1) and SM(2) showed small variations relative to the parental V79 cells. These variations may represent small deletions or point mutations resulting in slight alterations of the HPRT gene restriction pattern. SM(3) appeared to be the same as the parental cells. Thus, no gross deletions of HPRT were seen in the spontaneous HPRT− mutants. In 5 of the 10 VP-16-induced mutants—namely, SM(1), SM(2), SM(3), SM(10), and SM(10)—there was no hybridization of functional gene frag-
ments, but hybridization to the pseudogene fragment was the same as in parental cells. This suggests that there is a deletion of the entire functional gene in each of these mutants. In two of the VP-16-induced mutants [10(1) and 10(2)], there was only one fragment of approximately 14,500 bp seen. This pattern was also consistent with a partial deletion of a major portion of the gene. In one VP-16-induced mutant, 5(4), there was no hybridization to the predominant pseudogene fragment or to the HPRT gene. Examination with ethidium bromide and UV light showed DNA to be present in this lane in a concentration equal to that in other lanes. Furthermore, hybridization with other probes such as the gene for adenine phosphoribosyltransferase (APRT; not shown) also demonstrated DNA to be present in this lane. This suggests that in the 5(4) mutant, deletions occurred at the site of the pseudogene as well as within the HPRT gene. VP-16-induced mutant 5(5) showed loss of the 14,500-bp fragment. This suggests a partial deletion different from that seen in 10(1) and 10(2).

Fig. 2 shows the Southern blot analysis of DNA cut with Pst I. Three major bands are apparent. The predominant functional gene fragments seen with Pst I were approximately 9700, 9150, and 3200 bp. VP-16-induced mutants 5(1), 5(2), 5(3), 5(4), 10(3), and 10(4) were missing the 9700-, 9150-, and 3200-bp fragments, thus confirming deletion of the HPRT gene in these cell lines. VP-16-induced mutants 10(1) and 10(2), the spontaneous mutant SM(3), and the parental V79 cells exhibited identical hybridization to all of the functional gene fragments. In contrast, spontaneous mutants SM(1) and SM(2) showed hybridization to both 9700- and 9150-bp functional gene fragments and loss of the 3200-bp fragment. 5(5) exhibited a loss of the 3200-bp fragment. However, appearance of a new hybridizing fragment at a slightly higher molecular weight relative to the 3200-bp fragment was seen in 5(5), possibly indicating rearrangement of the functional gene.

Fig. 3 shows the Southern blot analysis of DNA cut with EcoRI, which revealed functional gene fragments of approximately 19,000 bp and 14,500 bp and a pseudogene fragment of 1200 bp. These results confirmed the “normal” pattern of hybridization in the V79 cells and spontaneous mutants and showed deletion of the HPRT gene in VP-16-induced mutants 5(1), 5(2), 5(3), 5(4), 10(3), and 10(4). 5(5) showed loss of the 14,500-bp fragment; in addition, it also showed a more diffuse hybridization pattern above 15,000 bp, possibly indicating a rearrangement of the functional gene. While 10(1) and 10(2) showed normal hybridization to the 19,000-bp fragment, an absence of hybridization to the 14,500-bp fragment was seen in these lines. Instead, hybridization to fragments < 14,500 bp was observed in both 10(1) and 10(2), possibly indicating partial deletions of the functional gene.

These results clearly show that VP-16-induced mutations in HPRT are associated with deletions of major segments of the HPRT gene, whereas spontaneous mutants demonstrate little or no deviation from the parental HPRT gene pattern.

**DISCUSSION**

We and others have previously shown that VP-16-induced cytotoxicity is clearly related to the formation of Topo II–DNA complexes (4, 9, 11, 19). These complexes initiate a process that ultimately leads to cell death; however, the complexes themselves are not the immediate cause of cell death. The basis for these statements can be summarized as follows: (i) VP-16 stabilizes the complexes between Topo II and DNA, which, under alkaline protein-denaturing conditions, yield frank DNA strand breaks or cleavable complexes (4, 19); (ii) increasing doses of VP-16 result in increasing numbers of cleavable complexes and increasing cytotoxicity (10, 11, 19); (iii) withdrawal of VP-16 results in rapid reversal of cleavable complexes, yet the cells die in ensuing generations (4, 9); (iv) VP-16 exposure during S phase causes maximal cytotoxicity, whereas cells undergo VP-16-induced cleavable complex formation during all phases of the cell cycle (20); (v) different cell lines show a poor correlation between VP-16-inflicted cytotoxicity and DNA strand breaks (10, 21); (vi) VP-16 is a potent inducer of SCE in a dose-dependent fashion (10, 13, 22); (vii) continuous exposure to VP-16 results in an increase in cleavable complex formation followed by a plateau after approximately 30 min, whereas SCE formation and cell killing continue to increase with time of exposure (10); and (viii) an excellent correlation exists between VP-16-induced cytotoxicity and SCE (10).

