Relationship Between Cytotoxicity and Site-Specific DNA Recombination After In Vitro Exposure of Leukemia Cells to Etoposide

Chun-Lin Chen, James C. Fuscoe, Qing Liu, Ching-Hon Pui, Hazem H. Mahmoud, Mary V. Relling*

Background: Etoposide, an inhibitor of the normal religation activity of the nuclear enzyme topoisomerase II, can induce a secondary acute myeloid leukemia characterized by site-specific DNA rearrangements. The schedule of drug administration appears to be a clinical risk factor for this devastating treatment complication. Purpose: We tested the hypothesis that prolonged exposure of leukemia cells in vitro to low concentrations of etoposide, compared with short exposures to high concentrations, could produce equivalent or greater desired cytotoxic effects, with decreased occurrence of undesired site-specific double-stranded DNA recombinational events (i.e., recombinogenesis). Methods: We used the frequency of V(D)J (variable-diversity-joining) recombinase-mediated deletions of exons 2 and 3 of the hypoxanthine phosphoribosyltransferase (HPRT) gene as a biomarker of etoposide-induced, nonhomologous, site-specific DNA rearrangement. A polymerase chain reaction-based technique was used to measure exon 2 + 3 deletions in human lymphoid leukemia CCRF-CEM cells 6 days after either 4-hour or 24-hour treatment with etoposide at clinically relevant concentrations, achieving equivalent (e.g., 95%) cytotoxicity (14.2 x 10^-7 versus 4.1 x 10^-7) or at equivalent etoposide concentrations (e.g., 1 μM (10.8 x 10^-7 versus 1.3 x 10^-7)). Thus, the ratio of desired cytotoxic to undesired recombinogenic effects was higher with the 24-hour schedule. After the treated cells were subcloned at limiting dilutions, the frequency of the exon 2 + 3 deletion increased from 16.3 x 10^-7 to 4.33 x 10^-3, indicating that the recombinational event is not necessarily lethal. Conclusion: For all drug concentrations and levels of cytotoxicity studied in CCRF-CEM cells, there was a greater ratio of cytotoxicity to genetic recombinogenesis following prolonged exposure to etoposide than following brief exposure. Implication: These data suggest that recombinogenesis is not intrinsically linked to cytotoxicity. If confirmed in the clinical setting, the use of prolonged dosage schedules may provide a means to decrease the risk of etoposide-induced acute myeloid leukemia without compromising treatment efficacy. [J Natl Cancer Inst 1996;88:1840-7]
bination-prone sequences, such as topoisomerase II cleavage sites (18-21), Alu repeats (19,22), χ-like elements [found at the (4;11) translocation breakpoint (23)], and V(DJ) (variable–diversity–joining) recombination recognition signal sequences (21,24,25). MLL gene rearrangements are also found in more than 80% of de novo infant leukemias (26,27). By contrast to the broad spectrum of mutations found in the hypoxanthine phosphoribosyltransferase (HPRT) genes of adults (28), most HPRT gene mutations observed in the lymphocytes of infants involve large DNA deletions that appear to result from V(DJ) recombination (29). Thus, there may be a tendency for increased nonhomologous recombination in the hematopoietic cells of infants. Moreover, the inappropriate use of V(DJ) recombination machinery has also been demonstrated in the translocation breakpoints associated with many lymphoid cancers (30). These observations suggest that a combination of the MLL gene being prone to rearrangement (31) and excessive nonhomologous recombination is etiologic in epipodophyllotoxin-associated leukemogenesis.