On the basis of the high degree of correlation between VP-16-induced SCE and cytotoxicity, we suggest that the Topo II–DNA crosslinks induced by VP-16 may function to induce SCE associated with aberrant DNA recombination. It is possible that aberrant recombination could lead to the interruption of genetic sequences by deletion and/or insertion. Since SCE are distributed throughout the genome, it is anticipated that aberrant recombination processes could affect both nonessential and essential genes. Interruption or elimination of an essential gene can then lead to cell death by
ultimately resulting in a cellular deficiency of that gene's product.

In our present experiments, we studied the effect of VP-16 on the HPRT gene as a marker for this hypothesis. Our results clearly demonstrate that VP-16 can cause genetic deletions resulting in loss of a functional gene's product. While these events were measured in the nonessential HPRT gene, it is likely that the same phenomenon happens throughout the genome in genes coding for both essential and nonessential genes.

On the basis of these studies, we propose the model shown below as contributing to the mechanism of VP-16-induced cell death.

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VP-16
↓
VP-16-Topo-II-DNA complex
                          ↓
Replication block
                          ↓
Replication block bypass
                          ↓
SCE
                          ↓
(Nonhomologous recombination)
                          ↓
Essential gene inactivation
                          ↓
Depletion of essential gene product
                            ↓
Death
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In this scheme, VP-16 is accumulated intracellularly and interacts with Topo II and DNA to form complexes that crosslink and stabilize the topological structure of DNA. During DNA replication, progress of the replication fork is blocked by a drug-stabilized complex, and the cell resorts to SCE as a bypass mechanism. This process may require several intermediate steps for the replication machinery as indicated by the multiple arrows in the scheme. Some of these SCE result in or are associated with aberrant or nonhomologous recombination in which segments of DNA are either added or deleted, leading to the interruption of normal genetic sequences and the ultimate deletion of essential gene products and consequent cell death. Since this manuscript was originally submitted, Han et al. published an abstract indicating that 40% of APRT- mutants, induced by treatment with the Topo II inhibitor VM26 in CHO-D422 cells, had clearly demonstrable gene deletions (23).

This hypothesis, that nonhomologous or aberrant recombination during the replication process contributes to the mechanism of VP-16-induced cell death, is further supported by the observation that DNA crosslinks are well established as a stimulus to SCE formation during DNA replication (24). In addition, this hypothesis explains the requirement for DNA replication to foster VP-16-induced SCE formation and cytotoxicity (25). Therefore, a dose-dependent increase in VP-16-induced ternary complexes may be associated with a dose-dependent obstruction to replication forks and a dose-dependent increase in SCE and aberrant DNA recombination. Thus, we have shown previously that VP-16-induced SCE are directly related to VP-16-induced cytotoxicity (10). This study clearly shows the deletion of genetic material in response to VP-16 treatment. On the basis of these experiments, it cannot be determined whether the extent of the deletions is dependent on the dose of VP-16. However, it is anticipated that the number of deletions and aberrations sustained in the genome increases in a dose-dependent fashion and may provide the mechanism for dose-dependent toxicity. The questions remain as to whether deletion size varies in a dose-dependent fashion and whether the deletions occur more or less frequently in specific genes, in actively transcribed versus nontranscribed regions of chromatin or at any specific base sequences. Any of these possibilities might contribute to tissue specificity of VP-16 and/or other determinants of its activity as well as to cellular resistance mechanisms. In addition, it will be important to determine whether similar effects occur with diploid genes and whether different Topo II-active agents show different sequence specificity or gene-targeting preference since the latter may provide a basis for developing more effective drug combinations.

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