Of the site-specific mechanisms of DNA rearrangements, inappropriate V(DJ) recombination can be quantified directly from surviving human cells by use of a polymerase chain reaction (PCR)-based assay of specific deletions of a nonphysiologic (i.e., endogenous but inappropriate) target gene, HPRT. The epipodophyllotoxins are known to cause large DNA deletions and rearrangements (32,33), but they have been shown only recently to increase site-specific V(DJ) recombinase-mediated deletions in the HPRT gene (34). In human lymphoid leukemia cells, a single treatment with etoposide at 0.5-10 μM caused an immediate, concentration-dependent increase in exon 2 + 3 deletions in HPRT (34). We hypothesized that etoposide’s inhibition of the normal recombination reactions catalyzed by topoisomerase II enhances inappropriate modes of DNA recombination and that V(DJ) recombinase-mediated deletions in a nonphysiologic substrate (HPRT) might be an informative biomarker of etoposide-induced nonhomologous recombination. Also, because exon 2 + 3 HPRT deletions were noted as little as 4 hours of treatment with etoposide (34)—a point at which cytotoxicity was not yet evident—these data suggested that cytotoxicity and recombination might not be inseparable effects. Therefore, we tested the hypothesis that one could achieve equivalent or enhanced cytotoxicity while inducing less nonhomologous recombination by exposing leukemia cells in vitro to low concentrations of etoposide for prolonged times, rather than to high concentrations of etoposide for short times.

Materials and Methods

Materials

Etoposide was a gift from Bristol-Myers Squibb, Wallingford, CT. Oligonucleotide primer A106 (5′-CTACTGCCCCTTTACATGAGACAC), which anneals at nucleotides 22718-22741 of the HPRT gene, oligonucleotide primer A107 (5′-CAGTTTCCCCTGTTCGG), which anneals at nucleotides 1835-1851, and oligonucleotide primer A115 (5′-GTGCGATGTTGAGGCTTC), which anneals at nucleotides 2094-2111, were synthesized and purchased from Biosynthesis Inc., Lewisville, TX. The oligonucleotide probe A217 (5′-TGGGGGACAGGTTTTGCT), which anneals at nucleotides 2166-2185, was obtained from Operon Technologies, Inc., Alameda, CA. Nucleotide numbering for the HPRT gene was as given by Edwards et al. (55). Primers A106, A107, and A115 were unpurified; primer A217 was purified by high-performance liquid chromatography (HPLC). Tris base, N,N,N′-nitro-N-nitrosoguanidine (MNNG), 2-amino-6-mercaptopurine (thioguanine), 6-mercaptopurine, Ficol 400, and human placenta genomic DNA were from Sigma Chemical Co., St. Louis, MO. 2′-Deoxyadenosine 5′-triphosphate (dATP), 2′-deoxyguanosine 5′-triphosphate (dGTP), 2′-deoxythymidine 5′-triphosphate (dTTP), and 2′-deoxyguanosine 5′-triphosphate (dGTP) were from Pharmacia Biotech, Inc., Piscataway, NJ. Taq polymerase was obtained from The Perkin-Elmer Corp., Norwalk, CT. Nusieve agarose was purchased from Nusieve (3:1) agarose from Life Technologies, Inc. (GIBCO BRL), Gaithersburg, MD. [γ-32P]dATP and a 5′-end-labeled primer were synthesized by and purchased from BioWhittaker, Inc., Walkersville, MD. Fetal bovine serum was obtained from BioWhittaker, Inc., Walkersville, MD. Fetal bovine serum was from Falcon, Becton Dickinson and Co., Lincoln Park, NJ, and was heated at 56 °C for 30 minutes before use. An OmniGene temperature cycler (Model TR3 SM2; National Labnet Co., Woodbridge, NJ) and TCO 100 mini-fluorometer (Hoefer Scientific Instruments) were used. Slide-A-Lyzer dialysis cassettes were from Pierce Chemical Co., Rockford, IL. GeneScreen Plus hybridization transfer membranes were from Du Pont NEN, Boston, MA. Human lymphoid leukemia CCRF-CEM cells were from the American Type Culture Collection, Rockville, MD.

Clinical Basis for Choice of In Vitro Treatment Schedule

Plasma pharmacokinetics were evaluated following low-dose oral and the more typical intravenous doses used in most treatment regimens. Children with acute lymphoblastic leukemia (ALL) were treated according to the St. Jude Children’s Research Hospital protocols after written informed consent was provided by the patient, parent, or guardian. Institutional review board guidelines, in accord with an assurance filed with and approved by the U.S. Department of Health and Human Services, were followed. Nineteen children with ALL in first remission received 300 mg/m² etoposide intravenously over a 2-hour period, and six children with relapsed ALL received 50 mg/m² etoposide orally. Blood samples (3 mL) were collected into heparinized, evacuated tubes prior to and at several time points after administration of etoposide. Plasma was frozen at −80 °C until the time of assay, and etoposide concentrations were measured by HPLC with electrochemical detection as described (36). Pooled data from each regimen were analyzed by use of a maximum likelihood estimation strategy (37), assuming a two-compartment pharmacokinetic model with first-order absorption (after oral dosage only), and the resulting pharmacokinetic profiles are depicted in Fig. 1. The time periods for in vitro etoposide treatment of human lymphoid leukemia CCRF-CEM cells (see below) were chosen because they approximated the time periods for which patients exhibit brief high (i.e., >10 μM) or prolonged low (i.e., >1 μM) plasma concentrations of etoposide in vivo after the short infusions previously associated with secondary AML (2,3) and the alternative low-dose oral regimens currently undergoing clinical evaluations (38,39), respectively.

Etoposide Treatment

Etoposide in dimethyl sulfoxide (DMSO) was added to CCRF-CEM cells in RPMI-1640 medium—10% heat-inactivated fetal bovine serum and 2 mM l-glutamine in logarithmic phase at 1 × 10⁶ cells/mL at 37 °C in 95% air-5% CO₂. The final etoposide concentrations were 0.0, 0.25, 1, 2.5, 5, and 10 μM for cells treated for 4 hours and 0, 0.025, 0.1, 0.25, 0.5, and 1.0 μM for cells treated for 24 hours (final concentrations of DMSO being 0.1% vol/vol). After either 4 hours or 24 hours, cells were either collected immediately or subcultured to a density of 3 × 10⁶/mL every 2 days for 6 days, at which times DNA was extracted from 10² or more cells by using a Blood & Cell Culture DNA kit according to the manufacturer’s protocol (QIAGEN, Chatsworth, CA), dialyzed against 10 mM Tris–HCl (pH 8.0)–1 mM ethylenediaminetetraacetic acid (TE), and quantified by fluorometry. Each etoposide concentration–exposure duration assessment was performed in duplicate (i.e., two separate flasks of cells) in two independent experiments for each time period of subculture (i.e., no subculture versus subculture for 6 days). The data from cells treated for 4 hours with etoposide have been reported previously (34). MNNG in DMSO was added as 1, 2.5, 5, 10, or 20 mM concentrated solutions to CCRF-CEM cells for
**Preparation of Genomic DNA**

DNA was extracted as described above, dissolved in and dialyzed against TE, and concentrated if necessary by use of Centricron-10 columns (Amicon Inc., Beverly, MA). The concentration of DNA was determined by fluorometry using calf thymus DNA as the standard, following the manufacturer's protocol.

**Detection and Quantitation of the Exon 2 + 3 HPRT Deletion**

A hemi-nested PCR approach was carried out in two consecutive rounds by use of modifications of previously reported procedures (28,29,40) to detect and quantify the deletion of exons 2 and 3 of HPRT. Primers A106 and A107 were used in the first round; 1 μL of the first-round product and primers A106 and A115 were used for the second round. DNA product is amplified only when exons 2 and 3 of HPRT have been deleted, with the class I deletions giving rise to a 595-base-pair product (Fig. 2) (29,44). Positive control reactions consisted of linearized plasmid DNA equivalent in amount to 2.7 × 10^6 cells. Twenty microliters of each PCR reaction was electrophoresed through 1.4% agarose gels in TBE (Tris-borate-EDTA buffer) and transferred to nylon membranes with 0.4 N NaOH-0.6 M NaCl. The membranes were hybridized with 32P-5'-end-labeled A217 (HPRT intron 1 oligonucleotide) in a solution of 5X SSPE (saline-sodium phosphate-EDTA buffer), 2% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 100 μg/mL salmon sperm DNA at 42 °C and washed in 2X SSC (saline-sodium citrate buffer) and then in 0.1% SDS-2X SSC at 42 °C. The membranes were exposed to Storage Phosphor Screens and scanned by use of a PhosphorImager, the pattern of radioactive hybridization signals was visualized using ImageQuant Software (all from Molecular Dynamics, Sunnyvale, CA). All replicates producing a PCR-amplified DNA fragment that hybridized with the HPRT-specific probe A217 were counted as positive, those that did not were considered null.

The fraction of replicates for each set of samples constituting an etoposide concentration and duration of exposure that was null for HPRT-specific PCR products (P0) was determined, and the average number of deletion mutants per reaction (x) was estimated by use of a Poisson model (41); i.e., \( x = \ln(1/P_0) \). We assumed that each cell contained 6.25 pg DNA. Because each replicate PCR reaction contained 2.5 μg DNA, the frequency of exon 2 + 3 deletion mutants per cell was estimated as equal to x divided by 4 × 10^6 cells. Results were based on the total number of PCR replicates for each concentration–duration of exposure condition, but essentially identical results were obtained if the fraction of positive replicates was taken as the average of the mean of the two experiments.

To assess whether the average DNA content per cell was affected by etoposide treatment, we disrupted known numbers of untreated or etoposide-treated cells by sonication in a cuvette and measured DNA concentration directly in the cuvette by fluorometric assay. The average DNA content for 4-hour-treated samples (6.5 pg/cell; n = 60), 24-hour-treated samples (6.8 pg/cell; n = 40), and untreated samples (6.4 pg/cell; n = 25) did not differ (P = .35, analysis of variance); thus, we assumed that the DNA content was constant in all experiments.

**Sequencing of HPRT 2 + 3 Deletion Junctions**

Several PCR products, each obtained from different experiments, were purified with QIAquick Gel Extraction Kit (QIAGEN) following the manufacturer’s protocol, then sequenced at the Center for Biotechnology, St. Jude Children’s Research Hospital, by use of a cycle-sequencing reaction that employs a fluorescence-tagged dye terminator (PRISM; Applied Biosystems, Foster City, CA). Sequencing was analyzed by use of the Genetics Computer Group (GCC 8.0) software package (The University of Wisconsin, Madison). We analyzed homologies of the unarranged HPRT locus to the V(DJ) recombinase heptamer ([CA/GAT/TGG]) and nonamer ([GGTTTTTTGT]) recognition sequences (42,43), γ-like elements ([GG/AT/GAT/G]) (44), topoisoerase II cleavage sites ([AG/GT/TC]-NCCNCNG(T/C/N)(GG)(GTT)/NCT/C(T/C)), with N representing any of the four possible nucleotides) (45) and ACG/T(A/G)CCCGG(C/T)/A(C/T)-GTI (46), and topoisoerase I cleavage site consensus sequences ([CC/GT/T]) (44). Using only PCR products without the nucleotide removal ("nibbling") or insertions characteristic of some V(DJ) recombinase-mediated rearrangements, we also analyzed homologies of the HPRT locus exhibiting the exon 2 + 3 deletions to topoisoerase II cleavage sites.

**Statistical Analysis**

We compared the IC50 values for the 4-hour- and 24-hour-exposure determinations whether their 95% confidence intervals overlapped. Inferences upon changes in deletion frequencies were based on the observed fraction of replicates positive for the exon 2 + 3 deletion. We used the exact Wilcoxon rank-sum test to assess whether the proportions of positive replicates were the same after either 4-hour or 24-hour treatment versus control (i.e., no treatment). We used the exact Manel–Haenszel test, with data stratified according to the drug dosage, to determine whether overall (global) differences in the proportion of cells positive for the exon 2 + 3 deletion existed between 4-hour and 24-hour treatments.

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*Fig. 1. Measured etoposide plasma concentrations following administration of 300 mg/m² etoposide intravenously over a 2-hour period (n = 19, circles) or 50 mg/m² etoposide orally (n = 6, squares) to children with acute lymphoblastic leukemia. Solid lines indicate the best fit from analyzing pooled data from each regimen. Dashed and dotted lines indicate the time period and concentrations associated with 99% cytotoxicity in our in vitro studies using 4-hour versus 24-hour exposures, respectively.*
**Set 1**

A) Hem additional chain reaction (PCR) approach, using primers A106 and A107 for the first round of PCR and primers A115 and A115 for the second round of PCR, allows amplification of a DNA product only when exons 2 and 3 of hypoxanthine phosphoribosyltransferase (HPRT) have been deleted. The most common class of exon deletions (class I) results in a final base-pair (bp) fragment. (B) Southern blot analysis indicating hybridization of an intron 1 HPRT oligonucleotide probe with seven of 10 replicates treated with 10 \( \mu \text{M} \) thionapte for 4 hours, one of 10 replicates treated with 1 \( \mu \text{M} \) etoposide for 24 hours, and none of 10 replicates from untreated control CCRF-CEM cells. (C) Sequences of a subset of PCR products encompassing the class I intron 1/intron 2 breakpoints following etoposide treatment. Mutation A was observed in three of nine and three of 13, mutation B in four of nine and four of 13, mutation C in one of nine and none of 13, mutation E in zero of nine and two of 13, mutation F in zero of nine and one of 13, and mutation G in zero of nine and one of 13 sequenced samples following treatment of cells with etoposide for 4 hours and 24 hours, respectively. Conserved heptamer and nonamer sequences are indicated in boxes; nucleotide insertions are italicized; putative P-nucleotides [i.e., the palindromic nucleotides that are frequently inserted in V(D)J—i.e., variable-diversity-joining—recombinase mediated rearrangements] are underlined. D) Mutations H and I were found exclusively after 24-hour treatment. Heptamer-like sequences are boxed; rectangular brackets indicate mismatched topoisomerase II cleavage site consensus sequences (45); braces indicate the three bases critical for V(D)J recombinase recognition.

**Set 2**

**Fig. 2.** A) A hemi-nested polymerase chain reaction (PCR) approach, using primers A106 and A107 for the first round of PCR and primers A115 and A115 for the second round, allows amplification of a DNA product only when exons 2 and 3 of hypoxanthine phosphoribosyltransferase (HPRT) have been deleted. The most common class of exon deletions (class I) results in a final 595-base-pair (bp) fragment. B) Southern blot analysis indicating hybridization of an intron 1 HPRT oligonucleotide probe with seven of 10 replicates treated with 10 \( \mu \text{M} \) etoposide for 4 hours, one of 10 replicates treated with 1 \( \mu \text{M} \) etoposide for 24 hours, and none of 10 replicates from untreated control CCRF-CEM cells. C) Sequences of a subset of PCR products encompassing the class I intron 1/intron 2 breakpoints following etoposide treatment. Mutation A was observed in three of nine and three of 13, mutation B in four of nine and four of 13, mutation C in one of nine and none of 13, mutation E in zero of nine and two of 13, mutation F in zero of nine and one of 13, and mutation G in zero of nine and one of 13 sequenced samples following treatment of cells with etoposide for 4 hours and 24 hours, respectively. Conserved heptamer and nonamer sequences are indicated in boxes; nucleotide insertions are italicized; putative P-nucleotides [i.e., the palindromic nucleotides that are frequently inserted in V(D)J—i.e., variable-diversity-joining—recombinase mediated rearrangements] are underlined. D) Mutations H and I were found exclusively after 24-hour treatment. Heptamer-like sequences are boxed; rectangular brackets indicate mismatched topoisomerase II cleavage site consensus sequences (45); braces indicate the three bases critical for V(D)J recombinase recognition.

**Set 3**

**Subculturing of Cells to Partially Clone the Exon 2 + 3 Deletion**

At 6 days after treatment with 5 \( \mu \text{M} \) etoposide for 4 hours, the cells from one flask were further subcultured in the presence of 10 \( \mu \text{M} \) 6-thioguanine for 9 days to partially select for exon 2 + 3 deletion-containing cells. These cells were then subcultured at limiting dilutions to sequentially select the exon 2 + 3 deletion-containing cells. DNA extracted from at least 10 separate subcultured aliquots was assayed by the exon 2 + 3 deletion assay (see above) for each dilution. The subculturing process was repeated eight times. Control CCRF-CEM cells (not treated with etoposide) were also subcultured in the presence of 6-thioguanine and assayed for exon 2 + 3 deletions.

**Results**

**Characteristics of HPRT Exon 2 + 3 Deletions**

Immediately following and 6 days after etoposide treatment, HPRT exon 2 + 3 deletions were elevated in treated cells relative to those in untreated control cells. The majority of the PCR-amplified DNA bands that hybridized with the HPRT-specific probe were approximately 595 base pairs in size, consistent with the most common class I HPRT exon 2 + 3 deletions cloned from the lymphocytes of infants (29). Sequencing of a subset of the PCR products revealed the existence of breakpoint sequences in the corresponding wild-type, unarranged HPRT
that were consistent with illicit V(DJ) recombination in the genesis of the majority of mutants following both 4-hour and 24-hour etoposide exposure (Fig. 2). Typical class I HPRT exon 2 + 3 deletion mutations (29) were confirmed in nine of nine and 11 of 13 of the 595-base-pair amplification products following 4-hour and 24-hour treatments, respectively: A heptamer (nucleotide matches at six of seven positions) was identified immediately adjacent to the intron 1 breakpoint, and a heptamer (nucleotide matches at four of seven positions) and nonamer (nucleotide matches at seven of nine positions) were identified immediately 5' to the intron 3 breakpoint; nibbling and insertions characteristic of V(DJ) recombinase activity (42) were also evident (mutations A, B, C, E, F, and G) (Fig. 2). A mutant (mutation D, not shown) of aberrant size (450 base pairs) did not show evidence of V(DJ) recognition signal sequences. Two unique 595-base-pair mutants (mutations H and I) with breakpoints different from those previously described for class I HPRT exon 2 + 3 deletion mutants (29) might also be caused by the illegitimate action of V(DJ) recombinase (Fig. 2), as evidenced by the presence of heptamers in intron 1 and in intron 3 immediately adjacent to the breakpoints. In both heptamers, the three nucleotides closest to the breakpoints, thought to be the most critical (47), are in agreement with the consensus heptamer. PCR amplification of DNA from two PCR replicates containing exon 2 + 3 deletions produced aberrantly sized products (mutations J and K, not shown) with unique breakpoints (at intron 1/intron 3 positions = 2238/21876 and 3000/22447, yielding 1010- and 1202-base-pair DNA fragments, respectively). Both of these mutations (J and K) were characterized by nucleotide sequences in unrearranged HPRT spanning all four breakpoints (across intron 1 and intron 3 in both mutations) that were homologous with topoisomerase II cleavage site consensus sequences (matches at either 15 or 16 of 18 nucleotide positions). For mutations H through K, the intron 3 breakpoints occurred in AT-rich sequences (85% for mutation J and 70% each for mutations H, I, and K).

Mutations A and B were present following both 4-hour and 24-hour treatments, mutations C and D (not shown) were found only after the 4-hour treatment, and mutations E through K were observed only after the 24-hour treatment. Thus, 22 of 25 mutations displayed breakpoint sequences consistent with illicit V(DJ) recombination, and two of 25 mutations displayed topoisomerase II cleavage sites spanning the breakpoints of the wild-type, unrearranged intron 1 and intron 3 sequences. We did not observe χ-like elements or topoisomerase I cleavage recognition sites.

There were no homologies to topoisomerase II cleavage sites observed in the nucleotide sequences at the breakpoints of the rearranged exon 2 + 3 deletion mutants themselves.

**Schedule Dependence of Exon 2 + 3 Deletions**

As expected, cytotoxicity increased with increasing etoposide concentration and time of exposure (Fig. 3). Initial frequencies of the exon 2 + 3 deletion (immediately after treatment) were elevated in treated cells relative to those in untreated control cells (P<.005 for both comparisons), with frequencies of 0.77 x 10^{-7} for the control and 2.63 x 10^{-7} for the 4-hour regimen and 4.80 x 10^{-7} for the 24-hour regimen at equivalent (95%) cytotoxicity (2.5 and 0.5 μM etoposide, respectively) (Fig. 4). By day 6, however, the frequency of the exon 2 + 3 deletion was higher after the 4-hour treatment (14.2 x 10^{-7}) than after the 24-hour treatment (4.06 x 10^{-7}) at equivalent degrees of cytotoxicity (global P value = .0003, Fig. 4). In fact, deletion frequencies at day 6 were also higher at any given etoposide concentration in cells that were treated for 4 hours rather than 24 hours (Fig. 3), despite lower cytotoxicity with the 4-hour exposures (IC50 [95% confidence intervals] = 0.65 μM [0.55-0.75 μM] and 0.124 μM [0.11-0.14 μM] for the 4-hour and 24-hour regimens, respectively). Deletion frequencies did not differ immediately after the 4-hour and 24-hour treatments (P = .32), but they were significantly higher after 4-hour exposure when assessed at day 6 (P = .0003). Initial deletion frequencies were related to etoposide concentration (and cytotoxicity) following both exposure durations, but the day-6 frequency was concentration related only for the 4-hour regimen (Fig. 4). Thus, it appears that more cells survive with sublethal DNA deletions following equally cytotoxic treatment that is delivered over a shorter time period.

**Separation of Cytotoxicity and Recombination**

CCRF-CEM cells were able to survive and grow with these sublethal DNA deletions of exon 2 + 3, further evidence that recombinogenic events do not necessarily lead to cell death. The frequency of the exon 2 + 3 deletion increased from 16.3 x 10^{-7} at day 6 following treatment with 5 μM etoposide for 4 hours to 115 x 10^{-7} as a result of a 9-day partial selection in 10 μM 6-thioguanine. Identical 6-thioguanine treatment of control CCRF-CEM cells resulted in no detectable deletions (i.e., <0.25 x 10^{-7}). Moreover, sequential subculturing of an aliquot of the 4-hour etoposide-treated cells at limiting dilutions (for the exon 2 + 3 deletion) has yielded deletion frequencies close to those predicted if the deletion conferred little growth disadvantage or advantage. This population of cells has an exon 2 + 3 deletion frequency of 4.33 x 10^{-3} after eight limiting-dilution steps.

If we assume that the deletion frequency is an indicator of the most serious adverse effect of the epipodophyllotoxins and that cytotoxicity represents the desired antitumor effect, the ratio of the percentage cytotoxicity to the deletion frequency could serve as an indicator of the therapeutic index of the two treatment schedules. At equivalent (95%) cytotoxicity, these ratios were 6.7 and 23.5 following the 4-hour and the 24-hour exposures, respectively, which suggests an improved therapeutic index with the prolonged exposure schedule.

**Fewer Exon 2 + 3 Deletions After Treatment With an Alkylating Agent Than After Treatment With Etoposide**

MNNG, a directly acting alkylating agent, causes a much higher frequency of overall HPRT mutations (primarily point mutations) than does etoposide (48). The IC50 of MNNG against CCRF-CEM cells was 5.8 μM (95% confidence interval = 5.1-6.5 μM). However, MNNG treatment caused a lower frequency (1.06 x 10^{-7}) of site-specific HPRT exon 2 + 3 deletions.
Fig. 3. Cytotoxicity (left ordinate, closed symbols), depicted as the mean percentage of surviving human lymphoid leukemia CCRF-CEM cells (treated versus untreated) at 6 days of subculture after 4 hours of treatment with etoposide (squares) versus 24 hours of treatment with etoposide (circles). Fitted lines indicate the best fit of nonlinear least-squares regression analysis of all four measurements of cytotoxicity at each concentration-exposure duration condition. The hypoxanthine phosphoribosyltransferase (HPRT) exon 2 + 3 deletion frequency (right ordinate, open symbols) was assessed at 6 days of subculture following 4 hours of treatment with etoposide (squares) versus 24 hours of treatment with etoposide (circles) and was based on the total number of replicates positive for the HPRT exon 2 + 3 deletion compared with the total number of replicates of $4 \times 10^7$ cells tested for the exon 2 + 3 deletion at each etoposide concentration-exposure duration condition tested.

than did similarly cytotoxic 4-hour etoposide treatment ($14.9 \times 10^{-9}$) ($P = .0003$).

Discussion

The occurrence of secondary AML in patients following epidophyllotoxin therapy is presumably related to topoisomerase II inhibitory effects. Identical 11q23 translocations have been reported to occur in patients (although less frequently) after the use of non-epidophyllotoxin agents (e.g., anthracyclines), which inhibit topoisomerase II to a lesser extent (1). Because etoposide inhibits the normal religation of double-strand breaks in DNA (49), it is not surprising that the agent would cause increased nonhomologous DNA recombination. The suggestion that the desired cytotoxic effects of etoposide are inextricably linked with its recombinogenic effects received support from a study showing that the best predictor of cytotoxic effects in a variety of cell lines was the level of sister chromatid exchange, not the concentration of cleavable complexes (4).

We have shown that the desired cytotoxic and undesired recombinogenic effects of etoposide are not necessarily inseparable, using V(D)J recombination in the HPRT gene (which is an endogenous but inappropriate substrate) as an indicator of this undesired recombination. Although associated with much greater cytotoxicity, the 24-hour etoposide exposure produced a significantly lower frequency of deletions at day 6 than did the same concentration of etoposide given over a 4-hour period. It seems unlikely that all of the cells with exon 2 + 3 deletions on day 6 were destined to undergo apoptosis, in part because those cells have subsequently been selected quantitatively with standard limiting-dilution techniques and have grown at approximately the same rate as untreated CCRF-CEM cells. Thus, etoposide-induced recombogenesis is not necessarily a lethal event, a finding with important implications for the development of secondary leukemia.

It is intriguing that recognition signal sequences consistent with the action of V(D)J recombination were evident at the majority of the breakpoints in the deletion mutants. The absence of clear topoisomerase II consensus sequences at the breakpoints in cells surviving etoposide treatment does not preclude the involvement of topoisomerase II in the nonhomologous recombination; however, its involvement is difficult to document. There is considerable heterogeneity in topoisomerase II cleavage sites, and the addition of nontemplated nucleotides following cleavage might obscure the recognition of cleavage sites present in the intact gene. Moreover, measuring topoisomerase II cleavage by the direct assessment of ternary drug–DNA–topoisomerase II complexes (20) would have the disadvantage that it is not possible to determine which breakpoints persist following the cell death that occurs as a result of etoposide treatment. However, it is certainly likely that topoisomerase II is
directly or indirectly involved in the recombinogenic processes leading up to leukemogenic in vivo. We also analyzed topoisomerase I cleavage sites because the functional deficiency in one topoisomerase might be compensated for by an increase in the function of the other; however, neither these sites nor recombinogenic-prone \( \chi \)-like elements were observed at the breakpoints.

It is noteworthy that identical mutations were observed from more than one independent experiment (for mutations A, B, and E; Fig. 2) and that the majority of the breakpoints (particularly those in intron 1 adjacent to the slightly mismatched heptamer) occurred reproducibly at the same site. Such “hot spots” for HPRT deletions have also been reported in lymphocytes of patients with Fanconi anemia (50).

The much lower deletion frequency following equally cytotoxic treatment with the alkylator MNG suggests that the nonhomologous site-specific recombination induced by etoposide is not a nonspecific response to DNA damage or to cell death, both of which occur with MNG treatment. It is interesting that MNG produces point mutations in HPRT more frequently than does etoposide (48). Furthermore, the molecular mechanism of alkylator-induced AML appears to differ from that of epipodophyllotoxin-induced AML, in that the former type of AML lacks the site-specific, balanced translocations typical of epipodophyllotoxin-induced AML (7). These findings further support the use of HPRT exon 2 + 3 deletions (rather than a more nonspecific test of mutagenesis) as a putative biomarker of the undesired recombinogenic effects of etoposide.

The recognition of the cell cycle specificity and proliferation dependence of etoposide (51) has led to development of more prolonged dosage schedules, with improved antitumor effects observed for equivalent total doses given over a period of several days rather than given as a single, short intravenous infusion (52). Prolonged, daily administration of relatively small doses (e.g., 50-100 mg/m\(^2\) per day) of oral etoposide has shown moderate to good activity, even in patients in whom treatment with epipodophyllotoxins given less often as brief, higher-dose infusions had previously failed (38,39). Although these prolonged schedules have been used in several studies (38,39,53,54) of patients with refractory or relapsed tumors, they have not been widely used to treat newly diagnosed patients. Given the numerous factors that affect drug concentrations in vivo (e.g., protein binding, distribution, and drug interactions (55)), one must exercise caution in extrapolating from in vitro data. However, our findings suggest that prolonged exposure to etoposide regimens merits further study as a means of achieving comparable or enhanced cytotoxicity while inducing less site-specific, nonhomologous DNA recombination, thereby possibly minimizing the leukemogenic potential of etoposide and other epipodophyllotoxins.

References


Notes

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